The phytochrome regulation of growth and extracellular peroxidase activity

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by

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Ph.D. Thesis: - Abstract

by Brigitte Simone Hurwitt

The phytochrome regulation of growth and extracellular peroxidase activity.

Changes in extracellular peroxidase activity may be mediated by phytochrome as a means of regulating growth rate changes. This was tested in the first internode of light grown mustard seedlings. A correlation between an extracellular anionic peroxidase isoform (A4), extracted by infiltration/centrifugation, was found to decrease in activity by 50% when growth rate was enhanced by a low R:FR ratio. The preparation of protoplasts revealed A4 to be more or less exclusively extracellular. The phytochrome-regulation of A4, apparent in the small percentage of the enzyme that could be extracted by infiltration/centrifugation, was not repeated when the major pool of A4 was examined. A light regulated increase in peroxidase activity was however found in mustard hypocotyls. The light-regulation of cucumber hypocotyl growth was tested and changes in a ionically bound (IB) cationic peroxidase fraction examined. More specifically the use of a long hypocotyl, phytochrome B deficient cucumber mutant (*lh*), enabled further speculation as to the phytochrome species involved in these changes. When examined, the IB peroxidase activity increased in activity within two hours of the addition of supplementary FR light (a low R:FR ratio), correlating with a change in the rate of growth that could be detected using a mm scale ruler. Whether changes in extracellular peroxidase activity constitute a primary mechanism in the phytochrome-mediation of growth rate changes in light-grown cucumber hypocotyls remained indeterminable. Speculations and possible importance of the observed correlations are discussed. In etiolated cucumber seedlings phytochrome has been shown to control growth within minutes of exposure to light. The extracellular peroxidase activity however, remained unaltered until two days after the commencement of de-etiolation. Thus it was postulated that there appears to be two separate mechanisms in cucumber hypocotyls by which phytochrome-mediated growth rate changes and associated changes in cell wall extensibility are regulated

Abbreviations:

2,4-D - 2,4-Dichlorophenoxyacetic acid
4CN - 4 chloronaphthol
ACC - 1-aminocyclopropane-1-carboxylic acid
AMPS - ammonium persulphate
B - blue
BAP - 6-Benzylaminopurine
B/UV-A - blue/ultraviolet-A
D - dark
ELISA - enzyme linked immunosorbent assay
FR - far-red
GA - gibberellic acid
h - hour(s)
min - minute(s)
MW - molecular weight
NAA - naphthalene acetic acid
NAD(P)H - nicotinamide-adenine dinuclueotide (phosphate), reduced form
PAL - phenylalanine ammonia lyase
PAR - photosynthetically active radiation
Pfr - the far-red light-absorbing form of phytochrome
pI - isoelectric point
PMSF - phenylmethanesulphonyl fluoride
Pr - the red light-absorbing form of phytochrome
Ptotal - total phytochrome i.e. Pr+Pfr
R - red
RER - rough endoplasmic reticulum
R:FR - quantum ratio of red and far-red light
SDS-PAGE - sodium dodecyl sulphate - polyacrylamide gel electrophresis
TEMED - NNN'N'- tetramethylethylenediamine
TMB - 3,3',5,5' tetramethyl benzidine
W - white
Wt - wild type

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CHAPTER ONE

CHAPTER ONE - Introduction

The family of peroxidase enzymes are involved in a wide range of physiological processes throughout the life cycle of the plant. In this study changes in the rate of stem extension brought about by changes in the light conditions are investigated in conjunction with any changes in cell wall peroxidase activity. To begin with the properties of the cell wall itself will be discussed and the properties of the peroxidase enzyme. This is followed by a summary of the relevant properties of the phytochrome photoreceptor family. Finally, possible interactions between the phytochrome regulation of growth and changes in peroxidase activity are considered.

1.2. The importance of the plant cell wall in the regulation of cell development

The cell wall imparts strength and structural integrity to the cell, conferring rigidity to the plant. Any mechanisms that are therefore able to alter the properties of the cell wall provide possible points at which the regulation of cell extension might be controlled, e.g. laying down of cell wall components and a change in activity of enzymes that cause or break cross-links between these components. Cell expansion is governed to a large extent by selective, closely controlled weakening of the cell wall (Fry, 1986). The wall itself constitutes a highly complex network of interwoven fibres that has a net negative charge due to a preponderance of uronic acid residues. These residues are chiefly the glutamic acid of pectins in the primary cell wall and glucuronic acid of xylans in the secondary cell wall. The overall net negative charge of the cell wall retards the movement of positively charged molecules through it. As such small neutral or positively charged molecules may act as cell signalling molecules. The 'signalling' by plant cell wall fragments (oligosaccharides) in plants is well characterized as a wound induced response eliciting a phytoalexin or protease inhibitor defence mechanism (Ryan, 1987). Plant cell wall fragments have also been identified

as signals for growth and development, e.g. a xyloglucan isolated from sycamore cell walls inhibits the auxin-stimulation of pea stem segments (York et al., 1984). The relatively small size of the pores between the cell wall components is also advantageous in the physical prevention of pathogen entry.

1.2.1. Structure of the plant cell wall

The primary cell wall is deposited by the daughter cells once the cell plate is complete and continues to be deposited as long as the cell surface is increasing in surface area. Layers that are laid down following the cessation of cell expansion comprise the secondary cell wall. All cell walls are composed of both a microfibrilar phase and a matrix phase. Cellulose microfibrils are laid down following the cessation of cell expansion on the inner surface of the existing wall. The matrix components (hemicelluloses, pectins and proteins) are added by intussusception into the existing wall. The cellulose microfibrils typically occupy 15 % of the volume and constitute 20-30 % dry weight of the wall (Varner and Lin, 1989). In mature higher plant cells, the microfibrils are very long and approximately 10 nm in width. The orientation of these microfibrils within the cell wall is initially determined by the microtubules. At deposition they are usually laid down in a transverse direction to the major direction of growth. As the cell elongates the outer microfibrils are pulled into parallel alignment with the axis of elongation.

Cellulose is an unbranched ß 1,4-glucan with approximately 15,000 sugar residues per molecule, although this degree of polymerization is lower in primary cell walls. These microfibrils are held in a crystalline lattice imparting a high tensile strength to the cell wall. This structure is stabilized by intra- and intermolecular hydrogen bonds between the ring oxygen of one glucose residue to a C3 hydroxyl hydrogen of adjacent residues within a chain and between the hydroxyl hydrogen and OH-O atoms on neighbouring chains.

The other major polysaccharide is xyloglucan, a hemicellulose that comprises 20 % of the dry weight of the primary cell wall of dicots and 2 % of monocots. All xylans have backbones of ß-4 linked xylosyl residues with different combinations of side chains attached to the second or third oxygen (Varner and Lin, 1989). The xyloglucan chains have an extremely long backbone compared with both other microfibrils and the spacing between microfibrils in the cell wall. It has been suggested that the two ends of a xyloglucan molecule may therefore H-bond to different microfibrils of cellulose. Thus acting to tether adjacent microfibrils contributing to the coherency of the cell wall (Fry, 1986).

The matrix phase is a chemically complex, non-crystalline phase that includes polysaccharides, proteins and phenolic compounds. Many of the structural cell wall proteins are glycosylated and contain an unusual amino acid, hydroxyproline, that is not generally found in proteins within the protoplast. Extensin, the most extensively studied cell wall protein, making up 1-10 % of the wall matrix, contains 40 % hydroxyproline and a high percentage of lysine and serine (Cassab and Varner, 1988). This confers a basic characteristic to the extensin protein. The tyrosine residues of extensin are involved in the formation of both intra and intermolecular covalent bridges forming cross links within the cell wall. In addition to the structural proteins, also present in the matrix phase are many other proteins which constitute an important part in the mechanism of regulation of both growth and defence. These include exoglucosidses (ß-glucosidase, ß-xylosidase, ß-galactosidase and α-galactosidase), endo-ß 1,4- glucanase and endo ß 1,3- glucanase, and many enzymes including peroxidase, invertase, cellulase, acid phosphatase, pectinase, pectin methylesterase, ascorbic acid oxidase and malate dehydrogenase.

1.2.2. Function of the plant cell wall during extension growth

Once a certain critical turgor pressure has been exceeded, the cell wall stretches in a plastic (non-reversible) manner. This is superimposed on the elastic (reversible) stretching of the wall. Obviously plastic extension must be closely controlled spatially

and temporally from within the plant (its developmental programme) and in response to the external environment, e.g. light and temperature, in order to permit co-ordinated growth. As cross-links between cell wall macromolecules are formed, the increased mechanical strength of the wall opposes the turgor pressure of the protoplast, restricting any increase in growth. The balance between these two forces therefore controls growth, extension occurring once a threshold turgor pressure has been reached. Thus in the short term the major point of control of cell extension is the cell wall.

The osmotic pressure of the protoplast is maintained by the biosynthesis of cell metabolites and by active uptake of inorganic ions, e.g. K^+. A balance between metabolic wall loosening events which accelerate extension, and metabolic as well as physical wall-stiffening that retard extension, combine to control extension growth. Wall expansion is therefore dependant upon the overall metabolic activity of the cell. New wall material deposited during cell expansion is from two sources: a) cellulose microfibrils that are synthesized at the plasma membrane and b) matrix polysaccharides that are synthesized intracellularly and transported to the wall in membrane bound vesicles. The bonding between new cell wall components and existing cell wall macromolecules is therefore important in determining the mechanical strength of the cell wall. The plastic and elastic properties of the cell wall are therefore seen to impose constraints to which the cell may extend. The cross-links formed between cell wall components and their catalysts will be discussed later.

1.2.3. The role of the epidermis

The mechanically rigid epidermis has been suggested to be the region of the stem most directly involved in the control of extension growth (Taiz, 1984). The wall extensibility of the epidermal cells is lower than that of the cortical cells, and hence the extensibility of the whole organ is limited by the relative inextensibility of the epidermis. Indole-3yl-acetic acid (IAA) induced growth is known to loosen the epidermal wall e.g. in maize coleoptiles and in third internode sections of pea seedlings

(Kutschera et al., 1987). The growth of intact pea sections was found to be twenty times more sensitive to IAA than peeled cortical cylinders (Kutschera and Briggs, 1988). The action of auxin in controlling the extension of stems is therefore thought to occur principally by altering the extensibility of the epidermal cell walls (Brett and Waldron, 1990). The epidermal zone also possesses abundant phenolics which may be tightened by the formation of peroxidase-catalyzed cross links (Fry, 1988), (see later). Thus localized action of cell wall peroxidase within the region would greatly alter cell extension. Further support that the epidermis is the main region controlling the extent to which the cell is able to extend has been found in mature cells of mung bean hypocotyls. Here changes in peroxidase activity were found to be most obvious in the epidermal cells, peroxidatic activity being restricted to the cell wall of elongated cells (Goldberg et al., 1986).

1.3. Peroxidases within the plant cell wall

1.3.1. General structure of the peroxidase enzyme

Peroxidases are glycoproteins that often constitute a significant proportion of the total protein content of the cell. They are widely distributed in the plant kingdom and also occur in the animal kingdom. Most higher plants have been found to contain a large number of peroxidase isozymes, often differing greatly in their isoelectric points. It is this large number that has led to both interest in the peroxidase enzyme family and the difficulty in assigning specific roles to any one isoform. The quaternary structure of peroxidase is made up of two domains, a distal and proximal region, separated by the haem group with two conserved histidines containing sequences inferred as the active sites. The polypeptide chain is usually a continuous V or U shape with predicted values as 25 % helix and 9 % extended. Plant peroxidases contain four disulphide bridges and combine two calcium ions each which aid stability to the enzyme and its activity. The sites of carbohydrate attachment are predicted to be turns in the structure (Welinder, 1985). The comparison of horseradish peroxidase c with turnip peroxidase

7 revealed only 49 % homology of their amino acid sequences suggesting that major alterations have occurred during evolution (van Huystee, 1987).

Generally, the peroxidase enzymes themselves are composed of an apoprotein moiety which is produced in the RER, a haem moiety from a glutamic acid precursor and glycosidic prosthetic groups which are added as the polypeptide is transported through the cell via the Golgi apparatus to the cell wall (van Huystee, 1987). Activity of the peroxidase enzyme is only established by association of the apoprotein with the haem moiety to form the holoenzyme (Chibbar et al., 1984). As such the haem moiety has a potential role as a regulator of the synthesis of the peroxidase apoprotein. Whilst this mechanism has not been established for the control of peroxidase synthesis, another haem protein, catalase, is known to be regulated by haem concentration (van Huystee, 1987).

Van Huystee (1978) reasoned that owing to the large haem pool that exists in cultured peanut cells the control of peroxidase release may involve the glycosylation process. This idea is supported by findings that the peptide chain appears to be synthesised in advance of the addition of the glycosidic prosthetic group. The glycosidic side chains form 15-17 % of the molecular weight of major horseradish peroxidase isozymes whilst minor isozymes contain only 7 % carbohydrates. It is noteworthy to highlight the possibility that different peroxidase isozymes that can be extracted from a particular plant tissue might infact be due to different phases of development of the glycoprotein.

1.3.2. Inferred functions of the peroxidase enzyme

Peroxidases appear to influence a great variety of physiological processes in plants. The general reaction they catalyse can be written as follows:

$$2AH + H_2O_2 \longrightarrow A2 + 2\,H_2O$$

$$AH_2 + H_2O_2 \longrightarrow A + 2\,H_2O$$

where A is an aromatic ring (Fry, 1988). Their roles within the cell wall include the generation of hydrogen peroxide (see 1.3.3), the catabolism/regulation of auxin (see 1.3.4), PAL like activity (di Biasi, 1986), induction of wilting (Lagrimini et al., 1990), organogenisis and cell wall edification, lignification and suberization (Gasper et al., 1991), the latter playing an important role in the defence against stress conditions and pathogen attack. A major function of cell-wall peroxidases and the subject of interest studied here, concerns their role in the alteration of cell wall extensibility, ostensibly via catalyzing the formation of cross links between cell wall macromolecules. Some of these suggested functions of peroxidase are outlined below, together with factors that are known to affect cell wall peroxidases (section 1.5). Their role in the formation of cell wall cross-links is discussed separately in section 1.4.

1.3.3. The production of hydrogen peroxide, a prerequisite for peroxidase catalyzed reactions

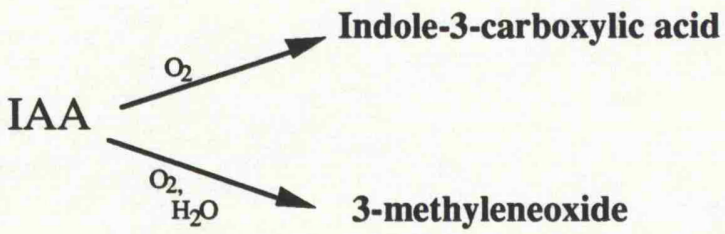
Many oxidoreductases are associated to the plasma membrane. Oxygen uptake, when NAD(P)H is supplied, is due mainly to the presence of peroxidase. This peroxidase catalyzed oxidation of NAD(P)H in the presence of a soluble phenolic and Mn^{2+} leads to the production of H_2O_2. In support of this a cell wall bound peroxidase from horseradish has been demonstrated to be responsible for the formation of H_2O_2 at the expense of NAD(P)H (Elstner and Heupel, 1976). More specifically different peroxidase groups have been ascribed to different roles. In *Nicotiana tabacum* the basic group of peroxidase enzymes are responsible for the formation of H_2O_2 whereas the acidic groups are believed to have a separate function as part of the same pathway leading to lignin formation, for which a supply of H_2O_2 is a prerequisite (Mader et al., 1980). An example where basic peroxidases have been suggested to be associated with the production of lignin is in the lignifying peach fruit endocarp (Abeles and Biles, 1991). Thus, the requirement for H_2O_2 may impose a mechanism of regulation of peroxidase catalyzed stem extension via the supply of NAD(P)H from metabolic

processes. Another cell wall-bound enzyme, malate dehydrogenase (MDH), which 'carries' its coenzyme NAD, may also play a crucial role in the production of H_2O_2. In the presence of malate, MDH generates NADH which via a peroxidase catalyzed reaction forms H_2O_2 (Fry, 1988).

1.3.4. The catabolism of auxin, regulation of the acid growth affect

Naturally occurring plant auxins are known to mediate wall loosening via an acid growth mechanism. The acid growth hypothesis postulates that high levels of auxin increase the activity of a H^+ ATPase enzyme in the plasma membrane. The increased acidity in the cell wall not only prevents the formation of cross-links between cell wall macromolecules but may also break existing bonds effectively lowering the tensile strength of the cell wall. Increased cell extension via IAA is a two stage mechanism, acidification increases stem extension for approximately 2 h followed by de novo synthesis of cell wall polysaccharides increasing growth for up to 24 h. Non-decarboxylative and decarboxylative oxidations are the main processes governing the amount of free IAA. Thus the regulation of these processes may alter cell extension by the indirect mediation of the acid growth effect. The decarboxylation of IAA appears to be catalyzed by peroxidase, whilst non-decarboxylative oxidation is not thought to be catalyzed by peroxidase. The possible routes of peroxidase mediated IAA decarboxylation is illustrated in Fig 1.1 below.

