Characterisation of gametophytic mutants affecting pollen function in *Arabidopsis thaliana.*

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by

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Δημητριου Φραγκουλη.

Abstract

Characterisation of gametophytic mutants affecting pollen function in *Arabidopsis thaliana*

Christos Michaelidis

The progamic phase of plant reproductive development involves events from pollen germination to gamete fusion. Physiological studies suggest that complex mechanisms are involved in this pathway. In order to identify gametophytic genes which function in this pathway, an insertional mutagenesis screen based on segregation ratio distortion was performed on a population of 4,094 independent *dSpm* transposon insertion lines. This screen did not produce any mutants of interest, but two mutants, *seth4* and *seth7*, were identified from a similar screen of *Ds* transposon insertion lines. *seth4* and *seth7* showed stably reduced segregation ratios arising from reduced gametophytic transmission and reciprocal crosses showed no (*seth4*) or severely reduced (*seth7*) transmission of the antibiotic resistance marker only through pollen. In both mutants, pollen morphology was normal but pollen germination was severely affected. In *seth4*, the insertion disrupted the coding region of a gene encoding an armadillo (ARM) repeat protein. In *seth7*, the transposon was inserted within the 3'-UTR of a gene encoding a putative serine/threonine protein kinase. A wild-type copy of *SETH4* complemented the *seth4* mutation and restored male transmission. *SETH4* is the founding member of a discrete *Arabidopsis* gene family that contains two *SETH FOUR-LIKE* genes, *SFL1* and *SFL2*. *SETH4* was found to be preferentially expressed in the male gametophyte while *SFL1* and *SFL2* were expressed exclusively in the sporophyte. SETH4, SFL1 and SFL2 proteins, when fused to GFP, suggest cytoplasmic localisation in transient expression assays. In this work two mutants identified as essential for the male gametophyte during the progamic phase have been phenotypically characterised. *SETH4* was analysed using genetic, molecular and bioinformatic analyses and is proposed to be part of a novel molecular pathway controlling cellular growth in the gametophyte.

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Table of contents **Page**

List of figures **Page**

Abbreviations

aa	amino acid
bp	base pair
°C	degrees centigrade
cM	centimorgan
cm	centimetre
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
g	gram
GUS	β-glucuronidase
h	hour
kb	Kilobase pair
kDa	Kilodalton
l	litre
M	molar
Mda	Megadalton
MES	2-(N-Morpholino)ethanesulfonic acid
µg	microgram
µl	microlitre
µm	micrometer
µM	micromolar
mg	milligram
ml	millilitre
mm	millimeter
mM	millimolar
MS	Murashige and Skoog
ng	nanogram
OD	optical density
ORF	Open reading frame

PCR	polymerase chain reaction
PNACL	The protein and nucleic acid chemistry laboratory
ppm	parts per million
psi	pound per square inch
RNA	ribonucleic acid
rpm	revolution per minute
rt	room temperature
RT-PCR	reverse transcriptase-PCR
TAE	tris-acetate EDTA
Tris	tris(hydroxymethyl)aminomethane
Triton X-100	(t-Octylphenoxypolyethoxyethanol)
U	enzyme units
V	volts
v/v	volume per volume
WT	wild type
w/v	weight per volume

Chapter 1

Introduction

Reproductive interactions in flowering plants are carried out in the sporophytic tissues of the pistil (stigma, style, and ovary). Pollen, the haploid male gametophyte, forms a tip growing extension called the pollen tube to deliver the sperm cells to the female gametophyte for the fertilisation to occur. Just before fertilisation, the female gametophyte exerts an effect on the pollen tube, guiding it to the micropyle. The mechanism of pollination and fertilisation in plants is a fundamentally interesting problem in cell biology. The processes underlying the plant reproduction have been described in several species at the morphological level over the last four decades. The number though of identified molecular components of plant reproduction is still low. Recent advances in plant molecular biology lead to the identification of a plethora of genes (in *Arabidopsis thaliana* 3,500 genes specifically expressed in pollen) expressed during plant reproduction (Honys and Twell, 2003). Assigning a role on these genes should identify novel pathways in all the aspects of cell biology and stimulate research for many years to come.

1.1 Pollen development

Pollen grains harbour the sperm cells. Pollen develops within the anther of the stamen, the male organ of the flower. Premeiotic diploid precursor cells, called microspore mother cells, undergo meiosis to produce tetrads of microspores which separate and vacuolate. An asymmetric mitosis (pollen mitosis I) follows, resulting in a vegetative and a generative cell. In species with a tri cellular pollen, the generative cell divides again (pollen mitosis II) to produce two sperm cells. Normal pollen development relies on the tapetum cells. The tapetum cells synthesise proteins, lipids, and flavonoids that are deposited on the microspores and comprise the outer strata of the pollen wall, the exine and tryphine layers. In the latter stages of pollen development, the pollen undergoes dehydration to some extent depending on the species, before being released from the anther into the environment as mature pollen grain (Twell 2001; 2002).

1.2 Progamic phase

The pistil, the female organ of the flower, comprises of the stigma (where pollen first alights), the style (a conduit between the stigma and ovary), and the ovary, which contains the ovules. When pollen comes in contact with the papilla cells on

the stigma, it adheres, hydrates, and germinates. The vegetative cell extends the pollen tube, a structure that carries the sperm cells and conveys them to the embryo sac and egg in the ovule.

The progamic phase has been described as the culmination of events from the initial recognition of the pollen on the stigmatic cells to the delivery of the sperm cells in the ovary and fertilisation of the egg and central cell. The progamic phase has been separated by researchers in four stages (Figure 1.1) (Kandasamy *et al.* 1994). A universal model for pollination, whether self-incompatible or compatible, has not emerged up to now. On a survey of the literature on pollen tube guidance, it becomes apparent how much variation there is in the mechanisms of pollination in the flowering plants. Plants have a variety of ways to accept compatible pollen, guide the sperm cells to the ovule as well as a variety of ways to reject self-pollen. One major obstacle to the research is the technical difficulties that arise in following the events *in vivo*. The sporophyte tissues constitute a barrier and hinder the morphological observation of the growth and guidance of the pollen tube in the style. Data on the pollen tube growth and chemotropism are usually from *in vitro* studies and are sometimes accompanied by genetic studies that implicate proteins in guidance. Thus far, unifying themes on directional guidance mechanism have not emerged (Cheung *et al.* 1993; Pruitt *et al.* 1993; Wilhelmi and Preuss 1999; Lord 2000; Palanivelu and Preuss 2000; Lord 2003), due to the different strategies utilised to navigate the different structural and chemical environments found in female tissues of the plant species studied. Nevertheless, lipids, sugars, and ovule-derived molecules are known to be involved.

1.2.1 Stage I of the progamic phase; the recognition, adhesion hydration and germination of the pollen grain on the stigmatic papillae.

The stigma is the first female tissue to receive the pollen grain and in the self-compatible species (which predominate in the angiosperms) pollen from the same flower adheres, hydrates, germinates a pollen tube, and the pollen tube grows into the style (Lord 2000; Lord 2003).

Stage	Tissue	Pollen tube length (μm)	Time
Stage I	Pollen-stigma interface	<5	15 min
Stage II	Papillar cells	100	40 min
Stage III	Stigma	200	50 min
	Style	500	120 min
	Ovary	2,000	2-10 hours
Stage IV	Funiculus micropyle	2,000	2-10 hours

Figure 1.1. The Progamic phase of *Arabidopsis* sexual reproduction.
The progamic phase has been arbitarily separated in four stages to ease the dissection of the complex events taking place during the interaction of the haploid gametophytes with the sporophytic tissues. The table from Kandasamy *et al.* (1994) should act as a guide for the spatial and temporal events taking place during the progamic phase.

1.2.1.1 Recognition adhesion

Initiation of pollination is dependent on the ability of the pollen grain to adhere to the stigmatic surface. The control of pollen adhesion is complex as adhesion is under polygenic control (Preuss *et al.* 1993). It is recognised as being a step for the recognition of self/not self pollen in the self-incompatible species. The pollen coat contains many molecules involved in the initial interaction with the stigma (Dickinson 2000). On contact with the stigmatic surface, the pollen wall, the sporopollenin exine, flows to form an adhesive foot at the papilla surface (Elleman and Dickinson 1986). The stigmatic papillae, the specialised receptive tissues, rapidly expand and loosen the outer layer of their stigmatic walls when a pollen grain has landed on them; an elevation in secreted calcium at the site of pollen adhesion on the papillar surface has also been observed in the *Brassica* species (Dickinson 1999). The interaction between the pollen and the papillae is species-specific. It has been demonstrated that *A. thaliana* stigmas bind *A. thaliana* pollen with much higher affinity than pollen from related species and such an interaction occurs within seconds of pollination (Zinkl 1999). A genetic screen to isolate mutants showing reduced pollen adhesion has resulted in the isolation of several *lap* (less adherent pollen) mutants (Zinkl and Preuss 2000). The *lap1* mutant shows gross defects in the exine structure. This mutant does not exhibit reduced fertility so even if the pollen coat is important in pollen adhesion, the adhesion process is independent of pollen hydration and growth.

1.2.1.2 Hydration

After adhesion, the pollen grain hydrates. The interaction between stigma and pollen results in changes in the pollen coat enabling hydraulic continuity between the pollen grain and the stigmatic papillae (Elleman and Dickinson 1986). The processes involved in pollen hydration are not well characterized. The diverse nature of the stigmatic surface in different angiosperm families leads to the suggestion that hydration mechanisms are divergent, although the reorganization of the vegetative cell resulting from the pollen hydration, appears to occur in all the species examined (Heslop-Harrison and Heslop-Harrison 1992). Controlling water flow into the grain from the stigma regulates hydration of pollen. The hydration of the pollen grain is aided by the presence of the outer wall of pollen

(pollen coat) that, in *Arabidopsis*, contains predominantly lipases and oleosins (Mayfield *et al.* 2001). The loss of an oleosin protein from the coat (GRP17–1) delays pollen hydration of the mutant pollen almost three-fold when compared to wild-type pollen speed of hydration. As the *grp17* pollen eventually hydrates, it has been speculated that the delay was due to a failure to interact with the stigma, rather than failure to absorb water. Whether GRP17 directly communicates with the stigma or is interacting with other molecules, as lipids, is not yet established (Mayfield and Preuss 2000). Lipids are also implicated in the hydration process. *Eceriferum (cer)6-2*, an *A. thaliana* mutant, is depleted in long-chain lipids in the pollen coat, and hydration is disrupted. In the case of *POLLEN-PISTIL INCOMPATIBILITY 1(pop1)*, which has been shown to have defects in lipid biosynthesis (Preuss 1993; Hulskamp *et al.* 1995; Fiebig *et al.* 2000) hydration is also affected. Application of one of the lipids from the pollen coat exudate, the long chain lipid trilinolein, enabled *pop1* mutants to germinate normally. It also enabled pollen to hydrate and germinate on leaf tissue following removal of the cuticle, which is normally a non receptive surface (Wolters-Arts *et al.* 1998). Identification of an aquaporin, a water channel protein, essential for pollen hydration, implicated in self-incompatibility response in *B. oleracea,* suggests that such molecules do play a role in pollen hydration in other species (Ikeda *et al.* 1997).

1.2.1.3 Germination

The reorganization of the vegetative cell resulting from the pollen hydration leads to the germination of the pollen grain and the formation of the pollen tube. In germinating pollen grains, the actin filaments assemble around the germination pore just before tube emergence (Gibbon *et al.* 1999). Secretory vesicles are transported by the actomyosin system to the apical region to deliver cell membrane and wall materials to support the growth of the pollen tube (Cheung *et al.* 2002; Cheung *et al.* 2003). The structure of the pollen tube is further discussed in section 1.3. Very little is known about the molecular components that act during the germination of the pollen grains. It has been noted that the number of pollen grains on the stigma stimulate the germination rate, the 'mentor effect', and a secreted peptide called phytosulphokine was implicated on this effect. This five

amino acid, sulphated peptide was found to induce germination in pollen populations of low number (Chen *et al.* 2000).

1.2.2 Stage II of the progamic phase; pollen tube growth through the stigmatic papillae.

Once a pollen tube has been formed, it will grow through the stigmatic papillae before reaching the stylar tissues and eventually the ovules. The initial penetration of the stigmatic surface has been studied in several species with dry stigmas and appears to be quite variable. In *A. thaliana* and *B. oleracea* the pollen tube penetrates the stigmatic cuticle and enters a space between the outer layer of the cuticle and the main body of the fibrillar cell wall. There it continues to grow until it reaches the base of the cell. It then enters the stigma transmitting tissue (Kandasamy *et al.* 1994; Derksen *et al.* 2002).

In the *Solanaceae*, lipids have been proposed to direct the pollen tube growth by controlling water flow to pollen (Wolters-Arts *et al.* 1998). The major components of the hydrophobic exudate in the Solanaceae are triglycerides (Lancelle *et al.* 1987). The hydrophobicity of the exudate is a critical factor in the ability of pollen to penetrate the stigma. This hydrophobicity establishes a gradient of water that is used as a guidance cue by the germinating pollen tube (Lush *et al.* 1998). In an *in vitro* reconstruction of the stigmatic environment it was revealed that the speed of pollen hydration and germination is related to the proximity between the aqueous phase and pollen grains suspended in the exudate/oil phase (Lush *et al.* 2000). The pollen tube in such an environment emerged closer to and was directed towards the aqueous phase. So the initial guidance mechanism of the pollen tube might be controlled by the physical environment in the stigma and not dependent on pistil components; a controversial proposal when first raised, it re-acquainted the scientific community with the notion that nature selects for the simple solutions firstly. When several oils were used as a substitute for stigmatic exudates, pollen did germinate, an observation supporting the suggestion above.

1.2.3 Stage III of the progamic phase; pollen tube growth through the style.

Following the penetration of the stigma, pollen tubes grow through the style towards the ovule. The style, an elongated structure, connects the stigma to the ovary. It can be an open canal, as in lily, or packed with cells, as in *Solanaceae* and *Arabidopsis*. The inaccessibility of the pollen tube through the female tissues has slowed down the research on the growth and guidance of pollen tubes through the style. Part of the problem is the complex nature of the style. The different organisation of stigmas and styles lead to the formation of different theories explaining the pollen tube growth and guidance in the style. Mechanical and biochemical gradients have been implicated in the guidance of the pollen tubes through the style as well as chemoattractants, even though the latter proposition has lost some ground lately (Lush 1999).

In some species, such as maize and pearl millet, the directionality of pollen tube growth was proposed to arise from the mechanical influences existing within the stylar tract (Heslop-Harrison and Heslop-Harrison 1985). In this model of tube growth, the cellular architecture of the transmitting tissue provides the necessary guidance cues required by the elongating pollen tube to reach the ovary. This type of mechanism has been suggested for the *Solanaceae*, where the tubes grow through the intercellular spaces between the parallel files of transmitting tract cells that comprise their solid styles (Lush *et al.* 2000). In solid stigmas and styles, enzymes are probably needed to facilitate entrance into the transmitting tract extracellular matrices (ECMs) and so facilitate this mechanical guidance towards the female gametophyte. Several of these enzymes have been described in pollen such as pectin esterase (Mu *et al.* 1994), glucanase (Doblin *et al.* 2001), polygalacturonase (Pressey and Reger 1989; Torki *et al.* 2000), and endoxylanase (Bih *et al.* 1999; Suen *et al.* 2003). All of these enzymes could serve to modify the ECM of the stigma and style as the pollen tube traverses the pistil and possibly the pollen wall itself but their exact role is yet to be fully characterised. From structural data it appears that the pollen tube and pistil ECM become as one entity where they join. The two matrices may be remodelled to form a third, containing components of both (Derksen *et al.* 2002).

Biochemical guidance cues within the style that attract pollen tubes and promote growth towards the ovary have been proposed as well in the case of the *Solanaceae*. In the style, the pollen tubes are in intimate contact with the components of the ECM of the transmitting tract. The ECM is comprised of a complex mixture of proteins, in particular arabinogalactan proteins (AGP), proline-rich glycoproteins and extensin-like proteins. AGPs, are the candidate molecules to provide nutrient, adhesive support, or lubrication to the pollen tube given their extraordinary sugar content (sometimes up to 95% of the protein molecular mass) and their adhesive properties (Mollet *et al.* 2002). A transmitting tissue-specific (TTS) AGP from *Nicotiana tobacum* has been found to stimulate pollen tube growth and attract pollen tubes (Cheung *et al.* 1995; Wu *et al.* 2000). TTS proteins display a gradient of increasing glycosylation along the length of the style that coincides with the direction of pollen tube elongation. The TTS proteins could promote pollen tube growth by serving as a nutrient and at the same time, by attracting the pollen tubes, provide them with the cues necessary for their guidance to the female gametophyte.

Extensin-like proteins have been shown to make an important contribution to the adhesion between the growing pollen tube wall and the ECM of the pistil across a wide range of plant families. The extensins form a class of hydroxyproline-rich glycoproteins that are found in plant cell walls and are characterized by the presence of numerous repeats of a Serine–Hydroxyproline-Hydroxyproline-Hydroxyproline-Hydroxyproline motif. In the case of *N. tabacum*, class III pistil-specific extensin-like proteins (PELPs), are known to interact with the pollen tube as it extends through the stylar canal. The PELPs are translocated from the matrix to the pollen tube wall (de Graaf *et al.* 2003). In the transmitting tissue of *Antirrhinum* pistils PT11, a similar type of protein is specifically expressed (Baldwin *et al.*, 1992). In *Zea mays*, a pollen specific extensin-like protein, Pex1, was identified that might potentially recognize and interact with molecules within the style (Rubinstein *et al.* 1995; Stratford *et al.* 2001).

In the case of species with a hollow style as in *Lilium longiflorum*, the proposed guidance mechanism is different. The theory is that pollen tube guidance and

growth rate through the style is dependent on a haptotactic (matrix adhesion-driven) mechanism (Lord and Sanders 1992; Lord 2000; Lord 2003). The pollen tubes adhere to the secretory cells that form the lining of the transmitting tract via components in the style ECM and are guided towards the ovary. The transmitting tissue was shown to be capable of translocating inert latex beads in a manner reminiscent of pollen tube growth (Sanders & Lord, 1989). An adhesive complex, composed of pectin and a small cysteine-rich protein from the style, has also been implicated in pollen tube growth and possibly in directional guidance (Mollet *et al.* 2000; Park *et al.* 2000). Identification of stylar components responsible for adhesion to the growing pollen was based on an assay examining the binding of pollen tubes to a nitrocellulose membrane in the presence of stylar extracts. The fractions that bound the pollen tubes on the nitrocellulose membrane contained a small 9 kDa stigma/stylar protein, identified as a cysteine-rich adhesin (SCA) homologous to plant lipid transfer proteins, that in conjunction with a pectic fraction, enabled pollen adhesion to occur (Lord 2003; Park and Lord 2003). Immunolocalization confirmed the localisation of SCA within the stylar ECM and in *in vivo* grown pollen tubes (Park *et al.* 2000). The other stylar component identified by the bioassay was a large molecule of approximately 1.5 MDa, a member of the pectin family. The bioassay also revealed that the two molecules bind to each other to promote pollen adhesion and to stimulate the rate of pollen tube growth in a pH-dependent fashion (Mollet *et al.* 2000). An increase in calcium in the ECM of the transmitting tract on pollination has been reported in styles of *Petunia hybrida* (Bednarska and Butowt 1995; Lenartowska *et al.* 2001) and may be a source of the divalent cation that has been shown to be essential for pollen tube growth *in vitro*.

1.2.4 Stage IV of the progamic phase; pollen tube growth at the funiculus

Chemotrophic factors probably play a bigger role in pollen tube guidance within the ovary where pollen tubes in many plant species have to sharply change their growth orientation to gain access into the ovules and fertilise the egg cell. The precise nature of these chemical attractants remain unknown as well as the precise way these cues emanate from the female gametophyte to guide the pollen tube. Sugar moieties and calcium, which exist in high levels around the ovules and within the synergids of the embryo sac, respectively, have often been postulated

to play chemotropic roles (Willemse *et al.* 1995; Higashiyama *et al.* 2001; Higashiyama 2002b; a; Higashiyama *et al.* 2003). In peach, secretions from both the ovary and individual ovules appear to have a role in pollen tube guidance and penetration of the ovule (reviewed in Herrero 2003).

Screens for genes affecting seed-set, revealed that guidance cues for the pollen tubes are emanating from the mature embryo sac and surrounding diploid ovule tissues and are critical for the guided entrance of pollen tubes into the ovule (Hulskamp *et al.* 1995; Ray *et al.* 1997; Palanivelu and Preuss 2000; Shimizu and Okada 2000). Similarly, studies in the *Arabidopsis magatama* (*maa*) mutant (Shimizu and Okada 2000) suggest for surface adhesives acting as guidance cues. In the *maa* mutant, that shows delayed female gametophyte development, pollen tubes appeared to lose their way just before entering the micropyle. The delayed ovules also showed a tendency to attract two pollen tubes rather than one. A 'monogamy' model has been proposed, where the female gametophyte emits the two attractants (a funiculus adhesion guidance cue and a micropyle guidance cue) and the pollen tubes emit repulsive forces to prevent polyspermy.

When a semisterile *A. thaliana* line, *TL-1*, was used that did not develop normal embryo sacs, the pollen tubes were guided only to the ovules containing normal embryo sacs but not to those with degenerated embryo sacs. This again leads to the suggestion that viable fully mature embryo sacs can provide the necessary signals to guide the pollen tube (Ray *et al.* 1997). Arresting ovule development in tobacco by inhibiting ethylene biosynthesis also results in the mistargeting of the pollen tubes to the ovary (De Martinis and Mariani 1999). Conclusive evidence that the female gametophyte provides the attraction cues for the pollen tubes was presented by Higashiyama and co-workers in an elegant experiment in 1998. Using a novel *in vitro* fertilization system they showed that the synergids themselves were the source of the chemotropic substance (reviewed in Higashiyama *et al.* 2001; Higashiyama *et al.* 2003). In more detail, the pollen tubes and naked embryo sacs of *Torenia fournieri* were co-cultivated on solid growth media. The pollen tubes were found to grow with a high degree of accuracy toward the micropylar end of the naked embryo sac. This behaviour was

completely abolished by experimental eradication of the ovules via laser ablation, providing further evidence in support of a role for proteins produced by the embryo sac in pollen tube guidance.

In 2003, identification of the gene responsible for the *pop2* mutation led to the proposal that a γ-amino butyric acid (GABA) gradient may be a critical signal for guiding the pollen tube to the female gametophyte. In *pop2,* which is self sterile when homozygous, pollen and ovule are morphologically normal, suggesting that the defect is in the interaction between the two. The mutant pollen tube can germinate and penetrate the stigma, but does not guide to the female gametophyte. *POP2* encodes a GABA transaminase. A gradient of GABA increasing from the stigma to the inner integument provides the pollen tubes guidance cue to reach the female gametophyte (Ma 2003; Palanivelu *et al.* 2003; Yang 2003).

1.3 The pollen tube: Structure and growth.

Pollen tubes are cylindrical cells with a hemispherical apical dome. Growth occurs only in the tip of the dome. Within the pollen tube the cytoplasm itself exhibits a high degree of polarised zonation. Growing pollen tubes, have a clear zone at the extreme apex, with large organelles and inclusions located several microns from the apex (Taylor and Hepler 1997; Geitmann and Emons 2000). In pollen tubes, a large central vacuole fills the bulk of the cell. The cytoplasm is restricted to a thin layer, except for the accumulation at the apex of the cell (Figure 1.2).

Growing pollen tubes form callose partitions that wall off the distal portions of the tube, keeping the cytoplasm and male germ unit (the two sperm cells and the vegetative nucleus) in the apical region. The elongating pollen tube thus leaves a trail of cell wall and a very thin layer of cytoplasm but keeps the bulk of the protoplasm moving forward at the tube apex (Derksen *et al.* 2002).

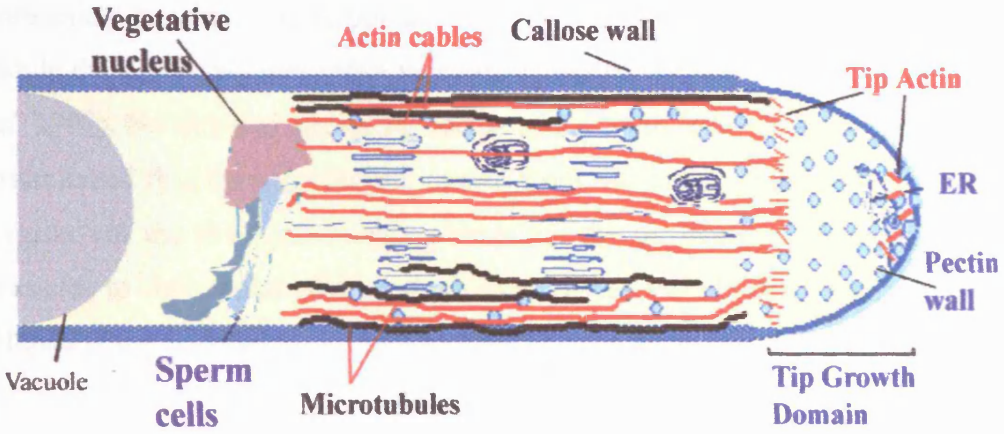


Figure 1.2. The structure of the pollen tube.
Pollen tubes are cylindrical cells with a hemispherical apical dome. Growth is occurs only in the tip of the dome. Within the pollen tube the cytoplasm itself exhibits a high degree of polarised zonation. Growing pollen tubes have a clear zone at the extreme apex, with large organelles and inclusions located several microns from the apex (Taylor and Hepler 1997; Geitmann and Emons 2000). In pollen tubes, a large central vacuole fills the bulk of the cell. The cytoplasm is restricted to a thin layer, except for the accumulation at the apex of the cell.

Coupled with the cytoplasmic organisation is the pattern of the cytoplasmic movement in pollen tubes. The rapid growth of the pollen tubes demands the presence of an active secretory system to provide for the extensive amount of material needed to support the forward progression of the cell (Cai *et al.* 2000; Parton *et al.* 2001; Parton *et al.* 2003). It is described as reverse fountain streaming, in which the acropetal lanes move forward along the edge of the cell, while the basipetal lanes move rearward in the central core of the cell (de Win *et al.* 1999). No directed streaming occurs in the tip of the cell. Even if it has been established that the vesicles are carried from the shank of the cell to the apical region via the reverse fountain streaming, the mechanism of delivery of the vesicles to their point of exocytosis has not been established yet (reviewed in Hepler *et al.*, 2001).

The cell wall, the product of secretion, exhibits gradients in composition and extensibility (Geitmann *et al.* 1995). Esterified pectin residues present in the mature pollen grains and are secreted at the tip of the pollen tube(Aouali *et al.* 2001). The enzyme pectin methyl esterase then increasingly de-esterifies them and the resulting carboxyl groups are targets for cross-linking by calcium, forming a gradient of esterified at the apex to de-esterified pectins at the base of the cell wall. The calsium uptake of the cell wall increases the strength of the structure thus creating an increasing gradient of the degree of cell wall rigidity and decrease in extensibility as the pollen tube elongates (Geitmann and Cresti 1997; Li *et al.* 2002). Cellulose and callose are also present, but usually several microns behind the tip (Heslop-Harrison 1987).

Pollen tube elongation is dependent on the close interactions with the female tissues (Cheung *et al.* 1996; Lord 2003). Pollen tubes secrete receptor molecules in the cell wall that interact with female signal and nutrient molecules (Cheung *et al.* 1995; Muschietti *et al.* 1998). There is a receptor kinase in tomato pollen tubes that is specifically de-phosphorylated by contact with stylar extracts and ligands for this transmembrane kinase were isolated via a yeast two hybrid system. Many of these potential ligants were secreted molecules from the stylar matrix. One candidate though, LAT52, occurs in the pollen tube wall itself and allows for the

possibility of a positive feedback signalling event taking place during pollen tube growth (Tang *et al.* 2002; Johnson and Preuss 2003). Cell wall glycoproteins, including arabinogalactan proteins (AGPs), proline-rich proteins, and extensins, although constituting only 10% of the cell wall material, may be important contributors to the formation and structure of this complex extracellular matrix.
The AGPs participate in the control of cell wall formation. By precipitating the AGPs using the Yariv reagent, inhibition of pollen tube elongation occurs without stopping secretion thus creating deformed deposits of pectin and callose that fail to incorporate properly into the cell wall (Roy *et al.* 1998; Mollet *et al.* 2002). AGPs have also been observed in periodic rings along the length of the tube together with demethylated pectins and these depositions appear to correlate with periodic phases of slower growth in an oscillating system (Geitmann and Cresti 1997; Roy *et al.* 1999; Mollet *et al.* 2002)

During the process of exocytosis at the tip of pollen tubes, excess membrane is secreted so recycling (endocytosis) is required to maintain the tube tip architecture and recycle the membrane (Derksen *et al.* 1995). Molecules that function in the exocytotic and endocytotic processes affecting the proper targeting of transported molecules in plants are, as expected, expressed in pollen tubes. Clathrin, implicated in endocytotic trafficking, has been localised in the plasma membrane at the tip of the *Lilium longiflorum* pollen tube (Blackbourn and Jackson 1996). By maintaining a balance between the rates of secretion and endocytosis, growth of the pollen tube is possible (Hepler *et al.* 2001).

Pollen tube exhibits pulsatile growth.

Pollen tubes exhibit oscillations in their rate of growth (20- to 30- sec period in lily as demonstrated by Pierson *et al.* (1996)). The underlying ion gradients and fluxes oscillate with the same frequency as the growth rate, although the phase relationships vary. In the case of the oscillations of the calcium ion fluxes, this led to two mechanisms proposed for the control of the tip growth in pollen tubes which might be coexistent (Messerli and Robinson 2003):

The cell wall consists of esterified pectin residues (Aouali *et al.* 2001) and calcium, protons and/or boron interact with it and control its mechanical properties. Therefore in a mechanistic proposal, changes in the extensibility of pectin, may control the pollen tube growth. Since the calcium gradient oscillated in phase with growth, the binding of calcium to cell wall components dominates the calcium influx signal (Holdaway-Clarke *et al.* 1997).

The other mechanism focuses on the experiments demonstrating that the elevations of calcium are uncoupled from secretion. The peak in calcium oscillation was calculated to precede the pulse of secretion and the associated extension of the tip by 4sec. Here, oscillatory changes in turgor pressure are the underlying event to which other the growth processes become linked (Messerli *et al.* 2000).

Actin and several actin-binding proteins are implicated in controlling the growth and directionality of the pollen tube (Hepler *et al.* 2001; Vidali and Hepler 2001; Vidali *et al.* 2001). Several models have been proposed over the years to explain the participation of the actin cytoskeleton in pollen tube growth. The simplest model, that actin only delivers secretory vesicles to the tip, is not valid since pollen tube elongation is more sensitive to actin-disrupting drugs than is the transport of secretory vesicles (Vidali *et al.* 2001). Another model proposes that the microfilaments act as a sieving mechanism by sorting out the secretory vesicles from the bulk endoplasm, a model that gained support by the finding of a cortical ring of F-actin in pollen tubes (Heslop-Harrison and Heslop-Harrison 1990; Kost *et al.* 1998).

Profilin, an abundant actin monomer-binding protein, inhibits the nucleation step in actin polymerisation and the incorporation of subunits to the pointed end of microfilaments. Pollen profilin is a cytosolic protein evenly distributed in the vegetative cytosol (Vidali and Hepler 1997). Excessive profilin was shown to inhibit growth faster than cytoplasmic streaming in pollen tubes and this function arises by maintaining a pool of monomeric actin ready for polymerisation and not

by interaction with poly- l-proline-binding as indicated in a very recent publication (Vidali and Hepler 2001; Vidali *et al.* 2001; McKenna *et al.* 2004).
The polarised elongation of microfilament bundles also involves pollen ROPs (Rac Of Plant where Rac stands for Ras-related-C3 botulinum toxin substrate). Pollen tube growth can be inhibited either by microinjecting antibodies against ROP or by the overexpression of a dominant-negative version of it (Kost *et al.* 1999; Li *et al.* 1999a). ROPs are concentrated at the tip of the pollen tube and their overexpression, or the expression of constitutively active mutants, modifies the cytological architecture of actin and generates isodiametric growth in the pollen tubes (Kost *et al.* 1999; Li *et al.* 1999a; Fu *et al.* 2001). These proteins probably act by polymerising the actin. Actin depolymerising factor (ADF)/cofilin, which enhances actin depolymerisation from the pointed end of the microfilament (Carlier *et al.* 1997; Carlier *et al.* 1999) was also identified in pollen (Kim *et al.* 1993; Lopez *et al.* 1996; Allwood *et al.* 2002). ADF, regulated by pH and phosphoinositides, is able to sever pre-existing filaments and bind to monomeric actin (Carlier *et al.* 1999). ADF controls actin dynamics and increasing levels of ADF can overcome the effect the constitutively active form of ROP creates in pollen (Carlier *et al.* 1997; Ressad *et al.* 1999; Hepler *et al.* 2001; Chen *et al.* 2002; Chen *et al.* 2003).

