THE ROLE OF CALLOSE AND β(1,3)-GLUCANASE IN MICROSPOROGENESIS

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

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May 1994

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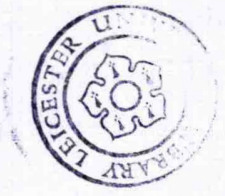

Acknowledgements

I would like to thank Rod Scott for coming up with the novel ideas for the experiments described in this thesis and for his help with the preparation of the aniline blue and TEM figures. I would also like to thank Wyatt Paul for handing over the *B. napus* A6 cDNA clone after his initial sequencing and database searches, and for supplying the A3, A9 and CaMV 35S promoter cassettes. I would also like to thank the following people for help with various experiments during the course of the project: Stefan Hyman for the preparation of anther sections for TEM and light microscopy shown in Chapter 6; George McTurk for preparing pollen for SEM; Caroline Dart for help with the locular fluid pH experiments; Gary Foster for providing the OG6 and OG7 oligonucleotides; Pierre de Wit for supplying the PR β(1,3)-glucanase antiserum and John Bailey for carrying out the squash of the meiocytes from a PR glucanase transformant. Thanks are also due to Garry Whitelam for inspiration and lots of helpful discussion. I would also like to thank Hugh Dickinson for providing time to help with the interpretation of anther and microspore TEMs.

I would also like to thank Diane Hird, Rachel Hodge and Wyatt Paul for their moral support and hours of discussion about a great variety of things, sometimes even about work. I would also like to say a big thankyou to Mike Roberts for the patience and support that he has provided during the last couple of years and for putting up with my thesis-induced mood swings. I'm also grateful to my Mum and Dad for the unfailing support that they have always given in all that I do. Last, but not least, thanks to all in the Botany Department for making my time in Leicester such an enjoyable one.

I would also like to acknowledge Nickerson International Seed Company (now Nickerson-BIOCEM limited) for their support of this research project.

The Role of Callose and β(1,3)-Glucanase in Microsporogenesis

Ph.D. Thesis by Dawn Worrall

The significance of the tetrad callose wall and its hydrolysis at microspore release has been investigated in *Brassica napus*. Callose wall breakdown was analyzed by measuring activities of β(1,3)-glucanase enzymes in relation to different stages of anther development. Assaying *B. napus* buds for endolytic and exolytic β(1,3)-glucanases established that activity peaked at the time of callose dissolution. Initial experiments to clone genes encoding these enzymes involved the use of probes related to PR β(1,3)-glucanases.

Sequencing of an anther-specific cDNA, A6, which was isolated from a *B. napus* 'sporogenesis' library, revealed similarity to previously characterized endo-β(1,3)- and β(1,3;1,4)-glucanases. Polyclonal antibody raised to the A6 protein identified a temporally-regulated ~60 kD band in *B. napus* buds which attained maximum levels at microspore release stage. Further analysis revealed that this ~60 kD band represented several proteins of different charge but similar molecular weight. A6 thus appears to be part of a family of immunologically related proteins. The sequence data and the temporal expression pattern suggest that A6 may be part of the callase enzyme complex involved in microspore release.

The role of the callose wall was investigated by engineering its premature removal *in vivo*. This was achieved by expressing a modified PR β(1,3)-glucanase in the anther tapetum of transgenic *Nicotiana tabacum*. These transgenic plants exhibited reduced male fertility, ranging from partial to complete male sterility. The microspores from the reduced fertility transgenic plants had an abnormal exine and the tapetum demonstrated hypertrophy. Male sterility appeared to result from bursting of the aberrant microspores after microspore release. These results suggested that premature callose hydrolysis is sufficient to cause male sterility and that callose is involved in exine formation in *N. tabacum*.

Some of the results in this thesis have been published in the following papers.

Worrall, D., Hird, D.L., Hodge, R., Paul, W., Draper, J., and Scott, R. (1992). Premature dissolution of the microsporocyte callose wall causes male sterility in transgenic tobacco. Plant Cell 4, 759-771.

Hird, D.L., Worrall, D., Hodge, R., Smartt, S., Paul, W., and Scott, R. (1993). The anther-specific protein encoded by the *Brassica napus* and *Arabidopsis thaliana* A6 gene displays similarity to β-1,3-glucanases. Plant J. 4, 1023-1042

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Chapter 1

Introduction

1.1 The Scope of this Thesis

In plants, β(1,3)-glucanase activity is both developmentally and environmentally regulated. Several β(1,3)-glucanases are expressed during pathogen attack and are regarded as typical pathogenesis-related (PR) proteins (Gianinazzi *et al.*, 1970; Bol *et al.*, 1990; Meins *et al.*, 1991). The soluble PR β(1,3)-glucanases often exist as isoforms within the same species and are commonly designated acidic or basic on the basis of their measured or deduced isoelectric point. These isoforms may also be located in different cellular compartments. In *Nicotiana tabacum*, basic isoforms tend to be located in the central vacuole and acidic isoforms in the intercellular fluid (for example Boller and Vögeli, 1984 and Parent and Asselin, 1984). All of these β(1,3)-glucanases are endolytic. Exolytic β(1,3)-glucanases have been found associated with cell walls in several species (Cline and Albersheim, 1981; Liénart *et al.*, 1986; Kurosaki *et al.*, 1989; Labrador and Nevins, 1989a). The function of these enzymes is not known, but a role in cell expansion is suggested (Labrador and Nevins, 1989a).

β(1,3)-Glucanase activities are also expressed during anther development, but these activities serve a very different function to the PR and cell wall associated types. These β(1,3)-glucanase activities, simply termed "callase" by Eschrich (1961), consist of endolytic and exolytic activity (Stieglitz, 1977). There is some evidence, but no definite proof, that callase is synthesized in the cells of the tapetum and then secreted into the locular space where it hydrolyzes the callose walls surrounding the tetrads of microspores (Echlin and Godwin, 1968a; Mepham and Lane, 1969; Stieglitz, 1977). Once the microspores are released into the locule they continue their development into pollen grains.

Whilst the anther β(1,3)-glucanases appear to be important in microspore release, it is unlikely that the dissolution of the callose wall alone is sufficient to cause microspore release in every case. Studies of microsporocytes in several species indicate that despite the accumulation of callose, the primary cell wall may persist, at least until tetrad stage (Waterkeyn, 1962; Echlin and Godwin, 1968b; Stieglitz, 1974; Bhandari *et al.*, 1981). A β(1,4)-glucan hydrolyzing activity in conjunction with β(1,3)-glucanase may therefore be necessary to effect microspore release in these situations.

The breakdown of the tetrad wall is temporally controlled and therefore the enzymes effecting microspore release must be under strict developmental regulation. Indeed mutants exist where there is a deviation in the timing of β(1,3)-glucanase activity and callose breakdown and these lines are male sterile (Frankel *et al.*, 1969; Warmke and Overman, 1972). The mechanism by which the appearance of hydrolyzing enzymes is coordinated with the appropriate developmental stage of the microspores is not known.

At the outset of the work described in this thesis very little was known about the anther β(1,3)-glucanases and no relevant cDNAs or genes had been isolated. Part of the

project described in this thesis was aimed towards the cloning of a gene or genes encoding these anther β(1,3)-glucanases. This was desirable in order to shed light on the interesting developmental control demonstrated by these anther-expressed enzymes. The first approach adopted to facilitate the isolation of an anther β(1,3)-glucanase was to utilize probes prepared to the PR β(1,3)-glucanases. A cDNA library, made from *Brassica napus* anther RNA at the appropriate stage in development, was available in the laboratory at Leicester, and it was hoped that screening of this library with various PR β(1,3)-glucanase probes would yield an anther-specific β(1,3)-glucanase cDNA. The isolation of an anther β(1,3)-glucanase cDNA would enable sequence comparison with the PR β(1,3)-glucanases and possibly answer questions on the evolutionary origin of the different types of β(1,3)-glucanase. Isolation of a gene corresponding to the cDNA would provide a means for studying the control of β(1,3)-glucanase expression in the anther.

The degradation of the callose wall via the hydrolytic action of β(1,3)-glucanase is one of the most cytologically obvious steps in the production of haploid microspores. There are several theories on the role of the temporary callose wall in microsporogenesis but none have been proven (see section 1.3.3). By removing the callose wall soon after its formation around the sporocytes it should be possible to obtain a clearer picture of the role of callose. To this end transgenic plants were generated which secrete a modified PR β(1,3)-glucanase from the anther tapetum before the appearance of the native callase enzymes. It was envisaged that these plants would give a better understanding of the role of callose in anther development. It was also quite likely that the resulting plants would be male sterile and thus be attractive for the plant breeding industry, to facilitate the production of hybrid seed.

1.2 Microsporogenesis and Microgametogenesis

After roots, stems and leaves have been established on the plant, flowers and then fruits and seeds may form, completing the life cycle and perpetuating the species. Most angiosperm species produce hermaphrodite flowers containing both the anthers and the pistil in which the male (pollen grains) and female (embryo sacs) gametophytes respectively are formed. The process of gametogenesis via meiosis is one of the most important processes during the development of plants, as it involves the change over from the sporophytic to the gametophytic generation and also provides the source of genetic variation via recombination.

The extremely reduced male gametophytes of angiosperms are formed within sporangia termed anthers and remain there during much of their development. In most angiosperms the anther consists of four lobes or locules separated by connective tissue. The anther wall has several layers, the epidermis, the endothecium, the middle layer and the

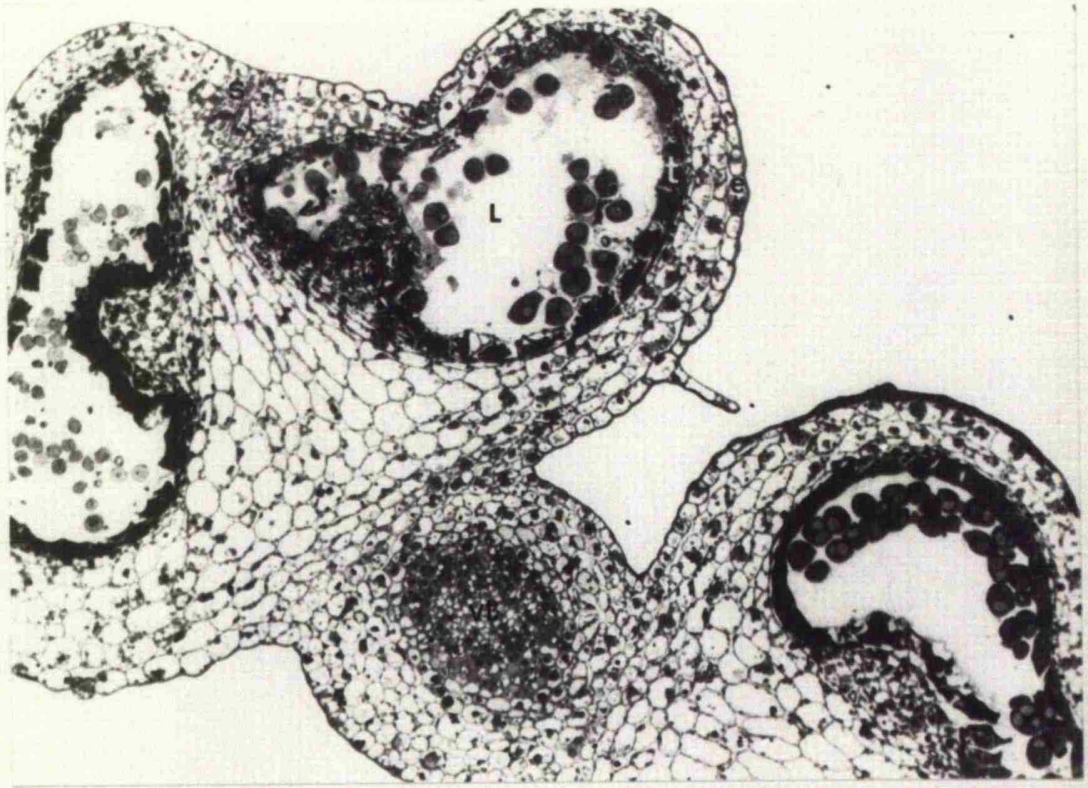
Figure 1.1
Transection of a *N. tabacum* Anther

(A) Semi-thin section (1 μm) of a wild-type *N. tabacum* anther stained with toluidine blue x160.

(B) Detail of (A) to show microspore tetrads within the callose wall and dense cytoplasm of the tapetum x400.

e, epidermis; **et**, endothecium; **L**, locule; **s**, stomium; **t**, tapetum; **vt**, vascular tissue.

A



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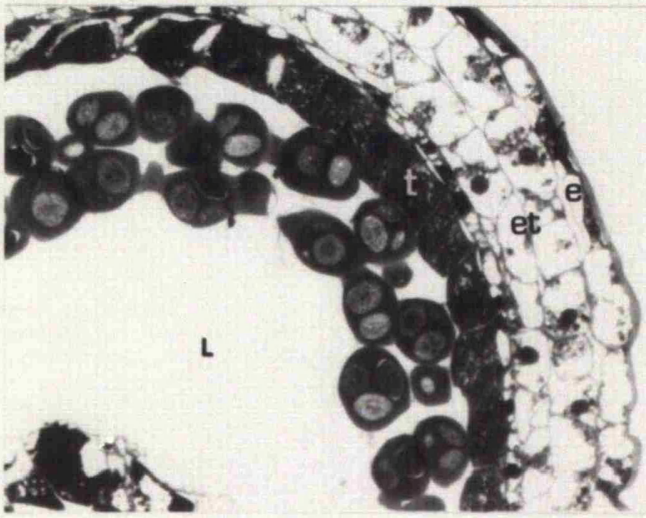


tapetum (Figure 1.1). The stomium is another specialized region of cells located in the anther wall between the locules and this is involved with the release of the pollen grains in response to environmental conditions. The middle layer of the anther shown in Figure 1.1 has almost been completely squashed by the expanding endothecium and tapetum. The tapetum is a specialized microsporangial tissue which is generally believed to be involved with the nutrition of the developing microspores and provision of materials for exine formation (Mascarenhas, 1975; Bhandari, 1984). Tapetal ontogeny is closely correlated with the development of the microspores and its importance is underlined by the fact that the failure of the tapetum will normally lead to the failure of the microspores (see Laser and Lersten, 1972).

Although relatively simple in structure, the male gametophyte provides a very interesting subject for the study of development, both at the cytological and molecular level. Because of its unique role, the microgametophyte has some very specialized functions. After anthesis, the gametophyte must exist as a free organism, the pollen grain, until it is carried to the stigma of the pistil by various vectors such as wind or insects. Once the pollen grain has reached the stigma, it germinates to produce a tube in which the sperm cells are transported to the embryo sac.

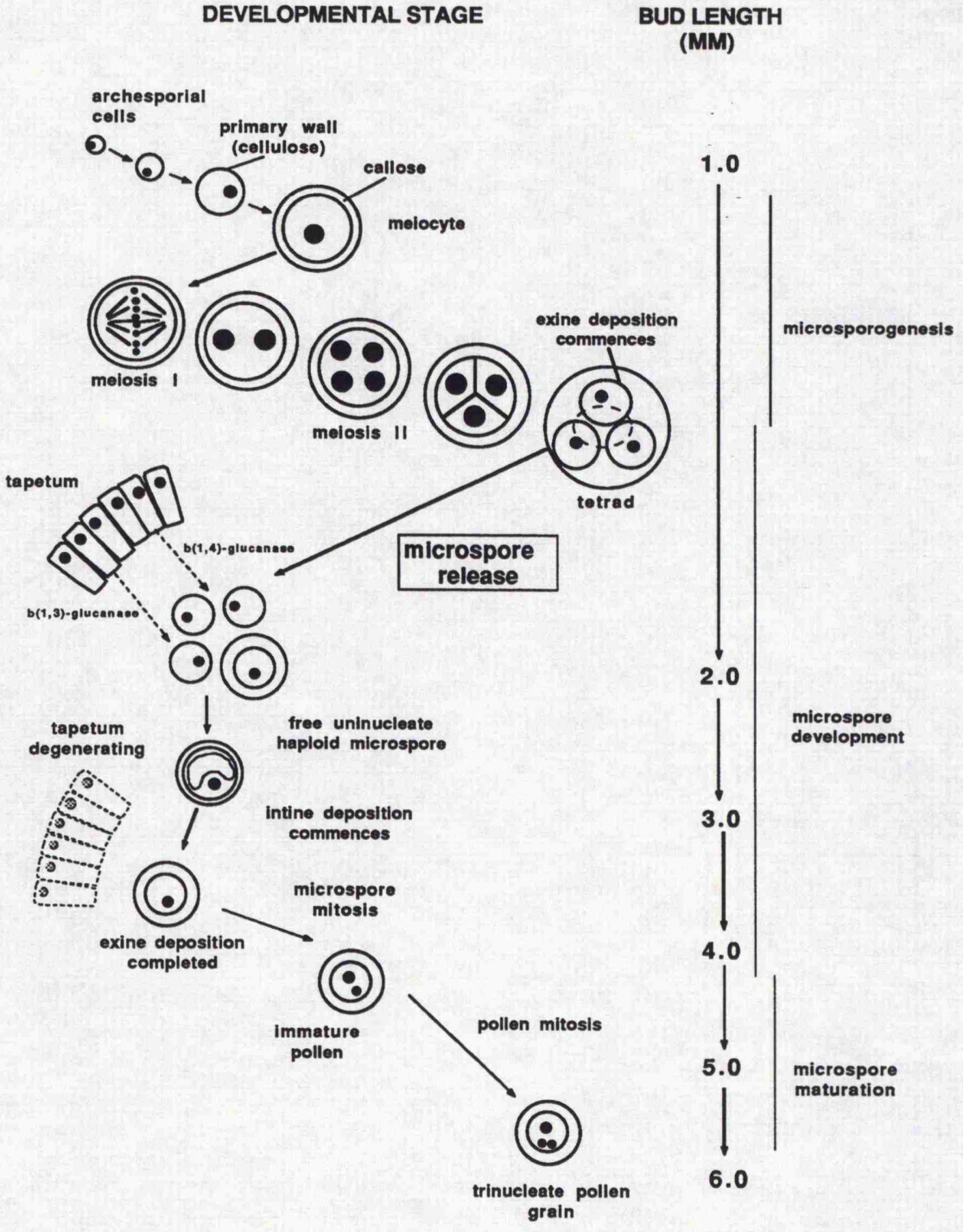
In *B. napus* and *Lilium* a very high degree of synchrony exists between the sporogenous cells of each flower and an excellent correlation exists between flower bud length and developmental stage (Stieglitz and Stern, 1973; Scott *et al.*, 1991a). This factor provides an important tool for the study of pollen development as it allows the rapid sorting of large amounts of buds into developmental stages. It also indicates that the control mechanisms for cell division are tightly linked to anther growth. The sequential stages of pollen development are shown in Figure 1.2. Very early during development a column of archesporial cells forms at each corner of the young anther. The anther wall layers are derived from periclinal divisions of the lateral archesporial cells and the pollen mother cells (pmc) or sporocytes are produced following several rounds of mitotic divisions of the inner archesporial cells.

The critical steps for the production of haploid microspores are two meiotic divisions. Just prior to these meiotic divisions a "special cell wall" consisting of callose, a β(1,3)-linked glucose polymer (Rowley, 1959; Heslop-Harrison, 1968a, b) is deposited around the sporocyte between the plasma membrane and cell wall. There are two types of cytokinesis involved with meiosis in the pmc, successive and simultaneous. One mode of cytokinesis tends to dominate in certain groups of plants:- simultaneous in dicots and successive in monocots, but there are too many exceptions to make this a general rule (Pandey, 1960). In the successive type of cytokinesis, for which *Lilium* provides an example, a cell plate is laid down after each of the two meiotic nuclear divisions (Stieglitz, 1974). In the simultaneous type (for example in *N. tabacum*), meiosis results in the production of four nuclei in a common cytoplasm, and it is only at the end of meiosis II that the haploid microspores are separated from one another by the cell plates (Horner, 1977).

Figure 1.2

Microsporogenesis and Microgametogenesis in *B. napus* Based on the Major Cytological Changes Occuring Within the Sporogenous Cells and Their Derivatives

The relationship between bud length and anther developmental stage is indicated on the right hand side of the figure.


DEVELOPMENTAL STAGE
BUD LENGTH (MM)
archesporial cells
primary wall (cellulose)
callose
meiocyte
meiosis I
meiosis II
exine deposition commences
tetrad
microsporogenesis
tapetum
b(1,4)-glucanase
b(1,3)-glucanase
microspore release
free uninucleate haploid microspore
tapetum degenerating
intine deposition commences
microspore mitosis
exine deposition completed
immature pollen
pollen mitosis
trinucleate pollen grain
microspore development
microspore maturation
1.0
2.0
3.0
4.0
5.0
6.0

The cell plates in many cases appear to be composed entirely of callose layers (for examples see Stieglitz, 1974; Horner, 1977; Waterkeyn, 1962; Echlin and Godwin, 1968a), but Skvarla and Larson (1966) in their studies of *Zea mays* found that the cell plates were predominantly composed of pectic substances which formed the framework upon which the internal callose walls were laid down.

After meiosis, the quartet of microspores are held together in a tetrad by the outer callose wall and the individual spores are separated by callosic cross walls. The daughter microspores are therefore completely isolated from each other and the surrounding cells by callose walls. Once meiosis is complete and synthesis of the elaborate pollen wall patterning has initiated beneath the callose wall on the surface of each microspore, the tetrad stage is ended by the enzymic dissolution of the tetrad wall. β(1,3)-Glucanase (or callase) activities which are thought to originate in the tapetum (Mepham and Lane, 1969; Stieglitz, 1977) show a sharp peak in activity during the time when the microspores are released from the tetrads into the sporangial locule (Stieglitz and Stern, 1973). These enzyme activities are therefore assumed to be the agent which brings about microspore release. It is not clear, however, whether callase is the sole agent which brings about microspore release. Several studies have shown that the primary cellulosic wall which originally surrounds the sporocyte persists at least until tetrad formation in some species (Bhandari *et al.*, 1981). This suggests that a cellulase activity in conjunction with callase may be required for microspore release in certain species.

After microspore release the microspores undergo both a rapid increase in size and further development of the pattern on the microspore wall. The microspore nucleus completes its extended interphase period and undergoes an unequal mitotic division to yield a small generative cell and large vegetative cell. The generative cell is delimited from the cytoplasm of the vegetative cell by two membranes enclosing a wall, which is thought to be pectic in nature due to its staining and solubility properties (Cresti and Tiezzi, 1990). At anthesis in angiosperms, the mature pollen grains can be either trinucleate or more commonly binucleate. In trinucleate pollen the generative cell divides into two sperm cells before anthesis, but in binucleate pollen the mature gametophyte contains only two cells. The final division of the generative cell into two sperm cells in this case occurs after the germination of the pollen grain on the stigma.

Anther development can be divided into three main phases according to the stage of the microgametophytes. (i) Microsporogenesis encompasses the stages where the sporocytes enlarge, become surrounded by the callose wall and eventually culminates in meiosis. (ii) Spore development begins when the microspores are still held together in tetrads by the callose wall. The beginning of this second phase is signalled by the start of microspore wall patterning directly after cytokinesis and continues after microspore release. (iii) Pollen maturation is generally considered to start after microspore mitosis and involves completion of the spore wall and storage of messages for germination and pollen tube growth.

The work reported in this thesis concentrates on the earlier stages of microgametogenesis, namely the formation of the callose wall through to its degradation at microspore release. The next few introductory sections therefore give some more detail on these aspects.

1.3 Callose

1.3.1 The Distribution of Callose in Plants

Callose synthesis is not unique to the microsporogenous cells, and occurs in numerous other situations in the plant. Despite this, callose is not generally considered part of the normal plant cell wall, but is often produced in response to wounding where it is thought to participate in the formation of a physical barrier against pathogen invasion (Goodman *et al.*, 1986). Staining with Sirofluor, a fluorochrome found as a contaminant in aniline blue, has shown that callose is also a major component of the pollen tube cell walls (Stone *et al.*, 1984; Rae *et al.*, 1985). A monoclonal antibody was raised to a β(1,3)-glucan and showed that the callose is located in the inner walls of the pollen tube of *Nicotiana alata* (Meikle *et al.*, 1991a). There is some evidence to indicate that the callose in pollen tube walls of *Lilium* exists as a heterogeneous substance, probably as a callosic glycoprotein (Reynolds and Dashek, 1976; Nakamura *et al.*, 1984). However, only small amounts of protein were detected in *N. alata* by Rae *et al.* (1985).

A more transient occurrence of callose has been found during megasporogenesis. The megaspores of plants which have a mono- or bisporic type of embryo sac are surrounded by a layer of callose for part of their development (Rodkiewicz, 1967; Bouman *et al.*, 1984). Callose has also been detected in the sieve elements of the phloem (see Cutter, 1978). The callose forms a sheath around the plasmodesmatal connective strands which penetrate the pores in the phloem sieve plate. Epidermal strips can be taken from the surface of a *Ophioglossum vulgatum* leaf, stained with aniline blue and viewed under fluorescence. The thickened walls adjacent to the pore, and also the walls between the guard cells of the stomata fluoresce. Lack of fluorescence when the same material is treated with β(1,3)-glucanase enzymes prior to aniline blue staining suggests that β(1,3)-glucan, probably callose is a component of the guard cell wall in some species (Peterson *et al.*, 1975).

1.3.2 Callose Deposition

Callose and cellulose production both appear to be catalyzed by plasma membrane associated enzymes. Substantial evidence points towards these β-glucan synthases being part of the same enzyme complex which interchanges between callose and cellulose

production in response to fluctuations in intracellular Ca^{2+} levels (Delmer, 1987). During wounding there is a transient rise in the level of intracellular Ca^{2+} and this is presumed to activate callose synthase activity (Kauss *et al.*, 1987). The purification of the membrane bound β-glucan synthase has been a slow process due to the fact that the complex loses activity when dissociated from the membrane. However, monoclonal antibodies have been raised to the complex isolated from *Lolium multiflorum* and used in immunoprecipitation experiments. SDS gel analysis showed that the complex consists of four major polypeptides and several minor ones. The immunoprecipitated complex was capable of synthesizing both β(1,3)- and β(1,4)-glucans (Meikle *et al.*, 1991b). The techniques of photolabelling and product entrapment have more recently allowed the identification of two polypeptides, of molecular weights 52 kD and 37 kD, which are likely candidates for the catalytic subunits of callose and cellulase synthase respectively (Li *et al.*, 1993). Although these polypeptides are the most likely candidates for the catalytic subunits of the β-glucan synthases, the authors indicate that several other polypeptides are also entrapped and photolabelled under conditions favouring β(1,3)-glucan synthesis. Other studies using several different approaches to isolate the catalytic subunit of callase synthase have identified polypeptides of various sizes (Read and Delmer, 1987; Mason *et al.*, 1990; Frost *et al.*, 1990; Dhugga and Ray, 1991a, b; Meikle *et al.*, 1991b). Although it is difficult to compare the results from different species, evidence for the isolation of the callose synthase catalytic subunit seems to be conflicting in many cases and more work is required in this area.

A class of protein which has a potential role in the regulation of callose synthase activity has been identified in cotton fibres (Andrawis *et al.*, 1993). A purified fraction of these proteins, which resemble annexins, binds to and inhibits the activity of a partially purified cotton fibre callose synthase. These annexins may therefore be involved with the control and localization of callase synthase activity.

In situations where callose is required to strengthen the cell wall after tissue injury, the production of callose by a plasma membrane located enzyme would appear the most effective method. However, in other circumstances this may be considered an over-simplification. Callose may also be deposited from golgi-derived vesicles during the formation of the cell plate, a process also thought to be under the influence of calcium (Helper and Wayne, 1985). It is possible that different mechanisms regulate callose deposition in different cells or even in the same cell at different times. In pollen tubes, vesicles may deliver the callose to the extending cell wall just behind the tip as this is the region containing the organelles and cytoplasm. Further away from the tip where the callose plugs form across the tube, the plasma membrane may mediate the callose deposition (Kaus, 1987). There seems to be a general consensus that callose containing vesicles are associated with the formation of the microsporocyte cell wall, though the exact origin of the callose is unclear. Dictyosomes and discrete vesicles have been observed in the cytoplasm of the meiocyte before the appearance of the callose wall. In *Zea mays,*

Skvarla and Larson (1966) noticed that just prior to callose deposition, the plasma membrane receded from the cell wall and large numbers of vesicles occupied the space. As no ER has been observed in the regions of callose deposition it is suggested that the dictyosomes are involved with the production of callose, which is in turn moved into the vesicles and then out to the cell wall (Heslop-Harrison, 1966). It is only more recently that the material inside the vesicles has been confirmed as callose (Blackmore and Barnes, 1988). Previously, due to the electron lucent nature of callose this was difficult to determine with the electron microscope.

The special callose wall secreted around the sporocyte prior to meiosis accumulates sequentially as a series of concentric layers (Waterkeyn, 1962; Skvarla and Larson, 1966). These layers are often indistinguishable when complete, but two main layers can often be recognized. The initial layer of callose which surrounded the sporocyte and therefore encloses all four microspores of the tetrad is usually obvious, as is the individual layer of callose that is frequently deposited by each of the daughter microspores (Longly and Waterkeyn, 1979). In *Pergularia daemia* there is no callose deposition before or after meiosis and separation of the tetrad of microspores does not occur. The pollen grains of this species are encased as permanent tetrads termed pollinia (Vijayaraghavan and Shukla, 1977).

1.3.3 Functions of Callose

The deposition of callose just before meiosis and its hydrolysis during early microspore development suggests that the callose wall layer has some special functions associated with these events. Waterkeyn (1962), proposed that the temporary wall fulfils the important biological role of separating the products of meiosis to prevent cell cohesion and fusion. The rapid synthesis and dissolution of the wall to release separate microspores is entirely consistent with this role. The callose wall has also been seen as providing a barrier between the microspores, the anther locule and the sporophytic tissue (Heslop-Harrison and Mackenzie, 1967). Labelled thymidine has been followed into the detached buds of *Lilium henryi* and the radioactive thymidine was not able to penetrate into the sporocytes or developing microspores until tetrad release (Heslop-Harrison and Mackenzie, 1967). This suggested that the callose wall prevented the passage of the radioactive thymidine and therefore acts as a molecular filter. However, these experiments do not rule out the possibility that the selectivity is provided by the plasma membrane. The role of callose as a molecular filter is not favoured by Mascarenhas (1975), as in other studies the callose wall did not prevent the penetration of a variety of reagents. Southworth (1971), demonstrated that glucose and sodium acetate pass through the callose wall of *Gebera jamesonii* but phenylalanine does not. Phenylalanine is a smaller molecule than glucose and would therefore be expected to enter. Colloidal iron passes readily through the callose wall of *Populus* and *Salix* tetrads (Rowley and Dunbar, 1970). The callose wall

also failed to prevent the penetration of tetrazolium salts used to visualize a staining reaction in *Paenia* (see Bhandari, 1984). Because of these observations, Mascarenhas (1975) suggested that callose walls must have some other important role in development of the male gametophyte besides that of a molecular filter.

Callose may also be involved with the establishment of the basic exine pattern on the pollen surface (Waterkeyn and Beinfait, 1970; Ford, 1971). Several workers (Heslop-Harrison, 1963, 1968a; Echlin and Godwin, 1968a) have observed that the exine pattern is laid down while the microspores are still embedded in the callose wall of the tetrad. Waterkeyn and Beinfait (1970) observed that the callose walls of *Ipomoea purpurea* show a regular geometric pattern and proposed that this constitutes a hollow template or mould for the primexine matrix. The distribution of the exine spines is directly related to the primary patterning of the spore chamber and therefore Waterkeyn and Beinfait (1970) suggest that the callose wall plays an essential role in the formation of the general exine pattern in *Ipomoea*.

1.3.4 Pollen Wall Formation

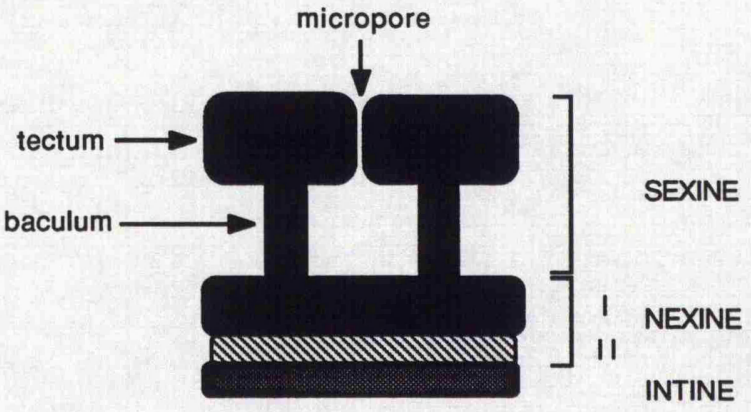
After the meiotic cell division, callosic cross walls are formed and following this, development of the pollen wall commences around each member of the tetrad (Dickinson, 1970; Heslop-Harrison, 1971; Sheldon and Dickinson, 1983). There are two main domains in the pollen wall: the exine, the outer patterned layer made of sporopollenin, and the intine, the inner smooth polysaccharide layer. In morphological terms there are many types of exine, the tectate and pilate types shown in Figure 1.3 are two of the more common examples. The diversity of pollen wall elaboration is vast and it is difficult to arrive at a set pathway for which similar features may be generated for different taxa. However, since the pioneering work of Heslop-Harrison (1963) there have been many studies on the ontogeny of the pollen wall and developmental models can be formulated. The first stages of pollen wall formation include positioning of the non-patterned apertural regions and formation of the primexine. After these primary events, the major layers of the wall are deposited sequentially at the microspore surface. Further modification and ornamentation of the pollen wall usually occurs after microspore release.

Whilst some types of pollen in monocotyledonous and dicotyledonous species do not have germinal apertures, most possess an area of wall devoid of exine, or covered by an operculum at which pollen tube emergence may occur upon successful pollination. The position and symmetry of the apertures appears to be determined very early in the development of the microspores. The meiotic cytoskeleton plays an important role in the formation of the single pore in *Triticum aestivum* (Dover, 1972). The pore forms in a position close to the poles of the meiotic spindle. Experiments with centrifugation (Heslop-Harrison, 1971; Sheldon and Dickinson, 1983) or colchicine (Sheldon and Dickinson, 1986) have also shown that in *Lilium*, the aperture and the symmetry of the

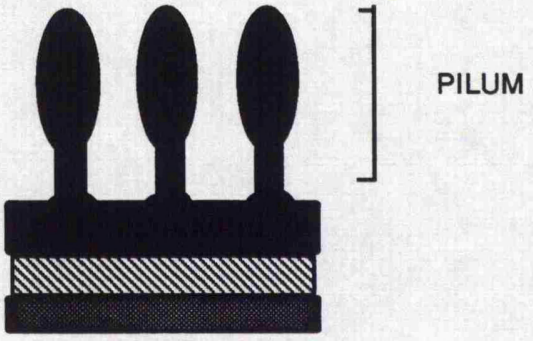
Figure 1.3
Diagram Interpreting the Organization of the Pollen Wall

Two types of exine stratification are shown. The nomenclature is based on that of G. Erdtman (diagram modified from Heslop-Harrison, 1968).

(A) Tectate Exine



(B) Pilate Exine



microspores within the tetrad are defined after the first meiotic division by the position of the meiotic spindles. The actual mechanism by which an area of the microspore plasma membrane becomes designated as an aperture is unknown. However, it appears that apertures are formed where a membranous shield consisting of sheets of endoplasmic reticulum is positioned close to the plasma membrane, thus sheltering the apertural region from wall forming activities.

The primexine is the first layer to be deposited beneath the callose wall. This layer is largely polysaccharide and is synthesized outside the plasma membrane of the young microspore (Blackmore and Barnes, 1990). The primexine is viewed as a matrix containing receptors for the exine wall polymer, sporopollenin (Heslop-Harrison, 1963). It is not easy to define when the term primexine ceases to apply, as its transformation into exine is a continuous process but Heslop-Harrison (1963) originally conceived that the primexine does not contain sporopollenin.

The tectum and bacula (sexine) and nexine I are laid down by the deposition of sporopollenin on the surface of membrane-like lamellae. The lamellae appear to originate near to the plasma membrane and were first observed by Rowley and Southworth (1967). Dickinson and Heslop-Harrison (1968) observed radially directed rods, the probacula, traversing the primexine. The early probacula consist of a folded lamellar structure upon which protosporopollenin is deposited. The sporopollenin produced by the microspores at tetrad period has a different staining capacity to sporopollenin which is later derived from the tapetum and was termed protosporopollenin by Dickinson (1971). It is possible that the protosporopollenin is in a different state of polymerization.

After the sexine is laid down within the primexine, the nexine I is deposited below the sexine, forming a foot layer which connects the probacula (Dickinson and Heslop-Harrison, 1968). The microspore continues to produce protosporopollenin which is built up on the surface of the microspore beneath the callose wall, making the initial wall more obvious. Microspore release occurs when the nexine I has become discernible and homogeneous. Once the callose wall is removed the microspore becomes exposed and the sexine, the outer layer, continues growth via the accumulation of tapetally derived sporopollenin. During this period the nexine II is laid down upon the characteristic lamellae beneath the nexine I (Rowley and Southworth, 1967; Dickinson and Heslop-Harrison, 1968). The pectocellulosic intine wall layer, present in all angiosperm pollen grains, generally begins to form after mitosis when the nexine II is completed (Blackmore and Barnes, 1990). The intine may contain stored substances involved with gametophytic incompatibility reactions (Knox and Heslop-Harrison, 1969).

Interesting developmental studies of the exine of *Artemisia* and other pollen types carried out by Rowley and associates at the University of Stockholm, have revealed the existence of a glycocalyx network on the outside of the plasma membrane. This network is attached to the microspore plasma membrane and passes through the nexine into the bacula and tectum. The exine network is shown to be fibrillar in nature and stains positively for

proteins and acidic polysaccharides (Rowley, 1975, 1978; Rowley and Prijanto, 1977). Subsequently, chemical etching techniques have provided an updated model for the glycocalyx network (Rowley *et al.*, 1981a, b). The new model for the exine is based on filamentous rod shaped subunits of 15-40 μm in diameter which are embedded in sporopollenin. Rowley *et al.* (1981a) propose that these non-sporopollenin macromolecules are glycocalyx units which function as sporopollenin receptors.

Pollen development has been studied in many species and a wide variety of ornamentation exists, but there are relatively few studies that discuss the origin of the different types of exine patterning. The mechanism which determines ornamentation has not yet been resolved. The exine pattern appears be determined before meiosis, as sterile pollen grains containing no nuclear material still exhibit patterned sporopollenin deposition (Rogers and Harris, 1969). This information suggested that patterning information must be transcribed in the microsporocytes, but only become conspicuous during primexine formation. This and other data led Heslop-Harrison to conclude that wall formation must be sporophytically controlled (for example Heslop-Harrison, 1971). Sheldon and Dickinson (1983) proposed that material is inserted into the plasma membrane to form contiguous 'plates'. These plates alter the membrane in such a way that the protrusions that constitute the first stages of wall formation only develop in the spaces between them. The plates therefore behave as a stencil for the formation of the primexine. The observations of Godwin *et al.* (1967), Rowley and Southworth (1967) Dickinson and Heslop-Harrison (1968) seem to suggest that the lamellae, which are formed outside the plasma membrane of the microspore, have a central role in the early growth of the pollen wall. The trilamellate structures termed white line centred lamellae (Rowley and Southworth, 1967) appear to occur in most groups of land plants, but they have not yet been observed in those members of the angiosperms in which the nexine II is absent (Blackmore and Barnes, 1987). However, in *Ipomoea* the inner surface of the callose wall shows the first signs of patterning even before the primexine is formed (Waterkeyn and Beinfait, 1970). In the pollen of *Compositae*, the position of the major spines also appear to be predetermined from the patterning in the callose wall (Barnes and Blackmore, 1986). Although the callose wall is clearly involved in exine pattern formation in these examples, it is still assumed that the plasma membrane mediates the pattern since this structure regulates both callose wall and primexine deposition (Blackmore and Barnes, 1990; Takahashi and Skvarla, 1991).

1.3.5 Microspore Release

As described above, the process of pollen patterning initiates while the microspores are still held within the callose wall. When the microspores are at the appropriate developmental stage the callose wall is broken down and the microspores are released into the locular space. The commonly accepted view of this process is that dissolution of the β(1,3)-glucan wall is mediated by a β(1,3)-glucanase activity (callase) which originates

from the tapetal cells (Echlin and Godwin, 1968b; Mepham and Lane, 1969; Stieglitz, 1977). This activity appears to be under strict developmental control. The enzyme activity is undetectable before meiosis, reaches a peak at microspore release and decreases again as the microspores mature (Frankel *et al.*, 1969; Mepham and Lane, 1969; Stieglitz and Stern, 1973). Anther β(1,3)-glucanases have been partially purified from *Lilium* (Stieglitz, 1977), but no cognate genes or cDNAs have been isolated.

In many species, microspore release also requires the action of β(1,4)-glucanases. Studies of pollen development frequently examine callose wall deposition around the sporocyte but fail to mention the effect this has on the original primary cell wall. The original primary wall often breaks down as the callose wall thickens (Heslop-Harrison, 1972; Horner and Rogers, 1974; Horner, 1977) but in *Heleborus foetidus* (Echlin and Godwin, 1968a) and *Olea europaea* (Pacini and Juniper, 1979), the primary wall can still be observed in a reduced form. Bhandari *et al.* (1981) showed with a histochemical stain that the primary wall persists until tetrad formation in *Alium tuberosum* and until telophase II in *Cyclamen persicum.* In *A. tuberosum,* the primary wall breaks down simultaneously with the callose wall at the time of microspore release. In *Lilium*, Stieglitz (1977) also observed that the primary wall is still intact at meiotic tetrad stage. The tetrad wall in many species therefore consists of a primary wall outlining the structure and a callosic layer which isolates each microspore. Microspore release in this situation must not only involve hydrolysis of the callosic wall, but also simultaneous disruption of the primary wall. A cellulase serologically related to the abscission zone cellulase has been shown to accumulate in the anthers of *Phaseolis vulgaris* (Sexton *et al.*, 1990; Campillo and Lewis, 1992). The role of this enzyme in anther development has not been confirmed, but an involvement in the dissolution of the tapetum or cells of the stomium is suggested (Campillo and Lewis, 1992). It may also be possible that this cellulase is involved with microspore release.

The correct timing of callase activity in anthers appears to be crucial to the normal development of the male gametophyte since faulty timing is associated with male sterility in several species (Frankel *et al.*, 1969; Warmke and Overman, 1972). Similarly a developmentally regulated cellulase enzyme may also be required for microspore release in some species and incorrect timing of this enzyme could also be associated with sterility. Many hypotheses attempt to explain the significance of the callose encasement (see 1.3.3), but the purpose of the persisting primary wall in many species has received little attention.

1.4 β(1,3)-Glucanases

1.4.1 Anther β(1,3)-Glucanases

Microspore release may require the coordination of several physiological factors. A correlation between β(1,3)-glucanase activity and microspore release has been observed in *Helleborus* (Echlin and Godwin, 1968a), *Petunia* (Frankel *et al.*, 1969), *Tradescantia*

(Mepham and Lane, 1969) and *Lilium* (Stieglitz and Stern, 1973). A strong relationship also exists between callase activity, pH of the anther locule and microspore release. A study in *Petunia hybrida* (Izhar and Frankel, 1971) showed that as microsporogenesis proceeds, the pH of the anther locular fluid drops. During meiosis when callase activity is undetectable, the pH of the anther locule is 6.8-7.0. Towards the end of the tetrad stage, the pH drops to 5.9-6.2. A burst of callase activity then follows and the tetrad callose walls are digested and the microspores are released into the locule. A fall in the locular pH of developing anthers was also reported by Linskens (1956) in *Lilium henryii*. Since the optimum pH for callase activity is pH 4.8-5.0, with activity undetectable above pH 6.3, Izhar and Frankel (1971) concluded that the pH drop was a precondition for callase activity. A further study by Izhar and Frankel (1973) showed that the concentration of free amino acids in the anther locule changes during development. The authors therefore suggested that pH change could be attributable to these fluctuations.

Stieglitz (1977) carried out a more comprehensive study of the role of β(1,3)-glucanase in microspore release and discovered that sharp peaks of both endolytic and exolytic β(1,3)-glucanase activity occurred at the time of callose wall breakdown. The activities were resolved chromatographically and found to be attributable to two different enzymes, an exo-β(1,3)-glucanase of molecular weight 62 kD and an endo-β(1,3)-glucanase of molecular weight 32 kD. The activities of these enzymes were found mainly in the somatic tissues (exo-β(1,3)-glucanase) or tapetum (endo-β(1,3)-glucanase). Stieglitz (1977) applied the β(1,3)-glucanase enzymes to isolated microsporocytes and observed their action on the tetrad wall by means of transmission and scanning electron microscopy. Stieglitz concluded from her study that in *Lilium* the immediate agent of callose breakdown was the endo-β(1,3)-glucanase. The precise role of the exo-enzyme is uncertain, but the most simple theory is that the exo-β(1,3)-glucanase functions to hydrolyze the oligosaccharides produced by the endo-β(1,3)-glucanase activity and thus provide readily metabolizable glucose for subsequent development (Stieglitz, 1977). Although Stieglitz (1977) carried out the semi-purification of anther β(1,3)-glucanases no further work on the isolation of any genes or cDNAs has been reported.

1.4.2 PR Endo-β(1,3)-Glucanases

Plants are able to defend themselves against fungal pathogens by a range of defence mechanisms among which the production of the PR proteins is one of the most extensively studied. Some of these PR proteins have endo-β(1,3)-glucanase or chitinase activity. Since many fungi contain chitin and β(1,3)-glucans as major components of their walls, the glucanohydrolases may play a role in defence against infection by pathogenic and

potentially pathogenic fungi (Abeles *et al.*, 1971; Pegg, 1977; Boller, 1985). There is good indirect evidence to support this view. Combinations of purified *P. sativum* chitinase and β(1,3)-glucanase inhibited the growth of fifteen out of eighteen fungi tested (Mauch *et al.*, 1988). Furthermore, glucan fractions produced after the breakdown of the fungal cell wall by β(1,3)-glucanase have been shown to act as elicitors of the defence response in plants (Keen and Yoshikawa, 1983).

Similar patterns of PR protein induction occur after the interaction of plants with different types of pathogens and each interaction in turn includes a broad range of defence reactions. If *N. tabacum* plants are infected with tobacco mosaic virus (TMV), they may be subsequently resistant to other unrelated viruses, fungi and bacteria. PR proteins can also be induced by abiotic elicitors such as salicylic acid (Hooft van Huijduijnen *et al.*, 1986) and this kind of treatment generally results in acquired resistance to pathogens.

β(1,3)-Glucanases have been purified from many plant species (see Bol *et al.*, 1990 and Meins *et al.*, 1991). All of the β(1,3)-glucanases that are induced by wounding and elicitor treatment are endohydrolases (Bol *et al.*, 1990) and often exist as isoforms within the same species. The different isoforms may be located in different cellular compartments. The basic isoforms accumulate predominantly in the vacuole of the cell (Boller and Vögeli, 1989; Mauch and Staehelin, 1984; Shinshi *et al.*, 1988; van den Bulcke *et al.*, 1989; Keefe *et al.*, 1990) and are present in healthy plants where they may accumulate to high concentrations in the lower leaves and roots (Felix and Meins, 1986; Castresana *et al.*, 1990). Although treatment with salicylic acid, ethylene and fungal elicitors causes up regulation of the basic endo-β(1,3)-glucanase, the proteins still remain in the central vacuole. Keefe *et al.* (1990) observed that despite ethylene treatment changing the cell type-specific distribution of the basic isoforms in *N. tabacum* leaves, the intracellular compartmentation remained constant. Following infection with bacterial or viral pathogens or treatment with salicylic acid or ethylene, the acidic isoforms of β(1,3)-glucanase accumulate in the intercellular fluid (ICF) (Parent and Asselin, 1984; van den Bulcke *et al.*, 1989; Payne *et al.*, 1990; Côté *et al.*, 1991; Linthorst *et al.*, 1990; Ward *et al.*, 1991). As acidic β(1,3)-glucanases are present in ICF, it is thought that they may serve as a first line of defence against invading pathogens whilst the basic β(1,3)-glucanases, stored in the vacuole, form a second line of defence. In *Solanum tuberosum,* the basic β(1,3)-glucanases are mainly found in the ICF, suggesting that they are secreted after fungal infection or elicitor treatment (Kombrink *et al.*, 1988).

Many experiments to determine the role of the PR proteins in defence have been carried out *in vitro* (for example Keen *et al.*, 1983), but it is important to discover the actual accumulation and localization of PR proteins *in planta* during active defence. Studies of an *N. glutinosa* x *N. debneyii* hybrid (Ahl and Gianinazzi, 1982) which is highly resistant to viral, bacterial and fungal pathogens are helping to elucidate the role of PR proteins during defence (Goy *et al.*, 1992). The high level of resistance of this hybrid is correlated to high

levels of chitinase, β(1,3)-glucanase, peroxidase and polyphenoloxidase. The parental species of the hybrid are much more susceptible to disease and do not contain high levels of hydrolase and oxidase activity. These observations reinforce the theory that the hydrolases and oxidases are important for disease resistance. Experiments with transgenic plants containing antisense constructs may also help to define the role for β(1,3)-glucanases. An antisense construct of a basic β(1,3)-glucanase under the control of the cauliflower mosaic virus (CaMV) 35S promoter was transformed into *N. sylvestris* (Neuhaus *et al.*, 1992). Constitutive and induced expression of the basic β(1,3)-glucanase was effectively blocked with the antisense construct without affecting the other acidic β(1,3)-glucanases. The transgenic antisense plants developed normally, were fertile and did not exhibit increased susceptibility to the fungus *Cercospora nicotianae*. This suggested that the basic β(1,3)-glucanase is not important for house keeping functions of the *N. sylvestris* cells or in defence against this particular pathogen. However, before the definitive role of the basic β(1,3)-glucanase can be determined, direct analysis of the β(1,3)-glucanase levels in putative target tissues is required. Antisense experiments may yet help to determine the role of the β(1,3)-glucanase isoforms in defence and in normal development.

1.4.3 Developmental Regulation of PR Endo-β(1,3)-Glucanases

The expression of a number of PR genes is developmentally regulated. For example as described above, basic β(1,3)-glucanase isoforms are present at high concentrations in the basal leaves and roots of healthy plants (Felix and Meins, 1986; Shinshi *et al.*, 1987; Castresana *et al.*, 1990). Similarly, the acidic and basic β(1,3)-glucanases are differentially regulated at the onset of flowering: expression of a basic β(1,3)-glucanase decreases (Felix and Meins, 1986; Neale *et al.*, 1990) but expression of the acidic isoform PR-2c begins (Fraser, 1981; Côté *et al.*, 1991). Lotan *et al.* (1989) extended these observations to show a spatial and temporal accumulation of PR proteins in a developmental manner. Antibodies raised to different classes of PR proteins were used to examine the distribution of these proteins in *N. tabacum* plants during normal growth. PR proteins were found in sepals, pistils, anthers and ovaries. Only the protein detected in the pistil was found to be serologically related to PR β(1,3)-glucanases. The β(1,3)-glucanases previously purified by Stieglitz did not appear to be detected with the acidic PR β(1,3)-glucanase antiserum used in the study by Lotan *et al.*

Two cDNA clones corresponding to the pistil PR acidic β(1,3)-glucanase were later isolated and sequencing revealed that the clones (termed sp41a and b) were 80% and 49% similar to the acidic and basic pathogen induced endo-β(1,3)-glucanases from the leaf (Ori *et al.*, 1990). The sp41 style-specific proteins, which constitute >12% of the transmitting tract tissue soluble protein, belong to a sub-family of the PR proteins. The sp41 isoforms

are unique in the fact that they are not induced by substances that induce other PR β(1,3)-glucanases. The function of this β(1,3)-glucanase in the style is not known, as a major endogenous substrate with β(1,3)-glucan linkages has not been defined in this tissue. However, as mentioned in section 1.3.1, β(1,3)-glucans have been detected in the cell walls and callose plugs of pollen tubes (Stone *et al.*, 1984; Meikle *et al.*, 1991a). It is possible that the stylar sp41 protein interacts with the β(1,3)-glucans of the pollen tube wall (Ori *et al.*, 1990).

Developmentally regulated PR β(1,3)-glucanases have also been detected in monocots (Simmons *et al.*, 1992; Høj *et al.*, 1989a). The appearance of β(1,3)-glucanase in germinating *H. vulgare* seeds, even though there is no endogenous substrate present, has prompted the idea that this β(1,3)-glucanase may protect the germinating seed from microbial attack. This β(1,3)-glucanase shares 52 % identity and thus an evolutionary origin with a *H. vulgare* β(1,3;1,4)-glucanase which is also expressed during germination (Høj *et al.*, 1989a). This β(1,3;1,4)-glucanase possibly functions to depolymerize the endosperm walls. β(1,3;1,4)-glucanases have not yet been identified in dicotyledonous species.

1.4.4 Structural Classification of PR Endo-β(1,3)-Glucanases

Amino acid sequences and/or cDNA clones have now been reported for a number of β(1,3)-glucanases from a variety of species. The greatest detail in sequence information is available for β(1,3)-glucanase isoforms from *N. tabacum*. The basic vacuolar β(1,3)-glucanase was the first to be represented by nucleotide sequence. Overlapping cDNA clones and sequenced tryptic peptides were reported by Shinshi *et al.* (1988) and the sequence was confirmed by more extensive protein sequencing by van den Bulcke *et al.* (1989). The primary structures of the acidic isoforms which are secreted into the ICF after TMV infection were established from comparison of partial amino acid sequence with the deduced sequence from cDNA clones (Payne *et al.*, 1990; Côté *et al.*, 1991; Linthorst *et al.*, 1990; Ward *et al.*, 1991). Two cDNAs have also been isolated for the β(1,3)-glucanase isoform expressed in the style (Ori *et al.*, 1990). Three additional cDNAs (GL153, GL161 and Ci30) which are likely to represent acidic β(1,3)-glucanases that are induced by TMV infection have also been identified (Linthorst *et al.*, 1990; Ward *et al.*, 1991).

Ward *et al.* (1991) arranged these *N. tabacum* β(1,3)-glucanases sequences into three structural classes based on their similarity. Class I includes the basic β(1,3)-glucanases that are located in the central vacuole. These isoforms diverge at <1 % of the positions (Shinshi *et al.* 1988). Class II includes the acidic isoforms (PR-2, N, O, Gl153, Gl161 and Ci30). This class differs from class I forms at a minimum of 48.4 % of the positions. The sole

representative of class III is PR-Q', which differs from both class I and class II by a minimum of ~43 %. There is a high level of overall similarity between the members of these three classes and this suggests that they are all related.

1.4.5 Processing of the PR Endo-β(1,3)-Glucanases

In *N. tabacum,* the acidic and basic β(1,3)-glucanases appear to be strictly compartmentalized. The former are secreted into the ICF and the latter sequestered into vacuoles. All of the known PR proteins are therefore synthesized as precursors with an N-terminal signal sequence. The N-terminal sequence is required for transfer to the ER and from here a default pathway results in secretion. Sorting of proteins from the ER to organelles such as the vacuole requires an additional signal (Chrispeels, 1991). The vacuolar isoforms of PR β(1,3)-glucanase are synthesized with both N- and C-terminal extensions (Shinshi *et al.*, 1988). Pulse chase experiments have shown that the first step in the processing of this protein is loss of the N-terminal signal peptide and the addition of an oligosaccharide side chain within the remainder of the polypeptide. The removal of the C-terminal extension is a late step and probably occurs in the vacuole (Sticher *et al.*, 1992). Treatment with tunicamycin (a glycosylation inhibitor) and endoglycosidase-H, a deglycosidase that removes sugar side chains, has shown that the enzyme has a single N-terminal glycan and this oligosaccharide is not required for targeting to the vacuole or for correct proteolytic processing (Sticher *et al.*, 1992). Voelker *et al.* (1989) suggested that C-terminal glycosylation may contribute to the stability of proteins. *Hordeum vulgare* lectin and wheat germ agglutinin are also vacuolar proteins that are synthesised as precursors with N- and C-terminal extensions (Bednarek *et al.*, 1990; Reikhel *et al.*, 1988). To test whether the C-terminal propeptide (CTPP) of the *H. vulgare* lectin contains sufficient information for vacuolar targeting, the coding sequence of the gene was fused to the C-terminus of a gene encoding a secreted protein, cucumber chitinase, and the resulting chimaeric gene introduced into *N. tabacum.* The fusion protein was redirected into the vacuole, confirming the role of the CTPP as a vacuolar sorting determinant (Bednarek and Raikhel, 1991). Van den Bulcke *et al.* (1989) therefore suggested that the basic β(1,3)-glucanase C-terminal extension may also function as a signal to direct the protein to the plant vacuole. Until recently there has only been partial amino acid sequence available for the acidic β(1,3)-glucanases (van den Bulcke *et al.*, 1989) but since the isolation of several cDNAs and genes (Linthorst *et al.*, 1990; Côté *et al.*, 1991; Ward *et al.*, 1991), sequence data has revealed that the secreted acidic β(1,3)-glucanase isoforms lack the C-terminal extension. This provides more evidence for the role of the basic β(1,3)-glucanase CTPP in vacuolar targeting.

1.4.6 Cell Wall Associated Exo-β(1,3)-Glucanases

Exo-β(1,3)-glucanase enzymes have been found in association with the cell walls from a variety of plant species including *Glycine max* (Cline and Albersheim, 1981), *Acacia verek* (Liénart *et al.*, 1986), *Daucus carota* (Kurosaki *et al.*, 1989) and *Zea mays* (Labrador and Nevins, 1989a). These hydrolases are strongly associated with the cell walls and in *D. carota*, the exo-β(1,3)-glucanases are maintained in the cell walls as high molecular weight complexes by acid-base interactions with polygalacturonide substances (Kurosaki *et al.*, 1992). The mechanism by which the other cell wall hydrolases are held in the wall has not yet been investigated.

The wall associated hydrolases generally have a molecular weight in excess of 60 kD (Cline and Albersheim, 1981; Liénart *et al.*, 1986; Kurosaki *et al.*, 1989) and the basic exo-β(1,3)-glucanases from *G. max* and *D. carota* appear to be glycosylated in their mature form (Cline and Albersheim, 1981; Kurosaki *et al.*, 1989). Deglycosylation experiments have shown that the oligosaccharide side chain accounts for 3 kD of the weight of the mature *D. carota* enzyme (Kurosaki *et al.*, 1991). As discussed previously, Shinshi *et al.* (1988) reported that the basic β(1,3)-glucanase from *N. tabacum* is initially synthesized as an N-linked glycoprotein, but this glycosylated intermediate is further processed with the loss of the oligosaccharide side chain. Different kinds of processing reactions thus take place during the synthesis of the two types of β(1,3)-glucanase.

The exo-β(1,3)-glucanases isolated to date appear to have both exo-β(1,3)-glucanase and β-glucosidase activities. In *D. carota* and *A. verek*, the levels of the two activities are comparable (Liénart *et al.*, 1986; Kurosaki *et al.*, 1991), but the enzyme isolated from *G. max* cell walls has a greater level of β-glucosidase activity (Cline and Albersheim, 1981). In contrast, the *Z. mays* enzyme displays a very low level of β-glucosidase activity (Labrador and Nevins, 1989a) but has the additional capacity to hydrolyze β(1,3;1,4) linkages of material isolated from the cereal cell walls. Although the *Z. mays* exo-β(1,3)-glucanase is unable to degrade β(1,4)-glucans, Labrador and Nevins (1989a) suggest that the capacity to hydrolyze the β(1,3;1,4) linkages must be due to a combination of exo-β(1,3)-glucanase and β-glucosidase activity.

The exact physiological role of these extracellular hydrolases has not yet been determined. It was reported that the exo-β(1,3)-glucanase isolated from *Z. mays* participates in the autolysis of cell walls (Huber and Nevins, 1979) and enhancement of the elongation of coleoptile sections (Labrador and Nevins, 1989a). Elongation was thought to be related to the degradation of β(1,3;1,4)-glucans, one of the major components of the cereal primary cell wall. Another possible function of the wall associated exo-β(1,3)-glucanase is in defence against pathogen attack. The expression of

exo-β(1,3)-glucanase in response to fungal infection has been investigated in *Triticum aestivum* (Sock *et al.*, 1990). Although four basic forms of the enzyme were found in ICF from infected leaves, the low level of exo-β(1,3)-glucanase activity did not increase in response to pathogen infection. These exo-β-(1,3)-glucanases therefore appear to be regulated in a different way to the PR endo-β-(1,3)-glucanases.

1.5 Callose and Male Sterility

Male sterility is a phenomenon which occurs under natural conditions and usually results in the total loss of male but not female gametes. In some mutants the failure of anther dehiscence can also result in male sterility even though the gametes are viable. The factors which cause the male sterility can be determined by the nuclear or cytoplasmic genome. Male sterile plants are used by plant breeders for the production of hybrid seed and are therefore of significant economical importance.

In certain male sterile lines of *Petunia* and *Sorghum*, the male sterility has been associated with mistiming of callase activity in the anthers. One *Petunia* cytoplasmic male sterile (cms) line has been identified which displays strong callase activity during early meiosis, before it is normally required (Frankel *et al.*, 1969). Similarly, in a cms line of *Sorghum*, premature callase activity was postulated as the cause of aberrant callose wall degradation (Warmke and Overman, 1972). Late appearance of callase activity has also been observed in both cms and genic male sterile (gms) lines of *Petunia*. It was therefore inferred by the above authors that any temporal deviation in callase activity can cause male sterility. Although these mutants provide good indirect evidence that aberrant callase activity is associated with male sterility they do not provide proof. As described in section 1.3.1, both callase activity and the pH of the anther locular fluid change during development. In the male sterile *Petunia* lines the pH drop remains tightly linked to callase activity whether the activity is precocious or delayed. These observations led Izhar and Frankel (1971) to assume that callase activity in the anther is pH dependent and that pH of the locular fluid must decrease before the callase enzymes become active.

The mistiming of callase activity is not the only factor which has been linked to the male sterility observed in *Petunia*. The accumulation of certain free amino acids in the anthers is different in fertile and sterile lines (Izhar and Frankel, 1973). Certain developmental patterns, normal or abnormal, were shown to be associated with a specific profile of amino acids. In particular, asparagine accumulation starting at prophase I was consistently associated with the breakdown of microsporogenesis. The changes in the balance of amino acids after a particular stage of microsporogenesis helped Izhar and Frankel (1973) to pinpoint when development was disrupted and may have indicated a breakdown in protein synthesis or degradation, or translocation of proteins in and out of the anther. Izhar and Frankel (1973) also suggested that the locular fluid pH change

observed during anther development could be attributable to fluctuations in amino acids.

In male sterile lines, alterations in tapetal morphology are often observed before the abortion of the microspores (Overman and Warmke, 1972; Laser and Lersten, 1972; Horner and Rogers, 1977; Warmke and Lee, 1977; Bino, 1985a,b). Frequently the first sign of tapetal degeneration is increased vacuolation and changes in the appearance of cytoplasmic organelles. Finally, the tapetal cells may enlarge and occasionally occlude the entire locule (Raj, 1969). The tapetum is the tissue in closest contact with the developing microspores and all nutritive materials must either pass through it or be metabolized by it before reaching the microspores (Echlin, 1971). The tapetum may also be important for enzymatic processes in the locule, including the release of callase. The tapetum is therefore thought to be intimately involved with the development of pollen in providing material for growth and for exine formation, and malfunctioning of the tapetum has often been regarded as the cause of microspore abortion. However, often the breakdown of microspore and tapetal function occur simultaneously, and it is therefore difficult to distinguish which is the primary factor.

The underlying cause of many of the cms phenotypes, including those of *Petunia* which display aberrant callase activity, is thought to reside in the mitrochondrial genome (Hanson and Conde, 1985). Regions of the mitrochondrial DNA that are associated with a cms trait have been identified by analysis of somatic hybrid lines that were generated by fusing protoplasts from a cms line with those from a fertile line. The resulting recombinant mitrochondrial genomes facilitated the isolation of a DNA region which segregated with male sterility in the somatic hybrids (Boeshore *et al.*, 1985). As a result of this work a novel mitrochondrial protein was discovered which was only present in the cms lines (Nivison and Hanson, 1989). This protein has a much lower abundance in fertile plants which carry the cms cytoplasm, but have a nuclear encoded fertility restorer gene. The nuclear gene must therefore modify expression of the novel mitrochondrial protein to maintain or restore fertility. In the male sterile plants it seems that the novel protein interferes with mitrochondrial energy production, specifically by interaction with the alternative oxidase pathway (Connet and Hanson, 1990). Presumably defects in the functioning of the mitrochondria result in the arrest of normal pollen development.

These studies suggest that there is a general breakdown of tapetum or microsporocyte cell function in the male sterile lines. It is difficult to dissect the complex observations made of the cms *Petunia* lines and determine what is the main cause of the male sterility. For example, what is the relative importance of the correct timing of callose wall breakdown in the context of all the other factors which must be taken into consideration? It was hoped that this complex situation could be simplified in transgenic plants by reproducing this one aspect of the *Petunia* RM cms line, the early appearance of callase activity, and thus determine the role of callose in microspore development. Such experiments will be described in Chapter 6.

1.6 Gene Expression During Anther Development

1.6.1 The Complexity of Gene Expression During Microsporogenesis and Microgametogenesis

As the processes of microsporogenesis and microgametogenesis are unique during the development of the plant, large numbers of genes must be specifically expressed at these stages. Using the technique of solution hybridization, Kamalay and Goldberg (1980) determined that during anther development in *N. tabacum* about 26,000 different transcripts are present and 11,000 of these are anther specific. A proportion of these transcripts are specific to the male gametophyte. Willing and Mascarenhas (1984) and Willing *et al.* (1988) have shown that 22,000-24,000 different mRNAs are expressed in mature pollen of *Tradescantia paludosa* and *Z. mays*, of which a maximum of 7,200 (30-40%) might be pollen specific. Mascarenhas (1989) estimated that this figure may actually be as low as 10%. Willing and Mascarenhas (1984) and Willing *et al.* (1988) also examined the levels of expression of genes in pollen compared to shoots. The mRNAs of mature *Tradescantia* pollen fall into three abundance classes and 75% of the transcripts occur in the two higher frequency classes. If this is compared to the situation in shoots where only 35 % of transcripts are abundant, it appears that pollen expressed genes produce much more prevalent mRNAs than the shoot genes. Many of the transcripts present in mature pollen represent genes that are activated in late pollen development. The accumulation of many of the RNA species may reflect a requirement for the rapid synthesis of proteins during pollen maturation and/or germination. At present there are no reports on the numbers of pollen expressed genes during the early stages of pollen development or on their similarity to the genes expressed later in pollen development. However several genes have been cloned which show expression after meiosis in the microspore development (uninucleate) stages (see section 1.6.2).

Specific sets of genes are therefore transcribed during male gametophyte development. About 80-90% of pollen sequences are shared with the sporophyte (Mascarenhas, 1989). Of the 10-20 % remaining sequences that are pollen specific, the expectation is that some will be unique because the pollen grain possesses a specialized structure not found anywhere else in the plant. However, in several examples, the pollen specific transcripts are the result of expression of genes encoding different isoforms of proteins present in the vegetative tissue. For example oleosins, proteins that form part of the membrane surrounding oil bodies, have been found in both seeds and pollen. The oleosin expressed in pollen is significantly different to the seed oleosin and is part of a different gene family to the genes expressed during embryogenesis (Roberts *et al.*, 1993a). β-Glucosidase in *Z. mays* is also an example of an enzyme that is encoded by different genes in pollen and sporophytic tissue (Frova *et al.*, 1987). This situation can make the

screening of libraries for anther/pollen specific genes more complex, as the sequence encoding these shared proteins might be similar and thus ignored in differential screening.

1.6.2 The Identification of Genes Involved with Anther Development

Several laboratories have constructed cDNA libraries to poly(A) RNA from anthers and differential screening of these libraries has produced two classes of cDNA. One class consists of cDNAs derived from anther-specific transcripts and the other class represents transcripts that are expressed at a high level in the anther and also at a much lower level in the vegetative tissue. The analysis of individual messages with regard to both temporal and spatial expression during anther development has allowed some generalizations to be made. During early gametogenesis, the large majority of mRNAs so far examined are expressed in the tapetum (Koltunow *et al.*, 1990; Smith *et al.*, 1990; Scott *et al.*, 1991a). The tapetum appears to dominate in the levels of transcript at these stages of development, and this seems to result in the high abundance of tapetum-specific clones in the libraries, and low levels of sporocyte messages. Another explanation for the rarity of sporocyte-specific transcripts in the whole anther libraries is that during the period of meiosis there is a general reduction in the amount of RNA, both messenger and ribosomal, found in the sporocytes (Porter *et al.*, 1983).

The profile of tapetum-specific cDNAs changes during the development of the tissue. The *B. napus* clones A3, A8 and A9 are the earliest expressed anther specific messages described to date (Scott *et al.*, 1991a). The mRNAs cognate to these cDNAs appear during the meiocyte stage soon after the tapetum has differentiated and the transcripts probably persist until just before tapetal degeneration. Other transcripts (for example 92b from *L. esculentum* and TA29 from *N. tabacum*) are present during the middle phase of tapetal development and persist until the tapetum degenerates (Smith *et al.*, 1990; Koltunow *et al.*, 1990). It is not clear whether these genes are down regulated at this stage or whether expression simply ceases as a consequence of tapetal degeneration.