Fig 1.1:



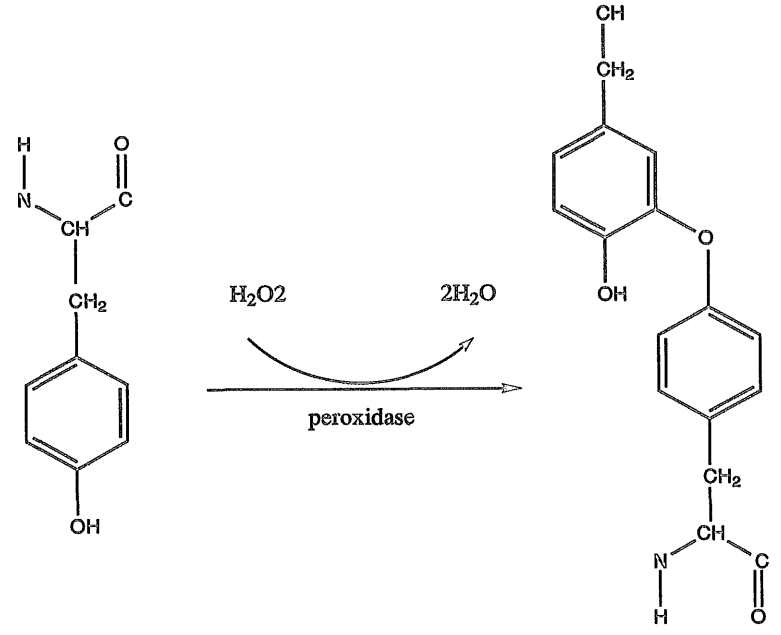
Auxin degradation is generally believed to be mediated by free, soluble peroxidases, with basic isoperoxidases being the most effective. The activity of these enzymes would therefore affect the level of auxin-mediated 'acid growth' effect (Gasper et al., 1991), contributing to a mechanism by which the growth rate of the cell might be regulated.

1.4. The mechanism of peroxidase action in the catalysation of cell wall cross-links

Cell wall peroxidase is believed to catalyse cross linking between cell wall monomers. This leads to an inhibition of cell extension via two separate mechanisms:

(a) The cross linking of extensin monomers via two tyrosine units joined by a diphenyl-ether bridge to form isodityrosine, (Fig 1.2).

Fig 1.2:

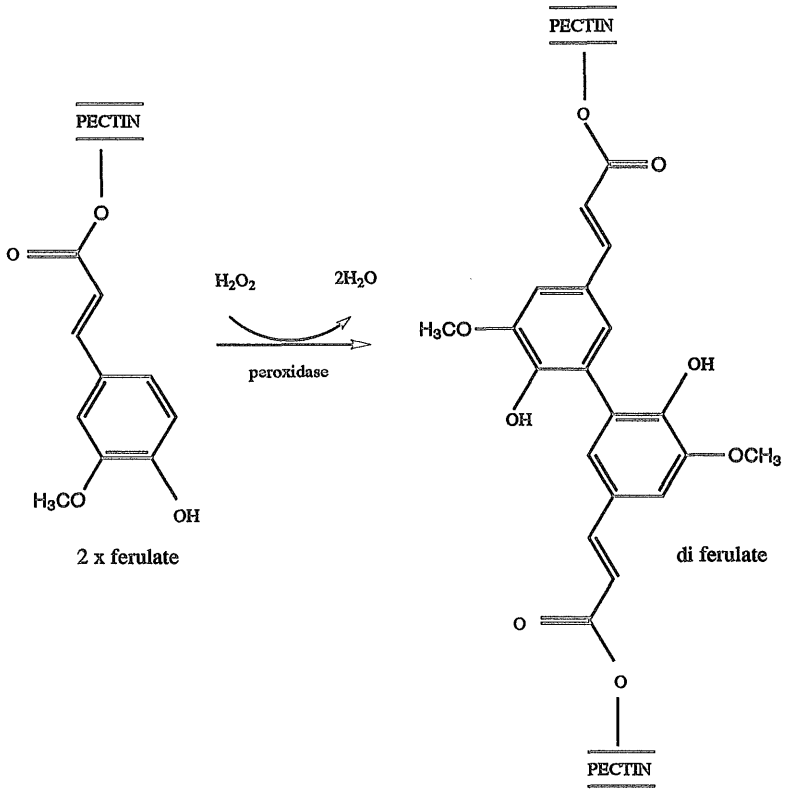


tyrosine

isodityrosine

(b) The cross-linking of pectin molecules by the oxidative coupling of their phenolic substituents. The second major class of wall-polymer bound phenolic groups are those biosynthetically derived from p-coumaric acid. The simplest ferulate coupling product being di-ferulate, (Fig 1.3).

Fig 1.3:



2 x ferulate

di ferulate

The formation of loops in the extensin chain, due to the peroxidase coupling of tyrosine residues, occurs near the pectin molecules in the cell wall. Pectin ionically binds both peroxidase and extensin bringing them together for free radical formation.

Therefore loops in the extensin occur near the pectin molecules due to the peroxidase coupling of tyrosine residues. Thus the peroxidase catalysed wall cross links can lower the extensibility of the wall altering extension growth rate. The control of phenolic cross links could be imposed at a number of points; a) synthesis and secretion of peroxidase, b) the supply of a reductant to convert oxygen to hydrogen peroxide, c) the supply of an enzyme that does (b), d) the number of phenolic groups per wall polymer, e) cell wall pH, f) the number of inhibitors to enzyme activity e.g. ascorbate (Biggs and Fry, 1990).

It has been suggested that wall rheology may be a complex function of three key variables: pH acid growth, extensin monomer levels and peroxidase activity (Lamport, 1986). When rapid cell extension is taking place a low pH, high extensin monomer levels and a low peroxidase activity are usually found supporting this idea. There are however numerous regulatory and moderating factors that may influence wall rheology outside of these parameters which need to be considered.

1.5. Factors other than light affecting the activity of plant cell wall peroxidases

Whilst the presence of substrates in the cell wall obviously influence the activity of extracellular peroxidase, there are many variables that have been considered to have a regulatory role on the activity of peroxidase enzymes. In some cases the activity of specific isoforms have been shown to be enhanced or decreased by specific factors. However the wide range and variability within this category makes elucidation and interpretation of the overall mechanism difficult. Amongst the known factors that alter the activity of peroxidase isozymes are the presence of metal ions (Barkardjieva, 1986, Barkardjieva and Christova, 1991), regulation by plant growth hormones e.g. gibberellic acid (GA), IAA and ethylene (see Fry, 1986), wounding of the tissue in which ethylene and 1-aminocyclopropane-1-carboxylic acid (ACC) have been

juxtaposed to exert an influence on the activity, and the glycosylation of the peroxidase enzyme itself (Hu and van Huystee, 1989).

Whilst dividing the individual responses into groups might ultimately prove to be arbitrary and even misleading, it is convenient and often necessary to study individual reactions inorder to ascertain a clearer overall appreciation of the whole picture. As such the mechanisms of possibly points of control will be discussed separately.

1.5.1. The presence of metal ions

That metal ions can determine the extent of the inherent functions of peroxidase activity has been suggested to be via both the regulation of its activity and by conferring heterogeneity to the enzyme itself (Barkardjieva, 1986). The peroxidase protein contains both Fe^{2+} and Ca^{2+} ions, (see 1.3.1.), the Ca^{2+} ions conferring stability to the enzyme and hence affecting its activity (Welinder, 1985). The physiological aspects of calcium will be discussed separately. In de-etiolated tissue Cu^{2+} and Ca^{2+} were found to strongly increase the photosensitivity of the enzyme, whereas Mn^{2+} only led to a slight increase and Zn^{2+} none at all, these responses were found to occur within different regions of the visible spectrum. In etiolated tissue a stronger and wider range of responses were observed to different visible light qualities (Bakardjieva and Christova, 1991). This indicates that not only can the presence of particular metal ions in the plant strongly influence the reaction of the peroxidase enzymes but that peroxidase itself may act as a photorecepting molecule. Thus implying a further role for light in the regulation of peroxidase activity. What influence and interactions this may infer onto and in conjunction with, phytochrome and any B/UV-A photoreceptor is undetermined.

1.5.2. The Ca^{2+}/H^{+} balance within the cell wall

The regulatory role of Ca^{2+} has been suggested through evidence that Ca^{2+} may act as an endogenous signal and secondary messenger. This may be due to the direct activation of the peroxidase enzyme, inactivation following binding of the enzyme to

receptor sites within the cell wall or secretion of the enzyme (Penel, 1986). The low levels of Ca^{2+} normally present within plant tissue are sufficient to initiate these responses. Alterations in the pH of the cell wall can lead to Ca^{2+} bridges between cell wall macromolecules being displaced. Calcium ions may also decrease tissue extensibility either in direct competition with H^+ ions or indirectly via a decrease in the activity of a cell wall enzyme (Brett and Waldron, 1990). Regulation by Ca^{2+} has however been argued against owing to its ubiquity. Another factor that argues against the regulation of Ca^{2+} as a means of controlling stem extension is that physiological levels of calcium are poorly regulated in the cell wall. However significant changes in the Ca^{2+}/H^+ ratio would induce modifications due to changes in the local pH and changes in the local pH are known to induce modifications in the activity of cell wall peroxidase (Demarty et al., 1984). Thus changes in local pH regulated by the relative concentration of H^+ and Ca^{2+} ions would alter the activity of the peroxidase isozymes present and hence cell wall extension. Support of this idea comes from Barcelo et al., (1989) who proposed that the cell wall bound verses the intercellular location of acidic isoperoxidases in lupin is a consequence of this ratio. The binding of the acidic isoperoxidase to the cell wall did not modify the activity but was thought to increase the stability of the enzyme inhibiting the irreversible denaturation that occurs at acidic pH.

1.5.3. Plant growth regulating hormones

Auxin is involved in the acidification of the cell wall and hence changes in the cell wall pH. Some matrix or microfibril-matrix interface bonds may be weakened by acid although whether it is ionic or covalent bonds that are affected remains uncertain. The balance between this and peroxidase activity involved in the decarboxylation of IAA provides a possible self-regulatory mechanism of growth control. To what extent these individual factors are mediated by other processes, including light, will determine the extent of their relative concentrations within the cell environment.

Gibberellic acid promotes growth and has been shown to influence the activity of peroxidase. In barley aleurone layers there are two peroxidase pools, a cytoplasmic, low pI pool and an apoplastic basic pI pool. The secretion of the low pI isozymes and the release of the high pI isoperoxidase enzymes have been shown to be influenced by GA (Jones, 1986). The secretion of peroxidase from spinach cell cultures has also been demonstrated to be inhibited by GA (Fry, 1980). This general suppression of peroxidase activity leads to an accumulation of extracellular phenolics present in an uncross-linked state in the cell wall (Fry, 1979). Higher levels of GA may therefore lead to an increase in growth by influencing the number of peroxidase catalyzed covalent cross links in the cell wall.

Ethylene and its correlation with peroxidase activity is a constituent of the wounding affect and will be discussed under that heading.

1.5.4. Wounding

A great variety of stresses received by plants e.g. attack by pathogens or wounding, lead to an increase in the activity of peroxidase enzymes. These peroxidases presumably being involved in the repair of the damaged cell wall (Lagrimini and Rothstein, 1987). Their known involvement in the processes of lignification and suberization processes enhances the concept of their role in a defence mechanism. An increase in peroxidase activity in response to stress/wounding has been reported in many tissues. In *Sedum album* leaves where the effect of ozone exposure was monitored (Castillo and Greppin, 1986, Castillo et al., 1984), a different response of anionic and cationic extracellular peroxidases was shown. The cationic peroxidases increased in activity within a few hours followed some hours later by an increase in the activity of an anionic isoform. In tobacco, different peroxidase isozymes were found to be selectively induced by different stimuli. Cationic and anionic subsets of peroxidase isozymes were induced by wounding and tobacco mosaic virus respectively (Lagrimini and Rothstein, 1987). In some instances specific peroxidase isozymes have been shown to be induced by wounding and stress factors e.g. in

cucumber cotyledons where during ethylene induced senescence an increase in the activity of a 33 and 60 kDa peroxidase protein was observed (Abeles et al., 1988, Abeles, personnel communication.).

The lignification of plant cells acts to both strengthen the walls during maturation of the cell and as a response to physical wounding of the cell. Lignin is a natural polymeric product derived from the dehydrogenative polymerization of three primary precursors, coniferyl alcohol, sinapyl alcohol and p-coumaryl alcohol. Wall bound peroxidases have been postulated to catalyse the dehydrogenation of p-hydroxycinnamyl alcohols in the presence of hydrogen peroxide (or other oxidizing species) to yield phenoxy radicals. The coupling of two radicals, one from the p-hydroxycinnamyl alcohol precursor and the other from a ready formed polymer, is considered to be the lignin growing step (Rolando et al., 1991).

1.6. Observed correlations between growth and peroxidase activity

Many examples have been demonstrated that support the hypothesis that changes in growth rate might be regulated via an alteration in cell wall peroxidase activity. The mechanisms by which this might occur have been discussed earlier. Much of this work has concentrated on inherent changes in, or cessation of elongation growth during the plants development. Direct relationships between peroxidase activity and elongation growth have been demonstrated in a number of tissues. In peanut hypocotyl segments, Zheng and van Huystee (1992) demonstrated the relationship between peroxidase activity and elongation by a number of methods. When elongation and any increase in protein content was inhibited by the addition of purified meta-flurotyrosine, an increase in cationic peroxidase activity was observed. The addition of purified peroxidases to the culture medium also led to a decrease in elongation and an increase in extension of the hypocotyl segments of 100 % was found to be induced by an anti-cationic peroxidase antibody 48 h after being added to the culture medium.

In contrast an anti anionic peroxidase antibody had no affect. This demonstrates not only a relationship between peroxidase activity and elongation but more specifically, the correlation between growth and the activity of a cationic ionically bound peroxidase isoform.

Two genotypes of the tall leaf fescue that differ in the length of their leaf elongation zone have been used to demonstrate that an increase in peroxidase activity occurs with the cessation of growth (Mac Adam et al., 1992 a,b). Cationic isoforms were found to increase in activity as growth decelerated, whereas the anionic isoforms only appeared to increase in activity during the onset of lignification (Mac Adam et al., 1992b). Goldberg et al., (1986), found that in young cells of mung bean hypocotyls most of the peroxidase activity was located in the cytosol, whereas in mature cells 73 % of peroxidase activity was bound to the cell walls. Importantly, the increase in peroxidase activity was concentrated below the zone of maximum elongation (Goldberg et al., 1987). In the epicotyls of *Cicer arietinum* the ionically bound peroxidase activity was lowest in those seedlings with the highest growing capacity remaining (Valero et al., 1991).

These changes in peroxidase activity have in some instances been correlated with specific or groups of specific isoforms. The assignment of specific peroxidase isoforms to changes in growth should help to further elucidate their exact role within the cell wall. It is of interest that in the cases cited here it is a change in the activity of the cationic peroxidase isoforms, or cationic plus anionic, that appear to correlate with any changes in growth rate.

1.7. Enzymes other than peroxidases and their suggested role in the mechanism of extension growth

The cell wall contains a variety of enzymes that act as cell wall components (see 1.2.1), all of which are hydrolases or oxidoreductases i.e. enzymes that operate with

simple substrates (H_2O, H_2O_2, O_2, etc). Many of these enzymes have been postulated to play a role in the regulation of cell extensibility. A brief summary of the major groups of cell wall enzymes and their proposed mechanism of action follows. Polysaccharide-hydrolysing enzymes fall into two categories: endo- and exo-hydrolases. The most numerous enzymes within the plant cell wall are the glycosidases. These exo-hydrolases are specific for the sugar residue they cleave from the non-reducing ends of polysaccharides. Whilst the nature of the action of glycosidases makes it unlikely that they play a direct role in cell wall loosening, the shortening of side chains might affect the physical properties of the wall. Endo-glycosidases cleave sugar-sugar bonds mid-chain in the polysaccharide e.g. pectinase, mannase, cellulase. These enzymes are therefore able to drastically alter the length of the polysaccharides and hence wall extensibility. (For further details see Ch 6-Fry, 1988).

Endotransglycosylases act by splitting the glycosidic linkage and transferring it to an alcohol e.g. another sugar, thereby altering the physical properties of the cell wall and allowing stem extension. For example, xyloglucan endotransglycosylase, a newly characterized cell wall enzyme cleaves xyloglucan molecules loosening the cell wall and allows molecular creep (Fry et al., 1992).

Oxidases use O_2 as an electron acceptor (instead of H_2O_2 for peroxidase) e.g. ascorbic acid oxidase and polyamine oxidase. The two common general reactions producing H_2O or H_2O_2. Some enzymes are able to catalyze both oxidatic and peroxidatic reactions. The second type of reaction may therefore provide a source of H_2O_2 required in the peroxidatic formation of isodityrosine, lignin, etc, and hence play an important role in the regulation of cell extension. (Fry, 1988).

1.8. Photomorphogenisis

When genetically identical seeds are grown in the light or the dark and the resulting morphology examined, the light-grown seedlings when compared with the dark-grown seedlings, are seen to have expanded cotyledon and true leaves, shorter hypocotyls and internodes, and pigmentation. It can therefore be concluded that the regulating factor of these differences must be light. The ability of the plant to respond to these different conditions lies in the ability to capture and relay the information in a manner which enables the appropriate developmental response. Amongst the known photoreceptors, phytochrome is probably the most extensively studied but others such as a blue light/UV-A receptor are also known to exist. In this work the main area of interest is concerned with the action of phytochrome, although the action of a B light receptor is also briefly examined.

1.8.1. Phytochrome types and distinguishing features

There are multiple forms of phytochrome that have been placed into different groups based on physiological, biochemical/immunological and molecular properties. To avoid confusion the segregation of discrete phytochrome species based on these techniques requires clarification of the nomenclature used. The varied range of R/FR light reversible reactions observed in higher plants has led to the conclusion that there are two physiologically and spectrophotometrically distinct pools of phytochrome, a light-labile and a light-stable pool.

In etiolated seedlings light-labile phytochrome accumulates at a very high level. This phytochrome is synthesized in the Pr form but is converted to Pfr following absorption of light. Following exposure to R light 95-99 % of the accumulated phytochrome (as Pfr) is lost within 24 h, which is 100 times faster than the turnover rate of Pr. The presence of a second discrete pool of phytochrome was supported by *in vivo* spectrophotometric studies of light-grown, but non-chlorophyllous, plant tissues (e.g. Jabben and Dietzer, 1979). In contrast to the phytochrome pool detected in etiolated

tissues, a phytochrome pool was detected that was stable following exposure to light, i.e. it was stable in the Pfr form. Using spectrophotometric analysis of light-mediated phytochrome degradation (destruction) in etiolated plant tissues, this light-stable pool was readily detected as a slow-degrading pool of phytochrome (e.g. Brockman and Schäfer, 1982).