1.4 Guidance of the pollen tube

The key feature of the pollen tube, paramount for the fertilisation, is its directionality. Pollen tubes responding to local cues, elongate from the stigma surface, transverse the style and guide towards ovules. The pollen tube responses to directional cues by the sporophyte/female involve modifications of the cytoskeleton and vesicular transport activities, especially membrane trafficking. Changes in ion fluxes and gradients are also observed, probably the first observable events that lead to the change of direction of the pollen tube (Taylor and Hepler 1997; Geitmann and Emons 2000; Lord 2000; Malhó *et al.* 2000; Palanivelu and Preuss 2000; Hepler *et al.* 2001; Vidali and Hepler 2001; Lord 2003).
The development of the pollen tube during the progamic phase led researchers to compare it with the directional guidance in axons and other cell migration systems

(Lord 2000; Palanivelu and Preuss 2000; Lord 2003). There are several different examples of cells exhibiting polarised growth, fungal hyphae, algal rhizoids, and pollen tubes, root hairs in plants. The tip focus growth, characteristic of these cells, permits the exploration the local environment and enables the acquisition of nutrients needed for the organism. Mechanical cues for signalling have been shown to be used by fungal hyphae, which, like pollen, also elongate by tip growth. Fungal hyphae have been shown to employ topographical guidance systems to determine where their appressoria form, in a thigmotropic response and this response involves calcium-dependent signalling (Watts *et al.*, 1998).

The pollen tube, by contrast to the other plant and fungal cell types, is well provisioned, and its explorations are focused on finding the ovule and delivering the sperm so that fertilisation can occur. Neurite cells also extend only at their apex, using processes that involve the cytoskeleton, grow invasively among other cells, and develop specific matrix associations. Moreover, calcium has been implicated in the directional guidance control mechanism of the neurite cells. Due to these similarities the pollen tube was compared with the neurite (Lord 2000, Palanivelu and Preuss 2000, Zheng and Yang 2000).

Pollen tubes, exhibit markedly polarised internal gradients and/or external fluxes of important ions, like calcium, protons, potassium, boron and chloride, which appear to play a fundamental role in the establishment of polarity and maintenance of such polarised growth. Several studies with intracellular indicators reveal that pollen tubes exhibit a tip-focused gradient of cytosolic calcium. It is assumed that this calcium gradient participates in the secretory process. Calmodulin, a calcium-binding protein involved in secretion, is present, but it is evenly distributed and not accumulated in the apical domain where the calcium consentration is high. The calcium gradient appears to be essential for growth. (Calder *et al.* 1997; Holdaway-Clarke *et al.* 1997; Roy *et al.* 1999; Hepler *et al.* 2001; Holdaway-Clarke *et al.* 2003).

Protons, like calcium, can have a profound effect on the process of growth (Hepler *et al.* 2001). Proton fluxes and pH gradients oscillate in relation to

growth. Because of the much greater mobility of protons, observation of the proton levels by elevated dye concentration can lead to dissipation of local gradients. This leads to decreased temporal resolution hindering observations. However, it has been observed that the extreme apex is slightly acidic, with the pH elevating nearly one pH unit and a distinct alkaline band forms toward the base of the clear zone, as well as the influx of protons in the tip to lag pollen tube growth by about 8 sec. In addition to the internal pH gradient, there are currents of protons in the extracellular medium surrounding the pollen tubes. Influxes are directed toward the apex of the cell with effluxes from the base of the clear zone, forming a current loop driven by proton ATPases on the base of the clear zone (Feijo *et al.* 1999).

1.5 Progamic phase genes identified in *Arabidopsis thaliana*

The progamic phase has been mainly studied over the years using species other than Arabidopsis. Physiological studies of pollen tubes have been performed on lily, tobacco, petunia or brassica. This is because pollen from these species can be easily grown and manipulated *in vitro* and self-incompatibility, an aspect of the plant sexual reproduction not addressed in this work since does not affect the *Arabidopsis*, has been extensively studied in the latter species. Recently though with the advent of the genome sequence project, *Arabidopsis* started to be utilised for the identification of genes affecting the male gametophyte. Mutant screens have contributed significantly to our knowledge of the female gametophyte development (Drews *et al.* 1998). Similar tests for the male gametophyte have resulted in the identification of comparably few mutants with defects in pollen tube elongation or guidance, allowing limited insights into the cellular and molecular processes governing pollen tube tip growth (Schiefelbein *et al.* 1993; Howden *et al.* 1998b; Grini *et al.* 1999; Procissi *et al.* 2001; Pacini and Hesse 2002; Palanivelu *et al.* 2003; Procissi *et al.* 2003; Lalanne *et al.* 2004). The genes identified, to date, playing a role in the male gametophyte in *Arabidopsis* during the progamic phase are presented in Table 1.1.

Mutant	Gene	Mutagen	AGI/Location	Protein Function	References
AtAPY1 and *AtAPY2*	*AtAPY1* and *AtAPY2*	Double T-DNA KO	AT3G04080 and AT5G18280	Apyrase	Steinebrunner et al., 2003
AtRac2	*AtRAC2*	DN, CA	At4g35950	GTPase	Kost *et al.*, 1999
AtRop1	*AtROP1*	OE, DN, CA	At3g51300	Rop GTPase	Li *et al.* 1999
AtRab1	*AtRAB1*		ND	ND	Cheung, A.Y.
kinky pollen (kip) ttd26/ttd34/ttd42	*KIP*	T-DNA	At5g49680	SABRE-like	Bonhomme *et al.* 1998
mad4	*MAD4*	EMS	Chr I 163cM	ND	Grini *et al.*, 1999
npg1	*NPG1*	T-DNA	At2g43040	CaM-binding protein	Golovkin and Reddy, 2003
ttd8	*ttd8*	T-DNA	At1g71270	ND	Bonhomme *et al.*, 1998
seth1	*SETH1*	Tn	At2g34980	GPI-N-acetylglucosaminyl transferase (GPI-GnT), subunit C	Lalanne *et al.* 2004
seth2	*SETH2*	T-DNA	At3g45100	GPI-N-acetylglucosaminyl transferase (GPI-GnT), subunit A	Lalanne *et al.* 2004
seth4	*SETH4*	Tn	At4g34940	ARM-repeat protein	Lalanne *et al.* submitted
seth7	*SETH7*	Tn	At2g41930	Ser/Thr protein kinase	Lalanne *et al.* submitted
spik	*SPIK*	T-DNA	At2g25600	K+ channel	Mouline *et al.* 2002
tip1	*TIP1*	EMS	ND	ND	Schiefelbein *et al.* 1993

Table 1.1. The *Arabidopsis* genes identified as acting during the progamic phase. Explanation of abbreviations for the mutagen: KO is Knock out; Tn is transposon; OE is over expression; DN is dominant negative expression; CA is constitutively active expression; EMS is Ethyl Methanesulfonate induced mutagenesis. ND is not determined.

1.6 The aim of the thesis

The number of molecular components identified as acting only during the progamic phase has been limited. The sporophytic tissues do not allow the morphological observation of the progamic phase events, hindering forward screens for their identification. The complexity of the interactions taking place during the progamic phase, at the genetic and molecular level, make a reverse genetics strategy an unattractive proposal for the elucidation of the molecular components of the progamic phase.

Identification and characterisation of essential genes acting during the progamic phase was the scope of this study.

In the first part of the project the aim was to identify genes with an essential role for the male gametophyte during the progamic phase. A forward genetics approach allowed the identification of genes playing an important role during the progamic phase, by screening and selecting for lack of transmission events in the transposon insertion mutagenised population. The Sainsbury Laboratory *Arabidopsis* Transposants (SLAT) collection was screened for progamic phase mutants. Analysis of 4,094 lines from the collection did not result in the identification of progamic phase mutants.

In the second part of this study the aim was the phenotypic, genetic and molecular characterisation of two progamic phase mutants identified in a segregation distortion screen done in parallel to the one performed on the SLAT lines. From the Cold Spring Harbour (CSH) collection two putative progamic phase mutants were handed over. The mutants were characterised genetically as essential for the male gametophyte and acting during the progamic phase. The mutants were defective in pollen tube initiation and germination. The insertion site of the transposons was identified. For *seth4*, the mutation was complemented by the introduction of a wild-type copy of the gene. For *seth7,* the insertion of the *Ds* element was in the 3'untranslated region of a putative protein kinase. The introduction of a wild type copy of the gene in *seth7* instead of restoring the male transmission, resulted in a more severe phenotype.

In the final part of this project the focus of the work was on the characterisation of the SETH4 protein and the two closest *Arabidopsis* homologues of SETH4, SFL1 and SFL2. Preliminary localisation and expression studies were performed and functional analysis of the novel proteins via the construction of double knock out lines were initiated.

A forward genetic screen for the identification of genes acting during the progamic phase, led to the identification of a novel three members family in *Arabidopsis* with gametophytic and sporophytic expression

Chapter 2

Materials and Methods

2. 1 Materials

Materials used in this work were obtained from the suppliers as follows: Chemicals: Sigma, Fisher Chemicals, GIBCO BRL, BDH, Fisons, Melford Laboratories Ltd; Molecular biology enzymes and reagents: GIBCO BRL, Boehringer Mannheim, Stratagene, Promega, Pharmacia, Perkin Elmer; Plant tissue culture salts and hormones: Flow Laboratories, Sigma; Agar and related products: Difco laboratories; Bacterial media: Sigma; DNA and RNA purification kits: Qiagen; Hybond membranes: Amersham; RT-PCR kit: Advanced Biotechnologies Ltd, Promega; Disposable plastic materials: Sterilin, Nunc and Sarstedt; Filters and Acrodiscs: Millipore and Calbiochem.

2. 2 Methods

Bacterial culture and storage

2 . 2. 1 Strains and genotypes

Escherichia coli (*E. coli*)

XL1-Blue: *recA 1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, *lac*, {*F'*, *proAB*, *lacIq*, *ZDM15*, *Tn10*, (tet^R)} (Bullock *et al.*, 1987).

DH5α: F-, ø80d*lacZ*DM15, D(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk-, mk+), *phoA*, *supE*44, l-, *thi*-1, *gyrA*96, *relA*1

Agrobacterium tumefaciens (*A. tumefaciens*)

GV3101: containing Ti plasmid pMP90

2. 2. 2 Antibiotics for bacterial selection

Ampicillin (100 mg/l), kanamycin, (50 mg/l) spectinomycin (100mg/l) and tetracyclin (10 mg/l) were used for growth and selection of *E. coli*.

Kanamycin (50 mg/l), spectinomycin (100mg/l) and rifampicin (50 mg/l) were used for growth and selection of *A. tumefaciens*.

2. 2. 3 Bacterial media and growth condition

Luria Bertani (LB): Bacto-tryptone 1% (w/v), bacto-yeast extract 0.5 % (w/v), NaCl 1% (w/v). pH adjusted to 7.0 with 1 M NaOH and autoclaved for 15 min at

120 °C. Solidified LB medium (LB agar) was prepared by incorporating 1.5% (w/v) agar into LB medium .

E. coli and *A. tumefaciens* cultures were grown at 37 °C and 28 °C respectively. Liquid cultures were grown on an orbital shaker at 240 rpm until the required optical density was obtained.

2. 2. 4 Long term storage of bacterial strains

A 0.45 ml aliquot of an overnight bacterial culture (*E. coli* and *A. tumefaciens*) was transferred to a cryogenic storage tube. 0.55 ml of filter sterilised 50% (v/v) glycerol was added, mixed and stored at -80 °C. To recover the single colonies of each strain a small portion of the frozen culture was streaked on solidified media containing selective antibiotic and grown as described in section 2. 2. 3.

2. 3 Bacterial transformation

2. 3. 1 Preparation of competent *E. coli*

$Ca^{2+}Mg^{2+}$ solution: Sodium acetate 40 mM, $CaCl_2$ 100 mM, $MnCl_2$ 70 mM. pH adjusted to 5.5 with 1 M HCl and filter sterilised.

Preparation of competent *E. coli* cells was performed as described by Hanahan (1983). A single colony of XL1-Blue or DH5α was grown in 25 ml of LB media at 30 °C. A 1-ml aliquot of the overnight culture was inoculated into 100 ml of pre-warmed LB media and grown until an OD_{600} of 0.2 was obtained. Filter sterilised 1 M $MgCl_2$ was added to a final concentration of 20 mM (2 ml) and the culture grown until an OD_{600} of 0.45-0.55 was obtained.

The bacterial culture was transferred to a 50 ml sterile tube, placed on ice for 2 hours and centrifuged at 3000 *g*, 4 °C, for 5 min. The supernatant was discarded, the bacterial pellet was gently resuspended in 50 ml of $Ca^{2+}Mg^{2+}$ solution, which had been pre-chilled to 4 °C and incubated on ice for 45 min. The bacterial culture was centrifuged as mentioned above, the supernatant was discarded and the pellet was gently resuspended in 5 ml of $Ca^{2+}Mg^{2+}$ solution containing 15% glycerol. The cells were quickly divided in 0.2 ml aliquots, transferred to 1.5 ml pre-chilled microfuge tubes, flash frozen in liquid nitrogen and stored at -80 °C.

2. 3. 2 Transformation of *E. coli*

An aliquot of competent *E. coli* (section 2. 3. 1) was thawed on ice, plasmid DNA (100-300 ng) was added, gently mixed by flicking the tube and incubated on ice for 30 min. The cells were heat shocked at 42 °C for 30 sec, 0.6 ml of LB (without antibiotic selection) was added in the 1.5ml microcentrifuge tube and grown on a shaker at 240 rpm for 1 h at 37 °C. Generally, two aliquots of the resulting cells were spread on LB agar plates containing antibiotic selection, allowed to dry and incubated overnight at 37 °C. The resulting transformants were identified by restriction digests (section 2. 6. 1) or colony PCR (section 2. 7. 4).

2. 3. 3 Preparation of competent *A. tumefaciens*

A single colony of A. *tumefaciens* was grown in 5 ml of LB media with selective antibiotic on a shaker (240 rpm) at 28 °C. The overnight culture was inoculated into 100 ml of LB media and grown until an OD_{600} of 0.5-1 was obtained. The bacterial culture was centrifuged at 3000 *g*, 4 °C for 5 min. The supernatant was discarded and the pellet was resuspended in 1 ml of pre-chilled (4 °C) 20mM $CaCl_2$ solution. The cells were quickly divided in 0.1 ml aliquots, transferred to 1.5 ml pre-chilled (4 °C) microfuge tubes, flash frozen in liquid nitrogen and stored at –80 °C.

2. 3. 4 Transformation of *A. tumefaciens.*

An aliquot of competent *A. tumefaciens* (section 2. 3. 3) was thawed on ice, 0.5-1 μg of plasmid DNA in was added and mixed by gentle flicking. The cells were incubated on ice for 5 min, frozen in liquid nitrogen for further 5 min and thawed at 37 °C for 5 min. After addition of 0.6 ml of LB (without antibiotic selection) in the 1.5ml microcentrifuge tube the cells were grown on a shaker at 240 rpm for 2-4 h at 28 °C. Generally, two aliquots of the resulting cells were spread on LB agar plates containing antibiotic selection, allowed to dry and incubated at 28 °C for 2 days.

2. 4 Isolation and purification of nucleic acids

2. 4. 1 Small scale isolation of plasmid DNA

Solution I: Glucose 50 mM, Tris-HCl (pH 8.0) 25 mM and EDTA (pH 8.0) 10 mM.

Autoclaved 15 min at 120 °C

Solution II: NaOH 0.2 N, SDS 1% (w/v) RNAse A (20mg/ml stock)

Solution III: Potassium acetate 5M, glacial acetic acid 11.5% (v/v). Autoclaved 15 min at 120 °C.

Phenol/chloroform: isoamyl alcohol (24:1): 100 ml phenol (Fisons), 100 ml chloroform and 4.16 ml isoamyl alcohol.

A 1.5 ml aliquot of an overnight culture (5 ml) grown from a single colony was transferred to a 1.5 ml microfuge tube and centrifuged at 14000 *g*, for 5 min. The supernatant was discarded and the pellet was resuspended in 100 µl of solution I. Following addition of 200 µl of solution II and 5 µl of RNAse A, the tube was gently inverted 5 times and incubated for 5 min on ice to allow lysis of the bacteria and RNA digestion. After lysis, 150 µl of solution III was added to the lysate, mixed and centrifuged at 14000 *g* for 5 min. The supernatant was transferred to a fresh tube, an equal volume of phenol chloroform was added, and the solution was vortexed and centrifuged at 14000 *g* for 5 min. The top aqueous layer was transferred to a fresh tube, 2 volumes of absolute ethanol was added and centrifuged at 14000 *g*, for 5 min at RT to precipitate the crude plasmid. The supernatant was removed, the pellet was washed with 100 µl of 70% (v/v) ethanol and centrifuged at 14000 *g*, for 1 min. The supernatant was discarded, the pellet was vacuum dried for 10-15 min and resuspended in 30 µl of sterile distilled water.

The method described above was also used to isolate plasmid DNA from *A. tumefaciens* except that the volume of overnight culture selected was increased to 4.5 ml.

2. 4. 2 Medium and large scale preparation of plasmid DNA

A 50 µl aliquot of an overnight bacterial culture grown from a single colony was inoculated into 50 ml of LB with selective antibiotic and grown as described in

section 2. 2. 3. The overnight culture was transferred to a 50 ml centrifuge tube and the bacterial pellet was recovered by centrifugation at 10000 g for 10 min at rt. The supernatant was removed and the pellet was resuspended in 2 ml of solution I and lysed with 3 ml of solution II by gently inverting the tube 3-4 times. Following lysis 2.5 ml of solution III was added mixed and the white bacterial debris was precipitated by centrifugation at 10,000 *g* for 5 min at rt. The supernatant was transferred through a Miracloth (Calbiochem) into a fresh centrifuge tube to remove the remaining debris. The resulting supernatant was mixed with an equal volume of isopropanol, centrifuged at 10,000 *g* for 10 min at 4 °C to precipitate the nucleic acid. The supernatant was removed, the pellet was resuspended in 500 µl of sterile distilled water and transferred to a 1.5 ml microfuge tube. To remove the RNA molecules 20 µl of RNAse A was added, briefly vortexed and incubated at 37 °C for 2 hours. Following RNAse treatment 500 µl of phenol/chloroform was added, the solution was vortexed and centrifuged at 14000 g for 5 min at rt. The top aqueous phase was transferred to a fresh microfuge tube and the phenol/chloroform step was repeated. To the resulting aqueous phase 100 µl of 5 M ammonium acetate and two volumes of absolute ethanol was added, the solution was vortexed and centrifuged at 14000 *g* for 5 min at rt to precipitate the plasmid. The supernatant was removed, the pellet was washed with 200 ml of 70% ethanol followed by a brief spin (1 min). The ethanol was removed, the pellet was vacuum dried for 10-15 min and resuspended in 50 µl of sterile distilled water. The resulting plasmid DNA was quantified by agarose gel electrophoresis (section 2. 5)

Bacterial cultures for large-scale isolation of plasmid DNA were essentially prepared as described in section 2. except that the volume of the cultures was increased to 400 ml, the bacterial pellet was recovered in 200 ml centrifuge tubes and 8, 12 and 10 ml of solutions I, II and III were used for cell lysis respectively. Following precipitation of the nucleic acid pellet with isopropanol the pellet was was resuspended in 3 ml of sterile distilled water and transferred to a 50 ml centrifuge tube. The supernatant was transferred to a fresh centrifuge tube, mixed with an equal volume of isopropanol and centrifuged as before. The supernatant was discarded, the resulting pellet was resuspended in 500 µl sterile distilled water and transferred to a 1.5 ml microfuge tube. RNAse A treatment,

phenol/chloroform extraction and ethanol precipitation of the plasmid DNA were performed as described in section 2. 4. 5.

2. 4. 3 Isolation of total RNA

Total RNA was isolated from roots, stems, leaves, flowers and pollen using TRIZOL reagent (GIBCO BRL) or TRI reagent (Sigma). Plant tissues were ground to a fine powder in liquid nitrogen, TRIZOL reagent was added to a volume that gave a TRIZOL: tissue ratio of 10:1 v/w and briefly vortexed to homogenise the sample. 1 ml of the homogenised sample was transferred to a 1.5 ml microfuge tube and incubated at 15-30 °C for 5 min. 0.2 ml of chloroform (per 1 ml TRIZOL) was added, vortexed for 15 seconds and incubated at 15-30°C for 2-3 min. The samples were centrifuged at 12000 *g* for 15 min at 2-8 °C to separate the total RNA in aqueous phase. The supernatant was transferred to a fresh tube, 0.5 ml of isopropyl alcohol (per 1 ml TRIZOL) was added, mixed and incubated at 15-30 °C for a 10 min. The samples were centrifuged at 12000 *g* for 10 min at 2-8 °C. The supernatant was discarded, the RNA pellet was washed with 1 ml of 75% ethanol (per 1 ml TRIZOL) and centrifuged at 7500 *g* for 5 min at 2-8 °C. The supernatant was discarded and the RNA pellet was vacuum dried for 5-10 min. Total RNA was partially dissolved in DEPC treated water by several pipetting, incubated for 10 min at 55-60 °C and quantified spectrophotometrically (section 2. 4. 6).

RNeasy plant mini kit

Total RNA was isolated from roots, stems, leaves, flowers and pollen using RNeasy plant mini kit (Qiagen) according to manufacturers instructions.

2. 4. 4 Purification of DNA fragments from agarose gels

DNA fragments required for cloning were purified from agarose gel using DNA purification kits QIAquick gel extraction kit (Qiagen) for DNA fragments less than 10 kb and Qiaprep miniprep kits (Qiagen) for DNA fragments larger than 10 kb according to manufacturers instructions.

2. 4. 5 Purification of DNA

Following enzymatic manipulation of DNA, to remove the proteins from DNA solutions, an equal volume of phenol/chloroform: isoamyl alcohol (24:1) (section 2. 4. 1) was added to the reaction mixture, vortexed and centrifuged at 14000 g for 5 min. The aqueous phase was transferred to a fresh tube, two volumes of absolute ethanol and sodium acetate (3.0 M, pH 5.2) to a final concentration of 0.3 M were added, vortexed and centrifuged at 14000 *g* for 20 min. The supernatant was removed, the pellet was washed with 1ml of 70% (v/v) ethanol and centrifuged at 14000 g for 1 min. The supernatant was removed, the pellet was vacuum dried for 10-15 min and resuspended in sterile distilled water.

2. 4. 6 Quantification of nucleic acids

The concentration of nucleic acid was determined by measuring the UV absorbance of a DNA or RNA sample at wavelengths of 260 nm and 280 nm using a Philips (PU 8740 UV/VIS) scanning spectrophotometer. The concentration of nucleic acid in the sample was calculated on the basis that an OD of 1 corresponds to 50 mg/ml of double-stranded DNA, 40 mg/ml of single stranded DNA and RNA, and 20 mg/ml of single stranded oligonucleotides.

2. 5 Agarose gel electrophoresis for separation of DNA

50x TAE buffer: Tris base 242 g, glacial acetic acid 57.1 ml, EDTA 0.5 M (pH 8.0) 100 ml.

Gel loading buffer: orange G 0.5% (w/v), glycerol 50% (v/v)

Agarose gels were prepared by melting the appropriate amount of agarose in 100 ml of 1x TAE buffer and cooled to 60 °C. The ethidium bromide was added to the molten gel to a final concentration of 0.5 μg/ml, mixed thoroughly and poured into a mould with an appropriate comb. The gel was allowed to set for 30 min at room temp, the comb was carefully removed and the mould was placed into an electrophoresis tank containing 1x TAE buffer.

The DNA samples were mixed with 10x gel loading buffer to a final concentration of 1x and loaded into wells. An aliquot of a standard DNA ladder was used as a control. A voltage of 8 V/cm was applied and the gel was run until

complete separation of the DNA fragments. After electrophoresis, DNA was visualised using a UVP UV transilluminator, a picture was captured using a Hamamatsu camera and a video graphic printer UP: 895CE (SONY). The size and quantity of the DNA fragments was determined by comparing with the known mass and size fragments of a 1 kb standard DNA ladder (NEB).

2. 6 Enzymatic manipulation and purification of DNA

2. 6. 1 Digestion of DNA with restriction endonucleases

DNA digests were normally set up using 10 U of restriction endonucleases in a total volume of 30 µl for 3-4 hours. The amount of enzymes used for digests was less than 0.1 volume of the final reaction mixture to avoid star activity and inhibition of enzyme activity by glycerol. The restriction endonuclease buffer and incubation temperature was selected according to manufacturers instructions. In cases where reaction condition was not favourable for simultaneous digests, DNA was purified (section 2. 4. 5) after the first digest and the condition was set up for the next restriction endonuclease.

2. 6. 2 Dephosphorylation of linearised plasmid DNA

10x dephosphorylation buffer:

$ZnCl_2$ 10 mM, $MgCl_2$ 10 mM, Tris-HCl (pH 8.3) 100 mM

To suppress self-ligation of the linearised plasmid DNA, the 5'-phosphates was removed using calf intestinal alkaline phosphatase (CIP). Following complete digestion of plasmid DNA (5-10 µg) with a desired restriction endonuclease, to a 50 µl reaction mixture, 0.5 unit of CIP, 10 µl of 10x CIP dephosphorylation buffer and 40 µl sterile distilled water were added to give a final volume of 100 µl. The reaction was incubated at 37 °C for 30 min. The dephosphorylated DNA was purified by phenol/chloroform extraction and ethanol precipitation (section 2. 4. 5).

2. 6. 3 Ligation of DNA fragments

Plasmid vector and insert required for a recombinant plasmid were ligated in a 10 µl ligation reaction using 2 units (0.5 µl) of T4 ligase (GIBCO BRL), 2 µl of 5X T4 ligase buffer with the concentration of termini of the insert approximately 3

fold higher than the plasmid vector. Approximately 100-200 ng of the vector DNA of vector was used in ligations.

The ligations were incubated overnight at rt and a 3-5 µl aliquot of each ligation was used for bacterial transformation as described in sections 2. 3. 2 and 2. 3. 4.

2. 7 Amplification of DNA by polymerase chain reaction (PCR)

2. 7. 1 General PCR

Standard PCR was performed to amplify the desired DNA fragments. The PCR reaction mixture consisted of 5-10 ng of purified DNA template, 5mM of primers, 2mM of dNTPs, 1x *Taq* buffer and 1.0 U of *Taq* polymerase (BIOLINE) was prepared in a 0.2 ml microfuge tube to a final volume of 20 µl. The PCR reaction was mixed and spun briefly. The DNA fragment was amplified for 30 cycles using the following thermal conditions as general: Denaturing DNA template 94 °C for 1 min, Primer annealing 5 °C below primer Tm of primer for 1 min, DNA synthesis 72 °C for 1 min. The PCR product was analysed by agarose gel electrophoresis (section 2. 5).

2. 7. 2 PCR using HiFi Polymerase

PCR amplification of genomic fragments subsequently utilised for cloning procedures was performed with a high fidelity proof reading DNA polymerase. The KOD HiFI polymerase from Novagen was utilised and the reaction conditions were according to manufacturers instructions.

2. 7. 3 Colony PCR

A small portion of a single colony was inoculated into a 20 µl PCR reaction (section 2. 7. 1) using a sterile toothpick. Initially, the bacteria were incubated at 95 °C for 5 min to lyse the cells and then the standard PCR amplification was performed as described in section 2. 7.1.

2. 7. 4 TAIL PCR

TAIL-PCR reactions were performed according to (Liu *et al.* 1995) with minor modifications.

The 3 prime *Ds*-flanking sequences were amplified using three *GUS*-specific nested primers (Lalanne *et al.* 2004) combined with the AD1, AD2 or AD3

degenerate primers (Liu *et al.* 1995). The 5' *Ds*-flanking DNA junction was confirmed by direct PCR amplification using *Ds5-1* and gene-specific primers

2. 7. 5 Reverse transcription and PCR amplification of RNA (RT-PCR)

RT-PCR was performed using the ABgene R*everse*-i T^{TM} One Step system RT-PCR Kit (AB-0845) to detect RNA transcripts in tobacco leaf and pollen. The RNA template used for RT-PCR was isolated with TRIZOL reagent (GIBCO BRL) as described in section 2 4. 3.

The RT-PCR reaction mixture consisted of 2x ReddyMix^{TM} Master Mix, 25 µl; RNA template, 1 µl (1 µg/ml); primer 1 µl, (10 mM); reverse transcriptase blend, 1 µl; was prepared in a 0.5 ml microfuge tube and made up to 50 µl with DEPC treated water. The RT-PCR reaction was mixed and spun briefly. The RNA was reverse transcribed and PCR amplified using the following thermal conditions: first strand cDNA synthesis 47 °C for 30 min (1 cycle), reverse transcriptase inactivation and initial denaturation 94 °C for 2 min (1 cycle), denaturation 94 °C for 20 sec, annealing 55 °C for 30 sec, extension 72 °C for 5 min (40 cycles), final extension 72 °C for 5 min (1 cycle). The presence of target template was detected by analysing an aliquot (10-20 µl) of the RT-PCR product using agarose gel electrophoresis (section 2. 5).

Two step RT-PCR conditions using Im Prom II reverse transcription kit for cDNA synthesis (Promega) with oligo dT primers were performed according to the instruction manual for increased sensitivity. The cDNA produced was diluted forty times and 1 µl was used as a template for standard PCR (2. 7. 1.)

2. 8 DNA sequencing

The purified extension products were analysed using ABI PRISM sequencer (ABI 373) by PNACL at Leicester University. The sequencing data obtained as chromatograms were analysed using the Sequence Editor 1.0.3 (Applied Biosystems) and Gene Jockey II (Biosoft) softwares.

2.9 Cloning using the GATEWAY PLANT TRANSFORMATION VECTORS

The promoter and ORF of *SETH4*, *SFL1* and *SFL2* were amplified from genomic DNA with the primers described in section 2.20. The attb1 and attb2 sites were

introduced in the sequence to be amplified in a two step adapter PCR. The Adapter PCR protocol allows for shorter primers to amplify attB-PCR products by utilizing four primers instead of the usual two in a PCR reaction.

In the first step template-specific primers containing 12 bases of attB are used in 10 cycles of PCR to amplify the target gene. A portion of this PCR reaction is transferred to a second PCR reaction containing universal attB adapter primers to amplify the full-attB PCR product. The PCR conditions for the secondary reaction are:

	Denature	94°C for 15 seconds
5 cycles	Anneal	45°C for 30 seconds
	Extend	68°C for 1 minute/kb of target amplicon
	Denature	94°C for 15 seconds
20 cycles	Anneal	55°C for 30 seconds
	Extend	68°C for 1 minute/kb of target amplicon

Afterwards, the primary reaction in the GATEWAY conversion technology (Invitrogen, Gaithersburg, MD, USA) is to recombine the amplified product with the attb1 and attb2 sites into the pDONOR201 vector and was done according to the manufacturer's instructions (INVITROGEN). The secondary reaction is the transfer of the amplified product to the destination vectors of interest. The vectors pK7WGF2, pK7FWG2 and pKGWFS7 were used for the C-terminal fusion of the eGFP to the gene of interest, the N-terminal fusion of the eGFP to to the gene of interest and the fusion of the promoter of interest to the eGFP:GUS fusion protein. The recombination reactions were done according to the manufacturer's instructions (INVITROGEN).

2. 10 Transient expression assay by microprojectile bombardment

Tobacco pollen was bombarded as described by (Twell *et al.* 1989) and onion cells were bombarded as described in (see method) using a BIO-RAD PDS-100/He particle delivery system.

2. 10. 1 Preparation of plant tissues

Tobacco Pollen

Pollen germination medium (PGM) (Tupy *et al.*, 1991): Sucrose 0.3 M, H_3BO_3 1.6 mM, Ca $(NO_3)_2$ 3 mM, $MgSO_4$ 0.8 mM, KNO_3 1 mM, MES 25 mM. pH adjusted to 5.9 with 1 M KOH and autoclaved 15 min at 120 °C.

Mature pollen collected as described in section 2. 14 was resuspended in liquid pollen germination medium (Tupy *et al.*, 1991) to a final concentration of 50 µg/ml. A 47 mm Whatman membrane filter was prewetted and placed in a 5 cm round petri dish. A 0.1 ml aliquot of the pollen suspension was spread onto surface of the membrane, allowed to set and used quickly for microprojectile bombardment.

2. 10. 2 Onion skin epidermis bombardment

Onions acquired from the local market were cut in quarters and the onion skins were separated. The onion skins were cut in 2.5 cm squares and placed in a 9cm round petri dish with a prewetted filter paper to keep moisture levels high.

2. 10. 3 Preparation of 1.6µm Gold microprojectiles

The 1.6 µm Gold microprojectiles were added to ethanol to a final concentration of 60 mg/ml. The slurry was vortexed, washed twice with sterile distilled water and resuspended in 50% sterile glycerol to a final concentration of 60 mg/ml. Aliquots of the gold particle suspension (25 µl) were done and kept at 4 °C.