The construction of pollen and anther libraries has yielded a variety of pollen-expressed and pollen-specific clones. These clones fall into two classes. The basis of this classification has been formed on the expression patterns of the individual mRNAs (see Mascarenhas, 1990), and on the profiles of extracted proteins and *in vitro* translated proteins from staged microspores and pollen of *T. aesticum* (Vergne and Dumas, 1988), *Z. mays* (Bedinger and Egerton, 1990) *Lilium* and *N. tabacum* (Schrauwen *et al.*, 1990). The early or class I transcripts are the least represented, but Bp4 of *B. napus* (Albani *et al.*, 1990) and apg from *Arabidopsis* (Roberts *et al.*, 1993b) are examples. The best characterized of the early expressed genes is *apg*. Promoter activity of *apg* is first detectable in uninucleate pollen and expression can still be detected at late binucleate stage. Down regulation of promoter activity occurs during maturation of the pollen grains (Twell *et al.*, 1993). The later, class II transcripts are expressed following microspore mitosis and

throughout pollen maturation. The *lat52* gene from *L. esculentum* and the gene represented by the Zmg13 cDNA from *Z. mays* share significant sequence similarity and are both expressed in the mature and germinating pollen grain (Twell *et al.*, 1989; Hanson *et al.*, 1989). The two classes of genes probably have very different roles. The class I genes are expressed during microspore development and the gene products could therefore participate in the construction of the pollen grain in processes such as pollen wall deposition. The class II genes are present at maximum levels at pollen maturity and anthesis. For some of these genes the transcript levels decrease markedly at the onset of pollen germination (Stinson *et al.*, 1987) and these are therefore thought to represent stored mRNAs which accumulate during pollen maturation for translation at pollen germination (see Mascarenhas, 1990). *In vivo* labelling of RNA formed during pollen germination has shown that transcription also occurs during pollen tube growth (Weterings, 1994)

1.6.3 The Function of Anther-Specific Gene Products

Newly isolated cDNAs are routinely sequenced and analyzed with various computer software. Comparison of the DNA or deduced amino acid sequence with DNA and protein sequence databases frequently reveals a functional identity for the gene product based on similarity to previously cloned sequences. Computer programmes can also yield information on the physical characteristics of the gene product such as molecular weight, isoelectric point, hydrophobicity and the presence of putative glycosylation sites. In this way all of the tapetum-specific transcripts have been found to contain putative signal sequences at the N-terminus of the sequence, for example TA29 and TA13 (Koltunow *et al.*, 1990) and A9 (Paul *et al.*, 1992). This suggests that these proteins are likely to be secreted from the tapetum, probably into the locule and thus supports the secretory role for the tapetum.

At the present time, the function of any of the cloned microspore-specific class I genes is unknown, with the exception of the *Bp19* gene of *B. napus*, which is speculated to have pectin esterase activity (Albani *et al.*, 1990). Of the later expressed anther- and pollen-specific genes, two *L. esculentum* clones, *lat56* and *lat59*, encode proteins with similarities to pectate lyases of *Erwinia* (Wing *et al.*, 1989) and are expressed in the pollen tube. These enzymes may function in the mobilization of reserves for pollen tube cell wall growth, or to aid in penetration of the stylar transmitting tissue. A similar function has been proposed for the *P2* gene product from *Oenothera organensis*, which is also present in germinating pollen tubes, and has a high degree of identity (54%) with *L. esculentum* polygalacturonase (Brown and Crouch, 1990). The *N. tabacum* gene TA56 (Koltunow *et al.*, 1990) appears to encode a thiol peptidase, and has been shown by *in situ* hybridization to be localized in the anther connective and stomium, maximally at the time of anthesis. This gene is thus implicated in the degradation of anther wall tissues leading to dehiscence.

1.7 An Overview of Thesis Aims

The overall aim of the project was to examine the significance of the tetrad wall in pollen production. Part of this investigation thus involved analysis of its degradation at the time of microspore release. Cloning a gene for an anther-specific β(1,3)-glucanase, an enzyme that appears to have a central role in this process was therefore desirable. *B. napus* and *N. tabacum* were analyzed with respect to timing of β(1,3)-glucanase activity in the bud or anthers. Having established that β(1,3)-glucanase activity peaked at the time during anther development when microspore release occurs, it was then feasible that the gene encoding this enzyme could be cloned from these species. It was initially thought that cloning could be aided by the use of probes prepared to the PR β(1,3)-glucanases. The first section of this thesis describes the use of such probes for this application. The second section looks at the isolation and characterization of an anther-specific cDNA called A6, which has significant sequence similarity to previously cloned PR β(1,3)-glucanases. Following on from this, an antibody was raised to the over-expressed A6 protein to try to characterize the peptide further. The relationship between A6 and anther β(1,3)-glucanases is discussed.

The second complementary part of the project was to investigate the role of callose in microspore development. To approach this, a modified PR β(1,3)-glucanase was expressed prematurely, before the appearance of the native callase, in the anther tapetum of transgenic plants. This experiment thus mimicked one aspect of the RM cms *Petunia* mutants described by Frankel *et al.* (1969). The information derived from these transgenic plants in relation to pollen wall formation and other aspects of microspore development is discussed.

Chapter 2

Materials and Methods

All solutions containing ingredients quoted as percentages are weight to volume or volume to volume ratios.

2.1 Sources of Molecular Biology Reagents, Enzymes and Plant Tissue Culture Chemicals

Chemicals and reagents were purchased from Sigma Chemical Company Ltd., or from BDH Ltd. Enzymes were obtained from BRL, Boehringer Mannheim, Stratagene, Perkin Elmer, Pharmacia or NBL. Tissue culture salts and hormones were purchased from Flow Laboratories. Agar and agar based media were from Difco Laboratories.

2.2 Plant Material

Material for nucleic acid isolation and protein extraction was obtained from *N.tabacum* SR1 plants grown under normal greenhouse conditions. To induce a wound response, *N.tabacum* plants were sprayed on two consecutive days with 5 mM salicylic acid (from a 0.25 M stock solution adjusted to pH 7.0 with potassium hydroxide). One day after the final spraying leaves were harvested, washed thoroughly with tap water, dried, frozen in liquid nitrogen and stored at -80 °C prior to nucleic acid extraction or used directly for protein extraction.

Material for protein extraction from *B. napus* Topaz was obtained either from field or greenhouse grown spring rape. To induce a wound response, *B. napus* plants were sprayed on two consecutive days with 5 mM salicylic acid (from a 0.25 M stock solution adjusted to pH 7.0 with potassium hydroxide) containing 0.05 % Tween 20 as a wetting agent. One day after the final spraying, leaves were harvested, washed thoroughly with tap water and used directly for protein extraction.

2.3 Protein Extraction and Analysis

2.3.1 Small Scale Crude Enzyme Extractions

Extraction Buffer:	50 mM	potassium phosphate buffer, pH 6.8
	1 mM	PMSF

Extracts were prepared from *B. napus* buds by grinding the buds in extraction buffer in an Eppendorf tube with a microhomogeniser. The suspension was then microcentrifuged for 2 minutes. The supernatant could then be used as crude extract or purified for use in test tube assays for the detection of β(1,3)-glucanase activity. Purification involved treating the supernatant with ammonium sulphate to 60 % saturation; the resulting precipitate collected

by centrifugation in a minifuge and resuspended in 50 mM potassium phosphate buffer pH 6.8, was dialysed against the same buffer overnight to remove contaminating carbohydrates. All steps were carried out at 4 °C.

2.3.2 Micro Protein Extraction

Extraction Buffer:	0.1 M	Tris-HCl, pH 8.0
	5 mM	EDTA,
	5 mM	DTT
	4 mM	PMSF.
3x SDS Sample Buffer:	200 mM	Tris-HCl, pH 6.8,
	6 %	SDS,
	30 %	Glycerol,
	15 %	β-mercaptoethanol,
	0.05 %	Bromophenol blue.

Protein was extracted from eight *N. tabacum* anthers dissected from buds at the appropriate developmental stage. Anthers were frozen with dry ice and stored at -80 °C prior to protein extraction. Frozen anther material was ground in an Eppendorf tube with a microhomogenizer and 90 µl of cold extraction buffer added. The samples were mixed, thawed, and then microcentrifuged for 3 minutes to remove the debris. The supernatant was combined with SDS sample buffer and boiled for 3 minutes prior to storage at -20 °C or separation of proteins by SDS-polyacrylamide gel electrophoresis (see section 2.3.8).

2.3.3 Large Scale Protein Preparations

Extraction Buffer:	0.1 M	sodium citrate, pH 8.0
	10 mM	sodium ascorbate
	1 mM	PMSF
	10 mM	β-mercaptoethanol

Total cell protein was extracted from plant material by grinding the tissue to a powder with a mortar and pestle cooled with liquid nitrogen. 4 ml of extraction buffer were added per gramme of tissue and ground with the frozen tissue. After thawing, the cell debris was pelleted from the homogenate by centrifugation at 15,000 x*g* for 20 minutes. The supernatant was treated with solid ammonium sulphate to 95% saturation, the resulting precipitate collected by centrifugation at 15,000 x*g* for 20 minutes and finally resuspended in 10 mM Tris-HCl, pH 7.5.

2.3.4 Extraction of Intercellular Fluid

Extraction Buffer:	5 mM	EDTA
	10 mM	sodium ascorbate
	1 mM	PMSF
	10 mM	β-mercaptoethanol

Intercellular fluid (ICF) proteins were extracted according to the method of Mauch and Staehelin (1989). Leaf material was washed and then cut into 2 cm strips and placed in a Petri dish. 100 ml of ice cold buffer was poured over the leaf strips to submerge them. The leaves were infiltrated in a vacuum desiccator for 10 minutes (a longer time period was sometimes required for thicker *B. napus* leaves). The leaf strips were then blotted dry, rolled up and put into the barrel of a 20 ml syringe. The syringes were put into tubes and centrifuged at 1,000 x*g* 10 minutes. Intercellular fluid was treated with solid ammonium sulphate to 95 % saturation. The precipitated proteins were collected by centrifugation at 15,000 x*g* for 20 minutes and resuspended in a small volume of 10 mM Tris-HCl, pH 7.5.

2.3.5 Approximate Protein Quantification via Coomassie Staining

Coomassie Stain:	0.1 %	Coomassie blue dye
	25 %	methanol
	10 %	acetic acid
Destain:	25 %	methanol
	10 %	acetic acid

Total protein concentration was determined via a Coomassie blue dot binding assay. 5 µl of sample was dotted at neat, 1:1 and 1:9 dilutions onto Whatmann 3 MM filter paper. A range of BSA standards at 1-10 mg/ml were dotted next to the samples. When dry the filter was immersed in Coommassie stain for 10-15 minutes on a slow shaking platform. The filter was destained for 10 minutes in destain. Protein concentrations were estimated by comparison to the BSA standards.

2.3.6 Protein Quantification via Bradford's Assay

Bradford's Solution:	600 mg/l serva blue G-250 stain in 2 % perchloric acid and filtered.

Assays were carried out in a microtitre plate using a method based on that of Bradford

(1976). Dilutions of BSA in the range 0-1.0 mg/ml were used as standards to calculate protein concentrations. 10 µl of protein sample was put into each well of the microtitre plate and then 100 µl distilled water and 100 µl Bradford's solution added. Protein concentrations were measured directly using a custom-written programme on a Dynatech MR 5000 microtitre plate reader.

2.3.7 β(1,3)-Glucanase Assays in Aqueous Solution

2.3.7.1 Standard Assay Procedure

Crude enzyme extracts were purified as described in section 2.3.1 and then an aliquot (typically 5 µl) of extract was incubated with 50 µl of 50 mM sodium acetate, pH 5 containing 1.5 mg/ml laminarin in a total volume of 100 µl for 45 minutes at 37 °C.

2.3.7.2 Detection of Total β(1,3)-Glucanase activity

Solution A: made by dissolving 40 g anhydrous sodium carbonate in 600 ml water then adding 16 g glycine and 0.45 g copper sulphate and making up to 1 litre.

Solution B: 0.12 g neocuprione. HCl dissolved in 100 ml water.

Total β(1,3)-glucanase activity was determined using a method based on that of Dygert *et al.* (1965). 200 µl of solution A followed by 200 µl solution B was added to the sample to be tested and then this was boiled for 10 minutes before rapidly cooling by putting the tubes under running tap water. Formation of a precipitate during heating indicated that insufficient amounts of reagents had been used. The volume was finally made up to 1 ml before the optical density (OD_{450}) of the sample measured on the Dynatech microtitre plate reader. Dilutions of glucose in the range 0-125 µg/ml were used as standards to calculate the amount of free reducing groups produced by β(1,3)-glucanase activity in the samples. The volumes of solution A and B were adjusted according to the table below.

Reagent volumes for various amounts of reducing sugar.

Glucose (µg)	Solution A (µl)	Solution B (µl)
5-25	100	100
26-50	200	200
51-75	300	300
76-100	400	400
101-125	500	500

The final values for β(1,3)-glucanase activity were expressed as μg glucose/hour/mg protein.

2.3.7.3 Detection of Exo-β(1,3)-Glucanase Activity

Assay Mixture:	200 μl of a 10 mg/ml 3,3',5,5'-tetramethyl benzidine (TMB) stock solution in 20 ml of 50 mM sodium acetate, pH 5.
Peroxidase/glucose oxidase enzymes:	PGO Powder capsules (Sigma) dissolved in water at a concentration of 100 mg/ml

Exo-β(1,3)-glucanase activity was determined by adding to the test solution 10 μl peroxide/glucose oxidase enzymes followed by 200 μl assay mixture. The reaction was stopped after 5-10 minutes by the addition of 40 μl 2.5 M sulphuric acid. The absorbance of the samples at 450 nm was measured in the Dynatech microtitre plate reader. Dilutions of glucose in the range 0-10 μg/ml were used as standards to calculate the amount of glucose produced by exo-β(1,3)-glucanase activity in the samples. The final values for β(1,3)-glucanase activity were expressed as μg glucose/hour/mg protein.

2.3.8 Separation of Proteins by Polyacrylamide Gel Electrophoresis

SDS Running Buffer:	25 mM	Trisma base
	192 mM	glycine
	0.1 %	SDS
Native Loading Buffer:	50 %	glycerol containing 0.05 % bromophenol blue

Proteins were separated on a variety of gel matrices. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) separates proteins according to molecular weight. SDS sample buffer (section 2.2.2) was combined with an aliquot of protein and the solution boiled for 5 minutes. The sample could then be stored at -20 °C or separated on a gel. Frozen samples required boiling for 1 minute prior to loading. Gel mixture was made up according to the following recipe. 15 ml of gel mix was sufficient to cast two gels in the Biorad Protean II protein system.

% Gel	Volume of Stock Solution (ml)			
	30 % Acrylamide/ 0.8 % Bisacrylamide	Water	1.5 M Tris-HCl pH 8.8	10 % SDS
10	5	6.1	3.75	0.15
11	5.51	5.59	3.75	0.15
12.5	6.25	4.85	3.75	0.15

100 µl ammonium sulphate (100 mg/ml) and 10 µl TEMED were added to polymerize the gel.

The percentage of acrylamide in the gel could be varied depending upon the expected size and /or the required resolution of the particular protein of interest. Once the resolving gel had been poured into the apparatus, propanol:water (1:1) was used as an overlay to exclude oxygen and help the gel to set. Once set, the propanol:water was rinsed off the top of the gel and the surface dried. The stacking gel, made according to the recipe below, was added and the well forming comb pushed into place.

Volume of Stock Solution (ml)			
30 % Acrylamide/ 0.8 % Bisacrylamide	Water	0.5 M Tris-HCl	10 % SDS pH 6.8
1.67	6.9	1.25	0.1

Denaturing polyacrylamide gels were run in SDS running buffer at 150 V until the dye front had reached the bottom of the gel. Molecular weight markers (Biorad, SDS-7) were included on the gel to determine the sizes of proteins of interest.

Proteins were stained by submerging the gel in Coomassie stain (section 2.2.5) for approximately 30 minutes on a rocking platform and then destaining for 1 hour. Alternatively, the proteins could be blotted from the gel onto an immobilizing membrane for immunoblotting experiments.

Non-denaturing polyacrylamide gels were made using the same recipe as above excluding the SDS. Native protein samples were combined with native loading buffer before separation on the gel.

2.3.9 Separation of Proteins by Isoelectric Focussing

Loading Buffer:	60 %	glycerol containing 4 % ampholytes (Sigma)
Anode Buffer:	20 mM	acetic acid
Cathode Buffer:	25 mM	sodium hydroxide

Native proteins can be separated according to their charge on isoelectric focussing gels. Prior to loading on the gel the protein samples were combined with an equal volume of loading buffer. The gel mixture was made up according to the following recipe based on the method of Robertson *et al.* (1987). This volume of gel mix was sufficient to cast two 5 % gels in the Biorad Protean II protein system.

Stock Solution	Volume (ml)
Water	14.0
30 % Acrylamide/ 1 % Bisacrylamide	4.0
50 % Glycerol	4.8
Ampholytes (Sigma)	1.2

This mixture was degassed for 5 minutes and then polymerised with 100 µl ammonium persulphate (100 mg/ml) and 40 µl TEMED. Once poured into the apparatus the well forming comb was pushed into place and the gel allowed to set for at least 1 hour. The anode and cathode buffers were cooled to 4 °C before use. Gels were run at 4 °C at 200 V for 90 minutes and 400 V for 90 minutes. Coloured isoelectric point markers (Biorad, range pI 4.7-10.6) were included on the gels.

Reverse IEF gels could also be run to allow proteins with a pI >10 to move into the gel. This was achieved by reversing the buffers and running the gel from anode to cathode.

Following separation on IEF gels, proteins could be assayed for β(1,3)-glucanase activity (see section 2.3.13) or transferred to an immobilizing membrane for immunolocalization experiments (see section 2.3.11).

2.3.10 Electroblotting Proteins After Separation by SDS-PAGE

Anode Buffer 1:	0.3 M	Trisma base
	10 %	methanol, pH 10.4
Anode Buffer 2:	25 mM	Trisma base
	10 %	methanol, pH 10.4

Cathode Buffer:	25 mM	Trisma base
	40 mM	β-aminohexanoic acid
	20 %	methanol, pH 9.4
Ponceau Stain:	0.6 %	Ponceau S
	1 %	acetic acid
TBS:	50 mM	Tris-HCl, pH 7.4
	200 mM	sodium chloride
TBS-Tween:	TBS containing 0.1 % Tween 20	

Proteins separated by SDS-PAGE were electroblotted onto a polyvinylidene fluoride (PVDF) membrane (Immobilon, Millipore) using a Milliblot SDE system (Millipore). A sandwich of Whatmann 3 MM filter paper gel and PVDF membrane was set up on the anode. Six pieces of filter paper were cut to the same size as the gel and two pieces were soaked in anode buffer 1 and placed on top of the anode, one filter soaked in anode buffer 2 placed on top followed by the pre-wet PVDF membrane and then the gel and finally three pieces of filter paper soaked in cathode buffer. Transfer was accomplished after 45 minutes at 250 mA (the current was increased if more than one gel was to be blotted at the same time). When using the Milliblot SDE system for electroblotting native proteins the methanol was omitted from the transfer buffers. The methanol is only required to acheive maximum binding of SDS proteins to the membrane

Correct transfer of proteins to the PVDF membrane could be determined by staining with Ponceau stain. The stain was poured on to the membrane and left for a few seconds. The stain was then rinsed off with water to reveal the bands of protein, the molecular weight markers were marked with a pencil and the stain was washed off with TBS-Tween.

2.3.11 Capillary Blotting Proteins After Separation by Native IEF

Transfer Buffer:	50 mM	Tris-HCl, pH 9.5

IEF gels were prepared for blotting onto PVDF membrane by equilibrating in the transfer buffer for 10 minutes. The transfer was carried out by capillary blotting as follows. The membrane was placed on top of Whatmann 3 MM filter paper on a sponge standing in a tray of transfer buffer. The gel was carefully placed on the membrane ensuring that no air bubbles were trapped underneath. The position of the isoelectic point markers were marked with a pencil. Another membrane was placed on the top of the gel and the position of the standards marked again. The remaining surface of the filter paper and sponge was covered with plastic cling film. Two pieces of filter paper soaked in transfer buffer were placed on

top of the membrane, and two dry pieces of filter paper on top of these. A stack of paper towels and a weight was laid over the gel. Transfer of buffer from the tray through the gel to the towels was left to occur over night. After transfer of proteins to the to the PVDF membrane, immunodetection experiments could be carried out as described in section 2.3.12.

2.3.12 Immunostaining

Blocking Solution: TBS-Tween containing 4 % Marvel milk powder.

Developing Solution: 0.5 mg/ml BCIP and 0.3 mg/ml NBT in 0.1 M Tris-HCl, pH 9.5 containing 1 mM $MgCl_2$

After blotting proteins onto the immobilizing membrane the membrane was put into blocking solution for 30 minutes. After blocking, the blots were incubated for 1-2 hour with antiserum (1/1000 dilution, unless otherwise stated) diluted in TBS-Tween containing 1 % Marvel milk powder. Blots were washed three times in TBS-Tween for 10 minutes, and then incubated in secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG, whole molecule, Dako) in TBS-Tween containing 1% Marvel milk powder (1/1000 dilution). After washing in TBS-Tween as before, the immunoreactive proteins were visualized by immersing the blot in 5 ml of developing solution. The reaction was stopped by washing thoroughly with distilled water. All treatments were carried out at room temperature.

2.3.13 Detection of β(1,3)-Glucanase Activity after Separation of Proteins in Polyacrylamide Gels

2.3.13.1 Direct Detection of Total β(1,3)-Glucanase Activity

Developing Solution: 200 ml 1 M sodium hydroxide containing 0.15 g 2,3,5-triphenyltetrazolium chloride

Proteins separated by native-PAGE or native-IEF could be asssayed directly for β(1,3)-glucanase activity with a tetrazolium agent essentially as described by Pan *et al.*, (1989). After electrophoresis the gel was rinsed with distilled water, equilibrated with 50 mM potassium acetate, pH 5.0 for 5 minutes with slow shaking, and then incubated for 40-60 minutes slowly shaking at room temperature in 70 ml of 50 mM potassium acetate containing 0.2 g laminarin. Control gels were incubated in 50 mM potassium acetate only, to detect proteins that did not have β(1,3)-glucanase activity but stained with this

procedure. Following incubation the gels were rinsed in distilled water, placed in a glass vessel and 200 ml preheated developing solution (this should be heated until the solution changed to a red colour) poured over. The gel was then heated in this solution in a microwave until red bands of activity appeared. Gels could be stored in 7.5 % acetic acid for several days; this solution also reduced the pink background slightly.

2.3.13.2 Detection of Exo-β(1,3)-Glucanase Activity

Exo-β(1,3)-glucanase activity was detected with an agarose overlay gel using a method modified from Sock *et al.* (1990). A solution containing 2 mg/ml laminarin and 2 % low gelling temperature agarose in 1M sodium acetate, pH 5.0 was heated then allowed to cool to 50 °C. 1 ml of 1M sodium acetate, pH 5.0 containing 100 mg/ml peroxide/glucose oxidase enzymes (see section 2.3.7.3) was added to the gel mixture and overlay gels 1.5 mm thick poured between prewarmed glass plates. After setting, the gel was incubated in a solution of 40 mg 4-chloro-1-napthol (dissolved in 5 ml DMSO) in 100 ml 1 M sodium acetate, pH 5.0. The separating gels were incubated in contact with the overlay gels overnight at 37 °C in the dark. Zones of enzyme activity appeared as dark blue bands on a clear background.

2.4 Bacterial Culture and Storage

2.4.1 Media for the Growth of Bacteria

NB and NA were made according to the manufacturers instructions and then bottled and autoclaved.

NZY medium: per litre

sodium chloride,	5 g
magnesium sulphate ($MgSO_4.H_2O$),	2 g
Bacto-yeast extract,	5 g
caesine hydrolysate,	10 g

Made to pH 7.5 with sodium hydroxide, bottled and autoclaved.

NZY medium was solidified by the addition of 1.5 % agar (technical number 3). The agar was dissolved by steaming the medium before autoclaving.

Top agar: the above NZY medium was solidified by the addition of 0.7 % agarose.

2xYT medium: per litre

Bacto-tryptone,	16 g
Bacto-yeast extract,	10 g
sodium chloride,	5 g

Made to pH 7.0 with sodium hydroxide, bottled and autoclaved.

2.4.2 Strains and Genotypes

2.4.2.1 *Eschericia coli* (*E. coli*):

XL1-Blue: *recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, lac, {F', proAB, lacI^q, ZDM15, Tn10, (tet^R)}*.
BB4: *e14$^-$(mcrA), hsdR514, supE44, supF58, lacY1 or D(lacIZY)6, galK2, galT22, metB1, trpR55 D(argF-lac) U169 {F', proAB, lacI^q, ZDM15, Tn10, (tet^R)}*.
HB101: *hsdR, hsdM, supE44, ara14, galK2, lacY1, proA2, rspL20, xyl-5, mtl-1, recA13, mcrB*. (pRK2013).
K38 (Tabor and Richardson, 1985).

2.4.2.2 *Agrobacterium tumefaciens* (*A. tumefaciens*):

C58C1 rif (pGV2260).

2.4.3 Antibiotics for Bacterial Selection

Name	Concentration (µg/ml) for:-	
	E. coli	*A. tumefaciens*
Ampicillin	100	-
Kanamycin	100	50
Rifampicin	100	50
Tetracyclin	12.5	2.5

2.4.4 Growth of Cultures

Using a sterile micropipette tip a single colony from a bacterial plate was transferred to 5 ml of NB or NZY medium containing the appropriate concentration of selective antibiotics (table in section 2.4.3) in a sterile universal. The culture was placed on an orbital shaker at 37 °C (*E. coli*) or 28 °C (*A. tumefaciens*) overnight or until the culture reached the required optical density.

Single colonies were obtained by dipping a flame sterilized and cooled loop into an overnight culture and streaking across the surface of solid medium which contained selective antibiotics. The medium was allowed to cool to 50 °C before the antibiotics were added and the plates poured. Plates were incubated upside down, at the appropriate temperature until colonies formed. Single colonies were also produced by spreading 100 µl of cell suspension over the surface of the selective medium with a flame sterilized and cooled glass spreader. Cultures could be kept on a plate sealed with Nescofilm for about a month if kept at 4 °C.

2.4.5 Long Term Storage of Cultures

750 µl of an overnight culture was mixed with 750 µl of 40 % glycerol in NB medium in a Cryogenic storage tube. After labelling the solution was flash frozen in liquid nitrogen and stored at -80 °C. To revive cells stored in this way, a portion of the frozen mixture was scraped off the surface using a flame sterilized scalpel blade and resuspended in 5 ml of medium and grown as described in section 2.4.4.

2.4.6 Congugation of Plasmid from *E. coli* to *A.tumefaciens*

pBin19 (Bevan, 1984) is a binary vector based on the wide host range replicon RK2 of pRK252. It can therefore replicate in both *E. coli* and *A. tumefaciens* which means that foreign DNA can be inserted into the vector and then screened in *E. coli* prior to transfer to *Agrobacterium.*

A helper plasmid pRK2013 (in *E. coli* strain HB101) was required to provide mobilization functions (Ditta *et al.*, 1980), to transfer pBin19 into the *A. tumefaciens* strain C58C1::pGV2260 (Debleare *et al.*, 1985). The transfer into *A. tumefaciens* therefore requires triparental mating which is outlined below.

A single colony of *E. coli* containing the correct pBin19 construct and the strain containing the helper plamid (HB101::pRK2013) was grown up in an overnight culture at 37 °C. A culture of the recipient *A. tumefaciens* (C58C1::pGV2260) was also set up to grow at 28 °C. 100 µl of each of these strains was pipetted onto a NA plate and spread with a glass spreader. Once dry, the culture was incubated overnight at 28 °C. A sterile loop was drawn across the culture and then streaked out on an NA plate containing ampicillin (50 µg/ml), kanamycin (50 µg/ml) and rifampicin (50 µg/ml). Transconjugent *A. tumefaciens* colonies were visible after two days incubation at 28 °C. After transconjugants had been selected the vector sequences were checked again to ensure that rearrangements or deletions had not occured during conjugation.

2.4.7 Small Scale Isolation of *A. tumefaciens* Total Nucleic Acids

TE:	10 mM Tris-HCl, pH 7.5 1mM EDTA, pH 7.5
Sakosyl:	5% Sarkosyl (Sigma, N-lauroylsarcosine, sodium salt) made up in TE.
Pronase:	5 mg/ml in TE
Phenol:chloroform:	Mixed in a ratio 1:1 phenol to chloroform containing 4 % isoamyl alcohol

Binary vectors are large and present at low copy numbers so it was necessary to carry out a total nucleic acid isolation from *A. tumefaciens* followed by Southern analysis to identify specific sequences. The method followed was essentially the same as that described by Draper *et al.*(1988).

A 5 ml culture of the *A. tumefaciens* strain was grown overnight from a single colony. Cells were pelleted from 1.5 ml of culture by microcentrifugation for 5 minutes, and resuspended in 300 µl of TE, to which was then added 100 µl of Sarkosyl. After mixing, 150 µl of pronase was added and the mixture incubated for 1 hour at 37 °C. The pronase treated cell suspension was then mixed with 500 µl of phenol/chloroform, by repeatedly passing through a 1 ml pipette tip. After centrifugation in a minifuge at 15,000 x*g* , the aqueous solution was submitted to this treatment a further 3 times. DNA was precipitated from the remaining supernatant by the addition of 1/20 th volume of 5 M NaCl and 3 volumes of ethanol. The nucleic acid was collected by microcentrifugation for 10 minutes, rinsed in 70% ethanol, dried, and dissolved in 50 µl of sterile distilled water.

2.5 Plant Tissue Culture

2.5.1 Media for Plant Cell and Tissue Culture

MSO: Based on MS salts (Murashige and Skoog, 1962). Preweighed packets marketed by Flow Lab contained all the ingredients for this medium with the exception of sucrose. This was added at a concentration of 30 g/l. The pH was adjusted to 5.8 and the medium bottled and autoclaved.

MSD4x2: MSO salts with the following additions, NAA (0.1 mg/l), 6-BAP (1 mg/l), sucrose (30 mg/l). The pH was adjusted to 5.8 and the medium bottled and autoclaved.

The above media based on MSO salts was solidified by the addition of 0.8 % agar which was dissolved by steaming the medium before autoclaving.

2.5.2 *N. tabacum* Transformation

Young *N. tabacum* (SR1) leaves were placed in a sterile Pyrex casserole dish and covered in 10 % bleach (Domestos). After 10-15 minutes the leaves were thoroughly rinsed with 4x 400 ml of sterile tap water. Working on a sterile white tile in a lamina flow cabinet the leaves were cut into 1 cm squares, avoiding the major veins, using a sterile scalpel blade and flame sterilized forceps. The leaf squares were put into a sterile Petri dish containg a solution of *A. tumefaciens* diluted 1/20 with liquid MSD4x2 medium for 20 minutes. After draining on the lid of the sterile Petri dish the squares were transferred, lower leaf surface against the agar, onto MSD4x2 medium. The plates were sealed with Nescofilm and incubated for 2 days under a low light intensity (2000 Lux, 16 hour photoperiod) at 28 °C. After this incubation period, when colonies of *A. tumefaciens* were visible, the leaf discs were transferred to MSD4x2 medium containing kanamycin (100 µg/ml) and augmentin (400 µg/ml), to select for transformed plant cells and inhibit the growth of *A. tumefaciens* . The plates were sealed with Nescofilm and returned to the culture room. After approximately 16 days, kanamycin resistant shoots and callus formed along the cut edges of the leaf disc explants. When the shoots had developed 2-3 well formed leaves, they were cut away from the callus with flame sterilized and cooled scalpel blade and forceps and the stem pushed into MSO medium with kanamycin (100 µg/ml) and augmentin (400 µg/ml), contained within small sterilized pots. The shoots were cultured under the same conditions as before until they had developed a good root system. They were then transferred to soil. The trays of young shoots were sealed in transparent plastic bags for 1-2 days to keep the atmosphere humid until they had become more established.

2.5.3 *In Vitro* Germination of *N. tabacum* Pollen

Pollen from dehisced anthers was germinated in a medium containing 15 % sucrose and 0.1 mg/ml boric acid. Germination was carried out at room temperature for 2-4 hours before microscopic examination.

2.5.4 Collection and Germination of Transgenic Seed

Transgenic *N. tabacum* flowers that had just opened were either crossed with wild type pollen, allowed to self naturally or forced to self and the flower labelled appropriately. The flower heads were then bagged to prevent pollination by other plants.

Transgenic seed was surface sterilized by submerging for 10 minutes in 10 % bleach (Domestos). The seeds were then rinsed thoroughly with sterile tap water before putting

onto sterile filter paper to dry. 70-100 seeds were then evenly spread over the surface of MSO medium containing kanamycin (150 μg/ml) in a sterile Petri dish.

2.6 Identification of Anther Specific mRNAs via cDNA Cloning

2.6.1 *B. napus* Anther cDNA Library

Scott *et al.* (1991a) determined that *B. napus* bud length is related to anther length and these lengths also correspond to the stage of male gametogenesis within the anther. Using this information a cDNA library from anthers, of 1.2-1.8 mm buds, was constructed which represented transcripts expressed during sporogenesis (Scott *et al.*, 1991a). This developmental stage encompassed the period in development where meiosis occurs and extends up to just before microspore release. The cDNA library was constructed in lambda ZAP I (Stratagene) according to the manufacture's instructions.

2.6.2 Plating out the Library

E. coli cells (strain BB4) competant for phage adsorption were produced by inoculating 50 ml of NZY medium (containing 0.2 % maltose and 10 mM magnesium sulphate) with an overnight culture. Once at an OD_{600} of 0.5 this culture was centifuged for 7 minutes at 1,000 x*g* and the pellet resuspended in 20 ml of 10 mM magnesium sulphate and stored for up to 3 days at 4 °C. The lambda phage was plated out on a lawn of competant cells by mixing 600 μl of cells with the desired number of plaque forming units (pfu) from the library and incubated at 37 °C for 15 minutes to permit adsorption of the phage particles to the cells. 7 ml of top agar at 48 °C was added to the cell/phage suspension and immediately poured onto solid NZY medium in a 14 cm sterile Petri dish, ensuring an even coating over the surface. Once dry the plates were inverted and incubated for 3 hours at 42 °C for immunoscreening the library or 8-16 hours at 37 °C for library screening with DNA probes.

2.6.3 Immunological Screening of the cDNA Library

Immunological screening is more effective when the plaque density is low as the colour produced by the chromogenic reaction is more intense at the expanding edge of the plaque. If the plaques are too crowded the border between neighbouring plaques is distorted. A plaque density of 2000 plaques per 15 cm plate was found to be the best plaque density. Nitrocellulose (NC) circles were soaked in 10 mM IPTG solution for a few minutes, then laid to dry on a piece of Whatmann 3 MM filter paper for about 1 hour. The plates were removed from the 42 °C incubator and quickly overlayed with the IPTG impregnated NC circles, ensuring that no air bubbles were trapped underneath. The plates were then

incubated at 37 °C for 3 hours to induce expression of the cDNAs. The plates were placed at 4 °C for a few minutes (to prevent the top agar peeling off) and then the orientation of each filter was marked by stabbing through it into the agar underneath and using a marker pen to indicate this position on the bottom of the Petri dish. The filter was removed with forceps and submerged in TBS-Tween (section 2.3.10) to remove any remnants of agar. The plates were stored at 4 °C until the results of the immunological screening was available. The NC filters were blocked and probed with antibody using the same protocol as described for immunostaining polyacrylamide gel blots. Duplicate filters were produced by overlaying the plates with IPTG impregnated NC filters and incubating the plates for a further 4 hours at 37 °C. The second set of filters were processed in exactly the same way as described previously.

2.6.4 Purification of Antiserum for Immunological Screening

Components of a polyclonal antiserum that react with antigens produced by *E.coli* could be removed by immunoadsorption. A 10 ml culture of *E.coli* (strain BB4) was grown up overnight and the cells harvested by centrifugation at 1,000 x*g* for 10 minutes. The cells were resuspended in TBS (section 2.3.10) and sonicated at full power for 3 periods of 30 seconds at 0 °C with 30 seconds cooling in between. The lysate was used to dilute the antiserum which had already been diluted with TBS-Tween and 1 % Marvel milk powder. 0.5 ml of lysate was added for every ml of diluted antiserum. The mixture was incubated for several hours at room temperature on a rotating platform and then used for immunological screening. The adsorbed antiserum was stored at 4 °C in the presence of 0.05 % sodium azide (care was taken to clearly label this solution, as sodium azide is poisonous).

2.6.5 Screening the Anther cDNA Library with DNA Probes

Denaturing Solution:	0.5 M	sodium hydroxide
	1.5 M	sodium chloride
Neutralizing Solution:	3.0 M	sodium chloride
	0.5 M	Tris-HCl, pH7.4
20x SSC:	3 M	sodium chloride
	0.3 M	sodium citrate, pH 7.0
50x Denhardts:	1 % Ficoll (type 400), 1 % PVP, 1 % BSA	

Hybridization Solution:	6x	SSC
	10x	Denhardts
	0.5 %	SDS
	6 %	PEG 6000
	0.5 mg/ml sheared herring sperm DNA	

Wash Solution A: 3x SSC, 0.5 % SDS
Wash Solution B: 0.5x SSC, 0.5 % SDS

Plates were cooled to 4 °C in the fridge to harden the top agar prior to plaque lifts being taken. Circles of "Hybond-N" (Amersham) nylon membrane were laid onto plates for 30 seconds, during which time orientation marks were made using a needle and marker pen as described in section 2.6.3 to enable later realignment of the filter to the plate. Membranes were laid plaque-side-up on filter paper soaked in denaturing solution for 7 minutes, then transferred similarly to paper soaked in neutralizing solution for 7 minutes. The membranes were finally rinsed for 2 minutes in 2x SSC, dried, and DNA bound to the filter by cross-linking on an ultra violet transilluminator (Ultra Violet Products inc.) which had previously been calibrated to show that 90 seconds was an optimum time for linking. Plaque lifts were prehybridized for at least 1 hour in hybridization solution at 55 or 65 °C (depending on the stringency required). Radioactively labelled probe was then added and hybridized overnight at the same temperature ensuring a good seal on the hybridization vessel. The filters were washed with several changes of preheated wash solution A at 55 or 65 °C for 20 minutes. Further washes with wash solution B were carried out if a more stringent wash was required. A hand held radiation monitor was used to measure the removal of radioactivity. After washing the filters were blotted dry, wrapped in Saran wrap and and exposed to X-ray film (3RX, Fuji) at -80 °C in a cassette with an intensifying screen. Autoradiograms were developed after 2-16 hours depending on the signal. Hybridizing plaques were lined up to the corresponding position on the original plate.

2.6.6 Storage of Isolated Phage Clones

SM buffer:	100 mM	sodium chloride
	17 mM	magnesium sulphate
	50 mM	Tris-HCl, pH 7.5
	0.01 %	gelatin

Clones were isolated by coring plaques out of plates using a sterile Pasteur pipette and eluting phage particles into 500 µl SM buffer containing a drop of chloroform to prevent bacterial growth. These phage suspensions can be stored for several years at 4 °C. In cases where cores were taken from densely plated phage, a second round of plating and

screening was carried out in order to obtain a single plaque.

2.6.7 Radioactively Labelling a DNA Probe for Hybridization

Oligolabelling Buffer: Mix buffers A, B and C in the ratio 2:5:3

Buffer A: Combine:-

625 µl	2 M Tris-HCl, pH 8.0
25 µl	5 M magnesium chloride
350 µl	distilled water
18 µl	β-mercaptoethanol
5 µl	each of 3 mM dATP, dGTP, dTTP

Buffer B: 2 M HEPES, pH 6.6

Buffer C: Random hexadeoxyribonucleotides (Pharmacia) suspended in:-

3 mM	Tris-HCl
0.2 mM	EDTA, pH 7.0

at 90 OD_{260} units /ml

Stop solution:

20 mM	sodium chloride
20 mM	Tris-HCl, pH 7.4
2 mM	EDTA
0.25 %	SDS

Oligolabelling (Feinberg and Vogelstein, 1984) utilizes DNA fragments purified from high gelling temperature agarose or within low gelling temperature agarose as a single stranded template for binding oligomers which act as primers for the Klenow fragment of DNA polymerase I. One of the deoxyribonucleotides used for "filling in " is labelled with ^{32}P to produce a radioactive probe. DNA probe stocks were boiled for 5 minutes to denature the DNA and 10 ng of this was added to the labelling reaction:-

0.6 µl	BSA (10 mg/ml)
3.0 µl	oligolabelling buffer
0.6 µl	Klenow
x µl	DNA
1 µl	dCTP

Sterile distilled water to 15 µl

The reaction was mixed and centrifuged briefly to collect the reactants to the bottom of the tube, then incubated for 1 hour at 37 °C. The reaction was stopped by adding 85 µl stop solution and an aliquot removed to measure the incorporation of radioactivity into the probe. The remaining probe was boiled for 5 minutes and cooled briefly on ice before adding to the hybridization solution.

2.6.8 Measurement of Radioactive Incorporation into DNA

Liquid Scintillant: 5 % POP in toluene

A known volume (usually 1 µl) of probe was dotted onto the centre of a Whatman GF/C glass fibre disc. An equal volume was added to 500 µl of 500 µg/ml herring sperm DNA in 20 mM EDTA and 125 µl of 50 % TCA added. This solution was chilled on ice for 5 minutes. The precipitate was collected by filtering through a GF/C disc in a filter tower. The filter was washed twice with 5 ml 10 % TCA, followed by 5 ml IMS and then both discs placed into vials containing 2 ml of liquid scintillant. The incorporation was calculated as the percentage of precipitated (incorporated) radioactivity over the total number of counts in the labelling reaction.

2.7 Analysis of Gene Expression by Northern Blotting

2.7.1 Micro Plant RNA Extraction

Extraction Buffer: 1:1 Phenol to
100 mM lithium chloride
100 mM Tris-HCl, pH 8.0
10 mM EDTA
1 % SDS

RNA was extracted from 15 *N. tabacum* anthers using a method by Verwoerd *et al.*, (1989). Plant material was collected in an Eppendorf tube and frozen with liquid nitrogen, before grinding to a powder with a microhomogenizer. 500 µl of hot (80 °C) extraction buffer (which was mixed before adding) was added and the sample homogenized for 30 seconds. 250 µl chloroform:isoamylalcohol (24:1) was added, mixed and microcentrifuged for 5 minutes. The water phase was removed to a fresh tube and 1 volume of 4 M lithium chloride added. The RNA was precipitated overnight and then pelleted by microcentrifugation for 10 minutes. The RNA was resuspended in 250 µl sterile distilled water and reprecipitated with 1/10 th volume 3 M sodium acetate, pH 5.2 and 2.5x volume of ethanol. The pellet was washed with 70 % ethanol, dried and resuspended in a small volume of sterile distilled water.

2.7.2 Scanning Spectrophotometry of Nucleic Acids

Nucleic acids absorb light maximally at ultra violet wavelengths, with an absorption peak at 260 nm. The measurement of OD_{260} can therefore be used to determine the concentration. An OD_{260} of 1.0 is equivelent to 37 µg/ml RNA solution or a 50 µg/ml double stranded DNA solution. Nucleic acid solutions were diluted into 300 µl of distilled water, placed in a quartz cuvette and scanned across the range 200-300 nm. A sharp peak at 260 nm preceded by a trough at 220-240 nm indicated a pure nucleic acid preparation.

2.7.3 Separation of RNA by Agarose Gel Electrophoresis

10x MOPS Buffer:	0.2 M	3-[N-Morpholino]-propane-sulphonic acid
	50 mM	sodium acetate, pH 7.0
	10 mM	EDTA
Loading Buffer:	50 % glycerol containing 0.1 mg/ml bromophenol blue	
TAE Buffer:	0.4 M	Tris-acetate, pH 8.0
	10 mM	EDTA

RNA was denatured at 65 °C for 5 minutes in the following solution:-

6 µl	RNA (5-10 µg)
12.5 µl	formamide (deionised)
2.5 µl	10x MOPS buffer
4 µl	formaldehyde (37 %)

The solution was chilled on ice before combining with 2.5 µl loading buffer and loading onto a 1.5 % agarose gel made with TAE buffer containing 0.2 µg/ml ethidium bromide. The gel tank, well forming comb and gel tray were cleaned thoroughly and wiped out with alcohol before use. The RNA was electrophoresed in TAE buffer until the bromophenol blue dye front was approximately 3 cm from the bottom of the gel. The ethidium bromide intercalates with the nucleic acid and therefore allows visualization by fluorescence when illuminated with a u.v. transilluminator. The RNA could therefore be visualized before capillary blotting onto Hybond-N.

2.7.4 Northern Gel Blotting

The ethidium bromide stained gel was equilibrated for 20 minutes in 20x SSC (section 2.6.5) before capillary blotting onto Hybond-N. A capillary blot was set up as follows: the

gel was placed on top of 2 pieces of Whatmann 3 MM filter paper on a sponge standing in a tray of 2x SSC buffer. The remaining surface of the filter paper and sponge was covered with plastic cling film. The Hybond-N membrane was then placed on top of the gel ensuring that no air bubbles were trapped underneath. Two pieces of filter paper soaked in transfer buffer were placed on top of the membrane, and two dry pieces of filter paper on top of these. A stack of paper towels and a weight was laid over the gel. Transfer of buffer from the tray through the gel to the towels was left to occur overnight. The filter was then dried and the DNA bound to the filter by u.v. cross linking for 90 seconds.

2.7.5 Hybridization of DNA Probes to RNA Gel Blots

20x SSPE:	3.6 M	sodium chloride
	0.2 M	sodium phosphate
	0.02 M	EDTA
	pH 7.7	

RNA Hybridization Solution:

50 %	formamide
5x	SSPE
5x	Denhardts (section 2.6.5)
0.5 %	SDS
0.1 mg /ml	herring sperm DNA

Filters were prehybridized in RNA hybridization solution for at least an hour before the addition of radiolabelled probes. Hybridization was carried out overnight at 42 °C. Washes were carried out as described in section 2.6.5. Hybridizing mRNAs were detected using autoradiography.

2.8 Restriction Enzyme and Southern Blot Analysis of DNA

2.8.1 Digestion of DNA with Restriction Endonucleases

Restriction enzymes were bought with an accompanying buffer and used as recommended by the manufacturers. Up to 2 μg of plasmid DNA was incubated for 1-16 hours at the appropriate temperature with the enzyme and buffer, and 0.05 μg RNase A. The enzyme activity could be removed by phenol/chloroform (section 2.5.3) extraction or denatured by heat.

2.8.2 Separation of DNA by Agarose Gel Electrophoresis

Loading Buffer:	0.25 %	bromophenol blue
	0.25 %	xylene cyanol
	25 %	Ficoll (type 400)

DNA fragments can be separated on agarose gel matrices. 2 µl loading buffer was added to the DNA sample prior to loading on the gel. The agarose gels were made by dissolving a variable concentration of agarose (0.8-1.5 %, depending on the size range of fragments expected) in TAE buffer (section 2.7.3), by heating to 95 °C. The agarose was allowed to cool before adding 0.2 mg/ml ethidium bromide and pouring into a casting tray. The gels were submerged in TAE buffer and run at 80 volts, with the appropriate molecular weight markers included on the gel. The separation of the DNA fragments could be monitored by visualization with a u.v. transilluminator.

2.8.3 Southern Blotting

Depurinating Solution:	0.25 M	hydrochloric acid

Before blotting, agarose gels were equilibrated by submerging in depurinating solution for 7 minutes, denaturing solution (section 2.6.5) for 30 minutes and neutralizing solution (section 2.6.5) for 30 minutes. The gel was rinsed in distilled water between treatments. The capillary blot was then set up as described for northern blotting (section 2.7.4). After u.v. cross linking for 90 seconds, hybridization with radioactively labelled probes was carried out as described in section 2.5.5.

2.9 Manipulation of DNA for Cloning of Fragments into Plasmid Vectors

2.9.1 Large Scale DNA Preparation

Lysis Solution:	50 mM	sucrose
	25 mM	Tris-HCl, pH 8.0
	10 mM	EDTA, pH 8.0
Alkaline-SDS:	0.2 M	sodium hydroxide
	1 %	SDS

A 500 ml overnight culture of the bacterial strain carrying the plasmid of interest was pelleted by centrifugation at 1,000 x*g* for 10 minutes. The cells were resuspended in 10 ml

of lysis solution. To this suspension 2 volumes of alkaline SDS were added and mixed by gentle inversion. To this, 1.5 (original) volumes of 3 M potassium acetate pH 5.2 were then added and the solution stored on ice for 5 minutes to precipitate protein and chromosomal DNA. This bacterial debris was spun off at 3,000 x*g* for 10 minutes and the plasmid precipitated from the supernatant with 0.6 volumes of cold isopropanol. Nucleic acids were collected by centrifugation at 3,000 x*g* for 10 minutes and resuspended in 0.5 ml distilled water per 100 ml culture.

2.9.2 PEG Purification of Plasmid DNA

PEG Solution:	1.6 M	NaCl
	13 %	PEG 6000

High molecular weight RNAs were precipitated by the addition of an equal volume of ice cold 5 M lithium chloride and removed by centrifugation at 3,000 rpm for 5 minutes. The remaining nucleic acids were precipitated with an equal volume of cold isopropanol, collected by centrifugation for 10 minutes, rinsed in 70 % ethanol and the pellet dried under vacuum. This pellet was resuspended in 100 µl of TE (section 2.5.3) containing 10 µg/ml RNase A and left for 15 minutes at 37 °C. The plasmid DNA was then precipitated by adding 100 µl of PEG solution and collected by microcentrifugaton for 5 minutes. The pellet was dissolved in 40 µl distilled water and the solution extracted once with phenol/chloroform (section 2.4.7). The DNA was finally precipitated by the addition of an equal volume of 10 M ammonium acetate and 2 volumes of ethanol. The plasmid was collected by microcentrifugation, rinsed in 70 % ethanol and dried before dissolving in the required volume of sterile distilled water. The concentration of plasmid DNA was calculated after the OD_{260} had been measured.

2.9.3 Purification of DNA From Agarose Gels

DNA fragments could be used for cloning after purification from agarose gel slices following electrophoresis. DNA was recovered from agarose using the "Geneclean II" DNA purification kit (Bio 101 Ltd). This involved binding the DNA to silica glass suspensions in high salt conditions, washing, and then eluting the DNA into water. The kit was used as per manufacturers instructions. Fragments purified in this way were ready for ligation into vectors, or enzyme modification prior to ligation.

2.9.5 Filling-In of Protruding 5-Prime Termini

In order to ligate fragments produced by restriction enzymes which leave a 5' overhang into blunt-ended vectors, the overhang was filled in by synthesizing a second strand with

the Klenow fragment of DNA polymerase I. DNA fragments were purified from agarose gels by the Geneclean method and resuspended in 16 µl of sterile distilled water. To this was added 2 µl of React 2 buffer (supplied with BRL restriction enzymes), 1 µl (2 units) Klenow enzyme and 1 µl of GTP extension mix (supplied with the Sequenase sequencing kit). The reaction was left at 37 °C for 1 hour, the Klenow enzyme was heat inactivated for 5 minutes at 70 °C and the DNA recovered by ethanol precipitation.

2.9.6 Ligation of DNA Fragments

Purified DNA was mixed in approximately equimolar ratios of vector and insert, 2 units of T4 DNA ligase and 5x ligation buffer (supplied with the enzyme from GIBCO) were added.

Typical Ligation Reaction:

Vector DNA	- x µl
Insert DNA	- y µl
5x Ligation Buffer	- 4 µl
T4 DNA Ligase	- 1 µl
Water	to 20 µl

Ligations were generally left at 10 °C overnight for sticky ended DNA and at 4 °C overnight for blunt ended, before transformation into competent *E. coli*.

2.9.6 Transformation of *E. coli* with Plasmid DNA

2.9.6.1 Preparation of Competent Cells

A single colony of the required host strain was looped into 5 ml liquid medium and grown overnight (see section 2.4.4). 1 ml of this was subcultured into 100 ml of fresh medium, and grown to an OD_{600} of 0.6 (3-4 hours) before the cells were collected by centrifugation at 1,000 x*g* for 10 minutes at 4 °C. The pellet was resuspended in 1/2 volume of ice cold 50 mM calcium chloride and allowed to stand on ice for 15 minutes before use (1 hour if the cells were to be stored). For long term storage, the cells were resuspended in 20 % glycerol, 50 mM calcium chloride and aliquoted into 100 µl batches. The aliquots of frozen cells were flash frozen in liquid nitrogen and stored at -80 °C.

2.9.6.2 Transformation Procedure

10x TCM buffer:	100 mM	Tris-HCl, pH 7.5
	100 mM	calcium chloride
	100 mM	magnesium chloride

A completed ligation reaction and 10 µl of 10x TCM buffer was added to a 100 µl batch of competent cells which had been thawed on ice. This mixture was incubated on ice for 30 minutes, at 37 °C for 2 minutes, followed by incubation at room temperature for 10 minutes. To the cells 1 ml of prewarmed NB (37 °C) was added and then the cells were heat shocked for 2 minutes at 42 °C. The cells were then shaken at 37 °C for 1 hour, pelleted in a microcentrifuge and resuspended in 100 µl of NB. Transformant colonies were produced by spreading this suspension on selective NA plates and incubating at 37 °C overnight.

2.9.7 Identification of Recombinant Plasmids in Transformed Colonies

A colour assay using X-GAL and IPTG is the simplest method of identifying colonies containing recombinant plasmids, but is only possible when using certain vectors and host cells. These vectors are those which contain a multiple cloning site within the *lacZ* gene. If the plasmid vector is present in a host strain which lacks the *lacZ* gene, the plasmid *lacZ* gene can complement for it. The *lacZ* gene product is a subunit of β-galactosidase and this facilitates the metabolism of the indigogenic substrate X-GAL when IPTG is present. The resulting blue product is easily visible in colonies. When the *lacZ* gene in the vector is interrupted by an insert, recombinant colonies are white and can thus be distinguished from non-recombinants. For colour selection, 20 µl of 40 mg/ml IPTG in water, and 20 µl of 40 mg/ml X-GAL in dimethylformamide were spread onto plates prior to spreading cells.

2.9.8 Colony Hybridization

When colour selection with IPTG and X-GAL was not possible, large numbers of colonies were screened by transferring them onto a Hybond-N nylon membrane, lysing the bacteria and probing with the required radioactively labelled DNA. A nylon membrane disc was laid onto the bacterial plate for 2 minutes, during this time orientation marks were made. The membrane was then transferred colony side up onto Whatman 3MM filter paper soaked in 2 x SSC containing 0.5% SDS and left for 2.5 minutes. To dry the membrane and fix the DNA to it, the membrane was baked in a microwave on full power for 2.5 minutes. The membrane was then hybridized as for plaque lifts (section 2.6.5). The colonies were recovered by replacing the bacterial plate in the 37 °C incubator for about 3 hours. Putative recombinants which gave a radioactive signal where checked by small scale isolation of DNA and subsequent restriction enzyme analysis.

2.9.9 Small Scale Isolation of Plasmid DNA

Recombinant colonies were picked into 5 ml of medium containing antibiotics and grown overnight. Cells were then pelleted from 1.5 ml of culture in a microcentrifuge, and

plasmid prepared by a scaled down alkaline lysis method as described in section 2.9.1. The cell pellet was resuspended in 100 µl of lysis solution and lysed by mixing with 200 µl of alkaline SDS and incubating ice for 1 minute. Cell debris and chromosomal DNA was precipitated with 150 µl of 3 M potassium acetate pH 5.2 and removed by microcentrifugation for 10 minutes. The remaining solution was extracted with phenol/chloroform (section 2.5.3) and the plasmid DNA precipitated by the addition of 2 volumes of ethanol. The nucleic acid was collected by microcentrifugation for 10 minutes, rinsed in 70 % ethanol and dried before dissolving in 50 µl of sterile distilled water. Restriction digestion analysis was carried out on 5 µl of the DNA solution.

2.10 Amplification of DNA by the Polymerase Chain Reaction

2.10.1 Amplification of Plasmid DNA

1x PCR Buffer:	44 mM	Tris-HCl, pH 8.8
	11 mM	ammonium sulphate
	4.5 mM	magnesium chloride
	7 mM	β-mercaptoehthanol
	4.5 mM	EDTA, pH 8.0
	1 mM	of each dNTP
	113 µg/ml	BSA

The PCR conditions used to amplify the mA6 fragment from the A6 clone in pBluescript plasmid were as follows:

Reactants :

Target DNA (1ng/µl)	-	1 µl
PCR Buffer (from an 11x stock)	-	1.8 µl
Primers (100 ng/µl)	-	2 µl+2 µl (primer concentrations equivalent to 1 µM)
Taq (Perkin Elmer) DNA - polymerase I (5U/µl)	-	0.3 µl
Sterile distilled water to 20 µl		

Cetus DNA thermal cycling machine programme:

The PCR programme consisted of 25 amplification cycles each consisting of:- 1 minute 20 seconds denaturing step at 94 °C; 1 minute 20 seconds annealing step at a temperature determined by the sequence of the primers used (40 °C in this case) and an extension step at 72 °C for 1 minute (timing adjusted for length of DNA to be amplified, 1 minute per kb DNA).

Reactions were carried out and the products analyzed by electrophoresis.

2.10.2 Production of a Modified PR β(1,3)-Glucanase cDNA from *N. tabacum* Leaf RNA

cDNA was synthezised from 5 µg of total *N. tabacum* leaf RNA using an Amersham cDNA synthesis kit. Synthesis was according to the manufacturers instructions with the following modifications. First strand cDNA was primed with the PR β(1,3)-glucanase C-terminal oligonucleotide (shown in Figure 5.1) and synthezised using murine reverse transcriptase. After second strand synthesis, the cDNA was purified by phenol/chloroform (section 2.5.3) extraction and precipitated with 0.05x volume sof 5 M sodium chloride and 2.5x volumes of ethanol. A linker, consisting of two complementary synthetic oligonucleotides, OG6 and OG7, was ligated to the cDNA. The sequences of the OG6 and OG7 primers are 5'-GGCCATGGAATTCATCTAGACC-3' and 5'-GGTCTAGATGAATTC-3', respectively. As synthetic oligonucleotides lack a 5' phosphate group this linker ligates only to the 5' end of the cDNA. Following linker ligation, the cDNA was diluted 1/50 and amplified by PCR using the OG6 and C-terminal oligonucleotides as primers. The PCR programme consisted of 25 amplification cycles, each consisting of a 1 minute in and 20 sec at 94 °C, 2 minutes at 55 °C, and 2 minutes at 72 °C. A second round of amplification was then carried out using the PR β(1,3)-glucanase N- and C-terminal oligonucleotides primers (see Figure 5.1) and under the conditions described above. All the primers were used at a concentration of 1 µM.

2.10.3 PCR Analysis of Transgenic *N. tabacum* Plants

PCR Extraction Buffer:	200 mM	Tris-HCl, pH 7.5
	250 mM	Sodium Chloride
	25 mM	EDTA
	0.5 %	SDS

A small piece of *N. tabacum* leaf was removed from the plant to be analyzed with the lid of an Eppendorf tube, and DNA was extracted by a rapid method described by Edwards *et al.* (1991). The leaf disc was homogenized with a disposable plastic rod for 15 seconds before 400 µl PCR extraction buffer was added. The solution was vortexed for 5 seconds and stored at room temperature until all of the samples were at this stage. The debris was pelleted by microcentrifugation for 1 minute and 300 µl of supernatant removed to a fresh tube. The DNA was precipitated by the addition of an equal volume of isopropanol and then pelleted, after a 2 minute incubation at room temperature, by centrifugation for 10 minutes. The DNA pellet was dried and resuspended in 100 µl of TE (section 2.5.3). 2 µl of the resulting DNA solution was then used in a PCR reaction, using the PR glucanase N- and C-terminal oligonucleotides as primers, both at a concentration of 1 µM. The PCR programme consisted of 30 amplification cycles, each consisting of 1 minute and 20 sec at

94 °C, 2 minutes at 57 °C, and 2 minutes at 72 °C. The amplified DNA was then visualized by ethidium bromide staining after electrophoresis of samples on a 0.8 % agarose gel (see section 2.7.2).

2.11 DNA Sequencing

2.11.1 Production of Single Stranded DNA Phagemids

The cell line harbouring the plasmid containing the DNA to be sequenced was grown in 2xYT (section 2.3.1) to an OD_{600} of 0.5-0.8. A 2 ml aliquot of this culture was removed and infected with helper phage M13KO7, at a multiplicity of infection of 10, by shaking at 37 °C for 1 hour. 400 µl of these infected cells were then transferred to 10 ml of medium containing 20 µg/µl kanamycin and grown at 37 °C overnight. The resulting culture produces phagemid DNA packaged as single stranded filamentous phage which is extruded from cells into the medium.

2.11.2 Purification of Single Stranded DNA

PEG Solution:	20 %	PEG 8000
	2.5 M	sodium chloride

The 10 ml culture resulting from above (section 2.11.1) was centrifuged for 10 minutes at 1,000 x*g* . The supernatant was removed to 3 Eppendorf tubes and microcentrifuged to remove all of the cells. The supernatant was poured into Eppendorf tubes containing 200 µl PEG solution and left at room temperature for 15 minutes. The phage particles were pelleted by microcentrifugation for 10 minutes, after which all traces of PEG solution was removed from the tube. The pellet was resuspended in 200 µl TE (section 2.5.3) and the phage coats broken open by the addition of 200 µl phenol and vortexing followed by 200 µl chloroform and vortexing. After 5 minutes centrifugation the aqueous layer was removed to a fresh tube and the nucleic acid precipitated with 1/20 th volume 5 M sodium chloride and 2.5x volumes ethanol. The pellet was finally resuspended in 40 µl distilled water and 3 µl of the DNA analyzed by electrophoresis. 7 µl of this DNA solution was generally used for sequencing.

2.11.3 Preparation of Double Stranded Template for Sequencing

Small scale DNA isolation provided material of sufficient quality for use in sequencing experiments. The DNA from 3-4 alkaline lysis mini preparations was combined to give a final volume of 100 µl, to this was added an equal volume of 8 M lithium chloride. After vortexing briefly the large RNA species were pelleted by microcentrifugation for 5 minutes

and the supernatant removed to a fresh tube. An equal volume of propan-2-ol was added and the solution microcentrifuged. The DNA pellet was resuspended in 100 µl sterile water and treated with 0.1x volumes of RNase (10 mg/ml) and incubated at 37 °C for 30 minutes. The DNA was precipitated by the addition of 1/20 volume 5 M sodium chloride and 2 volumes of ethanol and storage at -80 °C for 10 minutes. The DNA was pelleted by microcentrifugation for 10 minutes and resuspended in 100 µl sterile water before treating with phenol/chloroform. After vortexing and microcentrifugation for 5 minutes the aqueous layer was removed to a fresh tube and precipitated with an equal volume of 10 M ammonium acetate and 2 volumes of ethanol. After 10 minutes microcentrifugation the DNA pellet was finally resuspended in 20 µl sterile water. 2 µl of this DNA solution was analyzed by electrophoresis.

Approximately 10 µg of this DNA was denatured by the following method: the DNA solution was made up to 18 µl and 2 µl of 2 M sodium hydroxide was added. The solution was left to stand at room temperature for 5 minutes. The DNA solution was neutralized by the addition of 4 µl of 4 M ammonium acetate (pH 4.8). The denatured DNA was precipitated with 2.5x volumes of ethanol at -80 °C for 10 minutes. The denatured DNA was recovered by microcentrifugation and the pellet washed with 70 % ethanol. The pellet was resuspended in an appropriate volume of sterile water. 0.5-1.0 µg of the denatured DNA was used for annealing to the primer.

2.11.4 Sequencing Using Sequenase

10x TBE: per litre	121 g	Tris base
	53.4 g	Boric acid
	7.4 g	EDTA
	pH 8.3	
6 % Acrylamide solution:	6 %	acrylamide
	3 %	bisacrylamide
	7 M	urea
	1x TBE	
Ammonium persulphate solution:	250 mg/ml ammonium persulphate	

Templates were sequenced using Sequenase which employs dideoxynucleotide chain-terminating mixes, as described by Sanger *et al.* (1977). Templates primed with the appropriate oligonucleotide were sequenced with ^{35}S-labelled dATP, and the products separated by denaturing polyacrylamide gel electrophoresis. The Bio-Rad "Sequi-Gen" sequencing plates were sealed with 10 ml of acrylamide solution polymerized by the

addition of 50 µl of ammonium persulphate solution and 50 µl TEMED. Gels were made by polymerizing 50 ml of 6 % acrylamide solution with 50 µl TEMED and 50 µl ammonium persulphate solution. This mixture was poured into sealed plates and left to polymerize for 1 hour. The gel was pre-run to 50 °C in 1x TBE buffer before loading pre-heated samples. Gels were dried onto Whatman 3MM filter paper and exposed to autoradiographic film at room temperature overnight without the use of an intensifying screen.

2.11.5 Data Handling

Sequence data was compiled and analyzed on the Leicester University Computer Centre VAX mainframe, using the Wisconsin Genetics Computer Group (GCG) program suite (Devereux *et al.*, 1984) and other DNA analysis programs provided by the university molecular biology users group. Multiple alignments of deduced amino acid sequences were performed using the CLUSTAL programme (Higgins and Sharp, 1989).

2.12 Over-Expression of Proteins in *E. coli*

The pGEM (Promega) expression system is based on the T7 expression system developed by Studier and Moffat (1986). Sequences cloned into the pGEMEX-2 vector are expressed as T7 gene 10 fusion proteins. The bacterial host strain used was K38 harbouring pGP1-2, a plasmid containing a heat sensitive repressor (cl-857) of the T7 polymerase promoter (Tabor and Richardson, 1985). At 42 °C the repressor is unable to bind to the T7 promoter therefore allowing the expression of the T7 RNA polymerase and in turn the bacteriophage T7 gene 10 fusion protein.

2.12.1 Expression of Gene 10 Fusion Proteins

The recombinant pGEMEX-2 DNA was transformed into the bacterial strain containing the pGP1-2. A single colony was picked and grown up overnight at 30 °C in 5 ml of NZY medium containing 100 µg/ml ampicillin and 50 µg/ml kanamycin. 50 ml of fresh medium was inoculated with 2 ml of the overnight culture and grown at 30 °C for approximately 3 hours until the OD_{600} was at 0.6-1.0. During this period plasmid DNA was isolated from the remaining overnight culture and analyzed with restriction enzymes to ensure both the pGP1-2 and recombinant pGEMEX-2 plasmids were present. When the 50 ml culture had reached the required optical density a 1.5 ml aliquot was removed for a pre-induction control, and the remaining culture placed in a 42 °C shaking incubator for 20 minutes. The culture was then returned to the 30 °C incubator for 20 minutes before another 1.5 ml aliquot was taken.

2.12.2 Fusion Protein Mini Preparations

The 1.5 ml aliquots of culture were pelleted by microcentrifugation for 2 minutes. The pellet was resuspended in 50 µl of SDS Sample buffer (section 2.3.2) and boiled for 3 minutes. Approximately 20 µl of pre-induction protein and 15 µl of post induction protein were loaded on a SDS-polyacrylamide gel. Fusion proteins are often insoluble and accumulate in the inclusion bodies of the bacterial cell. It was therefore necesssary to carry out inclusion body preparations to enhance the isolation of the fusion protein.

2.12.3 Isolation of Inclusion Bodies

Lysis Buffer:	50 mM	Tris-HCl, pH 8.0
	1 mM	EDTA
	100 mM	sodium chloride
Lysozyme Solution:	10 mg/ml lysozyme in lysis buffer	
Inclusion Body Wash:	25 mM	Tris-HCl, pH 8.0
	10 mM	EDTA
	3 M	urea
Solubilizing Solution:	9 M	urea
	50 mM	glycine, pH10.8
	1 mM	EDTA
	20 mM	β-mercaptoethanol

The cells were harvested by centifugation at 3000 rpm for 5 minutes at 1,000 x*g* and then resuspended in 0.1x volumes of lysis buffer. For every 10 ml of suspension, 30 µl of 50 mM PMSF was added and 300 µl lysozyme solution. The suspension was incubated on ice for 20 minutes with occational stirring. Then for every 10 ml suspension 12 mg deoxycholic acid was added with stirring and the solution transferred to a 37 °C water bath. When the suspension became viscous 70 µl DNase (1 mg/ml) was added for every 10 ml and the solution incubated at 37 °C for 30 minutes or until the viscousness was lost. The inclusion bodies were pelleted by centrifugation at 1,000 x*g* for 5 minutes and then washed several times with lysis buffer containing 0.5 % triton X-100. The incusion bodies were pelleted again and then washed with inclusion body wash before finally resuspending the pelleted inclusion bodies in solubilizing solution. SDS sample buffer (section 2.3.2) was combined with approximately 15 µl of inclusion body protein and boiled for 3 minutes before analysis on SDS-polyacrylamide gels.

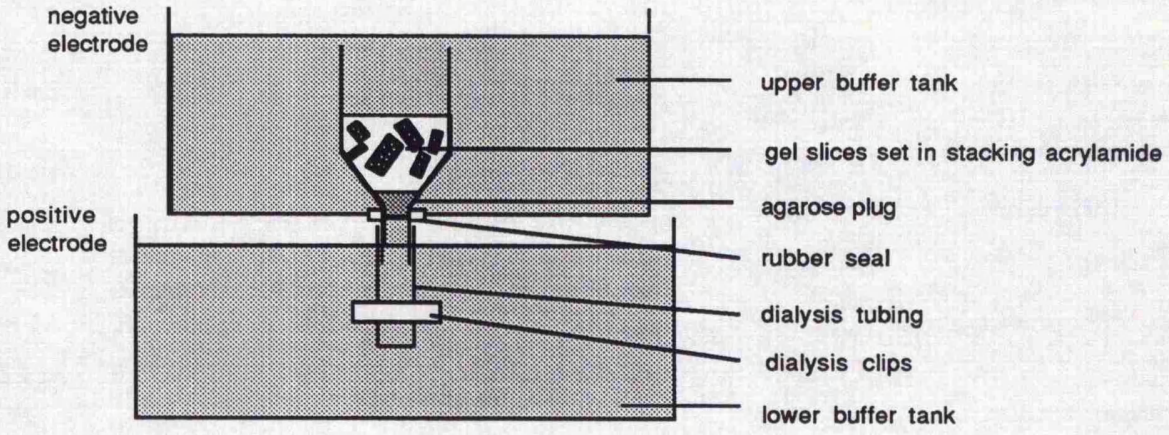
2.12.4 Purification of Fusion Protein by Electroelution

PBS: For 1 litre,	1 g	sodium chloride
	0.2 g	potassium chloride
	0.2 g	potassium dihydrogen phosphate
	1.2 g	disodium hydrogen phosphate
	pH 7.4	

Although bacterial inclusion body preparations enhanced the isolation of the fusion protein, the protein was not sufficiently pure for raising antibodies. Further purification involved electroelution of the fusion protein band from SDS-polyacrylamide gels. Large amounts of inclusion body protein were separated on two preparative scale SDS-polyacrylamide gels. The protein was briefly stained for 10 minutes in Coomassie stain (section 2.3.5) then destained. The fusion protein band was cut out with a scalpel blade and the gel pieces stored at 4 °C until the electroelution apparatus was set up.