Biochemical and immunological studies provided more direct evidence for the existence of multiple discrete molecular species of phytochrome. The form that predominates in etiolated tissues is also present in light-grown tissues but in much lower abundance and has been called Type I phytochrome. A residual pool that is more resistant to destruction as Pfr appears to be constitutively present at low abundance irrespective of light treatments and is referred to as Type II phytochrome (Abe et al., 1985, Tokuhisa et al., 1985).

The view that different phytochromes may be encoded by different genes has been clearly shown by Sharrock and Quail (1989). Using cDNA analysis, at least five phytochrome-related sequences have been isolated from *Arabidopsis thaliana* (Sharrock and Quail, 1989). These genes being named *PHY A-E* accordingly. Of these *PHYA, PHYB* and *PHYC* only show 50 % identity of their amino acid sequences with either of the other two (Sharrock and Quail, 1989). All previously obtained phytochrome-encoding sequences, from both monocots and dicots, were found to be more closely related to the deduced amino acid sequence of the *Arabidopsis PHYA* cDNA and have therefore been classified as *PHYA* homologues (Quail, 1991). Phytochrome A is light-labile (Somers et al., 1991) and it has also been established that the *PHYA* gene encodes the polypeptide moiety of this Type I phytochrome (Quail, 1991). Thus, Type I phytochrome can be re-defined as phytochrome A. The polypeptides of Type II phytochromes are presumably products of the other*PHY* genes.

1.8.2. Phytochrome - general properties and mechanism of action

All known phytochromes appear to be dimeric proteins. Each monomer contains a single, linear tetrapyrole chromophore linked to a cysteine residue via a thioether linkage in a highly conserved region of the apoprotein (Thomas, 1991). The molecular weight of the monomer is approximately 124 kDa. Whilst the exact mechanism of phytochrome action remains to be fully elucidated, it is well known that the basis of its action stems from its photochromic properties. The phytochrome molecule exists in two stable interconvertable forms, Pr that has a maximum absorption at approximately 660 nm and Pfr that has a maximum absorbance at approximately 730 nm. The conversion between the two phytochrome forms is commonly represented in its simplest form as below.

Both forms of phytochrome absorb in the R region but Pr absorbs little in FR, therefore below 730 nm or in polychromatic radiation there is a continuous cycling between the two forms and a dynamic photostationary equilibrium (Pfr/Ptotal) is established. It has been postulated that it is this photostationary state that determines the appropriate response, however, that Pfr is the active form of phytochrome is more generally accepted.

There are several intermediates that exist between Pr and Pfr and photoconversion by R and FR irradiation involve different pathways. The initial conversion of both Pr and Pfr to their respective excited states is a photoreaction, the subsequent conversion to other intermediates being thermal reactions (Kendrick and Kronenberg, 1986). At

high fluence rates, the rates of conversion of Pr and Pfr become limited by the thermal reactions of these intermediates, which therefore accumulate.

Type I and Type II phytochrome can be distinguished on the basis of their stability as Pfr. The level of phytochrome A, following the photoconversion of Pr to Pfr, is rapidly depleted upon exposure of the plants to light. Further loss of phytochrome A is due to light regulated reduced expression of the *PHY A* gene (Colbert, 1991), with in some cases, phytochrome itself mediating reduced *PHYA* expression. There is evidence that a light-stable Pfr is involved in mediating the down regulation of *PHYA* mRNA synthesis e.g. in pea seedlings (Furuya et al., 1991). Immunoblot analysis using monoclonal antibodies specific to phytochrome B and C, have shown that these phytochromes are present at low but equal amounts in etiolated and light treated tissue (Somers et al., 1991, López-Juez et al., 1992, Devlin et al., 1992). Thus adhering to the defined characteristic property of Type II phytochrome, that is stability of the Pfr form.

1.8.3. The action of the B/UV-A receptor

The B/UV-A photoreceptor has an action spectrum maxima between 370-380 nm (UV-A) and 400-500 nm (blue region). In higher plants responses to B light are known to include the inhibition of stem growth, phototropism, stomatal opening, chloroplast development and orientation, pigment, protein and enzyme synthesis and enzyme activation. The general characteristics of a B light mediated response are responsivity to a wide range of fluence rates from < 0.1 μmol m^{-2} s^{-1} to 400 μmol m^{-2} s^{-1}, relatively rapid responses and effectiveness mainly in the tissue being irradiated (Kendrick and Kronenberg, 1986). Very little is understood about the intervening chemical events from B light perception to the physical responses, in part because no one has as yet been able to isolate a B light receptor, of which there may be more than one type.

Phytochrome is known to affect certain responses to B light e.g. Pfr is thought to be a requirement for the B light mediated inhibition of de-etiolated cucumber hypocotyl elongation (Gaba et al., 1984). Although phytochrome is known to absorb in the B region (400-500 nm) and any synergism between R and B light responses constitute an important part in the regulation of a plants development, a separate B light receptor, from phytochrome, has been shown to exist by distinct responses to B light only. This has been demonstrated with pea epicotyl elongation which was found to be altered by changes in B fluence rate, whilst maintaining a near constant photostationary state throughout the duration of the experiment (Laskowski and Briggs, 1989). Furthermore, analyses of blue-light-response mutants of Arabidopsis have established the independence of the phytochrome and B light receptor systems (Liscum and Hangarter, 1993).

1.9. The family of phytochromes and their proposed roles

Phytochrome enables plants to respond to different light conditions throughout their physiological development from a seed to full maturity. Thus it has been postulated that different phytochromes may play specific regulatory roles.

1.9.1. The roles of Phytochrome action in etiolated seedlings

1.9.1.1. Responses

In etiolated seedlings phytochrome regulates a wide range of physiological and developmental responses, leading to de-etiolation and hence the attainment of a phototrophic state. The processes involved in de-etiolation and that are believed to be mediated by phytochrome include germination, inhibition of hypocotyl and internode extension, cotyledon and leaf expansion, flavonoid and photosynthetic pigment production, chloroplast biogenesis and the synthesis of enzymes (Smith and Whitelam, 1990).

The phytochrome-regulation of development in etiolated tissue has three physiologically distinguishable response modes:

(a) the 'very low fluence response' (VLFR)

(b) the 'low fluence response' (LFR)

(c) the 'high irradiance response' (HIR)

(a) VLFR is saturated at extremely low fluences. This response cannot therefore be reversed by FR, as both R and FR light can induce the response. It is difficult to assign to a particular function to the VLFR as saturation point is reached with R fluences as low as 10^{-7} μmol m^{-2}, equivalent to a few milliseconds of daylight (Smith and Whitelam, 1990). It is only seen in seeds and seedlings that have been grown in darkness.

(b) LFR are saturated at intermediate fluence rates, are fully reversible by R/FR and show full reciprocity. This forms the basis of what is considered to be the 'classical' phytochrome response. Characteristically the LFR displays a logarithmic relationship between response and the concentration of Pfr (Kronenberg and Kendrick, 1986).

(c) HIR is dependant on continuous long term irradiation and is often strongly dependant on the fluence rate. Maximum action normally occurs at wavelengths that maintain a low Pfr concentration for a long period of time. Both R and FR light are able to elicit a HIR type response, although the FR-HIR is restricted to etiolated seedlings only. The FR action maximum of the HIR of etiolated seedlings is usually considered to be the consequence of the rapid loss of (light-labile) phytochrome in the light. At the FR maximum a Pfr/Ptotal ratio is maintained that results in a Pfr concentration that is sufficient to induce the response, but insufficient to allow the loss of a significant amount of the (light-labile) phytochrome pool. Red light maintains a higher Pfr/Ptotal than FR light but Pfr is unstable and rapidly lost by destruction. The relative Pfr concentration is therefore lower and a lower than maximum response

action maintained. Upon de-etiolation the HIR shifts from FR-HIR to R-HIR, as the Pfr is depleted and the remaining pool is light stable. Blue light is also effective in the HIR via a separate B/UV photoreceptor. The HIRs show no R/FR reversibility and do not obey the reciprocity law between time and fluence rate. The HIRs are involved in seed germination, stem growth, leaf expansion and pigment synthesis. Different species and responses have different HIRs.

1.9.1.2. Phytochrome species and de-etiolation

That phytochrome A is abundant in etiolated seedlings and is rapidly lost upon exposure to light, has led to the idea that this phytochrome species is most likely to be responsible for mediating the process of de-etiolation. Biochemical and physiological experimental data provide evidence that phytochrome A may be responsible for the FR-HIR. However recent work with mutants of phytochrome A has cast doubts over the previous proposed simplicity on the action of phytochrome A. In light-grown transgenic tobacco plants, overexpressing an oat *PHYA* cDNA, a continuation of photoresponses are observed that are normally only present in etiolated seedlings such as the characteristic FR-HIR. This was demonstrated in the continued inhibition of extension growth in response to prolonged FR irradiation (Whitelam et al., 1992). This is consistent with the idea that phytochrome A is the phytochrome species responsible for mediating the FR-HIR. In contrast to these observations, *PHYA* null mutants grown in white (W) light, show a phenotype that is indistinguishable from that of their wild type (Wt) (Whitelam et al., 1993). Presumably another phytochrome species or an alternative light receptor, must be responsible for mediating the de-etiolation processes, questioning the defined role and importance of phytochrome A.

1.9.2. Phytochrome action in de-etiolated seedlings

When plants are growing in close proximity, in order to avoid being shaded by other plants and hence being subjected to poorer light conditions, a mechanism to monitor the change in the quality of the light received exists. One of the major roles of light-

stable or Type II phytochrome is the detection of other plants in the proximity and the regulation of appropriate 'shade avoidance' responses (Smith, 1986, Casal and Smith, 1989). In shade avoiding species the phytochrome detection of the R:FR ratio leads to a number of developmental responses. Included amongst these responses are the enhancement of stem and petiole extension, reduced chlorophyll production, enhanced apical dominance and reduced branching and reduced leaf development (Smith, 1986). These responses increase the plants capacity to compete for light by outgrowing its neighbours.

Although the way in which phytochrome status induces the appropriate responses has not been fully established, when photosynthetically active radiation (PAR) is constant an inverse linear relationship exists between the Pfr/Ptotal ratio and response e.g. internode extension (Morgan and Smith, 1978). This ratio between the amount of phytochrome present in the Pfr form and the total amount of phytochrome is established by the R:FR ratio. Therefore, a low R:FR ratio leads to a low level of Pfr and an increase in growth extension i.e. the shade avoidance response.

Although pulses of monochromatic light are not a natural occurrence in the environment, they have been extensively utilized as a means of examining specific light responses. To simulate the effects of changes in the R:FR ratio, end-of-day light treatments are often used. The provision of short irradiations at the end of the period during which PAR is provided, using either a R or FR light pulse, can determine the level of Pfr in the subsequent dark period (Casal and Smith, 1989), a simple relationship existing between the response of light grown plants and actinic light received.

Continuous irradiation with altered R:FR ratios, does however more closely resemble natural light conditions. Morgan et al. (1980), demonstrated that the stem extension of *Sinapis alba* increased with various wavelengths of additional FR to W, the extension rate being further accelerated by increased FR fluence rate. The results from *Sinapis*

alba and in general when continuous irradiation has been used, has generally been shown to be comparable to the responses induced using end-of-day light treatments.

1.10. Phytochrome deficient mutants, utilized to segregate the roles of different phytochromes

The assignment of different functions to any specific phytochrome species is hindered by the similarity in absorption spectra of light-labile and light-stable phytochrome (Tokuhisa et al., 1985, Abe et al., 1985). The use of photomorphogenic mutants modified in any of the phytochrome species has enabled some of these problems to be overcome by being able to study the effects of each phytochrome more specifically (Adamse et al., 1988). By comparing the different response modes of a mutant with its isogenic Wt should give an indication on the role of the missing component within the mutant. There are many photoreceptor mutants that have been postulated to be deficient in either a light-labile or a light-stable type phytochrome. The phenotype of these mutants should be indistinguishable irrespective of the point of mutation which may be due to either the lack of or a modified photoreceptor, or a modification in the transduction chain leading to a physiological response (Kendrick and Nagatani, 1991).

1.10.1. Mutations of phytochrome A

Amongst the plants that have mutations of phytochrome A, are the long-hypocotyl mutants of *Arabidopsis, hy8* (Parks and Quail, 1993), fre1 (Nagatani et al., 1993) and *fhy2* (Whitelam et al., 1993). So far however, only *fhy2* has been genuinely shown to have a mutation in the *PHYA* gene. When grown in FR the *fhy2* mutants remain almost indistinguishable from Wt plants grown in the dark, which is different from the findings with other *Arabidopsis* mutants (*hy1, hy2, hy3* and *hy6*). This mutant is now therefore called *phyA*. (Whitelam et al., 1993). Mutations of two other *Arabidopsis* loci, *fhy1* and *fhy3*, confer an elongated hypocotyl in FR but not in W light. Normal levels of phytochrome A are detected in both *fhy1* and *fhy3*, it has

therefore been speculated that these mutations specifically affect components of a phytochrome A signal transduction pathway (Whitelam et al., 1993).

A further mutant which has been the subject of much research is the *aurea* (*au*) mutant of tomato. This mutant, in comparison to its Wt does not de-etiolate properly and maintains a yellow-green appearance. It does however respond normally to end-of-day FR irradiation and low R:FR ratios by increasing elongation growth (Kendrick and Nagatani, 1991, Whitelam and Smith, 1991). *Aurea* seedlings possess normal levels of *PHYA* mRNA which is fully translatable in vitro. This finding together with the *au* and *PHYA* locus in tomato being located on different chromosomes, indicates that *au* is not a mutation of the *PHYA* gene. It has therefore been speculated that the *au* mutation may affect the accumulation or that it may be a chromophore synthesis/attachment mutant.

1.10.2. Mutations of Type II phytochrome

Amongst the light-stable phytochrome mutants are the *hy3* mutant of *Arabidopsis thaliana* (Nagatani et al., 1991, Somers et al., 1991), the *Brassica rapa ein* mutant (Devlin et al., 1992) and the cucumber *lh* mutant (Adamse et al., 1987, López-Juez et al., 1992).

The *Arabidopsis hy3* mutant displays an elongated phenotype and shows no inhibition of hypocotyl elongation in continuous R light, whilst displaying a similar phenotype to its Wt in continuous FR (Koornneef et al., 1980). The *hy3* mutant was therefore suggested to be deficient in a light-stable phytochrome (see Adamse et al., 1988b), with phytochrome B levels found to be approximately 2-5 % of the Wt levels (Somers et al., 1991). The diminished levels of phytochrome B are postulated to be responsible for the elongated phenotype. The *hy3* mutant does not show elongation growth responses to end of day FR light treatments (Nagatani et al., 1991).

A recently proposed phytochrome B like mutant is the *ein* mutant of *Brassica rapa*. This photomorphogenetic mutant, in comparison to its Wt does not show any response

to an end-of -day FR light treatment and only a small increase in first internode extension in a reduced R:FR ratio (Devlin et al., 1992).

The long hypocotyl (*lh*) cucumber mutant has been characterized by Adamse et al., (1987). The *lh*-seedlings are elongated compared to their wild type due to a prevention or retardation of cell maturation (Adamse et al., 1988a). Etiolated hypocotyls of the *lh*-mutant and the Wt seedlings show a similar rate of growth in the dark and both show R/FR reversibility of growth (Adamse et al., 1987). Similar amounts of phytochrome were found in etiolated tissue of the Wt and the *lh* (Nagatani et al., 1989). De-etiolated *lh* seedlings (compared with the Wt) show no response to R light and no R/FR reversibility of hypocotyl growth (Adamse et al., 1987). Light-grown *lh* seedlings also lack the end-of-day FR response of stem elongation (López-Juez et al., 1990). These observations are all consistent with the idea that the *lh* mutant is deficient in a light stable phytochrome. This was originally postulated by Adamse et al., 1988a, this theory later being substantiated by other workers using immunodetection techniques. It was established from this work that the *lh*-mutant does have a deficiency in a phytochrome B-like phytochrome (López-Juez et al., 1992).

1.11. Light regulation of cell wall peroxidase activity

As phytochrome performs fundamental roles in the regulation of plant growth and development, mechanisms by which these changes are controlled are the subject of much research. Cell wall enzymes that are able to alter cell extensibility by catalyzing the formation or breaking of cell wall cross links have therefore been postulated to be possible regulatory control points. Correlations between changes in growth rate and cell wall peroxidase activity have already been discussed (see 1.6). Whether phytochrome mediates changes in peroxidase activity as a means of controlling growth rate has been investigated in both maize and mustard (Kim et al., 1989 and Casal et al., 1990). In etiolated maize seedlings, phytochrome induces rapid and opposite

growth changes in the mesocotyl and coleoptile regions. Kim et al. (1989) using immunodetection techniques found that an anionic peroxidase (MW 98,000) isolated from the cell wall decreased in activity in the coleoptiles and increased in the mesocotyls. Phytochrome stimulates coleoptile elongation following a lag of approximately 15-20 min. The extractable level of an anionic cell wall peroxidase from this region is reduced by 50 % within 10 min. In the mesocotyl, growth is suppressed with a lag of 45-50 min, this is again preceded by a 40 % increase in the level of the anionic peroxidase which occurs within 30 min.