2. 10. 4 Preparation of macroprojectiles and bombardment of plant tissues

The gold microprojectile suspension aliquot (section 2. 11. 2) was vortexed for 1 min. 5 µg of plasmid DNA, 25 µl of 2.5 M $CaCl_2$ and 10 µl of 0.1 M spermidine free base were added separately, mixed by rapid pipetting and vortexing before adding the next solution. The mixture was vortexed for 3 min to allow for the precipitation of the plasmid DNA onto the gold microprojectiles. After precipitation the mixture was washed once in 200 µl of 70% (v/v) ethanol, once in 200 µl of absolute ethanol and resuspended in 80 µl of absolute ethanol. The particle suspension was vortexed for 1 minute and 10 µl were loaded on a macrocarrier and allowed to dry allowing for 4 shots per sample. The set-up of the gun was done according to the manufacturers instructions. The chamber was evacuated using a vacuum pump to a pressure of 25 inches of mercury. The rupture disk used was 1100 psi. The rupture of the rupture disk at 1100 to 1300 psi resulted in bombardment of plant tissue with the DNA coated microprojectiles.

After bombardment the vacuum was released and in the case of onion bombardments, the plate was sealed with Nescofilm and incubated under darkness for 24 hours at 25 °C. Then the epidermis layer was carefully removed, placed on a microscope slide and examined under an Axiophot 100 microscope (Carl Zeiss Inc.). In the case of tobacco pollen bombardment, the membrane was washed with 3 ml of PGM and removed from the petri dish, the petri dish was sealed with Nescoflim and incubated under constant illumination for 16 hours at at 25 °C.

2.11 Transformation of *Arabidopsis*

Infiltration medium: 2.165 g/l of 0.5x MS salts, 3.16 g/l of 1x Gamborg B5 vitamins, 0.5 g/l of 2-[N-Morpholino]ethanesulfonic acid (MES), 50 g/l of Sucrose and 10 µl/l ofBenzylaminopurine (1mg/ml)

A single transformed *Agrobacterium* colony was selected and inoculated 5 ml of LB containing the appropriate antibiotics and allowed to grow overnight at 28°C. A 4 ml aliquot of the overnight culture was diluted in 400 ml of fresh LB media with the appropriate antibiotics and the cells were grown in an orbital shaker for 24 hrs at 200 rpm at 28°C. The cells were centrifuged at 5000 rpm for 10 minutes and the pellet resuspended in standard infiltration medium. *Arabidopsis* plants to be transformed by floral dipping were grown under long day light regime. The primary bolt was clipped when the plants were approximately 2-5 cm tall. After a week, the siliques and fully open flowers were removed and the plants were used for transformation. Just before dipping, 300 µl of silwet L-77 were added per litre of culture. The above ground part of plant was dipped in *Agrobacterium* solution for one minute with gentle shaking. A plastic dome was kept on the dipped plants to keep the humidity high for one day. The plants were watered after two days.

2. 12 Plant samples and growth conditions

Tobacco pollen samples used for total RNA analysis and microprojectile bombardment were collected from greenhouse grown plants using a vacuum cleaner containing a 20 µm mesh (obtained from R. Cadisch and Sons, Arcadia Avenue, Finchley, London N32JZ) to retain pollen. Plant material were used directly or stored at –80 °C until required. *Arabidopsis* pollen was collected from greenhouse grown plants as described in (Honys and Twell, 2003) for RNA

analysis. For the *in vitro* germination assays, the anthers from five just opened flowers were collected from plants grown in greenhouse condition.

Arabidopsis thaliana ecotypes Landsberg *erecta* and Columbia were grown under greenhouse conditions with supplemented light allowing for 16 hours daylight at 22 °C.

2. 13 Arabidopsis pollen germination media

In vitro germination assays of *Arabidopsis thaliana* (ecotype Landsberg *erecta* and Columbia) pollen were performed according to the following:

AZAROV:

For 100ml of media use:

Media	Volume of 1% (w/v) stock
100 ml tap water	
7.81 mg $CaCl_2$	781 µl
12.72 mg $MgSO_4$	1272 µl
47.77 mg $Ca(NO_3)_2 + 4H_2O$	4777 µl
5.83 mg $K_2HPO_4 + 3H_2O$	583 µl
50 mg H_3BO_3	5000 µl
15 g sucrose	
3 g agar	

Dissolve media and apply a drop to a slide in inclined position to produce a thin layer. Apply pollen to the germination media and place slide pollen side down on bamboo sticks in a 15cm round petry dish with a moistened paper tissue and seal with millipore tape. Allow to grow for 10 –12 hours.

Pickert, M : 1% (w/v) agar in 20% (w/v) sucrose, 80 ppm boric acid, 10 ppm myoinositol

Place a few drops of medium onto a microscope slide. When set, tease apart anthers (just before dehiscence) on the agar surface.

Place the slide across plastic rods in the Petri dish. Put on the Petri dish lid.

Leave the dish for more than 6 hours in the light at 25 °C, 75% relative humidity

Fan et al., 2002 : The basic medium was: 5mM MES (pH5.8), 1mM KCl, 10mM $CaCl_2$, 0.8 mM $MgSO_4$, 1.5 mM boric acid, 1% Agar, 16.6% (w/v) sorbitol and 10 µg/ml Myo-inositol. The medium was prepared with distilled water, heated to 100°C for 2 min and 1.5 ml of the medium was applied in petri dishes forming a thin layer. The pollen from freshly dehisced anthers was applied onto the surface of the agar, and the dishes transferred to light chamber with 100% relative humidity (Fan *et al.* 2001).

Derksen et al,2002 : Pollen was collected by tapping open flowers on small pieces of dialysis membrane. The membranes were placed on semi-solid medium consisting of 0.01% H_3BO_3, 0.7% Bacto-Agar,0.07% $CaCl_2 \cdot 2H_2O$, 3.0% polyethylene glycol 4,000 and 20% sucrose. Pollen was germinated and grown for 6–24 h (overnight) in the dark at 27°C .

Stamens from five fully opened flowers were transferred into a well of a culture plate containing 100 µl of the liquid (no agar) pollen germination media (Derksen *et al.* 2002). The plate was sealed with Nescofilm and incubated at room temperature on a shaker (30 rpm) in the light.

2. 14 Callose staining

Fixative solution: 10% Acetic Acid Glacial, 30% Chloroform, 60% absolute Ethanol

Softening solution: 4 M NaOH

Aniline staining solution: 0.1M K_3PO_4 and 0.1% (w/v) Decolorised Aniline blue.

The tissue was placed in fixative solution for minimum of 2 hours. When the tissue was cleared, it was placed in softening solution for 5 minutes at 65 °C and carefully placed on a microscope slide mounted with the aniline staining solution. Callose deposition was examined under a Nikon Optiphot UV microscope.

2.15 4',6-diamidino-2 phenylindole (DAPI) staining

Pollen nuclei were visualised after staining with 4',6-diamidino-2 phenylindole (DAPI) as described by Park et al., 1998. Mature pollen were collected by placing 3-4 fully open flowers in a microfuge tube containing 300 µl of 10 µg/ml DAPI staining solution (0.1 M sodium phosphate, pH 7; 1mM EDTA, 0.1% Triton X-

100, 0.4 mg/ml DAPI). Pollen grains were released into the DAPI solution by brief vortexing and centrifuged briefly. Five µl of the pollen pellet were transferred to a microscope slide and viewed under epifluorescence microscope.

2. 16 FDA staining

Pollen viability stain. Viable pollen stains bright green (FDA), dead pollen stains red (EtBr).

FDA stain: 970 µl of 0.3 M Mannitol, 10 µl of 0.1 mg/ml fluorescein diacetate (F.D.A.) and 10 µl of 0.05 mg/ml ethidium bromide (EtBr)

A flower was placed in a 1.5 ml microcentrifuge tube and 50µl of the FDA stain were added to it. Using a gilson pipette, the middle of the flower was gently pressed down in the FDA stain and mixed carefully. The flower was removed and pollen was examined under Zeiss inverted microscope under UV, using low magnification.

2. 17 Alexander staining

Alexander stain: The stain was prepared by adding the following constituents in the order given below:

10ml of 95% alcohol; 10mg of malachite green; 50ml of Distilled water; 25ml of Glycerol; 5 g of phenol ; 5 g of Chloral hydrate; 5 mg of Acid fuchsin; 5 mg of Orange G; 4 ml of glacial acetic acid.

Pollen from each flower bud was liberated by squashing it directly in the fixation solution (ethanol:acetic acid, 3:1 v/v). After 30 min the liberated pollen was washed in 50 mM Tris buffer pH6.8. A fraction of the pollen grains was mixed directly on the microscope slide with Alexander stain (Alexander 1969). Dead pollen stained blue (cell wall staining) compared to living pollen which stained purple (cytoplasmic content staining).

2.18 Histochemical analysis of GUS expression

GUS extraction buffer: 0.05 M Sodium Phosphate at pH7.0, 0.01 M EDTA, 0.1% Triton X-100; 1 mM potassium ferricyanide, 1 mM potassium ferrocyanide

1 mM X-Glu.

To assay for GUS activity, *Arabidopsis thaliana* plants and *Arabidopsis* and tobacco pollen grains were immersed in a X-Gluc substrate solution for 2-4 hours at 37 °C. Assayed plants were cleared using ethanol to improve visibility of the blue GUS reaction product. Stained plant organs, pollen grains and pollen tubes were analysed by bright field transmitted light microscopy using an Axiophot 100 microscope (Carl Zeiss Inc.)

2. 19 Image processing

In order to visualise the localisation of GFP, pieces of bombarded pollen was removed from the Hybond N membrane (section 2. 11. 1), placed in 2-3 drop of liquid PGM (section 2. 11. 1) and spread over a microscope slide. Expression of GFP was detected using a Nikon Optiphot UV microscope with a 470-490 nm excitation filter and a 480 nm barrier filter. (Zeiss axiophot 100 microscope.) Images were captured as described in section 2. 21. Images were captured directly using a CCD camera (JVC KYF55B) and Imagegrabber software (Neotec).
The Openlab 3.1.5 software (Improvision, Coventry, UK) was used to measure pollen tube lengths from images captured with an Orca ER CCD camera (Hamamatsu Photonics, Japan).

2.20 Primers used

Primers used for identification of the site of *Ds* insertion.

General Primers

NAME	**TM (°C)**	**5-3'**
DS5-3	61.9	GGTCGGTACGGAATTCTCCC
GUS5-3	59.8	GATCCAGACTGAATGCCCAC
SETH4		
ORF5'	58	GTTTCTCATGGCGGATATTGTGA
1536DS5-3	55.8	AGCCCAAGCAACAACAGTTT
ORF3'	62	CATATATCCA TGGTACCCTC TTGACCC
SETH7		
1196seq3	56.8	CCAACAGTTAAAGAAAGT CTCGAA
1196GUS5-3	56.8	GCTATGATTATTGATCTGAGAGAG
1196rv	56.8	GCTATGATTATTGATCTGAGAGAG

Primers used for expression studies (RT-PCR)

SETH4		PRODUCT SIZE 762bp	
NAME		**TM (°C)**	**5-3'**
ORF5'	58		GTTTCTCATGGCGGATATTGTGA
1536DS5-3	55.8		AGCCCAAGCAACAACAGTTT

SFL1		PRODUCT SIZE 239bp	
NAME		**TM (°C)**	**5-3'**
SFL1FW	65		CGACTAGCAACGGTGTGAGCCAG
SFL1RV	60		CTTCTCAATCAGAACAGCGAAGC

SFL2		PRODUCT SIZE 298bp	
NAME		**TM (°C)**	**5-3'**
SFL2FW	64		CATCAATCGCGAAACGCGTCGG
SFL2 RV	66		CTTTGCACGCCGGTGAAGTGCG

At2g41930	PRODUCT SIZE 780bp	
NAME	**TM (°C)**	**5-3'**
SETH7FW	63	TCGTGCAATTGTGTGGGAAC
SETH7RV	63	CCTAACCATGGCCGCTTACC

Primers used the complementation construct

SETH4

NAME	**TM (°C)**	**5-3'**
1536fw	58	ACGCAAGAATGAAGGGACAG
1536rv	58	TCGCGGGTTTACTCTACGAT

SETH7

NAME	**TM (°C)**	**5-3'**
1196fw	60	GTCCGAACCAGATGAGAGGA
1196rv	56	GCTTTTGGTGGGTTTGTGAT

Primers used for GATEWAY constructs

General Primers:

attB1 adapter primer: GGGGACAAGTTTGTACAAAAAAGCAGGCT

attB2 adapter primer: GGGGACCACTTTGTACAAGAAAGCTGGGT

SETH4

NAME	5-3'
PROMOTER	
Prom6 Fw	AAAAAGCAGGCTACGCAAGAATGAAGGGACAG
Prom6 Rv	GAGTTTCTTG ATTGTTGTTT CTCACCCAGC TTTCT
C-TERMINAL eGFP	
Att1BC6.1	AAAAAGCAGGCTTCATGGCGGATATTGTGA AACAG
Att2BC6.1	GAGGGTCAAGAGGATTTCATTGAAACCCAGCTTTCT
N-TERMINAL eGFP	
Att1BN6.1	AAAAAGCAGGCTATGGCGGATATTGTGAAACAG
Att2BN6.1	GAGGGTCAAG AGGATTTCAT TACCCAGCTT TCT

SFL1

NAME	5-3'
PROMOTER	
Prom6.1 Fw	AAAAAGCAGGCTGGAGTGAG AATCCCATTG
Prom6.1 Rv	CCATTTGATC TGATATCGAC GACCCAGCTT TCT
C-TERMINAL eGFP	
Att1BC6.1	AAAAAGCAGG CTCGATGGCAGA CATCGTC
Att2BC6.1	CAAGGGGTTA CAATTTCAAT TGATACCCAG CTTTCT
N-TERMINAL eGFP	
Att1BN6.1	AAAAAGCAGG CTATGGCAGA CATCGTC
Att2BN6.1	CAAGGGGTTA CAATTTCAAT TACCCAGCTT TCT

SFL2

NAME	5-3'
PROMOTER	
Prom6.2 Fw	AAAAAGCAGGCTCCTATTATTTTGCTTGCATG TGTGT
Prom6.2 Rv	CGTAACCAGT TTCGTTATCC ACCCAGCTTT CT
C-TERMINAL eGFP	
Att1BC6.2	AAAAAGCAGG CTCCATGGGT GATCTAGCC
Att2BC6.2	GGCTAGATCA CCCATGGAGC CTGCTTTTT
N-TERMINAL eGFP	
Att1BN6.2	AAAAAGCAGG CTATGGGTGA TCTAGCCAAGC

Att2BN6.2 GGATCTAGAG GCTTTCACTT GTACCCAGCT TTCT

2.21 Bioinformatic analyses

InterPro (Mulder N.J. 2003)

Also used the INTERPRO domain search program at the MATDB website (http://mips.gsf.de/ proj/thal/db/search/search_frame.html) and InterProScan:. (Zdobnov 2001) InterPro is a tool that combines different protein signature recognition methods native to the InterPro member databases into one resource with look up of corresponding InterPro and GO annotation.

META-PP (Volker and Rost, 2003)

META-PP provides access to a selected set of high-quality servers in the areas of comparative modelling, threading/fold recognition, secondary structure prediction and more specialized fields like function prediction, homology modelling, secondary structure, threading and transmembrane helices.

PROSITE (Falquet L. 2002)

PROSITE is a database of protein families and domains. It consists of biologically significant sites, patterns and profiles that help to reliably identify to which known protein family a new sequence belongs.

PFAM

PFAM is a large collection of multiple sequence alignments and hidden Markov models covering many common protein families.

PRINTS (Attwood T.K. 2003).

Utilizes conserved motifs used to characterise protein families

Protein Domain Database (ProDom) (Corpet F. 2000)

ProDom is a set of protein domain families automatically generated from the SWISS-PROT and TrEMBL sequence databases

Simple modular architechture research tool (SMART) (Letunic I. 2002)

SMART is a web tool for the identification and annotation of protein domains, and provides a platform for the comparative study of complex domain architectures in genes and proteins. SMART allows the identification and annotation of genetically mobile domains and the analysis of domain architectures. The January 2004 release of SMART contains 685 protein domains families found in signalling, extracellular and chromatin-associated proteins. These domains are extensively annotated with respect to functional class, tertiary structures and functionally important residues. SMART now uses predicted proteins from complete genomes in its source sequence databases, and integrates these with predictions of orthology.

TIGRFAMs (Haft D.H. 2003)

Supports searches of protein sequence against a database of hidden Markov models (HMMs) based upon protein families.

PIR SuperFamily (Wu 2003).

The protein family classification system at the Protein Information Resource

SUPERFAMILY (Gough 2001)

The SUPERFAMILY database provides structural assignments to protein sequences. The database is based on a library of profile Hidden Markov Models that represent all proteins of known structure that was based on the SCOP classification of proteins.

Chapter 3
Identification of gametophytic genes affecting the progamic phase, via a transposon insertional mutagenesis approach.

3.1. Introduction

During the progamic phase, pollen is being recognised by the female tissues and allowed to form a pollen tube, a tip-only elongating structure which contains the sperm cells and its function is to transverse the female organs and deliver the sperm cells to the female gametophyte for the fertilisation to take place. In *Arabidopsis* it has been estimated that at least 5,000 genes are expressed in the mature pollen grain (Becker *et al.* 2003; Da Costa-Nunes and Grossniklaus 2003; Honys and Twell 2003). At this stage of development, the male gametophyte stores transcripts in preparation for the explosive growth that is to follow during the progamic phase. Identification of genes acting upon the progamic phase has been the aim of many laboratories for many years. Despite the growing success in the past decade in isolating gametophytically controlled genes that play a role during the progamic phase (Schiefelbein *et al.* 1993; Howden *et al.* 1998a; Grini *et al.* 1999; Procissi *et al.* 2001; Palanivelu *et al.* 2003; Procissi *et al.* 2003; Lalanne *et al.* 2004), progress has been slow. After the elucidation of the *Arabidopsis* genome (Arabidopsis Genome Initiative, 2000), reverse genetic approaches have been used for the identification of genes acting upon developmental phases or on specific tissues. Identification of important gametophytic progamic phase genes by these strategies, however, is not as easy. The diversity of cellular mechanisms underlying the post-pollination events hampers the identification of the function for the gene of interest. This situation is exacerbated by the inability to create a homozygous line for the gametophytic mutation of interest which leads to difficulties in identifying the mutated gametophyte, a problem aggravated by the inaccessibility of both the male and female gametophytic cells during the progamic phase (Wilhelmi and Preuss 1999).

A forward genetics approach allows the identification of genes playing an important role during the progamic phase by showing the phenotypic effect of a loss of function mutation by an insertion event in their open reading frame as loss of transmission of a selectable marker (Feldmann *et al.* 1997; Bonhomme *et al.* 1998; Howden *et al.* 1998a). Analysis of the gametophytic or sporophytic control of the gene can be identified by simple genetic tests. Insertional mutagenesis in *Arabidopsis* can be conveniently accomplished either by *Agrobacterium* mediated

T-DNA transformation, or by transposable element mobilisation. T-DNA Insertional mutagenesis has been extensively used in *Arabidopsis* and mutants affecting the gametophyte have been identified from such screens (McKinney *et al.* 1995; Park *et al.* 1996; Feldmann *et al.* 1997; Bonhomme *et al.* 1998; Howden *et al.* 1998a; Procissi *et al.* 2001; Mouline *et al.* 2002; Steinebrunner *et al.* 2003). T-DNA insertional mutagenesis is ideal for genome-wide saturating mutagenesis. For *Arabidopsis* the production of more than 250,000 independent insertion lines would be sufficient to cover all the genes and it has already been attained (Krysan *et al.* 1999; Pan *et al.* 2003). T-DNA though tends to be integrated in multiple arrays and create complex integration patterns and chromosomal rearrangements near the insertion making molecular analysis difficult. It was also thought that T-DNA integration in the genome would create transgene silencing phenomena but this theory was disputed recently (Stam *et al.* 1998; Lechtenberg *et al.* 2003).
The use of single stable transposon insertions as the tags could overcome this problem. Heterologous transposons from maize have been extensively used for transposon mutagenesis in Arabidopsis. This is because the genetic properties of the transposons are well understood and the timing, range and frequency of transposition of the maize transposons in *Arabidopsis* has been well characterised (Bancroft and Dean 1993b; Cardon, *et al.* 1993; Sundaresan 1995). Transposons typically integrate into the genome as simple, single-copy insertions. The transposon insertions have a greater bias for insertion near transcriptionally active sites. Transposed elements can be remobilised resulting in reversion and short-range transposition, allowing for verification of the linkage of the phenotype to the disrupted gene.

The maize *Enhancer-I/Suppressor mutator* (*En/Spm*) element is a small, genetically complex transposon (Cardon *et al.* 1993). It encodes a single transcript that gives rise to several mature transcripts by alternative splicing. Two *En/Spm*-encoded proteins, TnpA (68 kDa) and TnpD (131 kDa), participate in transposition (Frey 1990). TnpA binds to the sub-terminal repetitive regions of the *En/Spm* element, which contain multiple copies of sequence variants of the consensus TnpA binding site CCGACACTCTTA (Frey 1990; Cardon *et al.* 1993). The *En/Spm* element has the ability to transpose to unlinked locations

rendering selection for segregated transpositions efficient and the *En/Spm* transposase gene is transcribed and alternatively spliced in *Arabidopsis* resulting in high levels of transposition during development (Aarts *et al.* 1995). The independent transposition frequency (ITF) of the *dSpm* (the rate of unique insertion events) in *Arabidopsis* ranged from 7.8% to 29.2% (Aarts *et al.* 1995). The *En/Spm* transposon element has been successfully used as an insertional mutagen in *Arabidopsis* (Aarts *et al.* 1995; Wisman *et al.* 1998; Speulman *et al.* 1999; Tissier *et al.* 1999; Marsch-Martinez *et al.* 2002). Unfortunately, the transposition frequency of the *En/Spm* elements is affected by *cis-* and *trans-* acting mutations so changes affecting the sequence, the amount or the structure of proteins that are encoded by the *En/Spm* can affect the developmental timing and frequency of transposition (Frey 1990). Also, *En/Spm* elements are subject to various epigenetic phenomena associated with DNA methylation and in common with most other transposons in eukaryotic organisms, *En/Spm* have a tendency to transpose to closely linked loci. The *En/Spm* element has been used for the creation of insertion populations in *Arabidopsis* (Aarts *et al.* 1995; Tissier *et al.* 1999; Marsch-Martinez *et al.* 2002).

Another heterologous transposon from maize has been extensively used in Arabidopsis. The *Activator/ Dissociation* (*Ac/Ds)* elements were the first to be described in *Arabidopsis*, are easier to control and more useful for the single-copy insertion populations that were created (Bancroft *et al.* 1992; Grevelding *et al.* 1992; Bancroft and Dean 1993b; Sundaresan 1995; Long *et al.* 1997; Walbot 2000). The *Ac/Ds* elements are also less sensitive to epigenetic changes making them more suitable for studying gene regulation. The *Ac* transposase though, in contrast to the *En/Spm* transposase, has low transposition efficiencies since it has to be modified to relieve transcriptional and post-transcriptional constraints on expression in *Arabidopsis* (Grevelding *et al.* 1992).

3.1.2 The Sainsbury Laboratory Arabidopsis Transposants (SLAT) lines collection.

To accomplish the aim of producing stable independent insertions spanning the whole *Arabidopsis* genome, Tissier *et al.* (1999) used the *En/Spm* element as an insertional mutator. Soil-applied selectable markers were used for the positive and

negative selection. The markers allowed for the selection for stable transposition in soil in the greenhouse, facilitating the creation and identification of more than 70,000 plants containing stable single insertions. Both markers were carried on a single construct that also bears the non-autonomous defective element (*dSpm*) and the transposase genes.

The *dSpm* element carries the phosphinothricin (PPT) resistance gene (*BAR*) used to select for T-DNA integration and for transposon reinsertion. The *BAR* gene from *S. hygroscopicus* is a phosphinothricin acetyltransferase (Thompson 1987) which provides resistance to PPT (Lutz *et al.* 2001). PPT, an analogue of glutamate, is an irreversible inhibitor of the enzyme glutamine synthetase. Glutamine synthetase catalyses the ATP-dependent condensation of ammonium and glutamate to form glutamine, ADP, and free phosphate. Inhibition of the enzyme leads to the built up of toxic levels of ammonium in the plants. Phosphinothricin acetyltransferase converts PPT into a non-herbicidal acetylated form by transferring the acetyl group from acetyl CoA to the free amino group of PPT, thus conferring PPT resistance on transgenic tissues and regenerated plants (D'Halluin 1992).

The *dSpm* was placed between the 35S promoter and the ATG codon of a ß-glucuronidase (*GUS*) gene (Jefferson *et al.* 1987). Excision of the *dSpm* would lead to completely blue seedlings when stained for *GUS* activity. In the T-DNA carrying the *dSpm*, the transposase genes *TnpA* and *TnpD* were cloned under the control of a 35S promoter, the *Spm* promoter, or a meiosis-specific promoter (Tissier *et al.* 1999) in an efford to get the highest excision frequencies by one of the different constructs.

The negatively selectable marker *SU1*, a bacterial cytochrome P450, was placed in the T-DNA allowing selection against the transposase source. The *SU1* gene from *Streptomyces griseolus* catalyses the activation of the pro-herbicide R7402 (DuPont, Wilmington, DE) to its active form, sulfonylurea, inhibiting or reducing the growth of plants that contain it (O'Keefe and Tepperman 1994). Selecting for the transposon and against the T-DNA would allow for the identification of the T2

progeny that carry an independently transposed and stable element. Because loss of the T-DNA depends on segregation from the transposed element following meiosis, unlinked elements would be preferentially selected. Furthermore, loss of the T-DNA would ensure that the *dSpm* element is stably inserted. Of course, doubly resistant plants would either carry the transposon but not the T-DNA because the transposon has moved away or would have the *dSpm* element inserted in the *SU1* gene, disrupting its function (Figure 3.1) or not excised at all. In the SLAT lines, the frequency of doubly resistant plants in the T2 generation ranged from 5×10^{-5} to 2×10^{-3}. After selection by GUS staining against the unexcised dSpm elements the final number of independent insertions was calculated ranging from 0.006 to 0.037% (Tissier *et al.* 1999).

The lines with relatively low frequencies of double resistance gave rise to more independent events, whereas lines with a high frequency of doubly resistant plants produced few independent insertions, a phenomenon witnessed in other transposon insertion populations (Tissier *et al.* 1999; Marsch-Martinez *et al.* 2002). The use of the *dSpm* element as an insertional mutagen, the soil-applied selection for the transposon and the number of independent lined provided by the Sainsbury laboratory, made the SLAT lines collection ideal for the identification of gametophytic genes. The SLAT lines collection was provided to the scientific community via Nottingham *Arabidopsis* Stock Centre (NASC). The collection was organised as such. Seeds from plants were harvested in pools of 50 individuals. Approximately 1200 pools were generated and divided up as follows: 48 pools of 50 were assigned to a super pool; a total of 25 super pools were assembled (Tissier *et al.* 1999).

3.1.3 The Cold Spring Harbour (CSH) collection

The CSH collection was created for enhancer and gene trap transposon mutagenesis (Sundaresan *et al.* 1995). Enhancer trap reporter constructs have a minimal promoter in front of the reporter gene so there is expression only when the element is inserted near *cis*-acting chromosomal enhancers (Aarts *et al.* 1995). Gene trap reporter constructs (promoter traps) have no promoter, so expression of the reporter gene can occur only when the element inserts within a transcribed chromosomal gene, creating a transcriptional fusion.

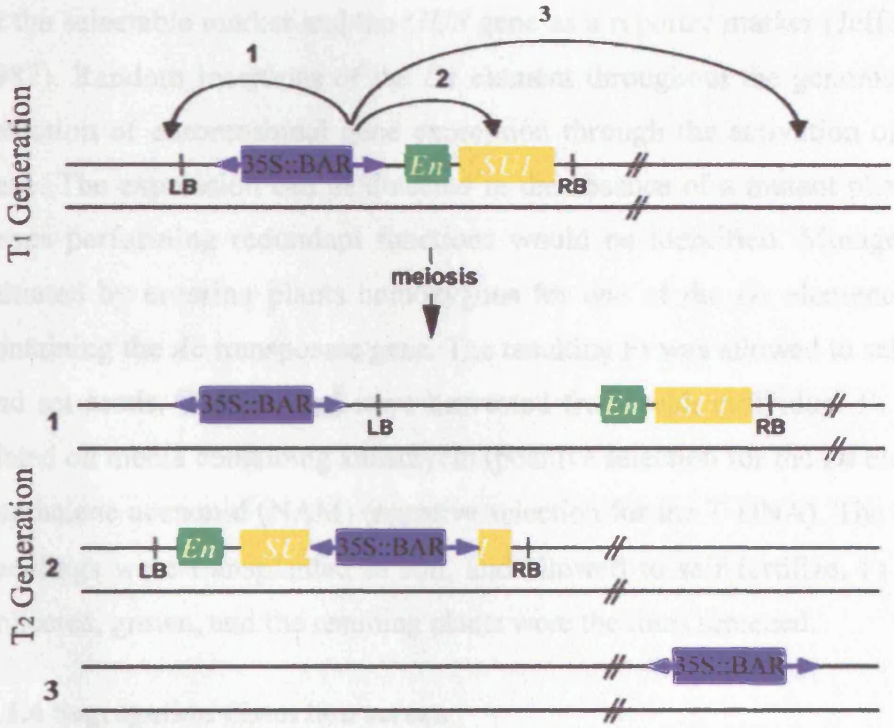


Figure 3.1. Selection for unlinked transposition of the *dSpm* element in the SLAT collection.

The T1 generation of plants transformed with the T-DNA carrying the *dSpm* cassette was allowed to self. By segregation during meiosis and selection for the transposition event, the *dSpm* element is expected to transpose out of its original location either outside the T-DNA (1), inside the T-DNA in a different location disrupting genes in the T-DNA (2) or in an unlinked to the T-DNA location (3). By selecting for the *dSpm* the PPT resistant plants and against the T-DNA by selecting plants resistant to the pro-herbicide, it is possible to eliminate the linked transposition events. This strategy does not allow the selection against scenario (2).

The two-element *Ac/Ds* system from maize (Sundaresan *et al.* 1995) was transformed into the Landsberg *erecta* (Ler) ecotype. The *Ds* elements carry the neomycin phosphotransferase (NPTII) gene (conferring resistance to kanamycin) as the selectable marker and the *GUS* gene as a reporter marker (Jefferson *et al.* 1987). Random insertions of the *Ds* element throughout the genome allow for detection of chromosomal gene expression through the activation of the *GUS* gene. The expression can be detected in the absence of a mutant phenotype, so genes performing redundant functions would be identified. Mutagenesis was initiated by crossing plants homozygous for one of the *Ds* elements to plants containing the *Ac* transposase gene. The resulting F_1 was allowed to self-pollinate and set seeds. The F_2 seed were harvested from each individual F_1 plant, and plated on media containing kanamycin (positive selection for the *Ds* element) and napthalene acetamid (NAM) (negative selection for the T-DNA). The Nam^r Kan^r seedlings were transplanted to soil, and allowed to self-fertilize. F_3 seed were collected, grown, and the resulting plants were the lines screened.

3.1.4 Segregation distortion screen

The selection for gametophytic mutations was based on the distortion from the Mendelian segregation of a trait, which in this case was due to the selection marker. In mutations not affecting the gametophytic genes, the expected ratio of resistant to sensitive plants is three to one. In the case of a transposon insertion into a gametophytic gene (either male or female) that causes a null mutation, however, the segregation ratio observed after a self-fertilisation would be one resistant to one sensitive plant (Feldmann *et al.* 1997; Bonhomme *et al.* 1998; Howden *et al.* 1998).

3.2 Results

3.2.1 SLAT lines Collection

Based on the segregation distortion screening strategy, 300 pools of 50 plants, as provided by the NASC, were analysed for the SLAT lines collection. From each individual pool, 20 plants resistant to PPT were selected and allowed to set seed, in an effort to select only for independent mutations. Due to the fact that not all plants managed to reach maturity and set seed it was only possible to collect seeds

from 4094 individual plants. Seeds from individual plants were screened for distortion of the Mendelian segregation of the PPT resistance to sensitivity ratio. Seeds (50 to 100 per line) were sown on soil, sub-irrigated with 50mg/l glufosinate (PPT analogue) and allowed to germinate under greenhouse conditions. After two weeks the segregation ratio of the lines was scored (Figure 3.2). The PPT selection was varied in several individual lines. Besides the resistant and sensitive to PPT classes, there was a class of seedlings exhibiting reduced resistance to the PPT (Figure 3.2 D). These lines exhibited the reduced resistant to PPT phenotype even when grown in decreased herbicide concentrations.

In addition to the search for the lines exhibiting a segregation distortion phenotype, a limited genetic characterisation of the SLAT collection was performed. The segregation ratios of single seed descent lines from 26 pools were gathered. From the 26 pools, 976 individual lines were derived. From this sub-section of the SLAT population screened it was derived that almost 60 % of the lines were homozygous for the *dSpm* element. The percentage of the population carrying one or more *dSpm* elements that did not affect the gametophytes was almost 30 % of the total and 2.5 % of lines was sensitive to PPT and classed as an escape (Figure 3.3). The single seed descent lines were classified according to their segregation ratio and plotted according to the pool they derived from (Figure 3.4). In some of the pools there was a misrepresentation of segregation ratio classes.