Before the electroelution apparatus was set up as in Figure 2.1 the narrow width dialysis membrane was equalibrated in SDS running buffer (section 2.3.8) at 60 °C for 1 hour. The dialysis chamber was created by sealing the bottom of the glass vessel with the dialysis membrane and Nescofilm, and adding 200 µl of 10 % sucrose solution upon which was floated 1 % agarose dissolved in SDS sample buffer. Once the agarose plug had set, the sucrose was flushed out with SDS running buffer. The glass vessel was put into place in the electroelution apparatus and then the dialysis chamber and membrane were filled up with the buffer and the end sealed with the dialysis clip (as shown in Figure 2.1). Prior to polymerization in stacking acrylamide, the gel slices were washed in water to remove the destain and then equilibrated in a small volume of SDS running buffer. Once the gel slices and 10 ml stacking acrylamide had been put into the glass vessel, the surface was sealed off with propanol water (1:1) to allow polymerization. The propanol water was washed off and the SDS running buffer in which the gel slices had been equilibrated was poured on to the top of the acrylamide. The buffer chambers were then filled up with SDS running buffer and the protein electroeluted overnight at 25 V. As the protein was Coomassie stained it could be seen migrating into the dialysis chamber. Once this process was complete the current was reversed for a minute to ensure that the protein was not bound to the dialysis membrane. Before disassembling the apparatus, it was important to remove as much of the buffer as possible to avoid diluting the eluted protein. Approximately 5 µl of the eluted protein was boiled in SDS sample buffer (section 2.3.2) for 3 minutes and then the concentration of the protein estimated by separation on an SDS polyacrylamide gel and comparison to the molecular weight markers. The protein was purified further by dialysis overnight against PBS.

Figure 2.1
Diagram of the Electroelution Apparatus


negative electrode
positive electrode
upper buffer tank
gel slices set in stacking acrylamide
agarose plug
rubber seal
dialysis tubing
dialysis clips
lower buffer tank

2.12.5 Raising Antibodies to the Purified Fusion Protein

Freunds complete adjuvant (FCA) was combined with 500 µg of the purified fusion protein in a total volume of 500 µl which was then used as a primary immunization injected subcutaneously into a New Zealand White rabbit. The rabbit was boosted subcutaneously at 14 day intervals with 100-300 µg protein in a total volume of 500 µl. FCA was not required for the boosts. Before each boost, a test bleed from the ear vein was carried out to monitor the antibody response. The rabbit was boosted five times and then left for a month before giving a final boost. After 6 weeks the rabbit was sacrificed and exsanguinated. Serum was prepared from the clotted blood. The rabbit immunizations and bleeds were carried out by Biomedical Services, Leicester University.

2.12.6 Affinity Purification of Antibodies From Fusion Protein Antiserum

Blocking Soluion:	4 %	BSA in TBS (section 2.3.10)
Diluting Solution:	1 %	BSA in TBS
Dissociating Solution:	100 mM	glycine-HCl, pH 2.5
Neutralizing Solution:	1 M	Tris-HCl, pH7.5

The serum produced after immunization of the rabbit contained anti-fusion protein antibodies. The anti-mA6 antibodies were purified from the heterogeneous serum by affinity immunoadsorption. Inclusion body proteins were prepared from a 250 ml culture of the recombinant and empty vector as described in section 2.12.3. The insoluble protein was washed and then resuspended in TBS. The protein was then treated with blocking solution and rotated for at least an hour. The blocked protein from the recombinant vector was collected by centrifugation at 1,000 x*g*. The insoluble protein was resuspended to a fine powder in dilution solution and added to 0.5 ml of antiserum. This solution was rotated overnight at 4 °C. The protein-antibody complexes were pelleted by centrifugation at 15,000 x*g* for 10 minutes. The pellet was washed 2x by resuspension and centrifugation with TBS-tween. The antibodies were dissociated from the complexes by fully resuspending the washed, drained complexes in 1 ml of dissociating buffer for 10 minutes at room temperature. The supernatant, containing the antibodies, was recovered by microcentrifugation for 5 minutes, and immediately neutralized by adding to 300 µl neutralizing solution. The neutralized supernatant was added to the insoluble inclusion body proteins from the empty vector which had been blocked and then resuspended in 1 ml of diluting solution. This was then mixed overnight at 4 °C or 3-4 hours at room temperature. The supernatant was recovered after centrifugation at 15,000 x*g* for 10 minutes. The supernatant represented affinity purified antibody for which a new working dilution had to be determined (the volume of the supernatant was approximately 3.5 ml and this represented the equivelent of 0.5 ml of the original serum).

2.13 Cytological Analysis of Anthers

2.13.1 Staining Callose

Anthers were dissected and locular contents were squeezed directly into a 0.005 % aqueous solution of water-soluble aniline blue made up in 0.15 M potassium hydrogen phosphate (K_2HPO_4). Maximum staining was achieved after 10-15 minutes. Preparations were observed using a Zeiss standard microscope fitted with a fluorescence attachment 2FL, and filter set 487709 (blue excitation) 450-490 nm.

2.13.2 Transmission Electron Microscopy

Anthers at a stage equivalent to microspore release, as determined by measuring anther length, were fixed and prepared for electron microscopy by Stefan Hyman (E.M laboratory, Leicester University) essentially as described by Grant *et al.* (1986).

Anthers were fixed in phosphate buffered 2.5 % glutaraldehyde, pH 6.8 overnight. During this incubation step, the anthers were contained within syringes to ensure that they remained completely submerged in the liquid. The anthers were washed twice in the above buffer for 5 minutes before post fixing in 1 % osmium tetroxide (in the above buffer) for a further 24 hours. The samples were then washed 3x in double distilled water for 5 minutes prior to dehydration treatment with the following ethanol series. 10 % ethanol for 20 minutes, 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 % ethanol, each for 30 minutes and 100 % ethanol 2x for 30 minutes. The samples were then incubated in propylene oxide for 2x 30 minutes and then propylene oxide: Spurr (1: 1) for 5 hours. Following this incubation, the cap was removed from the sample jar to allow the propylene oxide to evaporate overnight. The anthers were then incubated in 100 % Spurr for 7 hours and polymerised for 16 hours at 60 °C.

Semithin (0.2-0.4 μm) sections were mounted on glass slides and stained with 1 % toluidine blue in 1 % Borax (disodium tetraborate) for 30 seconds on a heating block. The stain was washed off with water and the sections dried on a heated block before viewing

2.13.3 Scanning Electron Microscopy

Pollen was sprinkled directly from a dehisced anther on to the surface of a stub which had been coated with a sticky tab. The pollen was then sputter coated with gold and viewed under a scanning electron microscope with the help of George McTurk (E.M. Laboratory, Leicester University).

2.13.4 Staining Pollen Grains with Vital Stain

Vital Stain Mixture:	95 %	alcohol (10 ml)
(Alexander, 1969)	10 mg	malachite green (1 ml of 1 % solution in 95 % alcohol)
	50 ml	distilled water
	25 ml	glycerol
	5 g	phenol
	5 g	chloral hydrate
	50 mg	acid fucsin (5 ml of 1 % solution in water)
	5 mg	orange G (0.5 ml of 1 % solution in water)
	2 ml	glacial acetic acid

Pollen grains fron dehisced *N. tabacum* anthers were mounted in a drop of stain, covered with a over slip, warmed briefly over a flame and then viewed with a Nikon microscope.

2.13.5 Staining Anther Contents with Aceto-Orcein

Aceto-Orcein Stain:	2 g	orcein added to 55 ml distilled water and 4 ml glacial acetic acid. warm on a heated stirirng block to dissolve, then filter.

Anthers were dissected from *N. tabacum* buds and the contents stained with aceto-orcein after breaking the anther up with a scalpel.

2.14 Measuring the pH of Locular fluid

These experiments were carried out in conjunction with Caroline Dart (Physiology Department, Leicester University). An ion-selective microelectrode was utilized for the measurement of pH of *N. tabacum* anther locular fluid. Anthers at different developmental stages were cut in half to expose the locular fluid. The half anthers were then supported in small squares of agarose gel (1 % agarose dissolved in water) so that the microelectrodes could be inserted into the locular fluid with the aid of a dissecting microscope. Three microelectrodes were used: an ion-selective microelectrode; a conventional microelectrode (which measures voltage) and a silver wire which served as a reference electrode. The pH reading for the locular fluid was considered to be the difference between the ion-selective microelectrode reading (minus the voltage electrode reading) and the reference electrode reading. For further information on the theory and use of microelectrodes see Dart (1991).

Chapter 3

Study of β(1,3)-Glucanases in *B. napus* Buds and *N. tabacum* Anthers

3.1 Introduction

One of the most obvious steps in microsporogenesis is the release of free haploid microspores from the meiotic tetrad. As described in Chapter 1, a key component in this step is a tapetally secreted β(1,3)-glucanase activity (callase) which hydrolyzes the thick callose walls surrounding the microspore tetrads. Work in *Petunia* (Frankel *et al*, 1969) and *Lilium* (Stieglitz and Stern, 1973; Stieglitz, 1977) showed that callase activity in anthers is under strict developmental control, first appearing when the microspores have synthesized the microspore wall, and rapidly disappearing again following callose wall dissolution and the release of the free spores. Correct timing of callase activity is critical, as early or late appearance of the enzyme activity can result in male sterility (Frankel *et al.*, 1969; Warmke and Overman, 1972). A major aim of the work reported in this thesis was to isolate genes encoding components of the callase activity expressed in the anther. A cDNA library was available in the laboratory which provided a route to the cloning of a cDNA representing a transcript expressed from such a gene (Scott *et al.*, 1991a). The library was constructed from anther mRNA of *B. napus* buds of length 1.2-1.8 mm. This size range of anthers corresponds to the developmental stage where meiocytes are present up to the stage just prior to microspore release from the tetrad. The library was therefore likely to contain cDNAs for transcripts encoding proteins involved in microspore release. Defence related β(1,3)-glucanases have been characterized more fully than the anther-specific enzymes and antibody and DNA probes were available (Shinshi *et al.*, 1988; Joosten and De Wit, 1989; Dong *et al.*, 1991). This chapter investigates the use of these probes in an indirect approach to the identification of a protein involved with microspore release.

3.2 Results

3.2.1 Changes in β(1,3)-Glucanase Activities During Bud Development in *B. napus*

A brief study of exo- and endo-β(1,3)-glucanase activity during microspore development in *B. napus* was undertaken to establish whether the profile of activity followed the same pattern as that observed in other plant species (Frankel *et al.*, 1969; Stieglitz and Stern, 1973). The length of *B. napus* buds can be correlated to the developmental stage of the microsporocyte or microgametophyte. Sorting the buds according to size provides a quick way of staging and avoids time consuming dissections (Scott *et al.*, 1991a). Crude enzyme extracts were made from the graded *B. napus* buds representing various stages of development and then partially purified by ammonium sulphate precipitation and dialysis. The protein concentration of these extracts was determined by Bradford's assay. Glucanase activities were determined by incubating an

aliquot of extract with a β(1,3)-linked glucan, laminarin, for 45 minutes at 37 °C. Total β(1,3)-glucanáše activity was detected by a method based on that of Dygert *et al.*, (1965), and involved the measurement of free reducing groups produced by enzyme activity whilst exo-β(1,3)-glucanase activity was determined by a micro-glucose oxidase/peroxidase assay which measures only free glucose. The final values obtained from the activity assays were standardized by taking into account the protein concentration. The profiles for total β(1,3)-glucanase activity and exo-β(1,3)-glucanase during microsporogenesis are shown in Figure 3.1 (the data for these graphs are given in Appendix A). The highest level of total β(1,3)-glucanase activity is observed in buds between 1.5-2.5 mm in length. This corresponds to the period of microspore release. The exo-β(1,3)-glucanase profile shows that the maximum level of activity was detected in the 2.5-3.5 mm buds which is equivalent to the period just after microspore release and the beginning of microspore maturation. The difference in reducing values between the two assays should provide a figure for endo-β(1,3)-glucanase activity. Although the micro-glucose oxidase/peroxidase assay for exo-β(1,3)-glucanase activity was capable of detecting ten fold more dilute concentrations of glucose standards than the Dygert determination, the assay for total β(1,3)-glucanase detected a much greater amount of activity than the assay for exo-β(1,3)-glucanase. It therefore seems that there is far more endo activity in *B.napus* buds. However, the exo-β(1,3)-glucanase activity in the extracts seemed to decrease more rapidly during storage between extraction and assaying than did the endo-β(1,3)-glucanase activity. This factor may indicate that the amount exo-β(1,3)-glucanase activity shown on the profile in Figure 3.1 is not a true representation of the amount of activity *in vivo*. Despite this problem the assays do suggest that there is an increase in β(1,3)-glucanase activity in *B. napus* buds at the time of microspore release.

3.2.2 An Investigation into the Relationship Between PR and Anther β(1,3)-Glucanases

One possible indirect method to identify a cDNA representing an anther-specific β(1,3)-glucanase was to utilize antibodies raised to PR β(1,3)-glucanases that were already characterized. Before such probes were used, an investigation was undertaken to establish whether the PR β(1,3)-glucanases were capable of hydrolyzing the callose wall surrounding the tetrads of microspores. This would prove a functional relationship between the anther and PR enzymes and therefore indicate whether PR β(1,3)-glucanase antiserum might recognize epitopes on the anther enzymes. To this end, *N. tabacum* plants were sprayed with salicylic acid to induce a wound response and then intercellular fluid (ICF)

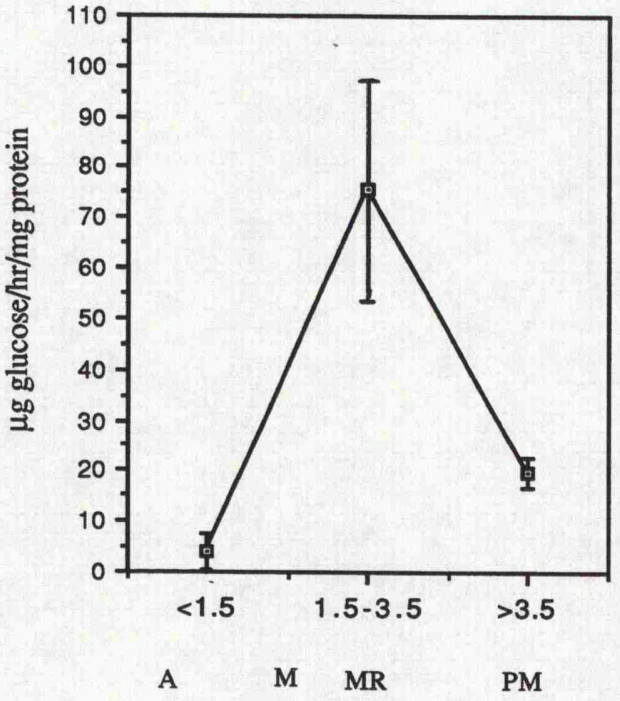
Figure 3.1

Changes in β(1,3)-Glucanase Activity During Development in *B. napus* Buds

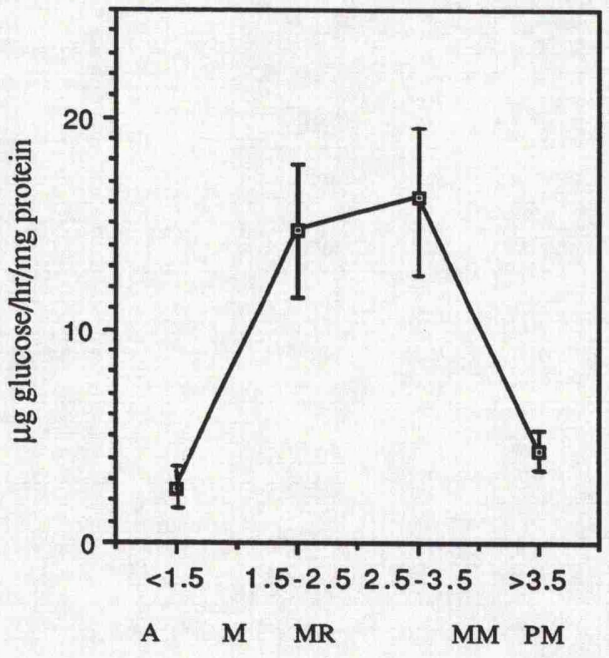
(A) Profile of total β(1,3)-glucanase activity during bud development in *B. napus*.
Reducing sugars were detected with a method based on that of Dygert *et al.*, (1965).
(B) Profile of exo-β(1,3)-glucanase activity during bud development in *B. napus*.
Glucose was detected with a micro-glucose oxidase/peroxidase assay.
The bars on each graph represent the standard error.
(The data is shown in Appendix A.1 and A.2)

Key to Developmental Stages
A, archesporial stage; **M**, meiocyte stage; **MR**, microspore release; **MM**, microspore mitosis; **PM**, pollen maturation.

A,


µg glucose/hr/mg protein
110
100
90
80
70
60
50
40
30
20
10
0
<1.5
1.5-3.5
>3.5
bud length (mm)
A
M
MR
PM
DEVELOPMENTAL STAGE

B,


µg glucose/hr/mg protein
20
10
0
<1.5
1.5-2.5
2.5-3.5
>3.5
bud length (mm)
A
M
MR
MM
PM
DEVELOPMENTAL STAGE

was extracted both from these leaves and control leaves. ICF was also extracted from the leaves of an interspecific hybrid of *N. glutinosa* x *N. debneyi* (Ahl and Gianinazzi, 1982). This hybrid produces PR proteins constitutively and therefore displays an increased resistance to pathogen attack. Equal amounts of these proteins were separated by SDS-PAGE and immunoblotted with antiserum raised to a 35 kD ICF acidic PR endo-β(1,3)-glucanase from *L. esculentum* (Joosten and De Wit, 1988) (Figure 3.2).

The antiserum had a very strong affinity for the proteins in the undiluted ICF from the interspecific hybrid of *N. glutinosa* x *N. debneyi*, but a more specific signal was obtained when the ICF was diluted ten fold. Immunoreactive proteins of approximately 36 kD were detected in the diluted ICF from the hybrid and also in the ICF from salicylic acid treated and control *N. tabacum* plants. The ICF from salicylic acid treated leaves showed a marked increase in the intensity of the larger molecular weight immunoreactive protein from that observed in the control. These immunoreactive proteins are likely to represent *N. tabacum* PR β(1,3)-glucanases which are known to be induced by salicylic acid (see section 1.4.2). The immunoreactive protein detected in the hybrid of *N. glutinosa* x *N. debneyi* exhibited a lower molecular weight than the main protein detected in *N. tabacum* and this is probably due to variation of the same protein between species. In addition to the main immunoreactive proteins detected in *N. tabacum* a lower molecular weight protein was also detected in the ICF protein from the salicylic acid treated plants, and may represent basic β(1,3)-glucanase isoforms which are located in the cellular vacuole, and this indicated that some cell leakage had occurred during the vacuum infiltration.

Having shown that ICF from *N. tabacum* leaves contained PR β(1,3)-glucanase enzymes, the effect of the ICF on the callose wall was investigated. *N. tabacum* anthers at an appropriate stage of development were dissected and the tetrads of microspores extruded. After washing, the tetrads were treated for an hour at room temperature with ICF that had been extracted from either salicylic acid treated *N. tabacum* plants or from the hybrid of *N. glutinosa* x *N. debneyi*. The tetrads were then stained with aniline blue and examined under the light microscope. Figure 3.3 compares untreated tetrads with tetrads incubated with *N. glutinosa* x *N. debneyi* ICF.

Control tetrads had a thick callose wall surrounding the microspores (Figure 3.3A) and this fluoresced bright yellow when visualized under blue light (Figure 3.3B). In contrast, tetrads treated with ICF appeared to lack the callose wall (Figure 3.3C). This was more obvious when tetrads of microspores were viewed under blue light (Figure 3.3D). The lack of callose wall following ICF treatment indicated that some component of the ICF (most likely the β(1,3)-glucanase enzymes) was capable of breaking down the callose wall.

It was interesting to note that the microspores were still held together in a tetrad structure even though the callose wall was absent (Figure 3.3D). Another observation was that the microspores within the tetrads that had been treated with ICF, and therefore lacking callose, were smaller relative to the control. This is apparent if Figures 3.3A and C are

Figure 3.2

Immunoblot of ICF Proteins Probed with the PR β(1,3)-Glucanase Antiserum

ICF proteins were separated on an 11 % SDS-polyacrylamide gel then electroblotted onto PVDF membrane before probing with the PR β(1,3)-glucanase antiserum. Lane 1, ICF from *N. glutinosa* x *N. debneyi* hybrid (Ahl and Gianinazzi, 1982); lane 2, 1/10 dilution of sample in lane 1; lane 3, ICF from *N. tabacum*; lane 4, ICF from a salicylic acid treated *N. tabacum* plant.

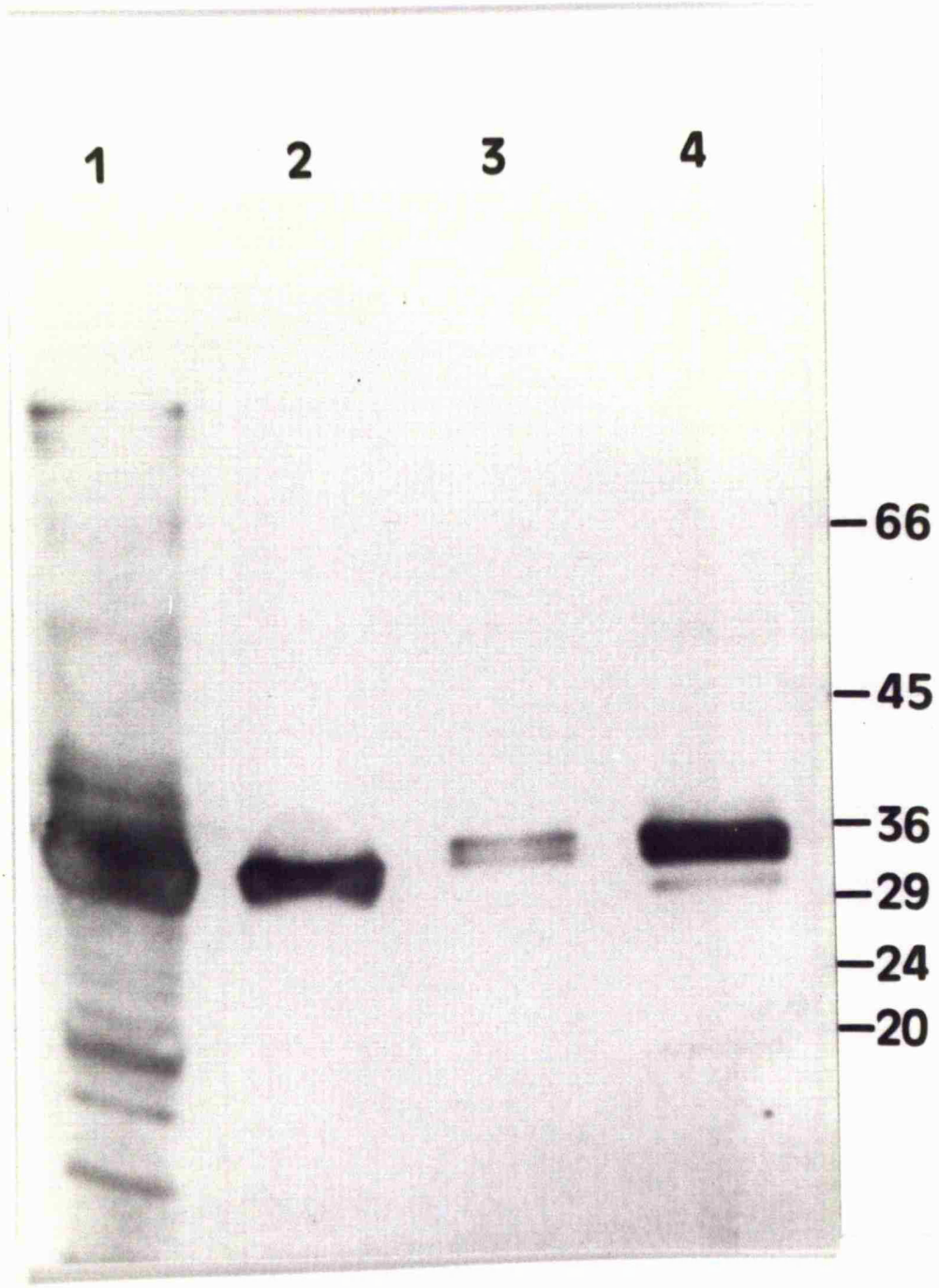

1
2
3
4
66
45
36
29
24
20

Figure 3.3

The Effect of ICF, Shown to Contain PR β(1,3)-Glucanases, on the Callose Wall of Isolated *N. tabacum* Tetrads

Isolated tetrads were treated with ICF extracted from a *N. glutinosa x N. debneyi* hybrid. These tetrads and control untreated tetrads were stained for callose with aniline blue. (A) and (B) Control untreated tetrads. (C) and (D) Tetrads treated with ICF. Magnification X400. In each case, the right hand column is shown under phase contrast and the left hand column displays the same view under blue excitation to highlight callose (yellow fluorescence). The photographic exposure time for aniline blue fluorescence in the ICF treated material was 3x that for the untreated.

A
B
C
D

compared.

3.2.3 A PR β(1,3)-Glucanase Antiserum Recognizes a High Molecular Weight Band in *B. napus* Buds and *N. tabacum* Anthers

An activity, most probably PR β(1,3)-glucanase, present in ICF from leaves was capable of hydrolyzing the callose wall surrounding the tetrads of microspores. It was thus feasible that the PR β(1,3)-glucanases would have epitopes in common with the anther enzymes and that the antiserum raised to the PR β(1,3)-glucanase from *L. esculentum* (described in section 3.2.2) may also be able to identify anther-specific β(1,3)-glucanases.

Protein extracts were made from *B. napus* buds of varying lengths corresponding to different stages of microsporogenesis as for the β(1,3)-glucanase assays:- <1.5 mm; 1.5-2.5 mm and >2.5 mm buds (refer to Figure 1.2 for developmental stages). ICF was extracted from the leaves of *B. napus*. Equal amounts of each protein extract was separated by SDS-PAGE then immunoblotted with the PR β(1,3)-glucanase antiserum (Figure 3.4A). A single temporally regulated immunoreactive band in the molecular weight range 50-60 kD was identified in the *B.napus* bud extracts. This band was present at maximum levels in the 1.5-2.5 mm buds, which are equivalent to the stage when microspore release occurs, decreased in abundance in the larger buds and was not detected at all in the smaller size class of buds. Several immunoreactive proteins with molecular weights between 30 and 40 kD were detected in the leaf extract and probably represent *B. napus* PR β(1,3)-glucanases.

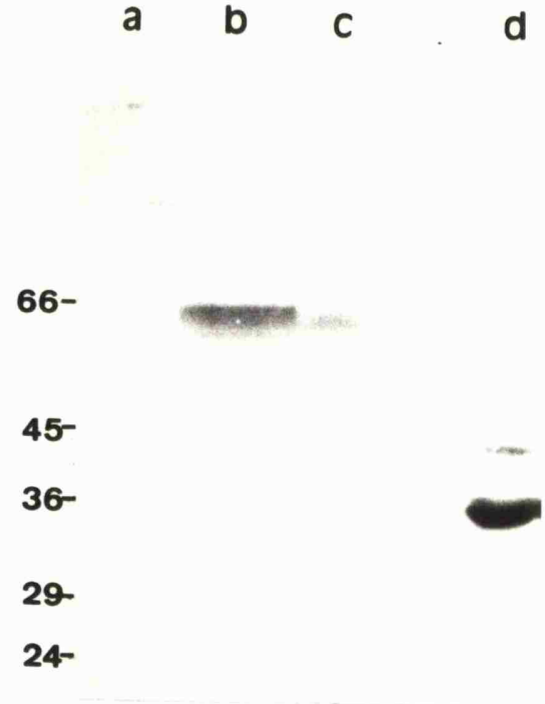
N. tabacum buds were dissected and the developmental stage of the microsporogenous cells within one of the five anthers determined by examination under the light microscope. Micro-protein extractions were carried out on the remaining anthers, which were categorized into:- cells undergoing meiosis, tetrads of microspores, immature microspores, or mature pollen. Protein extracts were also made from salicylic acid treated *N. tabacum* leaf material. Equal amounts of each protein extract were separated by SDS-PAGE then immunoblotted with the PR β(1,3)-glucanase antiserum (Figure 3.4B). An immunoreactive band in the molecular weight range 50-60 kD was present in all of the anther extracts but not in the *N. tabacum* leaf extract. Similar to the situation in the *B. napus*, immunoreactive proteins of approximately 35 kD in size were present in the extract from induced leaf material. These proteins probably represent the PR β(1,3)-glucanases.

Figure 3.4

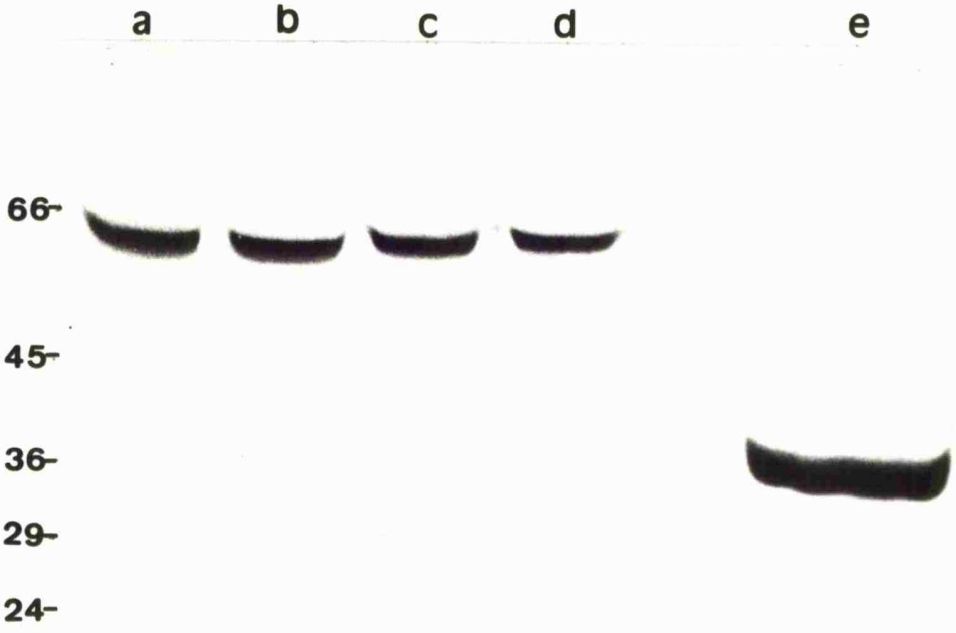
A PR β(1,3)-Glucanase Antiserum Recognizes a 60 kD Band in *B. napus* Buds and *N. tabacum* Anthers

Protein extracts were separated on 12 % SDS polyacrylamide gels then electroblotted onto PVDF membrane before probing with the PR β(1,3)-glucanase antiserum. The positions of the molecular weight markers (SDS-7, Biorad) are indicated at the side of each blot.
(A) Protein extracted from wild-type *B. napus*. Lane a, < 1.5 mm buds; lane b, 1.5-2.5 mm buds; lane c, >2.5 mm buds; lane d, leaf ICF.
(B) Protein extracted from wild-type *N. tabacum*. Lane a, anthers containing meiocytes; lane b, anthers containing tetrads; lane c, anthers containing immature microspores; lane d, anthers containing mature pollen; lane e, protein extracted from a leaf of a salicylic acid treated plant.

A



B



3.2.4 An Attempt to Determine the Relationship Between the Immunoreactive Band From *B. napus* Buds and β(1,3)-Glucanase Activity

Stieglitz (1977), apparently purified two different β(1,3)-glucanase activities attributable to proteins of 32 kD and 62 kD from the anthers of *Lilium*. The 62 kD protein differs significantly in size to the characterized PR enzymes which have molecular weights of 30-40 kD. The temporally regulated immunoreactive band detected in the *B. napus* bud extracts peaked in abundance at the stage when microspore release occurs and also had a molecular weight of approximately 60 kD, as estimated from SDS gels. However, Stieglitz (1977) used a Sephadex G-200 column to determine the molecular weights of the *Lilium* anther β(1,3)-glucanase enzymes and therefore direct comparison of proteins for which different methods of molecular weight determination have been used is not reliable. The temporally regulated immunoreactive band from *B. napus* buds did appear to possess the properties expected for a β(1,3)-glucanase involved with microspore release. However, this evidence was only circumstantial. Therefore it was important to show directly that the immunoreactive protein possessed β(1,3)-glucanase activity. For this purpose a method was used where β(1,3)-glucanase enzyme activity could be detected directly after separation of *B. napus* bud protein by native-IEF-PAGE .

Crude enzyme extracts were made from *B. napus* buds which had been sorted according to their size and therefore also to their developmental stage. These samples were electrophoresed on 5 % IEF gels with a pH range 3-10 and then assayed for β(1,3)-glucanase activity or capillary blotted onto immobilizing membrane for immunolocalization experiments. Total β(1,3)-glucanase activity was detected by incubating the gels in a laminarin solution and then staining the reducing sugars produced by enzyme activity within the gel with a tetrazolium agent. Scanning the activity gel with a gel scanner provided a sensitive method of visualizing the results of this assay, as often the gels were difficult to photograph.

Figures 3.5A and B show that the most prominent activity was present in all of the bud extracts and corresponded to an isoelectric point of approximately 10.6. Six other β(1,3)-glucanase activities were detected in the 1.5-2.5 mm size class of *B.napus* buds, and these had isoelectric points ranging between 4.75-10.6. Exo-β(1,3)-glucanase activity was detected in duplicate samples, separated on the same gel as above, by applying an agarose overlay containing laminarin, glucose oxidase, peroxidase and TMB and incubating overnight at 37 °C. One exo-β(1,3)-glucanase activity was detected in the extract from the 1.5-2.5 mm buds (Figure 3.5C). This activity had an isoelectric point between 8.3 and 10.6 and corresponded exactly with the β(1,3)-glucanase activity in the same isoelectric point range detected in the other assay with the tetrazolium agent (in other experiments another exo-β(1,3)-glucanase activity was also detected with a pI in the range 5.65-5.9 -

Figure 3.5

Detection of β(1,3)-Glucanase Activities in *B. napus* Buds by *In Situ* Gel Assay and Comparison with Immunoreactive Proteins.

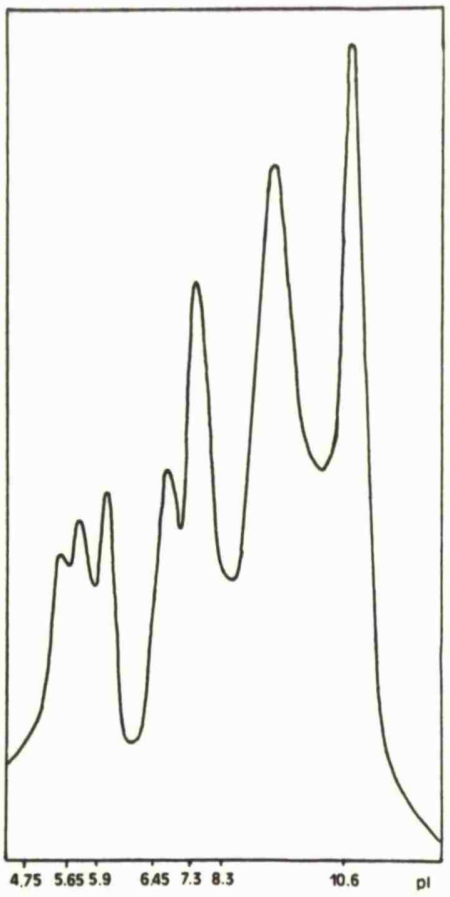
Protein was extracted from *B. napus* buds at various stages of development and separated on several duplicate reverse direction (see Materials and Methods Section 2.3.9) IEF gels under non-denaturing conditions. The positions of the pre-stained isoelectric point markers are indicated on the bottom of the scan. The *in situ* gel assays and immunoblots have been printed to the same size as the scan so that the peaks on the scan can be compared to the position of the activity bands or immunoreactive proteins. Lane1, <1.5 mm buds; lane 2, 1.5-3.5 mm buds; lane 3, >3.5 mm buds.

(A) Gel scan of total β(1,3)-glucanase activity gel. The scan was taken from lane 2 (1.5-3.5 mm buds) of the gel in (B).

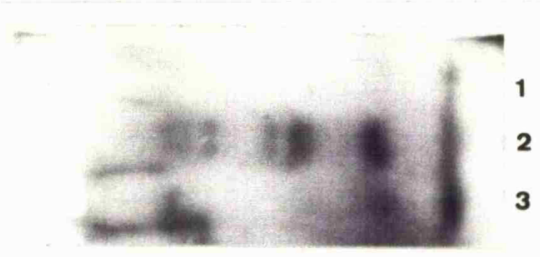
(B) *In situ* activity staining of total β(1,3)-glucanase activities.

(C) Activity staining of exo-β(1,3)-glucanase activities in an agarose gel overlay.

(D) Proteins were separated on a duplicate IEF gel, capillary blotted onto PVDF membrane and probed with the PR β(1,3)-glucanase antiserum.



A



B

C

D

see section 5.2.8). Replicate protein extracts that had been separated on the same IEF gels as those stained for β(1,3)-glucanase activity were blotted onto immobilizing membrane and probed with the PR β(1,3)-glucanase antiserum (Figure 3.5D). Five major immunoreactive proteins were detected from the 1.5-2.5 mm *B. napus* buds and the isoelectric points were at 5.6, 5.9, 5.9-6.45, 6.45 and 7.3. Minor immunoreactive proteins were also detected in this extract and these had isoelectric points between 8.3-10.6. An immunoreactive protein with a isoelectric point 6.45 was also present in the >3.5 mm buds.

3.2.5 An Attempt to Determine the Relationship Between the Immunoreactive Band From *N.tabacum* Anthers with β(1,3)-Glucanase Activity

Crude enzyme extracts were made from *N. tabacum* anthers which had been categorized according to their stage of microsporogenesis or microgametogenesis (meiocyte, tetrad, microspore release, mature pollen grains). These extracts were separated on IEF gels or by native PAGE and then assayed for β(1,3)-glucanase activity or blotted onto immobilizing membrane as described previously for *B. napus* bud enzyme extracts.

Several bands of β(1,3)-glucanase activity were detected with the tetrazolium agent in extracts separated by native PAGE (Figure 3.6A). The slower migrating protein appeared to be temporally regulated, with β(1,3)-glucanase activity present at a low level in the extracts from anthers containing tetrads and at a higher level in anthers containing newly released microspores. A faster migrating protein also detected by this method was present in all of the extracts. However, this activity was at a slightly different position in the extract from the anthers containing the mature pollen grains. The β(1,3)-glucanase present in the leaf ICF extract had a different mobility to any of the anther enzymes.

The PR β(1,3)-glucanase antiserum cross reacted with a protein which was in an equivalent position on the native polyacrylamide gel blot to the constitutive β(1,3)-glucanase activity detected with the tetrazolium agent (Figure 3.6B). A faster migrating protein present in the extract from anthers containing mature pollen grains also immunoreacted with the antiserum and appeared to correspond to an immunoreactive protein in the leaf ICF extract. Although there were several slower migrating immunoreactive proteins, none of these appeared to correlate with the temporally regulated β(1,3)-glucanase activity.

Crude enzyme extracts from the developmentally staged *N. tabacum* anthers were separated on native IEF gels and subsequently assayed *in situ* for β(1,3)-glucanase activity. Three bands of activity were observed (Figure 3.6C). Two activities had isoelectric points near to neutral and were temporally regulated with a peak of activity in the extracts from *N. tabacum* anthers in which microspore release was occurring. These proteins were not

Figure 3.6

Detection of β(1,3)-Glucanase Activities in *N. tabacum* Anthers by *In Situ* Gel Assay and Comparison with Immunoreactive Proteins.

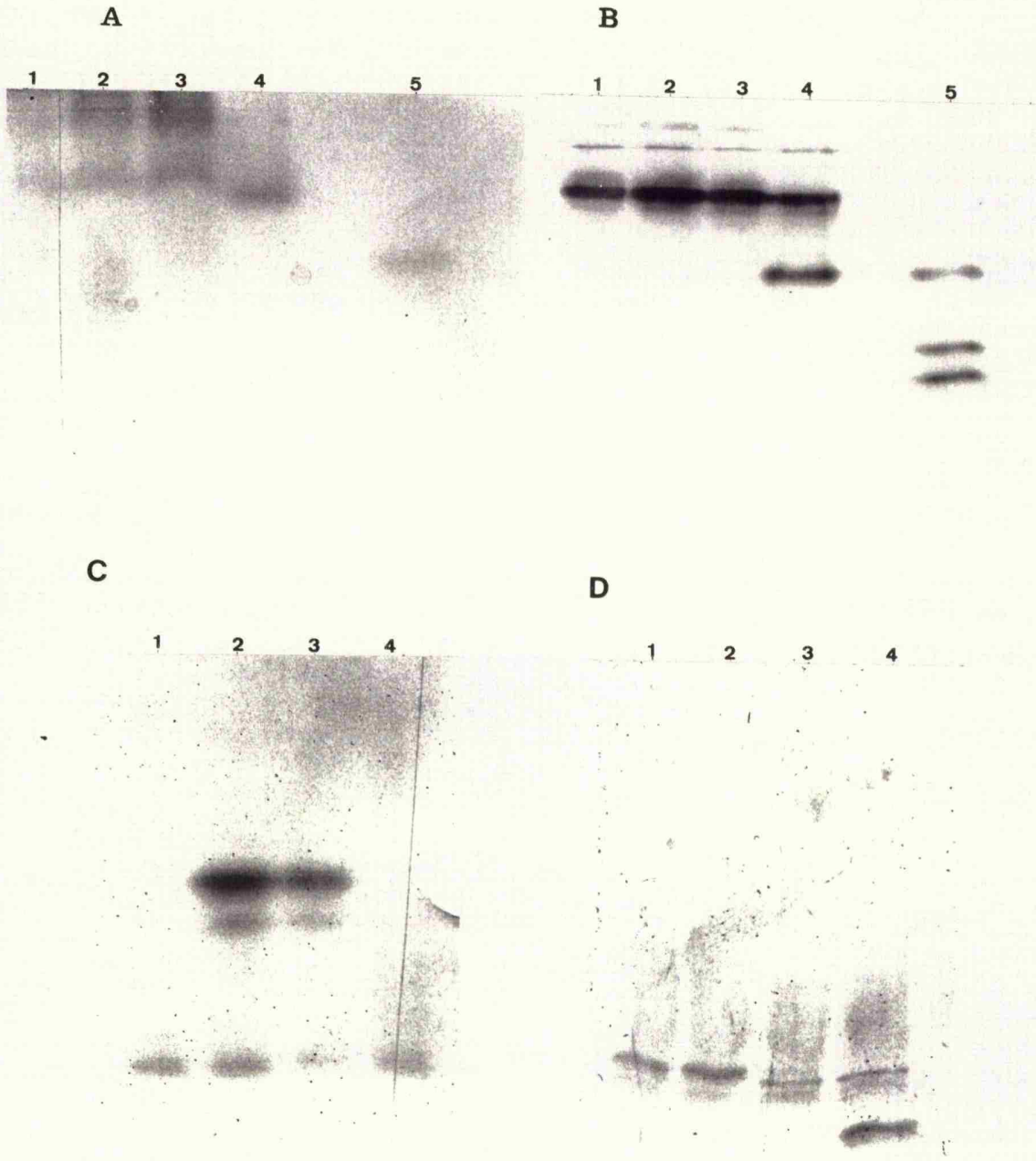
Protein was extracted from *N. tabacum* anthers at various stages of development and separated on duplicate polyacrylamide or IEF gels under non-denaturing conditions. Lane 1, meiocyte stage; lane 2, tetrad stage; lane 3, microspore release stage; lane 4, mature pollen grains; lane 5, leaf ICF.

(A) Protein separated by native-PAGE and assayed *in situ* for total β(1,3)-glucanase activity.

(B) Immunoblot of duplicate gel to (A) probed with the PR β(1,3)-glucanase antiserum.

(C) Protein separated on a native IEF gel and assayed *in situ* for total β(1,3)-glucanase activity.

(D) Immunoblot of duplicate gel to (C) probed with the PR β(1,3)-glucanase antiserum.


A
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present in the extracts from anthers in which mature pollen grains were present or in anthers which contained meiocytes. The third band of β(1,3)-glucanase activity had an isoelectric point of approximately 4.7 and was present in all of the samples. Duplicate protein samples, separated on the same gel as above, were capillary blotted onto immobilizing membrane and probed with the PR β(1,3)-glucanase antiserum. This immunoblot demonstrated that there were no immunoreactive proteins in the same region of the gel as the temporally regulated β(1,3)-glucanase activity (Figure 3.6D). However, there was an immunoreactive protein present in the acidic region of the gel in extracts from all of the developmental stages examined, and this may have therefore corresponded to the β(1,3)-glucanase activity with the isoelectric point of 4.7. An agarose overlay for the detection of exo-β(1,3)-glucanase activity was applied to a gel containing duplicate samples. Although a good level of exo-β(1,3)-glucanase activity was found in the laminarinase positive control, no activity was detected in the *N. tabacum* anther extracts. Further experiments have since determined that the exo-β(1,3)-glucanase activity is only detected in *N. tabacum* extracts if excess protein is loaded on the gel (D. Hird personal communication).

3.2.6 Screening a *B. napus* Anther-Specific "Sporogenesis" cDNA Library with a PR β(1,3)-Glucanase Antiserum

Since an immunoreactive band had been detected in extracts from *B. napus* buds with a PR β(1,3)-glucanase antiserum (section 3.2.3), it was anticipated that this antiserum would identify a protein expressed from a cDNA in the sporogenesis library which was available in the laboratory (Scott *et al.*, 1991a). The sporogenesis library was made from anthers of *B. napus* buds 1.2-1.8 mm in length and was therefore likely to contain cDNAs representing transcripts encoding proteins involved with microspore release. cDNAs inserted in the correct orientation and reading frame in a lambda library can be expressed to yield fusion proteins. The PR β(1,3)-glucanase antiserum recognized both native and denatured proteins in extracts from 1.5-3.5 mm *B. napus* buds which had been transferred from gels to immobilizing membranes. These proteins are likely to display many of the same epitopes as a fusion protein synthesized in bacteria.

For immunoscreening, the cDNA library was plated out on medium without IPTG and incubated at 42 °C to ensure that no fusion proteins toxic to the host were synthesized until plaque formation was underway. After 3 hours, filters impregnated with IPTG were laid on top of the developing plaques. This induced the production of fusion proteins and also provided an imprint of the plaque position on the plate. After further incubation the filters were probed with antibody.

The first immunological screening of the library produced a high background and all of the plaques apparently gave a positive signal. The antisera appeared to contain

components which reacted with antigens produced by *E. coli*. The diluted antiserum was therefore incubated with lysed *E. coli* cells to try to remove the contaminating antibodies by immunoadsorption. The diluted adsorbed antibody solution was used to screen the expression library again. Although the background was reduced by the antibody pretreatment, all of the plaques once again gave a positive signal. Therefore this approach was abandoned.

3.2.7 Screening of the *B. napus* Anther-Specific Sporogenesis cDNA Library with DNA Probes for PR β(1,3)-Glucanases

Three *A. thaliana* β(1,3)-glucanase genes (Dr F. Ausabel, Boston) and a *N. tabacum* PR β(1,3)-glucanase cDNA, pGL43 (Dr F. Meins, Basel), were kindly provided to the laboratory. Two of the *A. thaliana* genes were known to be induced in response to wounding and the other appeared to be under a different control mechanism (Dong *et al.*. 1991). This gene, BG1 and also the cDNA, pGL43 were used to screen the *B. napus* cDNA library. It was hoped that since *A. thaliana* is closely related to *B. napus*, the cDNA may identify an anther-specific β(1,3)-glucanase. However, the library screening failed to identify an anther-specific β(1,3)-glucanase cDNA clone.

3.3 Discussion

3.3.1 β(1,3)-Glucanase Activity in *B. napus* Buds

The work described in section 3.2.1 showed the temporal regulation of β(1,3)-glucanase activity in *B. napus* buds. This study has shown that there are two different β(1,3)-glucanase activities associated with the rise in callase activity during *B. napus* bud development. The endo-β(1,3)-glucanase appears to have significantly more activity *in vitro* than the exo-β(1,3)-glucanase, but maximum levels of activity for both enzymes was detected in extracts from buds close to microspore release stage of microsporogenesis (Figure 3.1). Since the endo-β(1,3)-glucanase activity appears to reach a maximum at microspore release stage, and is apparently the major activity in *B. napus* buds, this is good evidence that this activity is responsible for callose wall degradation (as shown by Stieglitz, 1977).

These experiments were carried out on extracts from whole buds and it is therefore possible that some of the β(1,3)-glucanase activities originated from tissue types other than the anthers. For example, in plant species with a monosporic type of embryo sac such as *B. napus*, a callose wall is also associated with megaspore development (Bouman, 1984).

The callose wall appears transiently just before meiosis in the ovules. A β(1,3)-glucanase activity is presumably also involved with the removal of this callose wall during megasporogenesis in *B. napus*. Therefore, a megaspore associated β(1,3)-glucanase activity could also account for some of the many bands observed in the gel assays described in section 3.2.4.

It is possible that the measurement of exo-β(1,3)-glucanase activity in this experiment is an over-estimation, as other glucose releasing enzymes could be present in *B. napus* buds, for example β-glucosidase. However, the two enzymes can be distinguished by their substrate specificities. Although both the exo-β(1,3)-glucanase and β-glucosidase can cleave glucose from dimers, trimers and tetramers, the exo-β(1,3)-glucanase acts more rapidly on the longer oligomers. Thus, by using laminarin, a 20-unit linear oligomer, as a the substrate it is unlikely that any significant amount of activity was due to β-glucosidase activity.

In studies of *Lilium* anthers, Stieglitz showed directly that the β(1,3)-glucanase activity was mainly due to exo- activity and not β-glucosidase, by conducting assays with various substrates. The endo- and exo-β(1,3)-glucanases of *Lilium* anther extracts were compared with respect to the rate of glucose release from a β(1,4)-dimer, cellobiose (a preferred substrate for β-glucosidase), and laminarin. The amount of glucose released from cellobiose was found to be only 7-9 % of that released from laminarin. This therefore supported the premise that the anther extracts contain primarily exo-β(1,3)-glucanase activity rather than β-glucosidase. A similar investigation could be carried out to determine whether there is any β-glucosidase activity in *B. napus* buds.

3.3.2 *In Vitro* Hydrolysis of the Callose Wall of Isolated Tetrads

The experiment in section 3.2.2 demonstrated that the callose wall was removed from isolated tetrads by treatment with ICF shown to contain PR β(1,3)-glucanases. The PR enzymes therefore appear to be capable of hydrolyzing the tetrad callose wall, demonstrating that the PR enzymes have a common substrate specificity with the anther callase. This experiment thus suggested that probes raised to PR β(1,3)-glucanases could be useful for identifying anther enzymes. In addition, the experiment also provided information on the nature and possible function of the tetrad wall.

The importance of the callose wall in isolating the developing microspores from changing osmotic conditions in the surrounding medium can be seen by comparing the ICF treated and untreated tetrads. At the same magnification, the microspores within the tetrads from each treatment are clearly different in size (Figure 3.3). Without the callose wall the microspores were smaller in size, probably indicating a loss of fluid through osmosis. In

support of this observation Barskaya and Balina (1971), whilst studying the effects of atmospheric drought on microsporogenesis, discovered that moderate drought in Sax beans causes considerable damage on cells which are not protected by a callose wall. The cells which have an intact callose wall do not plasmolyze.

In the absence of the callose wall, the ICF treated microspores were still held together in a tetrad, probably by the primary cellulosic wall. This primary wall must therefore be porous to large enzymes since the β(1,3)-glucanases obviously have access to the underlying callose layer. This observation also suggests that in addition to callase, microspore release in *N. tabacum* requires a cellulase activity. The cellulase activity would need to be under tight developmental control to avoid cellular damage.

It is interesting to speculate about the persistence of the primary cell wall around tetrads in species with a plasmodial type of tapetum. With this type of tapetum, the tapetal cell walls break down and the protoplasts move into the locule and fuse to form a coenocytic plasmodium. Dissolution of the tapetal cell walls can occur as early as the meiosis stage. One would expect that the dissolution of the tapetal cells in this case would also cause the lysis of the primary wall of the meiocytes.

3.3.3 Immunological Investigation into Anther β(1,3)-Glucanases

In section 3.2.3, immunoblotting proteins separated by SDS-PAGE with a PR β(1,3)-glucanase antiserum identified a band in the molecular weight range 50-60 kD in both *N. tabacum* anthers and *B. napus* buds. These findings were initially quite encouraging as no ~60 kD PR β(1,3)-glucanase had been identified previously, and this new immunoreactive protein found in anthers and buds could therefore have represented a new class of anther-specific β(1,3)-glucanase. However, the developmental profiles of the ~60 kD protein in *N. tabacum* anthers and *B. napus* buds were very different. In *B. napus*, the protein was temporally regulated peaking in abundance at microspore release stage, whereas in *N. tabacum* the protein was present at all stages investigated.

The temporal ~60 kD immunoreactive band detected in extracts from *B. napus* buds, appeared to be a good candidate for a protein involved with microspore release as it peaked at the expected time. The band had a diffuse appearance which is sometimes indicative of a glycosylated protein. However, the IEF gel blot (Figure 3.5D) revealed that there were several different immunoreactive proteins, with varying isoelectric points. Therefore the diffuse appearance of the immunoreactive band on blots of proteins separated by SDS-PAGE was probably due to the fact that there were several different proteins all with approximately the same molecular weight but different isoelectric points collecting at the same position on the gel. These proteins were all temporally regulated with respect to bud development and peaked in abundance in 1.5-3.5 mm buds.

In attempt to determine the relationship between immunoreactive proteins from *B.*

napus buds detected on IEF gel blots and β(1,3)-glucanase activity, duplicate IEF gels were assayed directly for total β(1,3)-glucanase activity or applied to an overlay gel and assayed for exo activity. The total β(1,3)-glucanase gel assays showed that there were many isoforms of the enzyme with a range of isoelectric points. Most of these activities peaked in 1.5-3.5 mm buds, which agreed with the results obtained for the β(1,3)-glucanase test tube assays and also with the blots. Only one exo-β(1,3)-glucanase activity was detected in the *B. napus* bud extracts and this activity was temporal and in the basic region, between 8.3 and 10.6, of the IEF gel. (In other experiments a different exo-β(1,3)-glucanase activity was detected with a pI in the range 5.65-5.9 - see section 5.2.8). This activity, with pI between 8.3-10.6, corresponded exactly with an activity detected with the total β(1,3)-glucanase gel assay. The simplest explanation for this result is that the exo-β(1,3)-glucanase activity is detected by both types of assay. This is quite possible since the tetrazolium agent reacts with all reducing sugars, including glucose. Another possibility is that a single protein possesses both endo- and exo-β(1,3)-glucanase activity or more unlikely, there could be two proteins with the same isoelectric point but different activities.

The picture of β(1,3)-glucanase activities in *B. napus* buds therefore appears to be complicated. There are many isozymes, most of which are subject to temporal regulation. Although the PR β(1,3)-glucanase antiserum also recognizes many ~60 kD proteins with varying isoelectric points it is difficult to prove that these proteins are β(1,3)-glucanases. Additionally because extracts from whole buds were used it is also possible that some of the immunoreactive proteins or β(1,3)-glucanase activities originated from tissue types other than the anther. The existence of many forms of β(1,3)-glucanase is expected in a species such as *B. napus* which is a hybrid between *B. oleracea* and *B. campestris*. The hybrid will have enzyme isoforms from both parents. However, perhaps if anthers were dissected from a temporal series of *B. napus* buds the pattern of immunoreactive bands would be simplified.

It seems very unlikely that the "constitutive" ~60 kD immunoreactive protein detected in *N. tabacum* anthers is involved in microspore release unless the protein is a zymogen, which becomes active at the stage when it is required due to the production of an activator or removal of an inhibitor. Another possibility is that this protein is present in anther locations other than the tapetum and locule and thus serves a different role altogether.

The information gained from the β(1,3)-glucanase gel assays did not support the zymogen theory. The immunoreactive protein detected on both native IEF and native polyacrylamide gels did not correspond to the temporal activity which had an isoelectric point of approximately 7. Instead the antiserum recognized an acidic "constitutive" protein. Thus, the temporally regulated callase-associated, β(1,3)-glucanase activity in *N. tabacum*

is immunologically distinct from the PR forms. Exo-β(1,3)-glucanase activity was not detected in extracts from *N. tabacum* anthers in test tube or gel assays. This was surprising as it was considered unlikely that *N. tabacum* would have a different system to *B. napus* and also *Lilium*. The observation that the exo-β(1,3)-glucanase activity in extracts from *B. napus* buds appeared to decrease after storage may also apply to the extracts from *N. tabacum* anthers. Perhaps this enzyme is very unstable and therefore very difficult to detect.

Screening the *B. napus* anther expression library with the PR β(1,3)-glucanase antiserum should in theory have resulted in the isolation of an anther-specific clone as this antiserum appeared to cross react with several proteins in extracts from buds. Perhaps if the problems with background had been overcome and more plaques had been screened a cDNA would have been isolated. However, this part of the project was put to one side when the A6 cDNA was isolated from the library (Scott *et al.*, 1991a). This cDNA was found to have regions of amino acid sequence similarity with PR endo-β(1,3)-glucanases (Dr W. Paul, Leicester University) and it was therefore thought that pursuing work with the A6 cDNA would be more rewarding.

3.4 Summary

The first route taken to clone an anther-specific β(1,3)-glucanase was to utilize probes raised to the PR β(1,3)-glucanases. Although this approach did not lead to the isolation of an anther-specific gene it did provide useful information on the relationship between the PR and anther enzymes. The observation that PR β(1,3)-glucanase enzymes were capable of breaking down the callose wall *in vitro* provided a tool for similar experiments *in vivo*. Removal of the microsporocyte callose wall prematurely using a PR β(1,3)-glucanase under the control of a tapetum-specific promoter would help to elucidate the role which callose plays during microsporogenesis (see Chapter 6).

β(1,3)-Glucanase activity was found to reach maximum levels in *B. napus* buds at microspore release stage of development. There were many isoforms of β(1,3)-glucanase, with different isoelectric points, which together made up this peak in activity. It proved difficult to establish how many, if any, of these isoforms were involved with microspore release. The PR β(1,3)-glucanase antiserum did recognize a high molecular weight temporal band on immunoblots of protein from *B. napus* buds, but it was not clear whether this band actually represented a β(1,3)-glucanase involved with microspore release. An added complication was that endo- and exo-β(1,3)-glucanase activity were both detected in *B. napus* buds. The interaction of endo- and exo-β(1,3)-glucanase in the process of microspore release still has to be determined.

The anther-specific β(1,3)-glucanases appear to be considerably different to PR

β(1,3)-glucanases and therefore the use of such probes to identify the anther clones may not be as straight forward as first envisaged. This approach was therefore put to one side when an anther-specific cDNA clone (A6) was identified which had regions of similarity to the β(1,3)-glucanase family.

Chapter 4

Identification of a cDNA Clone Representing a Putative Anther-Specific β(1,3)-Glucanase

4.1 Introduction

Differential screening of the *B. napus* anther "sporogenesis" cDNA library resulted in the isolation of a range of cDNA clones that displayed anther specificity (Scott *et al.*, 1991a). When sequenced, one of these clones (A6) had significant similarity to the plant β(1,3)-glucanase and β(1,3;1,4)-glucanase families (Dr W. Paul, Leicester University). The temporal regulation of the transcript represented by this cDNA during microsporogenesis appeared to be consistent with the timing expected for an anther-specific β(1,3)-glucanase involved with microspore release (Scott *et al.*, 1991a). This cDNA was identified at the same time as the library was being screened with the probes derived from the PR β(1,3)-glucanases. The library screening was therefore abandoned and work was concentrated on the further characterization of this clone. This chapter details the isolation and sequence analysis of the A6 cDNA clone.

4.2 Results

4.2.1 Characterization of an Anther-Specific cDNA that has Regions of Similarity to β(1,3)-Glucanases

4.2.1.1 Identification of an Anther-Specific cDNA

Anther-specific cDNAs were isolated (Scott *et al.*, 1991a) by differentially screening a sporogenesis library, made from anthers of *B. napus* buds 1.2-1.8 mm in length, with [^{32}P] - labelled first strand cDNA from anther RNA and also from seedling RNA. Isolated cDNAs were then used to probe dot blots of RNA from vegetative plant parts and reproductive tissues. Clones that displayed anther specificity were further characterized by high resolution RNA dot blots to investigate the temporal pattern of mRNA expression represented by the cDNA. The A6 cDNA was among several that had maximum hybridization to RNA from the tetrad stage of development on the high resolution dot blots (Scott *et al.*, 1991a). The transcript cognate to A6 is first detected during meiosis, reaches a peak of abundance at tetrad stage and levels decline again after microspore release. A northern gel blot determined that this pattern of expression was due to transcripts of approximately 1.6 kb (D. Hird, personal communication).

A Southern blot of DNA from various Brassicaceae species (*B. oleracea, B. campestris, B. napus* and *A. thaliana*) showed a complicated pattern of hybridization in all except *A. thaliana* (Dr R. Scott, personal communication). In *A. thaliana*, two or possibly three bands hybridized to the A6 probe, indicating the presence of two or three genes (depending upon the existence of restriction enzyme sites within the genes). The availability of an *A. thaliana* genomic library simplified the problem of identifying one of the many

genes represented by the bands to which A6 hybridized on the Southern blot of *Brassica* DNA, and screening this library provided two different genomic clones, G61 and G62 (Dr R. Scott, Leicester University). The genomic clone G62 was chosen for further analysis (D. Hird, personal communication).

4.2.1.2 Determination of the Nucleotide Sequence of the A6 cDNA, Prediction and Analysis of the Encoded Polypeptide

Sequencing can provide information on the possible function of the messages represented by cDNA clones by the similarity shown to characterized sequences. Therefore, sequencing of the A6 cDNA was started to determine the function of the corresponding message in anther development. Once the initial sequencing had been done, EMBL and Swissprot data base searches were carried out using the deduced amino acid sequence derived from the A6 clone (Dr W. Paul, Leicester University). Regions of significant similarity to the β(1,3)-glucanase and β(1,3;1,4)-glucanase families were discovered. Sequencing of the clone then became incorporated into this project. Subclones and single- or double-stranded templates were produced and the A6 clone sequenced to completion. The cDNA is 1534 base pairs in length and contains an open reading frame extending from 1 to 1424 bp suggesting that the clone is not full length (Figure 4.1), being truncated at the 5'- end. Later, comparison with the *A. thaliana* genomic clones revealed that there were possibly five amino acids, including the methionine missing from the N-terminus.

The predicted peptide for A6 has 474 residues giving an estimated molecular weight of 52.9 kD. The sequence contains a putative hydrophobic N-terminal sequence which conforms with the rules for hydrophobic signal sequences (von Heijne, 1983). If the N-terminus is removed the predicted molecular weight becomes 48 kD. An isoelectric point prediction was carried out on the putative A6 peptide: the full length peptide has a predicted pI of 9.23; if the hydrophobic signal sequence is removed from the N-terminus, the predicted pI drops to 8.94. Thus the peptide represented by A6 appears very basic. However, the prediction does not take into account folding or glycosylation which could alter the actual isoelectric point. The secondary structure prediction shown in Figure 4.2 demonstrates that there are eight possible N-glycosylation sites. This Figure also displays the hydrophobic nature of the N-terminus.

The predicted amino acid sequences for the A6 cDNA and the G62 gene were aligned with the primary structure of several previously described plant endo-β(1,3)-glucanases, a β(1,3;1,4)-glucanase and also an endo-β(1,3)-glucanase from *Saccharomyces cerevisiae* (Figure 4.3). There are representatives of each of the three classes of PR endo-β(1,3)-glucanase (described in section 1.4.4) included on the alignment. The most striking feature of the alignment is the long C-terminal region of the A6 and G62 sequences. The 114 amino acid extension is significantly longer than the C-terminal

Figure 4.1.
Nucleotide and Deduced Amino Acid Sequence of the A6 cDNA.

```
  F  F  L  F  T  L  V  V  F  S  S  T  S  C  S  A  V  G  F  Q  H  P  H  R  Y  I  Q  K  K  T
CTTTCTTCCTCTTCACCCTCGTCGTCTTTTCAAGTACAAGTTGCTCAGCGGTTGGGTTCCAACATCCGCACAGGTATATACAGAAAAAAA
       10        20        30        40        50        60        70        80        90

  M  L  E  L  A  S  K  I  G  I  N  Y  G  R  Q  G  N  N  L  P  S  P  Y  Q  S  I  N  F  I  K
CGATGCTAGAGTTAGCCAGCAAGATTGGTATTAACTATGGTAGACAAGGAAACAACCTACCATCTCCTTACCAATCGATCAATTTCATCA
      100       110       120       130       140       150       160       170       180

  L  I  K  A  G  H  V  K  L  Y  D  A  D  P  E  S  L  T  L  L  S  Q  T  N  L  Y  V  T  I  A
AACTCATCAAAGCCGGTCATGTCAAGCTCTACGACGCCGATCCAGAGAGTCTAACACTCCTCTCTCAAACCAATCTCTACGTCACCATAG
      190       200       210       220       230       240       250       260       270

  V  P  T  H  Q  I  T  S  L  S  A  N  Q  T  T  A  E  D  W  V  K  T  N  I  L  P  Y  Y  P  Q
CGGTGCCAACCCACCAGATCACTTCCCTCAGCGCCAACCAAACTACAGCTGAAGATTGGGTCAAAACCAATATCCTCCCTTACTACCCAC
      280       290       300       310       320       330       340       350       360

  T  Q  I  R  F  V  L  V  G  N  E  I  L  S  V  K  D  R  N  I  T  G  N  V  V  P  A  M  R  K
AAACACAAATACGATTTGTCCTTGTTGGAAACGAAATCCTCTCCGTCAAAGATAGGAACATAACCGGCAATGTCGTACCGGCAATGCGAA
      370       380       390       400       410       420       430       440       450

  I  V  N  S  L  R  A  H  G  I  H  N  I  K  V  G  T  P  L  A  M  D  S  L  R  S  T  F  P  P
AAATCGTGAACTCTCTCAGAGCCCATGGGATTCACAACATCAAAGTCGGTACACCTTTAGCTATGGATTCTCTTCGATCAACGTTTCCGC
      460       470       480       490       500       510       520       530       540

  S  N  S  T  F  R  G  D  I  A  L  P  L  M  L  P  L  L  K  F  L  N  G  T  N  S  Y  F  F  I
CGTCGAACTCAACATTCCGGGGAGATATCGCCTTACCGTTAATGTTGCCGTTGCTGAAGTTTCTCAACGGAACAAACTCTTACTTCTTTA
      550       560       570       580       590       600       610       620       630

  N  L  Q  P  Y  F  R  W  S  R  N  P  N  H  T  T  L  D  F  A  L  F  Q  G  N  S  T  Y  T  D
TCAATCTTCAACCTTACTTCCGTTGGTCAAGAAACCCTAATCACACCACGTTGGATTTCGCTCTGTTTCAAGGAAACTCAACTTATACCG
      640       650       660       670       680       690       700       710       720

  P  H  T  G  L  V  Y  H  N  L  V  D  Q  M  L  D  S  V  I  F  A  M  T  K  L  G  Y  P  Y  I
ATCCTCATACCGGTTTGGTTTACCATAATCTTGTAGACCAAATGTTGGATTCGGTTATCTTCGCCATGACCAAGCTCGGTTATCCATACA
      730       740       750       760       770       780       790       800       810

  R  I  A  I  S  E  T  G  W  P  N  S  G  D  I  D  E  I  G  A  N  V  F  N  A  A  T  Y  N  R
TCCGTATCGCAATCTCTGAAACCGGATGGCCTAACTCCGGCGACATCGACGAAATCGGAGCTAACGTTTTCAACGCCGCCACGTATAACC
      820       830       840       850       860       870       880       890       900

  N  L  I  K  K  M  T  A  T  P  P  I  G  T  P  A  R  P  G  S  P  I  P  T  F  V  F  S  L  F
GGAATTTGATCAAGAAGATGACCGCAACTCCACCAATCGGTACACCAGCTAGACCCGGTTCACCTATACCGACATTTGTTTTCTCCTTAT
      910       920       930       940       950       960       970       980       990

  N  E  N  K  K  P  G  S  G  T  Q  R  H  W  G  I  L  H  P  D  G  T  P  I  Y  D  I  D  F  T
TTAACGAAAACAAGAAACCCGGTTCGGGAACACAAAGACATTGGGGAATCTTGCATCCGGACGGTACACCAATCTACGACATTGATTTTA
     1000      1010      1020      1030      1040      1050      1060      1070      1080

  G  Q  K  P  L  T  G  F  N  P  L  P  K  P  T  N  N  V  P  Y  K  G  Q  V  W  C  V  P  V  E
CCGGTCAAAAACCCTTAACCGGTTTTAACCCTCTGCCTAAACCGACGAATAACGTTCCTTACAAGGGTCAAGTGTGGTGCGTACCGGTCG
     1090      1100      1110      1120      1130      1140      1150      1160      1170

  G  A  N  E  T  E  L  E  E  A  L  R  M  A  C  A  R  S  N  T  T  C  A  A  L  V  P  G  R  E
AAGGAGCCAACGAGACTGAGCTCGAGGAAGCTTTGAGGATGGCTTGTGCCCGAAGCAACACGACGTGTGCGGCTTTGGTTCCTGGCAGAG
     1180      1190      1200      1210      1220      1230      1240      1250      1260

  C  Y  E  P  V  S  V  Y  W  H  A  S  Y  A  L  N  S  Y  W  A  Q  F  R  S  Q  N  V  Q  C  Y
AATGTTACGAGCCGGTCTCTGTTTATTGGCACGCAAGCTACGCGCTTAACTCGTACTGGGCACAGTTCCGTAGCCAAAACGTCCAATGTT
     1270      1280      1290      1300      1310      1320      1330      1340      1350

  F  N  G  L  A  H  E  T  T  T  N  P  G  N  D  R  C  K  F  P  S  V  T  L  *
ACTTCAATGGATTAGCTCATGAGACCACGACTAACCCTGGAAATGATCGCTGCAAGTTTCCGAGCGTTACTCTGTGAGGAAGAACGCCTG
     1360      1370      1380      1390      1400      1410      1420      1430      1440

AAAGAGATTTAAGATGATCAAAGCTGGATTATTCGTATTTACTCATTCTAGATTTTCTGGTTTCTGTTTCGTGTGGCCTAATGTTGAGAA
     1450      1460      1470      1480      1490      1500      1510      1520      1530

AACT
1534
```

Figure 4.2.
Secondary Structure Illustration of the Deduced A6 Peptide Using the Chou-Fasman Prediction.