Casal et al. (1990) suggested that A4, one of the four anionic extracellular peroxidase isoforms extracted from the first internode of mustard seedlings, was specifically involved with phytochrome-mediated growth rate changes. These workers found that an end of day FR light treatment, leading to an increase in stem extension, coincided with a 50 % decrease in the activity of A4 that could be extracted by the infiltration/centrifugation technique. Phytochrome-regulated changes in the growth of mustard follows a lag period of 10 min after exposure to FR light whereupon a rapid acceleration of growth occurs (Child and Smith, 1987). A second phase of phytochrome regulated growth acceleration takes place after a lag of 35 min. Casal et al. (1990) suggested that in the pool of A4 being examined the decrease in peroxidase activity preceded the second but not the first phase of growth. This suggests that changes in the peroxidase activity may play a secondary role in the phytochrome-regulation of growth. The examples illustrated both show a light regulated change in extension growth that correlates with an increase in a particular fraction of peroxidase activity.

In cucumber hypocotyls B light is known to lead to a rapid inhibition of stem extension (Cosgrove, 1981, Gaba and Black, 1983, Gaba et al., 1984). This inhibition by B light of cucumber hypocotyl extension has been shown to inversely correlate with peroxidase activity (Shinkle and Jones, 1988). The B-inhibition could be reversed by 1mM ascorbate which completely inhibited peroxidase activity, thereby

providing another example in which a light-regulated inhibition of stem extension correlates with an increase in peroxidase activity. The findings of Shinkle et al. (1992) however, that the inhibitory effect of B light on hypocotyl elongation was faster than the observed change in total cell wall peroxidase activity, questioned the causal relationship between growth and peroxidase activity. This is discussed further in chapter seven.

1.12. This thesis

The role of phytochrome in the growth and development of plants is very complex. Here phytochrome-mediated growth rate changes have been used as a tool in the study of changes in growth and the role of peroxidase. This work investigates the hypothesis that phytochrome-regulated alterations in growth rate may be mediated by alterations in extracellular peroxidase activity. Further investigations have been carried out on the phytochrome-regulation of the anionic peroxidase isoform A4, previously studied by Casal et al., (1990). Correlations between the phytochrome-regulation of stem extension and the activity of extracellular peroxidase activity was tested in other plant species, namely cucumber seedlings, utilizing the availability of a photomorphogenic mutant, the cucumber *lh*-mutant. Each section of work is introduced in further detail at the beginning of the relevant chapter.

CHAPTER TWO

CHAPTER TWO - Materials and Methods

2.1. Plant material and growth conditions

2.1.1. Mustard

2.1.1.1. Light-grown tissue

Mustard seeds (*Sinapis alba* L.; Asmer seeds, Leicester, UK) were sown in Petersfield (Leicester, UK) No.2 Commercial range potting compost and grown under continuous white (W) light at 25°C for 14 days. Seedlings were either given a 20 min end-of-day R or FR light treatment prior to 2 h of darkness before centrifugate and homogenate extractions were made. These seedlings were used to provide first internode tissue.

2.1.1.2. Etiolated tissue

Mustard seedlings grown to compare the peroxidase activity of dark and light treated hypocotyls, were sown on damp tissue paper in plastic sandwich boxes and grown for 5 days in the dark, followed by 24 h in the dark or in continuous W. Any variations in the light treatments are indicated in the text.

2.1.2. Cucumber

2.1.2.1. Light-grown tissue

Cucumber seeds (*Cucumis sativus*) were either Burpless Tasty Green, Suttons, UK or the *lh*- mutant and its isogenic wild type (Wt), supplied by R.E. Kendrick, University of Wageningen, The Netherlands. All seeds were germinated individually in 5 cm^2 pots in Petersfield (Leicester, UK) Potting compost under continuous W light. In some instances the seeds were placed on moist tissue paper in sealed plastic sandwich boxes and kept in the dark at 4°C (vernalized) for 48 h before germinating as described. The light treatments given to the seedlings are indicated under the relevant

sections of text. All growth measurements were taken with a millimetre ruler. In order to measure the growth of sections down the length of the hypocotyl tobacco seeds were fixed to the hypocotyls with Vaseline and the individual sections measured following light treatment as indicated. This technique prevented the tissue from callusing which occurred when the tissue was marked with ink. This method was used to mark either 1 cm sections or one third sections of the cucumber hypocotyls. The seedlings were grown under low or high R:FR ratios and the respective areas of growth compared.

2.1.2.2. Growth of etiolated cucumber seedlings

Cucumber seeds were the *lh*-mutant and its isogenic wild type as supplied by R.E. Kendrick. Etiolated cucumber seedlings used to compare the effects of W, monochromatic R and FR light. In some instances the seeds were vernalized (see light-grown tissue, section 2.1.2.1.) for 48 h before germinating. The seeds were germinated in the dark on moist tissue paper for 72 h (Adamse et al., 1987) and then transplanted into individual 5 cm^2 pots in moist vermiculite and grown on in the dark for a further 24 h. The seedlings were then transferred to either monochromatic R or FR light or continuous W for up to 3 days. Light treatments, W and monochromatic R and FR, were the same as previously described for the mustard seedlings.

2.1.3. The *Brassica ein* mutant

Seeds of a normal, rapid-cycling line of *Brassica rapa* (syn. *campestris*) and the elongated *ein/ein* mutant were placed in Whatman No.1 filter paper moistened with BG11 mineral nutrient solution (Stanier et al., 1971) and chilled in the dark for 3 days. Seeds were germinated in the dark at 20°C for 1 day and then transferred to continuous W light (Devlin et al., 1992).

2.2. Light sources

Fluorescent W light growth rooms provided photosynthetically active radiation (PAR) = 130 μmol m^{-2} s^{-1}. The R:FR ratio treatment cabinets provided a photon fluence rate 400 - 700 nm of approximately 185 μmol $m^{-2}s^{-1}$ in both the high (7.12) or low (0.17) R:FR ratios. Red (R) light (total fluence rate 600-700 nm = 4.8 μmol m^{-2} s^{-1}) was provided by filtering the radiation from Thorn EMI (Birmingham, UK) Deluxe Natural 40-W fluorescent tubes through 1 cm deep copper sulphate solution (1.5 % w/v) and one red (No.14) Cinemoid sheet (Rank Strand, Isleworth, Middlesex, UK). Far-red (FR) light (total fluence rate 700-800 nm = 11 μmol m^{-2} s^{-1}) was provided by water-cooled 100-W incandescent bulbs with a black acrylic filter (Plexiglas Type FRF 700; West Lake Plastics, Lem, Penn., USA). Blue (B) light irradition (20-25 μmol m^{-1} s^{-1}) was provided by white flouresent tubes covered with blue cinemoid film (419 dark blue). All light measurements were made using a LI 1800/12 spectroradiometer (Li-Cor, Lincoln, NE, USA).

The effect on growth of B light was tested by growing seedlings under SOX lamp irradiation with or without the addition of B light. The SOX lamp irradiation was provided by 135W Thorn low-pressure sodium lamps.

2.3. Growth of cucumber seedlings

2.3.1. Region of light perception

Perception of different light qualities in the cotyledon leaves and the hypocotyl was tested by covering the cotyledon leaves with aluminium foil, uncovered seedlings were used as controls (Black and Shuttleworth, 1974). These seedlings (Wt and *lh*) were grown under different R:FR ratios, the Wt plants also being grown under SOX lamps to provide a B light-deficient environment. R:FR ratio light sources and SOX light irradiation were the same as described above.

2.3.2. Norflurazon-treated cucumber seedlings

Wild type and *lh*-mutant cucumber seeds were imbibed in 53 μm Norflurazon ([4-chloro-5-(methylamino)-2-(α,α,α–trofluro-m-tolyl)-3 (2H)-pyridazinone]; Sandoz AG, Basel, Switzerland) and afterwards watered in the same solution (Gaba et al., 1984). The bleached seedlings were excised and proteins extracted following the standard procedure described (2.4.3.).

2.4. Extraction of cell wall proteins

(Unless otherwise stated all chemicals used were of AnalaR grade and were either from Sigma, Poole, UK or BDH Limited, Warwickshire, UK)

2.4.1. Extracellular proteins from mustard

The first internodes of 14 day old light-grown mustard seedlings were excised 2 h after transfer to darkness this transfer being preceded by a 20 min pulse of either R or FR light. The method of extraction of cell wall proteins involving vacuum infiltration with $CaCl_2$ followed by centrifugation was that used by Casal et al., 1990, in which internode samples were placed into a plastic tube covered at the base end with muslin, and placed into a larger tube (eppendorf or test tube according to the size required) to collect the spun sample. The samples were vacuum infiltrated with 50 mM $CaCl_2$ and then blotted dry with tissue paper prior to gentle centrifugation at 650 g for 10 min In these experiments the number of internodes per replicate was 18-20. The parameters of the technique were tested, this included varying the $CaCl_2$ concentration of the infiltration medium between 0 and 1 M, the period of centrifugation (10 and 30 min) and the centrifugal force (650, 1,000, and 1,400 g). Extracts from leaf and second internode samples were prepared in the same manner. Sequential centrifugation steps were carried out by adding fresh 50 mM $CaCl_2$ solution to the syringe tubes and repeating the infiltration and centrifugation steps. Each centrifugation extract was stored separately.

2.4.2. Extraction of intracellular plus extracellular peroxidases from mustard internodes

To extract either the total peroxidase activity of the mustard internode sections or the remaining intercellular activity following vacuum infiltration and centrifugation together with the intracellular peroxidase activity, the upper half of the first internodes were ground in 0.1 M potassium phosphate buffer, pH 6.5, in an ice-cooled mortar at 4°C. The homogenate was centrifuged at 13,000 g for 10 min at 4°C and the supernatant used as the enzyme source. The peroxidase activity that could be extracted by homogenization was tested using different concentrations of $CaCl_2$.

2.4.3. Extracellular proteins from cucumbers

The top 1 centimetre section of Wt cucumber hypocotyls were vacuum infiltrated using a range of salts in the media, all at 50 mM. Sodium, lithium and calcium chloride were used. Each sample comprised five or six hypocotyl sections loaded into a stoppered syringe barrel. Each sample was vacuum infiltrated for 10 min, the vacuum being released twice within that period, and centrifuged at 650 g for 10 min. These samples, called the centrifugate (C), were either analyzed immediately or stored at -20°C.

Ionically bound peroxidases were extracted from both the *lh*-mutant and its isogenic Wt. Either the top 1 cm of the hypocotyl, 1 cm sections down the length of the hypocotyl, one third sections of the hypocotyl or the whole hypocotyl, were examined for peroxidase activity. In some experiments epidermal peels and the cortex from the top 1 cm of the hypocotyl were separated by mechanical stripping and the individual fractions analyzed. Extracts for the analysis of peroxidase activity were made from Wt and *lh* seedlings growing in low and high R:FR ratios and at the time periods indicated. The tissue was ground in extraction buffer (0.1 M MES, 0.2 % Triton X-100 and 1 mM Phenylmethylsulphonyl fluoride(PMSF)), using a pestle and mortar and centrifuged at 13,000 g for 10 min. The resulting supernatent was taken to represent extra and intracellular peroxidases and is referred to as the homogenate (H). The pellet was re-suspended in extraction buffer and centrifuged at 13,000 g for 10

min. This step was repeated until no peroxidase activity could be detected in the supernatant using 3,3',5,5' tetramethyl benzidine as a substrate. To extract ionically bound peroxidases the pellet was resuspended in extraction buffer containing 1 M NaCl and gently agitated at 4°C for 2 h, followed by centrifugation at 13,000 g for 10 min. This supernatant was examined for ionically bound (IB) peroxidase activity. The samples were either analyzed immediately or stored at -20°C. For the experiment discussed in section 5.2.3. an addition of 1 % bovine serum albumen (BSA) was added to the extraction medium.

2.4.4. Extraction of ionically bound proteins of *Brassica rapa*

The ionically-bound proteins from the upper half of the hypocotyls of *Brassica rapa* were extracted from 4 day old de-etiolated normal and *ein* mutant seedlings following the same procedure as that for extracts made from cucumber seedlings (2.4.3.).

2.5. Glucose-6-phosphate dehydrogenase assay

Glucose-6-phosphate dehydrogenase was assayed by a technique modified from Kojima and Conn (1982). Ten microlitres of centrifugate or homogenate sample, extracted from the upper half of the first internode of 14-day old light-grown mustard seedlings, was added to 800 µl of reaction mixture (50 mM Tricine, 4 mM $MgCl_2$, pH 8), 6 µM glucose-6-phosphate and 0.6 µM nicotinamide-adenine dinuclueotide phosphate (NADP). After 1 min the change in absorbance at 340 nm was read using an Amico DW2A spectrophotometer.

2.6. Preparation of protoplasts

The upper halves of the first internodes, from between thirty and forty 14 day old mustard seedlings, were longitudinally sectioned into quarters and placed into 15 ml of digestion medium: 0.5 M sorbitol, 1 mM $CaCl_2$, 5 mM 2-[N-morpholino] ethanesulfonic acid (MES-KOH), pH 6.0, 2 % (w/v) cellulase and 0.3 % (w/v)

macerozyme. After 2 h at 25°C the digestion medium was discarded and the tissue washed three times by gentle shaking with wash medium (0.5 M sorbitol, 1 mM $CaCl_2$, 5 mM MES-KOH, pH 6.0). After each wash the tissue was collected by pouring it through muslin. The washes were combined and filtered through a layer of Miracloth to remove vascular strands. The protoplasts in the combined washes were collected by centrifugation for 3 min at 50-100 g. The protoplast pellet was gently resuspended in 20 ml of 0.5 M sucrose, 1 mM $CaCl_2$, 5 mM MES-KOH, pH 6.0 and overlaid with 2.5 ml of 0.4 M sucrose, 0.1 M sorbitol, 1 mM $CaCl_2$, 5 mM MES-KOH, pH 6.0. This was further overlaid with 2.5 ml of wash medium to make a three step gradient. After centrifugation for 5 min at 300 g the protoplast band which collected between the upper two layers, was removed with a Pasteur pipette. The protoplasts were ruptured by freezing at -20°C and thawing to release the intracellular proteins.

2.7. Protein determination

Protein determination was carried out by the method of Bradford (1976). Ten millilitres of each extract was added to the assay mixture (50 % v/v dH_2O / Bradford reagent [600 mg/l Serva blue G-250 stain in 2 % perchloric acid and filtered]). The absorbance at 630 nm was read using a Dynatech microtitreplate reader. Alternatively the protein content of the samples was determined using a Bicinchoninic acid protein assay kit according to the manufacturers instructions (Sigma procedure No.TPRO-562), Sigma Chemical Co. Poole, U.K. Ten microlitres of sample was added to 200 µl of assay medium (0.5 % copper (II) sulphate in bicinchoninic acid) and the colour allowed to develop for 1 h at 37°C. The colour change at 600 nm was then read on a Dynatech microtitreplate reader. Bovine serum albumin was used to produce a standard curve.

2.8. Peroxidase assay in aqueous solution

Samples from the mustard seedlings were used undiluted, whereas samples extracted from cucumber hypocotyls were diluted 25 times in dH_2O prior to being assayed. Ten microlitres of each extract was added to 200 ml of assay mixture (0.1 mg ml^{-1} 3,3' ,5,5' tetramethyl benzidine and 0.002 % (v/v) hydrogen peroxide in 0.1 M citrate-acetate buffer, pH 5.0). Peroxidase activity was determined by measuring the change in absorption at 450 nm after 15 min. The reaction was stopped by the addition of 50 ml of 2.5 M H_2SO_4. The assay was carried out at 18 - 20°C. The absorbance was read on a Dynatech microtitreplate reader.

To examine for the pH of the assay mixture at which the peroxidase activity was at a maximum, the assay medium was tested at a range of pH (3-8). Centrifugate, homogenate and IB samples from the the apical region of Wt cucumber hypocotyls were examined.

2.9. Non-equilibrium pH gradient gel electrophoresis (NEPHGE).

The peroxidase isoforms were separated from the extracts according to charge by gel electrophoresis. This was carried out using a BioRad vertical mini gel kit following the technique of Robertson et al. (1987) as follows. The gel mixture was made up according to the following recipe: 14 ml distilled water, 4 ml 30:1 acrylamide:bisacrylamide, 4.8 ml 50 % glycerol and 1.2 ml ampholytes. This mixture was degassed for 3-4 min and then polymerized with 100 µl ammonium persulphate (AMPS) (100 mg/ml). Once poured into the apparatus the well forming comb was pushed into place and the gel allowed to set for at least 1 h. The samples were loaded in equal volumes with 60 % glycerol containing 4 % ampholytes and the gels run for 1.5 h at 200 V and 400 V for 1 h. The anode buffer (20 mM acetic acid) and the cathode buffer (25 mM sodium hydroxide), were both used at 4°C. The ampholytes

used were either full range (pI 3.5-10), high range (pI 8-10.5) or low range (pI 2.4 - 5) and coloured isoelectric point markers were from the Electran kits, range 4.7-10.6 or 2.4 - 5.65 . Following separation of the proteins, gels were washed in dH_2O for 15 min and stained for peroxidase activity with 40 ml of 0.2 mg ml^{-1} 4 chloronaphthol and 0.002 % (v/v) hydrogen peroxide in 0.1 M potassium phosphate buffer, pH 6.5. An alternative stain containning 0.1 mg ml^{-1} 3,3' ,5,5' tetramethyl benzidine and 0.002 % (v/v) hydrogen peroxide in 0.1 M citrate-acetate buffer, pH 5.0, was also used. The samples extracted using a 1 M salt solution were passed through a Sephadex column (G50-100) prior to the samples being run an a NEPHGE gel.

2.10. Tissue culture

2.10.1. Preparation and growth of sterile callus tissue.

Wt cucumber seeds were sterilized for 20 min in 20 % (v/v) thick bleach (Domestos) and then washed five times in double distilled water. The seeds were transferred to small kilner jars containing sterile moist vermiculite, the jars resealed and placed under continuous W light for 5 days. Solid Murashige and Skoog (MS) media was prepared with the addition of 30 g sucrose, 7 g agar per litre and a combination of hormones; 1) 0.5 mg^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) 2) 0.1 mg^{-1} 6-Benzylaminopurine (BAP) & 0.5 mg^{-1} 2,4-D 3) 0.5 mg^{-1} naphthalene acetic acid (NAA) 4) 0.1 mg^{-1} BAP & 0.5 mg^{-1} NAA. Four replica plates were prepared of each hormone combination (1-4) and five sterile explants were placed on each plate, each explant being a 0.5 cm section of hypocotyl. The plates were kept under continuous white light at 22°C. The growth of callus was examined for over the next 20 days.