From the 4094 individual lines screened, 35 lines were identified exhibiting segregation ratios ranging from 0.5:1 to 2:1. These lines, representing the 0.9 % of the population screened, were classed as putative (gametophytic) mutants and were further analysed. From each of the putative mutants, six F3 siblings were allowed to set seed for further analysis of the transmission of the segregation distortion ratio. Over 90 % of the isolated putative mutants showed no consistent inheritance of the segregation distortion trait in the F3 generation (Table 3.1).

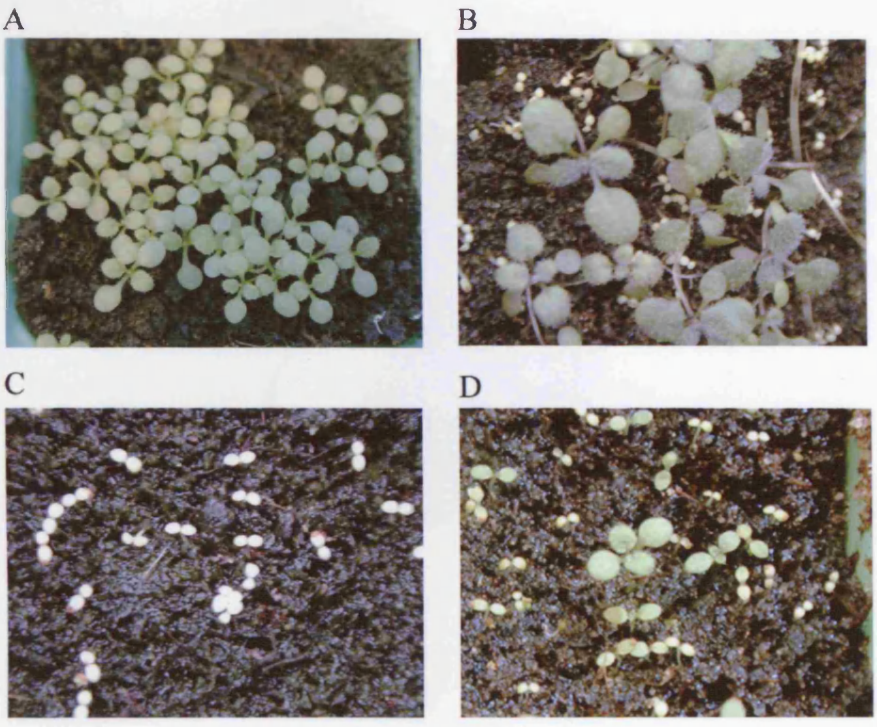


Figure 3.2 Segregation phenotype of the SLAT lines.
Selection for the *dSpm* was done by sub-irrigating with FINAL herbicide seedlings germinated and grown in soil under greenhouse grown conditions . After two weeks the plants were visually screened for resistance or sensitivity to the herbicide. Besides the resistant (A), segregating (B) and sensitive (C) lines, a number of individuals had varied resistance to the herbicide (D). These lines segregated to the resistant and sensitive classes but also a proportion of their population was partially resistant to FINAL, not bleaching but growing at a lower rate than the fully resistant individuals.

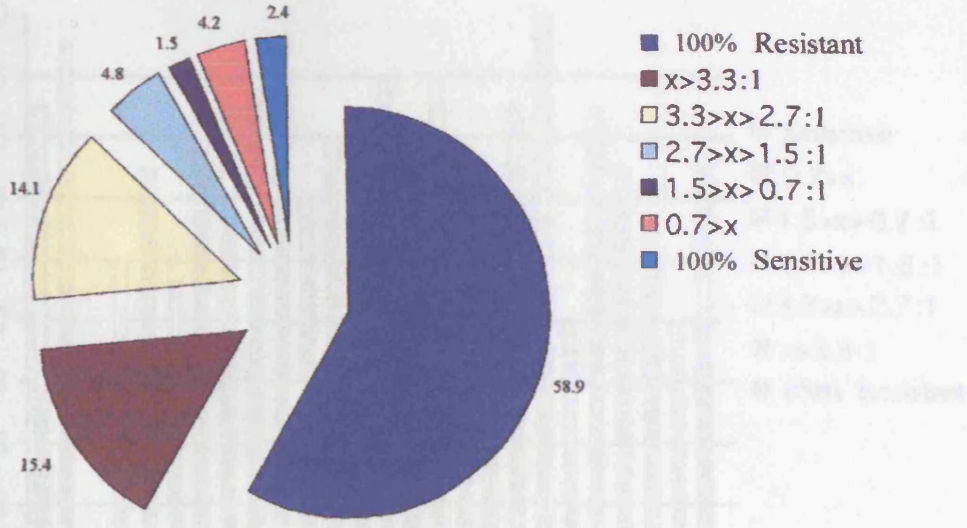


Figure 3.3. Summary of the segregation ratio classes for 976 SLAT lines.
The segregation ratios were categorised in seven classes: All resistant progeny; progeny segregation ratio more than 3.3 to 1; segregation ratio between 3.3 and 2.7 to 1; segregation ratio between 2.7 and 1.5 to 1; segregation ratio between 1.5 and 0.7 to 1, the class that should contain the mutants of interest; segregation ratio less than 0.7 to 1; all sensitive progeny.
x is the resistance to sensitive segregation ratio scored for a line when applying FINAL herbicide.

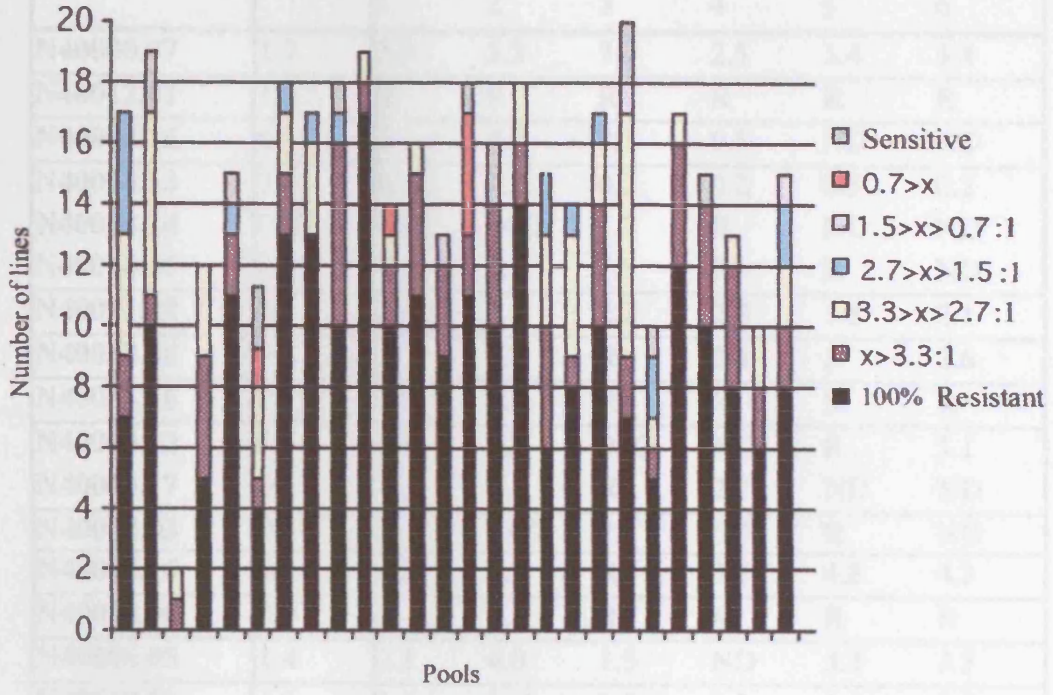


Figure 3.4. Segregation ratio distribution of independent lines within their respective seed pools.
The 976 lines were organised in the pools they derived from and categorised into 7 classes according to their segregation ratio (as in Figure 3.3). In the 26 pools the 976 lines were derived from, the representation of all the classes varied with most of the pools having the all resistant and segregating more than three to one the dominant classes.
x is the resistance to sensitive segregation ratio scored for a line when applying FINAL herbicide.

NASC code	F_2 (R/S)	F_3 (R/S)					
		1	2	3	4	5	6
N40000.07	1.7	3.5	5.2	3.7	2.5	3.4	3.4
N40012.01	1.4	R	R	R	R	R	R
N40013.16	0.5	5.3	4.6	7.1	9.5	ND	ND
N40014.13	1.0	0.5	0.2	0.2	0.2	0.3	0.2
N40014.14	0.02	ND	24.0	1.7	R	ND	ND
N40014.16	0.7	R	R	1.1	R	R	ND
N40021.08	0.1	2.6	4.2	2.2	2.8	1.8	2.1
N40032.08	0.2	R	1.8	R	2.3	R	2.6
N40056.16	0.2	ND	R	R	R	R	R
N40063.12	0.4	8.3	9.5	0.02	ND	R	3.1
N40063.17	0.02	R	2.2	R	2.7	ND	ND
N40064.03	0.2	R	11.0	7.3	6.0	R	ND
N40065.16	0.1	8.6	6.3	R	3.4	4.8	4.3
N40068.06	0.2	R	8.8	R	R	R	R
N40096.08	1.4	2.1	4.0	1.5	ND	3.2	3.3
N40118.03	1.3	1.6	3.3	3.8	1.2	1.4	1.0
N40123.18	0.4	3.3	5	1.4	2.1	2.9	4.4
N40146.19	0.6	0.4	4.3	6.9	11.6	1.5	1.3
N40146.09	0.1	2.2	3.4	1.7	1.4	2.6	2.6
N40153.02	0.3	2.7	2.4	2.7	1.6	2.3	2.8
N40161.14	0.4	5.5	5.8	R	R	R	R
N40181.15	0.1	2.8	2.6	3.0	3.0	3.6	ND
N40180.10	0.9	R	4.8	3.0	5.4	R	R

Table 3.1: The segregation ratio of putant gametophytic lines.
Analysis of the segregation ratios of 23 of the putants in the F_2 and the F_3 generations. The resistance (R) to sensitive (S) segregation when selecting for PPT was scored for the F_2 generation and subsequently, six PPT resistant individuals were allowed to self fertilise and set seed from each line. The resistance (R) to sensitive (S) segregation ratio when selecting for PPT was scored for these F_3 generation lines. Minimum number of seedlings scored was 150. ND is not determined.

The remaining lines together with some F_3 individual lines still exhibiting a segregation distortion ratio were allowed to self fertilise and were used as pollen donor and acceptors for reciprocal test crosses. No consistent inheritance of the segregation distortion trait in F_4 generation was seen and none of the putants exhibited reduced male transmission of the selection marker, therefore, further genetic characterisation of these lines was discontinued.

3.2.2 Cold Spring Harbour (CSH) Collection

3, 359 *Ds* transposon insertion lines were screened for segregation distortion of the kanamycin resistance marker by Lalanne, E., Patel, R., and Twell, D. Of the gametophytic putative mutants identified, as exhibiting segregation distortion ratio, two lines *seth4* and *seth7* ("*seth*" lines, named after the brother and murderer of the Egyptian fertility god Osiris,) from the gene-trap CSH collection of insertional mutants were offered to me for further analysis.

3.2.2.1 Genetic data

From the initial screen performed by Lalanne and co-workers, *seth4* and *seth7* were identified as fully penetrant male-specific gametophytic mutants showing a 1:1 ratio of resistant to sensitive seedlings. Genetic transmission through the male and female gametes was determined by reciprocal test crosses in which heterozygous mutants were crossed to wild type and the transmission of the resistance marker scored. Only a small reduction in the transmission through the female was observed for *seth7* (76%) whereas no or almost no transmission was observed through the male (transmission efficiency from 0.8% to 0%), indicating that these mutations act specifically on the success of the male gametophyte (Table 3.2). The genetic transmission of the *Ds* element through the male gametophyte was not possible even in non competitive pollinations where the amount of pollen grains placed on the stigma surface (20 to 30) would allow for all the pollen tubes emerging to fertilise the female gametophyte.

Co-segregation data for the *SETH* mutants.

Sixty *seth4* and *seth7* F4 siblings were allowed to set seed. The seed set from each one was plated on kanamycin plates and the resistance to sensitive ratio was

determined. None of the individual seed sets showed a resistance to sensitivity ratio of 3:1(results presented in figure 4.11 panel C and 4.13 panel B).

Line	Selfed Kan^R:Kan^S	Segregation Ratio R/S	Ler > female		Male> Ler	
			Kan^R:Kan^S	TE_{female}	Kan^R:Kan^S	TE_{male}
seth7	516/601	0.85	242/317	76.3 %	4/543	0.8 %
seth4	2580/2817	0.92	365/350	100 %	0/194	0 %

Table 3.2: The transmission efficiency (TE) through each gamete (TE_{male} and TE_{female}) describes the fraction of gametes successfully transmitting the transposon to the progeny relative to the number of gametes expected to carry the transposon. The ratio was calculated by scoring for the kanamycin resistant (KanR) and kanamycin sensitive (KanS) seedlings. The calculations were as described in Howden *et al.* (1998).

3.2.2.2 Phenotype of the *SETH* mature pollen grains.

Mature pollen from the *seth* mutants, when morphologically analysed appeared tricellular and similar to the wild type. More than 96 % of the pollen grains were viable and Alexander staining did not reveal cytoplasmic defects (Figure 3.5). Moreover, no increased levels of seed or ovule abortion were observed in either of the *seth* mutants. Their reduced male transmission and normal pollen morphology defines these as male gametophytic progamic phase mutations that act upon pollen germination, tube growth or guidance.

3.3 Discussion

3.3.1 SLAT lines collection.

The *Arabidopsis* genome sequence revealed about 26,000 genes that have been predicted by a variety of bioinformatics tools (The *Arabidopsis* Genome Initiative, 2000). However, the exact biological role for most genes is still unknown, requiring specific biological experiments to uncover their function. Reverse genetic screens would be a potentially rewarding strategy utilizing this current knowledge but about two-thirds of the genome is duplicated in the form of large chromosomal segments and about 4,000 genes are tandemly repeated as two or more copies (Blanc *et al.* 2000).

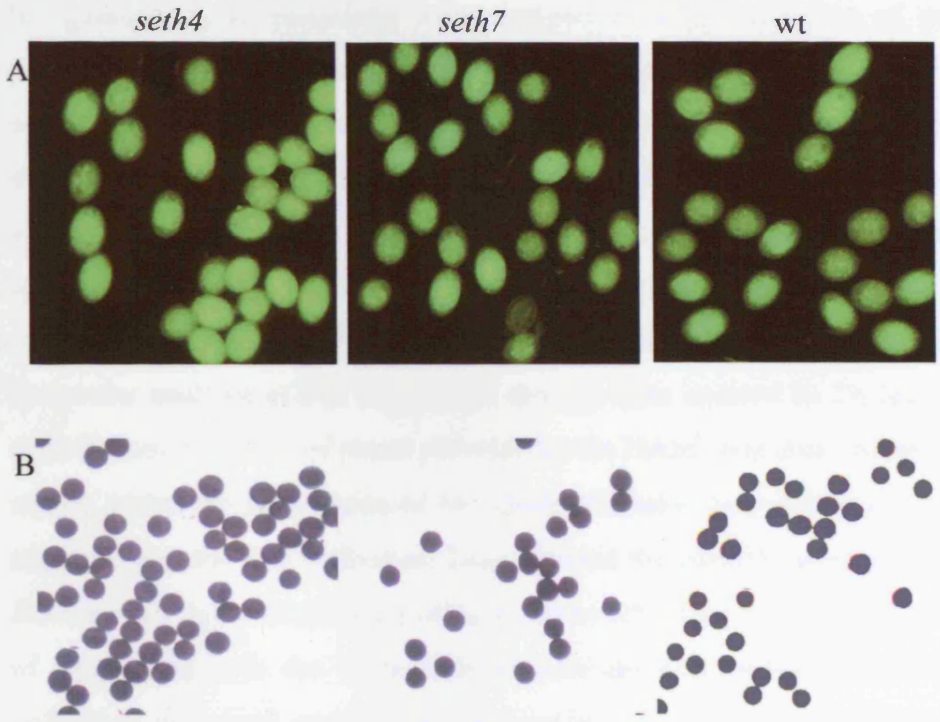


Figure 3.5. Morphological analysis of pollen from the CSH collection. Mature pollen from the *seth4* and *seth7* plants was analysed by Fluorescein Diacetate, a cell viability stain, (A) and Alexander, a cytoplasmic density stain, (B) for morphological defects but appeared identical to pollen from the wild type plants.

By doing a forward screen it is possible to identify genes that are essential for the progamic phase as fully penetrant mutations.

No gametophytic mutations were derived from the analysis of the SLAT population. According to frequencies of gametophytic mutations in T-DNA populations (Feldmann *et al.* 1997; Bonhomme *et al.* 1998; Howden *et al.* 1998a) the expected percentage of gametophytic mutations identified would be approximately 1%. In the population size screened it would be expected to have approximately 40 insertions in gametophytic genes. In order to understand the under-representation of gametophytic mutations in the SLAT collection, molecular analysis of 135 single seed descent lines isolated by Dr Igor Vizir and myself from the pools of plants provided by the NASC was done in the John Innes centre, under the supervision of Dr Jonathan Clarke. Sequencing of the flanking regions from the 135 individual lines allowed the identification of independent lines as well as the elucidation of the excision efficiency of the *dSpm.* Correlation of these data with the large-scale genetic analysis performed on the SLAT collection explained some of the reasons for the observed misrepresentation of gametophytic mutations in the SLAT collection.

Molecular analysis showed that there is a 51% level of unexcised *dSpm* transposons from the T-DNA start site. This shows that the negative selection applied was leaky and the active transposase could still excise the *dSpm* element creating unstable insertions as well as creating a high copy number of *dSpm* elements in the genome, a fact that explains the 60% of resistant to PPT lines in the SLAT collection and the further 33% of lines harbouring one or more *dSpm* elemants. The frequencies of excision and independent transposition events for *En/Spm* element were reported as high in *Arabidopsis* (Aarts *et al.* 1995). However, Tissier et al. (1999), reported germinal excision frequencies in the 10^{-2} of the population and unlinked transposition in the range of 2.5 to 10 x 10^{-4}. One of the theories formed to explain such a result was that the presence of the *BAR* gene and the bacterial spectinomycin resistance gene on the d*Spm* interfered with the transposition events. Assuming that all excision events give rise to insertions, the ratio between germinal excision and unlinked transposition frequencies (1:20

to 1:50) suggests that 8 to 20% of transpositions were to unlinked loci (Tissier *et al.* 1999). In the 4049 lines screened, eight lines would be expected to be having insertions in gametophytic genes if no further skewing of the gametophytic line representation took place. The level of redundancy in the SLAT lines isolated, according to the molecular data provided by the John Innes Centre, was approximately 20% (eight insertions out of 39 having one or more siblings). The way the independent lines were produced from the pools though (i.e. collecting only 20 siblings from approximately 100 grown) potentially artificially increased the level of non-redundant lines in the single seed descent lines collected.

The severely reduced level of redundancy witnessed indicates that some insertions are overepresented in the NASC SLAT pools. Preliminary tests on the JIC SLAT collection revealed a low level of redundancy (16 to 20%) (Tissier *et al.* 1999). The increase in the level of redundancy from the initial SLAT collection when compared to the NASC collection can only mean that there is a misrepresentation in the number of lines existing per pool in the current NASC collection and the data on the distribution of the segregating individual lines per pool in section 3.2.1 corroborates such an assumption. In fact, the increase in the redundancy level shows that during the bulking-up of the SLAT collection for distribution purposes, the number of individual independent insertions in the pools decreased. Since the SLAT collection is propagated in pools, a reduced number of plants grown per pool or a reduced amount of seeds collected per pool could limit the number of independent individuals per pool selected. Lines carrying deleterious insertions in gametophytic genes are expected to be even more affected by such an event. In lines affecting the gametophytes, only 50% of the seed set would be PPT resistant. Compared to the lines homozygous for the *dSpm* where all of the seed set would be PPT resistant and the lines carrying one or more insertions not affecting the gametophytes where 75% or more of the seed set would be PPT resistant. Based on this, in three generations, the number of gametophytic mutations in the population would have been reduced by a sevenfold. Finally since the d*Spm* elements are subject to various epigenetic phenomena associated with DNA methylation, it would be possible that some of the segregation

distortion ratios observed were due to epigenetic events that reverted in the next generations.

3.3.2 *SETH4* and *SETH7* are progamic phase mutants.

Since no gametophytic mutants were identified in the SLAT collection, it was decided to continue with the analysis of the gametophytic genes affecting the progamic phase identified in the CSH collection. The definition of *SETH7* and *SETH4* as progamic phase mutants was based on ratio of 1:1 resistant to sensitive seedlings in self progeny, reduced transmission of the resistance gene through the male and no morphological defect of the mature pollen after visual examination. The mutants are fully penetrant male-specific gametophytic, showing a 1:1 ratio of resistant to sensitive seedlings. The screening of progeny seeds from 60 kanamycin resistant progeny of each mutant line showed an exact correlation between the reduced transmission and antibiotic resistance, suggesting that these insertions are tightly linked to the reduced transmission phenotype. Genetic transmission through the male and female gametes was determined by reciprocal test crosses in which heterozygous mutants were crossed to wild type and the transmission of the resistance marker scored. Only a small reduction in the transmission through the female was observed for *SETH7* (76%) whereas no or almost no transmission was observed through the male for *SETH4* and *SETH7* (transmission efficiency from 0% to 0.8% respectively), for both mutants, indicating that these mutations act specifically on the success of the male gametophyte. By eliminating the competitive element of pollinations via applying a reduced number of pollen grains on the stigma surface, it was demonstrated that the *Ds* induced mutation causes a detrimental effect on the ability of the pollen to either create propagate or target a pollen tube. Inspection of the mature pollen grains of the *SETH* mutants, showed that SETH pollen grains are of normal cellular morphology.

Chapter 4

Molecular and phenotypic characterisation of the *seth4* and *seth7* mutants.

4.1 Introduction

The forward genetics approach followed for the identification of genes playing an important role during the progamic phase did yield two mutants (chapter 3) *seth4* and *seth7*. The mutants are fully penetrant, male-specific, showing a 1:1 ratio of resistant to sensitive seedlings. Progeny testing of 60 kanamycin resistant siblings for each line revealed complete co-segregation of the antibiotic resistance and segregation ratio distortion trait, indicating that the *Ds* insertions are tightly linked to, or responsible for, the reduced transmission.

4.2 Phenotype of the *seth* lines; Can the *seth* pollen form a pollen tube and maintain its growth?

4.2.1 *In-vitro* germination assay

In order to analyse the efficiency of the *seth* pollen grains to form a pollen tube an *in vitro* germination assay was performed. *In vitro* germination of pollen is a well established procedure. It is already well described and characterised in other systems and successfully used for the identification and analysis of proteins that are essential for the pollen tube growth and directionality. *In vitro* grown tobacco pollen tubes are the model for the study of ion exchange during pollen tube growth and for dissection of the molecular components controlling and interacting with the ion fluxes. The directionality and the molecular exchange controlling the recognition/interaction and subsequent change of direction of the pollen tube after an external signal (that being either ion, especially Calcium fluxes or female tissue derived molecules) has also been analysed in *in vitro* grown pollen tubes in the *Torenia fournieri (*Higashiyama *et al.* 2000*;* Higashiyama *et al.* 2001*)*. In Arabidopsis, *in vitro* pollen tube germination and growth is heavily ecotype specific. Germination rates of different ecotypes have been described to vary from 20 % of the total population to almost 90% of the population as reported in Taylor and Hepler (1997). In the ecotype Landsberg *erecta* pollen grains did not germinate efficiently with any of the germination media reported to work for the other ecotypes. The maximum pollen germination achieved was reported to be at 75% of the population by Fan *et al.* (2001) but it could not be reproduced experimentally. Recently, another *in vitro* pollen germination media has been used

and high rates of pollen germination have been reported (Derksen *et al.* 2002). Derksen and co-workers reported that there is a stimulation in pollen germination when the media is imbibed with pistil material. In closer examination of this phenomenon, the effect of different plant tissues on the germination of the pollen tubes was examined (Figure 4.1). Pollen germination without any plant material in the proximity was in the range of 20 to 30%. Wounded leaf material and pistil material caused the same increase in the pollen germination frequency, reaching 40 to 50% germination efficiency. The highest pollen germination efficiency though was observed with the anther material reaching 70% of the total population. Since the effect of the plant material was mostly local, it was decided to use liquid germination media and supplement it with anther material. The pollen germination with this method was reproducibly above the 65% levels. Utilisation of this protocol led to the pollen germination media currently used for the assay of the germination efficiency of the *seth* mutants (Lalanne *et al.* 2004).

4.2.2.1 Do *seth4* pollen grains germinate in vitro?

Anthers from *seth4/SETH4* plants were incubated in pollen germination medium and the percentage of pollen grains germinating in the population was scored (Figure 4.2). The pollen germination efficiency of Ler and the wild type sibling of *seth4* was examined simultaneously at 20 min, 40 min, one hour, two hours, four hours, six hours and eight hours. The experiment was repeated four times and the number of pollen grains counted per time point per line was in the range of 100 to 200. The level and rate of pollen tube formation was calculated for the wild type and mutant. In wild type plants, pollen germination started after one hour. For the period of one hour to six hours the number of pollen grains germinated quadrupled per hour (average) and after the 6 hours time point the germination rate levelled off. After 8 hours, 76% of the pollen grains have formed a pollen tube. For the *seth4* pollen germination did begin at 1 hour and the number of germinated pollen grains tripled per hour during the incubation period from 1 to 6 hours and then levelled off after 6 hours. After 8 hours of incubation, 30% of pollen grains had germinated.

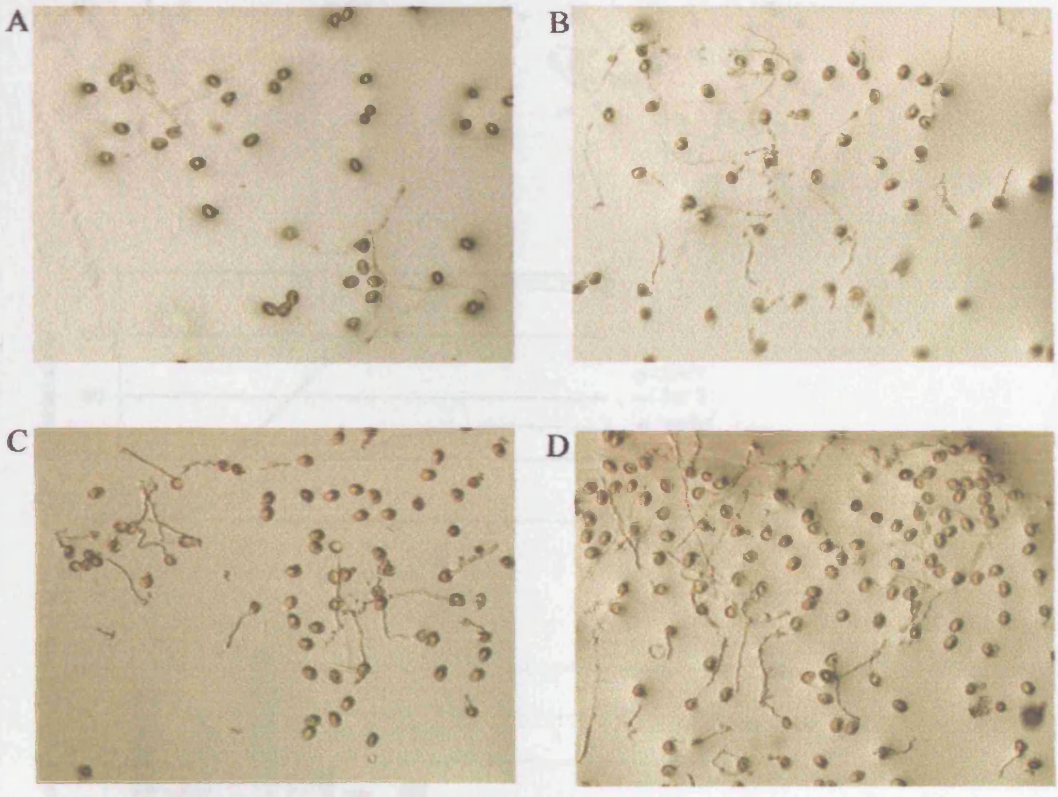


Figure 4.1. The effects of plant material in the *in vitro* pollen germination. Solid *in vitro* pollen germination media as described in Derksen *et al.* (2002) was used for the germination of Landsberg *erecta* pollen. Pollen from freshly dehisced anthers was placed on dialysis tubing membrane pre-wetted with *in vitro* germination media. The membrane pieces were placed on the solid media above no plant material (A), wounded leaf material (B), pistil (C) and anther (D).

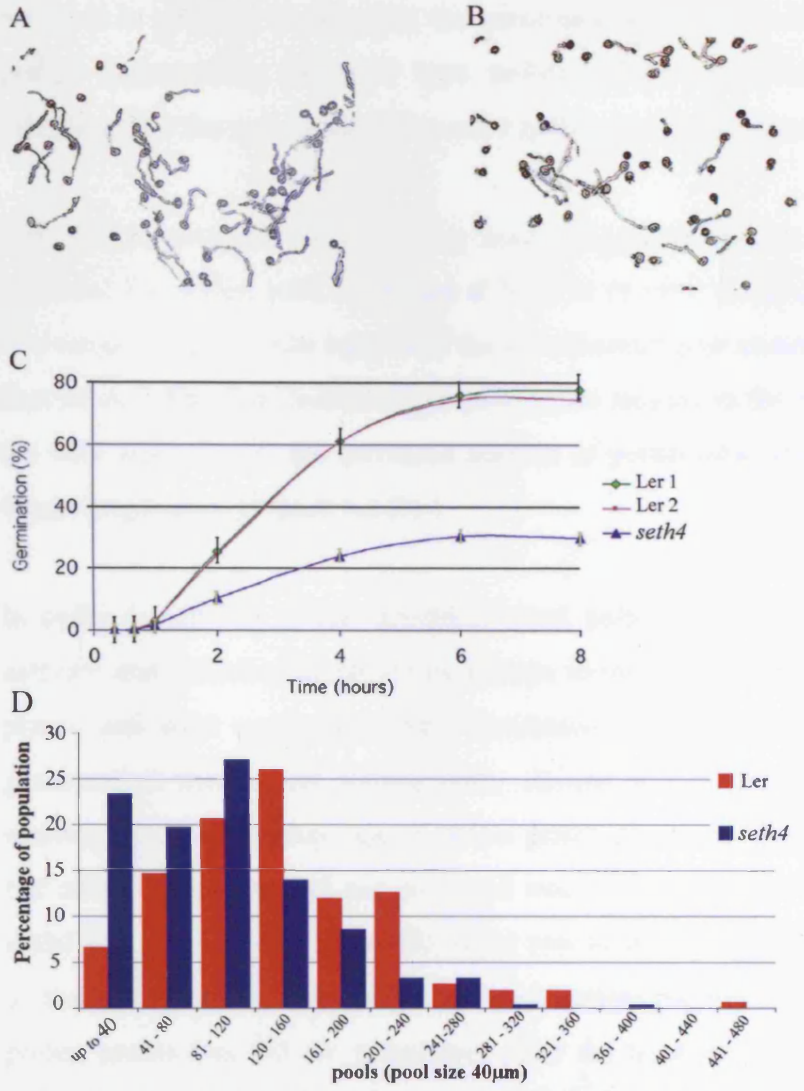


Figure 4.2. Analysis of the pollen germination of *seth4 in vitro*.
The ability of the *seth4* mutant pollen grains to germinate and extend a pollen tube was analysed in liquid *in vitro* germination media supplemented with anther material. Photos A (Ler) and B (*seth4*) are representative of the pollen population after six hours of incubation. Pollen grains start to germinate after 40 minutes of incubation and after six hours the maximum number of pollen grains have germinated . Pollen germination in the *seth4* hemizygous plants is 29.8 % and in the wild type pollen (Ler (Ler1) and wild type sibling of *seth4* (Ler2)) 77. 7 % of the population. (n = 250 pollen grains; Three replicate experiments; SE = 2%). The length of the pollen tubes was measured after 6 hours. The frequency of the *seth4* mutant and wild type pollen tube lengths distributed in pools of 40µm was plotted in (D). Ler1 is Ler; Ler2 is the wild type sibling of *seth4*.

Since the population of pollen grains in the *seth4* segregates 1:1 wild type to *seth4*, it is possible to calculate the germination efficiency for the *seth4* pollen grains (subtracting the wild type pollen grains germination efficiency as calculated for the wt). None of the *seth4* pollen grains did manage to germinate.

The pollen germination efficiency assay suggests that the *seth4* mutation is essential for pollen tube formation at least in *in vitro* pollen germination assay. The range of pollen tube lengths of the *seth4* hemizygote mutant and the wild type was scored. The distribution of the pollen tube lengths in the *seth4* was similar to the wild types except the increased number of pollen tubes distributed in the 0 to 40μm length pool (Figure 4.2 D).

In order to identify if the non-germinated pollen grains of *seth4* managed to activate and polarise (identified by callose formation), pollen from *seth4/SETH4* plants and wild type plants were incubated for two and six hours in pollen germination media and subsequently stained with aniline blue stain (callose staining dye). The percentage of pollen grains germinating, showing polarisation but non-germination and not polarised was counted for the wild type sibling of *seth4*, Ler and *seth4* (Figure 4.3). After two hours of incubation, 60 % and 40 % of the pollen grains from wild type and mutant respectively showed polarised pollen grains that did not germinate. After six hours of incubation, 40 % of the pollen grains of the *seth4* mutant did not germinate or show polarisation but only 20 % of the pollen grains from the wild type plants exhibited such a phenotype. From this result it can be calculated that 64 % ((40/50) x 100 x 0.8) of the *seth4* mutant pollen grain cannot activate.