Putative glycosylation sites are indicated with with a red circle. Hydrophilic and hydrophobic regions are represented with diamond and oval shaped symbols, respectively.

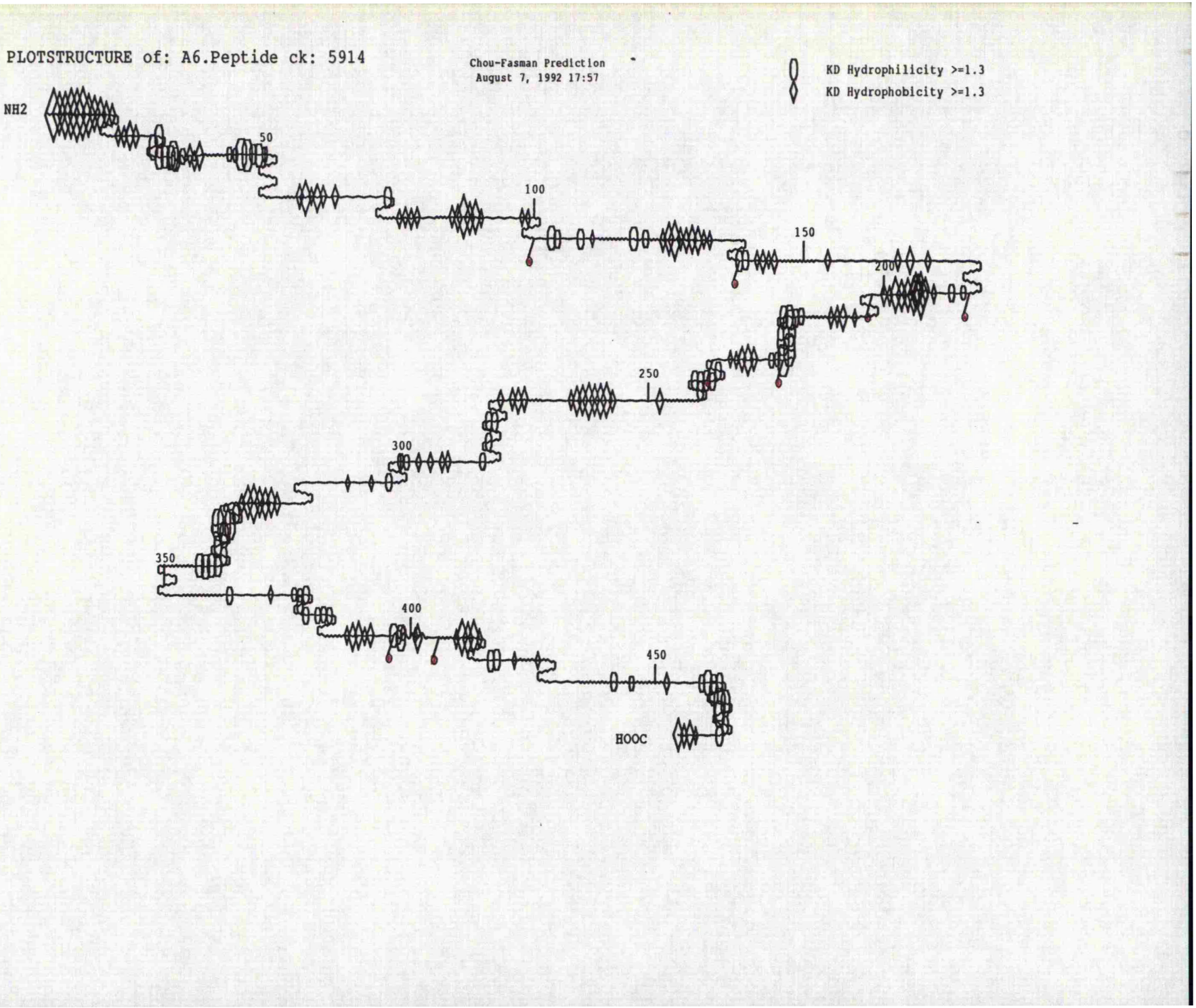

PLOTSTRUCTURE of: A6.Peptide ck: 5914
Chou-Fasman Prediction
August 7, 1992 17:57
KD Hydrophilicity >=1.3
KD Hydrophobicity >=1.3
NH2
50
100
150
200
250
300
350
400
450
HOOC

Figure 4.3.
Sequence Alignment of the Deduced A6 Protein with Selected Plant Endo-β(1,3)-Glucanases and an Endo-β(1,3;1,4)-Glucanase and a *Saccharomyces cerevisiae* Endo-β(1,3)-Glucanase.

The sequences compared are At 1, At 2 and At 3; β(1,3)-glucanases from *A. thaliana* (Dong *et al.*, 1991). Np; basic, class I β(1,3)-glucanase from *N. plumbaginifolia* (de Loose *et al.*, 1988). Pv; basic class I β(1,3)-glucanase from *Phaseolus vulgaris* (Edington *et al.*, 1991). Nt PR-Q'; class III β(1,3)-glucanase from *N. tabacum* (Payne *et al.*, 1990). Nt(II); acidic, class II β(1,3)-glucanase from *N. tabacum* (Linthorst *et al.*, 1990). HvB1314; class II β(1,3;1,4)-glucanase from *Hordeum vulgare* (Slakeski *et al.*, 1990). Hv; class II β(1,3)-glucanase from *H. vulgare* (Høj *et al.*, 1989a). AtG62; A6 sequence from *A. thaliana* (Hird *et al.*, 1993). BnA6; A6 sequence from *B. napus*. Sc; class II endo β(1,3)-glucanase from *S. cerevisiae* (Klebl and Tanner, 1989; Mrsa *et al.*, 1993).

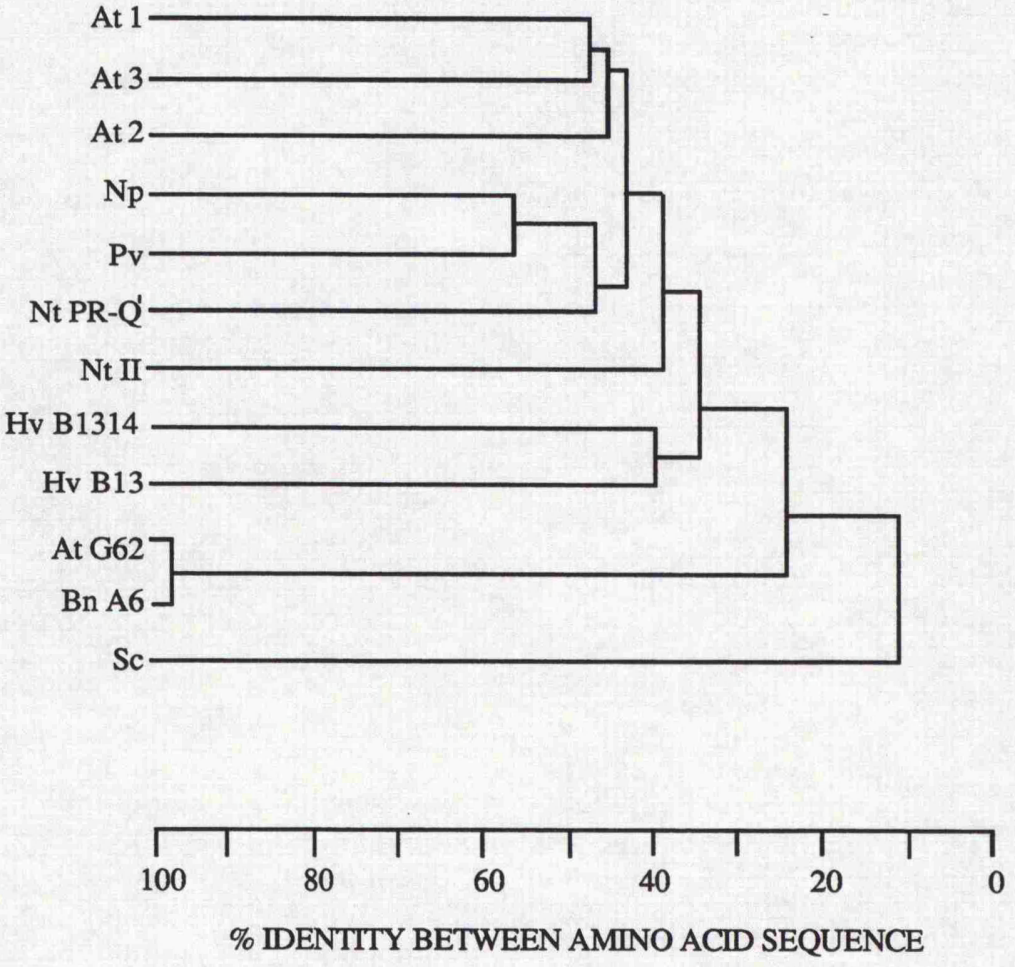
Regions A and B are thought to be involved with catalytic activity. Important conserved residues are shown in bold type . *, conserved residue; ·, conservative substitution.


At1
At3
At2 MLRDARRYLAKS
Np LQMAAIILLGLLVSSTEIVGAQS
Pv Q
NtPR-Q' QFLFSLQMAHLIVTLLLLSVLTLATLDFTGAQ
Nt(II) MTLCIKNGFLAAALVLVGLLICSIQMIGAQS
HvB1314 MAGQGVASMLALALLLGAFASIPQSMGEFFNFLLWQWNSATTLPGVES
Hv MARKDVASMFAAALFIGAFAAVPTSVQS
AtG62 MSLLAFFLFTILVFSSSCCSATRFQGH-RYMQRKTMLDLASK
BnA6 FFLFTLVVFSSTSCSAVGFQHPHRYIQKKTMLELASK
Sc MRFSTTLATAATALFFTASQVSA
At1 VG-VCYGRNGNNLPSPAETIALFKQKNIQRVRLYSPDHDVLAALRGSNIEVTLGLPNSYL
At3 IG-VCYGRNGNNLRPASEVVALYQQRNIRRMRLYDPNQETLNALRGSNIELVLDVPNPDL
At2 IG-RC-----GSLQTTKHPANA----------LYGPDPGALAALRGSDIELILDVPSSDL
Np VG-VCYGMLGNNLPPASQVVQLYKSKNIRRMRLYDPNQAALQALRGSNIEVMLGVPNSDL
Pv IG-VCYGMMGNNLPSANEVINLYRSNNIRRMRLYDPNGAALGALRNSGIELILGVPNSDL
NtPR-Q' AG-VCYGRQGNGLPSPADVVSLCNRNNIRRMRIYDPDQPTLEALRGSNIELMLGVPNPDL
Nt(II) IG-VCYGKHANNLPSDQDVINLYNANGIRKMRIYNPDTNVFNALRGSNIEIILDVPLQDL
HvB1314 IG-VCYGMSANNLPAASTVVNMFKSNGINSMRLYAPDQAALQAVGGTGVNVVVGAPNDVL
Hv IG-VCYGVIGNNLPSRSDVVQLYRSKGINGMRIYFADGQALSALRNSGIGLILDIGNDQL
AtG62 IG-INYGRRGNNLPSPYQSINFIKSIKAGHVKLYDADPESLTLLSQTNLYVTITVPNHQI
BnA6 IG-INYGRQGNNLPSPYQSINFIKLIKAGHVKLYDADPESLTLLSQTNLYVTIAVPTHQI
Sc IGELAFNLGVKNNDGTCKSTSDYET-ELQALKSYT----------STVKVYAASDCNTL
At1 QSVASSQSQANAWVQTYVMNYANGVRFRYISVGNEVK---I---SDSYAQFLVPAMENID
At3 QRLASSQAEADTWVRNNVRNYAN-VTFRYISVGNEVQ---P---SDQAASFVLPAMQNIE
At2 ERLASSQTEADKWVQENVQSYRDGVRFRYINVGNEVK---P---S--VGGFLLQAMQNIE
Np QNIAANPSNANNWVQRNVRNFWPAVKFRYIAVGNEVS---PVTGTSSLTRYLLPAMRNIR
Pv QGLATNADTARQWVQRNVLNFWPSVKIKYIAVGNEVS---PVGGSSWYAQYVLPAVQNVY
NtPR-Q' ENVAASQANADTWVQNNVRNY-GNVKFRYIAVGNEVS---PLNENSKYVPVLLNAMRNIQ
Nt(II) QSL-TDPSRANGWVQDNIINHFPDVKFKYIAVGNEVS---P-GNNGQYAPFVAPAMQNVY
HvB1314 SNLAASPAAAASWVRSNIQAY-PKVSFRYVCVGNEVA---GGATQN-----LVPAMKNVQ
Hv ANIAASTSNAASWVQNNVRPYYPAVNIKYIAAGNEVQ---GGATQS-----ILPAMRNLN
AtG62 TALSSNQTIADEWVRTNILPYYPQTQIRFVLVGNEIL---SYNSGNVSVN-LVPAMRKIV
BnA6 TSLSANQTTAEDWVKTNILPYYPQTQIRFVLVGNEIL---SVKDRNITGN-VVPAMRKIV
Sc QNLGPAAEAEGF---TIFVGVWPTDDSHYAAEKAALQTYLPKIKESTVAGFLVGSEALYR
At1 RAVLAAGLGGRIKVSTSVDMGVLGESYPPSKGSFRGDVM---VIMEPIIRFLVSKNSPLL
At3 RAV--SSLG--IKVSTAIDTRGIS-GFPPSSGTFTPEFR---SFIAPVISFLSSKQSPLL
At2 NAVSGAGLE--VKVSTAIATDTTTDTSPPSQGRFRDEYK---SFLEPVIGFLASKQSPLL
Np NAISSAGLQNNIKVSSSVDMTLIGNSFPPSQGSFRNDVR---SFIDPIIGFVRRINSPLL
Pv GAVRAQGLHDGIKVSTAIDMTLIGNSYPPSQGSFRGDVR---SYLDPIIGYLLYASAPLH
NtPR-Q' TAISGAGLGNQIKVSTAIETGLTTDTSPPSNGRFKDDVR---QFIEPIINFLVTNRAPLL
Nt(II) NALAAAGLQDQIKVSTATYSGILANTYPPKDSIFRGEFN---SFINPIIQFLVQHNLPLL
NtB1314 GALASAGLGH-IKVTTSVSQAILGVYSPPSAGSFTGEAD---AFMGPVVQFLARTGAPLM
Hv AALSAAGLGA-IKVSTSIRFDEVANSFPPSAGVFK---N---AYMTDVARLLASTGAPLL
AtG62 NSLRLHGIHN-IKVGTPLAMDSLRSSFPRSNGTFREEITG--PVMLPLLKFLNGTNSYFF
BnA6 NSLRAHGIHN-IKVGTPLAMDSLRSTFPPSNSTFRGDIAL--PLMLPLLKFLNGTNSYFF
Sc NDLTASQLSDKINDVRSVVADISDSDGKSYSGKQVGTVDSWNVLVAGYNSAVIEASDFVN
At1 LNLYTYFSYAGNIGQIRLDYALFTAPSGIVS-DPPRSYQNLFDAMLDAMYSALEKFGGAS
At3 VNNYPYFSYTGNMRDIRLDYILFTAPSTVVN-DGQNQYRNLFHAILDTVYASLEKAGGGS
At2 VNLYPYFSYMGDTANIHLDYALFTAQSTVDN-DPGYSYQNLFDANLDSVYAALEKSGGGS
Np VNIYPYFSYAGNPRDISLPYALFTAPNVVVQ-DGSLGYRNLFDAMSDAVYAALSRAGGGS
Pv VNVYPYFSYSGNPRDISLPYALFTSPNVVVR-DGQYGYQNLFDAMLDSVHAAIDNTRIGY
NtPR-Q' VNLYPYFAIANNA-DIKLEYALFTSSEVVVN-DNGRGYRNLFDAILDATYSALEKASGSS
Nt(II) ANVYPYFGHIFNTADVPLSYALFTQQEA-----NPAGYQNLFDALLDSMYFAVEKAGGQN
NtB1314 ANIYPYLAWAYNPSAMDMSYALFTASGTVV-QDGSYGYQNLFDTTVDAFYTAMAKHGGSN
Hv ANVYPYFAYRDNPGSISLNYATFQPGTTVRDQNNGLTYTSLFDAMVDAVYAALEKAGAPA
AtG62 LNVHPYFRWSRNPMNTSLDFALFQGHSTYTDPQTGLVYRNLLDQMLDSVLFAMTKLGYPH
BnA6 INLQPYFRWSRNPNHTTLDFALFQGNSTYTDPHTGLVYHNLVDQMLDSVIFAMTKLGYPY
Sc ANAFSYWQ----------GQTNQNASY-SFFDDINQALQVIQSTKGSTD

```
                    ____A_____                                          __B_
At1                 LEIVVAETGWPTGGGVD-TNIE--NARIYNNNLIKHVKNG----TPKRPGKEIETYLFAI
At3                 LEIVVSESGWPTAGGAA-TGVD--NARTYVNNLIQTVKNG----SPRRPGRATETYIFAM
At2                 LEIVVSETGWPTEGAVG-TSVE--NAKTYVNNLIQHVKNG----SPRRPGKAIETYIFAM
Np                  IEIVVSESGWPSAGAFA-ATTN--NAATYYKNLIQHVKRG----SPRRPNKVIETYLFAM
Pv                  VEVVVSESGWPSDGGFG-ATYD--NARVYLDNLVRRAGRG----SPRRPSKPTETYIFAM
NtPR-Q'             LEIVVSESGWPSAGAGQLTSID--NARTYNNNLISHVKGG----SPKRPSGPIETYVFAL
Nt(II)              VEIIVSESGWPSEGNSA-ATIE--NAQTYYENLINHVKSGA--GTPKKPGKAIETYLFAM
HvB1314             VKLVVSESGWPSAGGTA-ATPA--NARIYNQYLINHV--GR--GTPRHPG-AIETYVFSM
Hv                  VKVVVSESGWPSAGGFA-ASAG--NARTYNQGLINHV--GG--GTPKKRE-ALETYIFAM
AtG62               MRLAISETGWPNFGDIDETGANILNAATYNRNLIKKMSASPPIGTPSRPGLPIPTFVFSL
BnA6                IRIAISETGWPNSGDIDEIGANVFNAATYNRNLIKKMTATPPIGTPARPGSPIPTFVFSL
Sc                  ITFWVGETGWPTDGTNFESS--------YPS--VDNAKQFWKEGICSMRAWGVNVIVFEA
                    .   ..*.***. *.    .        *    .                ..    . .*.


                    __B_
At1                 YDEN---QKPTPPYVEKFWGLFYPNKQPKYDINFN
At3                 FDEN---SKQGPE-TEKFWGLFLPNLQPKYVVNFN
At2                 FDEN---KKE-PTY-EKFWGLFHPDRQSKYEVNFN
Np                  FDEN---NKNPE--LEKHFGLFSPNKQPKYPLSFGFSDRYWDISAENNATAASLISEM
Pv                  FDEN---QKSPE--IEKHFGLFKPSKEKKYPFGFGAQRMQRLLLMSSMQHIPLRVTCKLE
NtPR-Q'             FDED---QKDPE--IEKHFGLFSANMQPKYQISFN
Nt(II)              FDEN---NKEGDI-TEKHFGLFSPDQRAKYQLNFN
HvB1314             FNEN---QK-DNG-VEQNWGLFYPNMQHVYPISF
Hv                  FNEN---QKTGDA-TERSFGLFNPDKSPAYNIQF
AtG62               FNEN---QKSGSG-TQRHWGIFDPDGSPIYDVDFTGQTPLTGFNPLPKPTNNVPYKGQVW
BnA6                FNEN---KKPGSG-TQRHWGILHPDGTPIYDIDFTGQKPLTGFNPLPKPTNNVPYKGQVW
Sc                  FDEDWKPNTSGTSDVEKHWGVFTSSDNLKYSLDCDFS
                    . *               .*.       *


Pv                  PSSQSLL
AtG62               CVPVEGANETELEETLRMACAQSNTTCAALAPGRECYEPVSIYWHASYALNSYWAQFRNQ
BnA6                CVPVEGANETELEEALRMACARSNTTCAALVPGRECYEPVSVYWHASYALNSYWAQFRSQ


AtG62               SIQCFFNGLAHETTTNPGNDRCKFPSVTL
BnA6                NVQCYFNGLAHETTTNPGNDRCKFPSVTL
```

Figure 4.4.
Dendrogram Derived from the Percentage Identity Between the Sequences Shown in Figure 4.3.

Only the region of the aligned sequences corresponding to the mature β(1,3)-glucanases has been used for this dendrogram, i.e. the N- and C-terminal extensions were not included.


At 1
At 3
At 2
Np
Pv
Nt PR-Q'
Nt II
Hv B1314
Hv B13
At G62
Bn A6
Sc
100
80
60
40
20
0
% IDENTITY BETWEEN AMINO ACID SEQUENCE

extensions of the basic *N. tabacum* and *P. vulgaris* β(1,3)-glucanases (23 and 32 amino acids, respectively).

Although the *S. cerevisiae* and plant glucanases differ greatly in amino acid sequence, the alignment (Figure 4.3) enables the identification of amino acids that are conserved in all glucanases. As it is likely for sequences adjacent to essential amino acid residues to be conserved in related enzymes, important regions can easily be identified in such alignments. Two such regions labelled A and B are illustrated in Figure 4.3.

The program used for the alignment also generated data that could be used to produce a dendrogram of the percentage identity between the amino acid sequences. Figure 4.4 shows the dendrogram produced when the amino acid sequences of the central more conserved regions are used for the program. These are the regions corresponding to the mature protein of the PR β(1,3)-glucanases and therefore this dendrogram does not include the N-terminal regions or the regions beyond the last C-terminal residue of the mature glucanases. A dendrogram was also generated using the full length sequences and this demonstrated exactly the same order and thus relationship between the sequences, but obviously the level of identity between A6 and the glucanases in this case was much lower due to the long C-terminal extension.

4.3 Discussion

4.3.1 The Protein Derived from the A6 Nucleotide Sequence Has Regions of Similarity with Plant β(1,3) and β(1,3;1,4)-Glucanases

An anther-specific cDNA (A6) was isolated from a *B. napus* anther library. Searching the data bases with the amino acid sequence derived from the A6 cDNA revealed that the protein had regions of significant similarity to the plant endo-β(1,3)- and β(1,3;1,4)-glucanases. The alignment in Figure 4.3 shows that although both the *S. cerevisiae* endo-β(1,3)-glucanase and the A6 sequences display low overall similarity to the other plant glucanases, specific regions can be identified that are conserved throughout all of the sequences. Few gaps were introduced into the sequences to allow alignment of A6, demonstrating the similarity in spacing of the regions of amino acid sequence conservation between A6 and other glucanases. The two main conserved regions A and B contain 9/10 and 5/8 amino acids respectively, that are identical or conservative substitutions. The conserved regions are therefore likely to contain essential amino acids. These amino acids could be necessary for catalysis, glycosylation, enzyme - substrate interaction or the maintainance of secondary or tertiary structure.

Carboxy groups have been implicated in the activity of several β(1,3)-glucanases (Høj *et al.*, 1989b; Macgregor and Ballance, 1991). Two such groups are the glutamic acid (E) residues (equivalent to the codons at 828 and 996 on the nucleotide sequence of the A6

cDNA clone; Figure 4. 1) which are present in the conserved regions A and B in A6 and also occur in all four plant enzymes and in the *S. cerevisiae* β(1,3)-glucanase (shown in bold in Figure 4.3). Inhibitor studies with glucanases purified from *N. glutinosa* suggest that tryptophan (**W**) residues are also important for enzyme activity (Moore and Stone, 1972). The amino acid residue **W** (equivalent to the codon at 837 on the nucleotide sequence of the of the A6 cDNA clone, Figure 4.1), present in the conserved region A, is identical in the *S. cerevisiae* and plant glucanases (Figure 4.3).

The presence of conserved positions in glucanases from evolutionary distinct species of plants suggests that they are important in enzyme function. A6 also contains many of these conserved positions along the length of the peptide sequence therefore providing good evidence for the function of the A6 gene product.

Several of the sequences shown in the alignment have been inferred as β(1,3)-glucanases by comparison of deduced amino acid sequence with partial sequence of purified proteins. Some of the sequences have been shown directly to have β(1,3)-glucanase activity by over-expression studies in *E. coli* (Payne *et al.*, 1990; Castresana *et al.*, 1990). Other isoforms of β(1,3)-glucanase have been identified solely on the basis of comparison of sequence with previously characterized β(1,3)-glucanase sequences. That these deduced sequences are generally accepted as β(1,3)-glucanases supports the conclusion that A6 could be a glucanase despite the fact that enzyme activity has not yet been proven.

4.3.2 A6 May be a Member of a Distinct Class of β(1,3)-Glucanase

In this chapter indirect evidence is presented for the function of the A6 gene product as a β(1,3)-glucanase. This evidence is based on the presence of conserved amino acids, which although relatively few in number, are correctly positioned along the peptide to produce a protein which may have glucanase enzyme activity. Although the alignment allowed the identification of regions of amino acid conservation between A6 and the glucanases, it also highlighted the differences between the sequences. The overall difference between the sequences of the alignment can be observed more clearly in the dendrogram. If A6 is a β(1,3)-glucanase, the divergence of this sequence from PR endo-β(1,3)-glucanases is significant. In the dendrogram, A6 and G62 fall into a group which is distinct from the other glucanases. This raises the question as to whether A6 and G62 might define a new class of β(1,3)-glucanase.

A6 is not the only member of this putative new class of β(1,3)-glucanase, as A6 appears to be a member of a gene family in *B. napus*. Three other partial cDNA clones (A11, A20 and A28) showing nucleotide sequence similarity to the A6 cDNA (S. Smartt, personal communication) were isolated from the *B. napus* sporogenesis library by

differential screening (R. Hodge, personal communication). The cDNAs were 229, 565 and 127 bp in length respectively, and encode peptides of 32, 139 and 19 amino acids. Comparison of the nucleotide sequence of the three partial cDNAs, A6 and the G62 genomic clone over the region of the shortest clone (A28) shows that A11 and A28 are the most similar to each other (Figure 4.5). The A6 cDNA is more similar to G62 gene than any of the partial cDNAs. The G62 gene is therefore most likely to represent the *A. thaliana* homologue of the ***B. napus*** A6 gene rather than genes corresponding to any of the other cDNAs. The existence of many genes that are similar to A6 is confirmed by the complicated pattern of bands hybridizing to the *B. napus* sample on the genomic Southern blot. Some of these bands probably represent A11, A20 or A28.

The following paragraphs describe the features of A6 which separate this gene from previously identified glucanases. If the A6 cDNA does represent a β(1,3)-glucanase, the features of the A6 sequence and deduced peptide suggest that it does not fit into any of the existing classes of β(1,3)-glucanase described in the Introduction (section 1.4.4). The observation that the *A. thaliana* PR β(1,3)-glucanases (At 1, 2 and 3 on the alignment) are more similar to PR β(1,3)-glucanases from other species than to A6 and G62 further implies that A6 and G62 are significantly diverged or of separate origin to the PR enzymes.

The first feature which distinguishes A6 is the unique C-terminal extension which is longer than any possessed by the basic PR endo-β(1,3)-glucanases shown on the alignment. The basic endo-β(1,3)-glucanases of *N. tabacum* contain a C-terminal extension which is cleaved off to give rise to the mature protein. It is thought that this C-terminal extension may be involved with vacuolar targeting of the protein (van den Bulcke *et al.*, 1989). It is possible that the long C-terminal region of A6 also carries targeting information. The long C-terminal extension of A6 is a feature which clearly makes this sequence distinct from the rest of the glucanases.

The high molecular weight predicted for the A6 peptide is a consequence of the C-terminal region. The computer prediction gave a value of 52.9 kD for the molecular weight of the A6 peptide. This is significantly larger than the PR β(1,3)-glucanases, which have molecular weights of approximately 35 kD.

The expression pattern of the A6 gene is different to any of the PR endo-β(1,3)-glucanases. Promoter fusions to *β-glucuronidase* (*gus*) and the ribonuclease, *barnase,* coding sequences have been used to determine the spatial and temporal expression pattern of the gene equivalent to the A6 cDNA (Hird *et al.*, 1993). In transgenic *N. tabacum*, a sharp peak of GUS activity in the tapetum is observed immediately before microspore release, which subsequently declines. Later, during the pollen maturation stage, GUS activity was also found in the pollen but at a much lower level than in the tapetum. Transgenic *N. tabacum* expressing *barnase* under the control of the G62 promoter confirmed the results of the GUS data in that the plants were completely normal in

Figure 4.5
The A6 Sequence is Most Closely Related to the *A. thaliana* Genomic Sequence.

The table illustrates the percentage similarity at the nucleotide level between the *A. thaliana* G62 genomic sequence and the four *B. napus* cDNAs, A6, A11, A20 and A28. Only the regions of DNA common to all sequences are compared.

	At G62	Bn A6	Bn A11	Bn A20	Bn A28
At G62	-				
Bn A6	79	-			
Bn A11	70	78	-		
Bn A20	72	69	66	-	
Bn A28	70	78	98	67	-

appearance except for complete male sterility resulting from *barnase* expression in the tapetum. *B. napus* transformed with the same *gus* constructs had the same tapetum specific temporal pattern of expression but lacked expression of *gus* in the pollen.

PR β(1,3)-glucanases have not previously been detected in the anthers, despite attempts at probing protein gel blots with PR β(1,3)-glucanase antiserum (Lotan *et al.*, 1989). The only PR-type β(1,3)-glucanase demonstrating expression in floral organs is sp41 which is present in the pistil.

It is perhaps not a surprise that the anther has a specific version of β(1,3)-glucanase for the special function of tetrad callose wall dissolution. There are several examples of proteins present in pollen which are different isoforms of proteins that are also present in the vegetative tissue. It could therefore be possible for a separate isoform of β(1,3)-glucanase to be present in the anther to serve this specific purpose. It also seems that differentiation into classes is an evolutionary conserved feature of β(1,3)-glucanases since the same classes of the enzyme can be found in different plant species. This suggests that the different isoforms play different roles in plant growth and development.

If A6 and related sequences do represent a β(1,3)-glucanases the features of these proteins described above suggest that they fall into a completely new class. This class can be defined as containing anther-specific basic isoforms which have a high molecular weight and a characteristic C-terminal extension.

Chapter 5

Immunological Characterization of A6

5.1 Introduction

This chapter details experiments to determine whether the A6 cDNA represents an anther-specific β(1,3)-glucanase. One route taken for the further characterization of the A6 clone was to over-express part of the cDNA in *E. coli* and then use this protein for the production of an antibody. It was possible that the antibody would provide more information on the status of the mature A6 peptide. For example, the antibody may help to determine whether the long C-terminal region was retained in the mature protein.

Previously, anti-enzyme immune serum has successfully been used to inhibit the activity of an exo-β(1,3)-glucanase (Labrador and Nevins, 1989b) and other proteins e.g. nitrate reductase (Notton *et al.*, 1985) providing a direct association of a specific protein with an enzyme activity. Thus it was hoped that antibody raised to the over-expressed A6 protein could also be used in a similar way to inhibit β(1,3)-glucanase activity in extracts from *B. napus* buds. This would then provide more evidence that the A6 peptide represented a β(1,3)-glucanase involved with microspore release in the anther.

5.2 Results

5.2.1 Production of a "Mature" A6 Fragment for Over-Expression Experiments

The production of large amounts of protein in *E.coli* was useful for raising antibody for examining any possible processing of the A6 protein and for use in enzyme inhibition experiments. The alignment of the predicted primary structure for A6 clone with the previously isolated glucanases indicated that the protein encoded by A6 contained a hydrophobic N-terminal signal sequence and a long C-terminal extension. It was thought possible that both of these extensions could be cleaved off during processing to produce a putative "mature" form of the protein. Oligonucleotides complimentary to the A6 sequence were designed for use as primers to amplify a fragment that would encode a putative "mature" protein. The oligonucleotide for removing the N-terminal extension had the following sequence 5'- GGGGAATTCCATGGGCAAGATTGGTATTAACTATGG -3', which included a start codon and restriction enzyme sites *Eco* RI and *Nco* I. The oligonucleotide for removing the C-terminal extension had the following sequence 5'- CCCGGTACCGCGGCTAGGTAAAATCAATGTCG -3', which included a stop codon and restriction enzyme sites *Sac* II and *Kpn* I. Although not discussed in this thesis, the "mature" fragment was also thought to be useful if over expression experiments leading to the production of active protein were to be carried out.

The resulting PCR product was cloned and sequenced to check that the PCR had not introduced errors. The sequence was found to be 100 % identical to the A6 sequence. The

"mature" A6 (mA6), as an *Eco* RI/*Sac* I fragment from pBluescript KS- (Stratagene), was cloned into *Eco* RI/*Sac* I cut intermediate plasmid vector, pIC 19H (Marsh *et al.*, 1984). In-frame cloning of the mA6 fragment into pGEMEX could then be acheived by taking an *Eco* RI/*Hind* III fragment from the intermediate pIC vector and inserting this into *Eco* RI/*Hind* III cut pGEMEX vector.

The pGEM expression system (Promega) was available in the laboratory and although this vector was designed for the production of a fusion protein, which is unlikely to have enzyme activity, it was known to be useful for making large amounts of protein suitable for the production of antibody. The pGEM expression system is based on the T7 expression system developed by Studier and Moffatt (1986). Sequences cloned into the pGEMEX vector are expressed as T7 gene 10 fusion proteins. The bacterial host strain used was K38 harbouring pGP1-2, a plasmid containing a heat sensitive repressor (cl-857) of the T7 polymerase promoter. At 42 °C the repressor is unable to bind to the T7 promoter and therefore allows the expression of the T7 RNA polymerase and in turn the bacteriophage T7 gene 10 fusion protein.

Both recombinant and non-recombinant GEMEX-2 plasmid vectors were transformed into the heat-inducible cell line and cultures derived from single colonies used for the expression experiments. Figure 5.1A shows that a 20 minute induction at 42 °C was sufficient to allow the bacteriophage T7 gene 10 leader peptide to accumulate to great abundance. This protein was also detectable to a lesser extent in the protein extract from un-induced cells. The recombinant vector produced visible amounts of fusion protein after a 20 minute induction at 42 °C, but not to the same levels as the non-recombinant vector. The predicted size of the mA6 peptide is 36 kD, when expressed as a gene 10 fusion protein, this increases to approximately 60 kD, which is the size of the induced protein band shown in Figure 5.1A .

The production of recombinant proteins in the pGEM system often results in the formation of insoluble inclusion bodies containing the fusion protein. These inclusion body complexes can co-precipitate, in a denatured state, with ribosomes, nucleic acids and other cytoplasmic protein. Inclusion body preparations were carried out on the induced recombinant vector containing cells. Figure 5.1B shows that the inclusion body preparations enriched the isolation of the insoluble fusion protein.

5.2.2 Purification of the T7 gene 10-mA6 Fusion Protein for the Production of Antibodies

The inclusion body preparation was not sufficiently pure to use for the production of antibodies and therefore large scale preparative gels were used to separate large amounts of the inclusion body proteins. The T7 gene 10-mA6 fusion protein was then purified by cutting out the band from the gels and electroeluting the protein. The concentration of the purified fusion protein was estimated by comparison with molecular weight markers

Figure 5.1

Over-Expression of 'Mature' A6 (mA6) in *E. coli* and Subsequent Purification of the Peptide for Antibody Production

After separation on 12 % SDS-polyacrylamide gels *E. coli* proteins were stained with Coomassie blue.

(A) Fusion protein mini preparations from *E. coli* containing either non-recombinant or recombinant pGEMEX vector. Lane a, molecular weight marker (SDS-7, Biorad); lane b, pGEMEX; lane c, pGEMEX after 20 minute induction at 42 °C; lanes d, f, and h, independent transformants of pGEMEX-mA6; lanes e, g, and i, pGEMEX-mA6 transformants after 20 minute induction at 42 °C.

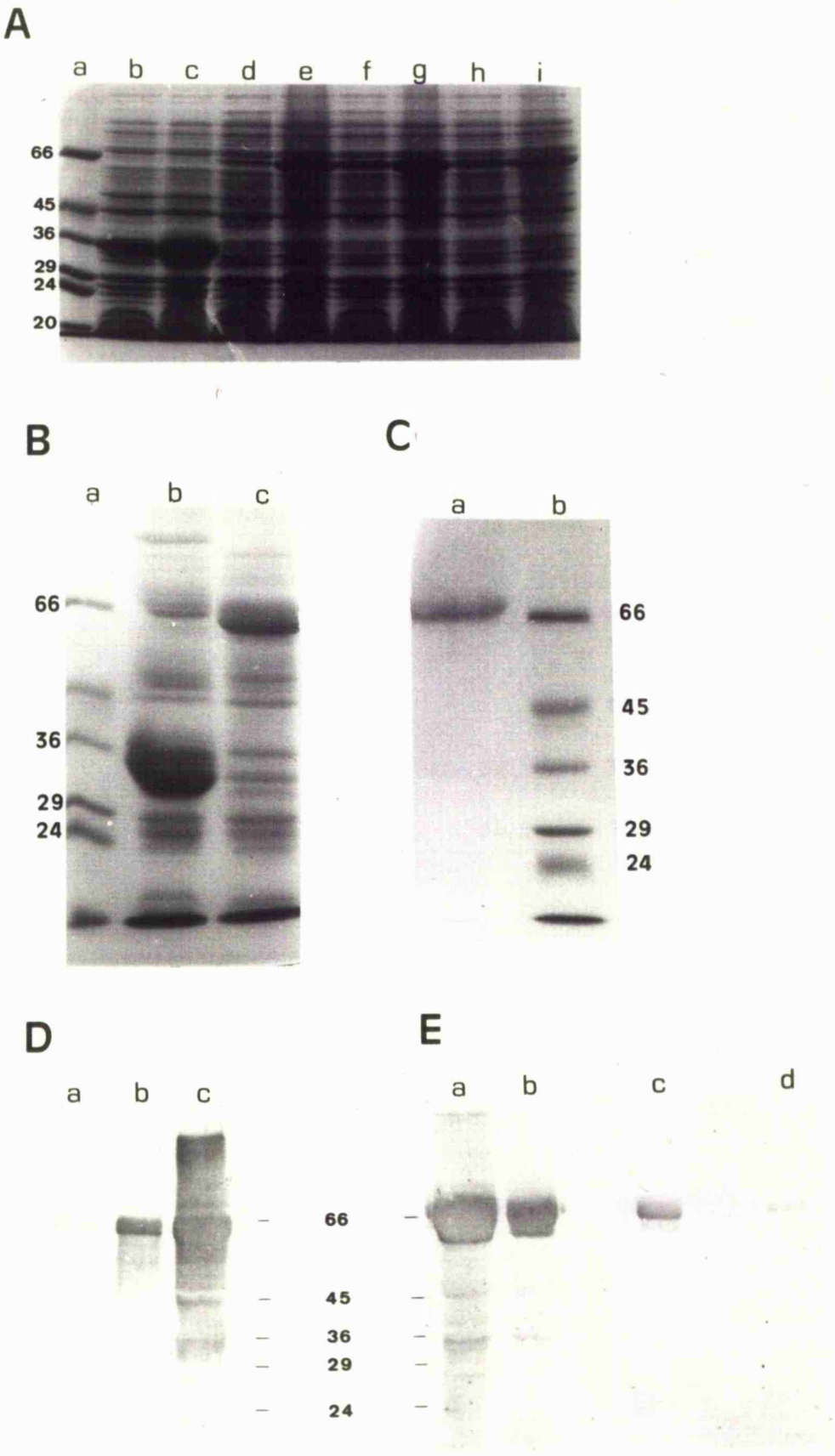
(B) Inclusion body preparations from *E.coli* containing pGEMEX and pGEMEX-mA6. Lane a, molecular weight markers; lane b, pGEMEX; lane c, pGEMEX-mA6.

(C) Purified T7 gene 10-mA6 fusion protein (lane a) compared to SDS-7 molecular weight markers (lane b).

After separation on 12 % SDS polyacrylamide gels inclusion body proteins from *E.coli* containing pGEMEX-mA6 were electroblotted onto PVDF membrane.

(D) Blot probed with 1/ 20, 000 dilution fourth bleed anti-mA6 serum. Lane a, 1/1, 000 dilution inclusion body protein; lane b, 1/100 dilution inclusion body protein; lane c, 1/10 dilution inclusion body protein.

(E) Blot probed with PR β(1,3)-glucanase antiserum. Lane a, 1/10 dilution inclusion body protein; lane b, 1/100 dilution inclusion body protein; lane c, 1/1,000 dilution inclusion body protein; lane d, 1/10, 000 dilution inclusion body protein.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separated on a SDS-polyacrylamide gel (Figure 5.1C). The fusion protein was purified further by dialysing against PBS, to remove SDS, before being used to immunize a rabbit.

5.2.3 Determination of Antibody Specificity and Subsequent Purification

The working dilution of the antibody raised to the mA6 fusion protein was determined. Different dilutions of inclusion body proteins were separated by SDS-PAGE and immunoblotted with varying concentrations of antiserum (Figure 5.1D). Dilutions of fourth bleed serum to 1 in 20, 000 and dilutions of inclusion body protein to 1 in 1, 000 gave a very specific recognition of the T7 gene 10-mA6 fusion protein. The antibodies raised to the introduced protein were therefore at a high enough concentration in the fourth bleed serum to justify their purification.

The accumulation of the T7 gene 10-mA6 fusion protein into insoluble inclusion bodies facilitated the purification of antibody by affinity immunoadsorption. The anti-mA6 antibody activity could thus be separated from the heterogeneous antiserum. The serum was incubated with the recombinant vector inclusion body protein, the antibody-inclusion body complexes spun down and then dissociated. This was followed by incubating the partially purified antibodies with non-recombinant vector inclusion protein, thus removing the antibodies raised to the gene 10 peptide. After purification, a new working dilution for the antibody was determined in the same way as previously described. The working dilution of the purified antibody was 1 in 1, 000. The purified antibody (anti-mA6 antibody) was used in all subsequent experiments.

5.2.4 PR β(1,3)-Glucanase Antiserum Recognizes the T7 gene 10-mA6 Fusion Protein

In an attempt to link the A6 cDNA with β(1,3)-glucanases, different dilutions of inclusion body proteins were separated by SDS-PAGE and immunoblotted with the PR β(1,3)-glucanase antiserum (described in section 3.2.2). Figure 5.1E shows that the antibody was able to recognize the T7 gene 10-mA6 fusion protein, even in the most dilute sample.

5.2.5 Purified Antibody Recognizes A Temporally Regulated Protein in *B. napus* Buds.

The first step towards the characterization of the *B. napus* bud protein represented by the A6 cDNA clone was to investigate the temporal pattern of protein accumulation in *B. napus* buds using the anti-mA6 antibody as a probe.

Protein was extracted from *B. napus* buds of varying lengths and thus different developmental stages. The buds were of the following lengths:- <1.5 mm, 1.5-2.5 mm,

2.5-3.5 mm, >3.5 mm (refer to Figure 1.2 for the developmental stages corresponding to these lengths). Total protein extracts were also made from *B. napus* leaves. The protein samples were separated by SDS-PAGE and duplicate blots probed with either the anti-mA6 antibody or the PR β(1,3)-glucanase antiserum. The immunoblots shown in Figure 5.2A and B demonstrated that both antibodies appeared to recognize a temporally regulated immunoreactive band of approximately 60 kD in extracts from *B. napus* buds. The immunoreactive band was first detectable in <1.5 mm buds, reached a peak in buds of length 1.5-2.5 mm, where microspore release occurs, then declined in abundance again in buds of >3.5 mm. PR β(1,3)-glucanase antiserum also recognized proteins in the *B. napus* leaf extracts. However, as indicated by the strength of the signal and the rapidity of immunostaining, PR β(1,3)-glucanase antiserum had a much stronger affinity for the leaf proteins than the bud proteins.

5.2.6 Investigation into the Spatial Distribution of the Protein Recognized by the Anti-mA6 Antibody

The previous experiment showed that the protein represented by the A6 cDNA was present in buds and that it was regulated in a temporal manner. To further characterize A6 an experiment was designed to determine spatial distribution of the protein within the floral organs.

Protein was extracted from anthers, carpels and petals plus sepals, dissected from three size classes of *B. napus* buds (2.0 mm, 3.0 mm and 5.0 mm). After separation by SDS-PAGE the proteins were immunoblotted with either anti-mA6 antibody or PR β(1,3)-glucanase antiserum. Figure 5.2C shows that the anti-mA6 antibody identified an immunoreacive band in anther, carpel, and the sepal plus petal extracts from the 2.0 mm *B. napus* buds. Immunoreactive bands were also detected in anther and sepal plus petal extracts from 3.0 mm *B. napus* buds. The immunoreactive proteins had a molecular weight of approximately 60 kD. The signal was also present but relatively weak in the carpel extracts from 3.0 mm buds and there was no signal in any of the extracts from the 5.0 mm buds.

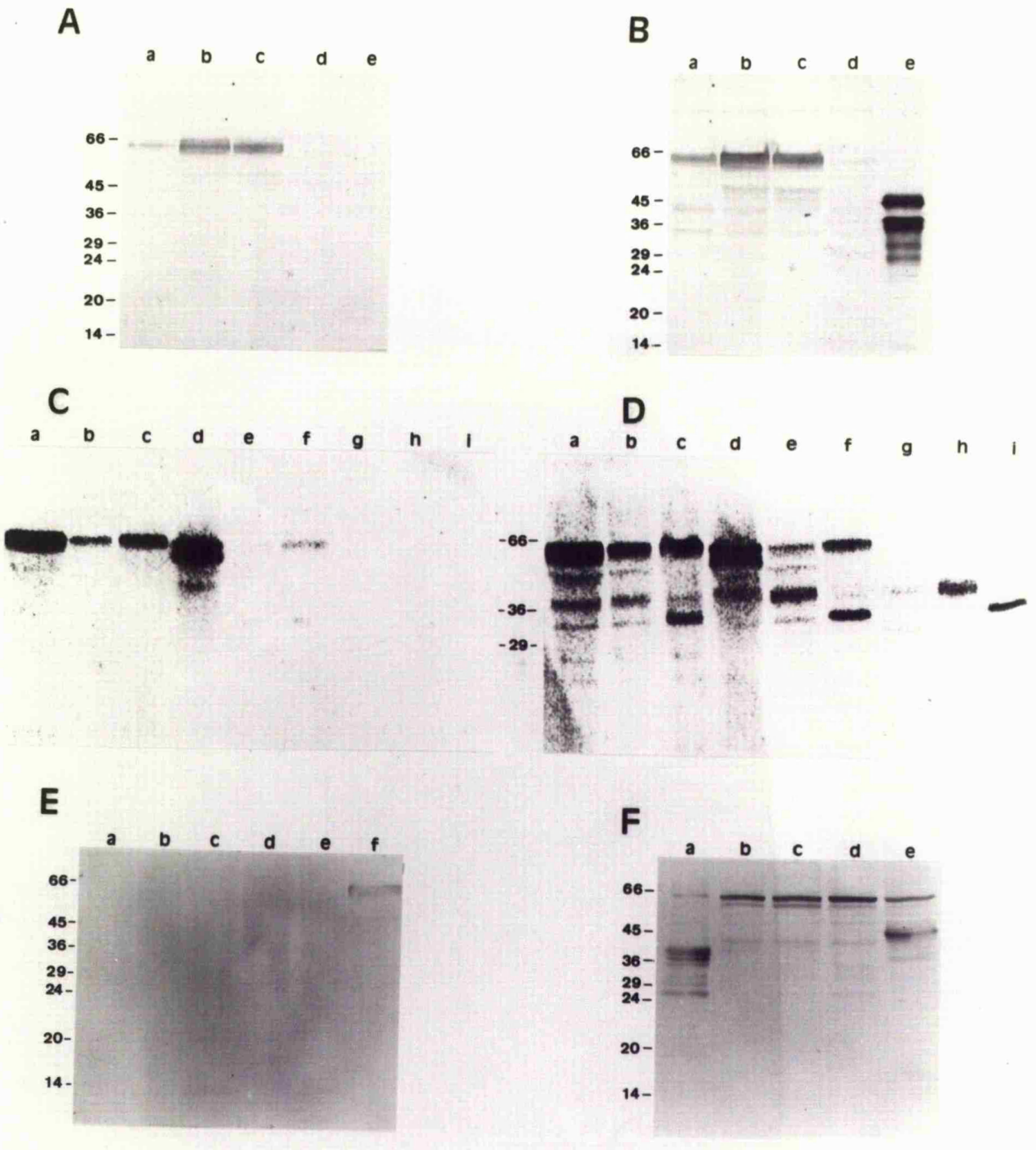
The duplicate blot probed with the PR β(1,3)-glucanase antiserum, demonstrated a much more complicated pattern of immunoreactive bands (Figure 5.2D). In the extracts from 2.0 mm and 3.0 mm *B. napus* buds, bands of approximately 60 kD were detected with the antibody, and these bands appeared to be the same as those identified by the anti-mA6 antibody. However, in this case the signal was relatively strong in the carpel and sepal plus petal extracts. In addition to these temporally regulated immunoreactive bands, there was another strong signal at approximately 30 kD in sepal plus petal extracts from all sizes of *B. napus* bud. The carpel extracts also contained proteins with epitopes for this antibody. In particular, a ~40 kD band was present in the extracts from all of the size

Figure 5.2

Anti-mA6 Antibody and PR β(1,3)Glucanase Antiserum Recognize a ~60 kD Temporally Regulated Protein in *B. napus* Buds.

Duplicate blots of 11 % SDS polyacrylamide gels were probed with the anti-mA6 antibody **(A)**, **(C)** and **(E)** or PR β(1,3)-glucanase antiserum **(B)**, **(D)** and **(F)**. The positions of the molecular weight markers (SDS-7, Biorad) are indicated at the side of each blot.

(A) and **(B)** Protein was extracted from wild-type *B. napus*. Lane a, < 1.5 mm buds; lane b, 1.5-2.5 mm buds; lane c, 2.5-3.5 mm buds; lane d, >3.5 mm buds; lane e, total leaf protein.
(C) and **(D)** Protein was extracted from dissected *B. napus* buds. Lane a, anthers from 2.0 mm buds; lane b, carpels from 2.0 mm buds; lane c, sepals plus petals from 2.0 mm buds; lane d, anthers from 3.0 mm buds; lane e, carpels from 3.0 mm buds; lane f, sepals plus petals from 3.0 mm buds; lane g, anthers from 5.0 mm buds; lane h, carpels from 3.0 mm buds; lane i, sepals plus petals from 5.0 mm buds.
(E) and **(F)** Protein was extracted from *N. tabacum*. Lane a, salicylic acid induced leaf protein; lane b, 2.0 mm anther; lane c, 2.5-3.5 mm anther; lane d, 5 mm anther; lane e, carpels; lane f, *B. napus* bud protein (positive control for blot probed with anti-mA6 antibody).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14
C
a b c d e f g h i
D
a b c d e f g h i
66
36
29
E
a b c d e f
66
45
36
29
24
20
14
F
a b c d e
66
45
36
29
24
20
14

classes of bud examined. An immunoreactive band of apparently the same molecular weight was also present in the anther extracts from the buds of 2.0 mm and 3.0 mm and to a lesser extent in the 5.0 mm buds.

5.2.7 Anti-mA6 Antibody Does Not Detect Proteins in *N. tabacum* Anthers, Carpels or Leaves

Once the anti-mA6 antibody had been used successfully to identify proteins in *B. napus* buds, the cross reactivity of the antibody with proteins from *N. tabacum* species was investigated to determine whether the protein was conserved in another species. Protein was extracted from a temporal sequence of *N. tabacum* anthers, from carpels at various stages of development and also from salicylic acid treated leaves. These protein extracts were analyzed for the presence of immunoreactive proteins after separation by SDS-PAGE by immunoblotting with either anti-mA6 antibody or PR β(1,3)-glucanase antiserum. The blot probed with the anti-mA6 antibody also contained protein extracted from *B. napus* buds as a positive control. It is clear from the blots probed with anti-mA6 antibody (Figure 5.2E) that although the positive control contains an immunoreactive band of the expected size (~60 kD) there were no detectable bands in any of the *N. tabacum* extracts.

In contrast, the duplicate blot probed with PR β(1,3)-glucanase antiserum contained immunoreactive bands in *N. tabacum* leaves, anthers and carpels (Figure 5.2F). In the leaf extract, the main group of immunoreactive bands had an approximate molecular weight of 36 kD, and probably represented the *N. tabacum* PR related β(1,3)-glucanase enzymes. These proteins were not detectable in the extracts from *N. tabacum* anthers or carpels. Instead, a high molecular weight immunoreactive band of approximately 60 kD was present in anther extracts from each stage of development and in the carpel extract (c.f. Chapter 3). A band of the same molecular weight was also present in the leaf extract, but to a much lesser extent. In addition to the ~60 kD immunoreactive band, the carpel extract also contained a 40 kD band which gave a strong signal with the PR β(1,3)-glucanase antiserum.

5.2.8 Does the A6 cDNA Clone Represent a β(1,3)-Glucanase?

The alignment of amino acid sequence encoded by the A6 cDNA with previously isolated glucanases suggested that the A6 gene may encode either the endo-β(1,3)-glucanase component of callase or some other type of anther-specific β(1,3) or β(1,3;1,4)-glucanase activity. The temporal and spatial regulation of the gene corresponding to A6 also supports this theory. One way to further test this hypothesis was to determine whether the anti-mA6 antibody could specifically inhibit exo- or endo-β(1,3)glucanase activity. The profiles of β(1,3)-glucanase activities during

microsporogenesis showed that activity was at a maximum in *B. napus* buds of lengths 1.5-3.5 mm. Microspore release occurs within buds of this range (see Figure 1.2). It was hoped that if the antibody raised to mA6 was added to 1.5-3.5 mm *B. napus* bud protein extracts, either endo- or exo-glucanase activity would be inhibited.

Figure 5.3A shows the profile of exo-β(1,3)-glucanase activity during the relevant interval of microsporogenesis in *B. napus* bud extracts that had been pre-incubated for 2 hours at room temperature with 2 µl of either: affinity purified anti-mA6 antibody, affinity purified anti-phytochrome antibody (provided by G. Whitelam, Leicester), or TBS/BSA solution. The antibodies were added on a working volume basis because the concentration of the antibodies had not been determined. The data shows that exo-β(1,3)-glucanase activity in the bud extracts which had been incubated with anti-mA6 antibody was reduced by more than half of the control activity.

In a different experiment, *B. napus* bud extracts were incubated with the PR β(1,3)-glucanase antiserum (Figure 5.3B). This antibody had a similar affect on exo-β(1,3)-glucanase activity, reducing this to less than half of the control activity

To test these results further, an antibody titration against exo-β(1,3)-glucanase activity was carried out. Varying dilutions of anti-mA6 or anti-phytochrome antibody were incubated with *B. napus* bud extract. After a 2 hour incubation at room temperature these samples and control samples, which had been treated in exactly the same way but lacked antibody, were assayed for exo-β(1,3)-glucanase activity. The reduction in exo-β(1,3)-glucanase activity, due to the presence of antibody, was expressed as a percentage of the activity in control extracts which lacked antibody (Figure 5.4). Although the anti-phytochrome antibody reduced activity slightly, the anti-mA6 antibody had a much greater effect, reducing the exo-β(1,3)-glucanase activity to 6.9 % of the control levels. A volume of 1-3 µl of antibody appeared to be sufficient for maximum reduction in activity.

Although experiments to inhibit endo-β(1,3)-glucanase activity with the antibody raised to mA6 were carried out, the experiments were not successful. This was mainly due to the difficulty of the assays, lack of time and availability of fresh plant material.

Preliminary results seemed to suggest that the anti-mA6 antibody was capable of reducing exo-β(1,3)-glucanase activity, thus providing more evidence that the A6 cDNA represents a β(1,3)-glucanase. It was thought that direct detection of β(1,3)-glucanase isozymes on isoeletrofocussing gels (as described in Chapter 3) and immunoblotting duplicate gels with the anti-mA6 antibody might provide a more informative way of identifying whether the major immunoreactive proteins corresponded to a specific band of β(1,3)-glucanase activity.

The computer predicted a pI of 9.23 for the peptide encoded by the whole A6 cDNA and 8.94 for the cDNA lacking the putative signal sequence (section 4.2.1.2). However, the prediction does not take into account any secondary or tertiary structure of the protein,

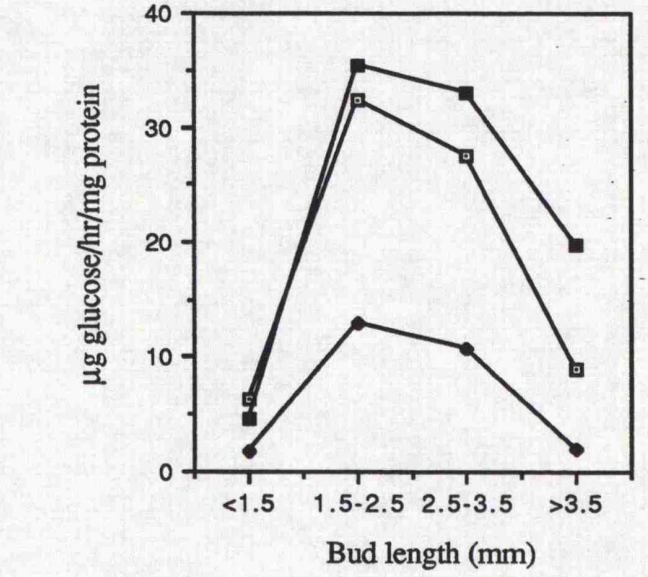
Figure 5.3

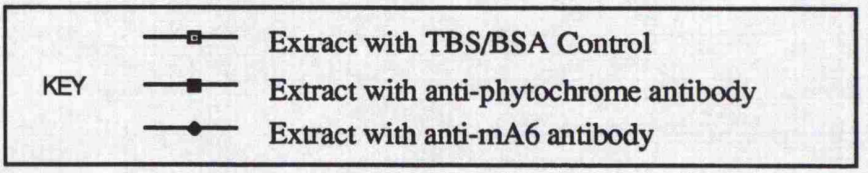
Immunoadsorption Experiments with Exo-β(1,3)-Glucanase Activity Extracted from *B. napus* Buds

(A) Anti-mA6 and anti-phytochrome (control) antibodies.

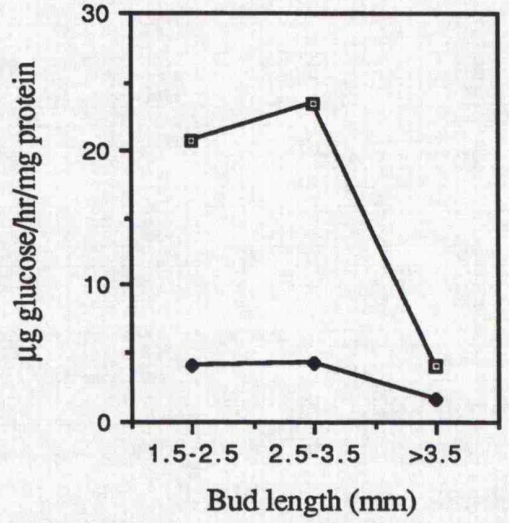
(B) PR β(1,3)-glucanase antiserum.

A,


40
30
20
10
0
µg glucose/hr/mg protein
<1.5
1.5-2.5
2.5-3.5
>3.5
Bud length (mm)


KEY
Extract with TBS/BSA Control
Extract with anti-phytochrome antibody
Extract with anti-mA6 antibody

B,


30
20
10
0
µg glucose/hr/mg protein
1.5-2.5
2.5-3.5
>3.5
Bud length (mm)

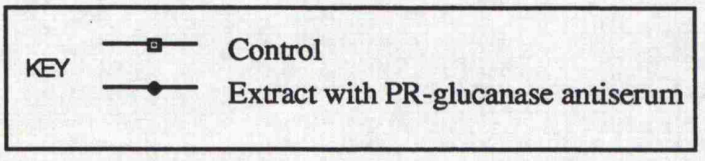
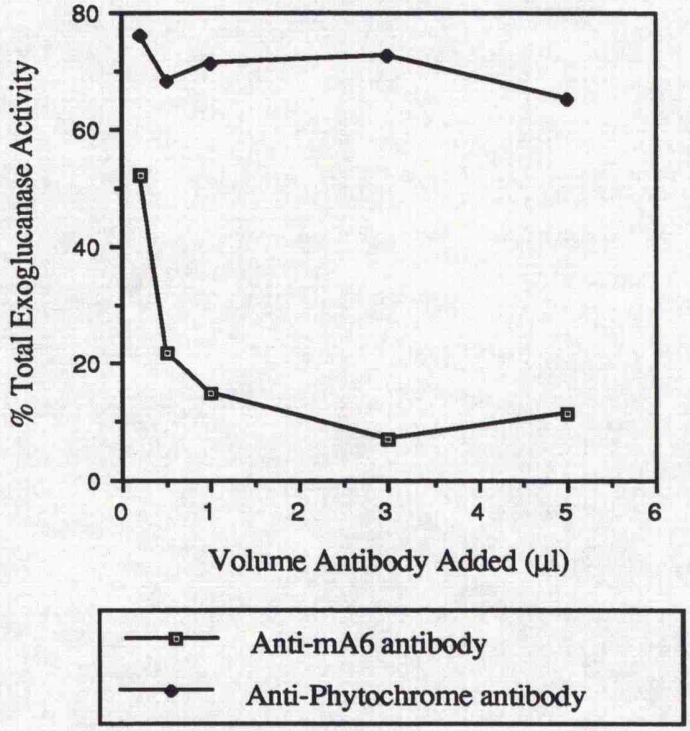

KEY
Control
Extract with PR-glucanase antiserum

Figure 5.4

Inhibition of Exo-β(1,3)-Glucanase Activity from *B. napus* Buds by Immunoadsorption to the Anti-mA6 Antibody

Immunoadsorption was carried out by adding various volumes of anti-mA6 or anti-phytochrome (negative control) antibody to an extract previously shown to have β(1,3)-glucanase activity. After incubation for 2 hours at room temperature, the samples were assayed for exo-β(1,3)-glucanase activity and compared to a control which had no antibody added.


80
60
40
20
0
% Total Exoglucanase Activity
0
1
2
3
4
5
6
Volume Antibody Added (µl)
Anti-mA6 antibody
Anti-Phytochrome antibody

and is therefore prone to errors. The isoelectric point for the protein could actually have been more basic than the prediction. IEF gels are arranged such that the acidic proteins must migrate all the way through the gel before they reach the correct isoelectric point at the bottom of the gel. Basic proteins have the least distance to travel and basic proteins that are not in the range of the ampholytes used in the gel may not enter the gel matrix. Initially, IEF gels were therefore only run for a very short period of time and in reverse orientation, to enable very basic proteins to enter and remain in the gel.

Figure 5.5 shows *B. napus* bud protein from partially purified extracts which were separated in this way and then either assayed for β(1,3)-glucanase activity or blotted onto PVDF membrane. Exo-β(1,3)-glucanase activity was restricted to *B. napus* buds in the size range 1.5-2.5 mm and 2.5-3.5 mm (Figure 5.5A) using the gel overlay technique described in Chapter 3.

Immunodetection experiments using either PR β(1,3)-glucanase antiserum or the anti-mA6 antibody also identified immunoreactive proteins that were restricted to the 1.5-2.5 mm and 2.5-3.5 mm *B. napus* bud extracts (Figure 5.5B and C). Two of these proteins were detected specifically by the anti-mA6 antibody, and the remainder (apparently four) cross reacted with both antibodies. As the IEF gels were run for a short period of time, the isoelectric point of these proteins could not be determined as the gel was not focussed. However, many of the immunoreactive proteins were located in the region of the gel that also contained exo-β(1,3)-glucanase activity.

In other experiments, crude enzyme extracts from sized *B. napus* buds were electrophoresed on reverse orientation IEF gels until equilibrium was reached, and then assayed for β(1,3)-glucanase activities or capillary blotted onto PVDF membrane. The β(1,3)-glucanase gel assays, scans and immunoblots of these samples are shown in Figure 5.6. Two major β(1,3)-glucanase activities were detected using the tetrazolium agent, which is specific for reducing sugars and therefore assays for both endo- and exo-β(1,3)-glucanase activities (see Chapter 3). The most prominent had a pI between 6.45 and 7.3 and the other had a pI of 5.65-5.9 (Figure 5.6A). Again, the β(1,3)-glucanase activities were concentrated in the 1.5-3.5 mm bud extracts, but were also present to a lesser extent in the <1.5 mm bud extracts. Only the more acidic activity was present in the larger bud extract (Figure 5.6B).

These *B. napus* bud extracts appeared to lack the major β(1,3)-glucanase activity (pI of 8.3-10.6) which was visible on the blot shown in Figure 3.5. However, a faint band was visible at this position and was detected by the gel scanner but it was too faint to photograph. There was one exo-β(1,3)-glucanase isoform detected in the gel overlay activity assay. This had a pI of 5.65-5.9 and corresponded exactly with the activity of the same pI detected with the tetrazolium agent. Although this activity band was too faint to photograph it was detected with the gel scanner (Figure 5.6A).

Figure 5.5

Detection of β(1,3)-Glucanase Activities in *B. napus* Buds by *In Situ* gel Assay and Comparison with Immunoreactive Proteins.

Protein was extracted from *B. napus* buds at various stages of development and separated for a short period of time on several duplicate reverse direction (see Materials and Methods Section 2.3.9) IEF gels under non-denaturing conditions.
Lane 1, <1.5 mm buds; lane 2, 1.5-2.5 mm buds; lane 3, 2.5-3.5 mm buds; lane 4, >3.5 mm buds and lane 5, laminarinase control.

(A) Activity staining of exo-β(1,3)-glucanase activities in an agarose gel overlay.

(B) Capillary blot of IEF gel probed with the anti-mA6 antibody.

(C) The same gel as in (B) capillary blotted on the reverse side and probed with the PR β(1,3)-glucanase antiserum.

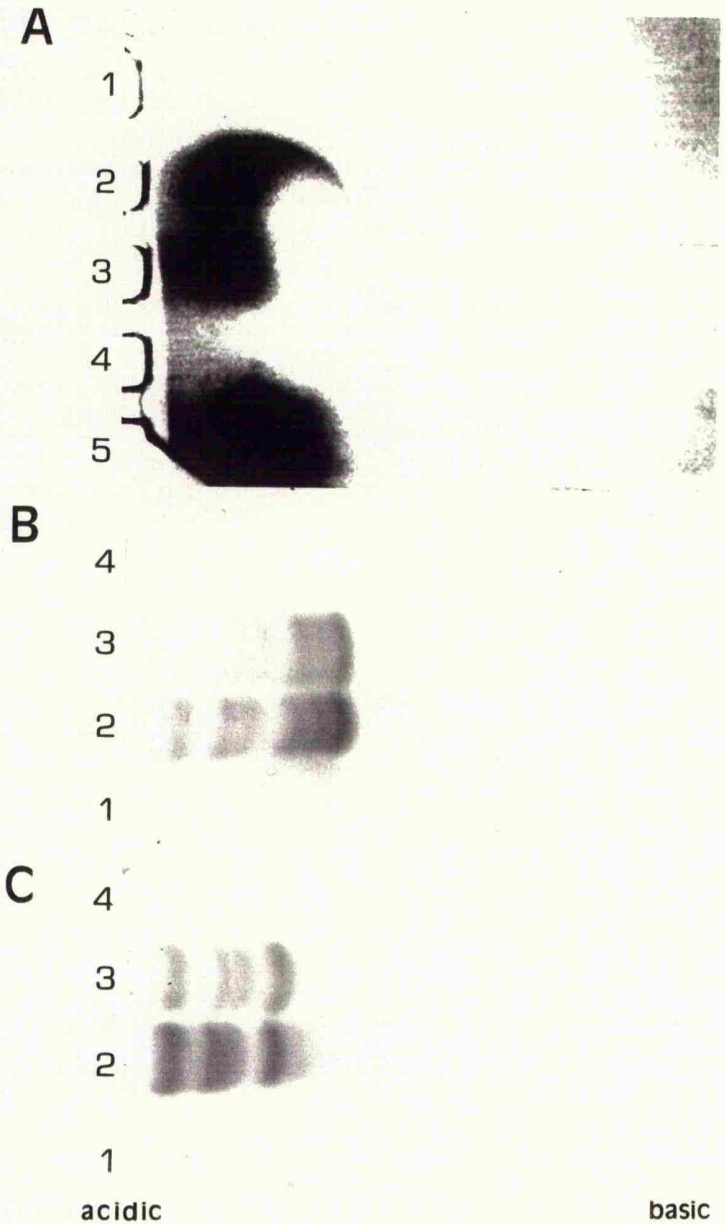

A
1
2
3
4
5
B
4
3
2
1
C
4
3
2
1
acidic
basic

Figure 5.6

Detection of β(1,3)-Glucanase Activities in *B. napus* Buds by *In Situ* gel Assay and Comparison with Immunoreactive Proteins.

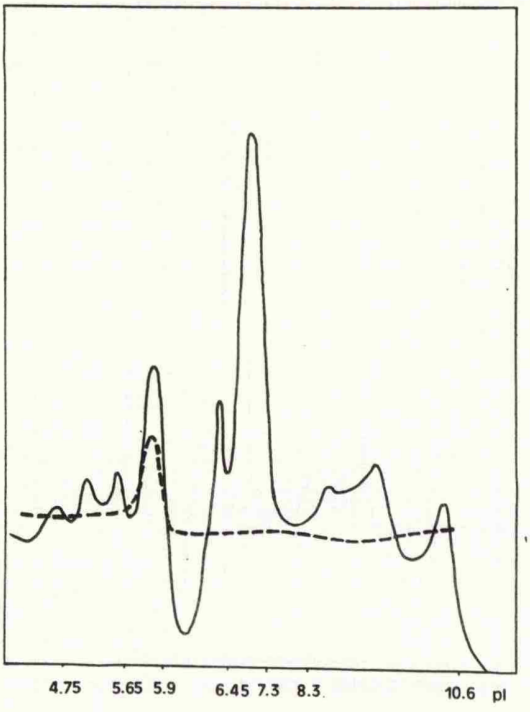
Protein was extracted from *B. napus* buds at various stages of development and separated on several duplicate reverse direction (see Materials and Methods Section 2.3.9) IEF gels under non-denaturing conditions. The positions of the pre-stained isoelectric point markers are indicated on the bottom of the scan. The *in situ* gel assays and immunoblots have been printed to the same size as the scan so that the peaks on the scan can be compared to the position of the activity bands or immunoreactive proteins. Lane 1, <1.5 mm buds; lane 2, 1.5-3.5 mm buds; lane 3, >3.5 mm buds.