2.10.2. Growth of cell suspension cultures.

Using the same hormone balance that produced the best callus growth (0.5 mg/l 2,4-D), liquid MS media was prepared with the hormone(s) added and 100 ml placed into 250 ml conical flasks. The callus tissue was transferred from the plates into the liquid medium and kept at 22°C. Callus tissue from three separate plates were kept as three

independant lines. The cultures were maintained by being sub-cultured every 14 days by a dilution of 1:10 into fresh MS medium. All work with cultures was carried out in sterile conditions.

2.10.3. Characterisation of the suspension culture.

At days 0, 4, 7, 11 and 14, 2 ml samples of the cultures were taken and centrifuged at 400 g for 5 min. The supernatant was taken as the 'medium' or 'non-bound' fraction. The resulting pellet was weighed, resuspended in extraction buffer (0.1M MES, 0.2 % Triton X-100 and 1 mM Phenylmethylsulfonyl fluride) and homogenized to release any soluble peroxidases. The extraction procedure then follows that of the extraction from whole cucumber tissue, the first supernatant being the 'soluble' fraction and the salt extraction producing the 'ionically bound' fraction.

2.10.4. Fresh weight, protein content and peroxidase activity of the cucumber cell suspension culture.

The fresh weight of the cells was taken as the weight of the first pellet spun down from 2 ml of suspension culture. The protein content of all fractions was determined using a Bicinchoninic acid protein assay kit. The peroxidase activity was assayed following the procedure previously described (see 2.1.4).

2.10.5. Addition of calcium to the suspension culture.

After subculturing the cultures were left for 24 h prior to 1 mM $CaCl_2$ being added to the medium. Samples were taken at 0, 1, 2 and 4 h after the addition of the Ca^{2+}, and again at day 11 of the cycle. The peroxidase and protein content were assayed as previously described.

2.11. Immunochemical studies

2.11.1. SDS-PAGE of cell wall protein

The samples were resolved by their relative molecular mass using sodium dodecyl sulphate - polyacrylamide gel electrophresis (SDS-PAGE) on a BioRad vertical mini

gel kit. A 12 % gel was used to resolve the samples. The resolving gel contained 4 ml 30:0.8 acrylamide:bisacrylamide, 1.25 ml resolving gel buffer (3.0 M Tris, HCl, pH 8.8), 0.5 ml 3 % ammonium persulphate (AMPS) and 4.14 ml dH_2O. This mixture was de-gased under vacuum for 3-4 min. 0.1 ml 10 % SDS was added and gently mixed in, followed by 5 µl of NNN'N'- tetramethylethylenediamine (TEMED). This mixture was transferred to the plates to 1 cm below the bottom of the comb, gently overlaid with a few drops of propanol/water to exclude oxygen and left to polymerize for approximately 30-60 min. The propanol/water was poured off and the top of the resolving gel rinsed in wash buffer (1.66 ml stacking gel buffer (0.5 M Tris-HCl, pH 6.8), 3.76 ml dH_2O, 60 µl TEMED). Stacking gel (0.83 ml 30:0.8 acrylamide:bisacrylamide, 1.66 ml stacking gel buffer, 0.33ml 1.5 % AMPS, 3.76 ml dH_2O, 60 µl 10 % SDS, 5 µl TEMED), was then poured onto the resolving gel, the comb inserted, and left to polymerize for approximately 15 min. Samples were mixed in equal volumes with 2X cracking buffer (50 % glycerol containning 0.05 % bromophenol blue) and boiled for 1 min. The gels were run in SDS running buffer (25 mM Trisma base, 192 mM glycine, 0.1 % SDS) at 150 V for 15 min and 200 V until the dye front had run to the end of the gel (approx. 45 min). Molecular weight markers (Biorad, SDS-7) were included on the gel to determine the size of the proteins.

2.11.2. Western blotting

Protein extracts resolved by SDS-PAGE were electroblotted onto nitrocellulose membrane (Hybond C, Amersham International, Amersham, UK). A transfer sandwich was set up as follows: three gel size pieces of Whatman 3MM paper were soaked in cathode, anode 1 or anode 2 buffers, [Anode buffer 1(0.3 M Trisma base, 10 % methanol, pH 10.4), anode buffer 2 (25 mM Trisma base, 10 % methanol, pH 10.4), Cathode buffer (25 mM Trisma base, 40 mM ß-aminohexanoic acid, 20 % methanol, pH 9.4), TBS (50 mM Tris-HCl,pH 7.4, 200 mM sodium chloride)], The cathode soaked paper was placed onto the cathode of the semi-dry blotter excluding air

bubbles and the gel placed on top. Nitrocellulose membrane (wet in distilled water) was laid over the gel, followed by the 3MM paper soaked in anode 2 and anode 1 respectivly. Placing the anode on top a constant current of 125 mA per gel was passed for 30 min.

Transfer of proteins to the membrane could be examined for by Ponceau Stain (0.6 % Ponceau S, 1 % acetic acid). The stain was poured over the membrane for a few seconds. The stain was rinsed off with water to reveal the stained protein bands, the molecular weight markers were marrked with pen and the stain washed off with TBS-Tween (TBS containning 0.1 % Tween 20).

2.11.3. Blotting native NEPHGE gels

The NEPHGE gels were prepared for blotting onto polyvinylidene difluoride membrane (Immobilon, Millipore) by equilibrating in transfer buffer (50 mM Tris HCl, pH 9.5) for 10 min. The transfer was carried out by capillary blotting by placing the membrane on top of Whatmann 3MM filter paper on a sponge standing in a tray of transfer buffer. The gel was carefully placed onto the membrane ensuring that no air bubbles were trapped underneath. The position of the isoelectric markers were marked with a pencil. Another membrane was placed on top of the gel and the position of the standards marked again. The remaining surface of the filter paper and sponge were covered with plastic cling film. Two pieces of filter paper soaked in transfer buffer were placed on top of the membrane, and two pieces of dry filter paper on top of these. A stack of paper towels and a weight was layed over the gel. This was left overnight to allow transfer of buffer from the tray through the gel to the towels. After transfer of proteins to the PVDF membrane immunodetection experiments were carried out following the protocol in section 2.4.4.

2.11.4. Immunodevelopment

The remaining protein-binding capacity of the blot was quenched with TBS-Tween and 5 % Marvel milk powder, with shaking overnight at 4°C. The primary antibodies

(the antisera raised to either the 33 or 60 kD peroxidases) were diluted at either 1:5,000 or 1:10,000 in TBS-Tween and the blot incubated in this solution for 1 h at 22°C with gentle agitation. The blot was thoroughly washed three times in dH_2O for 10-15 min each, followed by three 15 min washes in TBS-Tween, with or without sugars (50 mM xylose, manose and hexose). A 1:10,000 dilution of alkaline phosphatase-conjugated anti-rabbit antibody (Sigma, UK) in TBS-Tween was used as the secondary antibody and the blot incubated for a further 30-60 min. The blot was then thoroughly washed in both dH_2O and TBS-Tween as described above and then developed in substrate [0.1 M Tris, pH 9.5, 1 mM $MgCl_2$, 50 µl stock BCIP solution (5-Bromo-4-chloro-3-indolyl phosphate, p-toluidine salt: 50 mg/ml in 70 % (v/v) DMF)]. Development was stopped by extensive washing with dH_2O.

CHAPTER THREE

CHAPTER THREE - MUSTARD

3.1. Introduction

Light, acting via phytochrome and a separate B light receptor, is known to control extension growth in both etiolated and de-etiolated plants (Smith and Whitelam, 1990). In light-grown plants, phytochrome is responsible for two main adaptation phenomena; the shade avoidance response and photoperiod perception (Smith, 1986, Casal and Smith, 1989). A low R:FR ratio leads to a low level of Pfr and an increase in growth extension i.e. the shade avoidance response. To simulate the effects of changes in the R:FR ratio, end-of-day light treatments are often used, a R or FR light pulse can determine the level of Pfr, generally believed to be the active form of phytochrome (see Kronenberg and Kendrick, 1986), present in the subsequent dark period. In de-etiolated mustard seedlings, a FR light treatment to the first internode leads to a rapid increase in extension growth (Morgan et al., 1980, Child and Smith, 1987) and the reversibile nature of this induction indicating the involvement of phytochrome in the regulation of stem extension.

When etiolated plants are exposed to light the photomorphogenic pigments, including phytochrome and a B/UV-A photoreceptor, detect the quality, quantity and duration of the light source and initiate changes in the plants growth accordingly. When etiolated plants are exposed to light their rate of stem elongation is restricted and their stems increase in girth, providing greater support to the developing seedling. This de-etiolation of the seedlings also leads to an expansion of the leaves. Another phenomenon associated with light regulated de-etiolation, is that of pigmentation of the tissues due to the induction of photosynthetic and anthocyanin pigments. When etiolated mustard seedlings are treated with R, FR or B light extension growth is inhibited (Beggs et al., 1980). The predominant form of phytochrome in etiolated tissue is phytochrome A (Tokuhisa et al., 1985, Abe et al., 1985), following exposure

to light the level of phytochrome A rapidly diminishes. Phytochrome A is therefore thought to be the primary species active in the process of de-etiolation.

The growth rate of the plant is dependant upon the plasticity of the cell wall which governs the extent by which the cells are able to elongate due to the turgidity of the vacuole. The more extensively linked the cell wall macromolecules, the less plastic will be the properties of the cell wall (Fry, 1986). Therefore the apoplastic enzymes involved in the loosening and stiffening of the cell wall macromolecules e.g. peroxidase, pectin methylesterase and ascorbic acid oxidase, have in many instances been postulated to be regulated by factors that are known to regulate extension growth (Fry, 1986, Lin and Varner, 1991).

In previous experiments the phytochrome control of growth has been found to correlate with changes in extracellular peroxidase activity, e.g. in etiolated maize (Kim et al., 1989) and in light grown mustard seedlings (Casal et al., 1990). Casal et al. (1990) suggested that A4, one of the four anionic extracellular peroxidase isoforms extracted from the first internode of mustard seedlings, was specifically involved with phytochrome-mediated growth rate changes. These workers found that an end-of-day FR light treatment, leading to an increase in stem extension, corresponded to a 50 % decrease in the activity of A4 that could be extracted by the infiltration/centrifugation technique.

Experiments on cell wall enzyme activity often use the enzyme activity that is present in the apoplastic fluid to study comparative changes in activity. A commonly used method to obtain the extracellular fluid involves vacuum infiltrating the tissue with a weak buffer followed by gentle centrifugation, e.g. Barcelo et al. (1988), van der Berg and van Huystee (1984), Rohringer et al. (1983). The sample collected following centrifugation is believed to contain those enzymes that are freely soluble in the apoplastic fluid and those that are weakly bound to the cell wall. Investigations into the use and efficiency of infiltration/centrifugation as a means of extracting cell wall

enzymes has seen a variable range of results e.g. Terry and Bonner (1980), Li and McClure (1989).

The investigation undertaken here was to examine cell wall peroxidases in mustard seedlings, to determine whether the extracellular peroxidase activity was regulated by light. Initial experiments investigated the work of Casal et al. (1990) on the observed phytochrome-regulation of A4, an anionic peroxidase isoform in the first internodes of mustard seedlings. Following on from this idea, the cell wall peroxidase activity in mustard hypocotyls was examined for regulation by light and to ascetain whether the same isoforms were present in the hypocotyls as in the internodes. The work here questions the reliability of using the infiltration/centrifugation technique in the investigation of cell wall enzymes from *Sinapis alba*.

3.2. Phytochrome regulation of extracellular peroxidases in the first internode of *Sinapis alba*

3.2.1. Optimization of the infiltration/centrifugation technique

In order to characterize the A4 isoform, the activity of this enzyme needed to be at a maximum. Various parameters of the infiltration/centrifugation technique used were therefore tested in attempts to maximize the activity extracted. The relative binding strength of the enzymes to the plant cell wall would effect the efficiency of extraction depending on the ionic strength of the infiltration media used. A buffer of weak ionic strength would not be able to extract the highly ionically bound or covalently bound enzymes. As such it is important to ascertain the degree of binding to the cell wall, as any enzymes being extracted by a buffer below the ionic strength needed to free the enzyme from the cell wall might represent only a fraction of the total pool, possibly obscuring a true result. In order to test if the amount of peroxidase activity extracted increased with increasing ionic strength, the nature of the infiltration medium was examined using a range of ionic strengths from distilled water only to 100 mM $CaCl_2$ Whilst 0, 20 and 100 mM $CaCl_2$ all extracted similar low levels of apoplastic

peroxidase activity, 50 mM $CaCl_2$ yielded a much higher activity than both the lower and higher ionic concentrations, see Fig.3.1. Whilst it is difficult to explain why the extraction procedure would be less efficient using a higher ionic strength buffer it may be the higher ionic strength affects the activity, rather than the extraction, of the enzyme. As an infiltration medium of 50 mM $CaCl_2$ extracted the highest amount of peroxidase activity, this buffer continued to be used to extract apoplastic fluid from mustard internodes in subsequent experiments.

In previous studies on extracellular proteins using vacuum infiltration followed by gentle centrifugation, a force of 650 g is commonly used, e.g. Terry and Bonner (1980) and Casal et al. (1990). Other workers have however used faster spin speeds to extract the apoplastic fluid (Jupe and Scott, 1989, Federico and Angelini, 1986), whilst others have taken the intracellular washing fluid from tissue placed under repeated vacuum infiltration steps but no centrifugation step, as the soluble and weakly ionically bound apoplastic enzymes e.g. Li and McClure (1989). The effect of centrifuging the samples at different speeds was examined to see if the peroxidase activity, although extractable by 50 mM $CaCl_2$, still remained in any apoplastic fluid not extracted by the low speed of centrifugation (650 g). Three sequential centrifugations were carried out on three separate samples using speeds corresponding to 650 g, 1,000 g and 1,400 g. Similar levels of activity were found using 650 g and 1,000 g for all three sequential centrifugation steps (C1 - C3), whilst using 1,400 g the activity extracted at each stage was approximately double that of the lower speeds (Fig 3.2). However when the samples were tested for the activity of glucose-6-phosphate dehydrogenase, a cytoplasmic marker that would indicate the extent of cytoplasmic contamination, the samples extracted at 1,400 g had a higher level of activity compared with the samples from the lower centrifugation speeds neither of which showed any glucose-6-phosphate dehydrogenase activity (Fig 3.3).

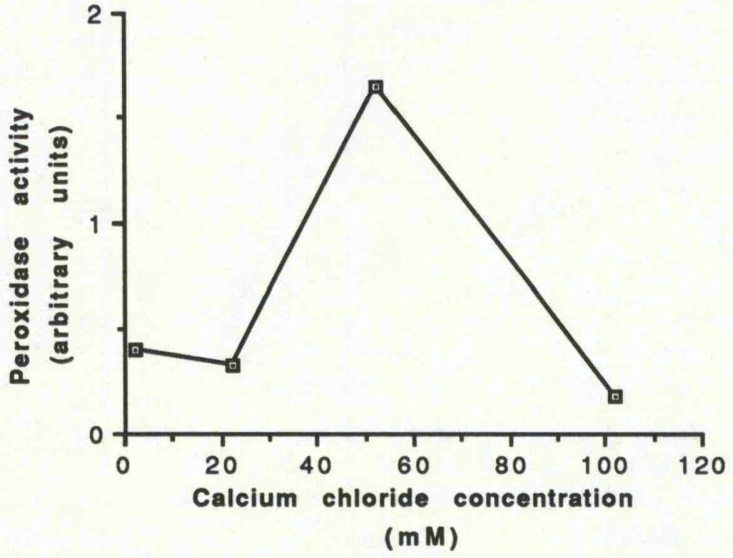


Fig 3.1: Peroxidase activity that could be extracted in the apoplastic fluid from the upper half of the first internodes of 14-day old de-etiolated mustard seedlings, using different ionic strength extraction buffers. The internode sections were vacuum infiltrated and then centrifuged at low speeds (650 g) to extract the extracellular peroxidase activity. The infiltration media used ranged in ionic strength from 0, 20, 50 and 100 mM.

Centrifugal force of spin	650 g	1,000 g	1,400 g
1st centrifugation	6.24	7.76	14.1
2nd centrifugation	4.25	3.45	7.35
3rd centrifugation	2.77	3.06	5.20
Total activity	13.3	14.3	26.6

Entries represent peroxidase activity (A450) per mg protein

Fig 3.2: Comparison of the specific peroxidase activity that could be extracted at different speeds of centrifugation, from the upper half of the first internodes of 14-day old de-etiolated mustard seedlings. The internode sections were vacuum infiltrated with 50 Mm $CaCl_2$ and then centrifuged at either 650, 1,000 or 1,400 g. Three sequential infiltration/centrifugation steps were carried out, and the peroxidase activity assayed, on the same samples (C1-C3).