4.2.2.2 Can *seth4* pollen grains form a pollen tube *in vivo*?

In vivo pollinations were performed on excised male-sterile (*ms1-1*) pistils (Wilson *et al.* 2001). The frequency of pollen grains germinating and initiating pollen tubes was scored 2 hours after pollination via aniline blue staining. Pollen tube formation in *seth4* is reduced to approximately 50 % of the wild type levels *in vivo*. The frequency of polarised pollen grains in the *seth4* were almost double that of the wild type populations (Figure 4.4A).

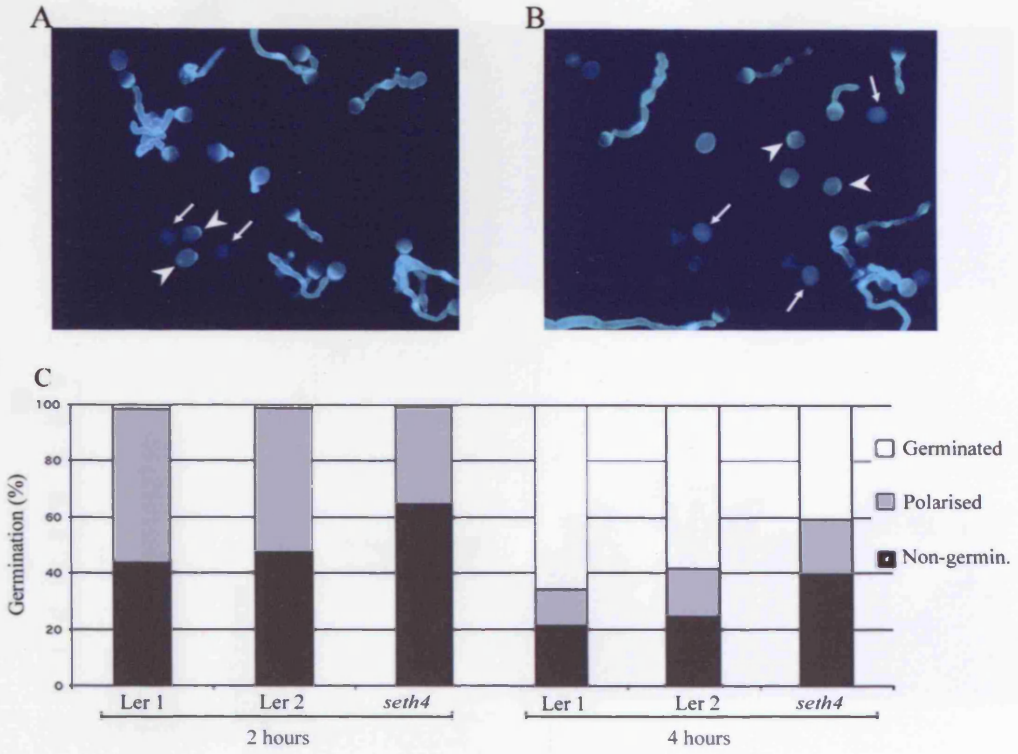


Figure 4.3. Analysis of the pollen tube formation of *seth4 in vitro*.
The ability of the *seth4* mutant pollen grains to form a pollen tube, its first outward sign being the callose deposition on the sides of the emerging pollen tube, was analysed after two and four hours of incubation in the *in vitro* germination media. Photos A (Ler) and B (*seth4*) are representative of the pollen population segregating in the germinated, polarised (arrowheads) and non-germinating (arrows) classes after four hours of incubation. In C the quantitative analysis of the ability of pollen from the *seth4* hemizygous plant and wt plants to form pollen tubes after two and four hours is presented. n = 750 pollen grains; SE = 3%; Ler1 is Ler; Ler2 is the wild type sibling of *seth4*.

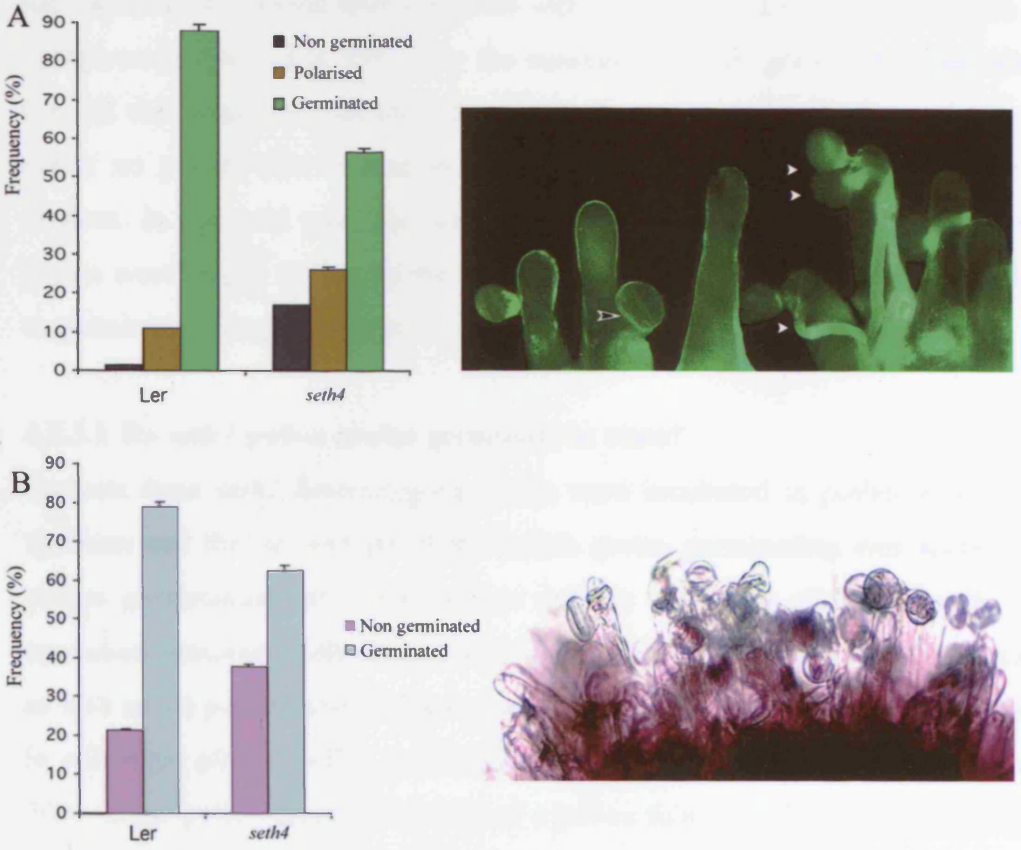


Figure 4.4. Analysis of the *in vivo* germination of *seth4*.
The pollen germination of *seth4 was* analysed *in planta* in limited pollination assays. Pollen tube formation was scored two hours after pollination via callose staining (A) and six hours after pollination via Alexander staining (B). (n = 500 pollen grains; SE = 2%) The images depict the staining pattern of Ler pollen grains: A after staining by aniline blue for callose (white arrowheads pollen grains forming pollen tubes, black arrowhead non polarised/germinated pollen grain). B after staining with Alexander stain a limited number of pollen grains placed on the stigma surface.

In vivo pollinations were performed on excised *ms1-1* pistils and the pollen tube formation was assessed after six hours with Alexander stain. Alexander stain is a cytoplasmic dye and in this assay the number of pollen grains which are empty (i.e. all the cytoplasm has moved to the pollen tube) and full of cytoplasm (i.e. either no pollen tube formation or the pollen tube formed is very small) are evident. In the wild type, six hours after pollination the majority of the pollen grains were empty (80%). In the *seth4* mutant, 60% of the pollen grains manage to germinate a long enough pollen tube (Figure 4.4 B).

4.2.3.1 Do *seth7* pollen grains germinate in vitro?

Anthers from *seth7* heterozygous plants were incubated in pollen germination medium and the percentage of the pollen grains germinating was scored. The pollen germination efficiency of Ler and the wild type sibling of *seth7* was examined simultaneously (Figure 4.5). The level and rate of pollen tube formation as well as the pollen tube length was calculated for the wild type and *seth7* plants. In wild-type plants, pollen germination started after one hour. After eight hours 70% of the pollen grains have formed a pollen tube. For the *seth7* mutant plant pollen germination begun after one hour and after eight hours of incubation 45% of pollen grains have germinated. The majority (approximately 60%) of the *seth7* pollen grains did not manage to germinate. The germination efficiency assay suggests that the *seth7* mutation seems to be very important for pollen tube formation at least in *in vitro* pollen germination assay. In the pollen tube length experiment there is a shift of the distribution of the individual pollen tubes according to their size. There is a high occurance of pollen grains not growing more than 160 μm (Figure 4.5 D).

4.2.3.2 Can *seth7* pollen grains form a pollen tube *in vivo*?

In vitro pollen germination assays showed that pollen germination was strongly reduced in *seth7* hemizygote. To monitor pollen germination efficiency *in planta*, an *in vivo* pollination assay was performed. This involved pollinating excised pistils from male-sterile (*ms1-1*) plants, and treating pistils with Alexander stain (Chapter 2.17) after four and six hours, to allow pollen germination and tube growth to be scored.

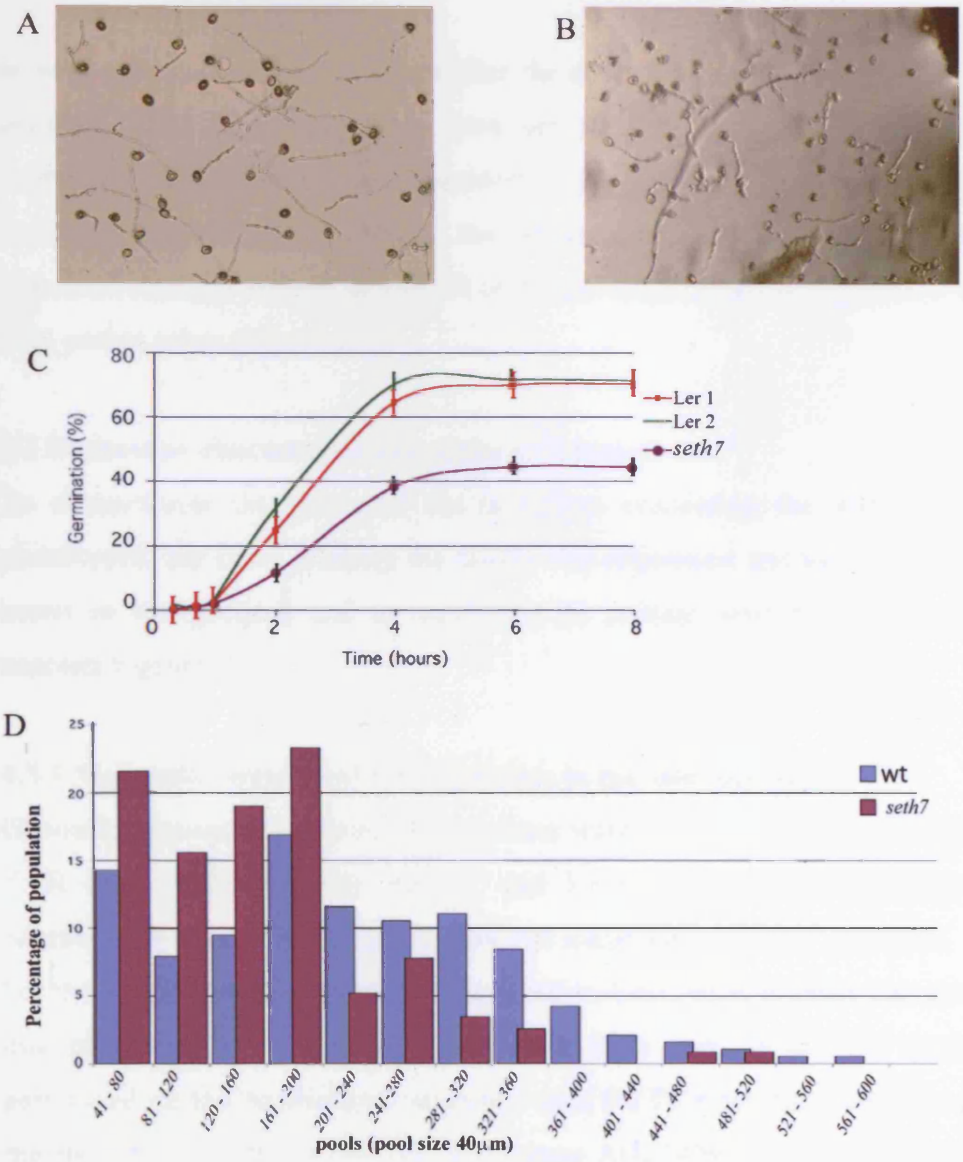


Figure 4.5. Analysis of the pollen germination of *seth7 in vitro*.
The ability of the *seth7* mutant pollen grains to germinate and extend a pollen tube was analysed in liquid *in vitro* germination media supplemented with anther material. Photos A (wt) and B (*seth7*) are representative of the pollen population after six hours of incubation. Pollen grains start to germinate after 40 minutes of incubation and after six hours the maximum number of pollen grains have germinated (C). In the *seth7* hemizygous plants 44 per cent of pollen do germinate after eight hours of incubation. For the wild type plants germination efficiency is 71 %. The length of the pollen tubes was measured after 6 hours. The frequency of the *seth7* mutant and wild type pollen tube lengths distributed in pools of 40µm was plotted in (D).(n = 300 pollen grains; Three replicate experiments; SE = 3% for *seth7*, 4% for Ler (Ler1) and 3.5% for the wild type sibling of *seth7* (Ler2)).

In wild type pollinations six hours after the deposition of the pollen grains on the papillae, 85% of pollen grains were not strongly stained, showing that the majority had germinated and transferred cytoplasm into the pollen tube. In contrast, approximately 26% of the pollen grains of the *seth7* hemizygote, remained strongly stained as a result of failure of germination or establishment of long pollen tubes (Figure 4.6).

4.3 Molecular characterisation of the *seth* mutations.

To characterise the nature of the insertions conferring the different mutant phenotypes, the DNA flanking the inserts was sequenced and used to position the insert in the genome and to determine its context with respect to adjacent annotated genes.

4.3.1 Molecular identity of insertion sites in the *seth* mutants.

Genomic sequences flanking *Ds* insertions were isolated for the *seth* mutants by TAIL-PCR (section 2.7.4). Both 5' and 3' *Ds*-genomic DNA junctions were confirmed by direct PCR amplification and sequencing of the 3' junctions.
For the *seth4* mutant a 2.2 kb tertiary reaction TAIL-PCR product was subcloned into pGEM-T EASY and sequenced from both ends. A BLAST search was performed on the *Arabidopsis* sequence with the PCR product sequence and the transposon is inserted in the ORF of the gene At4g34940 located on chromosome 4,553 nucleotide from the ATG (Figure 4.7). The putative *SETH4* gene encodes a novel protein of 664 amino acids containing five armadillo repeats.

From the *seth7* mutant a 700bp PCR product from the tertiary TAIL-PCR reaction was sent for sequencing. In *seth7* the *Ds* element was inserted into the 3'-UTR of the At2g41930 gene, 200 bp downstream of the stop codon (Figure 4.8). The putative *SETH7* gene encodes a putative Ser/Thr protein kinase of 351 amino acids containing a potential nuclear localization signal (*NLS*).

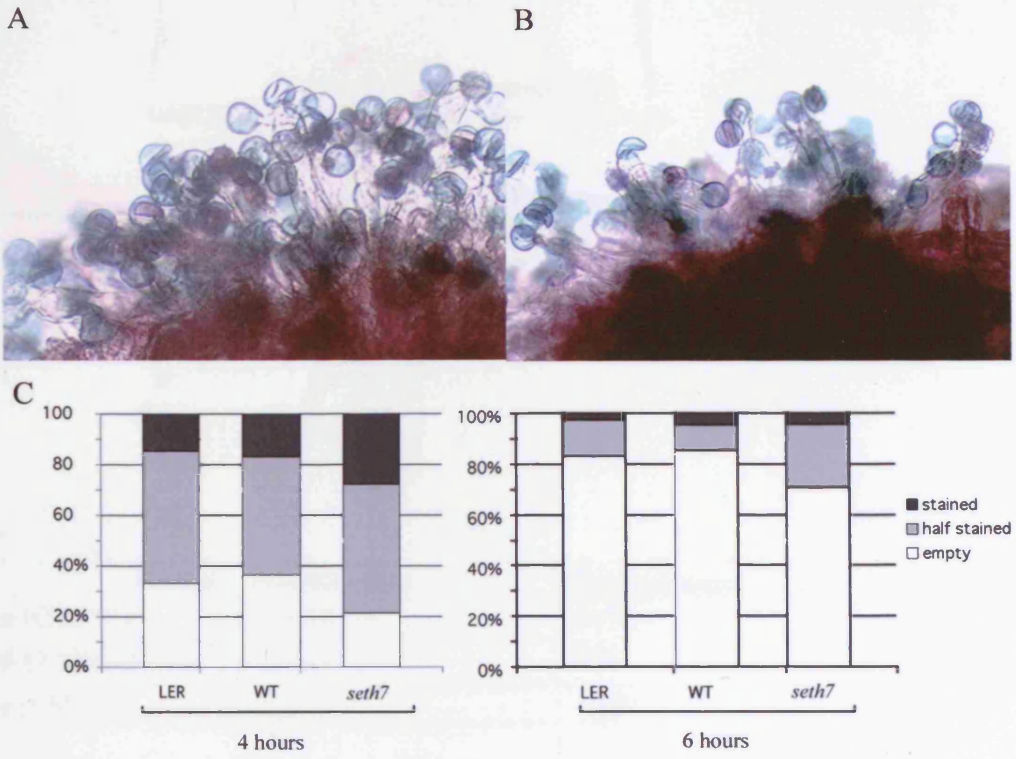
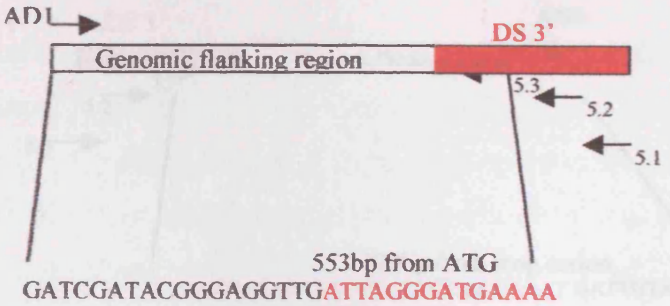
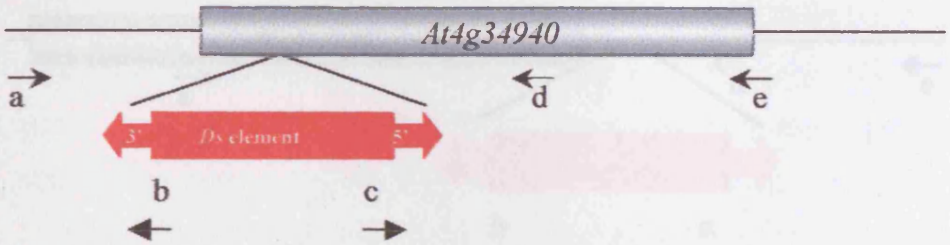


Figure 4.6 Analysis of the *in vivo* germination of *seth7*.
The pollen germination of *seth7* was analysed *in planta* in limited pollination assays. In A and B the pollen tube formation as visualised by Alexander staining can be seen for the wild type and *seth7* respectively. Pollen tube formation was scored four and six hours after pollination via Alexander staining (C). (n = 600 pollen grains; SE = 2%; WT is the wild type sibling of *seth7*).

A



B



C

Primer combination		Product size
a (ORF5')	**b** (GUS5-3)	1176
d (1536DS5-3)	**c** (DS5-3)	586
e (ORF3')	**c** (DS5-3)	1820

D

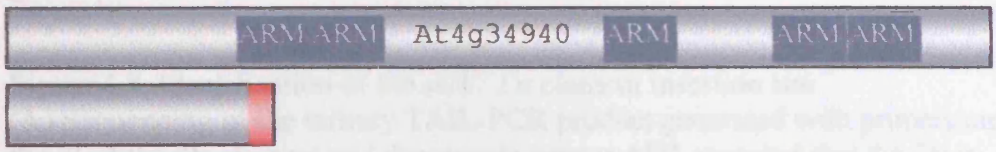
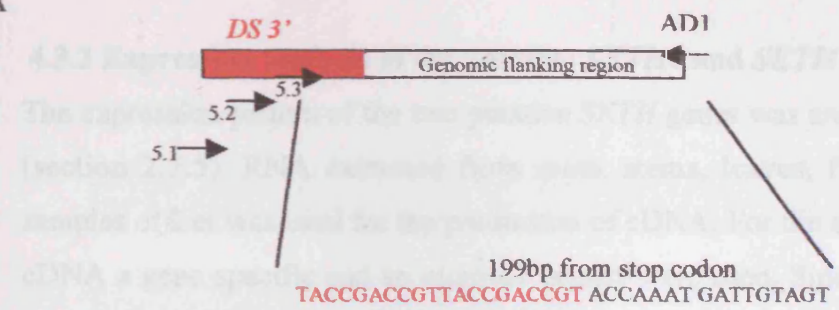
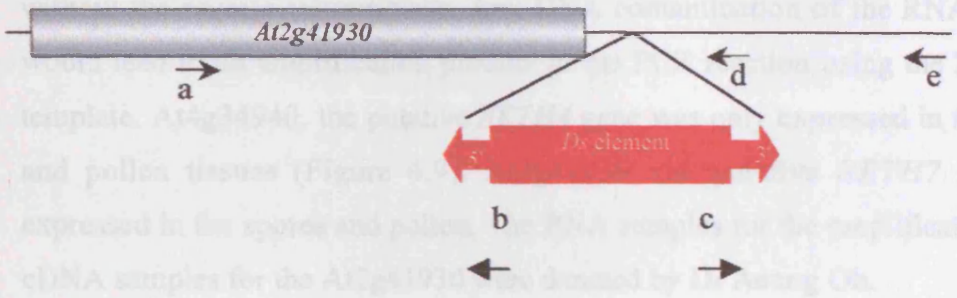


Figure 4.7. Identification of the *seth4 Ds* element insertion site.
Sequencing of the tertiary TAIL-PCR product generated with primers on the 3' of the *Ds* element and degenerate primer AD1 revealed that the *Ds* is inserted in the ORF of At4g34940 553bp from the start codon (A). In red is the terminal repeat of the *Ds* element and the junction with the At4g34940 ORF. The sets of primers designed and used for the verification of the insertion position of the *Ds* element are graphically depicted in B and the expected product, verified experimentally, is given in C. In D the conceptually predicted hybrid protein formed with the insertion of the *Ds* (in red) in the At4g34940 ORF is compared to the full protein. The positions of the ARM repeats are highlighted in the At4g34940 protein.

A



B



C

Primer combination		Product size
A (1196seq3)	**B** (DS5-3)	497 bp
D (1196GUS5-3)	**C** (GUS5-3)	684 bp
E (1196rv)	**C** (GUS5-3)	1457 bp

Figure 4.8. Identification of the *seth7 Ds* element insertion site.
(A) Sequencing of the tertiary TAIL-PCR product generated with primers on the 3' of the *Ds* element and degenerate primer AD1 revealed that the *Ds* is inserted in the 3' un-translated region of At2g41930 199 bp from the stop codon. The sequence in red is the terminal repeat of the *Ds* element and the junction with the At2g41930 un-translated region. The sets of primers designed and used for the verification of the insertion position of the *Ds* element are graphically depicted in B and the expected product, verified experimentally, is given in C.

4.3.2 Expression analysis of the putative *SETH4* and *SETH7* genes.

The expression pattern of the two putative *SETH* genes was analysed by RT-PCR (section 2.7.5). RNA extracted from roots, stems, leaves, flowers and pollen samples of Ler was used for the production of cDNA. For the amplification of the cDNA a gene specific and an oligo-dT primer were used. Since both At4g34940 and At2g41930 have no introns the cDNA synthesis reaction was done with and without the reverse transcriptase. Any DNA contamination of the RNA samples would lead to an amplification product in the PCR reaction using the RNA as a template. At4g34940, the putative *SETH4* gene was only expressed in the flower and pollen tissues (Figure 4.9). At2g41930, the putative *SETH7* gene was expressed in the spores and pollen. The RNA samples for the amplification of the cDNA samples for the At2g41930 were donated by Dr Aeong Oh.

4.4 Verification of the *SETH* genes

For the complementation, the ORF of the gene identified from the TAIL-PCR with one Kb of the 3' and 5' untranslated regions was reintroduced into the *seth* lines via floral dip transformation. The vector to used was pGreen0229 which confers resistance to Kanamycin in bacteria and resistance to PPT in plants. Restoration of fertility would be confirmed by reversion of the segregation distortion ratio of 1:1 to 2:1. The reason for the 2:1 expected segregation is due to the fact that the introduced DNA will most likely be unlinked to the *SETH* locus and a maximum of 25% of pollen grains are expected to carry both the SETH mutation and the complementing genomic fragment (Table 4.1).

4.4.1 Complementation of *seth4*

For the complementation of *seth4* two parallel strategies were followed. The complete gene containing 1 kb of 5'- and 3'-untranslated regions was amplified by PCR, subcloned to the pGreen0229 vector and the *seth4* plants were transformed by floral dip transformation. This is the most conclusive method of proving that the gene of interest causes the mutation.

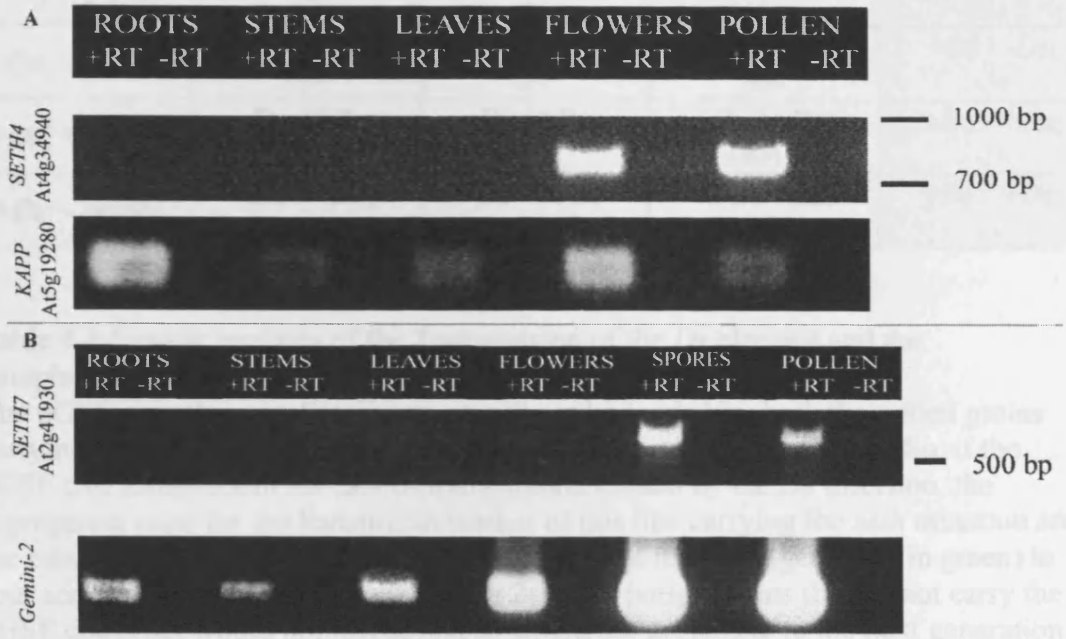


Figure 4.9. Expression analysis of the identified *Ds* disrupted genes in *seth4* and *seth7* mutants.
In the RT-PCR expression analysis of the two genes, cDNA was produced via amplification with a gene specific and an oligo-dT primer. The reaction was done with and without the addition of Reverse-Transcriptase. The PCR amplification of the cDNA produced followed, with gene-specific primers and both the plus and minus reverse transcriptase reactions were used as template for the PCR reaction to test for DNA contamination of the RNA samples.
(A) At4g34940, the gene identified as disrupted by the *Ds* insertion in *seth4*, a product of the expected size was amplified in the open flowers and pollen tissue. As a positive control, a 300bp fragment of the constitutively active *KAPP* gene was amplified under the same conditions. (B) At2g41930, the gene identified as disrupted by the *Ds* insertion in *seth7* was amplified in RNA derived from spores and pollen tissues. As a positive control for this reaction, under the same conditions, a 480bp fragment of the constitutive gene *Gemini-2* (identified in the laboratory, unpublished data) was amplified.

♀ \ ♂	*-Ds* ; +pGSE	*-Ds*; - pGSE	***+Ds***; + pGSE	***+Ds***; - pGSE
-Ds; + pGSE	***-Ds / -Ds;*** ***+/+***	***-Ds / -Ds;*** ***-/+***	***+Ds / -Ds;*** ***+/+***	***+Ds / -Ds;*** ***-/+***
-Ds; - pGSE	***-Ds / -Ds;*** ***+/+***	***-Ds / -Ds;*** ***-/+***	***+Ds / -Ds;*** ***+/-***	***+Ds / -Ds;*** ***-/+***
+Ds; + pGSE	***-Ds / +Ds;*** ***+/+***	***-Ds / +Ds;*** ***-/+***	***+Ds / +Ds;*** ***+/+***	***+Ds / +Ds;*** ***-/+***
+Ds: - pGSE	***-Ds / +Ds;*** ***+/-***	***-Ds / +Ds;*** ***-/-***	***+Ds / +Ds;*** ***+/-***	***+Ds / +Ds;*** ***-/-***

Table 4.1 Genetic analysis of the Transmission of the *Ds* element and the complementation construct (pGSE) in the *seth* mutants.
The pGSE complementation construct will not be carried by both the pollen grains carrying the *Ds* element *(+Ds)* when it is inserted in an unlinked locus. So, if the pGSE can complement for lack of transmission caused by the *Ds* insertion, the segregation ratio for the kanamycin marker of this line carrying the *seth* mutation and the complementing pGSE construct, would be eight resistant (genotype in green) to four sensitive plants (genotype in red) or 2:1. The pollen grains that do not carry the pGSE construct would not rescue and so would not propagate to the next generation (their genotype depicted in grey).

A lack of reversion of the segregation distortion phenotype of 1:1 to 2:1 from this construct though, does not necessarily signify that the gene of interest is not the cause of the phenotype observed since there is the possibility of errors in the PCR product amplification or the possibility that the untranslated region used for the complementation experiment was not sufficient. In order to sidestep this problem, a genomic clone containing the *SETH4* gene from the GARNet library screening service was used to complement the mutation. Complementation of the mutation by both methods would verify the role of the *SETH4* in the progamic phase development.

For the complementation of *seth4* with the PCR amplified gene, the PCR product was subcloned in pGreen0229 in two orientations (Figure 4.10), transferred to Agrobacterium and transformed into plants via the floral dip method (2.11). Seeds were harvested and the T_1 generation of transformants was screened for resistance to PPT. The resistant individuals containing the *Ds* element were allowed to self –pollinate and set seed. Seeds collected from these plants were screened on kanamycin plates and the segregation ratios scored (Figure 4.11).

The results suggest that there is reversion of the segregation distortion phenotype of 1:1 to 2:1 for both the genomic clone (pJSE4Y) and the constructs (pGSE4f and pGSE4r). Restoration of male fertility was analysed by using the plants that had progeny segregating 2:1 as pollen donors in reciprocal crosses to the wild type plants. Crossing results and T_2 segregation data are presented in table 4.2.

4.3.2 Complementation of *SETH7*.

Since there were no genomic clones from the GARNet library screening service that contained the At2g41930 gene, it was necessary to use the PCR product of the full gene (Figure 4.12). In order to minimize the effect of a PCR amplification error a high fidelity polymerase was used and two different PCR products were used in the constructs for the complementation of the *seth7* mutation. Identification of 21 independent PPT resistant lines and further characterisation of their self segregation showed no reversion of the segregation ratio. Instead all the PPT resistant transformants were exhibiting reduced segregation ratios (Figure 4.13).