(A) Gel scans of total and exo-β(1,3)-glucanase activity gels. Solid line represents total β(1,3)-glucanase activity and broken line represents exo-β(1,3)-glucanase activity. The scan was taken from lane 2 (1.5-3.5 mm buds) in each case.

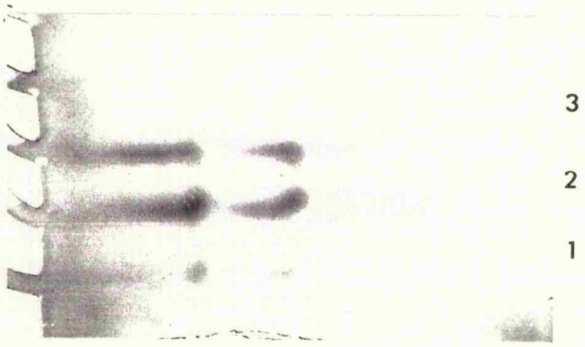
(B) *In situ* activity staining of total β(1,3)-glucanase activities.

(C) Proteins were separated on a duplicate IEF gel, capillary blotted onto PVDF membrane and probed with the anti-mA6 antibody.

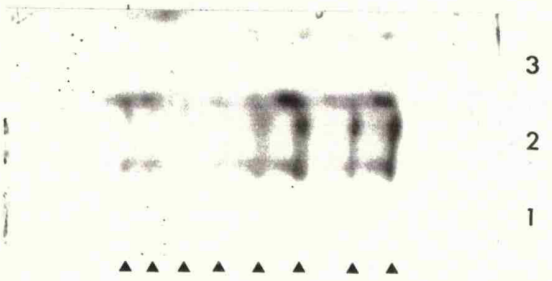
(D) The same gel as in (C) was capillary blotted on the reverse side onto PVDF membrane and probed with the PR β(1,3)-glucanase antiserum.



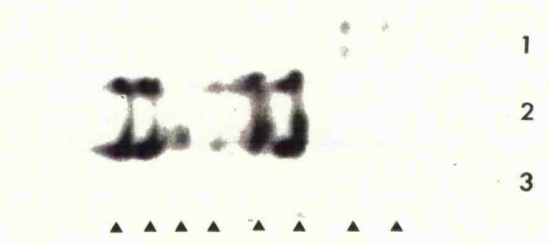
A



B



C



D

Immunostaining the protein blots with the anti-mA6 antibody or the PR β(1,3)-glucanase antiserum produced a complicated ladder of immunoreactive proteins (Figure 5.6C and D). The major cross reacting proteins were in the 1.5-3.5 mm bud extracts. Although, both antibodies appeared to recognize the same proteins, the PR β(1,3)-glucanase antiserum had a stronger affinity for the proteins with isoelectric points in the range 4.75-8.3 and the anti-mA6 antibody had a stronger affinity for the proteins with isoelectric points between 8.3-10.6.

Partially purified *B. napus* bud extracts were electrophoresed on normal direction IEF gels until equilibrium was reached and then assayed for β(1,3)-glucanase activities or capillary blotted onto PVDF membrane. These results are shown in Figure 5.7. Two isoforms of exo-β(1,3)-glucanase activities were evident from the gel overlay assay, one having an approximate isoelectric point of 5.65 and the other between 8.3 and 10.6 (Figure 5.7A and B). Both of these exo-β(1,3)-glucanase isoforms were only visible in the 1.5-2.5 mm bud extracts and both had a similar intensity on the gel scan. β(1,3)-Glucanase activity could not be detected in these extracts with the tetrazolium agent.

The immunoblots (Figure 5.7C and D) from duplicate gels demonstrated a complicated pattern of cross reacting proteins. These were present mainly in the 1.5-2.5 mm and 2.5-3.5 mm bud extracts, but also to a lesser extent in the smaller bud extract. The antibody raised to mA6 appeared to cross react strongly with a protein having an isoelectric point between 8.3 and 10.6 which cross reacted weakly with the PR β(1,3)-glucanase antiserum.

Another protein which was specifically detected with the PR β(1,3)-glucanase antiserum, had an isoelectric point of approximately 4.7, and was present in all four size ranges of *B. napus* buds. Interestingly this immunoreactive protein is not detectable on blots from IEF gels run in the reverse orientation. This is probably because the protein does not enter the gel matrix under these conditions as its isoelectic point is at the limits of the range used in the gel.

As the results of the IEF gel assays are very complex, these together with the results from Chapter 3 have been summarized as simply as possible in Table 5.1.

Figure 5.7

Detection of β(1,3)-Glucanase Activities in *B. napus* Buds by *In Situ* gel assay and Comparison with Immunoreactive Proteins.

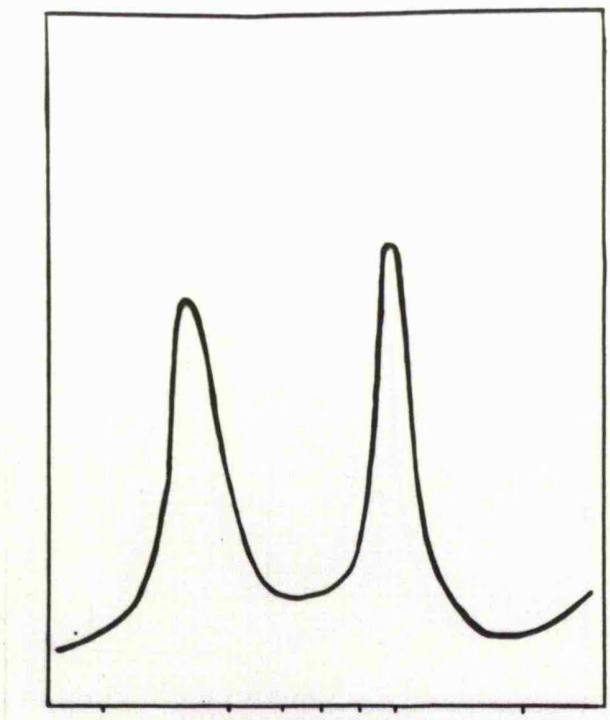
Protein was extracted from *B. napus* buds at various stages of development and separated on several duplicate normal direction IEF gels under non-denaturing conditions. The positions of the pre-stained isoelectric point markers are indicated on the bottom of the scan. The *in situ* gel assays and immunoblots have been printed to the same size as the scan so that the peaks on the scan can be compared to the position of the activity bands or immunoreactive proteins. Lane 1, <1.5 mm buds; lane 2, 1.5-2.5 mm buds; lane 3, 2.5-3.5 mm buds; lane 4, >3.5 mm buds.

(A) Gel scan of exo-β(1,3)-glucanase activity gel. The scan was taken from lane 2 (1.5-2.5 mm buds).

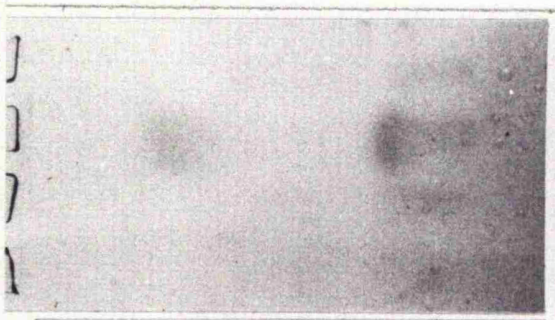
(B) Activity staining of exo-β(1,3)-glucanase activities in an agarose gel overlay.

(C) Proteins were separated on a duplicate IEF gel, capillary blotted onto PVDF membrane and probed with the anti-mA6 antibody.

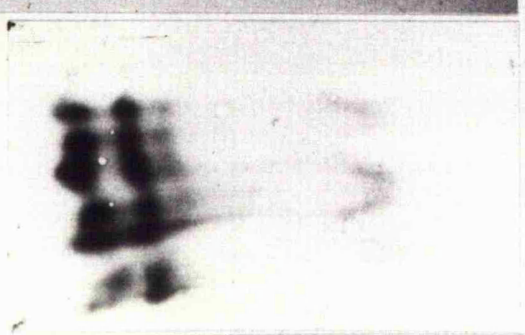
(D) The same gel as in (C) was capillary blotted on the reverse side onto PVDF membrane and probed with the PR β(1,3)-glucanase antiserum.



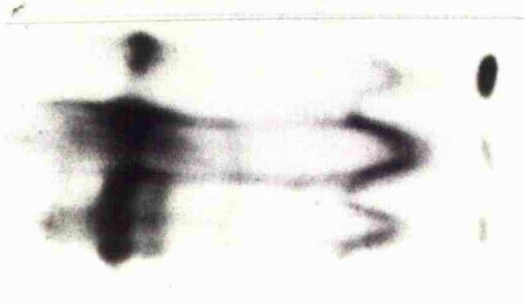
A



B



C



D

Table 5.1

Summary and simplification of the β(1,3)-glucanase activities from *B. napus* buds detected on IEF gels in Chapters 3 and 5.

Isoelectric Point	
Exo- β(1,3)-Glucanase Activity	Total β(1,3)-Glucanase Activity[a]
5.65-5.9	5.65-5.9
---	6.45-7.3
8.3-10.6	8.3-10.6[b]

a, The main peaks of β(1,3)-glucanase activity fall within these isoelectric point ranges determined by pre-stained standards. These main peaks sometimes consisted of 2-3 peaks clustered together.

b, The predicted isoelectric point for the A6 peptide is in this range.

5.3 Discussion

5.3.1 Both Anti-mA6 Antibody and PR β(1,3)-Glucanase Antiserum Detected a Temporally Regulated ~60 kD Band in *B. napus* Buds

In section 5.2.1 a putative "mature" fragment of the A6 cDNA was over-expressed in *E. coli* and antiserum was raised to the protein in rabbit. Initial characterization experiments with the purified antibody (anti-mA6 antibody) showed that a temporally regulated band of ~60 kD was recognized in extracts from *B. napus* buds (section 5.2.5). This band peaked in abundance in buds of length 1.5-2.5 mm, which corresponds with the microspore release stage of development. A ~60 kD band with the same temporal pattern of expression was also detected by the PR β(1,3)-glucanase antiserum in *B. napus* bud extracts. It was therefore assumed that both antibodies cross reacted with the same protein. This ~60 kD immunoreactive band had a slightly greater molecular weight than the computer estimated value of 52.9 kD for the A6 peptide. There are several possible explanations for this size discrepancy, such as the addition of oligosaccharide side chains to the peptide. This is discussed further in section 7.2.2.

Further analysis of proteins in *B. napus* buds was carried out by separation of extracts on native IEF gels and immunoblotting with the anti-mA6 antibody. This study revealed that the ~60 kD immunoreactive band identified on SDS-PAGE blots was actually

comprised of several different proteins with varying isoelectric points but approximately the same molecular weight. A similar pattern of immunoreactive proteins was also detected on duplicate IEF gel blots probed with the PR β(1,3)-glucanase antiserum. It therefore appears that A6 is part of a family of temporally regulated *B. napus* bud proteins.

5.3.2 Immunoreactive Proteins are not Restricted to Anthers of *B. napus* Buds

The spatial distribution of the A6 protein within *B. napus* buds was investigated by immunoblotting protein extracted from various organs of the bud at different stages of development (section 5.2.6). The anti-mA6 antibody recognized a ~60 kD band in extracts from the anthers and also from the sepals plus petals and carpels. These proteins all appeared to be developmentally regulated with the same temporal expression pattern. If the A6 clone does represent a β(1,3)-glucanase involved in microspore release the presence of the same or closely related proteins in the sepals plus petals is difficult to explain.

One possibility is that A6 is not involved in microspore release, but serves some other function. Wall bound exo-β(1,3)-glucanases have been isolated from several plant species (Cline and Albersheim, 1981; Liénart *et al.*, 1986; Kurosaki *et al.*, 1989; Labrador and Nevins, 1989a). These β(1,3)-glucanases appear to be different to the PR β(1,3)-glucanases in that the molecular weight is much greater, which is perhaps consistent with A6 being an exo-β(1,3)-glucanase. A role for these proteins in wall loosening to facilitate cell elongation and growth has been suggested (Labrador and Nevins, 1989a, b). If A6 is related to the exo-β(1,3)-glucanases then the presence of the protein in the anthers, carpels and sepals and/or petals could reflect a role in the rapid expansion of the bud as it develops and matures.

It is also possible that anther protein corresponding to A6 does represent an exo-β(1,3)-glucanase component of callase, and the proteins immunologically related to A6 detected in other floral organs serve some different function, such as cell wall loosening as suggested above. It is interesting to note that the anti-mA6 antibody does not cross react significantly with any proteins in extracts from *B. napus* leaf material and therefore seems to be a isoform that is unique to the floral organs.

The SDS-PAGE blot of dissected *B. napus* buds extracts probed with the PR β(1,3)-glucanase antiserum displayed a complicated pattern of immunoreactive bands. In addition to the band at ~60 kD which was also detected by the anti-mA6 antibody, this antiserum detected numerous other developmentally regulated and constitutive proteins. Many of these proteins were not detected on blots containing whole bud extracts, probably because they were diluted by more abundant proteins. Lotan *et al.* (1989) reported that PR proteins accumulate during flower development and it is therefore quite likely that some of the immunoreactive proteins may be organ-specific versions of the PR β(1,3)-glucanases.

The ~40 kD protein detected in carpels is probably a *B. napus* version of the 41 kD protein from *N. tabacum* (sp41) characterized by Ori *et al.* (1990) which represents a diverged, organ specific version of the PR β(1,3)-glucanase family thought to be associated with the transmitting tract of the style.

5.3.3 The Anti-mA6 Antibody Does not Detect Proteins in *N. tabacum* Anthers, Carpels or Leaves

An attempt was made to utilize the anti-mA6 antibody to identify a protein equivalent to A6 in *N. tabacum*. Despite carrying out immunoblots on extracts of anthers, carpels and leaf which had been separated by SDS-PAGE, no immunoreactive proteins were detected in *N. tabacum*. The PR β(1,3)-glucanase antiserum did cross react with proteins in *N. tabacum* anthers, carpels and leaves. However, the ~60 kD band identified in anthers and to a lesser extent in carpels and leaves is unlikely to be related to A6 or involved with microspore release as it is not temporally regulated. The ~40 kD band detected in the carpel tissue probably represents the sp41 protein described above (Ori *et al.*, 1990).

The PR β(1,3)-glucanase antiserum, which was raised to a *L. esculentum* protein, appeared to cross react with many of the same *B. napus* proteins as the anti-mA6 antibody, yet these antibodies did not recognize any temporally regulated proteins in *N. tabacum*. This may be a surprise since *N. tabacum and L. esculentum* are more closely related than *L. esculentum* and *B. napus*. Perhaps is would be informative to probe *L. esculentum* anther proteins with the two antibodies.

5.3.4 Is A6 a β(1,3)-Glucanase Involved with Microspore Release?

Duplicate SDS-PAGE blots of *B. napus* bud protein probed with either the anti-mA6 or PR β(1,3)-glucanase antiserum showed that both appear to recognize the same temporally regulated ~60 kD band. Subsequent IEF analysis showed that this band consisted of several different immunoreactive proteins, one of which is the A6 protein. There are several features of the A6 protein which suggest that it may represent a new class of β(1,3)-glucanase involved with microspore release. Firstly, the sequence data presented in Chapter 4 indicated that that although A6 contains amino acids essential for glucanase activity is very different to previously characterized glucanases. Secondly, the A6 protein is larger than any of the PR β(1,3)-glucanases so far isolated, but similar to the exo-β(1,3)-glucanase purified from the anthers of *Lilium* by Stieglitz (1977) and to other exo-β(1,3)-glucanases purified from plant cell walls (Cline and Albersheim, 1981; Liénart *et al.*, 1986; Kurosaki *et al.*, 1989). Unfortunately, no sequence data is available for these proteins and therefore the relationship between A6 and these plant exo-β(1,3)-glucanases cannot be established. Additionally, although there are proteins immunologically related to

A6 in several floral organs, the A6 gene is only expressed in the anther. The sequence data, spatial distribution and the size of A6 peptide provide indirect evidence for the possible function of the protein as a β(1,3)-glucanase involved with microspore release. In the latter part of this Chapter, the anti-mA6 antibody was used to specifically associate the A6 protein with β(1,3)-glucanase activity.

An immunological relationship between the A6 peptide and the endo-β(1,3)-glucanases was directly demonstrated. Dilutions of the over-expressed mA6 protein down to 1/1000 were still identified on blots probed with the PR β(1,3)-glucanase antiserum, indicating a strong antibody affinity for the protein.

Preliminary results with the anti-mA6 antibody seemed to indicate that exo-β(1,3)-glucanase activity from *B. napus* buds was inhibited by the presence of the anti-mA6 antibody (section 5.2.8). This provided more evidence that A6 may represent an exo-β(1,3)-glucanase. However, it may also be possible that the reduction in the amount of glucose detected was due to inhibition of β-glucosidase activity by the presence of the antibody. β-Glucosidase is a more general type of glucose releasing enzyme which has less specificity for the type of dimer linkage than exo-β(1,3)-glucanase (Reese *et al.*, 1968). As described in Chapter 3, Stieglitz (1977), compared β(1,3)-glucanase activity in *Lilium* anther extracts with respect to the rate of glucose production from laminarin and cellobiose, a preferred substrate for β-glucosidase because it is β(1,4)-linked. The conclusion drawn from this investigation was that the exo-β(1,3)-glucanase activity detected in anthers of *Lilium* was not attributable to β-glucosidase activity. The fact that laminarin was used as a substrate in the assays described in this thesis makes it unlikely that any exo-β(1,3)-glucanase activity was attributable to β-glucosidase activity. However, if β-glucosidase activity was detected in *B. napus* buds, cellobiose could be used as a substrate for IEF gel assays to distinguish between the two enzyme activities. Inhibitors of β-glucosidase activity such as β(1,5)-D-gluconolactone could also be used to distinguish between exo-β(1,3)-glucanase and β-glucosidase activity.

The presence of the PR β(1,3)-glucanase antiserum also appeared to reduce the exo-β(1,3)-glucanase activity in extracts from *B. napus* buds. This suggests that some level of identity exists between endo- and exo-β(1,3)-glucanases. Unfortunately, when experiments using the anti-mA6 antibody to inhibit endo-β(1,3)-glucanase were carried out no valid data were obtained.

It was anticipated that direct detection of β(1,3)-glucanase activity on IEF gels in conjunction with immunoblotting of duplicate gels would have given more insight into the relationship between the A6 clone and the β(1,3)-glucanase family. An added complication to this approach of trying to link immunoreactive bands with activity bands is that the

antibody will also recognize degradation products which may not have any activity. However, upon close inspection of the gels and blots it appears that both the anti-mA6 antibody and PR β(1,3)-glucanase antiserum do in some cases cross react with proteins in regions of the IEF gels that also contain β(1,3)-glucanase activity. For example, activity was detected in the isoelectric point range 8.3-10.6 with both β(1,3)-glucanase gel assays and immunoreactive proteins were also detected in this range (see table 5.1). Another observation, which was most noticeable on the unfocussed IEF gel blots (Figure 5.5), was that the anti-mA6 antibody appeared to cross react with these basic proteins more strongly than the PR β(1,3)-glucanase antiserum. Since the predicted pI for the A6 peptide is also very basic it seems very likely that the protein cognate to the A6 cDNA is amongst this group of basic proteins.

Similarly, the PR β(1,3)-glucanase antiserum recognized proteins not detectable by the anti-mA6 antibody on IEF gel blots. The clearest example of this is on Figure 5.6D where a very acidic protein was identified in all of the *B. napus* bud extracts examined. This result agrees with the data from the SDS gel blots of dissected *B. napus* bud extracts where an array of proteins were identified.

5.4 Summary

The putative mature region of the A6 peptide was over-expressed in *E. coli*. Once purified from inclusion bodies this protein was used to raise antibodies. The corresponding anti-mA6 antibody recognized a band of ~60 kD in *B. napus* buds, which appeared to be temporally regulated in accordance with microspore release. Separation of *B. napus* bud protein on IEF gels followed by immunoblotting showed that the 60 kD band actually represented several different proteins having various isoelectric points but approximately the same molecular weight. Although the anti-mA6 antibody appeared to cross react with several different proteins, the antibody favoured proteins in the more basic region of the IEF gels. This is consistent with the fact that a basic pI for the A6 peptide was predicted by computer. Although the anti-mA6 antibody also cross reacted with developmentally regulated ~60 kD proteins in extracts from *B. napus* carpels and sepals plus petals, no immunoreactive proteins were detected in anthers or carpels of *N. tabacum* flowers.

Preliminary attempts to try to identify the enzymic activity of the A6 peptide involved antibody inhibition experiments. Adding anti-mA6 antibody to extracts from *B. napus* buds appeared to inhibit exo-β(1,3)-glucanase activity. Further experiments are required to determine whether endo-β(1,3)-glucanase activity is also inhibited. β(1,3)-Glucanase activity gels and duplicate immunoblots were difficult to interpret due to the number of activity bands and immunoreactive proteins obtained. In some cases it appeared that immunoreactive proteins did co-migrate with β(1,3)-glucanase activity bands. However, further experiments are required to determine conclusively the function of the A6 protein.

Chapter 6

Investigating the Significance of the Tetrad Callose Wall

6.1 Introduction

Male sterility has been attributed to the premature appearance of callase, a β(1,3)-glucanase activity, in the anther locule of *Petunia* line RM cms (Izhar and Frankel, 1971). This early enzyme activity results in hydrolysis of the callose wall before the microspores are at the correct stage of development. Further studies of the same line of *Petunia* suggest that other aspects, besides premature appearance of callase, are the primary cause of the male sterility (Izhar and Frankel, 1973; Nivison and Hanson, 1989; Connet and Hanson, 1990). Thus this male-sterile line appears to have a complicated phenotype and conclusions about the role of callose in microsporogenesis cannot be drawn from it.

However, one way to analyze the role of the callose wall was to mimic one aspect of the RM *Petunia* line, namely the premature dissolution of the callose wall, by expressing a β(1,3)-glucanase activity earlier in development than the natural callase activity. Two components were required for the construction of a chimaeric gene capable of causing premature callose wall dissolution when expressed in plants. These were, a β(1,3)-glucanase that would be secreted from the tapetal cells into the anther locule and a promoter to drive the expression of this β(1,3)-glucanase prior to the appearance of normal callase activity in the locule.

Although the anther-specific β(1,3)-glucanase enzyme (callase) involved in microspore release had not been cloned, several PR β(1,3)-glucanases were well characterized at the time these experiments were conducted. In Chapter 3 it was demonstrated that ICF extracted from salicylic acid-treated *N. tabacum* leaves, containing PR β(1,3)-glucanase enzymes, was capable of breaking down the callose wall *in vitro*. Therefore, the PR β(1,3)-glucanases are able to hydrolyze the callose of the tetrad wall and might therefore substitute for callase *in vivo*. Shinshi *et al.* (1988) reported the nucleotide sequence of a *N. tabacum* basic vacuolar endo-PR β(1,3)-glucanase. It was anticipated that expression of this gene under the control of tapetum-specific promoters that are transcriptionally active during meiosis (Scott *et al.*, 1991a, b; Paul *et al.*, 1992) would cause premature breakdown of the callose wall and thus provide information regarding the role of the callose wall in microsporogenesis.

6.2 Results

6.2.1 Construction of Chimaeric Genes for the Expression of a Modified Basic PR β(1,3)-Glucanase in the Tapetum

Figure 6.1A shows that vacuolar isoforms of PR β(1,3)-glucanases of *N. tabacum* are initially synthesized as precursors with N- and C-terminal peptide extensions. The

Figure 6.1.

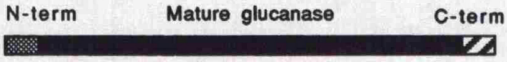
Construction of a Modified Basic β(1,3)-Glucanase Gene.

(A) Schematic representation of the coding region of the *N. tabacum* basic vacuolar PR β(1,3)-glucanase gene.

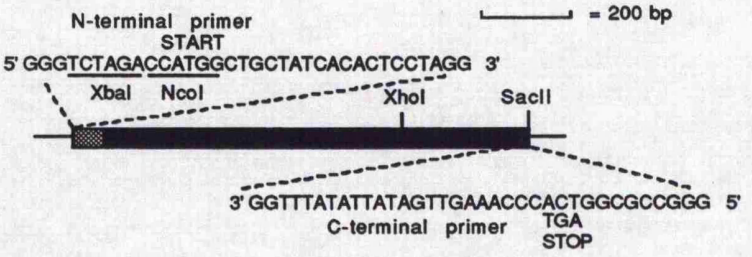
(B) Schematic representation of the modified PR β(1,3)-glucanase showing the sequence of the primers used for PCR amplification and relevant restriction enzyme sites.

(C) Diagram showing the strategy for cloning each promoter-β(1,3)-glucanase gene construct into pBin19 (Bevan, 1984). The transcriptional fusions, 35S PR and A9 PR , were both cloned as *Sac* I-*Eco* RV fragments into *Sac* I-*Sma* I-cut pBin19, and A3 PR was cloned as a *Sal* I-*Eco*RV fragment into *Sal* I-*Sma* I-cut pBin19. The translational fusion (A9(tl)PR) was cloned as a *Sac* I-*Eco* RV fragment into *Sac* I-*Sma* I-cut pBin19.

A Basic vacuolar PR glucanase (coding sequence)

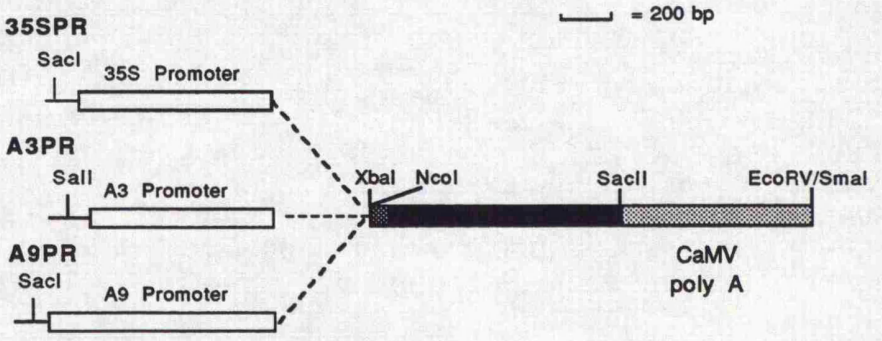


B PCR amplified glucanase

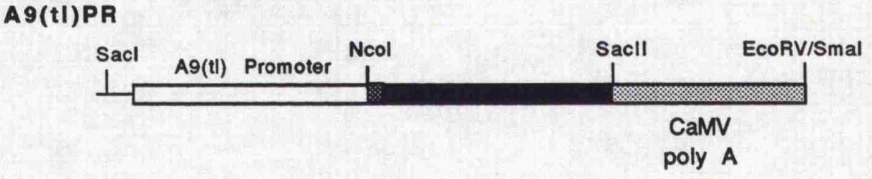


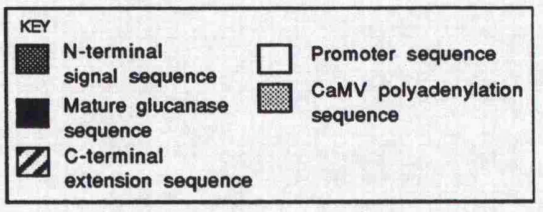
C Modified PR glucanase gene fusions

Transcriptional fusions



Translational fusion





glycosylated C-terminal propeptide (CTPP) and the N-terminal signal peptide are removed upon processing to the mature protein (Shinshi *et al.*, 1988). The CTPP of barley lectin was fused to cucumber chitinase, a protein that is normally secreted. When introduced into *N. tabacum* the fusion was redirected to the vacuole thus confirming that the role of the lectin CTPP is to direct the protein to the vacuole (Bednarek and Reikhel, 1991). van den Bulcke *et al.* (1989) suggested that the 22 amino acid basic vacuolar β(1,3)-glucanase CTPP may also be the signal that directs this protein to the vacuole. More recently, cDNAs have been isolated that encode acidic β(1,3)-glucanases found in the intercellular compartment of leaves (Linthorst *et al.*, 1990; Côté *et al.*, 1991; Ward *et al.*, 1991). These secreted forms of β(1,3)-glucanases lack a CTPP. This indirect evidence supports the idea that the basic β(1,3)-glucanase CTPP contains vacuolar targeting information.

On the basis of this information, synthetic oligonucleotides were designed complementary to the nucleotide sequence of the basic endo-PR β(1,3)-glucanase and used as primers to amplify a modified β(1,3)-glucanase that included the N-terminal signal peptide but lacked the CTPP (Figure 6.1B). This modification was presumed necessary to achieve secretion of the PR β(1,3)-glucanase enzyme from the tapetum to the locule. Several different PCR clones were cloned as *Xba* I and *Sac* II fragments into *Xba* I, *Sac* II-cut pBluescript (Stratagene), sequenced and compared to the published basic PR β(1,3)-glucanase sequence (Shinshi *et al.*, 1988). The final modified β(1,3)-glucanase gene was constructed from 2 clones (the 5' region of one clone from the *Xba* I site to the *Xho* I site and the 3' end of another clone from the *Xho* I to the *Sac* II) due to several frame shift mutations in the 3' region of one of the clones that would have prevented the production of an active protein (Figure 6.1B).

The sequence of the final modified β(1,3)-glucanase gene was identical to the equivalent region of the published basic PR β(1,3)-glucanase (Shinshi *et al.*, 1988), except for five nucleotide substitutions, as follows:- the codon starting at position 282 is CGA instead of GCA, altering the amino acid to arginine from alanine; the codon starting at position 498 is AGC instead of AAC altering the amino acid to serine from asparagine; the codon starting at position 864 is GTT instead of ATT altering the amino acid to valine from isoleucine and the codon starting at position 996 is CTC instead of CCC altering the amino acid to leucine from proline. The DNA sequence and the predicted peptide for the modified β(1,3)-glucanase, including the amino acid differences to the published sequence, are shown in Figure 6.2.

This modified β(1,3)-glucanase gene was then transcriptionally fused to a double cauliflower mosaic virus (CaMV) 35S promoter (Guerineau *et al*, 1988) forming the chimaeric gene (35S PR) depicted in Figure 6.1C. The modified β(1,3)-glucanase was also transcriptionally fused to the tapetum-specific promoters of the *A. thaliana* A3 and A9 genes (Scott *et al.*, 1991a, b; Paul *et al.*, 1992) (A3 PR and A9 PR constructs are shown in

Figure 6.2

Nucleotide and Deduced Amino Acid Sequence of the Modified β(1,3)-Glucanase Gene

The nucleotide substitutions and the corresponding amino acid changes from the published β(1,3)-glucanase sequence (Shinshi *et al.*, 1988) are highlighted.

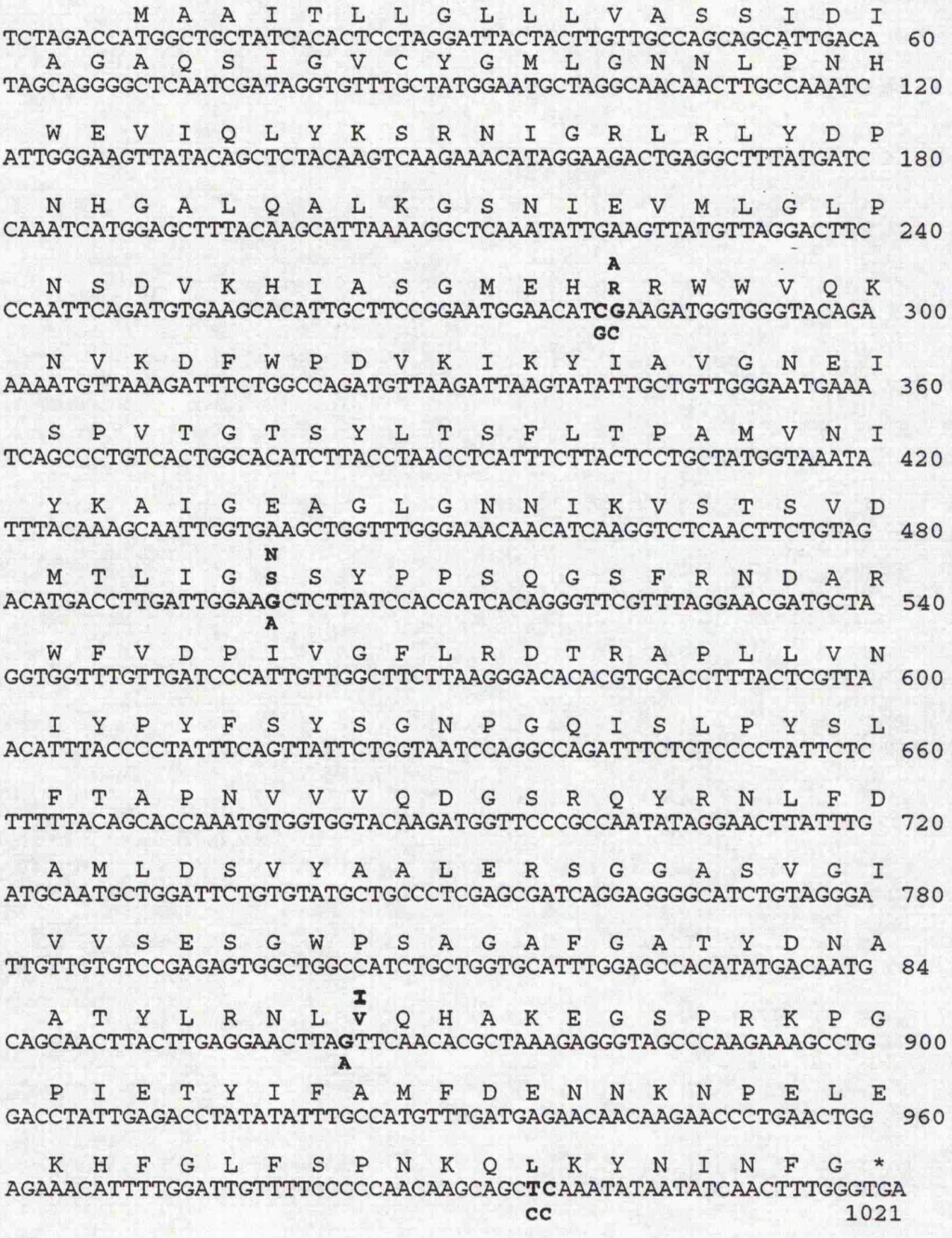

M A A I T L L G L L L V A S S I D I
TCTAGACCATGGCTGCTATCACACTCCTAGGATTACTACTTGTTGCCAGCAGCATTGACA 60
A G A Q S I G V C Y G M L G N N L P N H
TAGCAGGGGCTCAATCGATAGGTGTTTGCTATGGAATGCTAGGCAACAACTTGCCAAATC 120
W E V I Q L Y K S R N I G R L R L Y D P
ATTGGGAAGTTATACAGCTCTACAAGTCAAGAAACATAGGAAGACTGAGGCTTTATGATC 180
N H G A L Q A L K G S N I E V M L G L P
CAAATCATGGAGCTTTACAAGCATTAAAAGGCTCAAATATTGAAGTTATGTTAGGACTTC 240
A
N S D V K H I A S G M E H R R W W V Q K
CCAATTCAGATGTGAAGCACATTGCTTCCGGAATGGAACATCGAAGATGGTGGGTACAGA 300
GC
N V K D F W P D V K I K Y I A V G N E I
AAAATGTTAAAGATTTCTGGCCAGATGTTAAGATTAAGTATATTGCTGTTGGGAATGAAA 360
S P V T G T S Y L T S F L T P A M V N I
TCAGCCCTGTCACTGGCACATCTTACCTAACCTCATTTCTTACTCCTGCTATGGTAAATA 420
Y K A I G E A G L G N N I K V S T S V D
TTTACAAAGCAATTGGTGAAGCTGGTTTGGGAAACAACATCAAGGTCTCAACTTCTGTAG 480
N
M T L I G S S Y P P S Q G S F R N D A R
ACATGACCTTGATTGGAAGCTCTTATCCACCATCACAGGGTTCGTTTAGGAACGATGCTA 540
A
W F V D P I V G F L R D T R A P L L V N
GGTGGTTTGTTGATCCCATTGTTGGCTTCTTAAGGGACACACGTGCACCTTTACTCGTTA 600
I Y P Y F S Y S G N P G Q I S L P Y S L
ACATTTACCCCTATTTCAGTTATTCTGGTAATCCAGGCCAGATTTCTCTCCCCTATTCTC 660
F T A P N V V V Q D G S R Q Y R N L F D
TTTTTACAGCACCAAATGTGGTGGTACAAGATGGTTCCCGCCAATATAGGAACTTATTTG 720
A M L D S V Y A A L E R S G G A S V G I
ATGCAATGCTGGATTCTGTGTATGCTGCCCTCGAGCGATCAGGAGGGGCATCTGTAGGGA 780
V V S E S G W P S A G A F G A T Y D N A
TTGTTGTGTCCGAGAGTGGCTGGCCATCTGCTGGTGCATTTGGAGCCACATATGACAATG 84
I
A T Y L R N L V Q H A K E G S P R K P G
CAGCAACTTACTTGAGGAACTTAGTTCAACACGCTAAAGAGGGTAGCCCAAGAAAGCCTG 900
A
P I E T Y I F A M F D E N N K N P E L E
GACCTATTGAGACCTATATATTTGCCATGTTTGATGAGAACAACAAGAACCCTGAACTGG 960
K H F G L F S P N K Q L K Y N I N F G *
AGAAACATTTTGGATTGTTTTCCCCCAACAAGCAGCTCAAATATAATATCAACTTTGGGTGA
CC
1021

Figure 6.1C) and translationally fused to the A9 promoter (A9(tl)PR, Figure 6.1C). Promoter- β-glucuronidase (promoter-*gus*) fusions in transgenic *N. tabacum* showed that the A3 and A9 promoters become active in tapetal cells of anthers containing microsporocytes in the early stages of meiosis. Promoter activity reaches a peak around microspore release and disappears before the first microspore mitosis (Scott *et al.*, 1991b; Paul *et al.*, 1992). It was hoped that the tapetum-specific promoters would be suitable for the premature expression of β(1,3)-glucanase in the anther and that the CaMV 35S promoter would also produce some interesting results. The chimaeric genes were constructed (as described in Figure 6.1) and transformed into *N. tabacum*.

6.2.2 Expression of the Modified β(1,3)-Glucanase Gene from the Tapetum-Specific Promoters, but not from the CaMV 35S Promoter, Causes Male Sterility in Transgenic *N. tabacum*.

The preliminary analysis of the putative transformants involved confirming the presence of the transgene. DNA was extracted from kanamycin-resistant regenerated *N. tabacum* plants and analyzed for the presence of the modified β(1,3)-glucanase gene by the polymerase chain reaction (PCR) utilizing the N- and C-terminal β(1,3)-glucanase oligonucleotides or the M13 oligonucleotide as primers (see Figure 6.3). Consequently, PCR positive plants produced two fragments. The native PR β(1,3)-glucanase was also amplified by the PCR reaction with the gene specific primers, but the presence of an intron within this gene resulted in the production of a larger fragment that was easily distinguishable from the transgene (see Figure 6.3). Consequently, PCR positive plants produced two fragments. Plants that produced only the larger native PR β(1,3)-glucanase gene product upon PCR analysis were termed PCR negative.

Both PCR positive and negative plants were grown to maturity and the flowers and seed set analyzed. All transformants containing the modified β(1,3)-glucanase gene under the control of the A3 or A9 anther-specific promoters (a total of 29 transgenic plants) were normal in appearance but displayed varying degrees of reduced seed set. In contrast, all of the PCR negative plants were completely fertile. Transformants with a severe phenotype had small recessed anthers that were brown and lacked pollen grains (Figure 6.4A). In these plants, only flowers that had been cross-pollinated with wild-type *N. tabacum* pollen developed normal seed pods; all other flowers senesced. This confirmed that the reduction in seed set was due to male sterility rather than a general problem with the reproductive apparatus. Plants with incomplete sterility formed a variable number of small pods that contained few seeds in comparison to those of wild-type plants (Figure 6.4B). Forcing the flowers to self pollinate did not improve seed set and indicated that the reduced fertility was not due to the recessed anther phenotype.

Transformants containing the modified β(1,3)-glucanase gene under the control of the

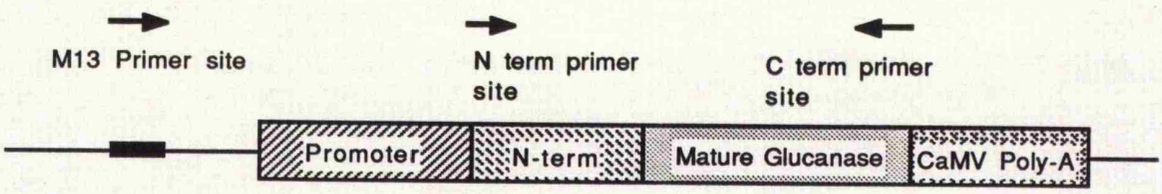
Figure 6.3

PCR Analysis Confirmed the Presence of the Introduced Modified β(1,3)-Glucanase Gene in the Regenerated *N. tabacum* Plants.

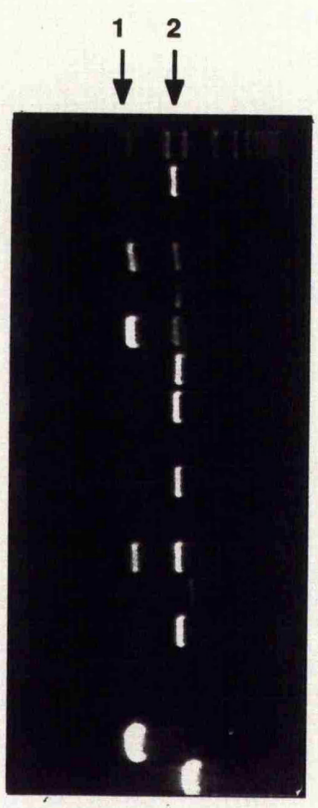
(A) Schematic diagram to illustrate the position of the primer sites on the modified β(1,3)-glucanase T-DNA.

(B) PCR products separated on a 0.8 % agarose gel.

A



B



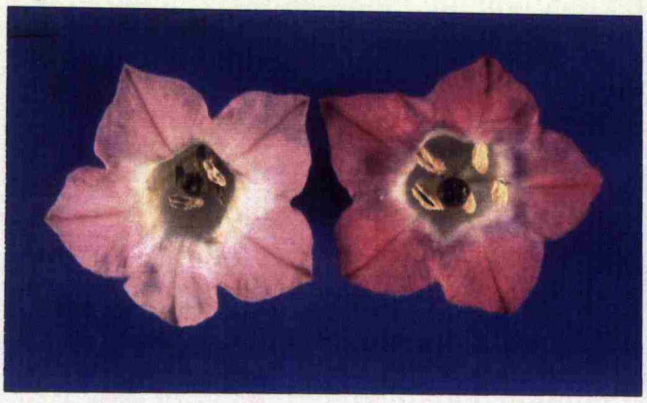
Molecular weight markers	
A3 PR #3	N + C term primers
	C term + M13 primers
A3 PR #2	N + C term primers
	C term + M13 primers
A3 PR #4	N + C term primers
	C term + M13 primers
A3 PR #2A	N + C term primers
	C term + M13 primers
A9 PR#10	N + C term primers
	C term + M13 primers
A9(t) PR#10	N + C term primers
	C term + M13 primers
Wild Type	N + C term primers
	C term + M13 primers
Plasmid	N + C term primers
	C term + M13 primers

1 = Transgene PCR product ~1 kb
2 = Native gene PCR product ~1·5 kb

Figure 6.4.
Reduced Fertility of the Transgenic *N. tabacum* Plants.

(A) Comparison of *N. tabacum* flowers from an A9(tl)PR transformant (left) and wild-type plant (right). Note lack of pollen on the anthers of the transgenic flower.
(B) Comparison of a seed pod from a wild-type *N. tabacum* plant (left) and a A9(tl)PR transformant (right).
(C) Bar chart comparing the average mass of seeds per pod between each transgenic *N. tabacum* line and wild-type plants (for data see Appendix B).

A

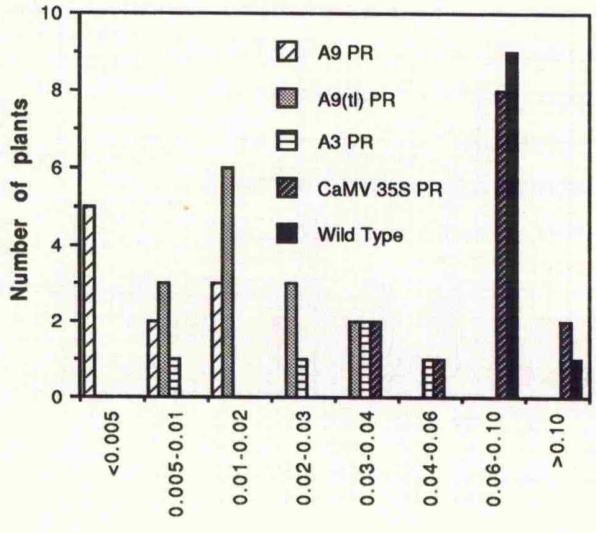


A9(tl)PR wt

B

wt A9(tl)PR

C



double CaMV 35S promoter were completely normal in appearance and significantly more fertile than the plants expressing the modified β(1,3)-glucanase from the tapetum-specific promoters. The fertility of 35S PR transformed plants was not appreciably different than that of wild-type plants. The bar graph in Figure 6.4C indicates the degree of fertility displayed by the transformants as judged by the average weight of seeds produced per pod (the data used to produce this graph is shown in Appendix B).

Plants containing A9 promoter fusions appeared less fertile than those containing the A3 promoter fusion, and the transcriptional fusion to the A9 promoter appeared more effective than the translational A9 fusion. These results are in accord with the relative strengths of these promoters as determined previously by *gus* fusion experiments which show that the A9 transcriptional promoter is approximately four times more active than the A3 promoter (personal communication, Dr. R. Scott).

The male sterility phenotype displayed by the T2 generation co-segregated with kanamycin resistance. An estimation of the number of T-DNA loci incorporated into the genome of the transgenic plants was achieved by plating the seeds (which were produced by crossing to wild-type plants) on a medium containing kanamycin. Kanamycin resistant seeds germinated to produce green seedlings whilst kanamycin sensitive seedlings bleached soon after germination. The table in Figure 6.5 provides an estimation of the T-DNA loci number in a selection of transgenic plants. This estimation assumes that the T-DNA loci are not linked.

The PCR amplification also gave a rough guide to the copy number of T-DNA inserts within the transgenic plants (see Figure 6.3). The intensity of the PCR product band from the transgene and the native β(1,3)-glucanase gene could be compared and as the copy number of the native gene was known the T-DNA copy number could be estimated. The native gene is approximately 500 bp larger than the transgene and was therefore amplified at a different rate, this factor also needed to be taken into consideration when estimating the copy number in this way. When the results of the PCR amplification were compared to the genetic segregation data there did seem to be a band intensity comparable with the loci number from the deduced ratio of kanamycin resistant to sensitive seedlings. For example, PCR analysis of transformant A3 PR 2 displayed a transgene band intensity that appeared to be approximately two fold brighter than the native gene. This suggested that two T-DNA loci were present in this transgenic plant. The ratio of kanamycin resistant to sensitive seedlings observed for this line also suggested the presence of two T-DNA loci.

The numbers of loci did not appear to correlate with the degree of male sterility displayed by the transgenic plants. For example, a transgenic plant with an estimation of 4 T-DNA loci (A9 PR 14) is in the lowest category of average mass of seeds per pod on the bar chart (Figure 6. 4C) whereas 2 plants with an estimation of 1 or 2 T-DNA loci (A3 PR 2 and A9(tl) 13) are also in this category of seed set.

Figure 6.5.
Table Showing Estimated Values for the Number of T-DNA Loci in Transgenic *N. tabacum* Plants as Judged by Kanamycin Resistance Segregation Analysis.

The seeds were the result of the application of wild-type pollen onto the transgenic plants. The estimation assumes no linkage.

TRANSFORMANT	NUMBER OF SEEDLINGS		APPROXIMATE RATIO	ESTIMATED NUMBER OF T-DNA LOCI
	BLEACHED	GREEN		
A9 PR 5	6	120	1:20	4
A9 PR 14	12	158	1:13	4
A9(tl) PR 13	91	88	1:1	1
A9(tl) PR 15	41	35	1:1	1
A3 PR 1	26	76	1:3	2
A3 PR 2	47	76	1:2	2
CaMV PR 7	4	119	1:30	5
CaMV PR 8	2	148	1:74	6

6.2.3 The Modified β(1,3)-Glucanase Protein was Detected in the Anthers of Transgenic *N. tabacum*

Anthers from the PCR positive plants were analyzed for the presence of the introduced β(1,3)-glucanase protein by immunoblotting with an antiserum raised against a PR β(1,3)-glucanase antiserum (described in section 3.2.2) which cross-reacts with the *N. tabacum* basic PR β(1,3)-glucanase. *N. tabacum* buds in the size range of 8-14 mm encompass the period of development where the A3 and A9 promoters are most active, as shown by promoter-*gus* fusion data (Scott *et al.*, 1991b; Paul *et al.*, 1992). The immunoblots shown in Figures 6.6A and 6.6B demonstrated that the modified β(1,3)-glucanase protein was present in the anthers of transgenic plants. The introduced β(1,3)-glucanase protein had a molecular mass of approximately 35 kD that appeared to have a slightly increased mobility in comparison to the immunoreactive β(1,3)-glucanase enzymes in wild-type *N. tabacum* leaf (this is most noticeable in Figure 6.6C). The PR β(1,3)-glucanase proteins present in leaf, represented by immunoreactive bands with molecular masses of approximately 35 to 37 kD, were not detectable in the anthers of control untransformed *N. tabacum* plants (Figure 6.6). Several transformants which were PCR positive, did not contain a detectable 35 kD immunoreactive band, for example transformant A9(tl)PR 17 (Figure 6.6B, lane 4), and were significantly more fertile than the other plants containing the A9 and A3 promoters shown in Figure 6.6. The presence of PR β(1,3)-glucanase protein, produced from A3 or A9 driven genes, therefore correlates with the male-sterile phenotype demonstrated by the transformed plants.

In transformants containing the β(1,3)-glucanase gene under the control of the CaMV 35S promoter the presence of the PR β(1,3)-glucanase protein did not correlate with male sterility (see bar chart in Figure 6.4C). The β(1,3)-glucanase protein detectable in the anthers of these plants was present at levels similar to those in transformants containing the other constructs but expression under the control of the 35S promoter does not lead to reduced fertility.

The immunoblot in Figure 6.6C shows that the introduced β(1,3)-glucanase protein accumulated in a temporal pattern consistent with the properties of the A9 tapetum-specific promoter. The modified β(1,3)-glucanase protein was first detectable during early meiosis, reached a peak at the stage when microspore release occurs, and then declined in abundance in anthers at a stage corresponding to microspore mitosis. Protein extracts made from wild-type anthers over the same developmental period did not contain any immuno-detectable ~35 kD protein (Figure 6.6D).

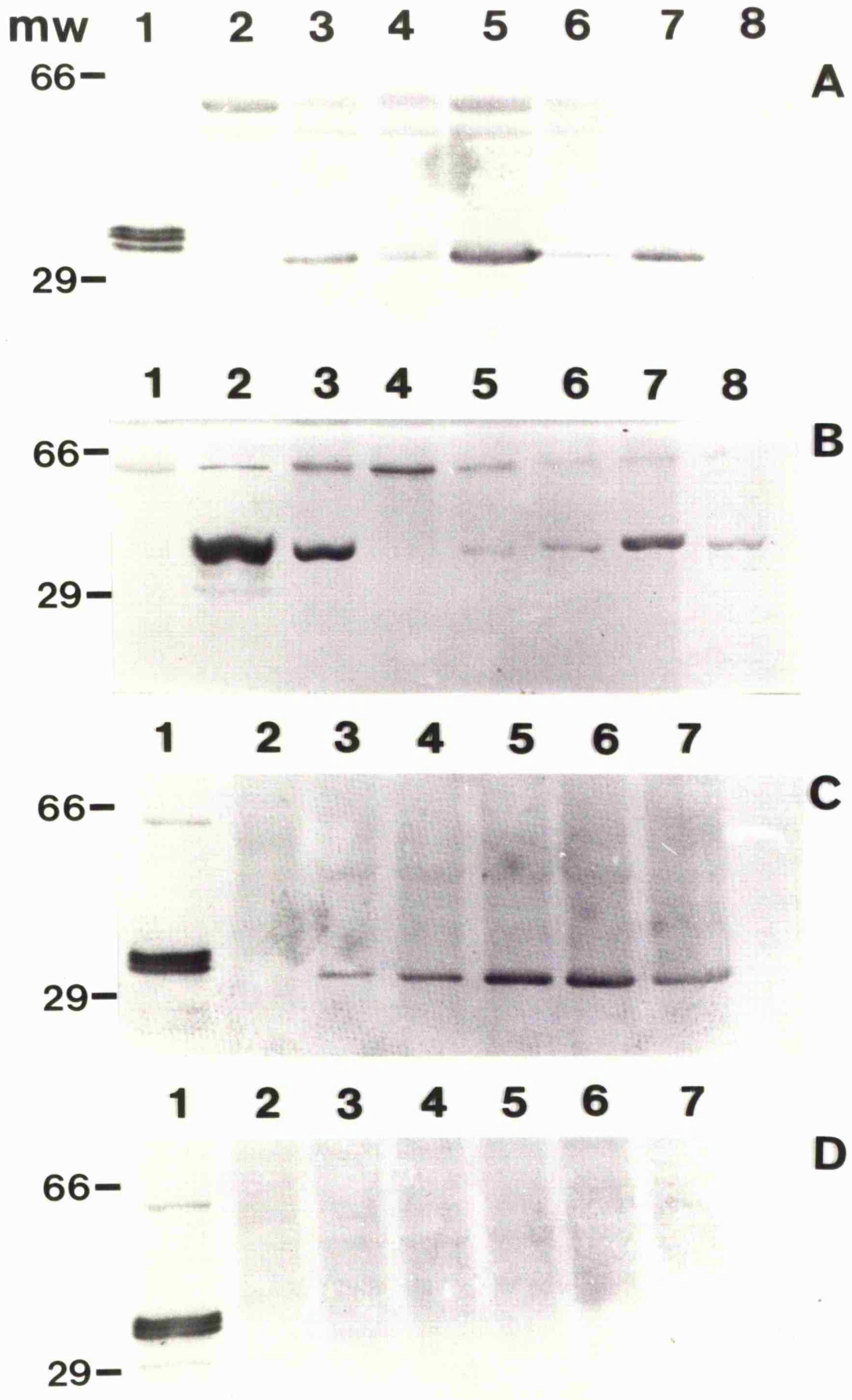
An interesting observation regarding extraction buffers was made from the blots shown in Figure 6.6. In Figure 6.6A and B, anther proteins were extracted in phosphate buffer and the 66 kD band (described in Chapter 3) was detected on the blots with the PR

Figure 6.6.

Modified β(1,3)-Glucanase Protein is Present in Anthers of Transgenic *N. tabacum* Plants.

Blots of SDS-polyacrylamide gels were probed with the PR β(1,3)-glucanase antiserum.
(A) Blot of an 11 % SDS-polyacrylamide gel. Lane 1 contains protein extracted from salicylic acid-treated wild-type *N. tabacum* leaves, and lanes 2-7, protein extracted from anthers dissected from buds in the size range 8-14 mm. Lane 2, wild-type plant; lane 3, A9 PR 1; lane 4, A9 PR 9; lane 5, A9(tl)PR 4; lane 6, A9(tl) PR 13; lane 7, A3 PR 2; lane 8, A3 PR 1.
(B) Blot of a 15 % SDS-polyacrylamide gel. Lane 2 contains protein extracted from salicylic acid-treated wild-type *N. tabacum* leaves. Lane 1 and lanes 3-8 contain protein extracted from anthers dissected from buds in the size range 8-14 mm. Lane 1, wild-type plant; lane 3, A9(tl) PR 14; lane 4, A9(tl)PR 17; lane 5, A3 PR 1A ; lane 6, 35S PR 2; lane 7, 35S PR 7; lane 8, 35S PR 14.
(C) Blot of an 11 % SDS-polyacrylamide gel. Lane 1 contains Protein extracted from salicylic acid-treated wild-type *N. tabacum* leaves and lanes 3-7 contain protein extracted from the anthers of the transgenic *N. tabacum* plant A9(tl)PR 1. Protein was isolated from anthers at the following developmental stages: archesporial, lane 2; meiocyte, lane 3; tetrad, lane 4; microspore release, lane 5. Lane 6 contains anthers of buds between 12 and 20 mm, and lane 7 contains anthers from buds between 20 and 30 mm.
Microspore release occurs at a bud length of 9-10 mm.
(D) Blot of an 11 % SDS-polyacrylamide gel. Lane 1 contains protein extracted from salicylic acid-treated wild-type *N. tabacum* leaves. Lanes 2-7 contain protein extracted from wild-type anthers at the same developmental stages and between the same size ranges as described in (C).

Molecular weight markers are given at left in kilodaltons.


mw 1 2 3 4 5 6 7 8
66–
29–
A
1 2 3 4 5 6 7 8
66–
29–
B
1 2 3 4 5 6 7
66–
29–
C
1 2 3 4 5 6 7
66–
29–
D

β(1,3)-glucanase antiserum. In Figure C and D, anther protein was extracted in tris buffer and the 66 kD band was not detected. This suggests that the 66 kD proteins are preferentially extracted in the phosphate buffer.

The level of modified β(1,3)-glucanase transcript was analyzed in the anthers from the transgenic *N. tabacum* plants which had undetectable levels of the protein and from the anthers of control plants. This would differentiate between two possible explanations for the absence of immuno-detectable modified PR β(1,3)-glucanase protein in these plants. The northern blot shown in Figure 6.7 showed that the plants which did not have a detectable level of protein had little or no modified β(1,3)-glucanase transcript in the anthers. The leaf extract has a slightly larger transcript than the anther extracts as this is the native transcript which contains the region encoding the C-terminal extension.

6.2.4 Male Sterility is Associated with Premature Disappearance of the Callose Wall

Once the modified PR β(1,3)-glucanase protein was detected in the anthers of transgenic plants, an investigation was carried out to determine the effect of the enzyme on the tetrad callose wall. A study of microsporogenesis was therefore undertaken in transformants containing the A9-glucanase translational fusion construct and untransformed control *N. tabacum* plants. Anthers at developmental stages between meiocyte and microspore release were dissected from buds and the locular contents stained for callose with aniline blue. Figure 6.8 shows the appearance of the callose wall in anthers of untransformed *N. tabacum* and in male-sterile *N. tabacum* anthers during the same developmental period. Microsporocytes of both sterile and fertile anthers appear normal before the initiation of meiosis. The meiocytes in both preparations have callose walls which fluoresce bright yellow when stained with aniline blue. The first observable difference between fertile and sterile anthers occurs during prophase I, when the thick callose wall that previously surrounded the meiocytes disappears in the transformed material. In wild-type tetrads, callose is deposited between the plasma membrane and the cell wall producing clear boundaries that separate the microspores. The microspores of sterile anthers appear to lack a callose wall but nevertheless remain held together as tetrads by some other material, most likely the primary wall, that does not show aniline blue fluorescence.

A second difference between tetrads of fertile and sterile anthers was that while tetrads squeezed out from fertile anthers separated easily, presumably due to a lack of intercellular cytoplasmic connections, tetrads from sterile anthers adhered in amorphous clumps.

Microspore release does occur in the male-sterile plants. The primary wall which holds the microspores together as tetrads in the transgenic plants is evidently broken down to

Figure 6.7.
Northern Blot of Anther RNA extracted from Transgenic Plants Probed with β(1,3)-Glucanase *Xba* I/*Sac* II Fragment.

RNA was extracted from anthers dissected from transgenic *N. tabacum* buds in the range 8-14 mm. Lane a; A9(tl)PR 1, lane b; A9(tl)PR 6, lane c; A9(tl)PR 8, lane d; A9(tl)PR 17, lane e; A9 PR 5, lane f; A9 PR 10, lane g; A3 PR 2, lane h; 35S PR 13, lane i; salicylic acid induced *N. tabacum* leaf
The autoradiogram was exposed for 1 month.

The transgenic plants A9(tl)PR 6, 8 and 17 did not contain any immuno-detectable modified β(1,3)-glucanase protein. The plant A9 PR 10 was PCR negative. The transgenic plants A9(tl)PR 1, A9 PR 5, A3 PR 2 and 35S PR 13 did contain immuno-detectable modified β(1,3)-glucanase protein.


a
b
c
d
e
f
g
h
i

Figure 6.8.
Comparison of the Callose Wall During Microsporogenesis in Wild-Type *N. tabacum* and those Transformed with Modified β(1,3)-Glucanase A9(tl)PR.

Both the wild-type and transformed *N. tabacum* developmental sequences are stained for callose with aniline blue. In each case, the left hand column is shown under phase contrast and the right hand column displays the same view under blue excitation to highlight callose (yellow fluorescence). With the exception of the meiocyte preparation, photographic exposure time for aniline blue fluorescence in the A9(tl) PR material was 3x that for the wild-type. Magnification x160

Non-transformed control
PR-glucanase
Meiocyte
Meiosis I (prophase)
Meiosis I (telophase)
Meiosis II (telophase)
Tetrad
Microspore release

cause microspore release. The timing of this event is equivalent to the timing of microspore release in wild-type anthers. The relationship between anther length and developmental stage remains the same in both male fertile and sterile plants, and thus the timing of the individual events that together constitute microsporogenesis appears unaffected by premature callose hydrolysis. However, soon after microspore release in the transgenic plants, many of the deformed microspores burst and this appears to be the factor which leads to reduced male fertility.

6.2.5 Meiosis is Normal in the Absence of the Callose Wall

In addition to the microsporocyte cell wall, callose also appears transiently around the megasporocyte during megasporogenesis at early meiotic prophase (Bouman, 1984). As the temporal and spatial distribution of callose is similar in both the male and female gametophyte, and callose is not a normal component of cell walls, it was considered possible that callose is involved in some aspect of meiosis in higher plants. To determine whether meiosis was normal in male-sterile plants, meiotic cells were dissected from male-sterile anthers and stained with aceto-orcein. In sterile anthers, despite premature dissolution of callose at early prophase I before the chromosomes have condensed, meiosis in the sporocytes was apparently normal (Figure 6.9). The four products of meiosis were observed as separate entities held together within tetrads. However, the four microspores within the tetrad are much more loosely associated than those seen in an untransformed control as often the nuclei of all four cells can be seen in the same plane. However, this observation may have been due to the pressure of the cover-slip squashing the tetrads. In the wild-type tetrads the callose wall is sufficient to prevent this. Aceto-orcein staining also demonstrated that the microsporocytes had the normal number of 24 bivalents (Figure 6.10) for *N. tabacum,* a natural amphidiploid (Kenton *et al.*, 1993).

6.2.6 Microspores Exhibit Aberrant Wall Development in the Absence of Callose

The callose wall is thought to play an important role in the establishment of the first exine patterning seen on the surface of the microspore. Various hypotheses have been proposed to explain the involvement of callose in laying down the pattern of primary exine (primexine). Larson and Lewis (1962) proposed that the callose wall is a source of glucose for the development of cellulosic primexine which provides the basic framework of the future exine. Waterkeyn and Beinfait (1970), suggested that the callose wall acts as a template or mold which is filled by primexine. As callose may play an essential role in the initial steps of microspore wall development, the structure of the microspore wall in one of the A9(tl)PR transformants was examined.

Transmission electron micrographs revealed abnormal wall development in sterile

Figure 6.9.
Meiosis is Normal in Transgenic *N. tabacum* Anthers.

Meiocytes were extruded from the anthers from a *N. tabacum* plant transformed with A9(tl) PR and stained with aceto-orcein.
(A) Early prophase I (zygotene).
(B) Prophase I (diakinesis).
(C) Metaphase I.
(D) Telophase I.
(E) Prophase II.
(F) Telophase II.
Magnification x1000.

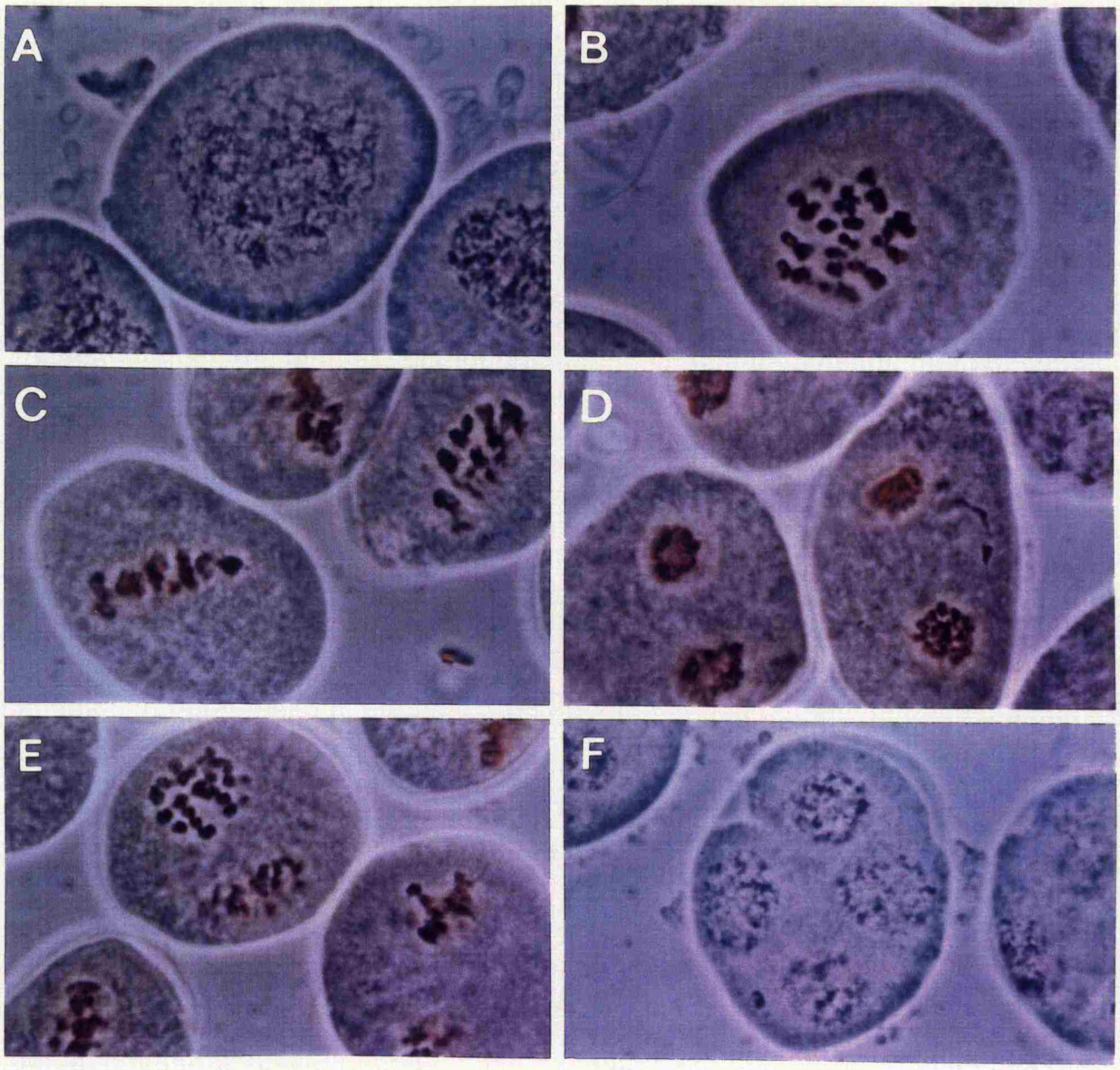

A
B
C
D
E
F

Figure 6.10.
Microsporocytes from the Anthers of Transgenic Plants Contain the Normal Number of Bivalents.

Prophase I (diakinesis) in a representative A9(tl) PR transformant. The contents of a anther at the appropriate stage in development were extruded out, stained with aceto-orcein and squashed. Chiasmata (points where non-sister chromatids have crossed over) are visible. Twenty-four bivalents were counted.
Magnification x1000.

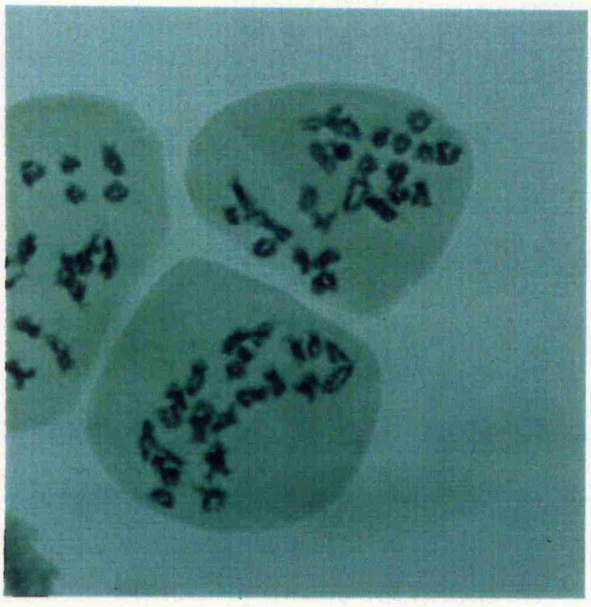


Figure 6.11.
Premature Callose Dissolution Causes Aberrant Microspore and Tapetal Development.

Transverse sections through anthers from a wild-type and a A9(tl)PR *N. tabacum* plant, viewed by transmission electron microscopy **(a-g)** or by light microscopy **(h-i)**. All anthers were at the tetrad stage of development.

(a) Microspore of a tetrad from a wild-type *N. tabacum* plant. The microspore exine (e) is well developed within the callose wall (cw) of the tetrad. Magnification x5000.

(b) Microspore from a A9(tl)PR transformant. The callose wall is absent and the microspore cell surface is electron dense and apparently lacks exine patterning. Magnification x5000.

(c) Developing microspore wall in wild-type *N. tabacum*. Exine wall formation occurs between the callose wall and the plasma membrane (pm) of the microspore. Laminations (l) are present beneath the regular deposits of sporopollenin that constitute the young tectum (t) and probacula (pb) of the developing exine. Several micropores (mp) are present within the tectum. Magnification x25000.

(d) Aberrant microspore wall development in A9(tl)PR transformed *N. tabacum*. Noncompressed laminations are apparent at the cell surface. Globular deposits of a material with the appearance of sporopollenin (sp) lie on the outside of the laminations. Several of the globules contain a very electron dense region (a) which abutts the cell surface and interupts the laminations. Magnification x25000.