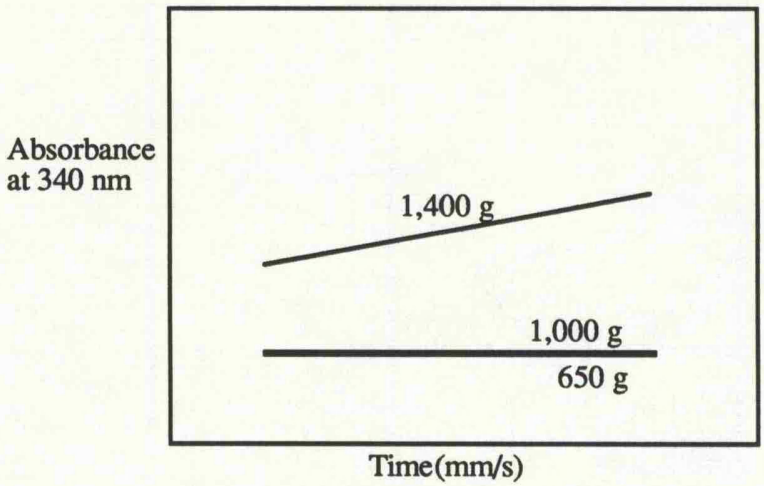


Fig 3.3: Glucose-6-phosphate dehydrogenase activity (A340nm), a cytoplasmic enzyme, present in the first centrifugate samples. All samples were extracted from the top half of the first internodes of 14-day old white light grown mustard seedlings. Following vacuum infiltration with 50 mM $CaCl_2$, the samples were centrifuged at either 650, 1,000 or 1,400 g. The glucose-6-phosphate dehydrogenase activity was examined to test to what extent the cells were being ruptured by the centrifugation step.

As at 1,400 g the cells appear to be rupturing yet little difference was observed between the activity extractable at 650 g and 1,000 g, further extractions were carried out at 650 g where least physiological damage to the cells is likely.

A further parameter that was investigated was the length of the centrifugation step. Initially a 10 min spin had been used following the procedure described by Casal et al., (1990). Increasing the length of the centrifugation might release more apoplastic fluid and hence further peroxidase activity, not released within 10 min. Spins of 10 min duration were compared with spins of 30 min for three sequential centrifugations. (Fig 3.4). The peroxidase activity after 30 min was significantly lower at each centrifugation step compared with the activity found after a 10 min spin. It seems odd that when the internode samples were spun for 30 min a lower enzyme activity was found when compared to samples spun for 10 min. It is assumed that the samples being centrifuged for 30 min, would contain approximately the same level of peroxidase activity after 10 min as the samples assayed at this point, the subsequent 'loss' of activity being difficult to explain. Although all the extraction procedures were carried out at 4°C the possibility remains that the longer centrifugation steps might have led to a decrease in the peroxidase activity through degradation and/or denaturation of the enzyme.

3.2.2. Distribution of A4

Having optimized the parameters of the centrifugation technique, the occurrence of A4 within different parts of the plant was examined to see whether this isoform was specific to the control of stem extension, as would be indicated by exclusive location in the stem region. Leaf centrifugate extracts were separated on a NEPHGE gel and compared to extracts from the first internode. A neutral isoform with strong activity was present in the leaf extract and the same isoform present but in less abundance in the internode. The acidic peroxidase isoforms that were found in the internode tissue were not however present in the leaf sample (Fig 3.5) and do not therefore appear to be involved in the growth and regulation of development of the leaf tissue.

Length of spin (min)	10 min	30 min
1st centrifugation	16.95	8.50
2nd centrifugation	16.73	12.36
3rd centrifugation	16.72	13.20
Total peroxidase activity	50.40	35.06

Entries are peroxidase activity per mg protein

Fig 3.4: The peroxidase activity extractable by increasing the length of time of the centrifugation step. The data illustrates the specific peroxidase activity (peroxidase activity (A450) per mg protein), present in the apoplastic fluid, when extracted using different lengths of centrifugation (10 and 30 min). Three sequential infiltration/centrifugation steps were carried out on each set of tissue. All extracts were from the first internodes of 14-day old white light grown mustard seedlings.

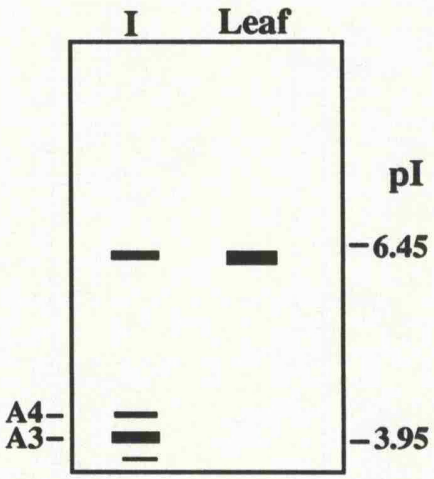


Fig 3.5: Diagrammatic representation to compare the peroxidase isoforms found in samples extracted from the internodes (I) and leaf of 14-day old de-etiolated mustard seedlings. The diagram represents a full range NEPHGE gel and the bands that could be stainned with 4CN.

The distribution of A4 was also examined in the first versus the second internodes, to determine if there was any developmental aspect of isoform specificity. Two sequential centrifugation steps were performed on each sample. The activities of A3 and A4 were similar in both the first and second internodes, both showing a decrease in peroxidase activity in the second centrifugation. (Fig 3.6). A1 and A2 were more clearly visible in the first internode samples in both the first and second centrifugates. Thus the anionic peroxidases being examined for correlations with phytochrome-mediated growth rate changes, in particular A4, are seen to be distributed specifically along the stem axis and might therefore be involved as part of an overall mechanism of stem regulation throughout the extension growth of the plant.

3.2.3. Subcellular location of A4

In order to examine whether any observed regulation of the A4 enzyme was likely to contribute a significant mechanism in the regulation of stem extension, the maximum amount of the A4 pool needed to be examined. To extract the maximal amount of A4 from the apoplastic fluid, five sequential infiltration/centrifugation steps were carried out on the same first internode sections. The same sections were homogenized to release any peroxidase activity from within the cells, or any further peroxidase activity located extracellularly which had not already been extracted. The samples were resolved by NEPHGE gel electrophoresis and stained for peroxidase activity. From Fig.3.7 it can be seen that anionic peroxidase activity, including A4, continues to be extracted even after five sequential infiltration/centrifugation steps and remains abundant in the homogenate of the centrifuged internodes. Also, although homogenizing the internodes in low ionic strength buffers yielded large quantities of A4, no further increase in A4 activity could be discerned by altering the ionic strength of the extraction medium; with 0, 20, 50 and 100 mM $CaCl_2$ all extracting approximately equal levels of peroxidase activity. With 1 M $CaCl_2$ only a

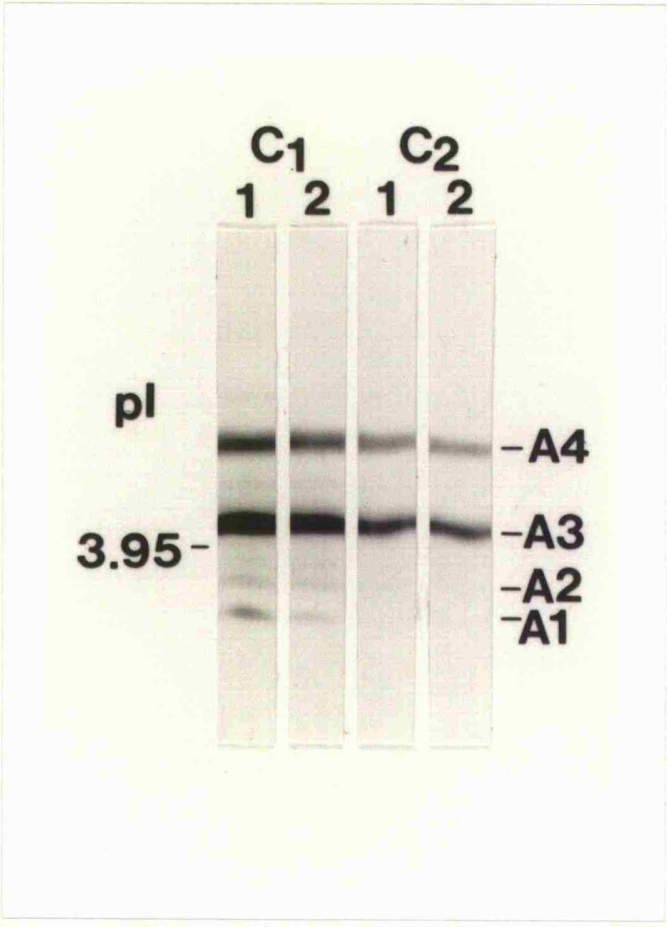


Fig 3.6: The presence of the same anionic peroxidase isoforms in the first and second internode. A low range NEPHGE gel (pI 2-5.5) of bands stained with 4CN, from two sequential centrifugation extracts (C1 and C2) from the first and second internodes of 14-day old white light grown mustard seedlings, labelled 1 and 2 respectively.

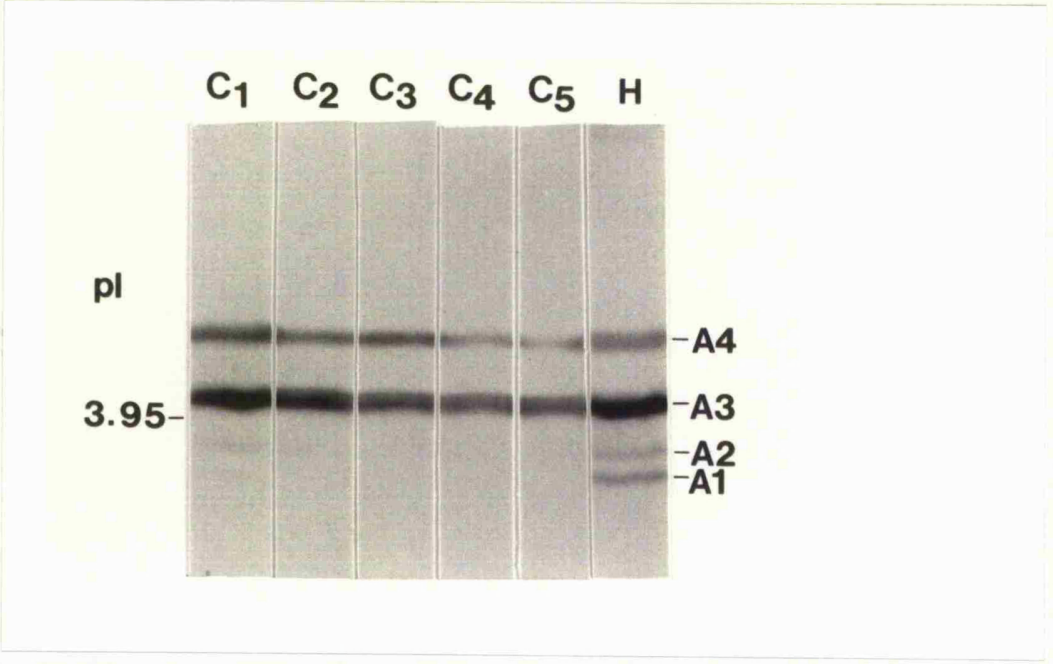


Fig 3.7: The continued presence of the anionic peroxidase A4, following five sequential centrifugation steps. A low range NEPHGE gel (pI 2-5.5) separating the extracellular anionic peroxidases extracted by sequential centrifugation of the upper half of the first internodes of 14-day old mustard seedlings. An homogenate extract of the same samples was compared to examine for intracellular and any remaining extracellular peroxidases. C1-5 = sequential centrifugations 1-5, H = homogenate.

third of the peroxidase activity that could be recovered using a single centrifugation step was found. Again, as in increasing the length of time the samples were centrifuged, a higher ionic strength medium would be expected to include the same level of peroxidase activity found with a lower ionic strength buffer. The reduction in activity found within these parameters is a phenomenon that as well as being difficult to rationalise, seems to question the reliability of this commonly used technique. As no significant increase in the peroxidase activity could be eluted from the tissue with increasing ionic strength, A4 does not appear to be ionically-bound to the cell walls of mustard internodes. This suggests that the continued presence of A4, and the other anionic peroxidases, in the sequential centrifugates may be the result of intracellular leakage from cells damaged by the centrifugation process. If this were true then the location of A4 as an apoplastic enzyme has to be questioned since any peroxidase activity observed in the centrifugates may have been due entirely to intracellular leakage. In order to determine if intracellular leakage caused the continuing presence of A4 in the centrifugates, glucose-6-phosphate dehydrogenase activity was used as a marker of cytoplasmic contamination (Li et al., 1989). From Fig 3.8 it can be seen that relatively little glucose-6-phosphate dehydrogenase activity is present in the sequential centrifugates but is abundant in the homogenate. If the infiltration/centrifugation technique had ruptured a significant number of cells, a relatively high level of glucose-6-phosphate dehydrogenase activity would have been expected in the centrifugate fractions. As this was not the case, the continuing presence of A4 in the sequential centrifugations cannot be readily attributed to cell breakage releasing an intracellular pool of A4.

The continued presence of A4 in the multiple centrifugation steps together with the sample to sample variation in the activities of A3 and A4 that could be recovered from mustard internodes, questions either the extracellular location of A4 as proposed by Casal et al.,(1990) or the efficiency of the infiltration/centrifugation procedure in extracting the extracellular proteins from mustard internodes. Further

Sample	NADH formed (nmol per min)
1st centrifugate	0.08
2nd centrifugate	0.12
3rd centrifugate	0.08
4th centrifugate	0.05
5th centrifugate	0.16
Homogenate	2.50

Fig 3.8: Intracellular contamination of the apoplastic fluid extracted by the infiltration/centrifugation technique. The activity of glucose-6-phosphate dehydrogenase, an intracellular enzyme, present in the five sequential centrifugate samples and subsequent homogenate extract (refer Fig. 3.7) as an indication of the extent of cellular breakdown caused by the centrifugation process.

experiments were therefore carried out on the first internode of mustard seedlings to determine the cellular location of the A4 isozyme.

In the absence of peroxidase isoform-specific antibody probes, an elegant and convenient method to determine the location of the enzymes being studied utilizes the preparation and analysis of protoplasts. By digesting the cell walls from whole plant cells any enzymes associated with the cell wall will be released into the digestion medium whilst the intracellular isoforms would be found within the protoplasts. A preliminary experiment was carried out to examine the most efficient way of preparing mustard internode sections to give the highest concentration of whole protoplasts. Longitudinal and horizontal sections were halved and quartered and placed into digestion medium for 2 h. The longitudinal quartered sections produced the greatest abundance of protoplasts (Fig 3.9) and therefore all subsequent protoplast preparations were carried out in this manner. The protoplasts were washed to ensure that as little contamination as possible from cell wall material was present in the protoplast fraction and the protoplasts then ruptured by freezing and thawing to release the intracellular proteins. The protoplast lysate and digest medium were resolved on a NEPHGE gel which was stained for peroxidase activity. An homogenate sample from intact internodes was used to compare the peroxidase isoforms found both intracellularly and extracellularly with those found separately in the two fractions. From Fig.3.10 it can be seen that A4 is undetectable in the protoplast lysates while large amounts of activity are present in the digestion medium and in the whole internode homogenate. The most abundant of the four anionic peroxidases, A3, was detectable in small amounts in the protoplast lysates and in abundance within the digestion medium and internode homogenate. The absence of detectable A4 within the protoplasts (intracellularly) suggests that it is more or less exclusively located in the apoplast of mustard internodes. On the other hand A3 appears to be located mainly extracellularly with a small proportion remaining within the cells. A neutral peroxidase

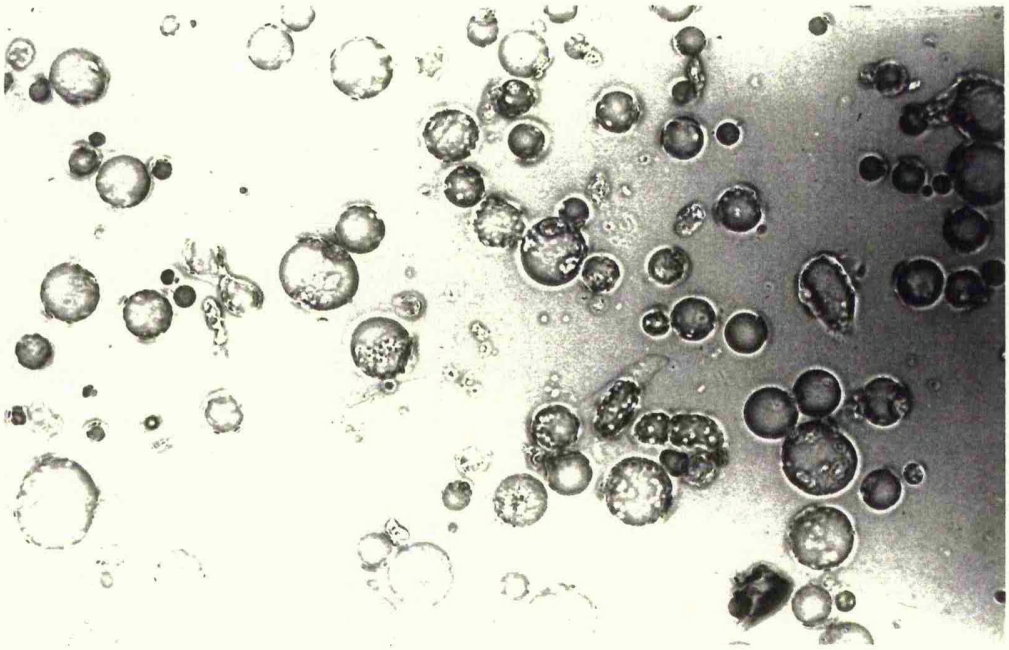


Fig 3.9: Protoplasts prepared from the upper half of the first internodes of de-etiolated mustard seedlings (x40 magnification).