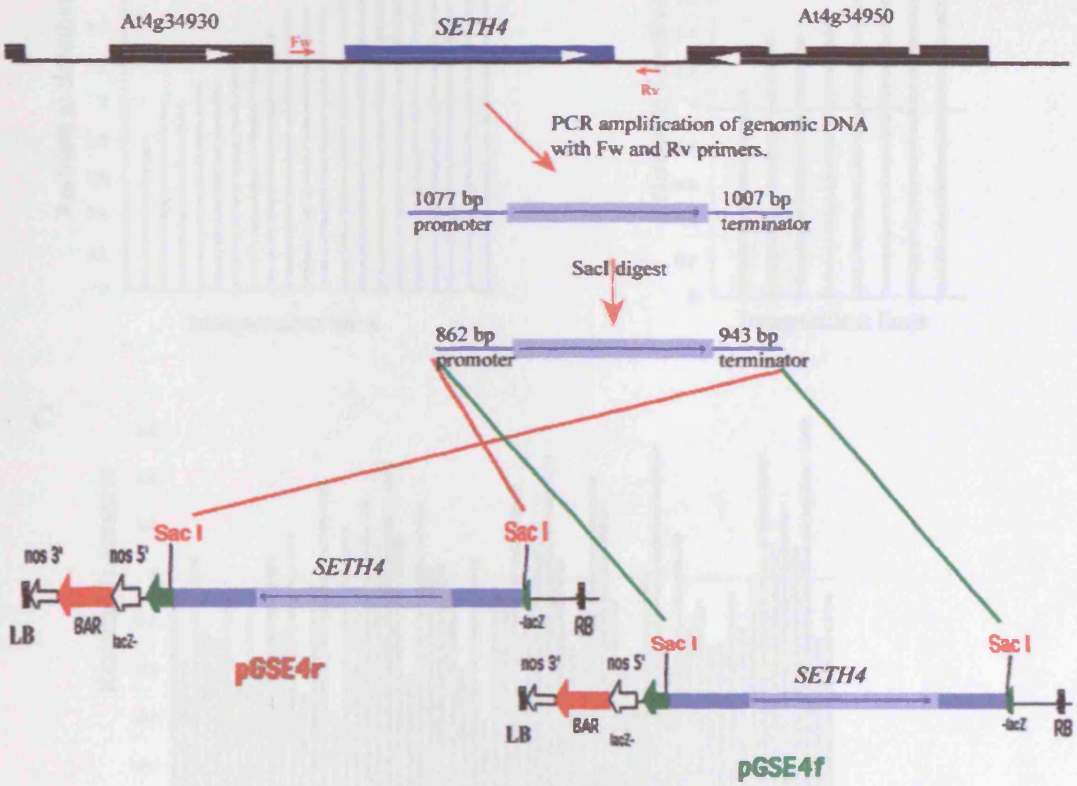
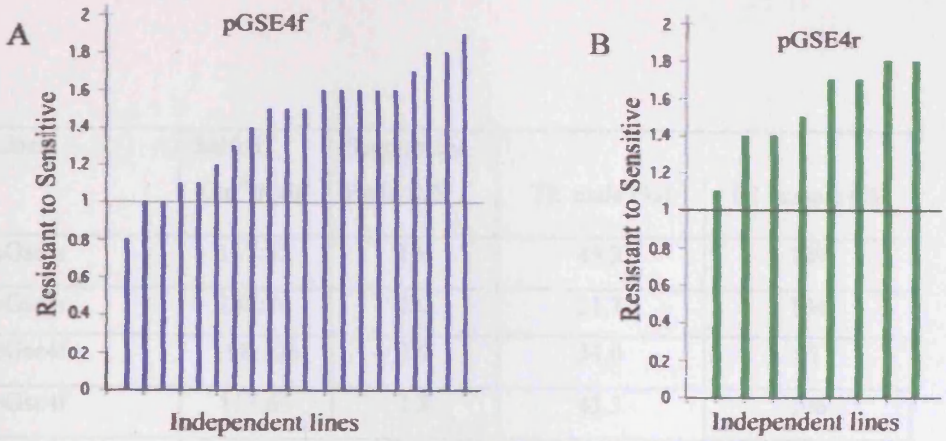
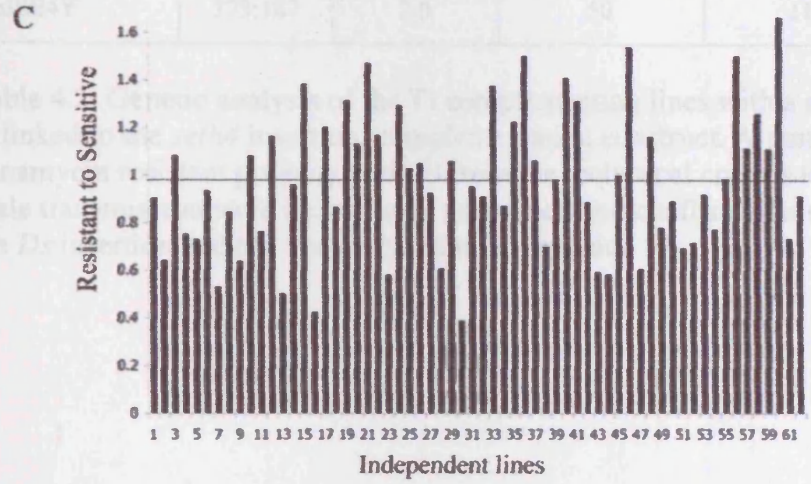


Figure 4.10. Constructs for the complementation of the *seth4* mutation.
The two constructs used for the complementation of the *seth4* were based on the pGreen229 binary plant transformation vector. Genomic DNA was PCR amplified, digested with *SacI* restriction enzyme and subsequently inserted in the linearised vector. After diagnostic digests of the constructs carrying the insertion to verify the orientation of the insertion the vectors were transformed into *seth4* plants.





D

Construct	Number of *seth4* plants carrying the construct	Number of plants segregating in the range of:		
		0.4 to 1.3 R:S	1.3 to 1.6 R:S	1.6 to 2.0 R:S
pGSE4f	20	7	10	3
pGSE4r	8	1	5	2
pJSE4Y	1	0	0	1

Figure 4.11. Segregation analysis of the *seth4* lines transformed with the pGSE4f and pGSE4r complementation constructs.
The segregation ratio for the kanamycin selection marker of the T2 generation of the pGSE4f, pGSE4r and pJSE4Y (JAtY clone) transformed *seth4* lines was analysed. The range of segregation ratios in the *seth4* population transformed with pGSE4f and pGSE4r is seen in A and B respectively. In C the natural range of segregation ratios recorded for the *seth4* mutant during the co-segregation experiment are presented. Plants that their progeny was segregating in the range of 1.6 to 2 to one (n=200) (D) were further analysed.

Lines	Selfed Kan^R:Kan^S	Segregation Ratio R:S	TE male (%)	TE female (%)
pGse4r	117:62	1.9	43.3	109
pGse4r	138:74	1.9	21.7	104
pGse4f	198:106	1.9	34.0	97
pGse4f	113:63	1.8	63.3	106
pGse4f	103:53	1.9	36.9	102
pJSE4Y	373:187	2.0	50	115

Table 4.2. Genetic analysis of the T_1 complementing lines with a single, unlinked to the *seth4* insertion, complementation construct. A portion of the kanamycin resistant progeny derived from the reciprocal crosses testing the male transmission were molecularly genotyped and confirmed as carrying both the *Ds* insertion and the complementation construct.

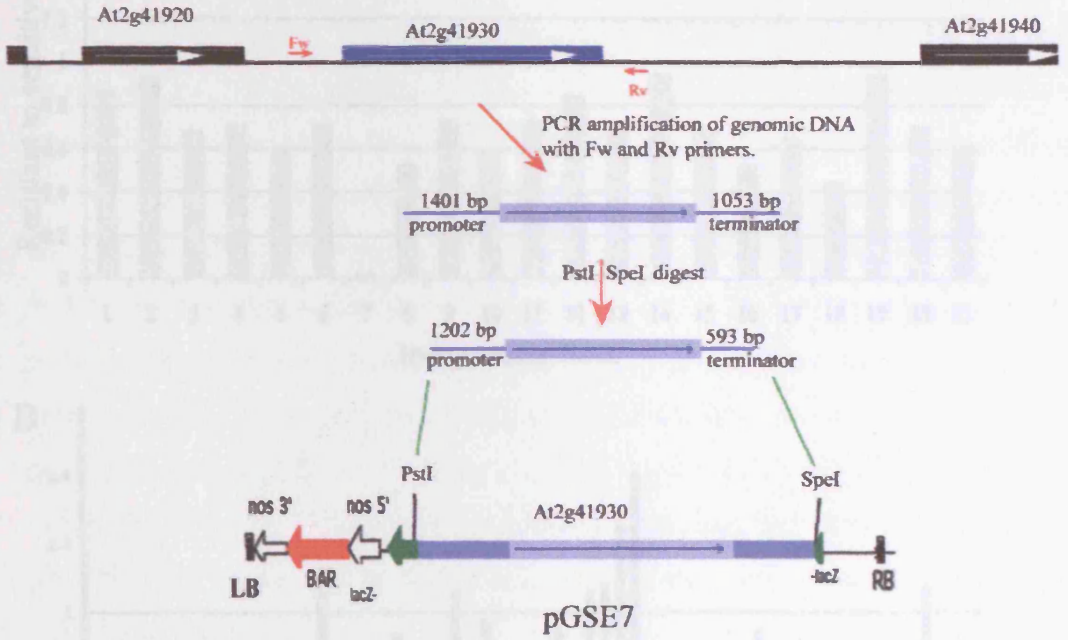
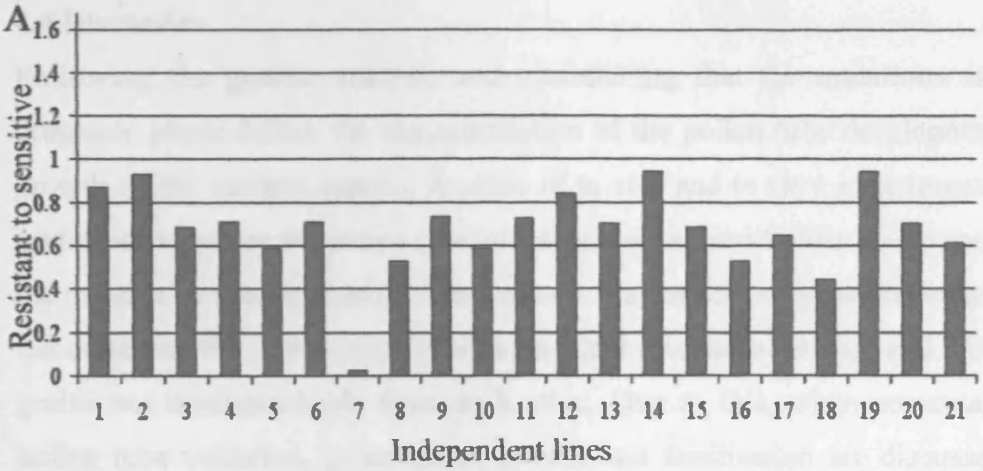
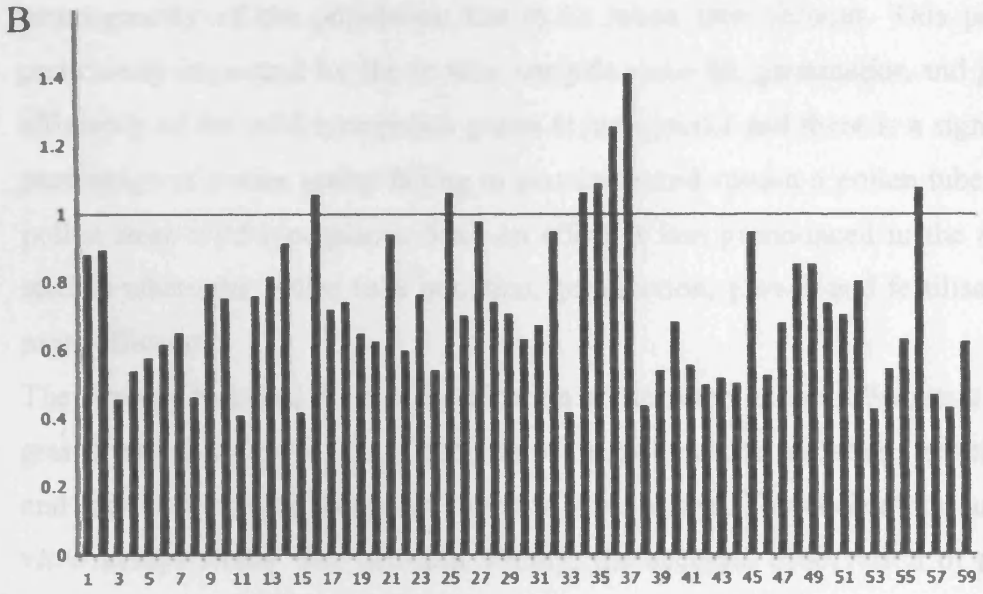


Figure 4.12. Construct for complementation of the *seth7* mutation.
The construct used for complementation of *seth7* was based on the pGreen229 binary plant transformation vector. Genomic DNA was PCR amplified, doubly digested with *PstI* and *SpeI* restriction enzymes and subsequently inserted in the linearised vector. After verification by diagnostic digests the vector was transformed into *seth7* plants.





C

Construct	Number of *seth7* plants carrying the construct	Number of plants segregating in the range of:		
		0.5 to 1.0 R:S	1. to 1.5 R:S	1.5 to 2.0 R:S
pGSE7	21	21	0	0

Figure 4.13. Results of the segregation of the T_1 generation of the *seth7* lines transformed with the pGSE7 complementation construct.
None of the lines exhibited a reversion of the segregation ratio to two to one resistant to sensitive siblings (A and C). In B the natural range of segregation ratios recorded for the *seth7* mutant during the co-segregation experiment are presented. The segregation ratio of all the transformed individuals transformed with pGSE7 was below one to one resistant to sensitive. (n=150)

4.4 Discussion

Following the genetic analysis and establishing that the mutations cause a progamic phase defect, the characterisation of the pollen tube development and growth on the mutants ensued. A series of *in vivo* and *in vitro* experiments were performed to assess the pollen tube initiation, growth and fertilisation potential of the mutants. It should be noted that since the mutants can only be hemizygous for the mutation, the pollen population is an equal mixture of mutant and wild type grains not distinguishable from each other. Due to this, when percentages of pollen tube initiation, germination, growth and fertilisation are discussed, the heterogeneity of the population has to be taken into account. This point is particularly important for the *in vitro* analysis since the germination and growth efficiency of the wild type pollen grains is not optimal and there is a significant percentage of pollen grains failing to germinate and sustain a pollen tube in the pollen from wild type plants. Such an effect is less pronounced in the *in vivo* studies where the pollen tube initiation, germination, growth and fertilisation is more efficient.

The strategy followed for the identification of the defect in the *seth* mutant pollen grains was as follows. Firstly analysis of the pollen tube initiation, germination and growth would be examined in the *in vitro* assays. The decision on using *in vitro* assays firstly was two-fold. Firstly, the accurate observation of a large number of pollen grains is straight forward in the liquid media, *in vitro* germination assay developed and used. Secondly, the *in vitro* assays eliminate the temporal variability of the pollen tube initiation and germination seen *in planta* since all the pollen grains have similar access to the *in vitro* germination medium whereas *in vivo* it is a common occurrence in heavy pollinations that pollen grains do not germinate uniformly due to limited access to the papilla cells. The reproducibility of the *in vitro* system, albeit with lower germination efficiency than observed *in vivo*, does allow for the identification of the defect in the mutants. So with the knowledge of that defect, targeted experiments can be done *in vivo* to verify and further examine the mutant phenotype. It must be noted that using this system defects in pollen tube initiation, germination and elongation can be examined directly. Indirectly, one can infer defects of the mutant pollen tube in cell to cell signalling as applied in the interactions of the pollen grain with the

female tissues. If the level of germination and growth of the mutant pollen tubes is similar to that of the WT, then the reason for lack of fertilisation and subsequently the reduced transmission of the transposon insertion must be failed guidance of the pollen tube to the ovules.

For both of the *seth* mutants, defects in the pollen tube germination were of varied severity. For *seth4,* blockage of pollen tube germination was almost total and of the failed pollen grains most did not activate as shown by the callose staining assays. In the case of the *seth7* mutant pollen grains, 70 % failed to germinate a pollen tube. Identifying that the pollen tube germination is the defective step in the two *seth* mutants, targeted experiments *in vivo* were carried out to verify the hindered pollen germination phenotype seen in the *in vitro* germination assays. Since the majority of the *seth4* mutant pollen grains fail to activate and subsequently germinate, the ability of the *seth4* pollen grains to activate and germinate *in vivo* was examined. Half of the *seth4* mutant pollen grains managed to germinate and the number of activated pollen grains in the *seth4* plant was similar to the wild-type levels.

For the *seth7* mutant, the *in vivo* experimental design was based on the higher germination efficiency the *seth7* pollen grains exhibited. The performance of the pollen tubes formed by the *seth7* pollen grains was examined. *seth7* mutant pollen grains are delayed in the formation of sufficiently long pollen tubes when compared to their wild type counterparts and a 26% of them even after six hours fail to form a long tube. It is evident from the *in vivo* tests that both mutants have a marked defect in the formation of a pollen tube. A number of mutant pollen grains appear to be able to germinate and form a pollen tube *in vivo.* The lack of fertilisation by the *seth* mutant pollen observed in the reciprocal crosses could be due to the *seth* mutant pollen tubes being out-competed by the wild type pollen tubes in the hemizygous *seth* plants. Non-competitive pollinations with pollen from the hemizygous plants were performed to address such a possibility. No *seth* individuals were identified from these crosses for either mutant, demonstrating that the *seth* pollen grains are unable to fertilise.

The forward genetics approach followed for the identification of genes playing an important role during the progamic phase did yield two mutants (chapter 3) *seth4* and *seth7*. The mutants are fully penetrant, male-specific, showing a 1:1 ratio of resistant to sensitive seedlings. The insertions are tightly linked to the reduced transmission phenotype. The transposon insertion site was identified via TAIL-PCR for both the lines.

Molecular characterisation of the *seth* mutants.

The sequence flanking the insertions was isolated by TAIL-PCR. For *seth4* the insertion in the ORF creates a loss of function phenotype that was confirmed by the complementation experiment. Introducing a PCR amplified, genomic fragment of *SETH4* with its 3' and 5' UTRs restored the *seth4* transposon insertion's male transmission. Insertional knockouts of the At4g34940 from the SALK collection exhibit the same segregation distortion ratio as *seth4* (resistant to sensitive ratio 1.2:1 (n= 640) male transmission of the T-DNA insertion 0% (n=123)). SETH4, a protein of unknown function contains five ARM repeats which are known to play a role in protein protein interactions and the phenotypic data suggest that SETH4 is necessary during the pollen tube formation.

A similar approach was followed for the *seth7* but the male transmission was not restored. Insertional knockouts from the SALK collections (not fully characterised yet) do not show a distorted segregation ratio (*seth7-1* F_3 generation Resistance to senitive ratio 1.2 (n=25) male transmission of the T-DNA insertion 44%. (n=112) ; *seth7-2* F_3 generation Resistance to senitive ratio 5.8 (n=34) so probably two T-DNA insertions are present and the line needs to be backcrossed and molecularly selected for the T-DNA insertion disrupting the putative *SETH7* gene). The transposon insertion in the *seth7* line, in 3'-UTR, 200bp from the stop codon could lead in either a loss of function or a gain of function phenotype. Due to the insertion of the Ds transposon the mRNA transcript could be either more or less stable than what it is in the wild type. The lack of complementation of the mutation via the insertion of the wild type copy of the gene in the *seth7* plants and actually the increase in the severity of the mutation, points out the possibility of a gain of function mutation or a very tight mutation to the transposon selection that was not identified in the co-segregation test.

Chapter 5

Molecular characterisation of SETH4 and the SETH FOUR LIKE proteins, SFL1 and SFL2

5.1 Introduction

In the recent chapters the genetic and phenotypic analysis of the two mutants identified by the screen for progamic mutants, *seth4* and *seth7*, was described. In both mutants, the *Ds* is not transmitted to the next generation via the male. The pollen grains are morphologically identical to the wild type but pollen germination is severely affected. Identification of the site of insertion of the *Ds* element in the *seth* mutants was performed via TAIL-PCR. For *seth4* the *Ds* element is inserted in the ORF of a pollen expressed gene which encodes for a novel protein of unknown function. In contrast to the results from *seth7*, *seth4* complementation proved that the gene responsible for the pollen tube phenotype is the gene with the identified transposon *DS* insertion. The unequivocal proof of the experiment and the interest it displays due to its putative function and the function of its sporophytic homologues made it a better and more interesting question for the further elucidation of its role.

5.2 Bioinformatic analysis of the SETH4 and putative SETH7 proteins.

5.2.1 Description of the SETH4 protein

The ORF, determined by conceptual translation, encodes a putative protein, of 664 aa. This putative protein contains five ARM repeats and a regulator of chromosome condensation factor (RCC1) signature (Figure 5.1). The regulator of chromosome condensation is a eukaryotic protein which binds to chromatin and interacts with Ran, a nuclear GTP-binding protein, to promote the loss of bound GDP and the uptake of fresh GTP, thus acting as a guanine-nucleotide dissociation stimulator. The interaction of RCC1 with Ran probably plays an important role in the regulation of gene expression. The RCC1-2 pattern identified in the SETH4 protein, is an eleven amino acid signature, derived from conserved positions in the C-terminal part of each repeat and detects up to five copies of the repeated domain. The pattern has a high rate of false positives according to the Interpro database.

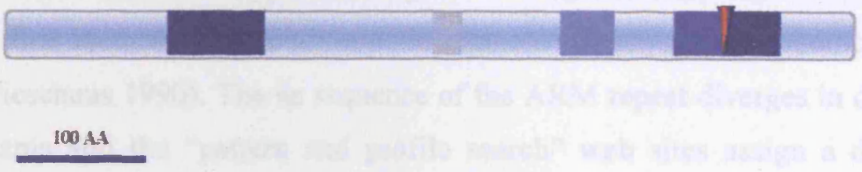


Figure 5.1. Domain structure of SETH4.
The ARM repeats are depicted as blue/grey rectangles. The confidence levels of the ARM repeat predictions are depicted by the depth of the blue colour. The RCC1-2 signature is depicted as the red triangle. The grey box represents the low complexity region in SETH4.

The low complexity region (consisting of mainly serine and glycine) of SETH4, at the middle of the protein, could play a role in the folding of the protein. The five armadillo (ARM) repeat recognised were assigned different levels of confidence by the pattern recognition programs (Zdobnov 2001).

5.2.2 ARM repeats

The ARM repeats are approximately 40 aa long tandemly repeated sequence motifs first identified in the Drosophila segment polarity gene armadillo (Peifer and Wieschaus 1990). The aa sequence of the ARM repeat diverges in different organisms and the "pattern and profile search" web sites assign a different consensus for the characterisation of an ARM repeat.

Due to this SETH4 has been assigned with three ARM repeats according to all prediction sites and two more ARM repeats which are bellow the threshold for some of the prediction program (Zdobnov 2001).

ARM repeats form three α helices H1, H2 and H3. Characterisation of the ARM repeats of Karyopherin-α and β-catenine by x-ray crystallography has identified at least 4 residues necessary for the formation of the 3-D fold of the ARM domain (Figure 5.2). Of course, certain residues at specific locations in the ARM repeats are important but not necessary (Andrade *et al.* 2001).

Analysis for ARM repeats of SETH4 was based on the facts above. The primary sequence of SETH4 was aligned with the signature for the ARM repeat from PFAM, SMART and INTERPRO databases . A CLUSTAL-W alignment was performed on the five SETH4 arm repeats identified with the signature repeats from the PFAM and SMART database (Figure 5.3). The character of all the required aa was conserved in the SETH4 ARM repeats. In order to further analyse the ARM repeats in SETH4, the secondary structure of the SETH4 protein was analysed by five prediction programs. PSIPRED, SAM-T99 and SAM-T02 are multi-search programs, using several databases for their analysis. The results of the helix predictions from these programs were aligned with the position of the predicted ARM repeats. The ARM repeats are characterized by the tertiary fold of their 3 helices. So for an ARM repeat to exist, I decided that the prerequisite three helices (and two turns) should be predicted by at least three out of the five secondary structure prediction programs (Figure 5.4).

A

```
Armadillo_seg          npenkqavveaGalppLvqLLs.pdeevqeeAawAlsNLaa
ARMADILLO   (PFAM)     NPENKQAVVEAGALPPLVQLLSPDEEVQEEAAWALSNLAA
ARM REPEAT  (SMART)    NDENKQAVVDAGGLPALVQLL  DEEVQKEAAWALSNLAS
```

B

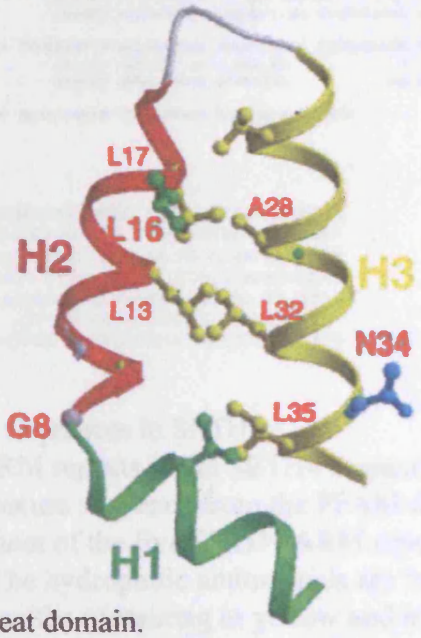


Figure 5.2. The ARM repeat domain.
(A) The ARM repeat is highly divergent. Different pattern recognition programs use slightly different signature for the ARM repeat . The three α-helices formed in the ARM repeat, interact to form a characteristic structural motif. This tertiary arrangement of the ARM repeat is derived from the crystal structure of β-catenin and α–importin. The amino acids highlighed in red in (A) are necessary for the 3-dimensional fold of the three helices as seen in (B).

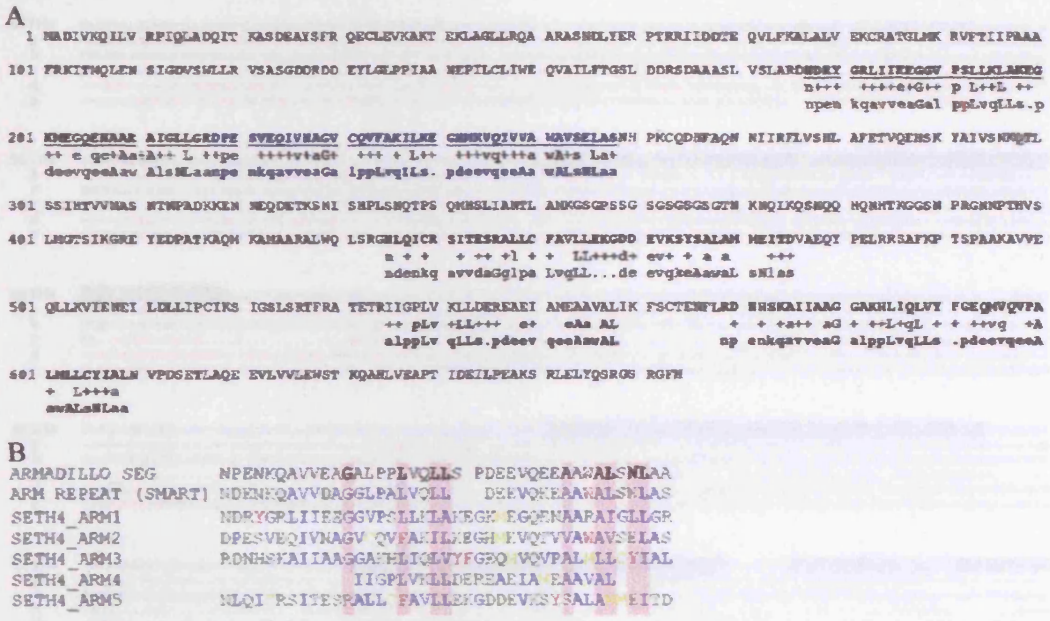


Figure 5.3. ARM repeats sequences in SETH4.
(A) The predicted five ARM repeats in the SETH4 sequence are aligned with the ARMADILLO_SEG signature sequence from the PFAM database.
(B) CLUSTAL-W alignment of the five SETH4 ARM repeats with the ARM repeat signature motifs. The hydrophilic amino acids are in green, the hydrophobic in blue, the sulfur containing in yellow and in red the aromatic amino acids. Highlighted in red are the conserved amino acids necessary for the tertiary fold of the ARM repeat.

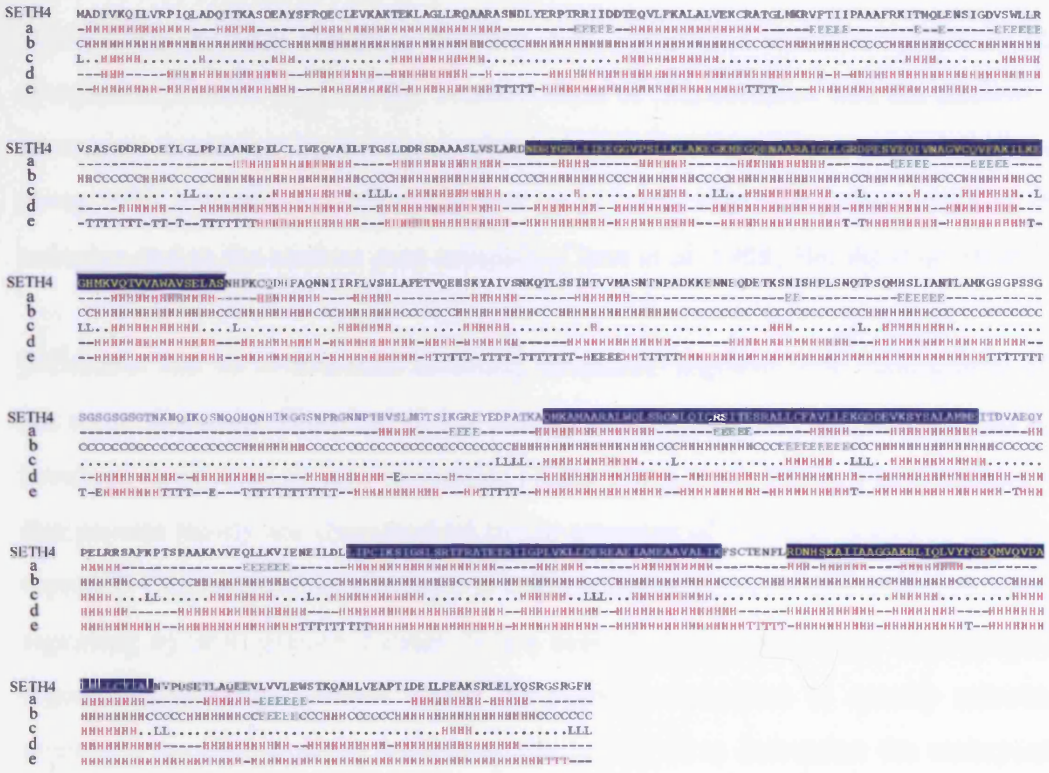


Figure 5.4. Secondary structure prediction of SETH4.
The SETH4 sequence was analysed by (a) jpred, (b) PSIPRED, (c) PROF, (d) SAM-T99 and (e) SAM-T02. The predicted helical regions are depicted as H, the coil regions as C the loop (mainly globular) regions as L, the regions not forming a structure as E and the turn regions as T. The predicted ARM repeats are highlighted in blue.

5.2.3 ARM repeat containing proteins.

The Karyopherin α (synonym importin α) acts as a nuclear import protein. Importin α recognises and binds to the NLS of nuclear proteins, via 10 Armadillo repeats which form the NLS binding site, forming a stable complex in the cytoplasm (Herold *et al.* 1998). Translocation of this complex into the nucleus is an energy dependent process and achieved with the aid of Ran, a small GTPase, along with homodimeric factor known as p10 and importin β that docks to the complex and to the nuclear pore complex (Conti *et al.* 1998; Herold *et al.* 1998).

β-Catenin and its invertebrate homolog Armadillo together with Plakoglobin and the more distantly related p120 ctn belong to a large family of proteins that are involved in diverse cellular processes (Rubenstein *et al.* 1997). The members of this protein family are characterised by the presence of multiple copies of the Arm repeat. β-Catenin besides playing a role in cadherin-mediated cell adhesion acts in signaling by *Wnt* growth factors. It is a central component of the Wnt/wingless signal transduction cascade which for example functions to specify anterior-posterior segment polarity in Drosophila larvae or to determine the embryonic dorso-anterior body axes in *Xenopus laevis* (Cox *et al.* 1999).

5.2.4 SETH4 Family

The two SETH4 like (SFL) proteins (At5g66200 and At4g36030 named as SFL1 and SFL2 respectively) were also identified in BLAST analysis of the *Arabidopsis* genome with the SETH4 protein sequence. They show globally 61% identity and 73% similarity with each other (Figure 5.5 A). The next highest homology to the SETH4 protein arises from Os_SFL (accession number OSJNBb0016M10.6), a rice protein showing 48% identity to SETH4 and 66% similarity. Another *Arabidopsis* protein shows the next highest similarity to SETH4, At3g26600. The level of similarity though between SETH4 and At3g26600 is 55% and the alignment of the two proteins is on their respective ARM repeats. SETH4, SFL1 and SFL2 are encoded by intronless genes. The SFL1 and SFL2 contain three to five putative Arm repeats. The ARM repeats are organised in a similar fashion to SETH4 (Figure 5.5 B). SFL1 encodes for a protein of 651aa of unknown function. SFL2 encodes for a protein of 670 aa of unknown function.