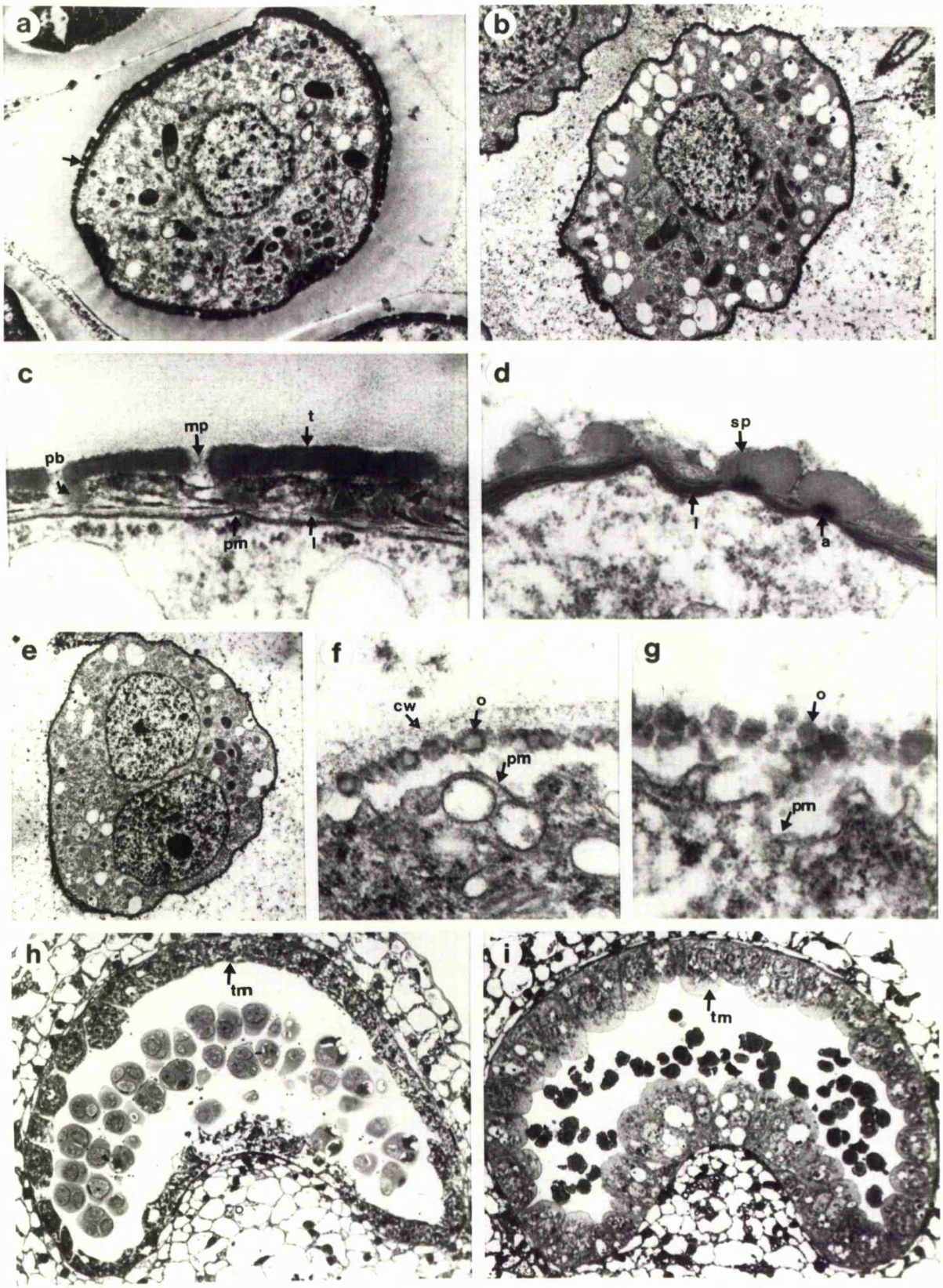
(e) A binucleate microspore from a A9(tl)PR transformed *N. tabacum* plant. Magnification x3000.

(f) Portion of the luminal surface of a tapetal cell in a wild-type *N. tabacum* plant. A line of orbicules (o) can be seen between the cell wall and the plasma membrane. Magnification x30000.

(g) Portion of the luminal suface of a tapetal cell in a A9(tl)PR transformed *N. tabacum* plant. The cell wall is absent and the orbicules have an irregular distribution and appear less homogenous than in the wild-type. Magnification x30000.

(h) Section through a wild-type anther showing the dimensions of the normal tapetum (tm). Magnification x160.

(i) Section through an anther from a A9(tl)PR transformed *N. tabacum* plant. The tapetum is highly vacuolated and shows signs of hypertrophy. Magnification x160.


a
b
c
mp
t
pb
pm
l
d
sp
l
a
e
f
cw
o
pm
g
o
pm
h
tm
i
tm

microspores during the period when the microspores are still embedded in the callose wall of the tetrad in control fertile plants. Figures 6.11a to e show electron micrographs of developing microspores from fertile and sterile *N. tabacum* anthers. The thick callose wall surrounding each microspore of the tetrads from control fertile anthers (Figure 6.11a) is clearly absent in sterile microspores (Figure 6.11b). The microspores from fertile anthers also appear to have a more regular outline than those from sterile anthers. The characteristic vaulted structure of the exine of control microspores (Figure 6.11c) is replaced by lamellae of varying thicknesses overlain by irregular deposits of electron dense material (Figure 6.11d). This material is likely to be sporopollenin. Some binucleate microspores were observed in the sterile anthers that may result from the failure of cytokinesis or subsequent fusion of the microspores (Figure 6.11e).

6.2.7 The Tapetum Exhibits Hypertrophy in Male-Sterile Anthers

In some types of male sterility, ultrastructural and histological examinations have revealed that the first signs of abnormality are found not in the microsporocytes or developing microspores, but in the tapetum (Horner and Rogers, 1974; Horner, 1977; Warmke and Lee, 1977; Bino, 1985a, b). A phenomenon termed hypertrophy occurs where the tapetum becomes enlarged and apparently invades the locular space. A general comparison of tapetal morphology was made between male fertile and sterile anthers to determine whether disruption of microspore development had any affect on the tapetum.

Sections were made from fertile anthers which contained tetrads and from male-sterile anthers at an equivalent stage. These were stained with toluidine blue. Comparison of Figures 6.11h and 6.11i shows that the tapetal cells of the male-sterile anther are larger and more vacuolate than those in the fertile anther. The locular side of the tapetal cells in the male-sterile anther is lightly stained, in comparison to the rest of the tapetum, and may indicate that the cytoplasm is more dilute in this region.

Electron micrographs of the tapetum also reveal differences in morphology between fertile (Figure 6.11f) and sterile anthers (Figure 6.11g). The outer wall appears to be absent and orbicules form irregular aggregations in the tapetum wall of sterile anthers. However, these features occur during normal tapetal development. A developmental sequence would therefore give a clearer indication of tapetal wall structure in the male-sterile plants.

6.2.8 Scanning Electron Microscopy Confirms that the Pollen From Dehisced Anthers of Male-Sterile Plants Has an Aberrant Exine

Sections of transgenic anthers viewed by transmission electron microscopy revealed that exine formation was disrupted at an early stage of microspore development. A further investigation of the pollen wall at a later stage in development was undertaken to determine the state of the exine. The powder from the surface of dehisced anthers of the T2 generation

Figure 6.12.
Pollen from 35S PR Transgenic *N. tabacum* Plants is Similar to Wild-Type Pollen.

(A) and (B) Scanning electron micrographs of pollen from dehisced anthers of a wild-type *N. tabacum* plant.
(C) and (D) Scanning electron micrographs of pollen from dehisced anthers of a T2 generation 35S PR transformant.

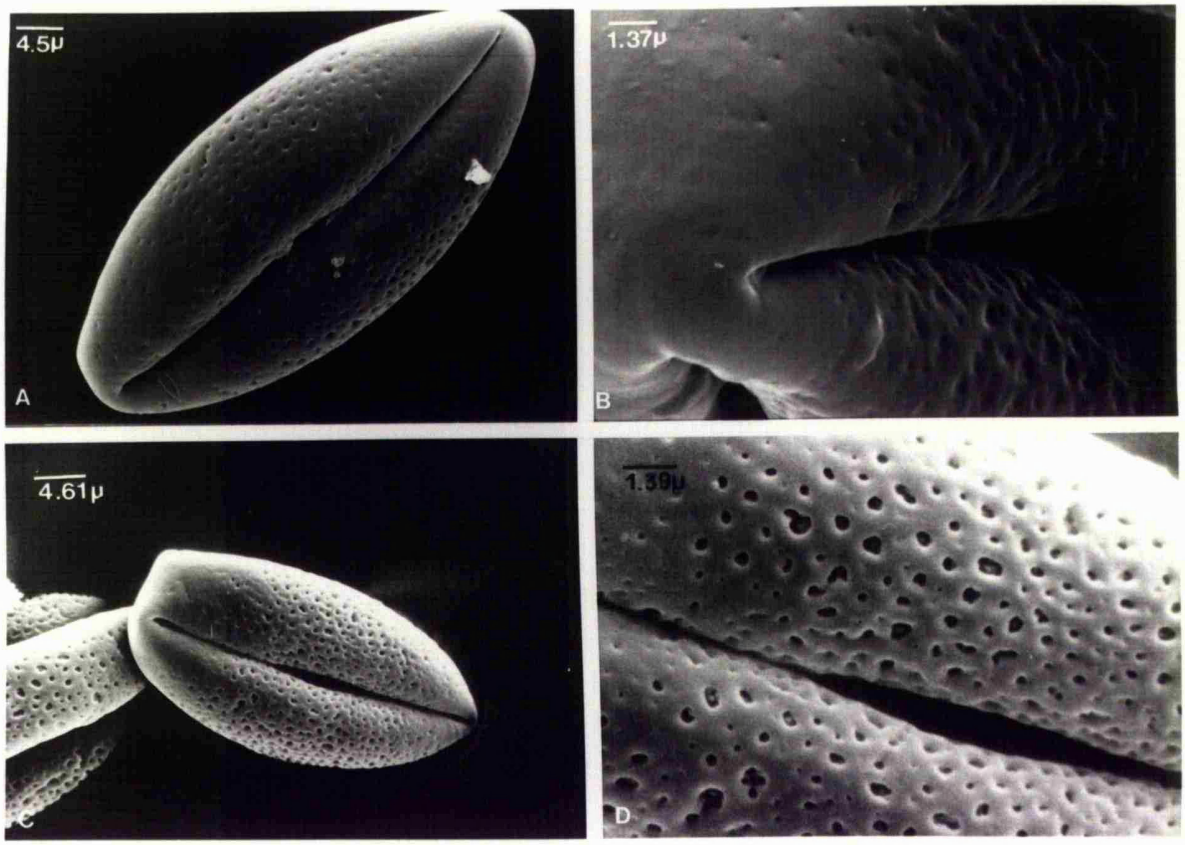
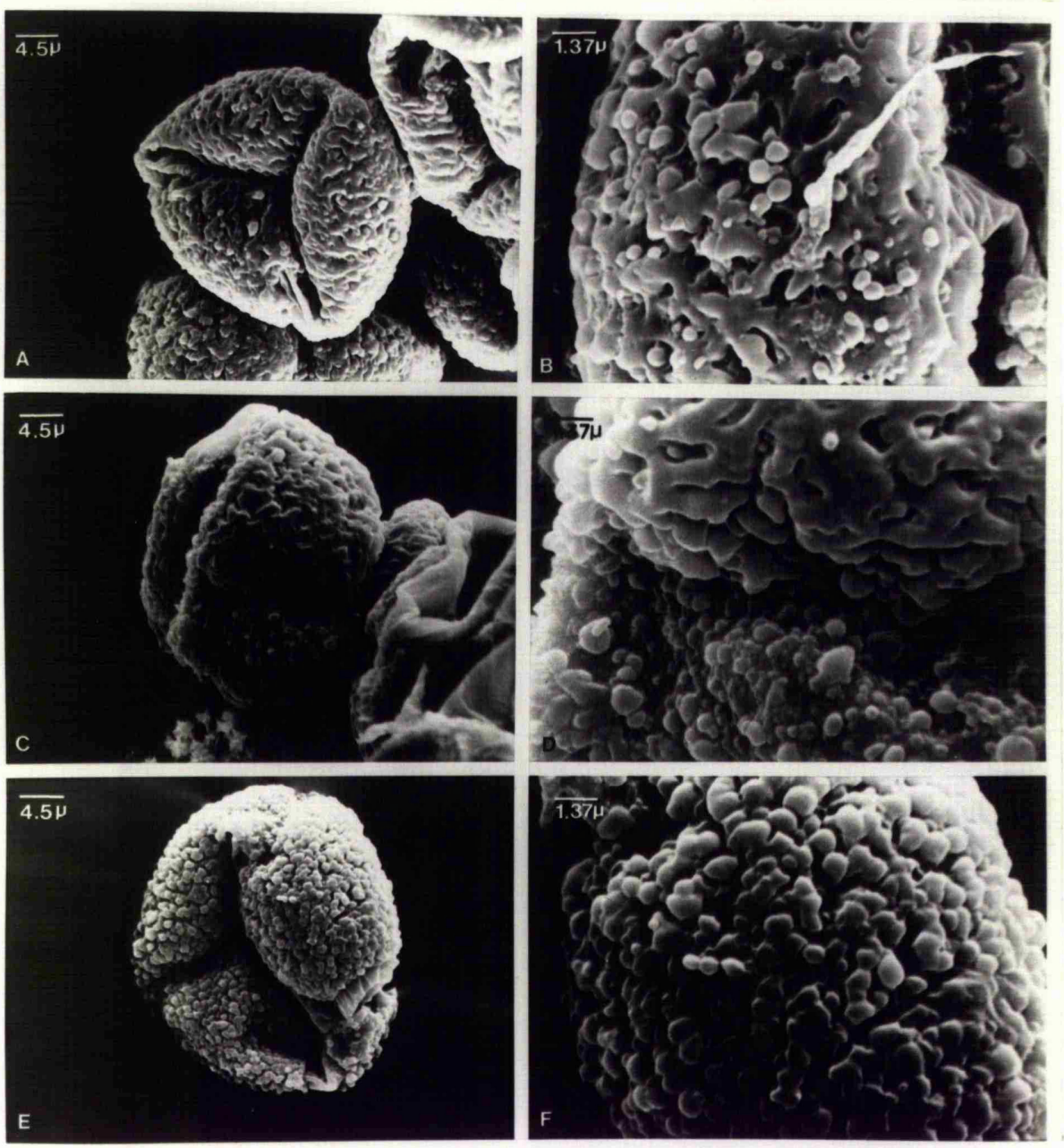

4.5μ
A
1.37μ
B
4.61μ
C
1.39μ
D

Figure 6.13.
Aberrant Exine of Dehisced Pollen from Transgenic *N. tabacum* Plants Expressing the Modified β(1,3)-Glucanase in the Anther Tapetum.

(A) and **(B)** Scanning electron micrographs of pollen from dehisced anthers of a T2 generation A3 PR transformant.
(C) and (D) Scanning electron micrographs of pollen from dehisced anthers of a T2 generation A9(tl)PR transformant.
(E) and (F) Scanning electron micrographs of pollen from dehisced anthers of a T2 generation A9 PR transformant.


4.5μ
A
1.37μ
B
4.5μ
C
37μ
D
4.5μ
E
1.37μ
F

of transgenic *N. tabacum* plants was analyzed by scanning electron microscopy. There was a small amount material on the surface of the sterile anthers in comparison to the anthers of plants containing the CaMV 35S construct and wild-type plants. The material on the sterile anthers appeared to be clumped together indicating that the aberrant pollen grains were unable to separate properly. The pollen grains of the plants containing the CaMV 35S promoter construct appear to be normal in appearance and abundance but there may have been larger micro-pores on the surface of these pollen grains compared to the wild-type (Figure 6.12).

The electron micrographs shown in Figure 6.13 appear to support the information gained from the transmission electron microscopy, in that there was irregular sporopollenin build up on the surface of the sterile microspores. The relative strength of each promoter seems to be reflected in the severity of pollen wall phenotype. Figure 6.13 has been arranged with the severity of phenotype increasing towards the bottom of the page. Accordingly, pollen from the A9 transcriptional fusion construct-containing plants is at the bottom. Many of the deformed pollen grains appear to be collapsed and the normal form of bacula and tectum is completely absent. The deformed pollen grains appear to have an apertures (colpi) in the expected location.

6.2.9 An Investigation into Pollen Viability

It was important to determine whether the collapsed pollen found on the anther surface of the transgenic *N. tabacum* plants was actually viable. Viability stains usually involve nuclear staining and therefore aborted pollen grains are not stained. The discovery of a stain mixture which was able to stain both the pollen wall and the protoplasm was therefore useful for this purpose (Alexander, 1969). Germination of the pollen *in vitro* would also determine whether the collapsed pollen was still functional.

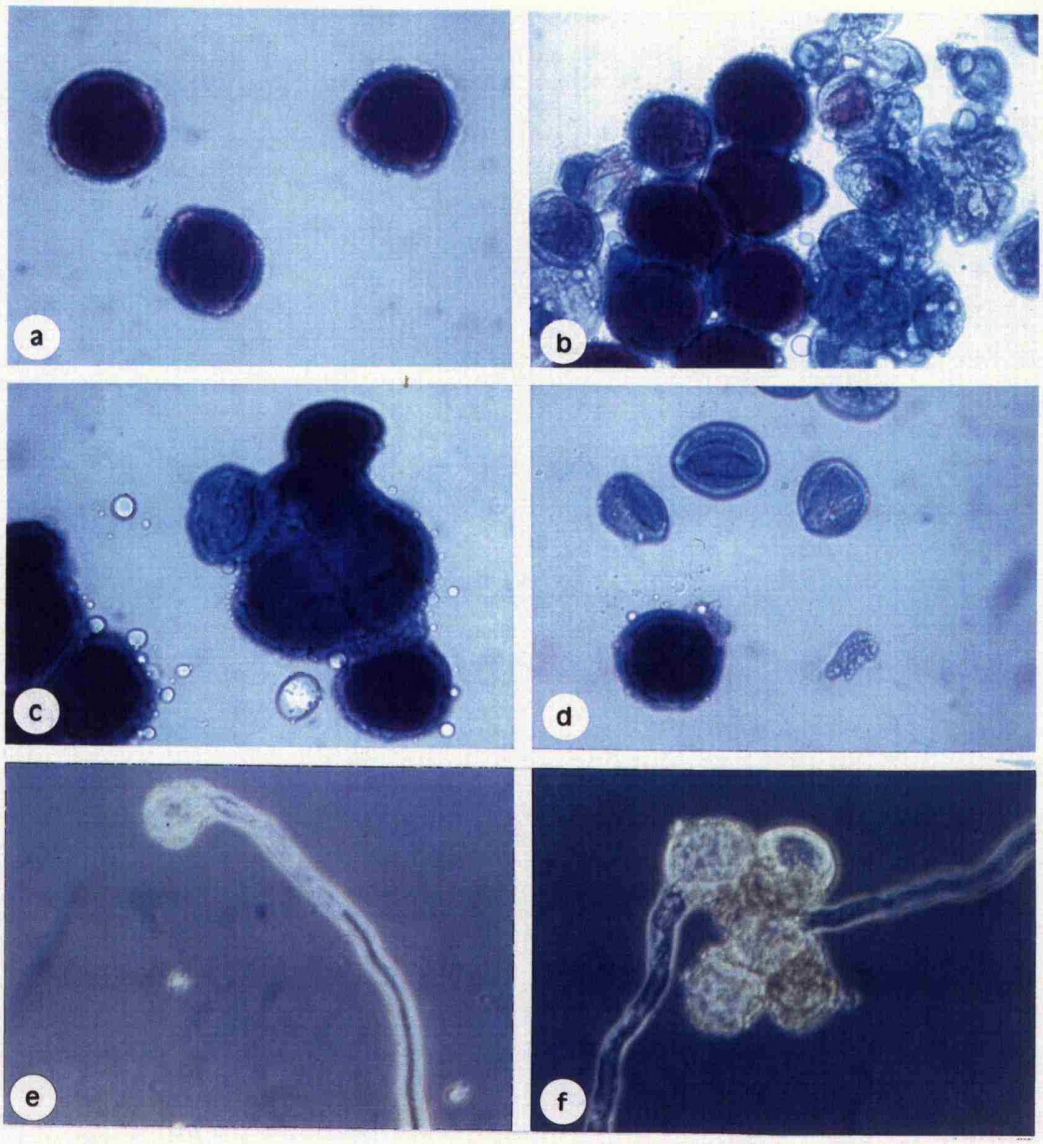
The viability stain mixture consisted of malachite green, which stains the pollen walls green-blue; acid fuchsin, for staining the protoplasm of non-aborted pollen and orange G to improve the differentiation. Figure 6.14 shows the results of staining wild-type pollen and pollen from the T2 generation of transgenic plants containing the modified PR β(1,3)-glucanase constructs with this stain. The wild-type pollen grains have a uniform shape and the pollen wall is stained green-blue and the protoplasm deep red (Figure 6.14 a). Many of the pollen grains from the transgenic plants lack viable cytoplasm and thus only the wall is stained. Occasionally, depending on the severity of the phenotype, there were grains with red stained protoplasm amongst the green-blue aborted pollen (Figure 6.14 b, c and d). The transgenic plants containing the A9 transcriptional fusion constructs demonstrated the lowest abundance of viable pollen grains, However, sometimes in a mass of fused pollen debris there did appear to be red staining viable cells (Figure 6.14 b).

Pollen was taken from anthers of wild-type and transgenic plants and put into pollen germination medium. After approximately 3 hours at room temperature the pollen was

Figure 6.14.

Viability of Dehisced Pollen from Transgenic *N. tabacum* Plants Expressing the Modified β(1,3)-Glucanase in the Anther Tapetum.

(a) Wild-type pollen stained with viability stain (Alexander, 1969). The cytoplasm is stained red with acid fuchsin. Magnification x400.
(b) Pollen from an A9 PR transgenic plant. The walls of aborted pollen grains are stained green-blue. Magnification x400.
(c) Fused pollen grains from an A9(tl) PR transgenic plant.
(d) Pollen from an A3 PR transgenic plant. Magnification x400.
(e) Germination of a pollen tube from a wild-type pollen grain. Magnification x200.
(f) Germination of pollen tubes from viable cytoplasm within an abnormal clump of pollen from an A9(tl)PR transgenic plant. Magnification x400.


a
b
c
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f

viewed under a light microscope (Figure 6.14 e and f). Although the pollen from the transgenic plants was often clumped together in fused masses, pollen tubes did grow out from amongst the debris.

6.2.10 Locular pH Changes During Microsporogenesis

The premature dissolution of the callose wall by the modified PR β(1,3)-glucanase required that conditions in the anther locule were conducive for enzyme activity before the normal appearance of callase. As described previously, studies in *Petunia* (Izhar and Frankel, 1971) and *Lilium* (Linskens, 1956) have shown that the locular fluid pH falls during microsporogenesis and that this event is correlated with a rise in callose activity. In *Petunia*, the pH of the locular fluid drops from pH 7.0 to 6.0 just prior to the onset of callase activity in both wild-type *Petunia* and in the premature or delayed callase activity mutants (Izhar and Frankel, 1971). It is difficult to determine what factors cause these pH changes and whether they have any direct association with the enzyme activity. However, the success of this experiment depended upon whether the pH conditions in the locule were suitable for activity between the onset of meiosis and the completion of meiosis II. An experiment was therefore carried out to determine if a pH drop occurs in the locule of wild-type *N. tabacum* anthers during development. This could then be compared to presence and timing of any fall of pH in the anther locules of the transgenic plants expressing a glucanase activity earlier in development.

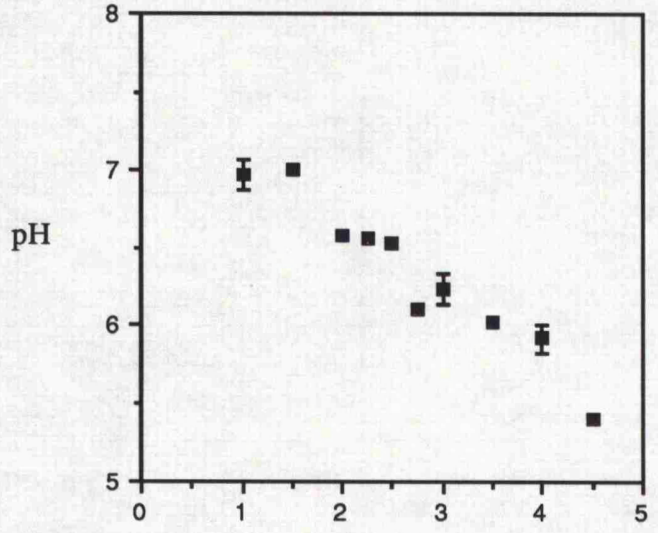
Microelectrodes were used to measure the pH of the locular fluid. This was achieved by selecting anthers at various stages of development (as determined by measuring the anther length), cutting these into two sections (transversely) and inserting microelectrodes directly into the locular fluid with the help of a dissecting microscope (see section 2.14 for more detail). Figure 6.15A shows that the pH of the locular fluid in wild-type *N. tabacum* anthers declines as development progresses (see Appendix C for data). The locular fluid is at pH 7.0 when archesporial cells are present (1 mm anthers) then falls steadily to pH 5.4 when the microspores are in early binuclear phase (4.5 mm anthers). Several locular fluid pH values from the anthers of transgenic plants are also shown on the scatter plot in Figure 6.15B. The locular fluid pH readings from 4 mm anthers of A9 PR and A3 PR transgenic plants are higher than readings from wild-type anthers. However, these data are preliminary.

Figure 6.15
Changes in Locular Fluid pH During Anther Development.

(A) Wild-type anthers. The bars represent the standard deviation.
(B) Wild-type and transgenic anthers.
The data for these scatter plots are presented in Appendix C.

Key to Developmental Stages.
A, Archesporial stage; **M,** meiosis; **T,** tetrad, **MR,** microspore release; **MI,** microspore interphase.

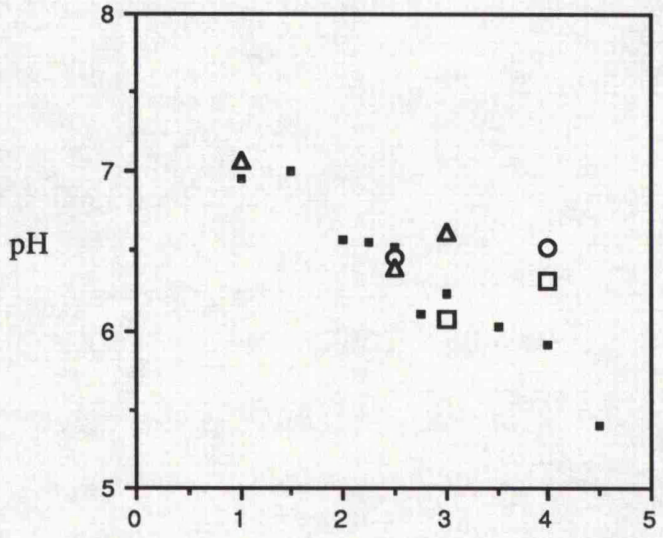
A



Anther length (mm)

Developmental stage

B



Key

- Wild-type
- A9 PR
- A3 PR
- 35S PR

Anther length (mm)

Developmental stage

6.3 Discussion

6.3.1 Removal of the Carboxy-Terminal Propeptide (CTPP) Directs the Modified β(1,3)-Glucanase to the Anther Locule

The premature hydrolysis of the tetrad callose wall required a β(1,3)-glucanase to be secreted into the locular space. Since a vacuolar β(1,3)-glucanase was chosen for the experiment, the coding region of the enzyme required engineering to direct secretion. A secreted β(1,3)-glucanase was synthesized from a vacuolar enzyme by removing the putative CTPP. The secretion of proteins after entry into the endoplasmic reticulum is generally accepted to be a default pathway, whereas proteins directed to organelles have specific targeting signals. van den Bulcke *et al.* (1989) therefore suggested that the CTPP of *N. tabacum* basic vacuolar β(1,3)-glucanase may function to direct the protein to the vacuole. A basic β(1,3)-glucanase lacking the CTPP was expressed in the anther tapetum of transgenic *N. tabacum* plants, therefore indirectly examining the role of the CTPP in vacuolar targeting. The deletion of the CTPP resulted in the secretion of the β(1,3)-glucanase enzyme into the locular space of the anther. The protein is therefore sorted differently when synthesized without the CTPP. This experiment provides indirect evidence that the CTPP is required for vacuolar targeting. However, the CTPP may not be the complete signal, further information could reside within the remaining β(1,3)-glucanase sequence. Targeting a non-vacuolar reporter protein to the vacuole by the addition of the basic β(1,3)-glucanase CTPP would confirm the suggested role.

6.3.2 Modified β(1,3)-Glucanase is Active in the Anther Locules of Transgenic *N. tabacum*

Premature or delayed callase activity has been linked to the male-sterile phenotype observed in some lines of *Petunia* and *Sorghum* (Frankel *et al.*, 1969; Warmke and Overman, 1972). The work discussed in this chapter details how phenocopies of these mutants were produced by expressing a modified basic β(1,3)-glucanase in the anther tapetum prior to the normal appearance of callase. The premature dissolution of the callose wall in this way required that the conditions in the anther locule at this earlier stage in development were conducive to β(1,3)-glucanase activity.

Izhar and Frankel (1971), established that in *Petunia, in vitro* activity of callase was optimum at pH 5.0 and that the enzyme was inactive above pH 6.3. They also measured *in vivo* locular pH at different stages of microsporogenesis and showed that locular pH falls from 6.8-7.0 to 5.9-6.2 just prior to the appearance of callase activity in normal, fertile

anthers and in the delayed callase activity mutants. Izhar and Frankel (1971), state that low pH is therefore a precondition for callase activity in *Petunia* and suggested that the timing of glucanase activity could be regulated by the drop in locular fluid pH.

The preliminary study on locular pH of wild-type *N. tabacum* anthers (section 6.2.10) indicated that the pH drops from 7.0 at archesporial cell stage to 6.55 at meiosis and then ~6.1 at microspore release stage. Therefore, the pH of the locular fluid at meiosis was likely to be too high for callase activity. The pH activity curve for the basic vacuolar glucanase used in this work is different to that of callase. This enzyme retains 26% of its maximum activity at pH 7.0 (Felix, 1984). Therefore, with hind-sight it was probably fortunate that the basic vacuolar glucanase was used for this work (even though it was the only sequence available at the time) as this enzyme retains sufficient activity for callose hydrolysis within the meiotic locule.

A preliminary study was carried out with the transgenic anthers to investigate the relationship between the premature appearance of β(1,3)-glucanase activity with pH of the locular fluid. Izhar and Frankel (1971) found that a pH drop was always associated with the appearance of callase in the anther locule of *Petunia*, even when callase activity was precocious in male sterile mutants. In the anthers of the plants generated in this study, the locular fluid pH appeared to remain the same as the wild-type pH during the period when the β(1,3)-glucanase first appeared. However, some of the locular fluid readings taken from A9 PR and A3 PR transgenic anthers after microspore release stage suggested that locular fluid pH did not decrease as development progresses. Thus it appears that the pH drop in observed in anthers is unlikely to be due to the appearance and activity of the β(1,3)-glucanase itself. It is still possible that callase could exert a different effect on the locular fluid environment, and cause a pH drop. The lack of a decrease in locular fluid pH in the transgenic anthers as development progresses is likely to be due to disruption of the anther and breakdown of the normal events.

The transgenic *N. tabacum* plants expressing the modified β(1,3)-glucanase from the tapetum-specific promoters displayed a range of reduced male fertility, as judged by the average weight of seeds produced per pod. It is likely that complete male sterility requires a high level of accumulation of β(1,3)-glucanase in the locules, especially if conditions are not favourable for activity. The experiment performed to determine the number of T-DNA loci indicated that position effects and strength of promoter have a much greater influence on the degree of male fertility than the number of T-DNA loci. The first sign of β(1,3)-glucanase activity, as demonstrated by lack of callose at the beginning of meiosis, correlates with the appearance of the immunoreactive band on the temporal blot. The β(1,3)-glucanase enzyme is therefore apparently active as soon as it appears in the locule, and since developing microspores synthesize callose, probably remains active throughout microsporogenesis. No clear correlation was observed on the immunoblots between the amount of modified β(1,3)-glucanase protein in the anther and the degree of male sterility

of the plant. This was probably due to the difficulty of collecting anthers that are precisely at the same developmental stage. However, as mentioned earlier, there did appear to be a correlation between the known strength of the tapetum-specific promoters and the degree of male fertility.

6.3.3 Transgenic Plants Expressing the Modified β(1,3)-Glucanase Under Control of the CaMV 35S Promoter Display No Obvious Phenotype and Are Male-Fertile

In contrast to the tapetum specific promoters, the fusion of the modified β(1,3)-glucanase gene to the CaMV 35S promoter led to the production of completely fertile plants. Plegt and Bino (1989) have shown with *gus* fusions that the CaMV 35S promoter has strong activity in the vascular cylinder of the anther and no activity in the tapetum or sporogenous cells during premeiotic, tetrad and microspore release stages. This information supports observations made in this study, which shows that the modified β(1,3)-glucanase protein is present in the anthers of transgenic plants, due to the signal obtained on immunoblots, but the absence of the male sterility phenotype indicates that expression is low or absent in the tapetum or microsporogenous cells at the relevant time of development. The signal obtained on the immunoblots was therefore most likely to be derived from modified β(1,3)-glucanase protein present in the vascular cylinder of the anther.

Previous studies have also identified large variations in the expression of *gus* genes under the control of the CaMV 35S promoter in different tissues (Benfey *et al.*, 1989; Jefferson *et al.*, 1987). Jefferson *et al.* (1987) transformed *N. tabacum* with the *gus* gene fused to the CaMV promoter, made stem sections and observed strong staining with the β-glucuronidase substrate in the vascular tissue. The sieve plates of phloem vessels contain callose (see Cutter, 1978), and therefore one would predict that glucanase expression under control of the CaMV 35S promoter would result in an abnormal phloem vessel phenotype. It must therefore be assumed that although the modified β(1,3)-glucanase was likely to be present in the vascular tissues of the transgenic plants because of the expression pattern of the CaMV 35S promoter (and shown by immunoblots of anther proteins), conditions were unfavourable for β(1,3)-glucanase activity in the phloem vessels, and thus no phenotype was seen. It was also possible that the callose substrate was not susceptible or accessible to the enzyme. This could be due to the callose existing as complexes with other carbohydrates.

6.3.4 Callose is Required for Pollen Wall Formation in *N. tabacum*

Several theories attempt to explain the role of the callose wall during

microsporogenesis, but its function still remains to be determined. Some of these theories suggest that callose may play a role in processes such as cytokinesis and exine formation or be involved with a mechanism for isolating the newly formed microspores. In light of the experiments described in this chapter, these theories can be reexamined.

Waterkeyn (1962) suggested that callose fulfils an important biological role acting as a temporary wall which is rapidly built up, to isolate the individual microspores, preventing cohesion and fusion. Later on in microsporogenesis the callose wall is rapidly hydrolyzed and the individual microspores are released. The importance of the callose wall as a mechanism to isolate the individual microspores can be shown in the study of plants from which the pollen is dispersed as permanent tetrads. The callose wall is often greatly reduced or absent in these plants (Vijayaraghavan and Shukla, 1977).

Examination of the transgenic plants generated in this study seemed to indicate that the callose wall was not required for cytokinesis since four separate microspores were formed and held together as a tetrad after meiosis in sterile anthers (section 6.2.4 and Figures 6.8, 6.9 and 6.11). However, analysis of the anther contents of the male-sterile plants after microspore release revealed that the abnormal pollen grains were often fused together in large clumps (Figure 6.14). Perhaps the callose wall is not essential for cytokinesis but is important in isolating the individual microspores to prevent fusion until the pollen wall is sufficiently formed.

It was interesting to observe that the microspores were still held together as tetrads despite the absence of the callose wall in the transgenic anthers. This suggested that additional material other than callose is involved with maintaining the tetrad structure in *N. tabacum*. Microspore release was observed in these anthers at the correct stage during development and thus enzymes other than β(1,3)-glucanase must normally be in operation for this event. The cell wall holding the tetrad together is likely to be the primary cell wall and therefore a temporal cellulase activity may also be involved with microspore release.

One of the possible roles for the callose wall, besides giving physical autonomy to the microspore in its early development, is its involvement in the establishment of the first exine patterning. Microspores are usually still embedded in the callose wall of the tetrad when the basic exine pattern is laid down. Dickinson and Heslop-Harrison (1968) and Waterkeyn and Beinfait (1970) both proposed models for the formation of exine patterning. The Dickinson and Heslop-Harrison theory suggests that whiteline bodies, initially composed of uniform layers of cellulosic material, sequentially condense to form a species-specific pattern of sporopollenin receptor sites. Sporopollenin, secreted by the microspore then accumulates at these sites beneath the callose wall. Waterkeyn and Beinfait (1970) focus more on the callose wall functioning as a template or mould for sporopollenin deposition.

The last stage at which microspores can be observed in the anthers of *N. tabacum* plants displaying a severe phenotype is just after microspore release. An abnormal wall consisting of a multi-laminate structure surrounds each microspore at this stage (Figure

6.11). This indicates that callose must play an important role in the correct formation of the microspore cell wall of *N. tabacum*. The multi-laminate wall appears to be an extreme form of the structures termed white line centred lamellae observed on the outside of microspores by Rowley and Southworth (1967) and Dickinson and Heslop-Harrison (1968). Interestingly, the laminate structure is also reminiscent of the tripartite lamellae involved with early exine development in liverwort spores (Brown and Lemmon, 1990). In the absence of callose it appears as though the cellulosic primexine has no surface to condense against and is therefore more obvious and exaggerated into layers or lamellations. The abnormality of the wall in sterile anthers is further illustrated by the thick and irregular deposits of sporopollenin seen on the surface of the abnormal microspores (Figure 6.11 and 6.13). In the absence of the callose wall, it seems that the formation of the proposed receptor sites for sporopollenin deposition is aberrant or may not occur. The situation may be further aggravated by the premature access of the microspore surface to tapetally derived sporopollenin afforded by the absence of the wall.

Wodehouse (1935) proposed that the arrangement of the apertures (colpi) of the pollen grain are related to the shape of the tetrad which is determined by the callose wall. More recent theories to explain how the position of the colpi is determined have arose from analysis of experiments where colchicine (Dover, 1972; Sheldon and Dickinson, 1986) or centrifugation (Heslop-Harrison, 1971; Sheldon and Dickinson, 1983) have been used to disrupt the meiotic spindle. These studies have shown that the position of the meiotic spindle influences both the tetrad symmetry and the position of the membranous shields which prevents the formation of primexine over the aperture region. The scanning electron micrographs generated from this study revealed that the material obtained from the surface of the dehisced anthers of the transgenic plants appeared to have apertures in the expected location on the surface of the sterile pollen grains. This supports the theory that the meiotic spindle controls the aperture position and that the callose wall has little, if any involvement.

6.3.5 Tapetum is Abnormal in Male-Sterile Transgenic *N. tabacum*

There appears to be a significant difference in tapetal morphology between fertile and male-sterile anthers. The most obvious abnormality is the increase in thickness of the male-sterile tapetal layer, a condition termed hypertrophy. This condition is often the first sign of abnormality in male-sterile anthers (Horner and Rogers, 1974; Horner, 1977; Warmke and Lee, 1977; Bino, 1985a, b). However, the degeneration of the microspores often occurs simultaneously with the proliferation of the tapetum, and therefore it is difficult to establish whether the abortion of the developing microspores causes the breakdown of the tapetum or *vice versa*.

In this experiment it is known that microspore development is the first to be disrupted, and a secondary effect is observed in the tapetum. The presence of tapetal hypertrophy in these plants indicates that this phenotype observed in many male-sterile lines may be due to

microspore abortion and not to tapetal malfunction. This tapetal thickening may indicate a retention of materials in the tapetum which would normally be transferred to the developing microspore or might be due to the reabsorption of sugars arising from the premature breakdown of the callose wall.

Increased vacuolation is also observed in the tapetum of the transgenic *N. tabacum*. The vacuole has many functions, transport and accumulation of substances and immobilization of toxic products are but a few. The formation of vacuoles in male-sterile plants may be a consequence of disturbances in one of these functions.

6.4 Summary

The cms lines of *Petunia* have been the subject of different types of investigation (Izhar and Frankel, 1971; Nivison and Hanson, 1989). The phenotype of these mutants is complicated and it is therefore difficult to determine the primary cause of the male sterility. By taking one aspect of the RM cms line of *Petunia*, the premature breakdown of the callose wall, the complicated phenotype has been dissected.

A modified basic PR β(1,3)-glucanase was expressed in the anther tapetum of *N. tabacum* to determine whether premature callose dissolution alone was sufficient to cause male sterility. The resulting transgenic plants displayed varying degrees of male sterility which is probably due to different levels of expression of the modified β(1,3)-glucanase. The modified β(1,3)-glucanase protein was secreted into the locular space, therefore indicating that the CTPP of the basic β(1,3)-glucanase is normally required for vacuolar targeting. Plants transformed with the modified β(1,3)-glucanase driven by the double CaMV 35S promoter were completely fertile, demonstrating that this promoter does not have a sufficient level of expression at the necessary time during development in the tapetum.

Removing the callose wall early during development gave an insight into the possible functions of callose in wild-type plants. A cytological study of meiosis revealed that the process was unperturbed by the lack of the callose wall. However, an investigation of the microspore wall by transmission and scanning electron microscopy showed a severely deformed lamellar structure coated with irregular deposits of sporopollenin instead of the regular vaulted structure of fertile microspores. In addition, the tapetum of transgenic male-sterile *N. tabacum* appeared to be abnormally vacuolate and hypertrophic. This indicated that the degeneration of the sporogenous cells led to a disruption of the tapetum. This highlights the close interaction which must occur between the developing microspores and the tapetum. The relative importance of tapetal abnormalities in the abortion of pollen are still open to question.

Chapter 7

Discussion

7.1 β(1,3)-Glucanase: an Enzyme with a Central Role in Microsporogenesis

Endo- and exo-β(1,3)-glucanases have many different functional roles. If the distribution of callose, the substrate for these enzymes, is analyzed a role in many different plant processes can be deduced, for example in fruit ripening (Hinton and Pressey, 1980), pollen tube growth (Ori *et al.*, 1990), coleoptile growth (Masuda and Wada, 1967), regulation of transport through the vascular tissue (Clarke and Stone, 1962) and cell division (Waterkeyn, 1967). β(1,3)-Glucanases also appear to be ubiquitous in nature; their presence in the secretions of bacteria and fungi are presumed to aid hydrolysis of plant debris and intracellular β(1,3)-glucanases in the digestive glands of invertibrates are supposed to aid with the decomposition of soil euglenids, protozoa and fungi. Of the plant endo-β(1,3)-glucanases, the PR β(1,3)-glucanases, particularly those from *N. tabacum*, have received the most attention since these proteins have been implicated in the defence response (Abeles *et al.*, 1971; Pegg *et al.*, 1977; Boller, 1985). This thesis is based upon a study of the microsporocyte callose wall and analysis of β(1,3)-glucanases which appear transiently during the development of the anther to hydrolyze this callose wall and aid microspore release. These anther-related β(1,3)-glucanases have been less frequently discussed in the literature.

7.1.1 β(1,3)-Glucanase Enzyme Activity Studies

In this thesis, two types of β(1,3)-glucanase enzyme activity, endolytic and exolytic, have been detected in *B. napus* buds and both show a periodicity that correlates with the breakdown of the microsporocyte callose wall. Maximum levels of endolytic and exolytic activity were detected in *B. napus* buds between the lengths of 1.5-3.5 mm, which includes buds likely to contain anthers in which microspore release is occurring. The temporal regulation of both activities parallels that reported in *Petunia* (Frankel *et al.*, 1969) and *Lilium* (Stieglitz, 1977).

In *Lilium,* Stieglitz (1977) found that although the endolytic and exolytic activity profiles are similar in that both show a distinct correlation with callose dissolution, there are appreciable differences between the two. The interval of endolytic β(1,3)-glucanase activity is much narrower than the exolytic, and is characterized by a two fold higher level of activity. Although the study in *B. napus* was not extensive enough to reveal differences in the periodicity between the two enzyme activities, it did provide sufficient data to show that the *B. napus* endo-β(1,3)-glucanase appeared to have a four fold greater level of activity over the exo-β(1,3)-glucanase (see section 3.2.1). Stieglitz suggested that the differences in the duration of activity in the anther could mean that each enzyme has a different

developmental significance in the process of microsporogenesis. Indeed, by fractionating anthers of *Lilium* at successive stages of development into different tissue types, Stieglitz also found that the two enzymes apparently have different patterns of spatial distribution. There was a relatively high level of exo-β(1,3)-glucanase activity situated in the outer somatic layer throughout the developmental period studied (from meiotic prophase to tetrad stage). Exo-β(1,3)-glucanase activity was also detected in the tapetal region, but even when this activity rose towards the end of the period analyzed, it did not reach that detected in the outer somatic layers. Stieglitz therefore concluded that the major proportion of exo-β(1,3)-glucanase is located in the somatic layers rather than in the tapetum and that the enzyme activity has a different profile in each tissue.

Endo-β(1,3)-glucanase activity was situated predominantly in the tapetum of *Lilium* anthers but was also detected in the outer somatic layers. In contrast to the exo-β(1,3)-glucanase activity, this enzyme was present at low levels in both tissues until late prophase when activity increased sharply. The tapetal tissue, in this case, contained the greatest level of activity.

Using isolated meiotic tetrads and semi-purified β(1,3)-glucanases Stieglitz also demonstrated that the endolytic activity alone was capable of callose degradation, and that the exolytic activity was not. Since the endo-β(1,3)-glucanase is in the immediate surroundings of the meiocytes and capable of callose degradation, Stieglitz proposed that the endo-β(1,3)-glucanase plays the primary role in callose breakdown by disrupting the long β(1,3)-glucan chains of callose. It was speculated that the resulting oligosaccharides then diffuse into the somatic layer where the exo-β(1,3)-glucanase can further hydrolyze them to monomers.

Perhaps the results described in this thesis enable Stieglitz's work to be reevaluated. Separation of *B. napus* bud protein on IEF gels and subsequent assays for β(1,3)-glucanase activity *in situ* revealed that there were many different endo-β(1,3)-glucanases and at least two exo-β(1,3)-glucanases present in the extract. Immunoblotting IEF gels with the PR β(1,3)-glucanase antiserum also showed that there were multiple bands despite only one band being detected on blots from SDS-polyacrylamide gels. If the antibody is immunoreacting with β(1,3)-glucanases in the extract it means that there are a number of isoforms of β(1,3)-glucanase of similar molecular weight. In support of the information gained from the test tube assays the majority of the β(1,3)-glucanase isoforms appeared to be temporally regulated with activity at the highest levels in the 1.5-3.5 mm buds.

Due to the purification procedures employed by Stieglitz, the β(1,3)-glucanases isolated from *Lilium* anthers were separated on the basis of molecular weight. It is possible that the activity peaks obtained from column fractions were due to multiple isoforms all having the

same molecular weight. Separation of *Lilium* anther extracts according to charge, as described in this thesis, is likely to reveal a more complex pattern of β(1,3)-glucanase proteins.

Whole *B. napus* buds were used in this study so it is not possible to establish which of the β(1,3)-glucanase isoforms originate in the anther tissue. Perhaps if the *in situ* gels had been carried out on extracts from anthers, the picture may have been simpler. Interestingly, Stieglitz identified two different profiles of exo-β(1,3)-glucanase activity from the different tissues of *Lilium* anthers. This could suggest that the activity is attributable to two different isoforms of the enzyme; one located in the outer somatic layer and one in the tapetal region. The existence of more than one form of exo-β(1,3)-glucanase in the anther is consistent with this study in *B. napus* where two exo-β(1,3)-glucanase enzymes were detected in extracts from buds (see section 5.2.8).

If there are two exo-β(1,3)-glucanases with different distribution patterns in anthers, the enzyme from the outer somatic layers could be involved with cell expansion or modification of the anther walls during development. The temporally regulated tapetally located enzyme would hence be implicated in the breakdown of oligosaccharide chains resulting from callose hydrolysis by the endo- activity. It is interesting to note at this point, that with regard to spatial distribution, A6 promoter activity is restricted to the tapetum. This has been demonstrated by fusions of a promoter fragment to the β-glucuronidase and barnase coding regions (Hird *et al.*, 1993).

In *N. tabacum* where anthers had been dissected from buds, only two endo-β(1,3)-glucanase activities with a periodicity expected for enzymes involved with microspore release were detected in the *in situ* gel assays. Both of these proteins had isoelectric points close to pI 7.0, but the predominant activity band was slightly more basic. The absence of detectable exo-β(1,3)-glucanase activity in *N. tabacum* anther extracts may be due to lability of this enzyme. The protein may be prone to denaturation and rendered inactive by the extraction and assay conditions. This seems highly likely as subsequent experiments have revealed the presence of one exo-β(1,3)-glucanase activity with a basic isoelectric point in the extracts from *N. tabacum* anthers. This activity is only detected when excess protein is loaded on the IEF gels (D. Hird, personal communication).

7.1.2 Regulation of Anther Hydrolytic Enzymes

The anther β(1,3)-glucanases have a very different role to the PR enzymes but a relationship was demonstrated between the two types of β(1,3)-glucanase in Chapter 3. The tetrad callose wall was hydrolyzed by intercellular fluid (ICF) extracted from *N. tabacum* leaves. The components of the ICF most likely to be responsible for this are β(1,3)-glucanases. Therefore, it seems that the PR β(1,3)-glucanases are capable of

substituting for callase *in vitro*. This experiment suggested that the relationship between the PR β(1,3)-glucanases and callase might be a simple one. For example, the enzymes could be related at the structural, as well as the functional, level. However, subsequent experiments made this doubtful.

The hydrolysis of the tetrad callose wall by PR β(1,3)-glucanases raises questions about the normal control of these PR enzymes in anthers. Since the mistiming of callase activity in certain lines of *Petunia* has been linked to male sterility (Izhar and Frankel, 1971), expression of PR β(1,3)-glucanase in response to pathogen attack in anthers must be under tight control. If wounding triggered systemic production of β(1,3)-glucanase in the anthers, microspore development would clearly be disrupted.

This experiment also clearly demonstrated that the *in vivo* substrate for PR β(1,3)-glucanases is callose. In response to pathogen attack, callose is produced at the wound site and PR β(1,3)-glucanases are induced or up-regulated. These two events appear to be antagonistic. The two processes must be regulated in some way during the wound response.

This study also revealed that microspore release in *N. tabacum* involves not only hydrolysis of the callose wall, but breakdown of the primary wall too. After treatment with the ICF the callose wall was removed but the microspores were still held together as a tetrad, presumably by the primary wall. In some studies of pollen development, examination of the primary wall around the sporocyte has shown that as the callose wall thickens the primary wall breaks apart (Heslop-Harrison, 1972; Horner and Rogers, 1974; Horner, 1977). However, in a few species the primary wall persists at least up until tetrad stage, and is then simultaneously broken down along with the callose wall to effect microspore release (Echlin and Godwin, 1968a; Stieglitz, 1977; Pacini and Juniper, 1979; Bhandari *et al.*, 1981). In these situations, several temporally regulated hydrolyzing enzymes must all appear at the same time in the anther locule to free the microspores from the tetrad.

If cellulases are involved in microspore release as seems likely, these enzymes would have to be under strict control to avoid damage to other cells of the anther. Alternatively, perhaps the primary cell wall of sporocytes has some special properties which distinguish it from other cells and thus enable it to be broken down without any damage to the rest of the anther. For instance, the sporocyte primary wall may have a different ratio of pectins, cellulose and other wall components. Observations of *Tradescantia* anther development suggest that the tapetum and sporogenous cell walls have a different constitution to the normal cell wall (Mepham and Lane, 1969). *Tradescantia* has a plasmodial type of tapetum and both the tapetal and sporogenous cell walls are hydrolyzed early in microsporogenesis. Mepham and Lane (1969) suggest that this early breakdown is due to the fact that the tapetal and sporogenous cell walls both contain a large amount of pectin. Their evidence for the pectic nature of the walls was based on staining with ruthidium red and digestion with

pectinase, which did not affect the walls of the parietal cells. The authors also suggest that the pectic walls of *Tradescantia* tapetum and sporogenous cells are different to that of *Helleborus* and *Cannabis,* which are more cellulosic. These plants both have a secretory tapetum. However, there have been inconsistencies in reports of the nature of the sporogenous cell wall of *Helleborus*. Some authors insist that it is mainly pectic (Waterkeyn, 1962) and others state that it is cellulosic (Echlin and Godwin, 1968a, b). Thus, although there appears to be conflicting information on the ratio of cell wall components of the sporogenous cells is does seem feasible that the walls may be different to other walls of the plant and this factor helps to facilitate microspore release without risking damage to other cells.

7.1.3 Immunoscreening for Anther β(1,3)-Glucanases

The ability of the PR β(1,3)-glucanases to hydrolyze the tetrad callose wall prompted the formation of a strategy to clone the anther β(1,3)-glucanases. If the PR enzyme could hydrolyze the same substrate as the anther enzymes then perhaps probes raised to the PR β(1,3)-glucanases would also detect the anther β(1,3)-glucanases. An antiserum raised to a PR β(1,3)-glucanase from *L. esculentum* (Joosten and De Wit, 1989) was donated to the laboratory and used to screen the *B. napus* anther cDNA library. Before the screening experiments were started, the ability of the antibody to recognize epitopes on the anther β(1,3)-glucanase proteins was tested. The preliminary results were encouraging as high molecular weight proteins of approximately 60 kD were detected in *B. napus* bud and *N. tabacum* anther extracts (section 3.2.3). The *N. tabacum* immunoreactive protein appeared to be constitutive, but the protein in *B. napus* had a temporal profile, peaking in abundance in buds at the microspore release stage.

Antibodies raised to *N. tabacum* acidic PR β(1,3)-glucanases have been used previously to probe protein extracts from anthers but no immunoreactive proteins were detected (Lotan *et al.*, 1989; Neale *et al.*, 1990). Antibodies raised to basic β(1,3)-glucanases involved with leaf excision in *Phaseolus vulgaris* have also been used for the same purpose without success (Campillo and Lewis, 1992). The immunoreactive proteins found in *B. napus* and *N. tabacum* were therefore the first proteins detected in anther tissue with a PR β(1,3)-glucanase antiserum. β(1,3)-glucanases have been detected in other floral tissues using PR probes (Lotan *et al.*, 1989; Ori *et al.*, 1990). One such protein, sp41 has 80 % amino acid similarity to the acidic class of β(1,3)-glucanase and appears to be developmentally regulated during flowering. This protein is not induced in response to stimuli that induce the PR β(1,3)-glucanases and thus may not be a true PR protein. It therefore appears that β(1,3)-glucanases belong to a large gene family, containing some members that are pathogen inducible and other members that exhibit

flower-specific developmental regulation.

Analysis of the *in situ* IEF gel β(1,3)-glucanase assays of *B. napus* bud extracts and the corresponding immunoblots probed with the PR β(1,3)-glucanase antiserum suggested that the immunoreactive proteins could have β(1,3)-glucanase activity. Since the proteins also appeared to be temporally regulated they seemed to be good candidates for components of the callase complex associated with microspore release. Screening the *B. napus* anther cDNA library should, in theory, have resulted in the isolation of sequences representing these immunoreactive proteins. However, this line of work was not pursued because the isolation of the A6 cDNA and the preliminary sequence analysis suggested that this cDNA could represent an anther β(1,3)-glucanase.

The ~60 kD immunoreactive protein identified in *N. tabacum* anthers and to a lesser extent in carpels and leaves had a constitutive pattern of expression in anthers and therefore was unlikely to represent a β(1,3)-glucanase involved with microspore release. IEF gels and duplicate immunoblots suggested that the immunoreactive protein did not have the same isoelectric point as demonstrated by the temporally regulated β(1,3)-glucanase observed on the *in situ* gel assay which is most likely to be the main callose degrading enzyme.

7.2 A6; an Anther-Specific β(1,3)-Glucanase?

7.2.1 cDNA Isolation

The differential screening of a *B. napus* anther cDNA library resulted in the isolation of several anther-specific clones (Scott *et al.*, 1991a). The anther-specific cDNA A6 was sequenced and the deduced protein was found to exhibit amino acid sequence similarity to the endo-β(1,3)- and β(1,3;1,4)-glucanase families. Despite low overall similarity of A6 to the other members of these families, the amino acid sequence alignments revealed that the A6 protein contained residues considered essential for catalytic activity of glucanase enzymes (Moore and Stone, 1972; Høj *et al.*, 1989b; Macgregor and Ballance, 1991). However, at present there is too little data to assign A6 to a particular class of glucanase on the basis of sequence alone. There are too few plant endo-β(1,3;1,4)-glucanase sequences available to implicate specific residues solely involved with this activity rather than β(1,3)-glucanase activity. It is therefore impossible to deduce from the alignments whether the A6 protein might be more likely to have β(1,3)- or β(1,3;1,4)-glucanase enzyme activity.

It would also be useful to compare the A6 sequence with a plant exo-β(1,3)-glucanase to determine whether any amino acids are shared. However, despite the purification of

several exo-β(1,3)-glucanases (Cline and Albersheim, 1981; Kurosaki *et al.*, 1989; 1991; Liénart *et al.*, 1986; Labrador and Nevins, 1989a), no sequence data is available to date. In contrast, several yeast exo-β(1,3)-glucanase sequences have been published (Vazquez de Aldana, *et al.*, 1991; Chambers *et al.*, 1993; Muthukumar *et al.*, 1993) but no significant amino acid similarity to either A6, or the plant and yeast endo-β(1,3)-glucanases is observed. Since some sequence conservation is observed between the plant and yeast endo-β(1,3)-glucanases, one might expect some degree of similarity between the yeast and plant exo-β(1,3)-glucanases. Since this is not the case, on this basis A6 is more likely to represent an endo-β(1,3)-glucanase.

If the A6 cDNA represents a β(1,3)-glucanase involved with microspore release, secretion of the protein into the locule to enable breakdown of the callose wall surrounding the microspore tetrads would be a feature expected of the protein. Significantly, fusion of the promoter region to *gus* and *barnase* have shown that the promoter is active in the tapetum (Hird *et al.*, 1993). Furthermore, the A6 protein derived from the nucleotide sequence has a putative signal peptide and therefore has the potential for secretion from the tapetal cells into the locular space. Both of these observations support the idea that A6 is a component of callase.

The Southern blot of *Brassica* species probed with the A6 cDNA revealed that there were multiple copies of the A6 gene in *B. napus*. Further differential screening of the *B. napus* cDNA library has also led to the isolation of three other cDNAs which are very similar to A6 (see section 4.3.2). This and the Southern blot data suggests that A6 is part of a closely related family of genes in *B. napus* which may represent a new class of plant glucanase restricted to anther development. At this stage in the project it appeared that A6 was a good candidate for a role in microspore release.

7.2.2 Production and Use of an Antibody Raised to A6 Protein

There were two obvious possibilities for the next step in characterization of the A6 clone, and both involved expression of A6 in *E. coli*. The most direct approach to determine whether A6 represented a β(1,3)-glucanase was to over-express the A6 clone and analyze the catalytic properties of the protein. Another approach was to generate antiserum to the over-expressed protein.

Since the predicted peptide for A6 contained a putative signal peptide, a possible long C-terminal extension and several sites which may be glycosylated in the mature protein, it was thought that the production of an active protein in *E. coli* may be difficult. To increase the chances of making an active enzyme, the A6 clone was modified by removing the sequences which encoded the putative signal sequence and C-terminal extension. This "mature" A6 fragment (mA6) produced by PCR was then used for over-expression experiments.

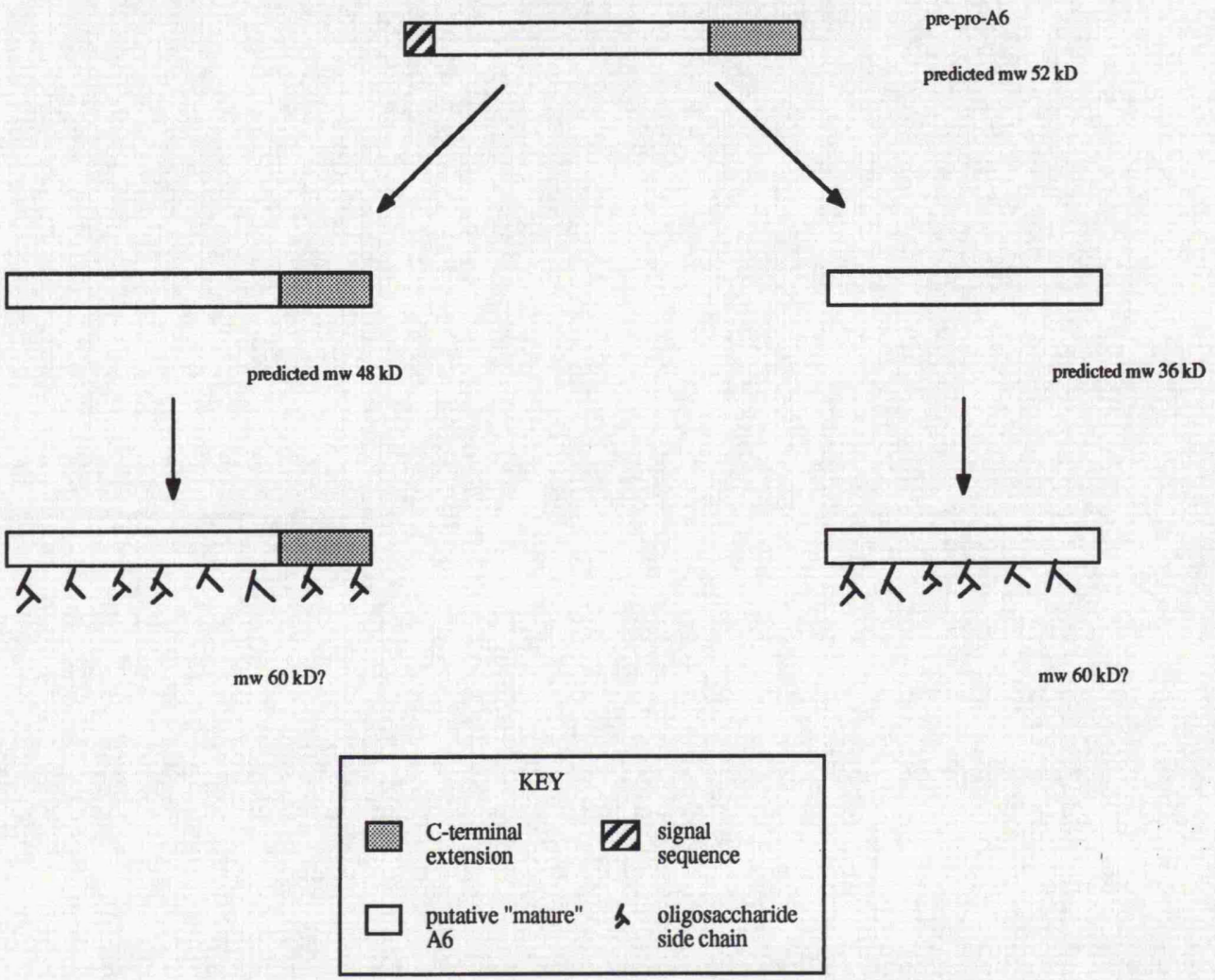
After the generation of the mA6 fragment several *E. coli* expression systems were investigated. The pGEM expression system (Promega) and the PT7-7 system (Tabor and Richardson, 1985) were available in the laboratory and therefore chosen as a starting point. The pGEM system was tackled first and worked effectively. The vector used in this system specifically results in the synthesis of T7 gene 10 fusion proteins and is thus not suitable for making active proteins but useful for generation of antibody. This part of the project was therefore pursued and the work involving production of active enzyme was passed over to D. Hird.

The anti-mA6 antibody was purified and then used to probe protein extracts from *B. napus*. Duplicate SDS-PAGE blots of *B. napus* bud protein probed with either the anti-mA6 antibody or PR β(1,3)-glucanase antiserum showed that both appear to recognize the same temporally regulated ~60 kD band. This band displayed a diffuse profile on the blots which is sometimes indicative of glycosylation. The molecular weight of this band was different to the value of 52.9 kD predicted for the A6 peptide. However, the predicted molecular weight included the putative hydrophobic signal sequence. When this was removed the size of the peptide decreases to 48 kD. Consequently, a discrepancy exists between the size of the protein detected by antibodies and the size of the A6 peptide inferred from amino acid sequence data. There are several possibilities to account for this. The addition of oligosaccharide side chains to the A6 peptide could account for the difference in molecular weight between the predicted value and the size of the protein detected on the blots. Analysis of the A6 peptide showed that it had eight possible sites for N-glycosylation. Six of these sites lie within the putative mA6 peptide and the other two sites are in the C-terminal region.

When aligned with representative proteins of the β(1,3)-glucanase family, the peptide predicted for the A6 cDNA appeared to have a very long C-terminal region. It is possible that this region serves as a sorting determinant. If this is the case there are several explanations to account for the size of the protein detected with the anti-mA6 antibody in *B. napus* bud extracts. These are illustrated in Figure 7.1. The oligosaccharide side chains of two yeast exo-β(1,3)-glucanases (Klebl and Tanner, 1989; Vazquez de Aldana *et al.*, 1991) and a carrot exo-β(1,3)-glucanase (Kurosaki *et al.*, 1991) add 3 kD to the molecular weight of these proteins. It is therefore possible that during processing the A6 peptide is cleaved down to the predicted "mature" form and heavily glycosylated, to give a protein with an approximate molecular weight of 60 kD.

An alternative is that the C-terminus is not removed and all of the eight predicted sites on the peptide are glycosylated. The resulting protein would probably be much bigger than 60 kD. As there is no evidence for the removal of the C-terminus during a processing event, until proven otherwise it must be assumed that the A6 protein is 48 kD as predicted and glycosylation increases the size to ~60 kD. If the A6 protein is involved in microspore release, then targeting of the protein to the vacuole (as is the case with the basic PR

Figure 7.1
Diagram Illustrating the Possible Processing Events of the A6 Peptide


pre-pro-A6
predicted mw 52 kD
predicted mw 48 kD
predicted mw 36 kD
mw 60 kD?
mw 60 kD?
KEY
C-terminal extension
signal sequence
putative "mature" A6
oligosaccharide side chain

β(1,3)-glucanases of *N. tabacum*) would be very unlikely as the enzyme would only be released upon destruction of the cell.

Immunoblotting IEF gels with both the anti-mA6 antibody and PR β(1,3)-glucanase antiserum revealed the presence of several cross reacting proteins in *B. napus* buds, despite only one band being detected on immunoblots from SDS-PAGE. Therefore, A6 is part of a family of immunoreactive proteins which all have different charges but the same, or similar, molecular weight. The A6 related proteins all appear to be temporally regulated, attaining maximum levels in buds between 1.5-3.5 mm. The existence of a family of A6 related proteins may have been expected if one considers the multiple banding pattern obtained on the Southern blot of *B. napus* DNA probed with A6.

It was envisaged that if *in situ* β(1,3)-glucanase IEF gel assays and immunoblots of duplicate samples were compared directly, an immunoreactive protein may have corresponded with an activity band and thus help to establish a role for the A6 peptide. The presence of multiple bands on the activity gels as well as on the immunoblots made this kind of analysis difficult. An additional complication was the fact that the antibody was likely to cross react, not only with active proteins, but also with non-active breakdown products. The table in Figure 7.2 attempts to indicate the presence of β(1,3)-glucanase activity or immunoreactive protein at particular isoelectric points. Despite the problems with this approach outlined above, the anti-mA6 antibody does appear to cross react with proteins in the same regions of the gel in which activity was detected, specifically in the isoelectric point range 8.3-10.6. But it is impossible to prove that the immunoreactive proteins definitely correspond to β(1,3)-glucanase activities even if they have the same mobility on IEF gels.

One way to test this technique further would be to pre-incubate the extracts with antibody before separation on the gel and then assay for β(1,3)-glucanase activity. Comparing these samples with non-treated extracts in relation to the presence or absence of particular activity bands may help to give a clearer result than duplicate activity gels and immunoblots.

Using the technique of immunoadsorption, preliminary experiments with the anti-mA6 antibody indicated that exo-β(1,3)-glucanase activity was inhibited in extracts from *B. napus* buds. Unfortunately when immunoadsorption experiments were carried out and total β(1,3)-glucanase activity assayed, no useful data was obtained. However, the association of A6 with exo-β(1,3)-glucanases is also supported by the molecular weight of the peptide, which is close to the size of both the exo-β(1,3)-glucanase purified from *Lilium* anthers and the cell wall associated exo-β(1,3)-glucanases (Cline and Albersheim, 1981; Liénart *et al.*, 1986; Kurosaki *et al.*, 1989). However, the amino acid sequence alignments of A6 with yeast exo- β(1,3)-glucanases did not display any significant identity, and thus, do not favour A6 representing an exo-β(1,3)-glucanase. The data appear to be conflicting and

Figure 7.2

Table to Summarize β(1,3)-Glucanase Activity Bands on *In Situ* Gel Assays and the Presence of Immunoreactive Proteins on Corresponding Blots

Each tick represents the presence of an activity band or immunoreactive protein. The cross indicates that no exo-β(1,3)-glucanase activity was detected in this isoelectric point range.

Isoelectric Point Range	Glucanase Activity		Protein Detected with Antibody	
	Total-Glucanase	Exo-Glucanase	Anti-mA6 Antibody	PR Glucanase Antiserum
5.65-5.90	✓✓✓	✓	✓✓✓	✓✓✓
6.45-7.30	✓✓	✗	✓✓	✓✓
8.3-10.60	✓	✓	✓✓✓	✓

further work is required to determine the function of the A6 peptide.

7.2.3 Floral Distribution of A6 and Related Proteins

The anti-mA6 antibody detected proteins in various floral organs of *B. napus*. It is debatable whether these other proteins result from expression of A6 in other tissues besides the anther. If one examines the phenotypic effect of *A6-barnase* expression in *N. tabacum*, only tapetal ablation and subsequent male sterility is discernible (Hird *et al.*, 1993). This suggests that the proteins detected in the carpels and sepals plus petals are related to A6, but are not products of the A6 gene itself.