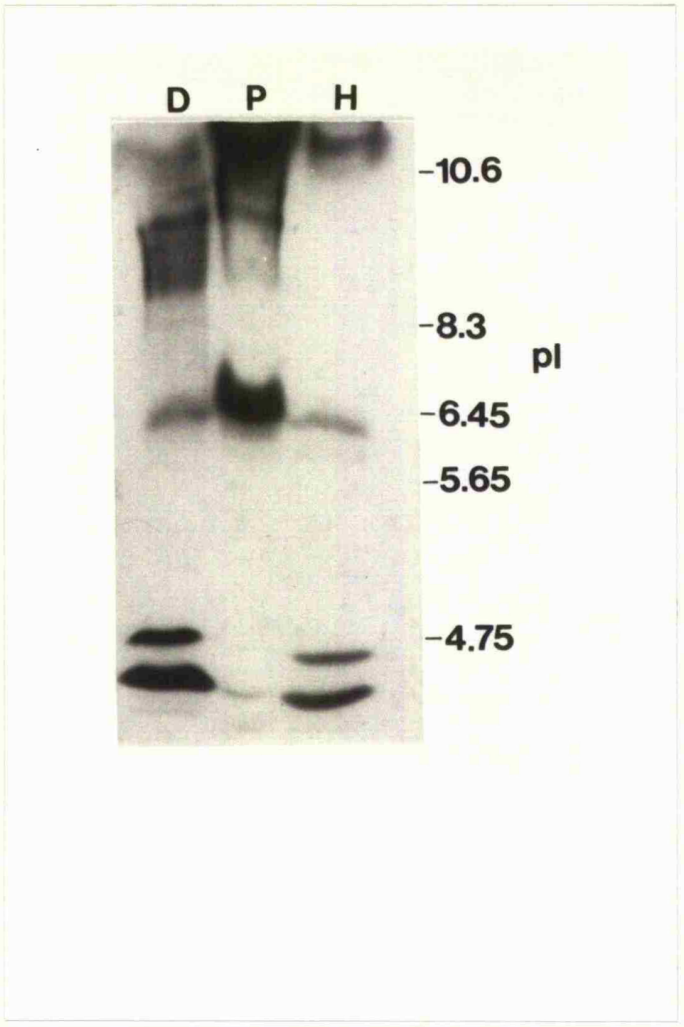


Fig 3.10: Examination of the location of the anionic peroxidase isoforms. Full range NEPHGE gel (pI 3.5-10) comparing the peroxidase isoforms found intracellularly (those within the protoplasts, P) with those found extracellularly (from the digested cell walls within the digestion medium, D) and those from a whole tissue homogenate, H.

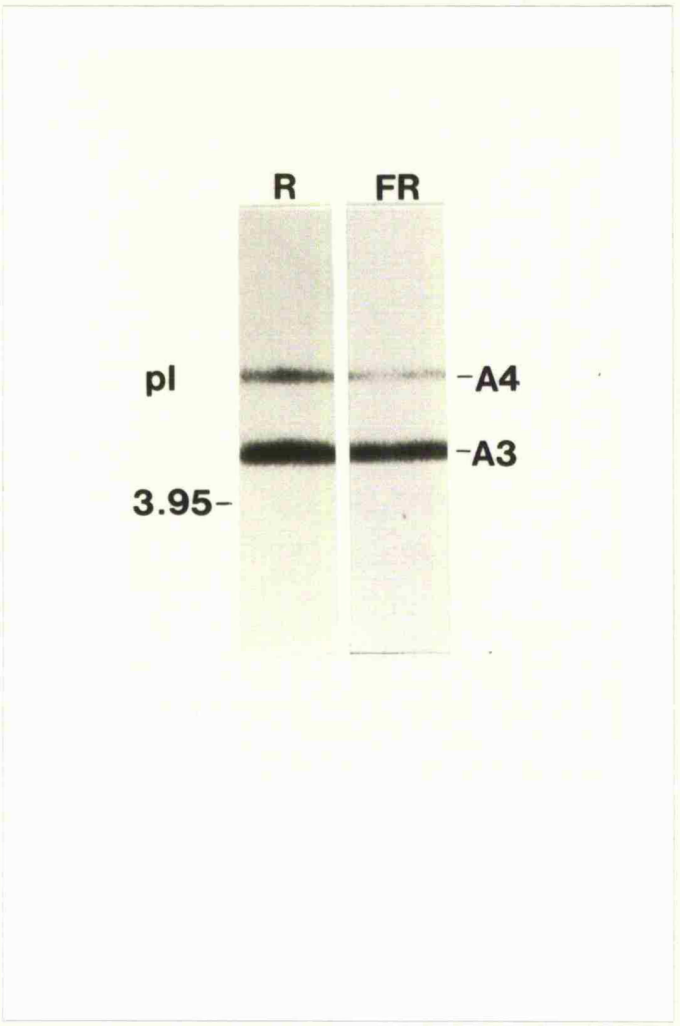
was also observed in each of the samples, but was most abundant in the protoplast lysate. Thus suggesting this to be an intracellular isoform and as such providing a possible marker for cytoplasmic contamination. The more or less exclusively extracellular location of A4, together with the problem of reduced activity found when the length of the centrifugation step and increased ionic strength of the extraction medium, further questions the efficiency of the infiltration/centrifugation technique as a means of extracting cell wall enzymes from mustard internodes.

3.2.4. Phytochrome regulation of growth and the maximum extractable A4 pool

Following the procedure used by Casal et al., (1990), the first internode of light grown mustard seedlings were treated with either a 20 min R or FR light treatment prior to transfer to darkness for 2 h. When the extracellular peroxidases were extracted by the infiltration/centrifugation technique, in accordance with the findings of Casal et al., (1990) the end of day FR light treatment led to a reduction of approximately 50 % in the activity of A4 (Fig 3.11). In order to quantify the change in activity of A4 and to reduce the variability in the activity of A4 observed through the infiltration/ centrifugation procedure, another anionic apoplastic peroxidase, A3, was used as an internal standard. This involves relating the activity of A4 to the activity of A3 within each experiment. This ratio was calculated by the relative density of the A3 and A4 bands scanned from NEPHGE gels. This method of quantifying the change in A4 activity utilizes the method of Casal et al. (1990).

Having established that A4 is almost exclusively extracellular and that the use of vacuum infiltration followed by gentle centrifugation is inefficient in extracting extracellular enzymes from mustard internodes, the percentage of A4 that can be extracted using a single centrifugation step was calculated. This was done by comparing the A4 activity present from a single centrifugation step, to the A4 activity present in the centrifugate and homogenate samples collectively, this was calculated to correspond to approximately 6 % of the total A4 pool. As such any observed

(a)



(b)

Light treatment	A3:A4 ratio
R	0.39
FR	0.19

Fig 3.11: Phytochrome-regulation of A4 activity. Low range NEPHGE gel (pI 2-5.5) of the extracellular anionic peroxidases of 14-day old, white light grown, mustard seedlings extracted after 2 h darkness following a 20 min R or FR light treatment . All extracts were made using a single centrifugation step; b) Comparison of the amount of the anionic peroxidase isoform A4, relative to A3, following the R or FR light treatment in 14-day old mustard seedlings.

differences in the activity of A4 extracted by a single infiltration/centrifugation step, is only considering a small fraction of the total enzyme activity. Therefore, the observed phytochrome-mediated 50 % reduction in A4 activity, only represents an alteration of ~3 % of the total A4 pool. As A4 is almost exclusively extracellular, extracts obtained by homogenizing the tissue produces a maximal amount of A4 enabling the previously observed phytochrome-regulation of A4 to be re-examined using the maximum possible pool of the enzyme.

Phytochrome-regulated changes in the growth of mustard follows a lag period of 10 min after exposure to FR light whereupon a rapid acceleration of growth occurs (Child and Smith, 1987). Any changes in the activity of A4 should correlate with this in order to be considered part of a primary mechanism in the altered rate of stem extension. A second phase of phytochrome regulated growth acceleration takes place after a lag of 35 min. Casal et al. (1990) suggested that in the small pool of A4 they were examining the decrease in peroxidase activity preceded the second but not the first phase of growth. Regulation of the major pool of this enzyme was therefore examined over a period of 2 h for regulation by phytochrome.

Over 2 h no significant difference could be observed in the activity of A4 within the homogenate sample (Fig 3.12). Thus, the previously observed phytochrome-mediation of A4 is not seen when the total pool is examined, with no change in activity of A4 being observed prior to either phases one or two of the phytochrome-mediated acceleration of growth. Many repetitions of this experiment showed varying levels of the activity of specific peroxidase isozymes but no consistent trend showing inverse correlations with growth were found. The gel shown however depicts a commonly seen representation.

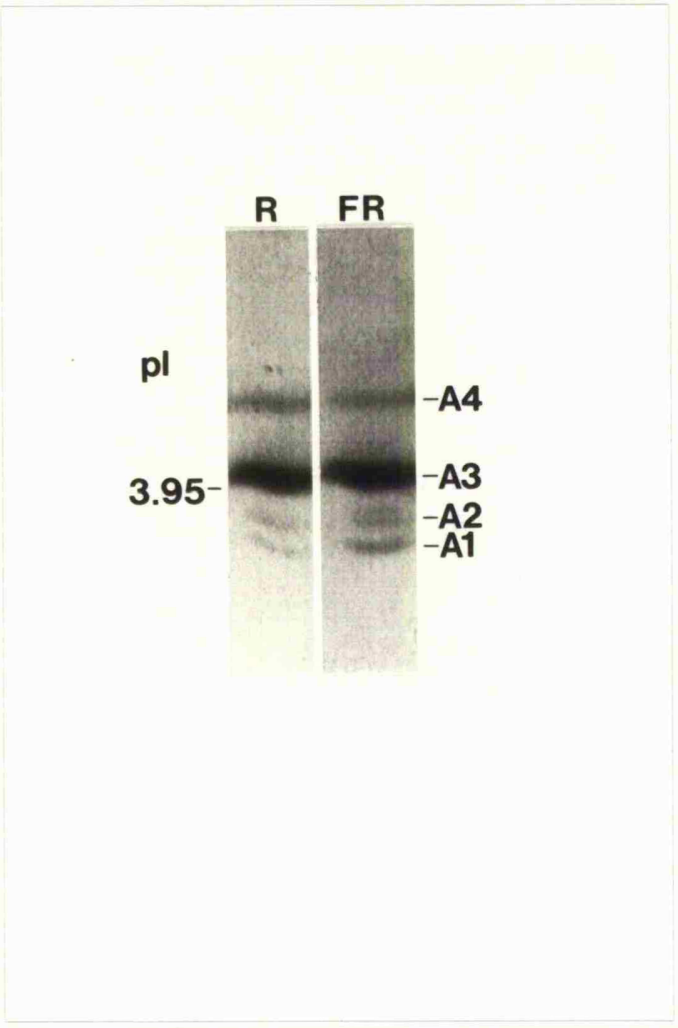


Fig 3.12: The phytochrome-regulation of anionic peroxidase activity, using homogenate extracts to examine the maximum amount of A4. Low range NEPHGE gel (pI 2-5.5) of the homogenate extracts from the first internode of 14-day old, white light grown, mustard seedlings after 2 h darkness following a 20 min R or FR light treatment.

3.3. Light regulation of peroxidase in mustard hypocotyls

3.3.1. Anionic peroxidase isoforms of mustard hypocotyls and the first internode

Mustard hypocotyls were then examined to see if a correlation existed between the light regulation of growth and extracellular peroxidase activity in this region. The extracellular anionic peroxidase isoforms from hypocotyls and the first internode were compared to see if the same isoforms were also present in other areas concerned with stem elongation. Earlier work concerned with the regulation of the mustard anionic peroxidase isozyme A4 (Casal et al., 1990) examined only those peroxidase isoforms associated with the first internode. Mustard hypocotyls were germinated in the dark for two days, followed by two days of either a dark (D) or white (W) light treatment. Samples were extracted from the hypocotyls by a simple homogenization step. Extractions by infiltration/centrifugation were tried but the hypocotyl segments, as well as being difficult to retain in a vertical position in the centrifugation tube, released very little apoplastic fluid by this method. To compare the isoforms present in the hypocotyls to those found in the first internode, a sample from the first internode of 14 day old W light grown plants was extracted by infiltration/centrifugation with 50 mM $CaCl_2$. All samples were separated on a low range NEPHGE gel and the peroxidase isoforms visualized by staining with 4CN. From Fig 3.13 it can be seen that the same anionic isoforms, A1, A3 and A4, that are present in the first internode sample were also present in the hypocotyl samples.

3.3.2. Regulation by light

Hypocotyl sections were examined for regulation by light of the total peroxidase activity and then further examined to see if any observed change in activity was due to any specific isoform. Two day old etiolated seedlings were treated with either D or W light for 2 days and samples prepared by homogenization. The peroxidase activity of the W light treated tissue was found to be approximately double that of the D-

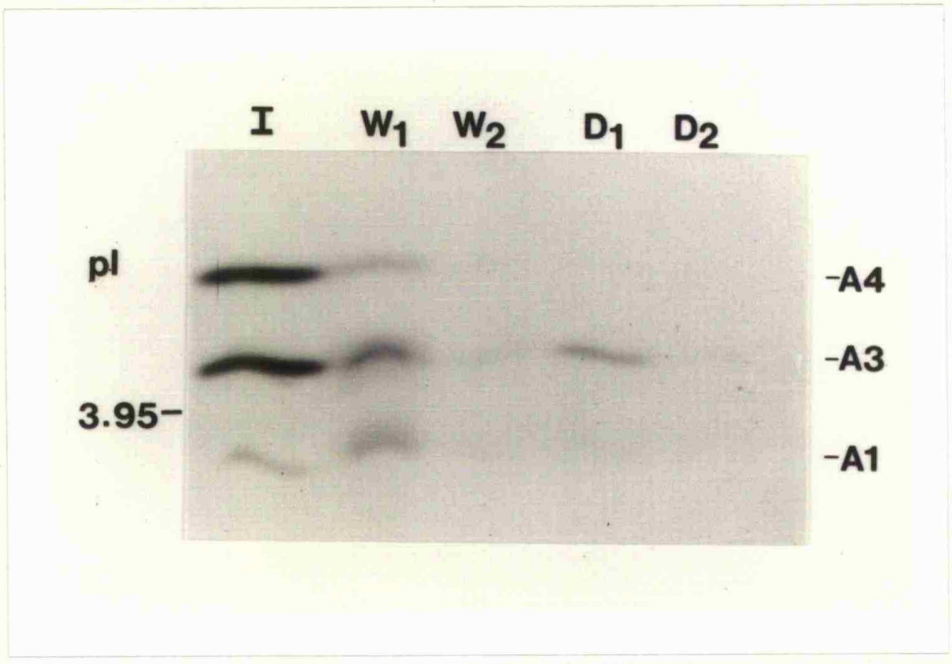


Fig 3.13: Anionic peroxidase isoforms present in mustard hypocotyls. A low range NEPHGE gel (pI 2-5.5) of the anionic isoforms present in extracts from the first internode of mustard (I) with extracts from 5-day old mustard hypocotyls. Extracts from 5-day old de-etiolated (W) and etiolated (D) mustard hypocotyls were also examined for light-regulation of peroxidase activity. Labels 1 and 2 represent the first and second centrifugations carried out on the same W or D hypocotyl samples.

grown tissue. Using total activities would obviously include the activities of any intracellular peroxidase isoforms present in hypocotyl tissue. However, no neutral or basic isoforms were observed when the samples were separated on a full range NEPGHE gel. This may be due to their low abundance within the samples extracted or the lack of cell specialization in this early growth phase, the role of any intracellular isoforms therefore remaining minimal. When the anionic isoforms were examined on a NEPHGE gel, both the A1 and A4 isoforms show a higher activity in the W compared to the D treated tissue (Fig 3.13).

Having established that extracellular peroxidase activity in mustard hypocotyls can be regulated by light, the rate of change of activity during the early stages de-etiolation was examined. Four day old etiolated mustard seedlings were either transferred to continuous W or maintained in D. Extracts from the hypocotyl tissue were made at 0, 1 and 2 h after transfer and peroxidase activity measured in a micro assay using a microtitre plate and TMB as a substrate. Taking the peroxidase activity at zero hours as the base point, in the dark treated tissue a slight increase in activity was observed over 2 h , whilst in the light treated tissue after just 1 h a three fold increase in peroxidase activity was found rising to over four times the initial activity after 2 h (Fig 3.14). Comparing the difference in peroxidase activity between light and dark treated seedlings at each time point, a two and a half fold increase was found after 1 h and a three fold increase after 2 h (Fig 3.14). Thus a dramatic increase in peroxidase activity is found to occur within 1 h of etiolated seedlings being exposed to W light.

In an attempt to increase the strength of the bands seen on the NEPHGE gel and to possibly visualize bands not previously seen, TMB, which was used in the plate based peroxidase assays, was compared to 4CN in gel staining. Whilst the same isoform pattern could be seen with either stain (Fig 3.15) the bands on the gel stained with TMB began to fade after a couple of hours and totally disappeared overnight. Therefore all gel staining was carried out using 4CN.

Time (h)	Dark grown	Light grown
0	100	100
1	120	300
2	130	434

Entries represent the relative increase in specific peroxidase activity (A450) per mg protein.

Fig 3.14: Rate of light-regulated changes in peroxidase activity of mustard hypocotyls. Data illustrates the relative change (%) in the peroxidase activity of 4 day old etiolated mustard hypocotyls at 0, 1 and 2 h, either following transfer to white light (light grown) or maintained in the dark (dark grown).