A

	SETH4	SFL1	SFL2	Os_SFL	At3g26600
SETH4		65% ID 77% SIM	60% ID 75% SIM	48% ID 66% SIM	37% ID 55% SIM
SFL1			61% ID 73% SIM	44% ID 59% Sim	37% ID 52% SIM
SFL2				42% ID 59% SIM	34% ID 52% SIM
Os_SFL					27% ID 42% SIM
At3g26600					

B

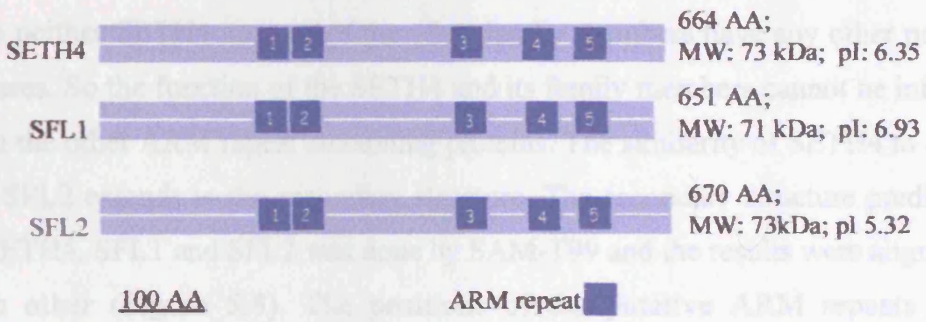


Figure 5.5. Close homologues of SETH4.
(A)The four closest homologues of SETH4 are plant proteins. SFL1 and SFL2 are Arabidopsis proteins exhibiting the highest similarity to SETH4 over the whole length of the protein. A rice protein, Os_SFL is the most similar protein to SETH4 after them. At3g26600 is mainly homologous to the SETH4 ARM repeats.
(B) SETH4, SFL1 and SFL2 are of similar size and the ARM repeats are in a similar spatial organisation.

The SFL family is speculated to have arisen from an ancient duplication event but then the proteins diverged. SETH4 is the most similar to the Os_SFL supporting the theory that SETH4 is the progenitor of this family. A CLUSTAL alignment of SETH4 with SFL1 and SFL2 visualised the global similarity between the family members. The similarity of the proteins was particularly high on the predicted ARM repeats and less so in the low complexity region recognised in the SETH4 protein (Figure 5.6) The SETH4 protein sequence was aligned with the SFL proteins and representative members of the ARM containing families and did not fall in any specific family (Figure 5.7). SETH4 and its family members are relatively small proteins with a small number of ARM repeats which are not placed in tandem as is the case in most of the ARM repeat containing proteins. Also neither SETH4 nor any of the other family members have any other protein features. So the function of the SETH4 and its family members cannot be inferred from the other ARM repeat containing proteins. The similarity of SETH4 to SFL1 and SFL2 extends to the secondary structure. The secondary structure prediction of SETH4, SFL1 and SFL2 was done by SAM-T99 and the results were aligned to each other (Figure 5.8). The positions of the putative ARM repeats were highlighted on the results. The similarity of the three proteins at the secondary structure is encouraging since it leads to the conclusion that the proteins have not diverged enough to have different functional roles in the cell.

5.2.5 Tertiary structure of SETH4, SFL1 and SFL2.

Predictions of the tertiary structure of SETH4, SFL1 and SFL2 were done via the EsyPred3D and SAM-T99 (Lambert *et al.* 2002). The tertiary predictions were visualised with the RASMOL program, and compared to the structure of β-catenin (Figure 5.9). The prediction programs work by aligning the sequence of interest to sequences with a determined tertiary structure. In the case of SFL1 and SFL2 the tertiary structure prediction span almost all of the protein. The series of canonical helices in SFL1 and SFL2 did form the superstructure created by the tandem ARM repeats described by Huber et al., in 1997 for the β-catenin. In the case of the SETH4 protein the tertiary prediction was only for the N-terminal part of the protein since the low complexity region does not allow the sequence comparison algorithms align more of the protein.

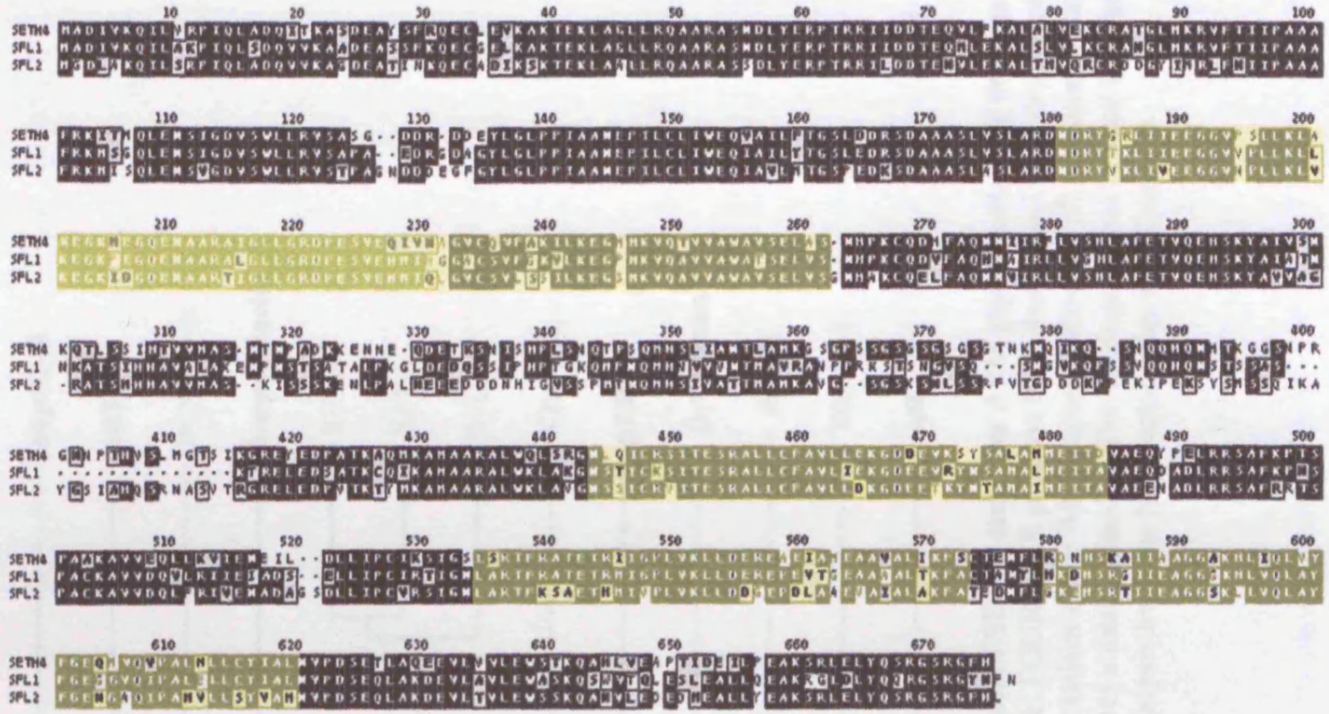


Figure 5.6.
CLUSTAL-W alignment of the SETH4 and the SFL1 and SFL2 proteins. Highlighted in yellow are the predicted ARM repeats.

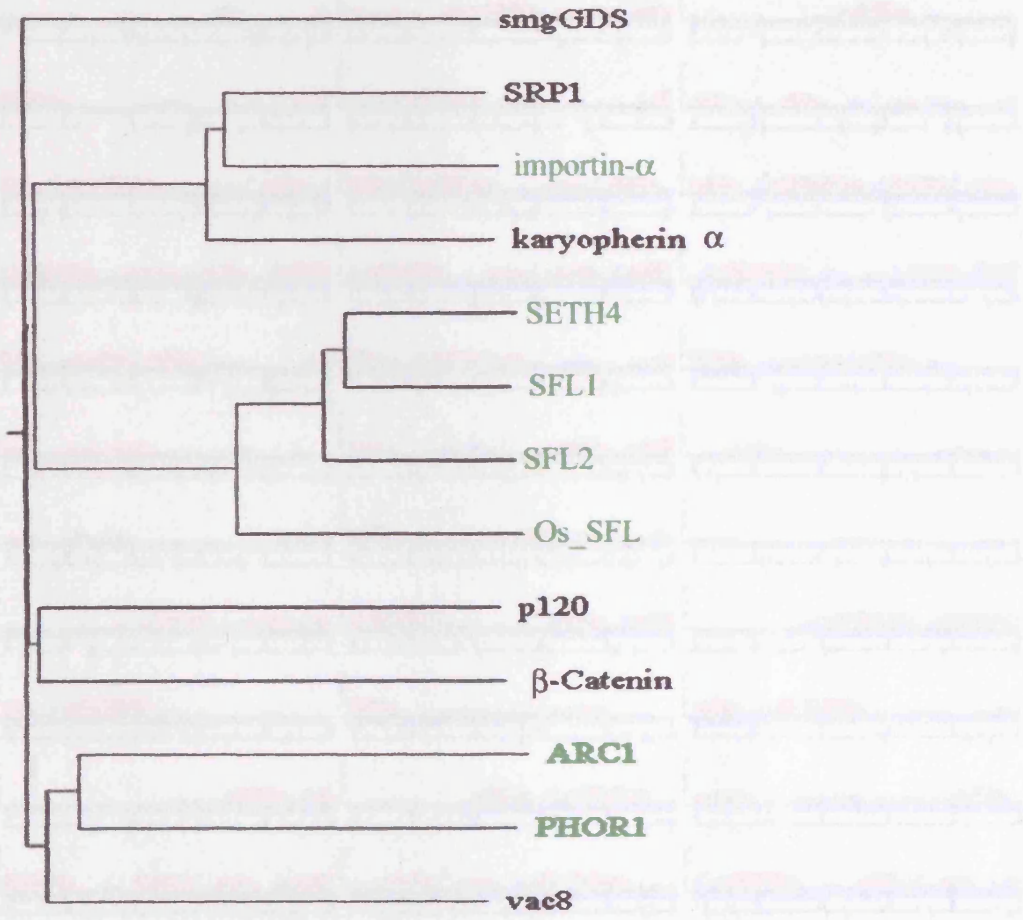


Figure 5.7. Dendogram of SETH4 and other ARM repeat containing proteins. SETH4 and the SETH FOUR LIKE proteins from *Arabidopsis* and rice when compared to representatives of the ARM repeat proteins of all kingdoms (plant proteins in green) form a distinct family. The dendogram was created through the CLUSTAL-W analysis from the MacVector program (Accelrys).

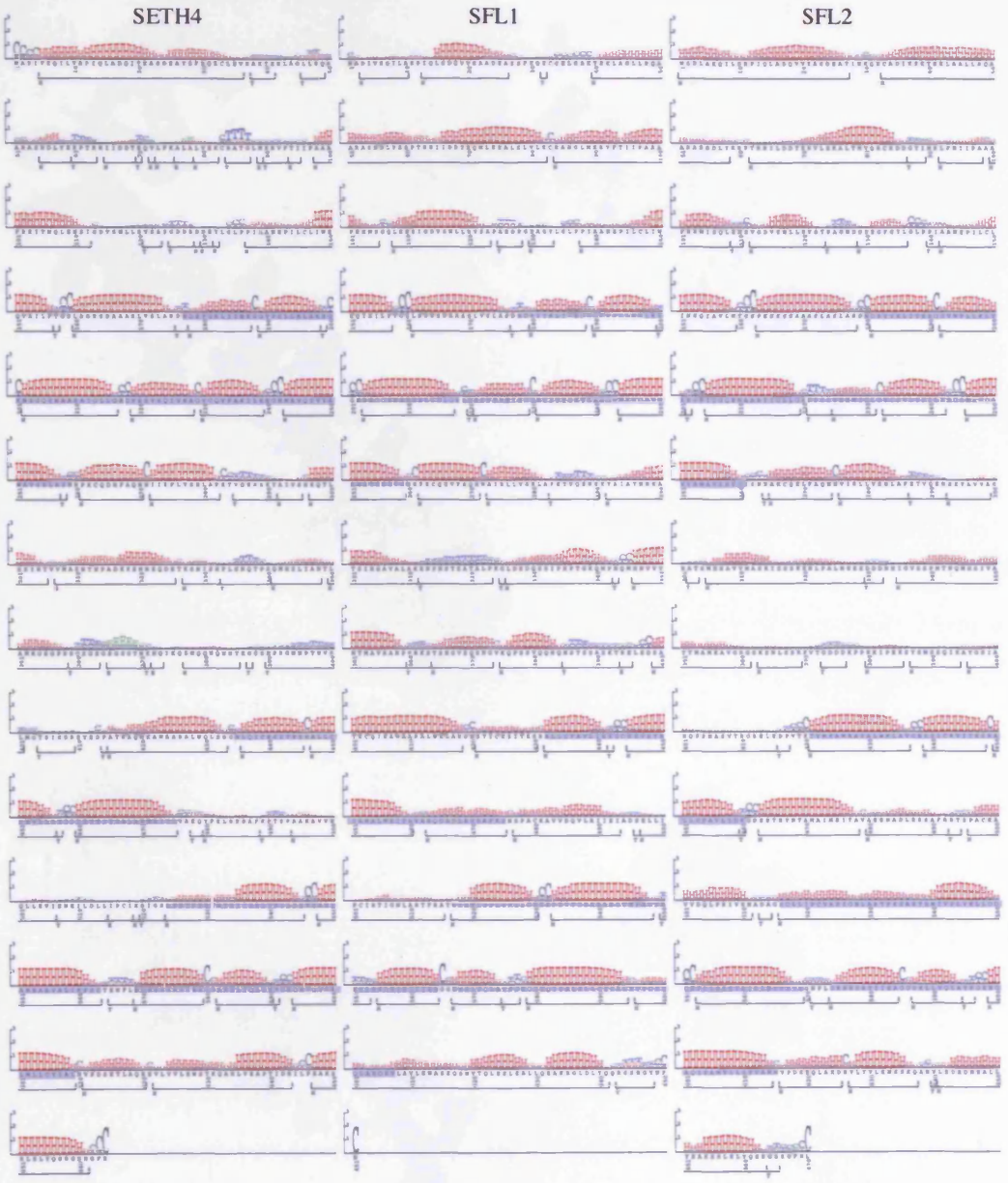


Figure 5.8. Secondary structure prediction of SETH4, SFL1 and SFL2.
Prediction of the secondary structure of SETH4, SFL1 and SFL2 was performed by SAM T-99.The position of the ARM repeats is highlighted in the sequence in blue. In the 4-character EBHTL alphabet used, E is a β–strand, H is helix, C is coil or loop and T is is a predicted turn.

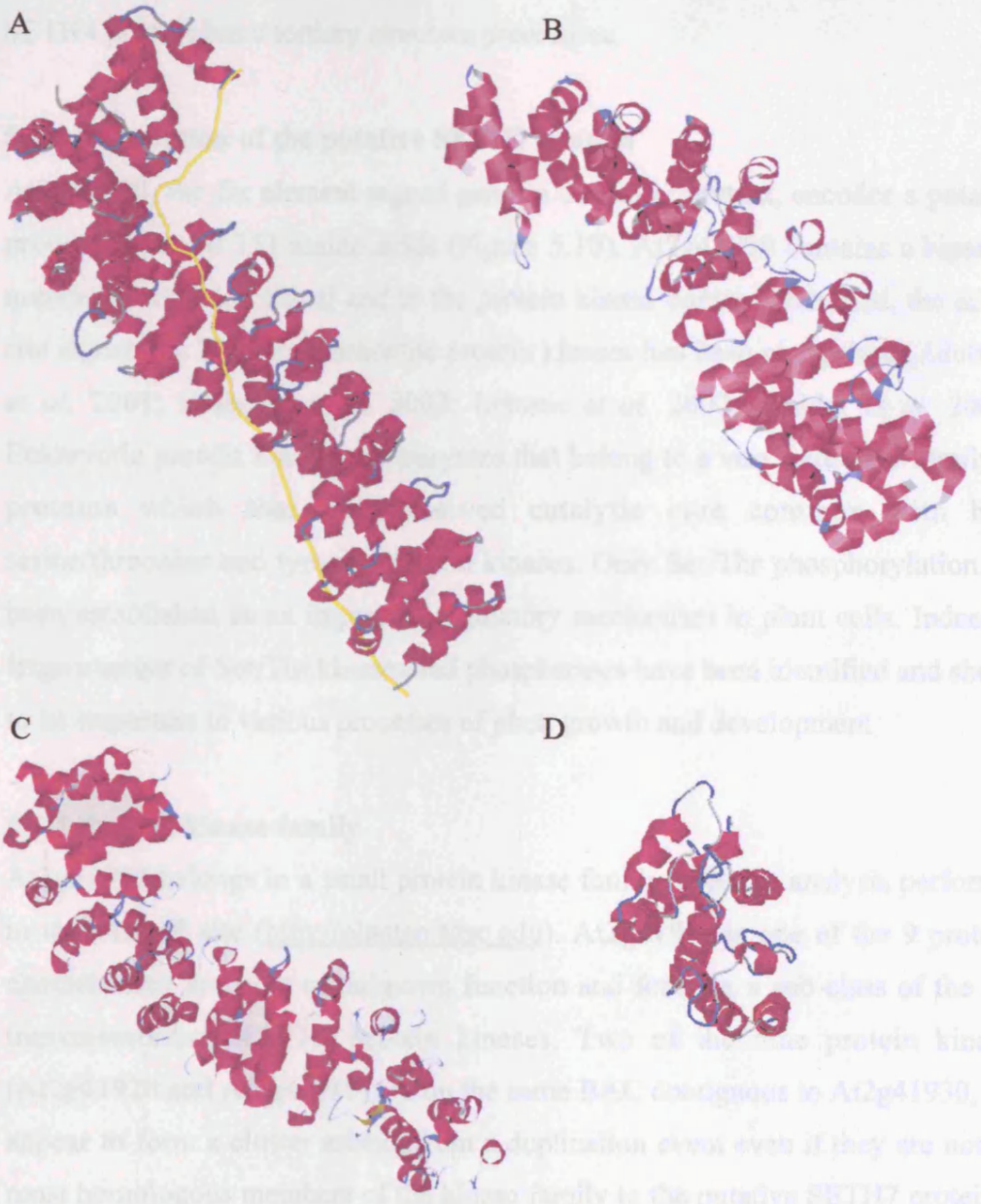


Figure 5.9. Predicted tertiary structure of SETH4, SFL1 and SFL2 .
(A) The tandem ARM repeats in β-CATENIN form a super helix that allows protein-protein interactions (yellow ribbon). In B,C,D, SFL1, SFL2 and SETH4 respectively.form this super structure to a lesser degree. The predicted tertiary structure of SETH4 is for the N-terminal half of the protein since the prediction programs (ESyPred3D in this case), for the time being, do not compensate for the low-complexity region present. The tertary structure was visualised by using the RasMol program.

In reality by deleting part of the low complexity region a bigger part of the SETH4 protein has a tertiary structure prediction.

5.2.6 Description of the putative SETH7 protein

At2g41930, the *Ds* element tagged gene in the *seth7* mutant, encodes a putative protein kinase of 351 amino acids (Figure 5.10). At2g41930 contains a bipartite nuclear localisation signal and in the protein kinase domain identified, the active site signatures for serine/threonine protein kinases has been recognised (Zdobnov *et al.* 2001; Bateman *et al.* 2002; Letunic *et al.* 2002; Mulder *et al.* 2003). Eukaryotic protein kinases are enzymes that belong to a very extensive family of proteins which share a conserved catalytic core common with both serine/threonine and tyrosine protein kinases. Only Ser/Thr phosphorylation has been established as an important regulatory mechanism in plant cells. Indeed, a large number of Ser/Thr kinases and phosphatases have been identified and shown to be important in various processes of plant growth and development

5.2.7 Protein kinase family

At2g41930 belongs in a small protein kinase family from the analysis performed in the PlantsP site (http://plantsp.sdsc.edu). At2g41930 is one of the 9 proteins characterised as being of unknown function and forming a sub-class of the non transmembrane MAP3K protein kinases. Two of the nine protein kinases (At2g41920 and At2g41910) are on the same BAC contiguous to At2g41930, and appear to form a cluster arising from a duplication event even if they are not the most homologous members of the kinase family to the putative SETH7 protein as depicted in Figure 5.11.

5.3 Molecular characterisation of *SETH4*, *SFL1* and *SFL2*.

5.3.1 Spatio /temporal expression of *SETH4*, *SFL1* and *SFL2*

Initial results from the RT-PCR work (4.3.2) showed that *SETH4* was expressed only in flower and pollen tissues. When the expression pattern of *SFL1* and *SFL2* was examined, using the same cDNA for the analysis as was used for the *SETH4*, it was revealed that they are sporophytically expressed (Figure 5.12).

50 AA

```
1   MEFVKVLGKG TYGSVELFSH KQNDGSLLYN AVKIMDSENY GSIDQEFRIL SELRGCPCIV
61  QLCGNSLVQG IDCNGKKVYM MSMEYAAAGT LTNFIKRNRT KLSDSVIKDF TRMILQGLVS
121 IHNHGYVHCD LKPDNILLFP LYDKDTWNCS YELKISDFGI STRAGDKSGC WRVDEPWVGT
181 SIYMSPESVS DGTTVEKTLD LWSLGCIVLK MYTGKRPWLG FEKDVKSLLL NQKAPEIPET
241 LPCDARLFLE KCFSRKPEER GSASELLLHP FLTGDEKKGS SVAGGERTGM VLRLRKPPPI
301 SKDIPTKPRK LKVISQKPQQ LKKVSNKPLK VKIVPPRPPR SDFVPVL
```

1. Eukaryotic protein kinase catalytic domain (hmmpfam (HMMER2.1.1))
2. Serine/Threonine protein kinases active-site signature (prosite_scan)
3 Bipartite nuclear localisation signal. (pfscan (pftools2.2))

Figure 5.10. Diagram of the features of the putative SETH7 protein.
The protein kinase domain of the putative SETH7 with the Serine/Threonine active site signatures is located at the N-terminal part of the protein. In the C-terminal part of the protein a bipartite nuclear localisation signal is predicted.

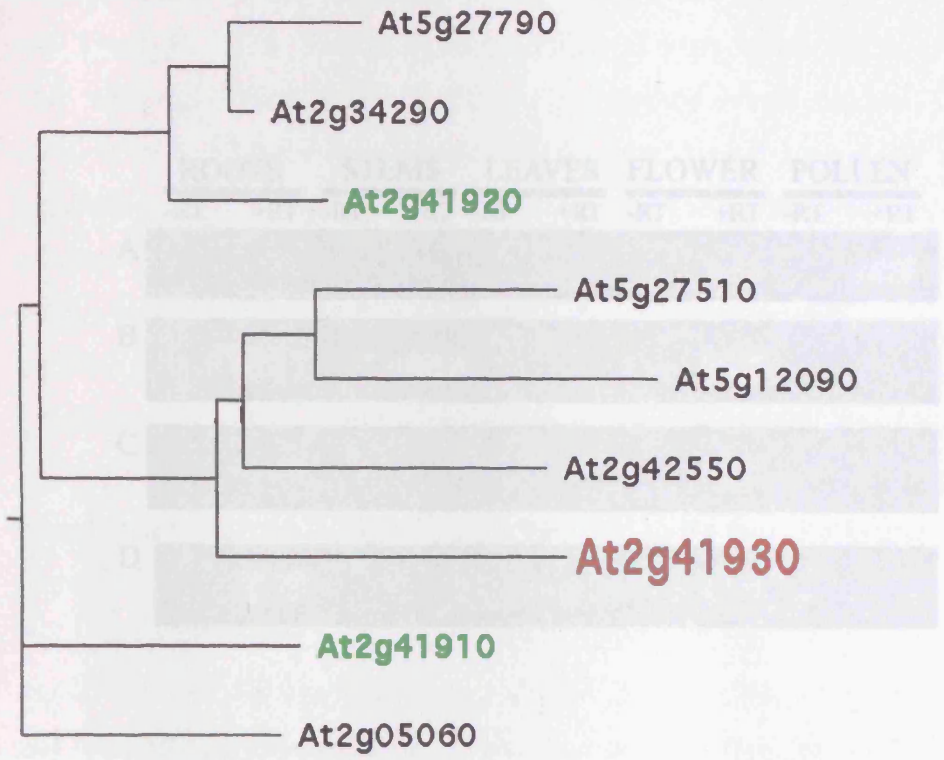


Figure 5.11. Dendogram of the putative SETH7 protein kinase family.
A dendogram of the nine putative protein kinases that form a sub group of the MAP kinase subfamily the putative SETH7 protein (in red) belongs to. At2g41920 and At2g41910, which belong in a physical cluster with the putative SETH7 on the same BAC show less than 50 percent identity to it.

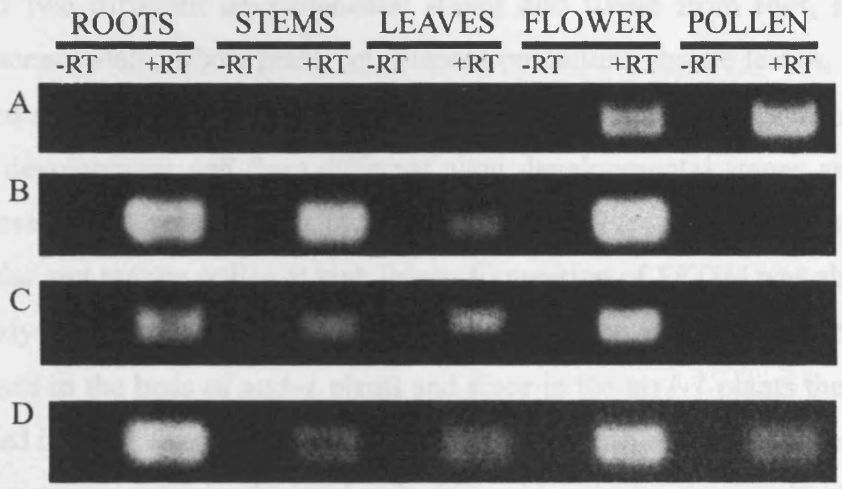


Figure 5.12 Comparison of the expression patterns of *SETH4* and the *SFL* genes by RT-PCR.
Analysis of the expression pattern of *SETH4*, *SFL1* and *SFL2* (A,B and C respectively) via RT-PCR. *SFL1* and *SFL2* show no expression in the mature pollen sample but are expressed in all the sporophytic tissues. The constitutive *KAPP* gene was used as a control for the reaction (D).

More detailed analysis of the SFL family protein expression pattern was brought out by the Affymetrics ATH1 GeneChip analysis. The data from several public experiments done by researchers in conjunction with the NASC centre were amassed and statistically processed by David Honys and presented in (Honys, D. and Twell, D. 2003). The data examined derived from probes of the Affymetrics chip with RNA from pollen in different developmental stages, *ms1-1* and WT buds at two different developmental stages and tissue from root, seedlings, inflorescence stalk, whole plant, cell suspension culture, rosette leaves, root hair-enriched samples and petiole. The data from the ATH1 Affymetrics chip during pollen development and from different plant developmental stages and tissues corroborated the pattern seen by the RT-PCR. *SETH4*, was expressed in the tricellular and mature pollen at high levels. Expression of *SETH4* was also seen in the early and late buds from *ms1-1* and wild type plants. Since *SETH4* is expressed in the buds of *ms1-1* plants and since in the *ms1-1* plants the pollen is degraded in the anther locule after release of the microspores from the tetrad stage the expression seen in the *ms1-1* buds could only be the result of *SETH4* expression in the sporophytic flower tissues (Wilson *et al.* 2001). Neither *SFL1* nor *SFL2* are expressed in mature and developing pollen grains. *SFL1* showed expression in all the tissues and developmental stages (Figure 5.13).

5.3.2 Spatio temporal expression assay of *SETH4* via a promoter eGFP::GUS fusion construct.

In order to further study the expression patterns of *SETH4* a 1000bp fragment from the promoter region of *SETH4* was PCR amplified and inserted in the pKGWFS7 Gateway plant vector, driving the expression of a eGFP::GUS gene fusion protein (section 2.9). Initial analysis of the kanamycin resistant individuals recovered from the infiltrated population was performed. The primary inflorescence was analysed by GUS histochemical staining (section 2.18) and then cleared in 70% ethanol. GUS expression could only be seen be seen in the pollen grains and the pollen tubes of the open flower. However analysis of earlier buds showed that at the –3 to –6 buds from the open flower GUS expression could be observed in the base of the anther filaments and the elongation zones of the pistil in the sub-epidermal region even if no clear structure could be recognised.

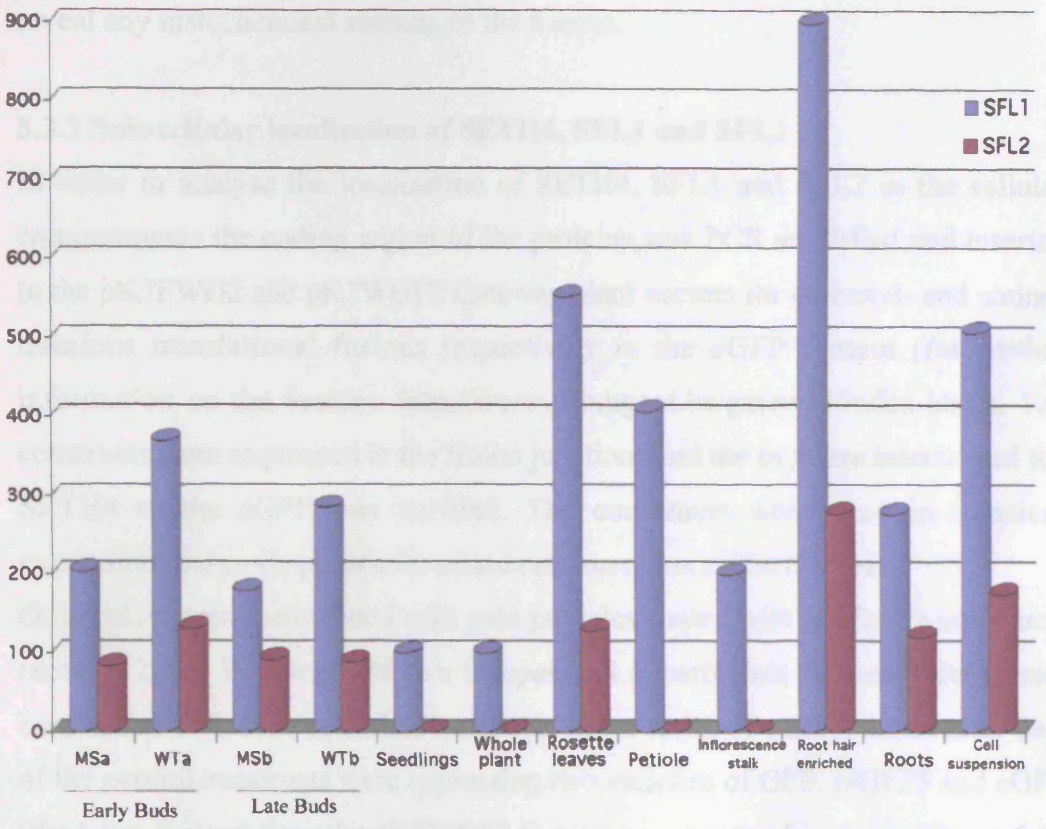


Figure 5.13. The sporophytic pattern of expression of the *SFL* genes. *SFL2* expression pattern, as analysed in the public domain organ tissue specific probes of the Arabidopsis Affymetrics ATH1 GeneChip, revealed that *SFL1* is constitutively expressed and at a higher level than *SFL2*. MS are buds collected from *ms1-1* plants.

In the flower buds after the –6 bud, GUS expression could only be observed in the anther filaments and the developing petals (Figure 5.14). An assay performed on the progeny of these lines for GUS expression in the roots and seedling did not reveal any histochemical staining of the tissues.

5.3.3 Sub-cellular localisation of SETH4, SFL1 and SFL2.

In order to analyse the localisation of SETH4, SFL1 and SFL2 in the cellular compartments the coding region of the proteins was PCR amplified and inserted in the pK7FWG2 and pK7WGF2 Gateway plant vectors for carboxyl- and amino-terminus translational fusions respectively to the eGFP protein (for further information on the vectors: http://www.psb.ugent.be/gateway/index.html). The constructs were sequenced at the fusion junctions and the *in frame* insertion of the SETH4 to the eGFP was verified. The constructs were used in transient expression assays via particle bombardment on onion epidermis cells.

Onion skins were bombarded with gold particles coated with the fusion constructs (section 2.10). In a series of two independent experiments the onion cells were bombarded with the two fusion constructs and a series of control constructs. Two of the control constructs were expressing two varieties of GFP. mGFP5 and eGFP (the latter derived from the pK7WGF2 Gateway vector can be seen in Figure 5.15 C) localisation in cells expressing the GFP with these two constructs was cytoplasmic and nuclear since the GFP protein on its own, at approx 33kDa, is small enough to pass through the nuclear pores. The other control construct was a GFP fused to the β-glucoronidase gene derived from the pCambia vectors. With this construct, the GFP signal in the transformed cells was expected and seen only in the cytosol since the fusion protein is too big to be passively diffused in the nucleus.

C-terminal fusions of SETH4 to eGFP

In two independent series of transient expression experiments done, eGFP fluorescence was only seen in 3 onion cells. The eGFP fluorescence signal was cytoplasmic only for two of the cells expressing eGFP. For the third cell expressing eGFP the fluorescence seen was punctuated and localised to the cell surface (Figure 5.15A).