Recently, transgenic *B. napus A6-barnase* plants have been generated. These plants also display male sterility, but in contrast to *N. tabacum*, some also show reduced female fertility and other floral abnormalities. It is possible that these features are the result of *barnase* expression from the A6 promoter. The fact that the anti-mA6 antibody also detects proteins in extracts from carpels and sepals plus petals of *B. napus* tends to further substantiate this. However, the immunoreactive proteins in the other floral organs of *B. napus* appear to be as abundant as the anther proteins. If the amount of immunoreactive protein reflected the activity of the A6 promoter in the anther, and *barnase* expression in the anther results in complete tapetal ablation, one would also expect the other floral tissues to be completely ablated since even low levels of barnase are extremely cytotoxic. Since this was not the case, the immunoreactive proteins in the carpel and sepal plus petals are unlikely to be products of the A6 gene but could represent other members of the A6 family. The fact that no floral abnormalities occurred in *A6-barnase N. tabacum* plants could mean that there is differential regulation of A6 related genes between *B. napus* and *N. tabacum*. In support of this interpretation, there appears to be more than one case where expression of *barnase* under the control of tapetum-specific promoters has resulted in floral abnormalities. When Denis *et al.* (1993) analyzed *B. napus* transformants containing *barnase* under the control of TA29, a tapetum specific promoter, floral abnormalities were also observed. Thus it is possible that the expression of *barnase* in one tissue of the anther may disturb communication between different parts of the flower and thus result in aberrations in other aspects of floral development.

Several features of the A6 peptide and the expression pattern of the A6 gene suggest that A6 is a good candidate for a component of callase. Alignments of the deduced peptide with previously characterized glucanases indicate that A6 could represent a β(1,3) or β(1,3;1,4)-glucanase. The size of the A6 peptide and preliminary experiments using the anti-mA6 antibody to inhibit enzyme activity favour an exolytic β(1,3)-glucanase role for the A6 protein. However, further experiments are required to clarify this.

If A6 does represent an exo-β(1,3)-glucanase involved with microspore release then a

yet uncloned gene encoding the endo-β(1,3)-glucanase must exist. The endo-β(1,3)-glucanase protein detected by Stieglitz in *Lilium* had a molecular weight of approximately 35 kD. The anti-mA6 antibody and PR β(1,3)-glucanase antiserum did not cross react with any protein of this molecular weight in *N. tabacum* or *B. napus*. Neither did the antibodies used in this study appear to cross react with the temporally regulated proteins of pI 7.0 detected in *N. tabacum* anthers using the *in situ* total β(1,3)-glucanase gel assays. Perhaps therefore, this activity corresponds to an endo activity similar to that detected by Stieglitz in *Lilium*.

The failure of existing probes to recognize this protein suggests that the endo-β(1,3)-glucanase is highly diverged in amino acid sequence from previously characterised β(1,3)-glucanases and A6. Thus, screening with existing β(1,3)-glucanase probes will not identify this endo-β(1,3)-glucanase. Therefore, the only means to be sure of cloning the gene encoding the endo-β(1,3)-glucanase involved with microspore release would be to purify the protein from anthers and carry out amino acid sequencing to generate oligonucleotide probes. These probes could then be used to screen a library or for PCR amplification.

Purification of the anther β(1,3)-glucanases would also enable comparison of the sequence with A6 and therefore shed light on the function of this peptide. If A6 does represent part of the callase complex, it will provide a means of discovering more about the mechanism by which callase secretion and the developmental stage of the microspores is coordinated.

The timing of callose breakdown during microgametogenesis is crucial and if incorrect male sterility may result. This suggests that callose plays an important role in microsporogenesis. In the past, observations of microspore development have led to speculation about the role of callose but as of yet there has been no definite proof for any of these hypotheses. One way of probing the significance of the callose wall in microspore development is to remove it prematurely and study the effects on spore development. This approach was utilized in the work described in the second part of this thesis.

7.3 An Investigation into the Role of the Callose Wall

The study of plants with mutant phenotypes is helping in the investigation of many different aspects of plant molecular biology, biochemistry and physiology. The rationale behind the use of mutants involves the identification of defined points in complex processes which when disrupted help analysis of the way in which the processes normally progress. Mutations can be spontaneous, or chemically and physically induced. In addition to mutations generated by these means, insertion mutants created by T-DNA transformation can also utilized.

Naturally occurring mutants exist where there is a deviation in the timing of β(1,3)-glucanase activity and callose wall hydrolysis in the anther and these lines are male-sterile (Frankel *et al.*, 1969; Warmke and Overman, 1972). These lines have therefore been studied in the hope of being able to dissect some of the processes of anther development and also for their uses in plant breeding. However, their phenotype appears to be very complex and it is difficult to establish whether the male sterility is a direct result of aberrant callose breakdown or whether this is secondary to other features such as degeneration of the tapetum (Bino, 1985a, b) or mitrochondrial dysfunction (Connet and Hanson, 1990).

7.3.1 PR β(1,3)-Glucanase Hydrolyzes the Tetrad Callose Wall *In Vivo*

Since it is difficult to dissect the complex observations of the natural male-sterile mutants, the work described in part of this thesis involved the generation of transgenic plants in which just one aspect of the *Petunia* RM mutants was reproduced, this being aberrant timing of β(1,3)-glucanase activity. There were two possibilities for the production of phenocopies of the male-sterile line, these were: (1) premature or (2) delayed timing of β(1,3)-glucanase activity in the anther locule. The experimental strategy chosen was the premature expression of β(1,3)-glucanase activity, as this avoided the need to suppress native β(1,3)-glucanase activity and might also provide more information on the role of callose in pollen development. As there were no sequence data available for the native anther β(1,3)-glucanase, an alternative enzyme was chosen for the experiment. Preliminary investigations (Chapter 3) had shown that some component of leaf intercellular fluid, most likely the PR β(1,3)-glucanases, was able to hydrolyze the callose wall around isolated tetrads *in vitro*, and this suggested that these enzymes may be able to substitute for anther β(1,3)-glucanase *in vivo*.

The experiments in Chapter 6 describe how a basic vacuolar endo-β(1,3)-glucanase was modified for secretion and expressed in *N. tabacum* under the control of tapetum-specific promoters. The resulting transgenic plants had anthers containing microsporocytes which lacked callose walls. The callose walls disappeared soon after promoter activity initiated, and this resulted in the plants displaying various degrees of reduced male fertility. The modified PR β(1,3)-glucanase was therefore successfully secreted from the tapetum into the locular space where it could exert its effects on the tetrad callose wall. This experiment therefore proved that an PR endo-β(1,3)-glucanase was able to substitute for the endo component of callase *in vivo*.

This experiment also provided evidence for the role of the C-terminal extension of the native vacuolar β(1,3)-glucanase. Previously the vacuolar β(1,3)-glucanase have been compared with other vacuolar PR proteins, such as chitinase and thus the C-terminal region

was assumed to be involved with vacuolar targeting. However, this had not been proven. The modified β(1,3)-glucanase which lacked the C-terminal region was secreted into the locular space of the anther, thus indicating that the C-terminal propeptide of the basic β(1,3)-glucanase contains information required for vacuolar targeting. However, further experiments are required before this can be demonstrated conclusively.

It was probably fortunate that basic vacuolar β(1,3)-glucanase was chosen for the experiment as even though the pH optimum for this enzyme is pH 5.5 it retains a significant level of activity at a neutral pH (Felix, 1984). The study of locular fluid pH in the anther indicated that pH is neutral earlier in development and becomes more acidic as development progresses. The PR β(1,3)-glucanase enzyme was therefore capable of callose degradation even though conditions were probably not favourable for maximum levels of activity.

7.3.2 A Role for the Tetrad Callose Wall in *N. tabacum*

The data generated from the transgenic plants in which β(1,3)-glucanase was expressed prematurely in the anther has also helped to give a clearer picture of the role of the callose wall in *N. tabacum* microspore development. In the past, several hypotheses have been put forward to explain the significance of the callose wall. Many of these ideas are based on the attempts to associate particular features of pollen morphology with specific functions. A different approach pioneered by Heslop-Harrison (1963) was to look at the development of the pollen grain and assess the biological significance of a certain feature. If a particular feature appears to be involved with several different cytoplasmic constituents then it must be important to the developing pollen grain. The idea that major features of the pollen grain such as arrangement of the apertures were related to tetrad geometry imposed by the callose wall has been discussed frequently (Godwin *et al.*, 1967; Heslop-Harrison, 1968a; 1971). These types of observations are perhaps limited and need to be proved experimentally. For example, disruption of cells at specific stages of development and then permitting them to mature is an informative way to discover the involvement of particular organelles in pollen development.

Disruption by centrifugation (Sheldon and Dickinson, 1983) or colchicine treatment (Sheldon and Dickinson, 1986) has provided insight into the mechanism by which pollen wall patterning is established, in particular the positioning of the colpus. This work strongly suggests that it is the meiotic spindle which influences the the tetrad geometry and the positioning of the membranous shields at the sites of developing apertures. The study described in this thesis involved the *in vivo* disruption of development in pollen. SEMs of pollen produced from transgenic plants revealed that although pollen wall patterning is severely affected, the position of the apertures remains constant. Observations of isolated tetrads from these transgenic plants also indicated that the microspores are more loosely

associated within the tetrad structure and do not form the typical tetrahedral shape. This appears to correlate with the work of Sheldon and Dickinson in that it is the organisation of the microtubular cytoskeleton which determines the apertural positioning and that the callose wall has little if any involvement with this process.

A frequently discussed possible role for the callose wall is to isolate the young microspores from each other and from the influences of the tapetum. Before meiosis in the anther, the sporocytes in effect share a common cytoplasm since they are all attached via cytoplasmic channels, forming a kind of giant coenocyte. After the first or second meiotic division, depending upon whether cytokinesis is successive or simultaneous, the connections are severed by deposition of the cell plate and the rapid accumulation of callose. It seems that upon the formation of the new haploid nuclei, genetic identity is required, perhaps to prevent competition between the gametes. At this time the callose wall is completed and thus can be seen as serving the purpose of sealing each microspore within its own unit of cytoplasm. The anthers of the transgenic plants described in this thesis frequently contained clumps of abnormal microspores. It appears that in the absence of the callose wall, separation of the microspores after meiosis was not maintained and therefore aggregations of abnormal microspores form. Although genetic isolation was achieved initially because cytokinesis occurs in the transgenic plants, it is subsequently lost. The callose wall therefore appears to be required to attain genetic and physical separation of the microspores. However, it is also possible that the microspores fuse together after microspore release since the pollen walls are so poorly formed.

One suggested role for the callose wall is to provide sugars, through its hydrolysis, for the continuation of pollen development. Observations of isolated tetrads and transgenic plants in which the callose wall has been removed prematurely has helped to form ideas for how the callose wall may be involved with osmosis in the anther locule. Soon after microspore release in the transgenic plants, many of the abnormal spores appear to burst. This could be due to the incomplete pollen wall failing to protect the spore against an increase in cell size due to water uptake via osmosis. In Chapter 3 it was shown that when isolated tetrads were treated with intercellular fluid and the callose wall removed, causing increased sugar concentration, the treated spores decreased in size considerably in comparison with the untreated spores. Perhaps under normal conditions in the locule, when the callose wall is broken down the sugars are moved into the spores followed by water through osmosis to maintain turgidity. This causes the spores to increase in size and the correctly formed pollen wall prevents the cells from bursting. In the experiment in Chapter 3 with the isolated tetrads, the normal mechanism by which this occurs is presumably disrupted and therefore the spores lose water by osmosis. In the transgenic pollen, one can envisage a situation in which sugars have been absorbed by the spores and thus water moves in by osmosis, but since the pollen wall has not formed correctly the microspores burst.

Another possible function of the callose wall which has received less discussion in the

literature involves its direct participation in the development of pollen wall ornamentation. Godwin *et al.* (1967) and Waterkeyn and Beinfait (1970) described the action of the callose wall as a template defining the position of the apertures and the deposition of the primexine in *Ipomoea*. Observations of freeze dried tetrads of *Scorzonera hispanica* suggests that the complex echinophate pollen wall pattern is also partly determined by the callose wall (Barnes and Blackmore, 1986). Well before the primexine is formed the callose walls have a pattern resembling the spacing of the ridges and spines of the mature wall.

It is unknown how this patterning is produced at present but two explanations have been suggested. It is possible that the final stages of callose wall deposition occurs differentially on the outside of the plasma membrane or that the inner surface of the callose wall is modified by β(1,3)-glucanase activity secreted from the microspore. Dickinson and Heslop-Harrison (1968) suggested a model for exine formation where the folding of a lamellar structure after primexine formation provides receptor sites for sporopollenin accumulation, but the involvement of the callose wall was not mentioned in this theory. In the Dickinson and Heslop-Harrison theory, if the lamellae lie flat against the microspore surface the sporopollenin is laid down in a flat layer and if the lamellae fold perpendicular to the surface forming radial protrusions then the sporopollenin is built up on the top forming the probacula.

Close study of the pollen from the transgenic plants generated in the experiments described here clearly indicate the importance of callose in pollen wall formation. In the absence of callose, the pollen wall appears to consist of a thick multi-laminate structure upon which irregular masses of sporopollenin are deposited. In the abnormal transgenic pollen, the mechanism of pollen wall development is severely disrupted. It is unclear however, whether this is because normally the callose layer makes a solid surface against which the lamellae are compressed or whether a callose template for exine sculpturing is absent. It is also possible that normally the callose acts as a source of glucose for primexine formation.

Sheldon and Dickinson (1983) updated the earlier model of Dickinson and Heslop-Harrison (1968) for exine formation and proposed that some kind of proteinaceous material is inserted into the plasma membrane to form contiguous plates. Cytoplasmic vesicles fuse with the plasma membrane and deposit the proteinaceous material into it. Once within the membrane this material organizes itself into juxtaposed plate structures or continuous sheets. The simple reticulate pattern of *Lilium* exine, they suggest, could be created by shrinkage at the surface of a matrix (as suggested by Wodehouse, 1935), or by hydrophilic/hydrophobic mixtures such as oil droplets on water. The natural shape resulting from even shrinkage is an array of regular polygons such as that formed when mud cracks. A similar pattern would be produced if the contents of the juxtaposed plates were not entirely lipophilic. They would then aggregate into circular plates within the fluid mosaics of the membrane. In either of these two mechanisms the baculae would form by movement of the sporopollenin precursors through the plasma membrane and into the

spaces formed by the cracks or juxtaposed plates.

How then does the situation in the transgenic *N. tabacum* pollen fit into this model? The normal *N. tabacum* pollen exine is stratified into tectum, bacula and foot layer and therefore, the patterning of the baculate layer is normally hidden from view by the tectum. In the transgenic pollen, the baculae are exposed. In the normal situation, it is possible that the sporopollenin precursors initially flood out of the microspore between the juxtaposed plates at a fast rate so that it rapidly spreads out over the whole surface of the spore into the more or less continuous layer of the tectum, before it has chance to polymerize. The polymerizing agents could be limited due to the excess amounts of sporopollenin precursors. After tectum formation, the extent of sporopollenin precursor diffusion is slower and thus the polymerizing agents can regulate the pattern of deposition in a more ordered manner. Perhaps the callose layer functions to provide a solid surface against which the tectum forms and also aids with the lateral diffusion of the sporopollenin precursors. In the transgenic plants where the callose layer is removed, the sporopollenin has no limit to its spread in a outwards manner and therefore it moves outwards instead of spreading over the surface of the spore to form the tectum. The sporopollenin can be observed in large amorphous lumps on the surface of the transgenic pollen, where tectum formation has broken down.

Although the transgenic plants produced in this study indicate that the callose wall is essential for pollen wall formation in *N. tabacum*, this is not always the case. Some species exist where there is no callose deposition before or after meiosis and microspore release does not occur. Pollen is dispersed as tetrads, or pollinia (Vijayaraghavan and Shukla, 1977). The exine is sparse and irregular in such species thus supporting the supposed function of the callose wall. Any model accounting for the importance of the callose wall in exine formation must also account for why patterning is possible in species that do not produce callose such as *Pandanus odoratissimus* (Periasamy and Amalathas, 1991) or form the exine after microspore release as in *Sorghum* (Christensen *et al.*, 1972). It seems reasonable to assume that in some instances the role of callose is absent or diminished but it is at its most significant in the generation of walls with elaborate architecture, complex stratification and patterning. To conclude, perhaps the callose wall is primarily adapted for isolation of the microspores and has gradually been developed for the fixation of tetrad geometry and the control of primexine deposition and hence exine patterning.

7.4 Male Sterility and Plant Breeding

The aims for the selection of improved crop plants have remained relatively constant for many years. Plants which have greater yield, resistance to pests and diseases and environmental stresses are selected. Apart from the direct selection of the best plants from existing populations, plant breeding at the simplest level involves the deliberate

hybridization of two parental plant genotypes to produce a novel recombinant genotype.

In fully controlled breeding it is essential that the male and female parents are protected from contamination by foreign pollen and since most flowering plants bare hermaphrodite flowers it is necessary to remove the anthers from flowers born on the female parent. This process of emasculation is often carried out by hand and is very time consuming and can result in damage to the flower. In addition, this process is not feasible for agricultural crops because of the scale.

The existence of male-sterile lines are of considerable use because they avoid the necessity of emasculation and provide the breeder with valuable material for the production of hybrid seed. In many crop plants, male sterility is controlled by a single nuclear gene and the condition can be maintained by crossing the recessive male-steriles with heterozygous female lines, giving half male-sterile and half male-fertile progeny. Another type of male sterility, cytoplasmic male sterility (cms), has been studied more extensively. This is usually associated with mitrochondrial dysfunction, but a phenotype is only manifested in the pollen. The male-sterile trait is inherited maternally. Plants displaying cms are therefore used as the female in crosses so that any seed produced on the cms parent will be the result of cross-pollination.

However, natural male-steriles may not always be available for plant lines carrying desirable features. This problem can be overcome by the advances of genetic engineering. Not only is it possible to introduce genes from other organisms (viruses, bacteria, algae) into plants, but also alter development or growth of the plant by manipulating the plant's genetic constitution. Previously, male-sterile plants have been generated by the expression of barnase (Mariani *et al.*, 1990; Paul *et al.*, 1992) or diphtheria toxins (Koltunow *et al.*, 1990) in the anther tapetum. Although these toxic proteins serve their function extremely well, transgenic crop plants expressing toxins may not be acceptable to the agricultural authorities or the consumer.

The production of plants which express a non-toxic protein of plant origin is obviously more appealing. The transgenic plants generated in this study may be useful for this purpose and have therefore attracted industrial interest (Nickerson Seeds, now part of Limagrain). The main aim of Nickerson Seeds was to introduce the modified PR β(1,3)-glucanase constructs into *B. napus*, a species important for its seed which is rich in oils. This has been carried out and the resulting plants display the same characteristics of male sterility as the *N. tabacum* transgenic plants. Obviously for the production of seed crops, the male sterility phenotype has to be reversed. Restorer genes are employed for this purpose. The term restorer gene is taken from the cms systems where the cms phenotype is a result of nuclear and cytoplasmic interactions. The nuclear encoded 'restorer' gene is able to over-ride the male sterility factor and thus confers fertility. When cms lines are used for breeding the cms female is crossed with a male which has normal fertility and a dominant restorer gene. The progeny are heterozygous for the restorer gene and therefore fertile and produce fruit and seed. This restorer approach could be extended for use with the PR

β(1,3)-glucanase expressing *B. napus* plants. One possibility could be the use of an antisense β(1,3)-glucanase for the restorer gene. If crosses were carried out where the female parent with premature β(1,3)-glucanase activity in the anther was crossed with a male parent homozygous for antisense β(1,3)-glucanase, the F1 hybrid would be heterozygous for the restorer and therefore be fertile and hopefully produce normal levels of seed.

7.5 Future Work

7.5.1 Further Characterization of A6

Preliminary experiments using the anti-mA6 antibody suggest that exo-β(1,3)-glucanase activity in extracts from *B. napus* buds was specifically inhibited by the presence of the antibody. Although the size of the A6 protein is consistent with that of the exo-β(1,3)-glucanase previously isolated from *Lilium* anthers (Stieglitz, 1977), alignment of the A6 peptide with other plant endo-β(1,3)- and β(1,3;1,4)-glucanases indicated a relationship with these enzymes. Because of this conflicting data several different approaches are currently being carried out to determine what type of glucanase activity, if any, the A6 peptide possesses. These approaches are detailed below.

Fusion of the complete coding region of A6 to the A9 promoter has resulted in the expression of A6 in the anther tapetum of transgenic *N. tabacum* plants before the appearance of the native callase enzymes (D. Hird, personal communication). It was anticipated that if A6 encoded a β(1,3)-glucanase, premature breakdown of the tetrad callose wall would result as with the PR β(1,3)-glucanase experiment. Unfortunately, the transgenic plants displayed no obvious phenotype. Immunoblotting experiments using the anti-mA6 antibody have determined that the A6 peptide is present in the locular fluid of anthers at microspore release stage in these transgenic plants (D. Hird, personal communication). This proves that the signal sequence at the N-terminus of the A6 cDNA is functional and that the A6 peptide is secreted from the tapetum into the locular space. The size and isoelectric point of the A6 protein expressed in *N. tabacum* is the same as that predicted for the A6 peptide. Despite this, the A6 peptide does not appear to exhibit callose hydrolysis.

Endo- and exo-β(1,3)-glucanase *in situ* gel assays and duplicate immunoblots were carried out on anther extracts from these transgenic plants but no activity appeared to be associated with the immunoreactive protein. Perhaps the A6 protein is unstable or requires a different β(1,3)-glucan substrate to the one used in the assay. If A6 does represent an exo-β(1,3)-glucanase it may be that a β(1,3)-linked glucan dimer such as laminaribiose would be a better substrate than laminarin, which has long chains of glucans.

Over-expression experiments of the A6 sequence in *E. coli* should also be pursued to try to obtain active enzyme. Previously, a basic PR endo-β(1,3)-glucanase from *N. plumbaginifolia* has been over-expressed in *E. coli* and active enzyme produced (Castresana *et al.*, 1990). The modified PR β(1,3)-glucanase used in the experiments described in Chapter 6 should be cloned into the expression vector and used along side the A6 expression vector as a good positive control to determine whether the protein extraction and assay systems were working effectively. Experiments to establish enzyme activity are crucial to determine whether the A6 peptide is part of the callase complex involved with microspore release. Although all the data so far suggests that A6 is a good candidate, the exact enzymic nature of the protein remains elusive.

The production of the anti-mA6 antibody was useful for investigating the size and distribution of A6 and related proteins in *B. napus*. However, it was not possible to determine what post-translational modification the protein undergoes to produce the mature form. In section 7.2.2, several mechanisms for modification of the A6 peptide which would result in a ~60 kD protein are discussed. It is possible that the long C-terminal region of the peptide is not present on the mature protein and only required for some processing event such as vacuolar targeting, similar to the situation found in the basic β(1,3)-glucanases of *N. tabacum*.

Since there are eight putative glycosylation sites on the A6 peptide sequence it seemed reasonable to assume that the increase in size from the predicted 52.9 kD to the actual ~60 kD was due to the addition of oligosaccharide side chains. In order to investigate the state of the mature A6 peptide, deglycosylation experiments have subsequently been carried out (D. Hird, personal communication). When treated with endoglycodidase F, the A6 peptide is only slightly smaller than the untreated protein. This result indicates that the most or all of the C-terminal extension must be retained on the A6 protein and glycosylation of one or more of the possible sites on the peptide causes the increase from the predicted molecular weight. Alternatively, since endoglyosidases are only capable of cleaving high mannose glycans it is possible that the A6 C-terminal extension is removed and the mature protein glycosylated with complex glycans.

7.5.2 Future Use of the Modified β(1,3)-Glucanase in Transgenic Plants

The production of transgenic *N. tabacum* and *B. napus* plants which prematurely express a β(1,3)-glucanase has helped to answer some interesting biological questions about the role of callose in microsporogenesis. These plants may also provide breeders with a useful tool for the production of F1 hybrid seed. Before the transgenic plants can be used for this purpose several criteria must be satisfied. The ability for a single copy transformant to cause complete male sterility is essential for breeding. The production of a homozygote of this plant would then mean that all of the progeny would contain a copy of

the gene. Segregation analysis of the T1 seed from the *N. tabacum* transgenic plants to suggests that the site of integration of the T-DNA rather than the copy number was the important factor in determining the level of expression of the modified β(1,3)-glucanase in anthers. Thus, it was possible that plants with a low copy number, possibly a single copy, could be completely male sterile. Southern blot analyses and further investigation of self seed set are required before this can be determined conclusively.

Complete male sterility in the modified β(1,3)-glucanase transgenic plants occurred very infrequently. It appeared that even in plants which displayed the most extreme phenotype some viable pollen-like structures survived. Perhaps if the amount of β(1,3)-glucanase protein in the anthers was increased, complete sterility would result. Insertion of a translational enhancer sequence, such as the omega enhancer from tobacco mosaic virus (Gallie *et al.*, 1987), in front of the β(1,3)-glucanase sequence may be one way of achieving this.

Expression of the modified PR β(1,3)-glucanase in the anther tapetum severely disrupted pollen development. Tapetal origin of the β(1,3)-glucanase component of callase has always been assumed in the literature since this is the major secretory tissue of the anther. Origin of β(1,3)-glucanase enzymes in the microspore itself is not generally considered. Barnes and Blackmore (1986) suggest a possible mechanism for the way in which pollen wall patterning is determined by the callose wall. This involves modification of the callose wall in specific areas by localized secretion of β(1,3)-glucanase. This hypothesis could be tested by expression in plants of the modified PR β(1,3)-glucanase under the control of a microspore-specific promoter which is active up until tetrad stage if such a promoter were available.

Callose is present in other locations, besides the tetrad wall, during the life cycle of the plant. For example, during the formation of pollen tubes, callose forms in localized deposits along the length of the none growing region of the tube. The function of these plugs is not known. Fusion of the modified PR β(1,3)-glucanase to a promoter which is active in pollen tubes could help to determine the function of these callose deposits. The *lat52* promoter from *L. esculentum* (Twell *et al.*, 1989) is active in pollen tubes, and *lat52-PR β(1,3)-glucanase* fusions are currently under test in transgenic *N. tabacum* plants.

7.5.3 A Method for Investigating Possible Interactions Between the Anther Tapetum and Developing Microspores

A very delicately balanced functional and biochemical relationship must exist between the tapetum and sporocytes throughout microsporogenesis so that viable pollen is produced. Disturbances in this harmony can result in cell abnormalities and male sterility. The temporal patterns of endo- and exo-β(1,3)-glucanase activity in anthers further indicate

that a direct relationship exists between the tapetum and microsporocyte during callose wall dissolution. It is interesting to speculate that the process of microsporogenesis is influenced by events in the tapetum via some kind of cell-cell signalling system. The production of transgenic plants where tapetal or microsporogenous cells are disrupted provides us with a possible method for looking at such signalling between the microsporocytes and the tapetum. Transgenic plants expressing *A6-gus* could be crossed with plants in which the microspores are ablated using an early microspore-specific promoter fused to *barnase*. The progeny from this cross could then be analyzed with respect to timing of GUS activity in the tapetum as compared to plants with normal fertile microspores. If the disruption of the microspores also affects tapetal functioning, a deviation in the timing of *gus* expression under control of the A6 promoter would be expected. Since an early microspore-specific promoter has not yet been identified, the transgenic plants generated in the work described in Chapter 6 of this thesis could also be used for this experiment. However, although microspore development is disrupted in these transgenic plants this disruption is initially due to expression of a modified PR β(1,3)-glucanase gene in the tapetum. Therefore, it may be difficult to establish whether any disruption in the tapetum, as detected by a change in the pattern of *gus* expression, is a result of microspore dysfunction or just due to the initial expression of the transgene in the tapetum. Interestingly, the tapetum in the transgenic plants expressing the modified PR β(1,3)-glucanase does display abnormalities in the form of hypertrophy. This type of experiment may not determine the exact nature of the mechanism by which the tapetal and sporogenous cell development is coordinated, but it may prove that some interaction does occur.

7.6 Summary and Conclusions

This thesis describes an investigation of the significance of the tetrad callose wall and the involvement of endo- and exo-β(1,3)-glucanases in the hydrolysis of this wall at microspore release. Endo- and exo-β(1,3)-glucanase activity were found to be temporally regulated during *B. napus* bud development, with maximum activity close to the time of microspore release. Charge dependent separation of *B. napus* bud protein on IEF gels determined that these peaks in activity were due to several different isoforms of β(1,3)-glucanase enzyme each having a unique isoelectric point.

Differentially screening a *B. napus* anther cDNA library resulted in the identification of a tapetum-specific gene termed A6. Sequence analyses of the A6 cDNA revealed similarity to plant endo-β(1,3)- and β(1,3;1,4)-glucanases. However, the A6 sequence is significantly diverged from the previously characterized glucanases, particularly due to the presence of the long C-terminal region. An antibody was raised to the central region of A6 which represented a putative mature A6 peptide. This antibody appeared to recognize the

same proteins as an antibody raised to a PR endo-β(1,3)-glucanase from *L. esculentum* in extracts from *B. napus* anthers and buds. Both antibodies recognized a band of approximately 60 kD on SDS-PAGE blots. This band actually represented several proteins all having approximately the same molecular weight but different isoelectric points. The anti-mA6 antibody appeared to have a greater affinity over the PR β(1,3)-glucanase antiserum for proteins with basic isoelectric points. This seems to agree with the computer predicted isoelectric point for the A6 peptide. Preliminary immunoadsorption experiments with the anti-mA6 antibody suggested that exo-β(1,3)-glucanase activity in *B. napus* buds was inhibited. Attempts to align the A6 sequence with exo-β(1,3)-glucanases isolated from *S. cerevisiae* resulted in insignificant matches. This data thus conflicts with the antibody experiments. Further work is still required to determine the enzymic role of the A6 peptide. However, the A6 sequence data coupled with the temporal and spatial expression data, seems to suggest that A6 may be part of the complex of enzymes involved with microspore release.

The significance of the callose wall was more directly investigated by prematurely removing this layer surrounding the microsporogenous cells. This was achieved by creating transgenic plants which secreted a modified PR β(1,3)-glucanase from the anther tapetum prior to the appearance of the native enzyme in the locule. Plants expressing this β(1,3)-glucanase exhibited reduced male fertility. Callose deposition appeared to be normal until prophase I whereupon it is prematurely degraded. After the removal of the callose wall the spores initially remain held together as a tetrad, presumably by the primary wall, and are later released. Meiosis is unaffected by premature callose removal, but the deposition of the pollen wall is severely disrupted. The resulting microspores appear to have completely lost their normal tectate perforate wall sculpturing and are covered by irregular deposits of sporopollenin. The tapetum appears to exhibit hypertrophy and is thus likely to be disrupted in its development. The reduced male fertility of these transgenic plants is likely to be due to bursting of the deformed spores at a time soon after microspore release. The results in this thesis suggest that premature callose degradation is sufficient to cause male sterility and that the callose wall is essential for correct pollen wall formation in *N. tabacum*.

Appendices

Appendix A
Glucanase Activities in *B. napus* Buds

Appendix A.1

		Bud Length (mm)		
		<1.5	1.5-2.5	>3.5
Total-glucanase activity (µg glucose/hr/mg protein)		0	87.70	18.08
		19.1	114.29	25.60
		0	125.70	19.14
		0	26.66	19.10
		0	24.00	16.00
	Average	3.82	75.67	19.58
	Standard Error	3.82	21.46	1.61

Appendix A.2

		Bud Length (mm)			
		<1.5	1.5-2.5	2.5-3.5	>3.5
Exo-glucanase activity (µg glucose/hr/mg protein)		0	6.20	7.25	2.16
		2.13	7.73	8.78	2.39
		5.46	19.80	22.51	7.51
		2.38	18.79	19.15	5.12
		2.57	20.72	23.39	4.01
	Average	2.508	14.65	16.21	4.35
	Standard Error	0.87	3.16	3.43	0.94

Appendix B

Seed Set Data For Investigating Male-Fertility in Transgenic Plants

Appendix B.1

Transformant	Number of Selfed Pods	Weight of Seeds per Plant	Average Weight of Seeds per Pod
A9(tl)PR#1	2	0.0566	0.0283
#4	2	0.0423	0.0212
#5	5	0.0810	0.0162
#6	6	0.0850	0.0142
#7	3	0.0590	0.0197
#8	62	1.2210	0.0197
#9	1	0.0165	0.0165
#10	12	0.0630	0.0053
#11	4	0.0320	0.0080
#13	7	0.0360	0.0051
#14	7	0.1190	0.0170
#15	4	0.1239	0.0310
#16	2	0.0575	0.0285
#17	20	0.7960	0.0390

Appendix B.2

Transformant	Number of Selfed Pods	Weight of Seeds per Plant	Average Weight of Seeds per Pod
A9PR#1	39	0.5460	0.0140
#2	2	0.0085	0.0043
#4	3	0.0310	0.0103
#5	0	0	0
#7	8	0.1160	0.0145
#8	3	0.0453	0.0151
#9	9	0.0410	0.0046
#2A	11	0.0320	0.0029
#4A	10	0.0482	0.0048
#14	1	0.0085	0.0085

Appendix B.3

Transformant	Number of Selfed Pods	Weight of Seeds per Plant	Average Weight of Seeds per Pod
A3PR#1	42	1.9000	0.0452
#2	2	0.0175	0.0088
#1A	11	0.3980	0.0362
#1B	34	0.9560	0.0281
#5	35	1.0500	0.0300

Appendix B.4

Transformant	Number of Selfed Pods	Weight of Seeds per Plant	Average Weight of Seeds per Pod
CaMv 35SPR#1	46	3.720	0.081
#2	59	5.896	0.100
#5	35	3.047	0.090
#6	41	3.935	0.100
#7	2	0.128	0.064
#8	28	1.420	0.050
#9	2	0.154	0.080
#10	10	0.373	0.037
#11	14	1.029	0.070
#12	15	0.568	0.040
#13	17	1.218	0.070
#14	14	1.870	0.130
#15	14	1.628	0.120

Appendix B.5

Plant	Number of Selfed Pods	Weight of Seeds per Plant	Average Weight of Seeds per Pod
#1	36	3.248	0.090
#2	16	1.936	0.121
#3	26	2.461	0.095
#4	24	2.113	0.088
#5	32	2.946	0.092
#6	20	1.883	0.094
#7	27	2.676	0.099
#8	39	2.857	0.073
#9	33	2.538	0.077
#10	28	2.517	0.090

Appendix C
Locular Fluid pH Data

Appendix C.1 Wild-Type Anthers

Anther Length (mm)	Developmental Stage	Locular Fluid pH	Average pH	Standard Deviation
1.0 1.0 1.0	Archesporial	6.92 7.07 6.9	6.963	0.093
1.5	Archesporial	7.0	7.0	-
2.0 2.0 2.0	Meiosis	6.64 6.48 6.62	6.55	0.013
2.0-2.5	Meiosis	6.55	6.55	-
2.5 2.5	Tetrad	6.52 6.54	6.53	0.014
2.5-3.0	Microspore Release	6.1	6.1	-
3.0 3.0 3.0 3.0	Microspore Release	6.13 6.32 6.3 6.17	6.23	0.094
3.5	Free Microspores	6.02	6.02	-
4.0 4.0 4.0	Microspore Mitosis	5.84 6.02 5.89	5.92	0.093
4.5	Early Maturation	5.4	5.4	-

Appendix C.2 Transgenic Anthers

Transformant	Anther length (mm)	Locular Fluid pH	Average pH
A9 PR	2.5 4.0 4.0	6.46 6.52 6.52	6.46 6.52
A3 PR	3.0 4.0 4.0	6.08 6.25 6.39	6.08 6.32
35S PR	1.0 2.5 3.0	7.07 6.4 6.62	7.07 6.4 6.62

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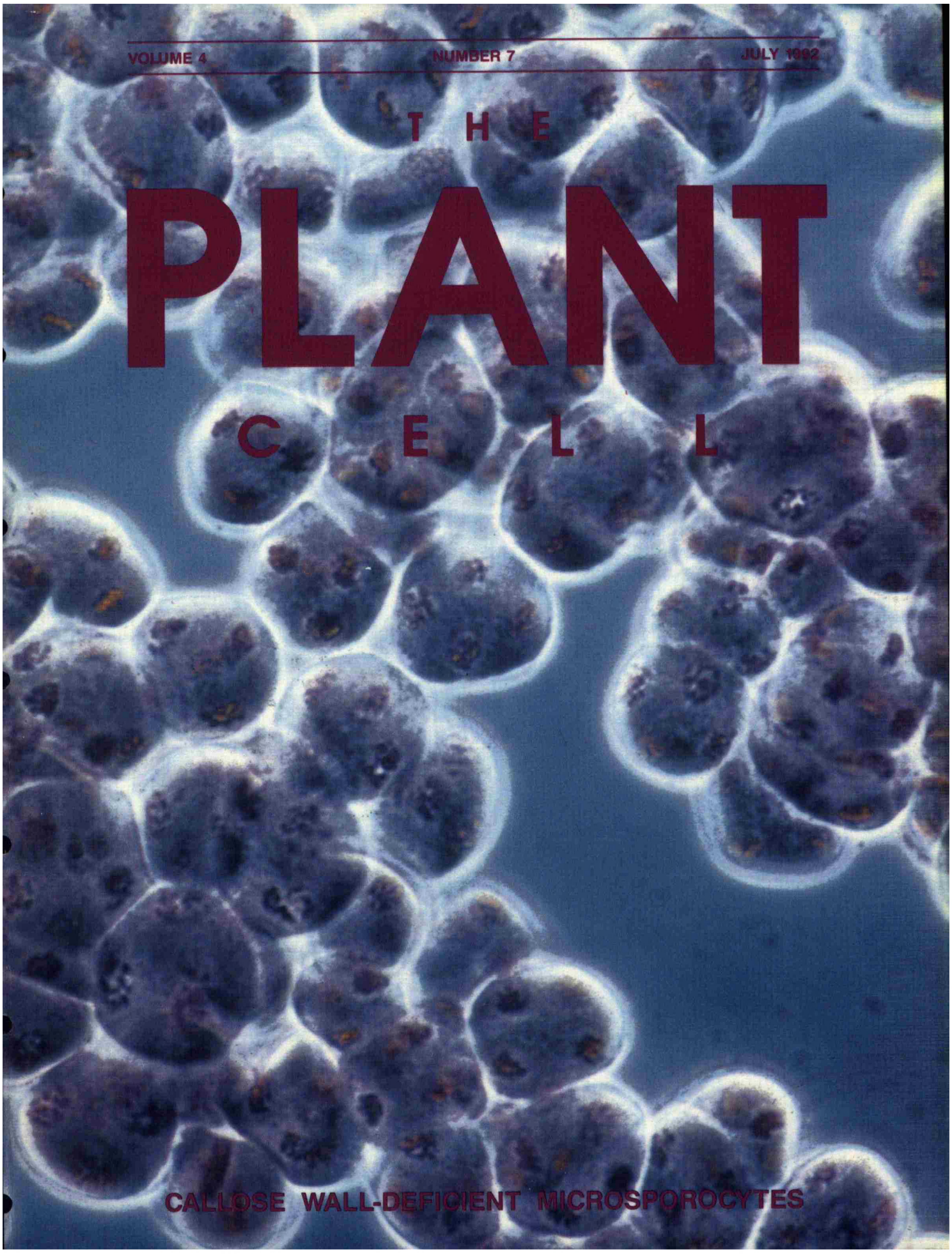
THE PLANT CELL



CALLOSE WALL-DEFICIENT MICROSPOROCYTES

The Plant Cell, Vol. 4, 759–771, July 1992

Premature Dissolution of the Microsporocyte Callose Wall Causes Male Sterility in Transgenic Tobacco

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Male sterility in a petunia cytoplasmic male sterile line has been attributed to the early appearance of active callase, a β-1,3-glucanase, in the anther locule. This leads to premature dissolution of the callose walls surrounding the microsporogenous cells. We have mimicked this aspect of the petunia line in transgenic tobacco by engineering the secretion of a modified pathogenesis-related vacuolar β-1,3-glucanase from the tapetum prior to the appearance of callase activity in the locule. Plants expressing the modified glucanase from tapetum-specific promoters exhibited reduced male fertility, ranging from complete to partial male sterility. Callose appearance and distribution are normal in the male sterile transgenic plants up to prophase I, whereupon callose is prematurely degraded. Meiosis and cell division occur normally. The resultant microspores have an abnormally thin cell wall that lacks sculpturing. The tapetum shows hypertrophy. Male sterility is probably caused by bursting of the aberrant microspores at a time corresponding to microspore release. These results demonstrate that premature callose degradation is sufficient to cause male sterility and suggest that callose is essential for the formation of a normal microspore cell wall.

INTRODUCTION

Before meiosis in angiosperms, microsporocytes synthesize a special cell wall consisting of callose, a β-1,3–linked glucan, between the cellulose cell wall and plasma membrane. Callose deposition continues through meiosis so that each of the products of meiosis, the tetrad of microspores, is also surrounded by callose. After the completion of meiosis and the initiation of microspore exine wall formation, the callose wall is broken down by callase, a tapetally secreted β-1,3-glucanase activity (Steiglitz, 1977), releasing free microspores into the locular space. In anthers of petunia and lily, callase activity follows a tight pattern of developmental regulation (Frankel et al., 1969; Steiglitz and Stern, 1973; Steiglitz, 1977). A relatively low level of activity is present in anthers during the first meiotic division, but once the second meiotic division starts, this rapidly increases and peaks at the time of microspore release.

In anther locules of several cytoplasmic male sterile (cms) petunia lines, callose wall dissolution occurs earlier (Rosy Morn [RM] cms) or later (partially restored cms) than normal due to the premature or delayed appearance of callase activity (Izhar and Frankel, 1971). It has been suggested that mistiming of callose wall degradation may be a primary cause of male sterility in these lines (Izhar and Frankel, 1971) and also in male sterile sorghum lines (Warmke and Overman, 1972). However, it is not known whether this is the only factor that contributes to male sterility. Changes in the abundances of amino acids in the locule (Izhar and Frankel, 1973) and alterations in tapetal morphology (Bino, 1985a, 1985b) precede callose wall degradation in the RM cms line. Because the molecular basis of the phenotype appears to be the expression of a novel mitochondrial protein (Nivison and Hanson, 1989), which may affect mitochondrial energy production (Connett and Hanson, 1990), there may be a general breakdown of normal tapetal or microsporogenous cell function leading to male sterility. These male sterile plants, therefore, provide only circumstantial evidence that the callose wall has a vital function in microsporogenesis.

Callose is not part of the normal cell wall; however, when produced in response to wounding (Goodman et al., 1986), it participates in the formation of a physical barrier against pathogen invasion. Delmer (1987) termed this callose "useful" and suggested that callose produced in other contexts, for example, in the cell plate and pollen tube wall, is an "accidental," nonfunctional product of callose synthase activity induced by elevated Ca^{2+} concentrations associated with other cellular processes. Thus, in the absence of clear evidence of an essential role, microsporocyte callose could be considered "accidental." However, several theories on the biological functions of this special callose wall have been advanced. Waterkeyn (1962) suggested that callose fulfills an important biological role in acting as a temporary wall that both isolates the products of meiosis to prevent cell cohesion and fusion and, upon its dissolution, results in the release of free cells. Heslop-Harrison (1964) proposed that the callose wall functions as a molecular filter isolating the developing microspores from the influence of the surrounding diploid tissue or sister spores (Heslop-Harrison and Mackenzie, 1967). These authors also

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suggested that the wall may prevent premature swelling of the microspores. Finally, Waterkeyn and Beinfait (1970) suggested that the callose wall provides a template or mold for the formation of the species-specific exine sculpturing patterns seen on mature pollen grains.

We have sought to mimic the natural RM cms phenotype by creating transgenic tobacco plants that secrete a modified pathogenesis-related (PR) vacuolar β-1,3-glucanase from the tapetum before the appearance of callase activity in the locule. As well as being attractive for plant breeding purposes to facilitate the production of hybrid seed (Mariani et al., 1990), the resulting phenocopies, it was envisaged, would give a better understanding of the role of callose in microsporogenesis. The results obtained demonstrate that premature callose dissolution is sufficient to cause male sterility in transgenic tobacco and that tapetal hypertrophy can be a consequence of a disruption in microspore development. Aberrant microspore cell wall formation observed in the male sterile plants also suggests that the callose wall is required for the correct formation and surface patterning of the microspore exine wall.

RESULTS

Construction of Chimeric Genes for the Expression of a Modified Basic PR β-1,3-Glucanase in the Tapetum

Two components are required for the construction of a chimeric gene that will cause premature callose wall dissolution when expressed in plants. These are a β-1,3-glucanase that can be secreted from the tapetal cells into the anther locule and a promoter to drive the expression of this β-1,3-glucanase prior to the appearance of normal callase activity in the locule. Although the anther-specific β-1,3-glucanase enzyme (callase) responsible for microspore release has not been cloned, several PR glucanases have been characterized. We have demonstrated that intercellular fluid extracted from salicylic acid–treated tobacco leaves, containing PR glucanase enzymes, is capable of releasing microspores from tetrads in vitro (R. Scott, unpublished results). Therefore, the PR β-1,3-glucanases are able to hydrolyze callose and might substitute for callase if expressed appropriately in vivo. Shinshi et al. (1988) have reported the nucleotide sequence of a tobacco basic vacuolar PR glucanase. We hoped that expression of this gene under the control of tapetum-specific promoters that are transcriptionally active during meiosis would cause premature breakdown of the callose wall and therefore induce male sterility.

Figure 1A shows that vacuolar isoforms of PR β-1,3-glucanases of tobacco are initially synthesized as precursors with N- and C-terminal peptide extensions. The glycosylated C-terminal propeptide (CTPP) and the N-terminal signal peptide are removed upon processing to the mature protein (Shinshi et al., 1988). Removal of the CTPP of barley lectin showed that this extension is necessary for vacuolar targeting (Bednarek et al., 1990). Without the CTPP, lectin follows the default pathway and is secreted from the cell. van den Bulcke et al. (1989) suggested that the 22–amino acid basic vacuolar glucanase CTPP may be the signal that directs this protein to the vacuole. More recently, cDNAs have been isolated that encode acidic glucanases found in the intercellular compartment of

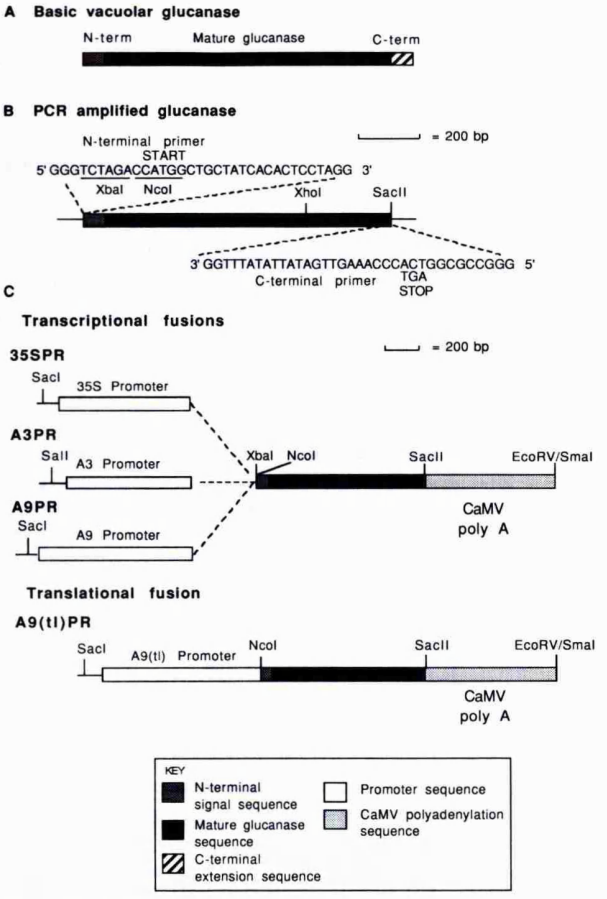

Figure 1. Construction of a Modified Basic PR β-1,3-Glucanase Gene.

(A) Schematic representation of the coding region of the tobacco basic vacuolar PR glucanase gene.

(B) Schematic representation of the modified PR glucanase showing the sequence of the primers used for PCR amplification and relevant restriction enzyme sites.

(C) Diagram showing the strategy for cloning each promoter-glucanase gene construct into pBin19 (Bevan, 1984). The transcriptional fusions, 35S PR and A9 PR, were both cloned as SacI-EcoRV fragments into SacI-SmaI-cut pBin19, and A3 PR was cloned as a SalI-EcoRV fragment into SalI-SmaI-cut pBin19. The translational fusion (A9(tl)PR) was cloned as a SacI-EcoRV fragment into SacI-SmaI-cut pBin19. The A9 translational fusion promoter, unlike that of the A9 transcriptional promoter, includes the entire untranslated A9 leader with the sequence around the initiating ATG of A9 mutated to an NcoI site (Paul et al., 1992).

leaves (Côté et al., 1991; Ward et al., 1991). These secreted forms of β-1,3-glucanases lack a CTPP. This indirect evidence supports the idea that the basic glucanase CTPP contains vacuolar targeting information.

On the basis of this information, we designed synthetic oligonucleotides complementary to the nucleotide sequence of the basic PR β-1,3-glucanase and used them as primers to amplify a modified glucanase that included the N-terminal signal peptide but lacked the CTPP (Figure 1B). This modification was presumed necessary to achieve secretion of the PR glucanase enzyme from the tapetum to the locule. The sequence of the final modified glucanase gene was identical to the equivalent region of the published basic glucanase (Shinshi et al., 1988), except for five nucleotide substitutions (see Methods).

This modified glucanase gene was then transcriptionally fused to a double cauliflower mosaic virus (CaMV) 35S promoter (Guerineau et al., 1988), forming the chimeric gene (35S PR) depicted in Figure 1C. The modified glucanase was also transcriptionally fused to the tapetum-specific promoters of the Arabidopsis A3 and A9 genes (Scott et al., 1991a, 1991b; Paul et al., 1992) (A3 PR and A9 PR constructs in Figure 1C) and translationally fused to the A9 promoter (A9(tl)PR, Figure 1C). Promoter–β-glucoronidase (promoter–GUS) fusions in transgenic tobacco have shown that the A3 and A9 promoters become active in tapetal cells of anthers containing microsporocytes in the early stages of meiosis. Promoter activity reaches a maximum during meiosis, and this level is sustained until shortly before the first microspore mitosis (Scott et al., 1991b; Paul et al., 1992). Thus, the CaMV 35S promoter and the tapetum-specific promoters should be suitable for the premature expression of glucanase in the anther. The chimeric genes were constructed and transformed into tobacco as described in Figure 1 and Methods.

Expression of the Modified Glucanase Gene from the Tapetum-Specific Promoters, but Not from the CaMV 35S Promoter, Causes Male Sterility in Transgenic Tobacco

DNA was extracted from kanamycin-resistant transgenic tobacco plants and analyzed for the presence of the modified glucanase gene by the polymerase chain reaction (PCR) utilizing the N- and C-terminal glucanase oligonucleotides as primers. The native PR glucanase was also amplified by the PCR reaction, but the presence of an intron within this gene resulted in the production of a larger fragment that was easily distinguishable from the transgene. Plants that produced only the larger native PR glucanase gene product upon PCR analysis were termed PCR negative.

Both PCR positive and negative plants were grown to maturity, and the flowers were analyzed. All transformants containing the modified glucanase gene under the control of the A3 or A9 anther-specific promoters were normal in appearance but displayed varying degrees of male fertility. In contrast, all of the PCR negative plants were completely fertile. Transformants with a severe phenotype had small recessed anthers that were brown and appeared to lack pollen grains. In these plants, only flowers that had been cross-pollinated with wild-type tobacco pollen developed normal seed pods. Only one of these plants, A9 PR 5, was completely male sterile; the rest formed a variable number of small pods that were either empty or contained few seeds in comparison to those of wild-type plants. Transformants containing the modified glucanase gene under the control of the double CaMV 35S promoter were completely normal in appearance and significantly more fertile than the plants expressing the modified glucanase gene from the tapetum-specific promoters. The fertility of 35S PR transformed plants was not appreciably different from that of wild-type plants. The bar graph in Figure 2 indicates the degree of fertility displayed by the transformants as determined by the average weight of seeds produced per pod. Plants containing A9 promoter fusions appeared less fertile than those containing the A3 promoter fusion, and the transcriptional fusion to the A9 promoter appeared more effective than the translational A9 fusion. These results are in accord with the relative strengths of these promoters in GUS fusion experiments, which show that the A9 transcriptional promoter is approximately four times more active than the A3 promoter (R. Scott, unpublished observations).

The transmission of the male sterile phenotype to progeny following cross-pollination with wild-type pollen was followed

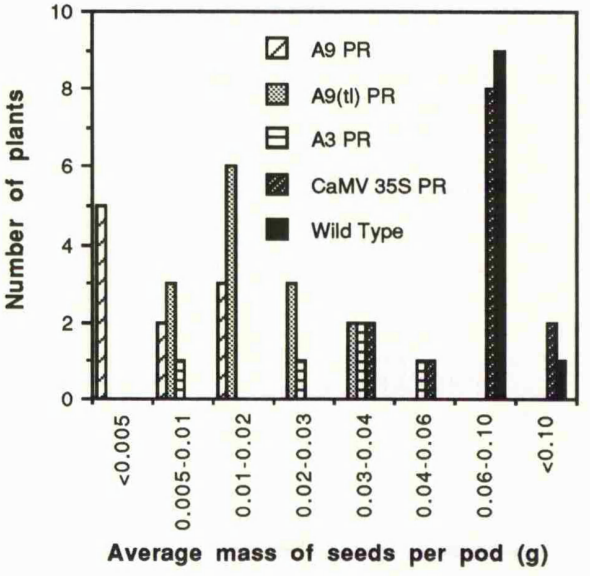

Figure 2. Fertility of Transgenic and Wild-Type Tobacco Plants.

Bar graph showing the average mass of seeds per pod in wild-type tobacco and each transgenic tobacco line (100 wild-type tobacco seeds weigh approximately 0.009 g).

for a number of transformants. For example, kanamycin-resistant seedlings of A9 PR5 × wild type (1 in 20 seedlings was kanamycin sensitive) were grown to maturity, and all exhibited the male sterile phenotype (data not shown).

Anthers from the PCR-positive plants were analyzed for the presence of the introduced glucanase protein by immunoblotting with an antiserum raised against an acidic PR glucanase from tomato that cross-reacts with the tobacco basic PR glucanase. Tobacco buds ranging in length from 8 to 14 mm encompass the period of development where the A3 and A9 promoters are most active, as shown by promoter GUS fusion data (Scott et al., 1991; Paul et al., 1992). The immunoblots shown in Figures 3A and 3B demonstrate that the modified glucanase protein is present in the anthers of transgenic plants. The introduced glucanase protein can be seen on the protein gel blots as an immunoreactive band with a molecular mass of approximately 35 kD that appears to have a slightly increased mobility in comparison to the immunoreactive glucanase enzymes in wild-type tobacco leaf; this is most noticeable in Figure 3C. The PR glucanase proteins present in leaf, represented by immunoreactive bands with molecular masses of approximately 35 to 37 kD, are not detectable in the anthers of control untransformed tobacco plants (Figure 3D). One transformant, A9(tl)PR 17 (Figure 3B, lane 4), which was PCR positive, did not contain a detectable 35-kD immunoreactive band and was significantly more fertile than the other plants containing the A9 and A3 promoters shown in Figure 3.

The presence of PR glucanase protein, as detected on immunoblots, correlates with the male sterile phenotype demonstrated by the transformed plants, except in the case of transformants containing the glucanase gene under the control of the CaMV 35S promoter (Figure 2). The glucanase protein detectable in the anthers of these plants is present at levels similar to those in transformants containing the other constructs, but the lack of a male sterile phenotype suggests that expression in the tapetal cells is either absent or too low to cause significant callose degradation. The glucanase accumulated in these anthers is likely to result from expression of the CaMV 35S promoter in other anther cell types.

The immunoblot in Figure 3C shows that the introduced glucanase protein accumulates in a temporal pattern consistent with the properties of the tapetum-specific promoter. The modified glucanase protein is first detectable during early meiosis, reaches a peak at the stage when microspore release occurs, and then declines in abundance again in anthers at a stage corresponding to microspore mitosis.

Male Sterility Is Associated with Premature Disappearance of the Callose Wall

A study of microsporogenesis was undertaken in transformants containing each of the promoter–glucanase constructs and untransformed control tobacco plants. Anthers at developmental stages between meiocyte and microspore release were dissected from buds and the locular contents stained for callose with aniline blue. The development and dissolution of the microsporocyte callose wall in all CaMV 35S PR plants examined were identical to that in wild-type plants. In contrast, the microsporocyte callose wall was prematurely degraded in the A9 PR, A9(tl)PR, and A3 PR plants that exhibited reduced male fertility. Preliminary studies found no significant difference in the extent and timing of callose degradation in these plants.

Figure 4 shows the appearance of the callose wall in anthers of untransformed tobacco and in male sterile tobacco anthers of an A9(tl)PR plant during the same developmental period. Microsporocytes of both sterile and fertile anthers appear normal before the initiation of meiosis. The meiocytes in both preparations have callose walls, seen as bright yellow fluorescence (Figure 4A). The first observable difference between fertile and sterile anthers occurs during prophase I, when the thick callose wall that previously surrounded the meiocytes disappears in the transformed material (Figure 4B). In wild-type tetrads, callose is deposited between the plasma membrane and the cell wall producing clear boundaries that separate the microspores (Figure 4E). The microspores of sterile anthers appear to lack a callose wall but still remain held together as tetrads by some other material that does not show aniline blue fluorescence. A second difference between tetrads of fertile and sterile anthers was that, whereas tetrads squeezed out from fertile anthers separate easily, presumably due to a lack of intercellular cytoplasmic connections, tetrads from sterile anthers adhered in amorphous clumps. Microspore release does occur in male sterile plants (Figure 4F), but very soon afterward, a large proportion of the deformed microspores burst. The relationship between anther length and developmental stage remains the same in both male fertile and sterile plants, and thus the timing of the individual events that together constitute microsporogenesis appears unaffected in the male sterile plants.

Meiosis Is Normal in the Absence of the Callose Wall

In addition to the microsporocyte cell wall, callose also appears transiently around the megasporocyte during megasporogenesis at early meiotic prophase (Bouman, 1984). Because the temporal and spatial distribution of callose is similar in both the male and female gametophyte, and callose is not a normal component of cell walls, it was considered possible that callose is involved in some aspect of meiosis in higher plants. To determine whether meiosis was normal in male sterile plants, meiotic cells were dissected from both male fertile and male sterile anthers and stained with aceto-orcein. In sterile anthers, despite premature dissolution of callose at early prophase I before the chromosomes have condensed, the sporocytes still undergo apparently normal meiosis. The four products of meiosis can be seen as separate entities held together within tetrads (Figure 4E). However, the four microspores within the tetrad are much more loosely associated than those seen in the untransformed control tetrad as often the nuclei of all four cells can be seen in the same plane. Aceto-orcein staining demonstrated that the microsporocytes had normal numbers of bivalents and chiasmata (results not shown).

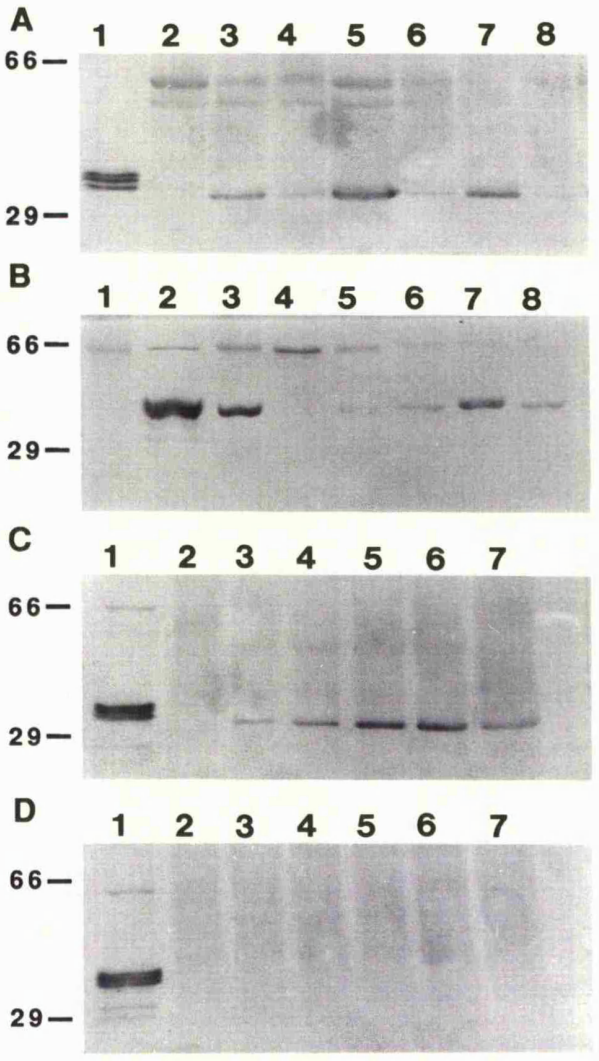

Figure 3. Modified Glucanase Protein Is Present in Anthers of Transgenic Tobacco Plants.

Protein gel blots of SDS–polyacrylamide gels were probed with an antibody raised against a tomato acidic PR glucanase.

(A) Protein gel blot of an 11% SDS–polyacrylamide gel. Lane 1 contains protein extracted from salicylic acid–induced wild-type tobacco leaves, and lanes 2 to 8 contain protein extracted from anthers dissected from buds ranging in length from 8 to 14 mm. Lane 2, wild-type plant; lane 3, A9 PR 1; lane 4, A9 PR 9; lane 5, A9(tl)PR 4; lane 6, A9(tl)PR 13; lane 7, A3 PR 2; lane 8, A3 PR 1.

(B) Protein gel blot of a 15% SDS–polyacrylamide gel. Lane 2 contains protein extracted from salicylic acid–induced wild-type tobacco leaves. Lane 1 and lanes 3 to 8 contain protein extracted from anthers dissected from buds ranging in length from 8 to 14 mm. Lane 1, wild-type plant; lane 3, A9(tl) PR 14; lane 4, A9(tl)PR 17; lane 5, A3 PR 1A; lane 6, 35S PR 2; lane 7, 35S PR 7; lane 8, 35S PR 14.

(C) Protein gel blot of an 11% SDS–polyacrylamide gel. Lane 1 contains protein extracted from salicylic acid–induced wild-type tobacco leaves, and lanes 3 to 7 contain protein extracted from the anthers of the transgenic tobacco plant A9(tl)PR 1. Protein was isolated from anthers at the following developmental stages: archesporial, lane 2; meiocyte, lane 3; tetrad, lane 4; microspore release, lane 5. Lane 6 contains anthers of buds between 12 and 20 mm in length, and lane 7 contains anthers from buds between 20 and 30 mm. Microspore release occurs at a bud length of 9 to 10 mm.

(D) Protein gel blot of an 11% SDS–polyacrylamide gel. Lane 1 contains protein extracted from salicylic acid–induced wild-type tobacco leaves. Lanes 2 to 7 contain protein extracted from wild-type anthers at the same developmental stages and between the same size ranges as described in **(C)**. Molecular mass markers are given at left in kilodaltons.

Microspores Exhibit Aberrant Wall Development in the Absence of Callose

The callose wall is thought to play an important role in the establishment of the first exine patterning seen on the surface of the microspore. Various hypotheses have been proposed to explain the involvement of callose in laying down the pattern of primary exine (primexine). Larson and Lewis (1962) proposed that the callose wall is a source of glucose for the development of cellulosic primexine, which provides the basic framework of the future exine. Waterkeyn and Bienfait (1970) suggested that the callose wall acts as a template or mold, which is filled by primexine. Because callose may play an essential role in the initial steps of microspore wall development, we examined the structure of the microspore wall of the A9(tl)PR transformants.

Electron micrographs revealed abnormal wall development in sterile microspores during the period when the microspores are still embedded in the callose wall of the tetrad in control fertile plants. Figures 5A to 5E show electron micrographs of developing microspores from fertile and sterile tobacco anthers. The thick callose wall surrounding each microspore of the tetrads from control fertile anthers (Figure 5A) is clearly absent in sterile microspores (Figure 5B). The microspores from fertile anthers also appear to have a more regular outline than those from sterile anthers. The characteristic vaulted structure of the exine of control microspores (Figure 5C) is replaced by lamellae of varying thicknesses overlain by irregular deposits of electron dense material (Figure 5D). This material is likely to be sporopollenin. Some binucleate microspores were observed in the sterile anthers that may result from the failure of cytokinesis or subsequent fusion of the microspores (Figure 5E).

The Tapetum Exhibits Hypertrophy in Male Sterile Anthers

In some types of male sterility, ultrastructural and histological examinations have revealed that the first signs of abnormality are found not in the microsporocytes or developing microspores, but in the tapetum (Horner and Rogers, 1974; Horner,

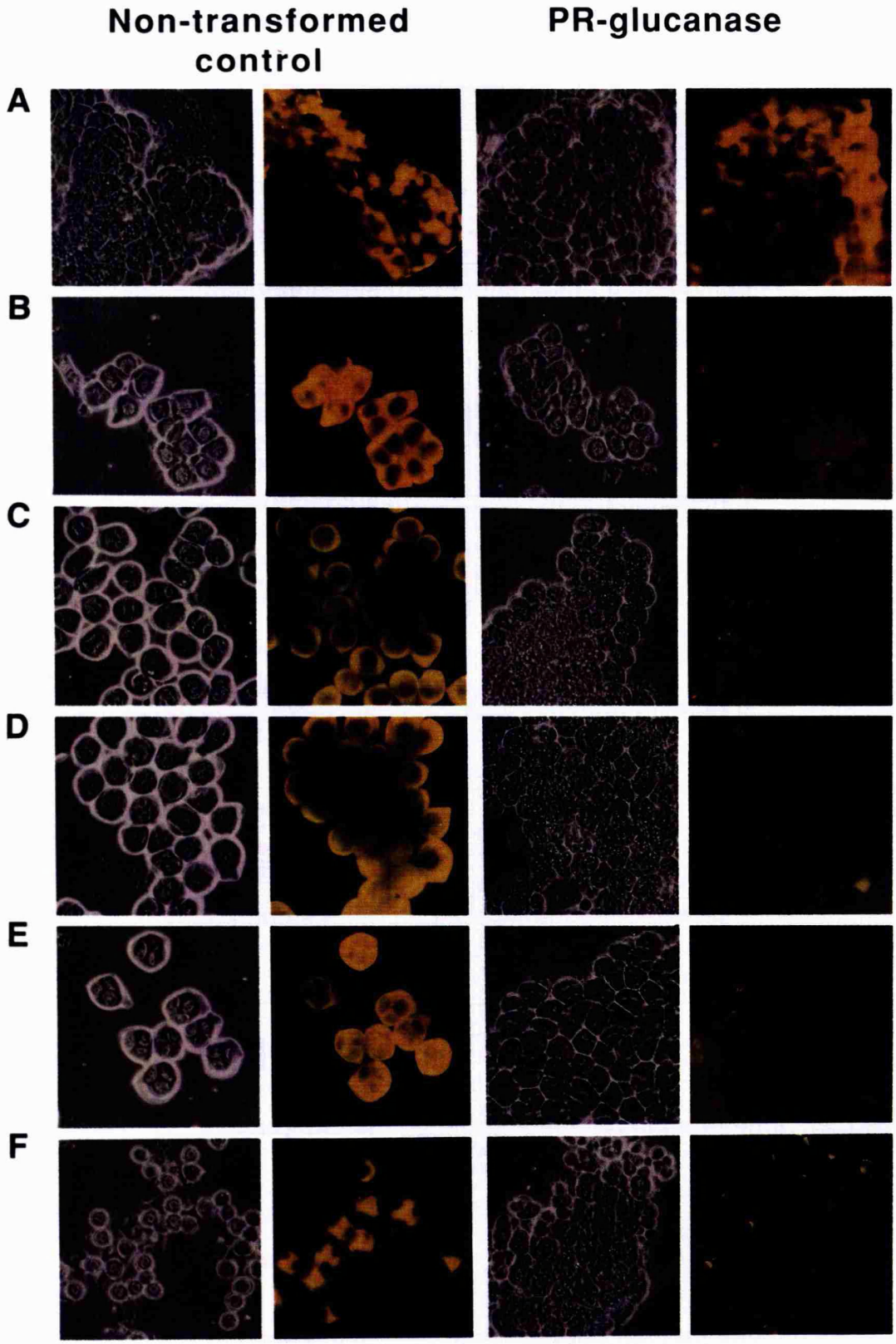

Figure 4. Comparison of the Callose Wall during Microsporogenesis in Wild-Type Tobacco and Those Transformed with Modified Glucanase A9(tl)PR.

1977; Warmke and Lee, 1977; Bino, 1985a, 1985b). However, degeneration of the developing microspores often occurs simultaneously with the proliferation of the tapetum, and therefore it is not clear whether the abortion of the developing microspores stimulates proliferation of the tapetum or whether the proliferating tapetum actually interferes with microspore development. A general comparison of tapetal morphology was therefore made between male fertile and sterile anthers to determine whether disruption of microspore development had any effect on the tapetum.

Sections were made from fertile anthers that contained tetrads and from male sterile anthers at an equivalent stage. These were stained with toluidine blue. Comparison of Figures 5H and 5I shows that the tapetal cells of the male sterile anther are larger and more vacuolate than those in the fertile anther. The locular side of the tapetal cells in the male sterile anther is lightly stained. The observed enlargement and apparent invasion of the locular space by the tapetal cells, a phenomenon termed hypertrophy, are often found in association with male sterility.

Electron micrographs of the tapetum also reveal differences in morphology between fertile (Figure 5F) and sterile (Figure 5G) anthers. The outer wall appears to be absent, and orbicules form irregular aggregations in the tapetum wall of sterile anthers. However, these features occur during normal tapetal development. A developmental sequence would therefore give a clearer indication of aberrant tapetal wall structure in the male sterile plants.

DISCUSSION

Secretion of a Modified Glucanase from the Tapetum Causes Male Sterility

The observation that male sterility appeared to be associated with mistiming of callase activity in cms lines of petunia suggested a novel route to the production of male sterile plants and a way to determine the role of callose in microsporogenesis. Ideally, phenocopies of the petunia mutants should express authentic callase activity in the anther locule either prematurely or late relative to the appearance of activity of the native callase. We chose to try and mimic the premature expression of callase because this avoids the need to suppress native callase activity. In addition, these mutants were more likely to yield information on the role of callose. In our laboratory, we have screened anther cDNA libraries with PR glucanase DNA probes and antibodies raised to PR glucanases in an attempt to clone the cDNA encoding callase. This approach proved unsuccessful, suggesting that callase is significantly diverged from other β-1,3-glucanases. However, the observation that PR glucanases could degrade callose surrounding tetrads in vitro suggested that these enzymes might substitute for callase in vivo. Therefore, a basic vacuolar β-1,3-glucanase was modified for secretion and expressed in tobacco from a CaMV 35S promoter and from tapetum-specific promoters. Transgenic plants expressing the modified glucanase gene from the tapetum-specific promoters exhibited moderate to complete reduction in male fertility. This phenotype was associated with the premature disappearance of callose from the microsporocyte walls, indicating that the modified glucanase is secreted from the tapetum and is active within the anther locule.

Removal of the C-Terminal Propeptide Directs Modified Glucanase to the Anther Locule

The secretion of proteins after entry into the endoplasmic reticulum is generally accepted to be a default pathway, whereas proteins directed to subcellular compartments contain specific targeting signals (Chrispeels, 1991). van den Bulcke et al. (1989) suggested that the CTPP of tobacco basic vacuolar glucanase may function to direct the protein to the vacuole. A basic glucanase lacking the CTPP has been expressed in the anther tapetum of transgenic tobacco plants, thereby indirectly examining the role of the CTPP in vacuolar targeting. The deletion of the CTPP resulted in secretion of the mature glucanase enzyme into the locular space. This experiment provided indirect evidence that the CTPP is required for vacuolar targeting. However, the CTPP may not be the complete signal because

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Figure 4. (continued).

(A) to **(F)** The extruded locular contents of wild-type and transformed tobacco anthers at a series of developmental stages were stained for callose with aniline blue. In each case, the panels of the left-hand column show the material under phase contrast and the panels of the right-hand column display the same view under blue excitation to highlight callose (yellow fluorescence).

(A) Meiocyte.

(B) Prophase of meiosis I.

(C) Telophase of meiosis I.

(D) Telophase of meiosis II.

(E) Tetrad of microspores.

(F) Microspore release.

With the exception of the meiocyte preparation, photographic exposure time for aniline blue fluorescence in the A9(tl)PR material was three times that for the wild type.

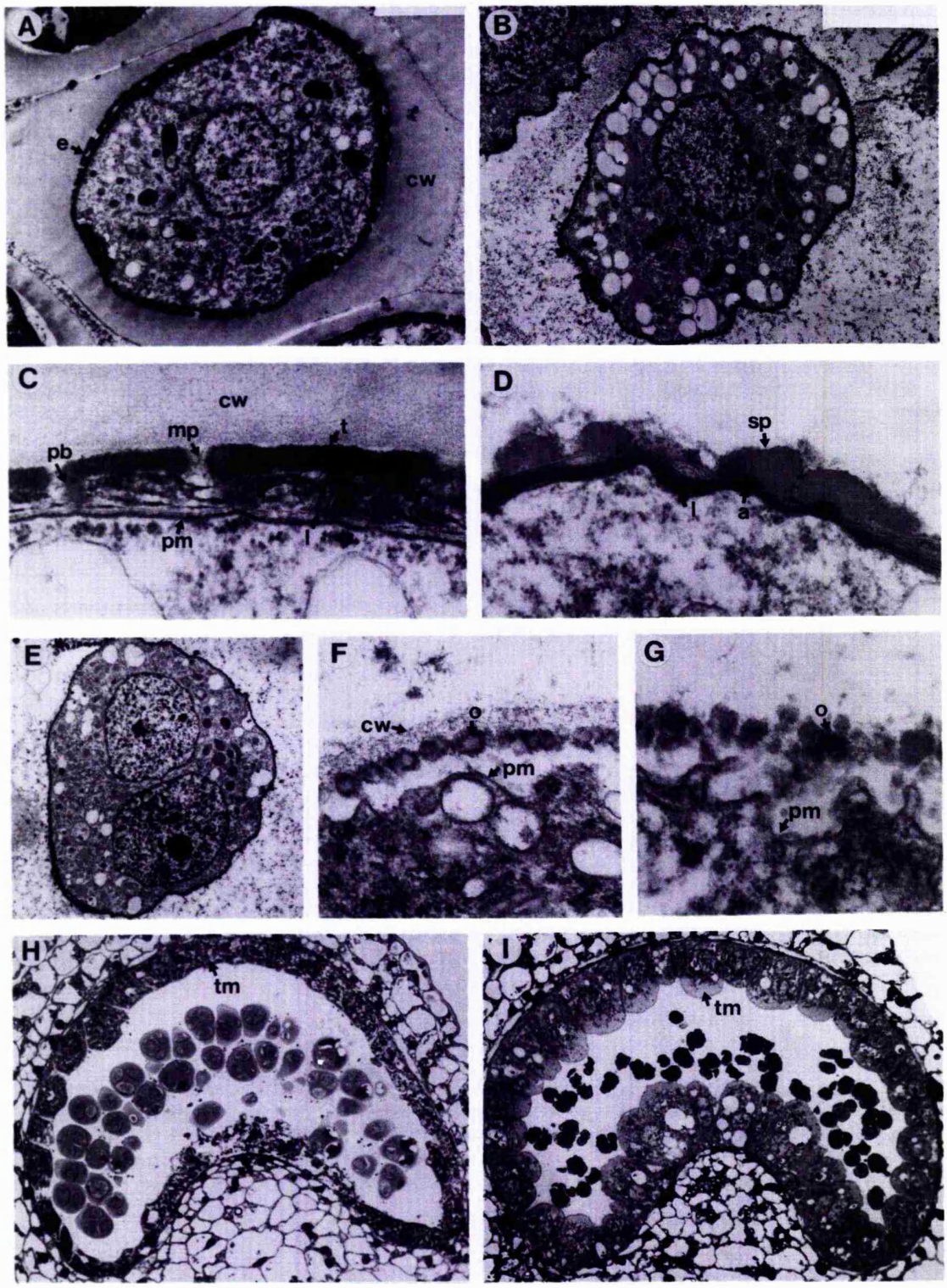

Figure 5. Premature Callose Dissolution Causes Aberrant Microspore and Tapetal Development.

further information could reside within the remaining glucanase sequence. Targeting a nonvacuolar reporter protein to the vacuole by the addition of the basic glucanase CTPP would confirm the suggested role.

Modified Glucanase Is Active in the Anther Locules of Transgenic Tobacco

Premature dissolution of callose requires that conditions in the anther locule, prior to normal callase activity, are conducive for glucanase activity. Studies in petunia (Izhar and Frankel, 1971) and lily (Linskens, 1956) have shown that the locular fluid pH falls during microsporogenesis. In petunia, the pH drops from 7.0 to 6.0 immediately preceding the onset of callase activity in the locule both in wild-type petunia and in the mutants with early or delayed callase activity (Izhar and Frankel, 1971). Because the optimum pH for callase activity is between 4.8 and 5.0, with activity undetectable above 6.3, Izhar and Frankel suggested that the timing of callase activity could be controlled by pH. Preliminary results suggest that a similar pH fall occurs during microsporogenesis in tobacco (D. Worrall, unpublished observation). Determination of the presence and timing of any fall in pH in the male sterile tobacco locules should determine whether the pH fall is independent of callase activation and callose degradation as predicted by Izhar and Frankel.

The pH activity curve of the basic vacuolar glucanase is significantly different from that of callase. At pH 7.0, where callase has no activity, the basic vacuolar glucanase retains 26% of its optimal activity, measured at pH 5.0 (Felix, 1984). This difference may have been crucial to the success of this work because in the absence of a coordinate pH drop, premature expression of callase may prove ineffective in causing premature callose degradation.

The transgenic plants expressing the modified glucanase gene from the tapetum-specific promoters displayed a range of male fertility. It is likely that complete male sterility requires high level accumulation of the modified glucanase in the locule because the pH of the locule is not optimum for enzyme activity. No clear correlation was observed on protein gel blots between the amount of modified glucanase in the anther and the degree of male sterility of the plant. This is probably due to the technical difficulty of collecting anthers that are precisely at the same developmental stage. However, there appeared to be a correlation between the known strength of the tapetum-specific promoters and the degree of male fertility.

Transgenic Plants Expressing the Modified Glucanase Gene from the CaMV 35S Promoter Do Not Show an Aberrant Phenotype and Are Male Fertile

Unlike the fusions to the tapetum-specific promoters, the fusion of the modified glucanase gene to the CaMV 35S promoter leads to the production of fertile plants. Plegt and Bino (1989) have shown with GUS fusions that the CaMV 35S promoter has strong activity in the vascular cylinder of the anther, but activity is undetectable in the tapetum and sporogenous cells during premeiotic, tetrad, and microspore release stages. This information supports the view that the modified glucanase protein accumulated in anthers of 35S PR plants is synthesized in cell types other than the tapetal or microsporogenous cells. Expression of the modified glucanase gene in the tapetum is either too low or occurs at a time in development inappropriate to cause male sterility.

Despite high levels of expression of the modified glucanase gene in the 35S PR plants, they appear to be phenotypically normal. It is possible that these plants will only display a phenotype when subjected to conditions that induce callose formation such as wounding or pathogen attack.

Figure 5. (continued).

Transverse sections through anthers from a wild-type and an A9(tl)PR tobacco plant, viewed by transmission electron microscopy (**A** to **G**) or by light microscopy (**H** to **I**). All anthers were at the tetrad stage of development.

(A) Microspore of a tetrad from a wild-type tobacco plant. The microspore exine (e) is well developed within the callose wall (cw) of the tetrad. ×3600.

(B) Microspore from an A9(tl)PR transformant. The callose wall is absent, and the microspore cell surface is electron dense and apparently lacks exine patterning. ×3600.

(C) Developing microspore wall in wild-type tobacco. Exine wall formation occurs between the callose wall (cw) and the plasma membrane (pm) of the microspore. Laminations (l) are present beneath the regular deposits of sporopollenin that constitute the young tectum (t) and probacula (pb) of the developing exine. Several micropores (mp) are present within the tectum. ×37,500.

(D) Aberrant microspore wall development in A9(tl)PR-transformed tobacco. Noncompressed laminations (l) are apparent at the cell surface. Globular deposits of a material with the appearance of sporopollenin (sp) lie on the outside of the laminations. Several of the globules contain a very electron dense region (a) that abuts the cell surface and interrupts the laminations. ×37,500.

(E) A binucleate microspore from an A9(tl)PR-transformed tobacco plant. ×3400.

(F) Portion of the luminal surface of a tapetal cell in a wild-type tobacco plant. A line of orbicules (o) can be seen between the cell wall (cw) and the plasma membrane (pm). ×34,500.

(G) Portion of the luminal surface of a tapetal cell in an A9(tl)PR-transformed tobacco plant. The cell wall is absent, and the orbicules (o) have an irregular distribution and appear less homogenous than in the wild type. pm, plasma membrane. ×34,500.

(H) Section through a wild-type anther showing the dimensions of the normal tapetum (tm). ×220.

(I) Section through an anther from an A9(tl)PR-transformed tobacco plant. The tapetum (tm) is highly vacuolated and shows signs of hypertrophy. ×220.

Callose Is Required for Normal Microspore Wall Formation in Tobacco

Although the RM cms line has been the subject of genetic (Nivison and Hanson, 1989), biochemical (Izhar and Frankel, 1971), and ultrastructural (Bino, 1985a, 1985b) studies, no detailed examination of the consequence of early callose dissolution has been undertaken. This may be due in part to the complex phenotype of this mutant; premature callose dissolution is probably only one manifestation of a general breakdown in microsporogenesis. A complicating aspect of this picture is that Bino (1985a), working with petunia lines apparently similar to those used by Izhar and Frankel (1971), could not detect premature callose wall dissolution. Therefore, the possibility exists that the ultrastructural studies of Bino (1985a, 1985b) may have been performed on a different petunia line.

The creation of transgenic tobacco plants that mimic only the premature callose dissolution observed by Izhar and Frankel (1971) has unambiguously determined the effect of premature callose dissolution on microsporogenesis. It appears that the callose cell wall is not required for meiosis or cytokinesis because four separate microspores are formed and held together in a tetradlike structure in the male sterile anthers. This suggests that material other than callose is capable of holding the microspores in a tetrad. Release of the microspores from the tetrad is also observed in the male sterile anthers, indicating that this process may normally require factors in addition to callase (Steiglitz, 1977; Sexton et al., 1990). One could speculate that the residual wall forming the tetrad may be cellulosic and that microspore release also requires the secretion of a cellulase. Callose is however required for the formation of a normal microspore cell wall. In its absence, the exine wall lacks regular sculpturing and is composed of an unusual multilaminate structure overlain by apparently random deposits of a material that is probably sporopollenin. The multilaminate wall appears to be an extreme form of the structures termed lamellae observed on the cell surface of developing microspores (Rowley and Southworth, 1967; Dickinson and Heslop-Harrison, 1968). Interestingly, this multilaminate structure is almost identical to those observed in early exine development in liverwort spores (Rowley and Southworth, 1967; Brown and Lemmon, 1990). In our material, sporopollenin is apparently deposited directly on the lamellae (Figure 5D) in accord with the model of Rowley and Southworth (1967).

The first step in the formation of the microspore exine wall appears to be the deposition of a cellulosic matrix between the cell plasma membrane and the callose wall. Dickinson and Heslop-Harrison (1968) suggest that the patterning of the exine is a consequence of the folding of the lamellae that appear after primexine formation. The lamellae lie either parallel to the microspore surface and subsequently form the tectum, or fold perpendicular to the cell surface forming radial protrusions that determine the position of the probacula. Sporopollenin, secreted by the microspore, then accumulates on the lamellae surface beneath the callose wall, the lamellae protrusions eventually forming the columnlike bacula of the exine. Following callose wall dissolution, the tapetum also contributes to sporopollenin deposition, leading to the completion of the intricate exine sculpturing of the mature pollen grain.

In the model of Waterkeyn and Beinfait (1970), the callose wall acts as a mold that is filled by the primexine. Subsequent sporopollenin deposition on the primexine leads to the final patterned exine wall. The internal surface pattern of the callose wall could arise by local differences in growth rate or callose degradation. In support of this model, the internal surface of the callose wall of *Ipomoea purpurea* was shown to have a regular hollow geometric pattern visible in phase contrast or fluorescence (Waterkeyn and Beinfait, 1970). Also, Vijayaragheven and Shukla (1977) showed by electron microscopy that microspores from *Pergularia daemia*, a species with no callose wall, develop a very sparse exine lacking typical surface patterning. In opposition to this model, a study of *Bougainvillea spectabilis* has shown that the plasma membrane rather than the callose wall is more likely to be the initiator of exine patterning (Takahashi and Skvarla, 1991). Thus, any model that proposes a role for callose in the formation of the microspore exine wall must explain why exine patterning is possible in the few species that do not produce callose, e.g., *Pananus odoratissimus* (Periasamy and Amalathas, 1991), or form exine after microspore release as in sorghum (Christensen et al., 1972).

In the transgenic sterile plants, the lamellae lie parallel to the microspore surface and do not fold to form probacula. This apparently explains the lack of surface sculpturing. However, at present our results do not help to determine whether callose forms a template for primexine patterning, provides a uniform surface against which primexine is deposited, or simply acts as a source of glucose for primexine formation. Further work is required to determine whether the primexine is actually formed in the mutants. Because the microspores burst shortly after release from the tetrad, perhaps due to an osmotic imbalance in the locular fluid, the possibility exists that microspores transferred to normal conditions would form unsculptured but viable pollen grains.

Tapetum Is Abnormal in Male Sterile Transgenic Tobacco

Apparently significant differences in tapetal morphology exist between fertile and male sterile anthers. The most obvious abnormality is the increase in volume and vacuolation of the male sterile tapetal layer. This may be caused by a retention in the tapetal cells of materials that would normally be utilized by the developing microspores or might be due to reabsorption of sugars arising from the premature degradation of the callose wall. Irrespective of the cause of the observed tapetal hypertrophy, this result indicates that tapetal hypertrophy observed in many male sterile plants may simply be due to microspore abortion rather than tapetal malfunction.

In conclusion, we have demonstrated that premature dissolution of the callose wall formed in microsporogenesis leads to male sterility in tobacco. This result supports the suggestion that the premature appearance of callase activity in RM cms petunia plants is sufficient to cause sterility in these lines. In the absence of the callose wall, an abnormal microspore wall is formed and microspores burst shortly after their release from tetradlike structures. In fertile plants, microspores rapidly increase in volume after microspore release. It is possible that this process is initiated in the male sterile plants, but because the microspore wall is abnormal the cells burst. Further work on these male sterile plants should clarify the role of callose in microsporogenesis.

METHODS

Plant Material

Material for nucleic acid isolation was obtained from *Nicotiana tabacum* SR1 plants grown under normal greenhouse conditions. To induce a wound response, tobacco plants were sprayed on two consecutive days with 5 mM salicylic acid (from a 0.25 M stock solution adjusted to pH 7.0 with KOH). One day after the final spraying, leaves were harvested, washed thoroughly with tap water, dried, frozen in liquid nitrogen, and stored at −80°C prior to nucleic acid extraction. Leaf material was ground to a fine powder with a pestle in a mortar that was cooled with liquid nitrogen, and RNA was isolated using a phenol-based method as described previously (Draper et al., 1988).

Production of Plants Containing a Modified Glucanase Gene

cDNA was synthesized from 5 μg of total tobacco leaf RNA using a cDNA synthesis kit (Amersham). Synthesis was according to the manufacturer's instructions with the following modifications. First-strand cDNA was primed with the pathogenesis-related (PR) glucanase C-terminal oligonucleotide (Figure 1B) and synthesized using murine reverse transcriptase. After second-strand synthesis, the cDNA was purified by phenol/chloroform extraction and ethanol precipitation. A linker, consisting of two complementary synthetic oligonucleotides, OG6 and OG7, was ligated to the cDNA. The sequences of the OG6 and OG7 primers are 5′-GGCCATGGAATTCATCTAGACC-3′ and 5′-GGTCTAGATGAATTC-3′, respectively. Because synthetic oligonucleotides lack a 5′ phosphate group, this linker ligates only to the 5′ end of the cDNA. Following linker ligation, the cDNA was diluted 1/50 and amplified by polymerase chain reaction (PCR) using the OG6 and C-terminal oligonucleotides as primers. Twenty-five amplification cycles were performed, each consisting of 1 min and 20 sec at 94°C, 2 min at 55°C, and 2 min at 72°C. A second round of amplification was then carried out using the N- and C-terminal oligonucleotide primers (Figure 1B) and under the conditions described above. All the primers were used at a concentration of 1 μM. Several different PCR products were cloned as XbaI and SacII fragments into XbaI, SacII-cut pBluescript KS- (Stratagene) and sequenced. The resulting PCR clone was found, by comparison to the published basic glucanase sequence (Shinshi et al., 1988), to have several frameshift mutations in the 3′ end that would have prevented the production of an active protein. Sequencing of several other PCR clones identified one that was free of mutations in this region. An XhoI site in the 3′ end enabled the construction of a composite gene from the two clones. Comparison with the published basic PR glucanase sequence (Shinshi et al., 1988) revealed five nucleotide substitutions that are as follows: the codon starting at position 282 is CGA instead of GCA, altering the amino acid to arginine from alanine; the codon starting at position 498 is AGC instead of AAC, altering the amino acid to serine from asparagine; the codon starting at position 864 is GTT instead of ATT, altering the amino acid to valine from isoleucine; the codon starting at position 996 is CTC instead of CCC, altering the amino acid to leucine from proline. Gene constructs were made as illustrated in Figure 1C, transferred to *Agrobacterium tumefaciens* pGV2260, and transformed into tobacco as described previously (Draper et al., 1988).

PCR Analysis of Kanamycin-Resistant Plants

A small piece of tobacco leaf was removed from each plant with the lid of a 1.5-mL microcentrifuge tube, and DNA was extracted by a rapid method described by Edwards et al. (1991). One one-hundredth of the resulting DNA solution was then used in a PCR reaction, using the N- and C-terminal oligonucleotides as primers, both at a concentration of 1 μM. Thirty amplification cycles were performed, each consisting of 1 min and 20 sec at 94°C, 2 min at 57°C, and 2 min at 72°C. The amplified DNA was then visualized by ethidium bromide staining after electrophoresis of samples on a 0.8% agarose gel.

Protein Extraction and Electrophoresis

Protein was extracted from eight anthers dissected from buds at the appropriate developmental stage. Anthers were frozen with dry ice and stored at −80°C prior to protein extraction. Frozen anther material was ground in an Eppendorf tube with a microhomogenizer, and 90 μL of cold extraction buffer (0.1 M Tris-HCl, pH 8.0, 5 mM EDTA, 5 mM DTT, and 4 mM phenylmethylsulfonyl fluoride) was added. The samples were mixed, thawed, and then centrifuged for 3 min to remove the debris. The supernatant was combined with SDS sample buffer. After boiling for 3 min, a proportion of each sample was loaded onto a 11% or 15% SDS–polyacrylamide gel to give equal amounts of protein in each lane.

Total cell protein was extracted from salicylic acid–treated tobacco leaves by grinding tissue to a powder in a mortar cooled with liquid nitrogen. Four milliliters of buffer (0.1 M sodium citrate, pH 8.0, 10 mM sodium ascorbate, 1 mM phenylmethylsulfonyl fluoride, and 10 mM β-mercaptoethanol) was added per gram of tissue and ground with the frozen tissue. After thawing, the cell debris was pelleted from the homogenate by centrifugation at 10000 rpm for 20 min. Proteins in the supernatant were precipitated with ammonium sulphate to 95% saturation and finally resuspended in 10 mM Tris-HCl, pH 7.5. An aliquot of protein was combined with SDS sample buffer, and protein was loaded onto SDS–polyacrylamide gels to give the same concentration as that produced for the anther extracts.

Immunoblotting and Immunostaining

Protein extracts resolved by SDS–polyacrylamide gels were electroblotted onto a polyvinylidene fluoride membrane (Immobilon; Millipore,

Bedford, MA) using a Milliblot SDE system (Millipore) according to the manufacturer's instructions. The blot was blocked with 4% (w/v) Marvel milk powder in TBS (Tris-HCl, pH 7.4, 200 mM NaCl) containing 0.1% Tween 20 (TBS-Tween) for 30 min. After blocking, the blots were incubated for 1 to 2 hr with tomato acidic anti-β-1,3-glucanase antiserum (1/1000 dilution) in TBS containing 1% (w/v) Marvel milk powder. Blots were washed three times in TBS-Tween for 10 min and then incubated in secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IgG, whole molecule [Boehringer]) in TBS containing 1% Marvel milk powder (1/1000 dilution). After washing in TBS-Tween as before, the blots were developed by immersing in a solution of 0.5 mg/mL 5-bromo-4-chloro-3-indolyl-phosphate and 0.3 mg/mL nitro blue tetrazolium in 0.1 M Tris-HCl, pH 9.5, 1 mM $MgCl_2$. The reaction was stopped by washing with distilled water. All treatments were carried out at room temperature.

Cytological Analysis of Callose

Anthers were dissected, and locular contents were squeezed directly into a 0.005% (w/v) aqueous solution of water-soluble aniline blue (Smith and McCully, 1978) made up in 0.15 M K_2HPO_4. Maximum staining was achieved after 10 to 15 min. Preparations were observed using a Zeiss standard microscope fitted with a fluorescence attachment 2FL and filter set 487709 (blue excitation). Anthers at a stage equivalent to microspore release were fixed and prepared for electron microscopy as described by Grant et al. (1986). Semi-thick (0.2 to 0.4 μm) sections were mounted on glass slides and stained with 1% toluidine blue in 1% Borax (disodium tetraborate).

ACKNOWLEDGMENTS

We thank Pierre de Wit for supplying the antiserum raised against tomato acidic β-1,3-glucanase, Stefan C. Hyman for preparing sections for light and electron microscopy, and Gary Foster for providing the OG6 and OG7 oligonucleotides. We also thank Hugh Dickinson for helpful discussion of the results and Dave Twell for critical reading of the manuscript. This research was supported by the Nickerson International Seed Company (Cambridge, U.K.) and a Science and Engineering Research Council case award to D.L.H.

Received February 10, 1992; accepted May 6, 1992.

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The Plant Journal (1993) 4(6), 1023–1033

The anther-specific protein encoded by the *Brassica napus* and *Arabidopsis thaliana* A6 gene displays similarity to β-1,3-glucanases

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Summary

An anther-specific *Brassica napus* cDNA, A6, and two corresponding *Arabidopsis thaliana* genes have been isolated. Sequence analyses of A6 revealed similarity to β-1,3-glucanases. The deduced A6 protein differs from other β-1,3-glucanases in the possession of a long C-terminus. Immunoblotting using an antibody raised to the A6 protein detects a temporal 60 kDa protein in *B. napus* buds, suggesting that the long C-terminal region is present in the mature protein. *A6 promoter–GUS* and *RNase* fusions demonstrate that the A6 gene is tapetum-specific and temporally expressed with a peak in activity when the plant normally expresses callase (a complex of endo- and exo-β-1,3-glucanase activities). The sequence similarity of A6 to other β-1,3-glucanases, coupled with the temporal and spatial expression data, suggests that A6 may be part of the callase enzyme complex.

Introduction

β-1,3-glucanases are a diverse family of hydrolytic enzymes that have been extensively characterized in plants. Basic isoforms of the enzyme are primarily found in the cell vacuole (Boller and Vögeli, 1984) and the acidic β-1,3-glucanases are secreted into the extracellular compartment (Parent and Asselin, 1984). In addition to having characteristic isoelectric properties, β-1,3-glucanases are also classified into two groups depending on the nature of their enzymatic action on β-1,3-glucans. Endoglucanases cleave this substrate into short chain reducing sugars whereas exoglucanase hydrolysis releases single glucose units from the reducing ends of the substrate. These different end products are the basis of assays which can distinguish between endo- and exoglucanase activities.

β-1,3-glucanases are most frequently associated with a role in plant defence and were first described as belonging to the pathogenesis-related family of proteins which accumulate rapidly in response to pathogen attack or stress (van Loon and van Kammen, 1970). However, recently an additional class of β-1,3-glucanases has been identified that is constitutively expressed in the roots and floral tissue of healthy plants (Memelink *et al.*, 1990). The accumulation of particular β-1,3-glucanases in floral tissue is developmentally regulated and unrelated to the defence response (Lotan *et al.*, 1989). β-1,3-glucanases can be found in the sepal, ovary, pedicel, style and anther (del Campillo and Lewis, 1992; Lotan *et al.*, 1989). The function of most of these β-1,3-glucanases is unknown. However, the constituent glucanases of callase, expressed in the anther, represent an important exception. The glucanase activity is responsible for dissolution of the tetrad callose wall and the release of the young microspores into the anther locules (Frankel *et al.*, 1969).

During pollen development, archaesporial cells in the anther give rise to microsporocytes and tapetal cells. The microsporocytes undergo meiosis to form tetrads of haploid microspores. The tapetum forms a single layer of cells around the anther locule in which the microspores develop. In almost all higher plants the tetrad of microspores is surrounded by a callose wall, a β-1,3-glucan polymer, which is secreted between the cell membrane and the primary cell wall, initially by the microsporocyte. As meiosis occurs, callose also accumulates along the cellular plates formed during cytokinesis, until each individual microspore of the tetrad is enclosed in a thick callose shell. At a critical development point, the callose is degraded by the β-1,3-glucanases of callase (Frankel *et al.*, 1969) which are secreted by the tapetal cells (Stieglitz and Stern, 1973). Work performed in lily indicates that callase consists of two β-1,3-glucanase enzymes, a 32 kDa endoglucanase and a 62 kDa exoglucanase (Stieglitz, 1977). *In vitro* experiments showed that digestion of the tetrads with the endoglucanase alone resulted in a dramatic reduction in the size of the callosic walls, whereas exoglucanase had no discernible effect. It was, therefore, suggested that the exoglucanase is responsible for degrading oligosaccharides, produced by the endoglucanase, into readily metabolizable glucose for use by the developing microspores (Stieglitz, 1977).

Following the degradation of the primary and callosic walls of the tetrad, the microspores are freed into the anther locule and continue their development into mature pollen grains. The build-up of callose around the microsporocytes and tetrads and its subsequent rapid dissolution is one of the most cytologically dramatic, and clearly distinguishable events in microsporogenesis. The

Received 15 April 1993; revised 29 July 1993; accepted 10 August 1993.
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developmental importance of correct callase expression is underscored by the occurrence of mutants in petunia and sorghum where premature (Frankel *et al.*, 1969; Warmke and Overman, 1972) or delayed (Izhar and Frankel, 1971) callase activity results in microspore abortion and male sterility. The timing of callase activity and the resulting callose breakdown is, therefore, of critical importance to the developing microspores. Work by Worrall *et al.* (1992) also showed that premature expression of an engineered β-1,3-glucanase transgene in the tapetum resulted in partial or total male sterility in tobacco.

To understand more about microspore development, a series of anther-specific cDNAs were isolated from a *Brassica napus* cDNA library encompassing the period of microsporogenesis (Scott *et al.*, 1991). In this paper one of the cDNAs, A6, isolated by Scott *et al.* (1991), is shown to encode a 53 kDa protein with low, but significant similarity to published plant β-1,3-glucanase sequences. Using the A6 cDNA as a probe, two *Arabidopsis thaliana* genomic clones were isolated. A promoter fragment from one of these was used to determine the temporal and spatial expression patterns of the A6 gene. The results of these experiments are consistent with the idea that A6 may encode a component of the callase enzyme complex.

Results

Sequence analysis of the A6 cDNA and genomic clone

The *B. napus* A6 cDNA is 1534 bp long and contains an open reading frame (ORF) from position 1–1424 bp, encoding a potential polypeptide of 474 amino acids. Two genomic clones, G61 and G62, which hybridized to the A6 cDNA, were isolated from an *A. thaliana* genomic library (Roberts *et al.*, 1993). Initial sequencing of the hybridizing regions of G61 and G62 revealed 96–98% identity at the nucleotide level within both the coding and upstream regions. Figure 1 shows a 2.7 kb contiguous sequence of the G62 genomic clone, which includes 885 bp upstream of the coding region. Comparison with the *B. napus* A6 cDNA suggests that the A6 gene encodes a protein of at least 53 kDa with a calculated isoelectric point of 9.2. The genomic clone contains two small introns within the coding region; one of 84 bp close to the N-terminal end and one of 77 bp adjacent to the C-terminal end. The introns contain the consensus splice junction sequences (Brown, 1986). There is a putative hydrophobic signal sequence of 21 amino acids, with the cleavage point between Ala-21 and Thr-22, according to the criteria set out by von Heijne (1983). A possible TATA box occurs at sequence position 787–793 bp (99 bp upstream of the ATG initiation codon). Alignment of the two coding regions shows that the *B. napus* A6 cDNA and the *A. thaliana* G62 genomic clone are 86% similar to each other at the DNA level.

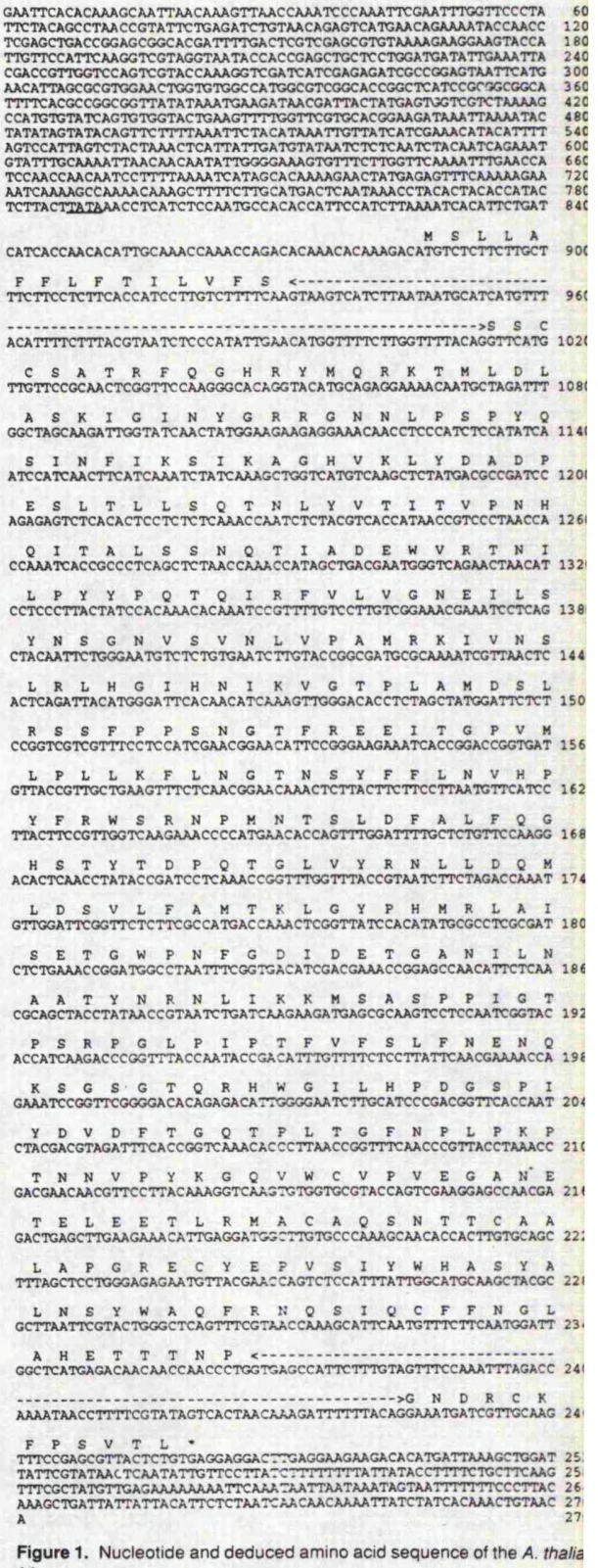

Figure 1. Nucleotide and deduced amino acid sequence of the *A. thalia* A6 gene.
The sequence includes an 885 bp upstream region and two short introns 84 bp and 77 bp. The position of the introns is marked with arrows abc the text. A potential TATA box is underlined.

Three other cDNAs (A11, A20 and A28) from the *B. napus* cDNA library (Scott *et al.*, 1991) were sequenced and found to be similar to the A6 cDNA. A11, A20 and A28 are partial cDNAs of 229, 565 and 197 bp, respectively, encoding peptides of 19, 32 and 139 amino acids. Comparison of the nucleotide sequences of the four cDNAs (Figure 2), illustrates that A11 and A28 are almost identical to each other and therefore possibly allelic. Also, the *A. thaliana* A6 gene shows a slightly greater percentage similarity to the *B. napus* A6 cDNA than to the other *B. napus* cDNAs (A11, A28 and A20). This suggests that the *A. thaliana* gene is likely to represent the gene homologue of A6, rather than one of the other cDNAs and that the *A. thaliana* gene homologues of A11/A28 and A20 are also likely to be present in *A. thaliana*.

Figure 3 shows a genomic DNA blot probed with the A6 cDNA under stringent conditions. In the diploid *A. thaliana* there appear to be three hybridizing bands (the upper band is a doublet on the original autoradiograph). In the hybrid, *B. napus* (swede and rape) there are at least six bands. The progenitors of *B. napus, B. campestris* (turnip) (data not shown) and *B. oleracaea* (cabbage and Brussels sprout) also contain several bands which hybridize to the A6 cDNA probe. Therefore, A6 appears to be a member of the large gene family in *Brassica*.

The deduced A6 protein sequence is similar to other plant glucanases

Screening the EMBL and SwissProt databases with the *B. napus* and *A. thaliana* A6 protein sequences revealed similarity of A6 to plant β-1,3-glucanases and β-1,3;1,4-glucanases. Figure 4 shows an alignment of the *B. napus* and *A. thaliana* A6 protein sequences with several plant endo-β-1,3-glucanases, plant endo-1,3;1,4-glucanases and a yeast endo-β-1,3-glucanase sequence. The A6 sequence display low, but significant, similarity to the other glucanase sequences. Alignments of β-1,3-glucanases allow the detection of conserved regions which are potentially important in enzyme structure and function. Two such regions thought to be important in glucanase activity, a and b (Meins *et al.*, 1991), are highlighted in Figure 4(a). Carboxyl amino acids, such as the two E (glutamate) residues conserved within regions a and b, are thought to be necessary for catalytic activity (Høj *et al.*, 1989b; Macgregor and Ballance, 1991). Also, inhibitor studies have shown that Y (tyrosine) and W (tryptophan) residues are required for catalytic activity (Moore and Stone, 1972). Conserved Y and W residues are highlighted in Figure 4(a) and are present in the *B. napus* and *A. thaliana* sequences. Therefore, the alignment strongly suggests that A6 and G62 are β-1,3-glucanases or β-1,3;1,4-glucanases. However, the dendrogram (Figure 4b), constructed using the sequences shown in Figure 4(a), shows that A6 is significantly diverged from the other members of the alignment. Moreover, A6 (and probably A11, A28 and A29) contains an additional C-terminal region of approximately 114 amino acids that is absent from the other glucanase sequences.

A6 gene expression commences immediately prior to microspore release in tobacco

The finding that A6 is similar to plant β-1,3-glucanases, coupled with previous data showing that the *B. napus* A6 cDNA is present in anthers prior to microspore release (Scott *et al.*, 1992), suggested that A6 may be part of the callase enzyme complex. Callase activity in anthers is

	At A6	Bn A6	Bn A11	Bn A20	Bn A28
At A6	—				
Bn A6	79	—			
Bn A11	70	78	—		
Bn A20	72	69	66	—	
Bn A28	70	78	98	67	—

Figure 2. The *A. thaliana* genomic sequence is most closely related to the A6 cDNA sequence.
A table illustrating the percentage similarity at the nucleotide level between the *A. thaliana* A6 genomic sequence and the four *B. napus* cDNAs, A6, A11, A20 and A28. Only the regions of DNA common to all sequences are compared.

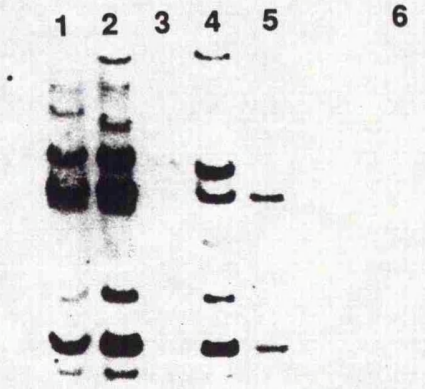

Figure 3. Determining the approximate number of A6-related genes in *Brassica* and *A. thaliana*.
Southern blot analysis of genomic DNA from several species cut with *Hind*III restriction enzyme. The blot was probed with ^{32}P-labelled A6 cDNA. Lane 1, *B. oleracea* var. gemmifera (Brussels sprout); lane 2, *B. oleracea* var. capitata (cabbage); lane 4, *B. napus* (swede); lane 5, *B. napus* var. olifera (oil seed rape); lane 6, *A. thaliana*.

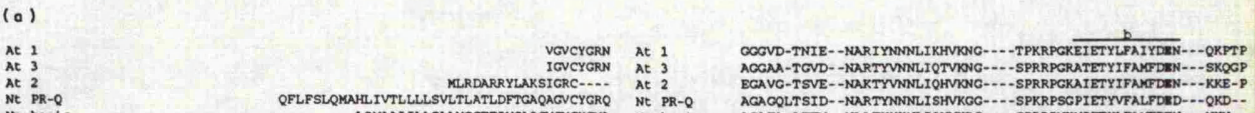

Figure 4. Sequence alignment of the deduced A6 protein with selected endo-β-1,3-glucanases, an endo-β-1,3;1,4-glucanase and a yeast endo-β-1,3-glucanase.

(a) The sequences compared are At1, At2 and At3; β-glucanases from *A. thaliana* (Dong *et al.*, 1991). Nt PR-Q; class III β-1,3-glucanase from *N. tabacum* (Payne *et al.*, 1990). Np basic; class I β-1,3-glucanase from *N. plumbaginifolia* (de Loose *et al.*, 1988). Pv β13: class I β-1,3-glucanase from *Phaseolus vulgaris* (Edington *et al.*, 1991). Nt stylar; stylar sp41 β-1,3-glucanase from *N. tabacum* (Ori *et al.*, 1990). Nt acidic; class II β-1,3-glucanase from *N. tabacum* (Linthorst *et al.*, 1990). Hv β1314; class II 1,3;1,4-β-glucanohydrolase from *Hordeum vulgare* (Slakeski *et al.*, 1990). Hv β13; class II 1,3-β-glucan endohydrolase from *H. vulgare* (Høj *et al.*, 1989a). At A6; *A. thaliana* A6 sequence. Bn A6; *B. napus* A6 sequence. Yeast: class II endo-β-1,3-glucanase from *Saccharomyces cerevisiae* (Klebl and Tanner, 1989; Mrsa *et al.*, 1993).

Regions a and b are thought to be involved in catalytic activity. Important conserved residues are shown in bold type. These are conserved in the *B. napus* and *A. thaliana* A6 sequences. *Conserved residue; ., conservative substitution.

(b) Dendrogram alignment of the sequences shown in (a) showing the A6 sequences to be less similar to other plant glucanases.

tightly regulated with a very sharp peak of activity coordinate with microspore release (Frankel *et al.*, 1969; Stieglitz and Stern, 1977). To determine whether the A6 gene is expressed in a similar pattern, an 885 bp A6 promoter fragment was linked to the *β-glucuronidase* (*GUS*) reporter gene (Bevan, 1984) and to the *RNase barnase* gene (Paul *et al.*, 1992). These constructs were transformed into tobacco and *B. napus*. Histochemical (data not shown) and fluorometric studies were performed on the tobacco *A6–GUS* transformants and a *B. napus A6–GUS* transformant (Figure 5). GUS activity was seen in the tapetum of the tobacco anthers and was also present in whole *B. napus* buds. Additional GUS activity was detectable in the mature microspores and pollen of the tobacco, but not in microspores or pollen of the *B. napus* transformant. Fluorometric assays showed a sharp peak of GUS activity in tobacco anthers (Figure 5a)

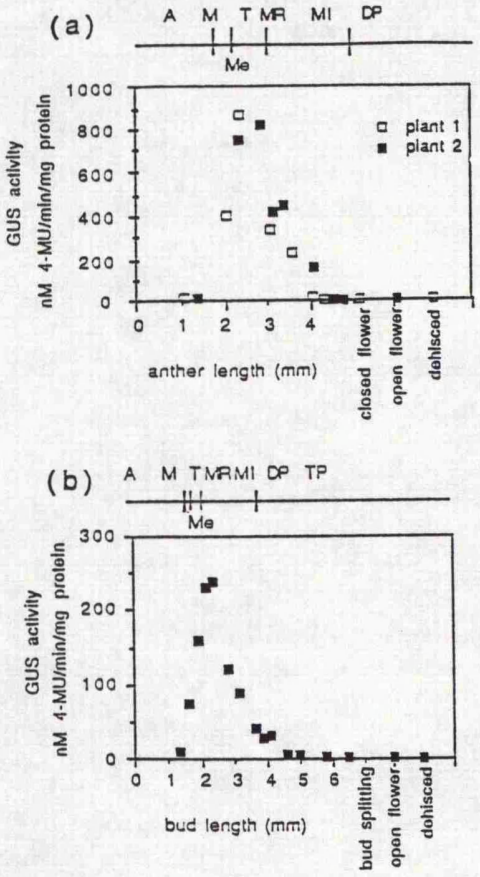

Figure 5. Fluorometric analyses of GUS activity in tobacco anthers (a) and *B. napus* buds (b) transformed with the *A6 promoter–GUS* fusion. Fluorometric assays were performed as described in Experimental procedures. The graphs show data obtained from representative transformants. GUS activity (measured in nM 4-MU min^{-1} mg $protein^{-1}$) is plotted against anther length and developmental stage. Abbreviations: A, archaesporial cells; M, meiocytes; Me, meiosis; T, tetrads; MR, microspore release; MI, microspore interphase; DP, dinucleate pollen; TP, trinucleate pollen.

and *B. napus* buds (Figure 5b) immediately before microspore release. GUS activity subsequently declined in both plants. The fluorometric data showed that the GUS activity seen in mature microspores and pollen of tobacco is extremely low in comparison with the levels of tapetal GUS activity.

Tobacco plants containing the *A6–barnase* construct were completely male sterile, but otherwise phenotypically normal. Dehisced anthers were empty with no trace of pollen. The male sterile phenotype was linked to kanamycin resistance and was heritable, since wild-type pollen crosses gave rise to male sterile, kanamycin-resistant plants. Figure 6 shows a comparison of anther development in a wild-type and a representative *A6–barnase* plant. The early stages of anther development appear identical in both the wild-type and *A6–barnase* plants; both have a distinct tapetal layer, and the microsporocytes have synthesized callose and completed meiosis. The first developmental differences are apparent in the 2.5–3.0 mm anthers. At this stage in the wild-type (Figure 6b) each individual microspore is surrounded by a thick callose wall and the tapetal layer is clearly visible as a darkly staining cell layer. In *A6–barnase* anthers (Figure 6a), tetrads are also visible, but these are less distinct from one another compared with tetrads in the wild-type plant. There are differences in the severity of phenotype in the *A6–barnase* anthers (presumably due to different levels of barnase expression between the buds of one plant). In the less extreme phenotype, a thick callose wall is visible around the tetrads, which is comparable with that of the wild-type plant. In addition, some *A6–barnase* tetrads apparently contain traces of callose between the individual microspores. Figure 6(c) shows an example of the more extreme *A6–barnase* phenotype; in this case the cell walls between the microspores are incomplete and there is no evidence of callose deposition by the microspores. The tapetum of all the *A6–barnase* plants is less organized and does not stain to the same degree as the wild-type tapetum (Figure 6d) suggesting that degeneration is underway. In 4.0 mm anthers (Figure 6f), the wild-type tetrads have broken down releasing the microspores into the locule (many spill out upon sectioning so there are many more than the photograph suggests). The tapetal layer, although much reduced in thickness, is still discernible. In the *A6–barnase* anther (Figure 6e), the tapetum has completely degenerated and callose degradation has failed to occur.

Two out of four of the *A6–barnase B. napus* transformants were also completely male sterile, female fertile and otherwise phenotypically normal. This again suggests that the A6 gene is tapetally expressed. However, the remaining two transformants, in addition to being male sterile, displayed floral abnormalities including reduced female fertility.

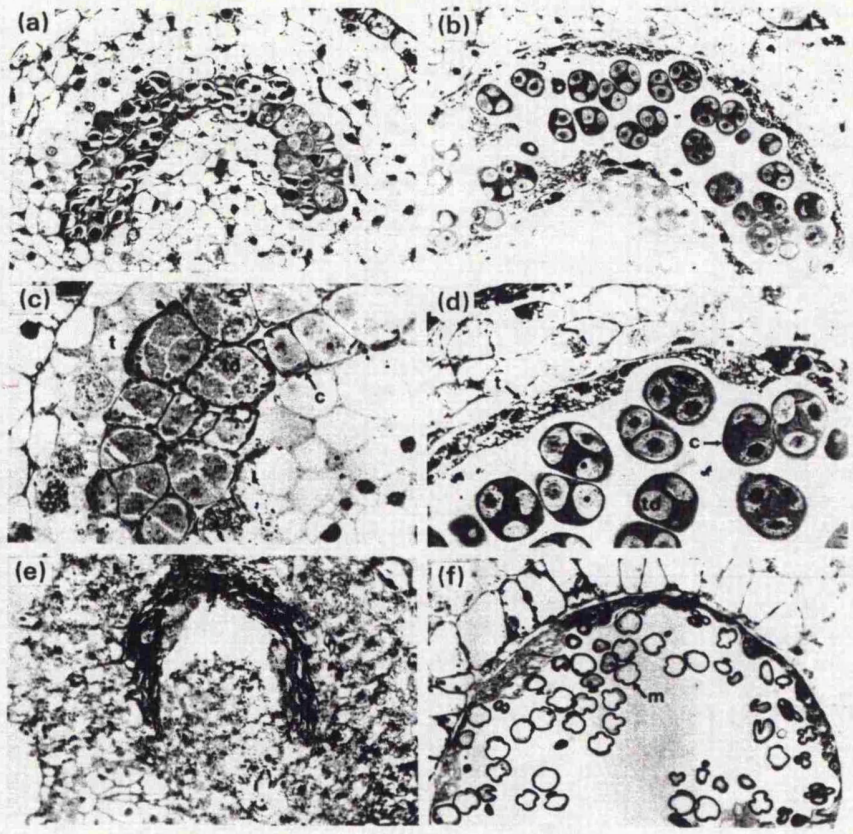

Figure 6. Transverse sections of wild-type (b, d and f) and *A6–barnase*-transformed (a, c and e) tobacco anthers.
These show the effects of tapetal RNase expression on microsporgenesis.
(a) *A6–barnase* (2.4 mm) anther, 65×; (b) 3.0 mm wild-type anther, 65×; (c) 2.5 mm *A6–barnase* anther, 160×; (d) 3.0 mm wild-type anther, 160×; (e) 3.5 mm *A6–barnase* anther, 65×; (f) 4.0 mm wild-type anther, 65×.
Abbreviations: c, callose wall; m, microspore; t, tapetum; td, tetrad.

The *A6–GUS* and *A6–barnase* promoter fusions demonstrate that the A6 promoter is active in the tapetum after the completion of meiosis but immediately prior to, or concomitant with, microspore release.

Immunodetection of the A6 protein family in B. napus *anthers*

Stieglitz (1977) partially purified two β-1,3-glucanases from lily anthers whose temporal activity peaked at microspore release; a 32 kDa endo-β-1,3-glucanase and a 62 kDa exo-β-1,3-glucanase (estimated by column chromatography). The estimated molecular mass of the A6 protein (53 kDa) falls between the two, although closer to the exoglucanase value. Therefore, it was of interest to determine whether the A6 protein undergoes any cleavage or modifications and to determine the size of the mature A6 protein in *B. napus* anthers. To address this question, a truncated version of the A6 protein was overexpressed in *Escherichia coli* as a gene 10–A6 fusion protein and used to raise anti-A6 polyclonal antibody in rabbit (see Experimental procedures). An antibody raised against a tomato acidic PR-β-1,3-glucanase (kindly donated by Pierre de Wit), was also used to investigate the size and distribution of the A6 protein in *B. napus*. Initially the PR antibody was used to probe an immunoblo of the G10–A6 fusion protein (see Experimental proce dures). The PR antibody cross-reacted with the G10–A6 fusion protein, but not with the G10 protein alone, thus providing evidence that A6 is immunologically related t β-1,3-glucanases. The PR and A6 antibodies were used to probe duplicate immunoblots of *B. napus* total bud pro tein samples (Figure 7). Both antibodies cross-reacted with a temporally regulated protein of approximately 6 kDa. The immunoreactive protein was just detectable i <1.5 mm buds, reached a peak in 1.5–2.5 mm buds where microspore release occurs, then declined in th 3.5–4.5 mm buds. The fact that the A6 antibody cross reacts with a 60 kDa band throughout the tempora sequence of anther development suggests that the A protein may retain most or all of the long C-termina region. The increase in size from the predicted 53 to 6 kDa could be the result of glycosylation. This size increas brings the A6 protein into line with the size of the lily exo-β 1,3-glucanase.

In an attempt to confirm that the immunoreactive 6 kDa band corresponds to the A6 gene product, protein from *B. napus* buds were separated on an isoelectr focusing (IEF) gel and probed with the A6 and PR antibo ies (Figure 8). Several developmentally regulated protein

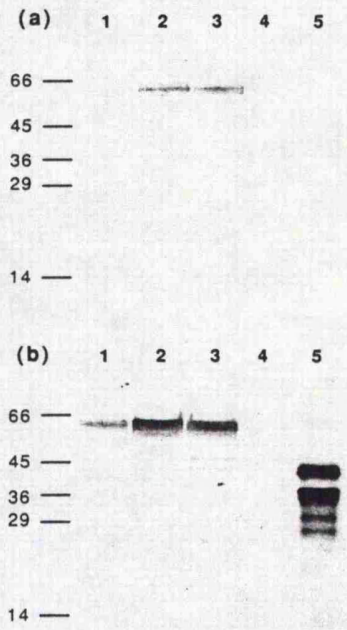

Figure 7 The A6 and PR antibodies cross-react with a temporally regulated 60 kDa protein in *B. napus* buds.
Duplicate protein gel blots of SDS–polyacrylamide gels probed with an anti-A6 antibody (a) and an anti-PR-β-1,3-glucanase antibody (b). Protein was extracted from wild-type *B. napus* buds. Lane 1, <1.5 mm buds; lane 2, 1.5–2.5 mm buds; lane 3, 2.5–3.5 mm buds; lane 4, 3.5–4.5 mm buds; lane 5, wild-type leaf protein. The position of molecular size markers is indicated on the left of each blot.

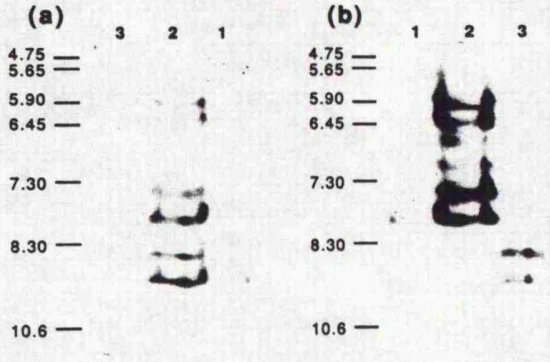

Figure 8. The A6 antibody has greater affinity for two basic proteins in *B. napus* buds.
Duplicate protein gel blots of an isoelectric focusing gel probed with an anti-A6 antibody (a) and an anti-PR β-1,3-glucanase antibody (b). Protein was extracted from wild-type *B. napus* buds. Lane 1, <1.5 mm buds; lane 2, 1.5–3.5 mm buds; lane 3, >3.5 mm buds. The position of pI markers is indicated on the left of each blot.

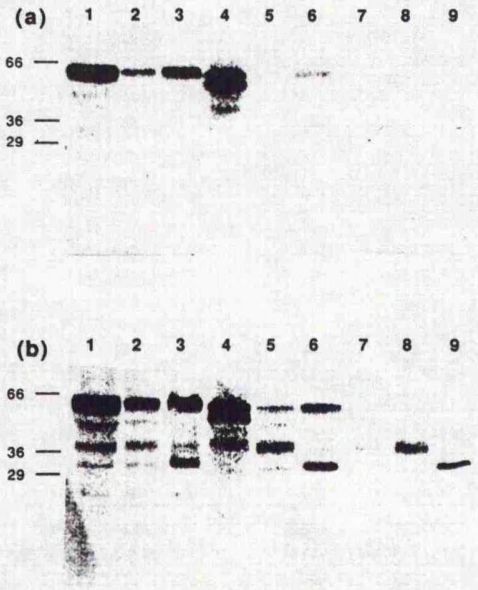

Figure 9. A6-related proteins are expressed in several floral organs in *B. napus*.
Duplicate protein blots of SDS–polyacrylamide gels probed with an anti-A6 antibody (a) and an anti-PR-β-1,3-glucanase antibody (b). Protein was extracted from dissected wild-type *B. napus* buds. Lane 1, anthers from 2.0 mm buds; lane 2, carpels from 2.0 mm buds; lane 3, sepals and petals from 2.0 mm buds; lane 4, anthers from 3.0 mm buds; lane 5, carpels from 3.0 mm buds; lane 6, sepals and petals from 3.0 mm buds; lane 7, anthers from 5.0 mm buds; lane 8, carpels from 5.0 mm buds; lane 9, sepals and petals from 5.0 mm buds.

cross-react with the antibodies. In particular, two bands are strongly highlighted by the A6 antibody. These proteins have pIs of about 8.5–9.0, which is consistent with the predicted pI of the A6 protein after removal of its putative signal peptide. The IEF blots show that *B. napus* may express several glucanases related to A6. To investigate the distribution of A6-like proteins further, *B. napus* buds were dissected and the tissues probed separately with the A6 and PR antibodies. Figure 9 shows duplicate immunoblots of anthers, carpels and sepals plus petals probed with the A6 and PR antibodies. In addition to cross-reacting with a 60 kDa protein in anthers, the A6 antibody cross-reacts with a temporal 60 kDa protein present in carpels and sepals plus petals. It is interesting to note that the expression pattern of the 60 kDa protein is similar in all three samples.The PR antibody also cross-reacts with several constitutive proteins. The data in Figure 9 suggest that members of the A6 gene family are expressed throughout the floral tissue in *B. napus*.

Discussion

The anther-specific *B. napus* cDNA, A6, and its corresponding *A. thaliana* genomic clone, G62, have been sequenced and shown to encode a protein with similarity to β-1,3-glucanases. Three partial cDNAs (A11, A20 and A28) isolated from the same library are closely related to the A6 sequence (Figure 2). This, in addition to the Southern blot data (Figure 3), suggests that there is a family of A6-related genes in *Brassica*.

The alignment of the *B. napus* and *A. thaliana* A6 protein sequences to glucanases (Figure 4) provides strong indirect evidence that A6 is a glucanase. Although sequence alignments differ slightly depending on the computer program used (Figure 4 was constructed on the CLUSTAL program and differs in some places to others (Meins *et al.*, 1991; Neuhaus, personal communication)) similar observations and conclusions can be made about the levels of similarity seen between the various sequences. Residues thought to be critical for catalytic activity (Høj *et al.*, 1989b; Moore and Stone, 1972; Macgregor and Dallance, 1991) are conserved in the A6 sequences, suggesting that A6 has all the necessary residues to function as a glucanase enzyme. Unfortunately at present, too few plant glucanase sequences are available to allow the identification of residues which distinguish β-1,3-glucanases from β-1,3;1,4-glucanases. Therefore, it is impossible to say whether the A6 protein has β-1,3-glucanase or β-1,3;4-glucanase activity. The dendrogram in Figure 4(b) illustrates that, apart from yeast glucanase, the A6 sequence has the lowest level of similarity to other members of the alignment. The A6 family may, therefore, represent a distinct class of plant glucanase. This idea is based both on the lower similarity throughout the mature coding region of the A6 sequences and on the presence of a unique C-terminal tail which extends 114 amino acids beyond the mature protein C-termini of other glucanases. Many glucanases contain a short C-terminal extension which is required for vacuolar targeting and it is possible that the extensive A6 C-terminal tail is also cleaved to form a mature protein. However, the A6 and PR antibodies detect an immunoreactive protein of 60 kDa throughout anther development. It is possible that this apparent increase in size from the predicted value of 53 kDa could be due to the retention of most or all of the C-terminal extension, coupled with glycosylation at one or more of the eight possible glycosylation sites within the A6 sequence.This is supported by the observation that A6 protein incubated with the deglycosylation enzyme, endoglycosidase F, is only slightly smaller than the untreated A6 protein (data not shown).

Stieglitz (1977) demonstrated that the callase enzyme complex in lily consists of two enzymes, a 32 kDa endoglucanase and a 62 kDa exoglucanase. If callase is conserved between *B. napus* and lily, then on the basis of size alone, A6 is likely to be an exoglucanase. This suggestion is supported by the fact that most plant exo-β-1,3-glucanases isolated to date are approximately 60 kDa in size; a 63 kDa exoglucanase has been identified in carrot (Kurosaki *et al.*, 1991), a 62 kDa exoglucanase in *Acacia* (Liénart *et al.*, 1986), a 68 kDa exoglucanase in soybean (Cline and Albersheim, 1981) and a 40 kDa exoglucanase in maize (Labrador and Nevins, 1989). In contrast, plant endoglucanases generally have a molecular mass of between 25 and 30 kDa (Meins *et al.*, 1991).

The published yeast exoglucanase sequences (Chambers *et al.*, 1993; Muthukumar *et al.*, 1993; Vazquez de Aldana *et al.*, 1991) show no significant amino-acid sequence similarity to plant or yeast endoglucanases despite utilizing the same substrate. Yet sequence similarity does exist between plant and yeast endoglucanases (Figure 4), indicating a degree of sequence conservation between endoglucanases. Assuming that this also is the case with plant and yeast exoglucanases, it suggests that plant exo and endoglucanases would not show sequence conservation. This reasoning favours the idea that the A6 protein is an endoglucanase. Exo and endoglucanase assays have been performed to try to determine directly the enzymic nature of the A6 protein. To date, however, no glucanase activity has been associated with the A6 protein.

Neither the A6 nor PR antibody cross-reacted with a developmentally regulated 32 kDa protein in *B. napus* anthers. This suggests that the endoglucanase component of the callase complex is significantly diverged from both A6 and other plant β-1,3-glucanases as to be seriologically distinct. Recently del Campillo and Lewis (1992) demonstrated the presence of two temporally regulated β-1,3-glucanases in bean anthers, neither of which cross-reacted with an antibody raised to a bean β-1,3-glucanase. This also implies a divergence between the enzymes of the callase complex and other β-1,3-glucanases.

In tobacco, the expression of the A6 promoter is apparently restricted to the anther, since the only discernible phenotypic effect of *A6–barnase* expression is tapetal ablation (Figure 6) and male sterility. In contrast, whilst *B. napus A6–barnase* transformants are also male sterile, some also display reduced female fertility and other floral abnormalities. This is likely to be the result of low-level *A6-barnase* expression in other floral organs. The fact that the A6 and PR antibodies cross-reacted with 60 kDa proteins in the carpels and sepals plus petals with similar temporal patterns to the anther protein (Figure 9) suggests that this might be the case. However, barnase is regarded as an extremely potent cytotoxin. If the amount of cross-reacting protein detected in the carpels and sepals plus petals reflected the activity of the A6 promoter then this would almost certainly result in ablation of these tissues. As this was not the case, the bulk of the immunoreactive protein in these tissues is presumably not the product of the A6 gene, although these proteins could represent members of the A6-related gene family. The anther-specific nature of *A6–barnase* expression in tobacco suggests that the low levels of *A6–barnase* expression in other floral tissues of *B. napus* do not occur in tobacco. This may be a result of differential regulation of the A6 promoter in these two species.

Wall-bound exoglucanases such as those isolated by Labrador and Nevins (1989) and Kurosaki *et al.* (1991) are thought to be involved in cell wall loosening (Labrador and Nevins, 1989). During microsporogenesis, all the floral tissues undergo rapid cell expansion, so it is possible some of the glucanases cross-reacting to the A6 and PR antibodies may be involved in cell wall loosening to facilitate cell elongation. In addition to cross-reacting with a temporal 60 kDa protein in all three tissue samples, the PR antibody also cross-reacts with several constitutive proteins. For example, the immunoreactive 40 kDa band present in the carpel extracts may correspond to the stylar β-1,3-glucanase isolated by Lotan *et al.* (1989).

Any component of callase activity must be secreted into the locule to act on the callosic walls of the tetrads. Therefore, callase proteins, synthesized in the tapetum, should contain a signal peptide to mediate translocation into the endoplasmic reticulum lumen. The deduced A6 protein sequences contain a putative signal peptide, therefore, it is possible that A6 is secreted into the locule of the anther.

The spatial and temporal expression of the A6 promoter in tobacco and *B. napus* is consistent with it being a component of callase, as described by Stieglitz (1977). However, in tobacco there is also low GUS activity in pollen which may be due to aberrant expression of the *A. thaliana* promoter in tobacco. Uknes *et al.* (1993) demonstrated ectopic expression of *GUS* in tobacco pollen from a *PR-1a–GUS* construct, casting some doubt as to the validity of *GUS* as a reporter gene in this tissue. Alternatively, since the mature *B. napus* pollen grain is trinucleate and the tobacco pollen grain remains binucleate until after germination, the difference in A6 promoter expression may be due to differences in pollen development in the two species.

Tobacco anther sections show tapetal ablation resulting from *A6–barnase* expression. The plants are male sterile and otherwise phenotypically normal. The anther sections (Figure 6), suggest that the A6 promoter is active in the tobacco tapetum after the completion of meiosis and initial callose wall formation. The tobacco *A6–barnase* sections can be compared with those of a tapetum-specific promoter of similar transcriptional strength, A9 (Paul *et al.*, 1992). The A6 promoter is expressed later than the *A. thaliana* A9 promoter, since the microsporocytes do not enter meiosis or form callose walls in the *A9–barnase* tobacco transformants. The timing of *A6–barnase* activity mirrors the onset of callase activity as described by Stieglitz (1977).

The occurrence of male sterile plants with premature or delayed callase activity (Izhar and Frankel, 1971; Kaul, 1988; Warmke and Overman, 1972) underlines the importance of the timing of callase activity in anthers, yet the control mechanisms involved are unknown. Perhaps the coordination of callase secretion by the tapetal cells with the development of the microspores involves communication between the two cell types. The A6 gene product looks a likely candidate for a component of the callase enzyme complex in *B. napus*. Thus, further characterization of the A6 gene and protein may help to uncover the specific roles of β-1,3-glucanases in microspore development and shed light on the type of communication required for the control of this crucial developmental process.

Experimental procedures

Libraries

The construction and screening of the *Brassica napus* cDNA library is described in Scott *et al.* (1991). The *Arabidopsis thaliana* genomic library (Roberts *et al.*, 1992) was screened as described in Paul *et al.* (1992).

DNA sequence and protein sequence analysis

Sequencing reactions were performed using a Sequenase kit (United States Biochemical). Contiguous sequences were assembled and analysed using the GCG (Devereux *et al.*, 1983) and Staden software packages. Alignments of nucleotide and amino acid sequences were carried out using the CLUSTAL program (Higgins and Sharp, 1989) using default parameters.

Production of the A6 antibody

Two oligonucleotides were designed to amplify a fragment from the A6 cDNA which displays sequence similarity to other β-1,3-glucanases. The N-terminal oligonucleotide, 5'-GGGGAATTC-CATGGGCAAGATTGGTATTAACTATGG-3' (Figure 1, position 109–130 bp), incorporates an ATG initiation codon and *Eco*RI and *Nco*I restriction sites. The C-terminal oligonucleotide 5'-CCCGGTACCGCGGCTAGGTAAAATCAATGTCG-3' (Figure 1, position 1082–1067 bp), incorporates a stop codon and *Sac*II and *Kpn*I sites. The resulting A6 PCR product (mA6) was cloned into pBluescript (Stratagene) as an *Eco*RI/*Sac*I fragment. This fragment was then cloned via an intermediate vector, pIC19H (Marsh *et al.*, 1984), into pGEMEX-2 (Promega) as an in frame *Eco*RI/*Hin*dIII fragment. The pGEMEX-2 expression system allows the expression of a gene 10 fusion protein and was used according to the manufacturer's instructions. SDS–PAGE gels were used to separate the inclusion body proteins. The gene 10–mA6 fusion protein was purified by electroelution from gel slices and then extensively dialysed against phosphate-buffered saline (140 mM NaCl, 1.5 mM KH_2PO_4, 2mM KCl, 7.5 mM Na_2HPO_4). Freunds complete adjuvant was combined with 500 μg of protein (total volume 500 μl), and injected into a New Zealand white rabbit. Booster injections were given at 14-day intervals, each comprising 100–300 μg protein in a total of 500 μl. A final boost was given 1 month later and the serum was collected 6 weeks later. The serum was incubated with recombinant vector inclusion body protein and the mA6 inclusion body complexes were spun down and dissociated. The partially purified antibody was then incubated with the non-recombinant G10 fusion protein to remove antibody raised to the G10 protein. The working dilution for the A6 antibody is 1 in 1000.

Protein extraction and immunoblotting

All measurements refer to bud length from the pedicel–bud junction to the tip of the longest sepal. Protein was extracted from fresh anthers and whole buds at appropriate developmental stages. The extractions were performed essentially as previously described (Worrall *et al.*, 1992), but using 50 mM KPO_4 pH 6.8, 1 mM phenylmethylsulphonyl fluoride, as the extraction buffer. Protein extracts for the IEF protein gels were added to an equal volume of IEF loading buffer (60% glycerol, 4% ampholytes) before loading. IEF gels were performed as described by Robertson *et al.* (1987), but run at 4°C rather than at room temperature.

SDS–PAGE gels were immunoblotted as previously described (Worrall *et al.*, 1992). IEF gels were immunoblotted on to polyvinylidene fluoride membrane (Immobilon, Millipore) using a capillary blot method with 25 mM Tris, pH 10.2, as the transfer buffer. The membrane was prewetted in methanol, washed in distilled water and then soaked in buffer prior to transfer. A membrane was placed above and below the gel giving duplicate, mirror image blots. The immunostaining and development of the blots was performed as previously described (Worrall *et al.*, 1922).

Construction of A6 promoter–GUS and A6 promoter–RNase fusions

An 885 bp *Eco*RI/*Nsp*HI fragment was isolated from the G62 genomic clone. The putative ATG initiation codon is within the *Nsp*HI restriction site. The promoter fragment was blunted with Klenow enzyme to destroy the ATG site. The blunt fragment was then cloned into *Sma*I cut pBluescript (Stratagene), and sequenced to confirm that the ATG codon had been destroyed. A *Bam*HI/*Sal*I cut promoter fragment was cloned into *Bam*HI/*Sal*I cut pB101.1–GUS (Jefferson *et al.*, 1987) to create an *A6 promoter–GUS* fusion. This was cloned, in both orientations, into pBin19 as a 3.5 kb *Eco*RI fragment. No difference in promoter activity was observed between the two constructs. To create the *A6–barnase* construct, the same blunted promoter fragment was cut out of pBluescript as a *Kpn*I/*Sba*I fragment.This fragment, and the *Xba*I/*Xho*I Barnase and Barstar containng fragment of pWP127 (Paul *et al.*, 1992) were cloned into *Kpn*I/*Sal*I cut pBin19 in a three way ligation.

Plant transformation

Nicotiana tabacum was transformed with *Agrobacterium* pGV2260 as described by Draper *et al.* (1988). *B. napus* transformation was performed at Nickerson Biocem, Cambridge, UK.

Histochemical and fluorometric analysis of transgenic plants

Histochemical assays were performed using 5-bromo-4-chloro-3-indoyl-β-D-glucoronide (X-Gluc). Fluorometric assays used 4-methylumbelliferyl glucuronide (4-MUG). Both assays were performed essentially as described by Draper *et al.* (1988). Protein concentrations were calculated using Bradford's reagent (Bradford, 1976).

Cytological analysis of anthers

Anthers were fixed (Grant *et al.*, 1986) and 0.25 μm sections taken for light microscopy. Sections were mounted on a microscope slide and stained with 1% toluidine blue in 1% borax (disodium tetraborate).

Acknowledgements

The authors thank Pierre de Wit for the tomato PR-β-1,3-glucanase antibody, Stefan Hyman for preparation of samples for microscopy, and Jean-Marc Neuhaus for alignments of glucanase sequences. We also thank Tina Barsby, Judy Freeman, Melanie Craze and Shona McLennan (Nickerson Biocem) for the *B. napus* transformations. Finally we are grateful for an SERC–CASE award to DLH and Nickerson Biocem, Cambridge, UK, for their support of this research.

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EMBL Data Library accession numbers X69887 (A6), X69888 (A11), X69889 (A20), X69890 (A28) X70409 (G62).