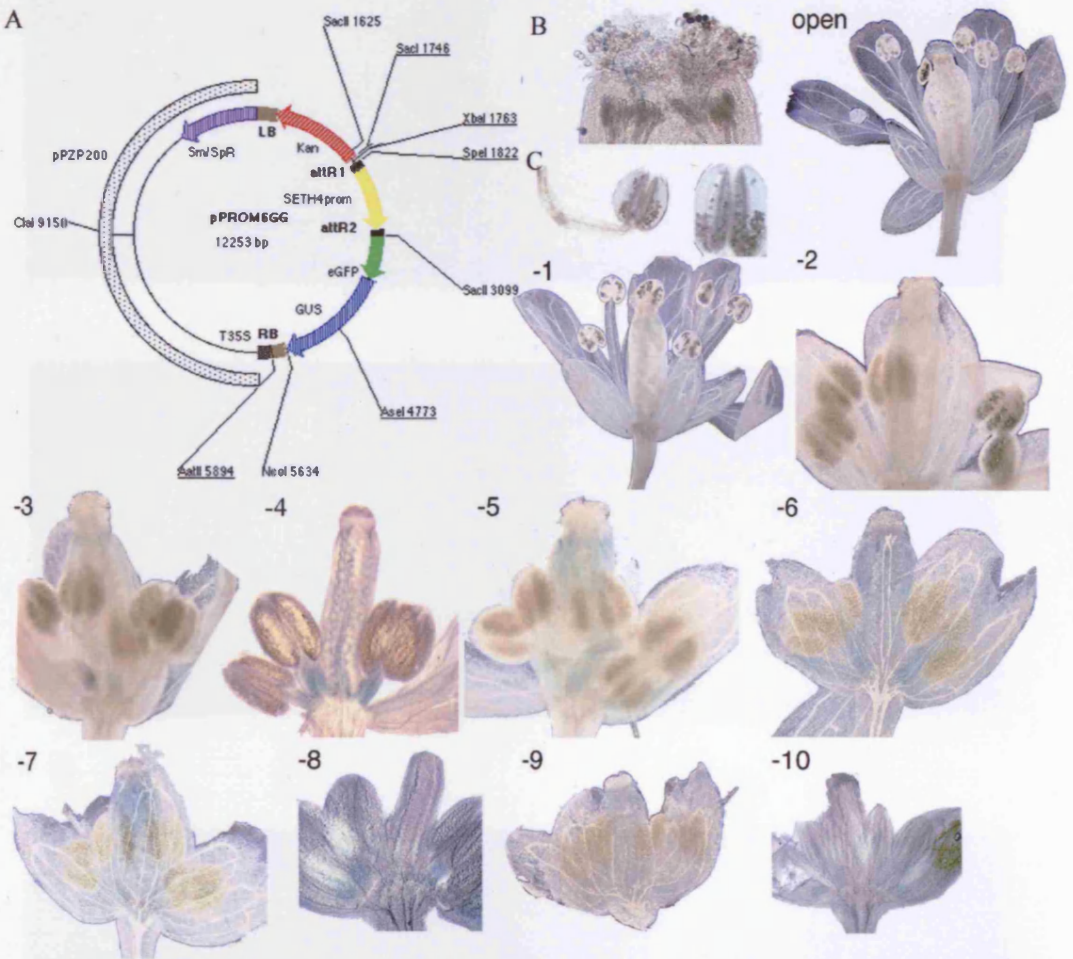
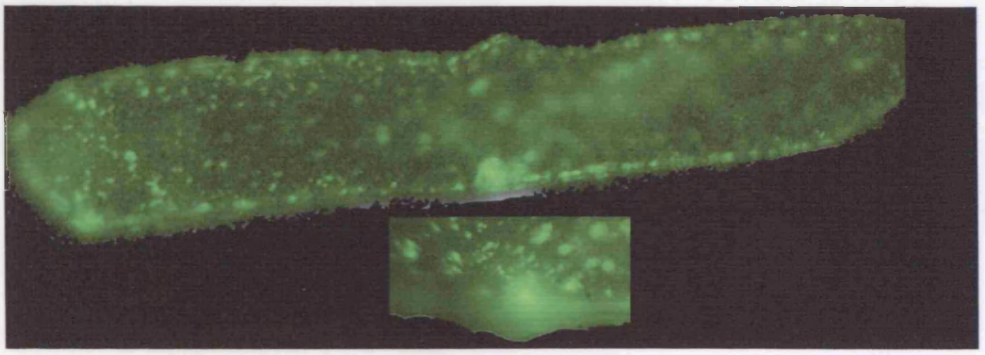


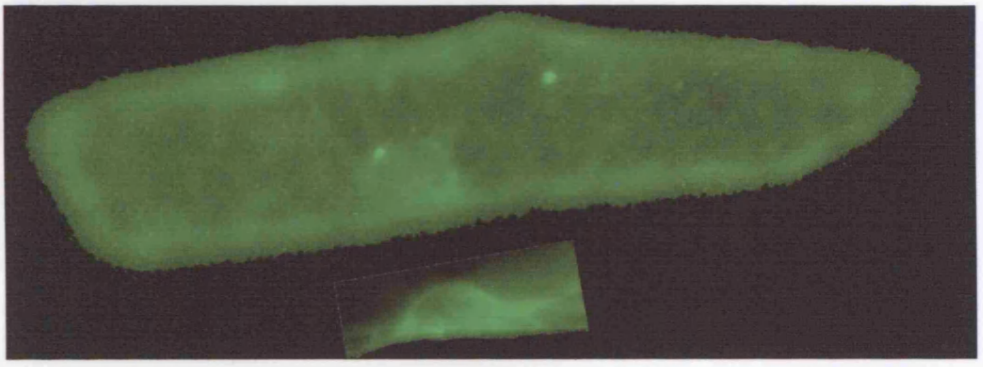
Figure 5.14. Promoter analysis of *SETH4* revealed male gametophyte expression of the eGFP:GUS protein.

The spatio-temporal expression of *SETH4* was examined by the stable transformation of *Arabidopsis* with the transcriptional fusion of the *SETH4* promoter to the eGFP:GUS fusion protein from GATEWAY (A). Expression of the eGFP:GUS fusion protein was inflorescence specific. The open flower and the subsequent ten buds (labelled respectively open and then -1 to -10) were opened, stained for GUS expression and cleared. GUS staining was localised in the emerging pollen tube (B) and in the mature pollen on the anther (C) and in the immature buds in the rapidly elongating zones of the pistil and petals.

A



B



C

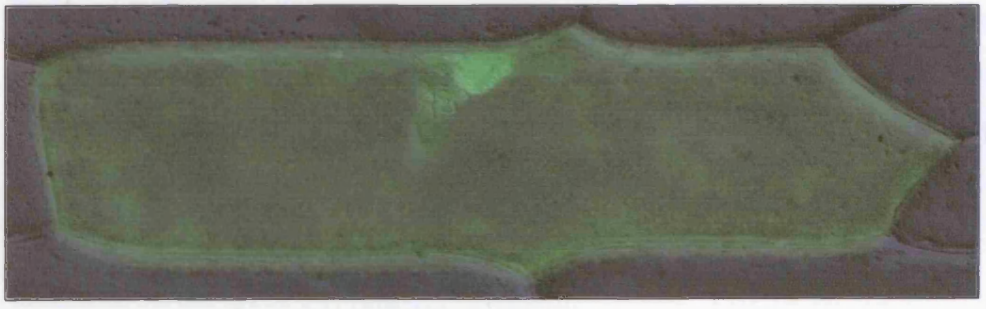


Figure 5.15.
Analysis of the SETH4 localisation pattern via transient expression of the C and N terminal eGFP translational fusions (A and B respectively) of SETH4 with eGFP from the GATEWAY Vectors in onion skin cells. In C the localisation pattern of the control GATEWAY vector pK7WGF2 that expresses only the eGFP protein, is presented.

N-terminal fusions of SETH4 to eGFP

In two independent series of transient expression experiments done, eGFP fluorescence was seen in 45 onion cells. In the 43 of the cells exhibiting eGFP fluorescence the eGFP signal localised in the cytoplasm only. In the other two cells with eGFP fluorescence the localisation of the signal could not be established (Figure 5.15b).

C-terminal fusions of SFL1 to eGFP

In the transient expression experiments done, eGFP fluorescence was only seen in 29 onion skin cells. The eGFP fluorescence signal was cytoplasmic for 28 of the cells expressing eGFP. For the 29th cell expressing eGFP the fluorescence seen was punctuated and localised to the cell surface (Figure 5.16A).

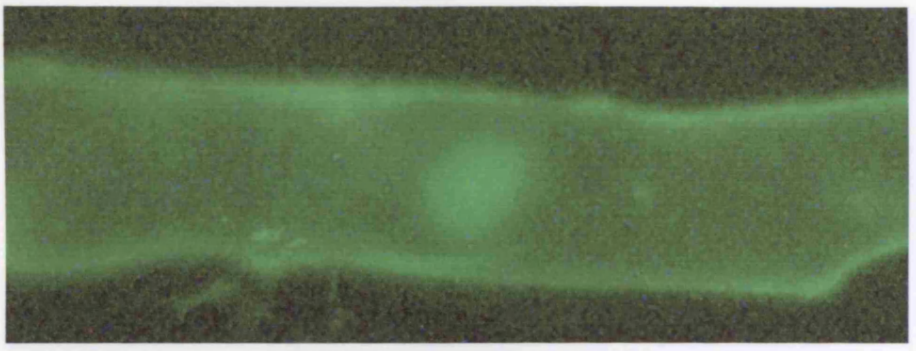
N-terminal fusions of SFL1 to eGFP

In the transient expression experiments done, eGFP fluorescence was seen in 102 onion skin cells. In the 88 of the cells exhibiting eGFP fluorescence the eGFP signal localised in the cytoplasm only. In 12 cells with eGFP fluorescence the localisation of the signal was both nuclear and cytoplasmic. In the other two eGFP expressing cells, the localisation pattern was either punctuated and localised to the cell surface or cytoplasmic and potentially localising on the cytoskeleton (Figure 5.16B).

C-terminal fusions of SFL2 to eGFP

In the transient expression experiments done, eGFP fluorescence was seen in nine onion skin cells. The eGFP fluorescence signal was cytoplasmic for three of the cells expressing eGFP. In three cells with eGFP fluorescence the localisation of the signal was both nuclear and cytoplasmic. One cell was showing cytoplasmic localisation and punctuated cell surface fluorescence. For two cells expressing eGFP the fluorescence seen was punctuated and localised to the cell surface (Figure 5.17A).

A



B

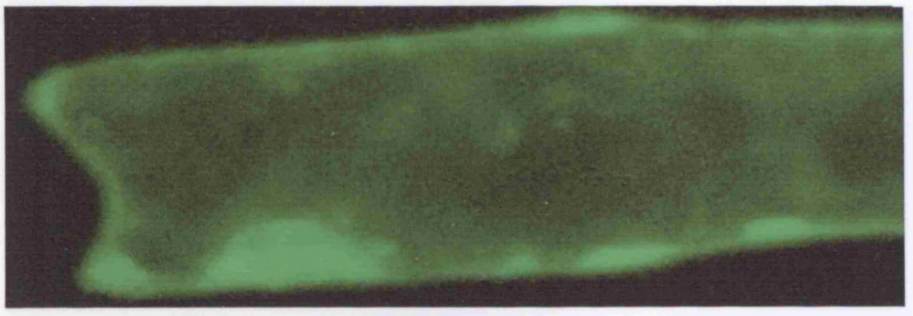
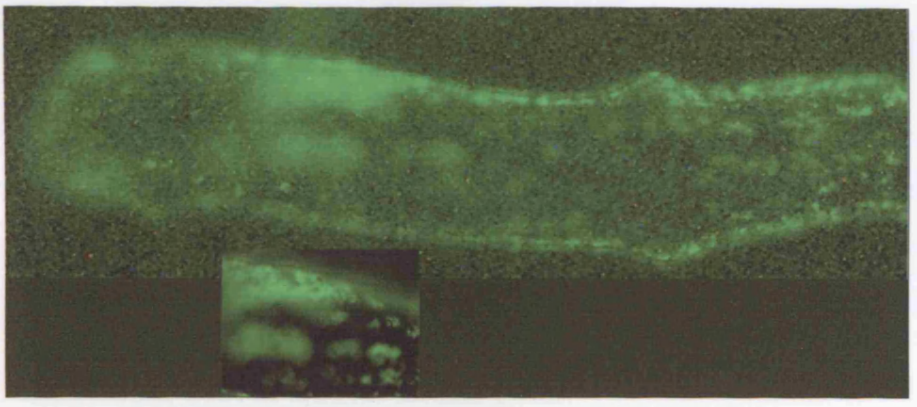


Figure 5.16. Analysis of the SFL1 localisation pattern via transient expression of the C and N terminal translational fusions (A and B respectively) of SETH4 with eGFP from the GATEWAY Vectors in onion skin cells.

A



B

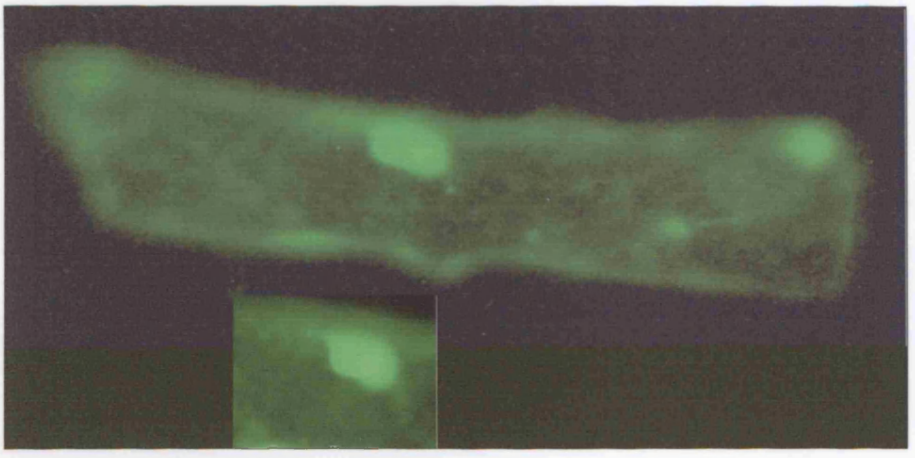


Figure 5.17. Analysis of the SFL2 localisation pattern via transient expression of the C and N terminal translational fusions (A and B respectively) of SETH4 with eGFP from the GATEWAY Vectors in onion skin cells.

N-terminal fusions of SFL2 to eGFP.

In the transient expression experiments done, eGFP fluorescence was seen in one onion skin cell. In the cell with eGFP fluorescence the localisation of the signal was both nuclear and cytoplasmic (Figure 5.17B)

5.4.2 Stable expression of the fusion protein in Arabidopsis roots.

Arabidopsis (ecotype Ler) was transformed with the two SETH4 N- and C-terminal eGFP fusion constructs and the construct expressing the eGFP. Root samples from resistant individuals from this generation were picked out and examined microscopically

From the N-terminal fusions of SETH4 to eGFP T_0 generation thirteen kanamycin resistant individuals were recovered. Analysis of the eGFP expression in the roots of the progeny of those plants revealed a mainly cytosolic localisation pattern.

From the C-terminal fusions of SETH4 to eGFP T0 generation, two kanamycin resistant individuals were recovered. No eGFP expression could be seen though. From the plants transformed with the control pK7WGF2 vector, strong nuclear localisation was seen (Figure 5.18)

5.6 Discussion

After the phenotypic and molecular characterisation of *seth4* and *seth7* the focus of the work was based on the analysis of the function of *SETH4*, *SFL1* and *SFL2*. The *SETH FOUR LIKE (SFL)* family presented a very interesting proposal. *SETH4* is essential for pollen tube germination and tube growth. The function of the protein cannot be elucidated from a bioinformatic analysis and the only close homologues are two unknown proteins, which share a high degree of similarity in the primary, secondary and tertiary structure, and the same organisation of the ARM repeats. Preliminary analysis of the expression patterns of *SFL1* and *SFL2* showed that they are expressed in all the tissues bar pollen.

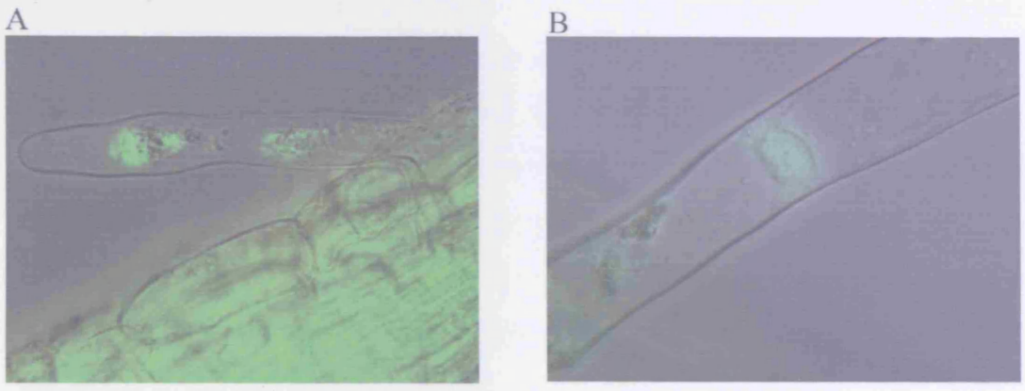


Figure 5.18. Stable transformations of the C- and N- terminal translational fusions of SETH4 to eGFP.
Root hairs from arabidopsis T2 plants transformed with the C- and N- terminal fusions and the GFP control vector resistant to kanamycin were examined for GFP expression and localisation. Plants transformed with the GFP fused to the N- terminal of the SETH4 showed cytoplasmic localisation of the GFP (A) whereas no expression of GFP was seen in the plants transformed with the GFP fused to the C- terminal of the SETH4. The plants transformed with the GFP control vector showed only nuclear localisation of the GFP (B).

This result gave rise to the hypothesis that SETH4 and the SFL family have the same function in the plant but SETH4 has a specialised role in the male gametophyte. SFL1 and SFL2 show similar expression pattern in the RT-PCR analysis. Of particular interest is that *SFL1* expression is at least half as much in the bud stages in the wild type samples as it is in the male sterile (*ms1-1*) samples. The difference in the expression is not due to the pollen expression since *SFL1* is not expressed there. This difference in expression level could be explained either due to the degeneration of the tissues it is expressed in the *ms1-1* plant or that *SFL1* expression is controlled by *MS1* (At5g22260). This induced expression in the wt buds is only observed in the early bud stage for the *SFL2*. The expression of *SFL2* is almost identical for the *ms1-1* and wt buds at the later stage. The expression pattern for the *SFL2* differs from the *SFL1* profile in other aspects as well. *SFL2* is expressed, according to the Affymetrix data, only on the rosette leaves, the root and root hair enriched samples and cell suspension samples; these are the samples at which *SFL1* is expressed the highest. It has been known that having multiple genes encoding similar proteins under the control of different expression signals enables the plant to respond with increased flexibility to developmental and environmental signals. In addition to that, non identical but very similar proteins that are expressed simultaneously in cells could allow for greater functionality and stability of the processes these proteins are involved.

The Spatio/temporal expression of *SETH4* is not exclusively gametophytic.

Results from the GUS expression pattern and the Affymetrix data show that SETH4 is expressed in the gametophyte in the tricellular and mature pollen and in the pollen tube. The results also reveal though that SETH4 is expressed in the sporophyte as well. GUS expression was seen in the rapidly elongating tissues of the flower but not in any other tissues. From this expression pattern it could be proposed that SETH4 might be necessary for the establisment of growth in plant cells.

The localisation of the SETH4, SFL1 and SFL2 proteins

The SETH4, SFL1 and SFL2 eGFP fusion proteins were all mainly localised cytoplasmically. In the SFL2 and SETH4 fusions there were occasions that the

localisation patterns did exhibit stricking patterns. Even if the exclusion of artefacts in the transient expression assays cannot be done it is interesting that two different constructs in separate experiments showed this punctuated expression of GFP on the cell wall. Especially when dealing with ARM repeat containing proteins, localisation pattern seen is not necessarily indicative of the sub-cellular localisation of the proteins in vivo. Comparisons could be made with the shuffling of the ARC1, RHOR1, α-importin, vac8 and β-catenin, all of which are ARM repeat containing proteins and are known to function and localise to the cytoplasm, nucleus, vacuole and cell plate.

Chapter 6
General Discussion

6.1 The male gametophyte during the progamic phase.

After landing on the surface of a compatible stigma, pollen grains germinate and form a pollen tube, which penetrates the female reproductive organ. Vegetative pollen tube cells elongate rapidly and in a strictly polar manner, as they grow through pistil tissue and transport male sperm cells enclosed in their cytoplasm to the egg apparatus located within ovules. Pollen tube mediated fertilisation is essential for sexual plant reproduction as well as for seed production, and depends on unknown signalling mechanisms that guide growing pollen tubes along their path. Elongating pollen tubes are an excellent model system to study the mechanism, the intracellular regulation, and the extracellular control of polar plant cell growth, a process that contributes essentially to cellular and organ morphogenesis throughout plant development. Pollen tube growth is characterised by the exclusive incorporation of new cell membrane and cell wall material in a small area at the requiring massive tip-directed targeted secretion, endocytic membrane recycling and cytoskeleton-mediated rapid organelle movement ("cytoplasmic streaming") (Hepler *et al.* 2001). Ca^{2+} ions (Messerli *et al.* 2000) and Rac type small GTPases (Kost, B. *et al.* 1999; Li *et al.* 1999b), which activate a phosphatidylinositol monophosphate kinase (PIP-K) that produces phosphatidylinositol 4,5 bisphosphate (PI 4,5-P_2) (Kost, B. *et al.* 1999), have been identified as key intracellular regulators of polar pollen tube tip growth. Additional factors with possible functions in the control of this process include a sub-apical alkaline band (Feijo, *et al.* 1999) and cAMP (Moutinho *et al.* 2001).

6.2 Identification of genes affecting the progamic phase.

Despite decades of intense research it has remained unclear

- how growth polarity is established and maintained in pollen tubes,
- how secretion, endocytic membrane recycling and cytoplasmic streaming are functionally integrated in these cells,
- how Ca^{2+}, Rac and possibly other signalling pathways interact with each other to coordinate the different intracellular processes that bring about tip growth, and
- how external factors control these intracellular signalling pathways to ensure pollen tubes stay on track on their way to ovules.

Current knowledge of the mechanism and the regulation of pollen tube tip growth derives largely from cell biological studies supported by biochemical experiments and by the analysis of effects of over-expressing pollen tube genes. Recent investigations of the pollen transcriptome have indicated, that 5000 genes are expressed in mature *Arabidopsis* pollen at the onset of pollen tube growth (Honys, and Twell 2003). Around 1000 of those genes were estimated to be specifically expressed in male reproductive cells (Honys and Twell 2003).

Such a wealth of information though, cannot be exploited at the moment. Reliable techniques that allow down regulation of the expression of specific genes in pollen tubes are not available to date. Reverse genetic strategies based on the information above might not lead to the identification of a phenotype/function for the gene(s) of interest due to redundancy issue (Steinebrunner *et al.* 2003). Mutations that disrupt the development of haploid male reproductive cells are difficult to screen for, because plants homozygous for such mutations cannot be generated. Plants heterozygous for such mutations produce large numbers of normal pollen tubes that overgrow mutant pollen tubes and complicate their identification. Screening for pollen tube mutants therefore requires the analysis of the sexual transmission of genetic markers linked to introduced mutations. *Arabidopsis* mutant screens have resulted in the identification of comparably few mutants with defects in pollen tube elongation or guidance, which allowed insights into the cellular and molecular processes governing pollen tube tip growth (Schiefelbein *et al.* 1993; Howden *et al.* 1998b; Grini *et al.* 1999; Procissi *et al.* 2001; Pacini and Hesse 2002; Palanivelu *et al.* 2003; Procissi *et al.* 2003; Lalanne *et al.* 2004).

When the current work has commenced, the SLAT lines were considered to be the best population for genome coverage and the use of the *En* element for the insertional mutagenesis was supposed to lead to a higher proportion of independent insertions that were also less genetically complex than the T-DNA integrations in the genome. For a genetic screen as the one undertaken such options are of paramount importance. The genetic and molecular characterisation of the putants would be easier as well as the identification of the site of insertion. Also the selection on soil facilitated the screening procedures particularly for this search for gametophytic mutations. Of course no gametophytic mutations were

identified from the screen due to a combination of events like the leaky counterselection for the launch–pad and the propagation of the independent lines in pools. An improved screen for *Arabidopsis* male progamic phase mutants, would be based on insertion mutagenesis using a T-DNA construct carrying a constitutively expressed BASTA resistance gene and a cDNA coding for a GFP::GUS fusion protein, under the control of Lat52 promoter. T-DNA insertion mutagenesis using this construct would allow for the re-screening of the lines showing reduced sexual transmission of BASTA resistance for pollen tube defects by staining for GUS activity. The defective male gametophytes would carry the GFP::GUS fusion protein and it would be possible to follow the defective pollen tubes and efficiently characterize the phenotype of the insertion.

Another approach for the identification of progamic phase genes would be a very targeted reverse genetics strategy based on existing data on the expression profile of the mature pollen or the germinating pollen grain, the latter an envisioned assay utilising the Affymetrix GeneChip. The aim of this experiment would be the identification of genes that are constitutively expressed during pollen tube elongation. By examining the expression profile of *Arabidopsis* pollen tubes grown *in vitro*, the genes expected to be up-regulated or down-regulated are going to be only a sub fraction of the genes expressed in the pollen tube *in planta*. The proteins necessary for the nurture and the elongation of the pollen tube cell should be expressed. In addition to these genes, a more detailed experiment could reveal genes activated earlier on during pollen tube germination. It has to be taken into consideration though that since only 70 % of the pollen do germinate there is going to be a percentage of pollen grains that are out of synch with the rest of the population and do not/will not progress through the pollen tube initiation and elongation.

An interesting class of mutants to be examined with the approach described above would be the ARM repeat containing family of proteins. Recent studies for ARM repeats, based on searches of the entire protein database, have revealed that at least one in 500 proteins contains ARM repeats (Andrade et al.,2001). The frequent occurrence of the ARM repeat is suggestive of important ability of the ARM repeat motif to allow for diverse protein-protein interactions. In the complete *Arabidopsis* genome 105 proteins were identified carrying the Arm

repeats by combining the results from the search protein by features from the TAIR website, PIR SuperFamily website, SUPERFAMILY website, PRINTS website, PROSITE website and PFAM website since the different profiles used by these pattern search sites should identify all the putant Arm repeat containing proteins. Identification through bioinformatic approaches of ARM repeats in *Arabidopsis* has been the scope of two laboratories during 2003 (Coates 2003; Mudgil *et al.* 2003). After CLUSTAL-W analysis of the 105 proteins a dendogram representing their relative similarities was constructed and the proteins could be classified in eleven groups. Utilising the expression data from Honys et al, 2002, the gametophytic or sporophytic specificity of these proteins was assigned as well as the any other known features of the proteins (Figure 6.1). Such an exercise should reduce the possibility for genetic redundancy affecting the reverse genetic screens making it an interesting proposal.

6.3 Perspectives and concluding remarks

The *seth4* and *seth7* mutations are male specific, affect the progamic development and the mutant pollen grains are defective in pollen tube germination and growth and are unable to fertilise the ovules. The *Ds* transposon insertion sites were identified and the genes affected from these insertions analysed further. The spatio/temporal expression of the genes was examined and both genes are expressed only in the pollen and flower tissues.

SETH4 encodes for a novel function protein. It is part of a three-member protein family in *Arabidopsis* and has a homologue in rice. SETH4, SFL1 and SFL2 (the other members of the *Arabidopsis* family) and the SETH4 homologous protein from rice have five ARM repeat motifs similarly spatially organised.

Figure 6.1. Characterisation of the 105 ARM repeat containing proteins in *Arabidopsis.*

The Arabidopsis proteins containing ARM repeats were identified through the TAIR, SMART, INTERPRO and Superfamily databases. Through CLUSTAL-W analysis of identifed proteins via the MacVector program, the dendogram depicted on the right was produced to visualise the groups of similar proteins in the extended ARM repeat containing family. Other known domains contained in the proteins are written on the side of the proteins. The expression profile of the genes (based on the data from the ATH1 affymetrix genechip) was presented as well. The genes expressed only in the male gametophyte are in the blue parallelogram whereas the sporophyte specific ones in the red parallelogram. The genes in the grey typeface have no expression data present in the ATH1 genechip. SETH4, SFL1 and SFL2 form their own sub-family present on the top of the figure.
U-box: a motif associated with proteasomal functions; C2 domain: phospholipid-binding domain; Kinesin: kinesin motor domain; BTB/POZ: Present in a fraction of zinc finger proteins, the BTB/POZ domain mediates homomeric dimerisation; HECT: (Homologous to the E6-AP Carboxyl Terminus) involved in ubiquitin-protein ligase activity.


AT3G26600
AT1G18500
AT5G65920 u box
AT3G49830 u box
AT3G09340 u box
AT4G36550 u box
AT1G67530 u box
AT1G24330 u box
AT1G27910 u box
AT5G65200 u box
AT5G62560 u box
AT3G47820 u box
AT5G40140 u box
AT4G21350 u box
AT5G58680 none
AT3G01400 none
AT3G54590 u box
AT2G23140 u box
AT5G42330 u box
AT3G54850 u box
AT3G46510 u box
AT2G28830 u box
AT1G71020 u box
AT1G23030 u box
AT5G07360 u box
AT5G06180 u box
AT1G29340 u box
AT1G06190 u box
AT1G10560 u box
AT1G08320 bZIP
AT1G76300 u box
AT1G28780 u box
AT5G58900 none
AT2G45720 none
AT1G01830 none
AT2G05810 none
AT1G61350 none
AT1G77460 C2 domain
AT1G44120 C2 domain
AT5G14530 none
AT4G12710 none
AT3G03440 none
AT4G35180 none
AT2G25130 none
AT4G03940 none
AT3G09350 none
AT3G53800 none
AT5G52000 importin a
AT5G49510 importin a
AT3G06720 importin a
AT1G09270 importin a
AT4G02150 importin a
AT1G02690 importin a
AT3G06570 importin a
AT5G03070 importin a
AT5G02580 HECT
AT5G37290 none
AT4G16490 none
AT1G51350 none
AT3G54870 KINESIN
AT1G12430 KINESIN
AT1G01950 KINESIN
AT4G03940 none
AT3G60350 β-CATENIN
AT2G44900 β-CATENIN
AT3G20170 none
AT5G19330 none
AT5G13060 BTB/POZ

ARM repeats form protein-protein interaction domains well characterised in other species and *Arabidopsis* (reviewed in Coates 2003). Proteins containing ARM repeats are implicated in cell signalling, localising proteins to organelles and acting as transcription regulators. For instance a protein involved in self-incompatibility is a well characterised ARM repeat. ARM Repeat Containing 1 (ARC1) protein contains five potential ARM repeats that bind an S-locus receptor kinase (SRK) (Stone *et al.* 2003). The kinase domain of SRK has been shown to bind to and phosphorylate ARC1 *in vitro*. A mutation in the kinase domain of SRK abolishes its binding to ARC1, indicating that the phosphorylation is essential for the interaction. *ARC1* expression is restricted to the stigma, where the self-incompatibility response occurs, and silencing of the *ARC1* gene results in loss of self-incompatibility, suggesting that it is an essential component of signaling downstream of SRK. The organisation of the ARM repeats in SETH4 and its family members, does not conform to the ARM repeat pattern identified in the other ARM containing proteins suggesting for novel function for the proteins. The expression pattern of *SFL1* and *SFL2* is complementary to the expression pattern of *SETH4*. Plants homozygous for either the SFL1 or the SFL2 knockout do not show any mutant phenotype suggesting a redundancy in their function. Based on the essential role of SETH4 during the progamic phase a double homozygous plant for both SFL1 and SFL2 knockouts would be expected to show a severe or lethal phenotype. Creation of such a double homozygote is underway. The cytoplasmic localisation pattern of SETH4, SFL1 and SFL2 seen in the carboxyl and amino terminal fusions of the proteins to the eGFP is not necessarily indicative of the sub-cellular localization of the proteins in vivo. In the *Arabidopsis* PHOR1, another ARM repeat containing protein, nuclear localisation of the protein is dependent on GA and resembles regulated nuclear localization of the armadillo/β-catenin proteins. Protein sorting studies with deletion mutants of the PHOR1 protein fused to GFP or to the GFP-GUS fusion demonstrated that it is the armadillo repeat that mediates nuclear sorting of the protein, whereas the N-terminal CPI domain is involved in cytoplasmic retention of the protein (Amador *et al.* 2001). So even if the localization pattern for the SFLs observed is cytoplasmic only, there is the possibility that through an interaction with another

protein/signaling molecule alter their localisation a phenomenon possibly witnessed in the transient localisations of the proteins to the cell wall.

To further characterise this novel family, a series of experiments would have to be done.

Cross-complementation of the *seth4* phenotype with the SFL1 and the SFL2 genes driven by the native *SETH4* promoter. This experiment would prove the functional redundancy of the family members. Until the exact phenotype the double knockout line for the sporophytic genes is known it is difficult to reflect on the possible experiments done. But it is very likely that through the identification of a novel gene essential for the pollen development, a novel pathway of interacting components would be identified in the sporophyte.

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