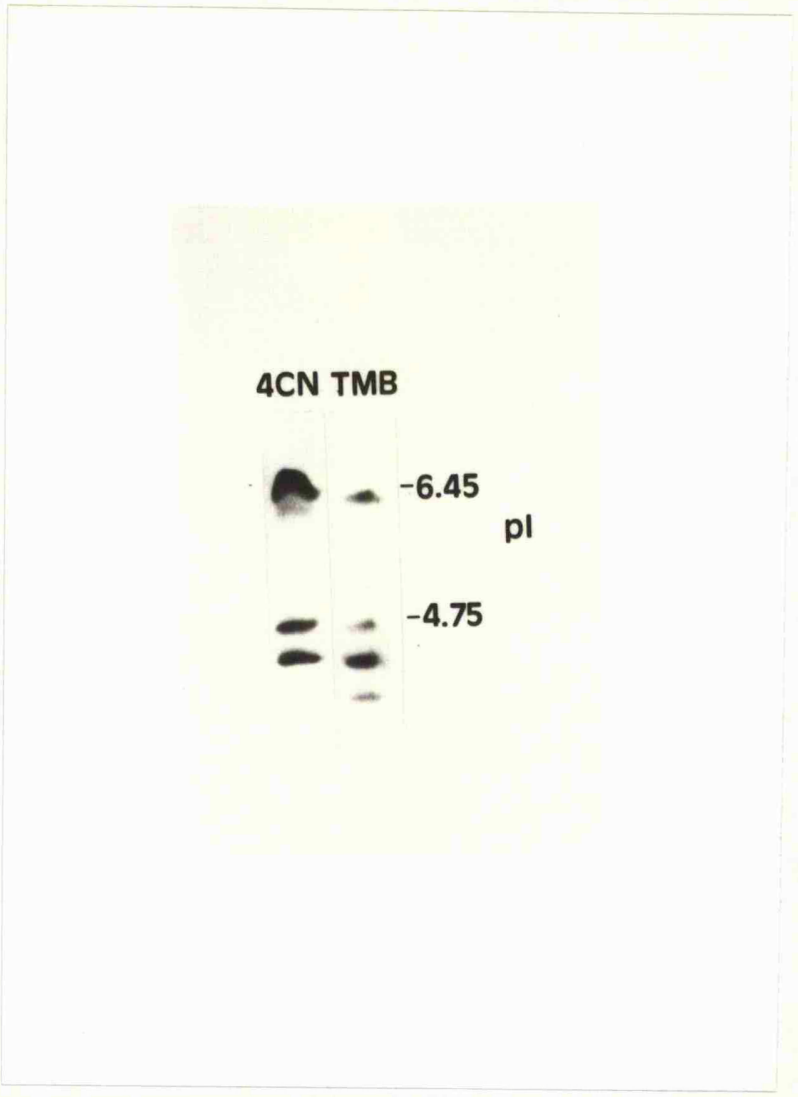


Fig 3.15: Comparison of the peroxidase isoforms highlighted and the relative intensities of, using different stains. Full range NEPHGE gel comparing the staining pattern observed using TMB or 4CN. The extract analyzed was an homogenate sample from the upper half of the first internode of 14-day old white-light grown mustard seedlings.

3.4. Discussion

3.4.1. Examination of the infiltration/centrifugation extraction technique for use with *Sinapis alba*

Having optimized the parameters of the infiltration/centrifugation technique for the internodes of mustard, i.e. length and speed of centrifugation and the ionic strength of the infiltration medium, this method was still found to extract only ~6 % of the extracellular peroxidase isoform A4. As A4 is almost exclusively extracellular and A3 mainly extracellular, there are factors and quirks that question the general reliability of this technique, these include; the variation in activity of the peroxidase that could be recovered from the mustard internodes after a single centrifugation step, the continued presence of A3 and A4 after multiple infiltration/centrifugation sequences and the abundance of A4 activity that could then be recovered by homogenizing the tissue, the lower peroxidase activity that could be recovered following an increase in the length of the centrifugation step and also by increasing the ionic strength of the buffer. The continued presence of A4 in the centrifugate samples is unlikely to be due to the continued synthesis of the enzyme throughout the course of the experiment as all stages of the extraction procedure were carried out at 4°C. Further confusion arises when considering that an increased centrifugation time led to a lower recovery of peroxidase activity and yet sequential infiltration/centrifugation clearly shows that A3 and A4 are still present after multiple 10 min centrifugation steps. The lower level of peroxidase activity that could be recovered with high ionic strength buffers negates the possibility of A4 not being extracted due to its binding properties. It is also interesting to note that when homogenizing the tissue a higher ionic strength buffer again led to a decrease in the enzyme activity extracted compared to lower strength buffers. The high ionic strength buffers may cause a reduction in the amount of activity recovered by denaturing the peroxidase enzyme. The possibility of continued activity being due to an intracellular pool of the enzyme being released into the medium from cells damaged by the centrifugation process can be excluded by the lack of a cytoplasmic

enzyme, glucose-6-phosphate dehydrogenase, being present in the centrifugate. In conjunction with this, the protoplast preparation indicates that A4 is more or less exclusively apoplastic and A3 is mainly apoplastic, therefore the low recovery of these enzymes in the first centrifugate cannot be explained by intracellular location. Terry and Bonner (1980), in a study on the use of centrifugation as a method of extracting apoplastic fluid from pea epicotyl tissue, found cytoplasmic contamination increased with an increase in the speed of centrifugation but interestingly different proteins and sugars were found at different times possibly reflecting their relative abundance within the cell.

Vacuum infiltration/centrifugation is a widely practised technique in the study of extracellular proteins. However the use of this technique and the inefficiency found when extracting the apoplastic A4 peroxidase from mustard internodes, imparts caution as to the accuracy of any findings made in this manner. The large difference observed between the whole enzyme pool and that which is extractable by infiltration/centrifugation causes doubt on the general applicability of this procedure. However where the activity of an apoplastic enzyme extracted by the infiltration/centrifugation technique is representative of any changes that are occurring in the total pool of that enzyme, there is no reason why this method cannot be used providing that its possible limitations are realized.

3.4.2. Phytochrome-regulation of an extracellular anionic peroxidase, A4

Changes in activity of A4 were recorded as the differential extractability of A4 versus A3. The isoform A3 was used by Casal et al. (1990) as an internal standard as it did not appear to be regulated by light. When the extracellular enzymes were extracted by infiltration/centrifugation, a 50 % decrease in the activity of A4 was observed when the seedlings were treated with an end-of-day FR light treatment compared to an end-of-day R light treatment. When compared to the total activity of the maximum A4 pool that could be extracted from the first internodes, the infiltration/centrifugation

technique was calculated to extract approximately 6 % of the total extracellular A4 pool. As the phytochrome-regulated change in activity was 50 %, the overall change in activity only represents approximately 3 % of the total A4 pool. To examine whether phytochrome mediated stem extension correlated with a phytochrome regulated change in A4, the maximum pool of the same isoform was investigated using extractions from the tissue homogenate. However, no relationship between stem extension and A4, or any of the other anionic peroxidase isoforms could be found.

The anomaly found in the phytochrome-regulation of A4, suggests that phytochrome is only mediating a change in a small percentage of the enzyme that can be extracted in a single infiltration/centrifugation step. There are a number of ways in which phytochrome might be mediating this change; e.g. a change in the binding of the enzyme to the plant cell wall, a change in the rate of synthesis or degradation of the enzyme, denaturing the enzyme and/or altering the activity per unit enzyme. The exact cause of this change in A4 activity remains, as yet, undetermined. In contrast to these results the whole pool of A4 showed no regulation by phytochrome prior to either phases of change in growth rate, 10 and 35 min (Child and Smith, 1987). This questions whether the reduction in A4 previously seen in the small pool of the enzyme, that proportion which can be extracted by a single centrifugation step, is of primary significance in the regulation of stem extension.

It may be the case that the 3 % change in extractable A4 activity does represent an important regulatory mechanism in the control of stem extension. There are a number of possibilities that might account for the lower activity extracted and hence potential mechanisms used to regulate the activity of extracellular peroxidase activity. These include a phytochrome induced alteration in the binding qualities of the enzyme to the cell wall, either due to an alteration of the enzyme itself or the binding sites of the peroxidase substrates, which might reduce the quantity of peroxidase enzyme that is extractable with 50 mM $CaCl_2$. This increased binding possibly altering the potential for growth extension. Another means by which a 3 % change in activity might be of

importance could be to do with the activity of the enzyme itself, so rather than a change in extractability of A4 its activity might be being regulated by phytochrome by degradation or denaturation of the enzyme. Despite the possibilities that can be postulated concerning the small change in the activity of A4, it seems implausible that such a small fraction of one peroxidase isoform would constitute a mechanism of primary responsibility for such major alterations in growth when 97 % of the enzyme activity remains unaltered. The original hypothesis of this work, that phytochrome-mediation of growth correlates with an inverse extracellular peroxidase activity in mustard hypocotyls (Casal et al., 1990) would not therefore appear to have been substantiated.

3.4.3 Light-regulation of peroxidase activity in mustard hypocotyls

An examination of the light regulation of extracellular peroxidase activity in etiolated mustard hypocotyls however, revealed that the peroxidase activity of both A1 and A4 peroxidase isoforms shows a higher activity following exposure of the seedlings to light. As the rate of stem extension decreased, due to the effect of light, the extracellular peroxidase activity increased, thus an inverse relationship exists between the rate of stem extension and peroxidase activity. In agreement with these findings there are many examples when light regulation of growth has been found to inversely correlate with peroxidase activity. For instance Kim et al. (1989) working with etiolated maize seedlings found that phytochrome induces rapid and opposite growth changes in different regions, stimulating the elongation of coleoptiles and inhibiting that of mesocotyls. An anionic peroxidase in the cell wall was found to decrease in the coleoptiles and increase in the mesocotyls preceding the phytochrome regulated growth rate changes. An increase in peroxidase activity has also been found to correlate with the B light induced growth inhibition of *Cucumis* hypocotyls. The B-light inhibition being reversed by 1 mM ascorbate which completely inhibited peroxidase activity (Shinkle and Jones, 1988). Although a correlation was found here in mustard

hypocotyls between the light regulation of growth and extracellular peroxidase activity, further experiments would be required to test specifically for regulation by phytochrome. However the relatively low peroxidase activity and low protein content that could be recovered from the hypocotyls (often less than 0.01 mg/ml), coupled with the difficulties inherent with the use of small seedlings, promoted the examination of an alternative species to further test for a correlation existing between phytochrome-regulated growth rate changes and extracellular peroxidase activity. Cucumber seedlings were chosen owing to the existing correlations found between B light regulation of the hypocotyl growth rate and peroxidase activity, the larger sized seedlings making experimentation easier and the availability of a constitutivly tall phytochrome B deficient cucumber mutant.

CHAPTER FOUR

CHAPTER FOUR - The control of cucumber hypocotyl extension by light

4.1. Introduction

Cucumber seedlings show rapid and large alterations in their growth rate due to changes in their light environment making them ideal experimental tools with which to study related changes in enzyme activity. The phytochrome regulation of cucumber growth has been well characterized by amongst others Black and Shuttleworth (1974), Gaba and Black (1985) and Adamse et al. (1987). A general finding has been that treatment with FR light leads to a rapid increase in extension growth. Blue light is also known to inhibit the elongation of cucumber hypocotyls (Cosgrove, 1981, Gaba and Black, 1983, Gaba et al., 1984). Many examples have been put forward to support the hypothesis that peroxidase levels in the plant cell wall correlate with changes in stem extension growth (Morrow and Jones, 1986, Fry, 1986, Jupe and Scott, 1989, Kim at al., 1989). Shinkle and Jones (1988) working with cucumber hypocotyls found that a B light induced peroxidase activity correlated with a decrease in cell wall extensibility. The B light inhibition could be reversed by 1 mM ascorbate which completely inhibited peroxidase activity. This existing correlation found between changes in the growth rate activity of cucumber hypocotyls and extracellular peroxidase activity, further enhances the potential use of cucumber seedlings.

Mutants of other plant species have been utilized to investigate differences in cell wall enzyme activities. In the slender pea phenotype, the enhanced stem elongation compared to the dwarf phenotype which is due to an increase in internode length, correlates with an altered peroxidase activity. The soluble and salt extractable peroxidase activities of the slender phenotype were significantly lower than those of the dwarf phenotype giving further evidence for a peroxidase mediated growth response of stem extension (Jupe and Scott, 1989).

The long hypocotyl cucumber mutant (*lh*) was first characterized by Adamse et al. (1987). Etiolated hypocotyls of the *lh*-mutant and the Wt seedlings show a similar rate of growth in the dark, show R/FR reversibility of growth (Adamse et al., 1987), and contain similar amounts of phytochrome (Nagatani et al., 1989). De-etiolated *lh* seedlings (compared with the Wt) show no response to R light and show no R/FR reversibility of hypocotyl growth (Adamse et al., 1987). Light-grown *lh* seedlings also lack the end-of-day FR response of stem elongation (López-Juez et al., 1990). The phytochrome status of the *lh*-mutant was originally postulated to be a deficiency in light-stable phytochrome (Adamse et al., 1988) and this theory later substantiated by other workers using immunodetection techniques who established that the *lh*-mutant does have a deficiency in a phytochrome B-like phytochrome (López-Juez et al., 1992). Therefore any differences observed in the regulation of growth and correlations with changes in enzyme activity, between the Wt and the *lh*-mutant, are likely to be attributable to phytochrome B.

A phenomenon that has so far only been characterized in cucumber seedlings is the phytochrome regulation of hypocotyl extension via the foliar cotyledons. Red light is perceived in the cotyledon leaves and an inhibitory action on growth transmitted to the hypocotyl. This transmissible element accounting for approximately 60 % of the phytochrome regulated hypocotyl growth inhibition (Shuttleworth and Black, 1974). This phenomenon observed in cucumber seedlings appears to be mediated by phytochrome as covering the cotyledon leaves has no effect on the inhibition of hypocotyl extension when the seedlings are irradiated with B light.

It is important in the monitoring of any changes in enzyme activity, to know where the main area of growth extension is occurring as any differences that are occurring should be at a maximum in this region. Preliminary growth experiments on de-etiolated seedlings were undertaken to examine the effect of changes in the R:FR light ratio, the region of extension growth, the location of R light perception and the effect of B light. The photosynthetic requirement of the phytochrome-regulation of hypocotyl extension

was examined by producing white seedlings by bleaching the tissue with Norflurazon (NF). The effect of covering the cotyledon leaves was also examined in the *lh*-mutant and its Wt, to observe what proportion of the regulation of cucumber hypocotyl extension via the cotyledons and from the hypocotyl itself, can be attributed to phytochrome B. The changes in extracellular peroxidases of de-etiolated cucumber hypocotyls whose rate of stem extension has been mediated by phytochrome, have been studied to investigate if a correlation exists between the two, included in the study where of aid, is the use of the *lh*-mutant cucumber. Further growth experiments were then carried out using etiolated seedlings placed into W light to compare the rate of de-etiolation and corresponding changes in the peroxidase activity. The peroxidase data relating to different growth experiments are detailed in chapter five.

4.2. De-etiolated cucumber hypocotyls

4.2.1. Phytochrome control of cucumber growth

To characterize the change in potential of phytochrome regulation of hypocotyl growth with time, cucumber seedlings were grown under continuous W light for 7 days and then either maintained in continuous W light or transferred to and grown under continuous W light with supplementary FR light. Cucumber seedlings were transferred to W+FR at days 7, 10, 12 and 14 to see whether the phytochrome regulation of hypocotyl extension looses potential with increasing physiological age of the seedling. Cucumber seedlings grown and maintained in W light show very little increase in hypocotyl length between day 7 and day 20. However at any point during this time lowering the R:FR ratio led to a rapid increase in hypocotyl growth eventually reaching a plateau of extension growth rate. With increasing hypocotyl age, a progressively smaller increase in growth is induced by low R:FR ratio, Fig 4.1. Fig 4.2 illustrates the increase in hypocotyl growth observed after 24 h supplementary FR light.

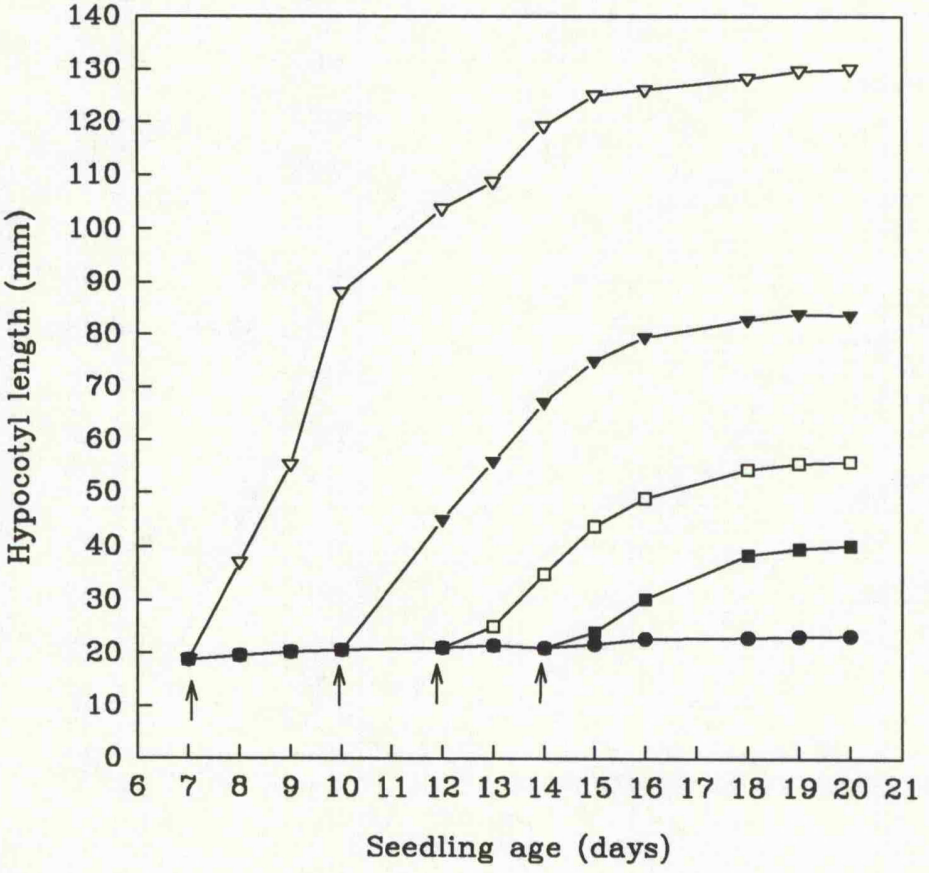


Fig 4.1: Phytochrome-regulation of hypocotyl extension in cucumber seedlings grown under continuous illumination. Seedlings growing in a high R:FR ratio were transferred to a low R:FR ratio at the times indicated, and their growth measured. All results are the means of a minimum of ten plants. SE are < 1 and not therefore shown.

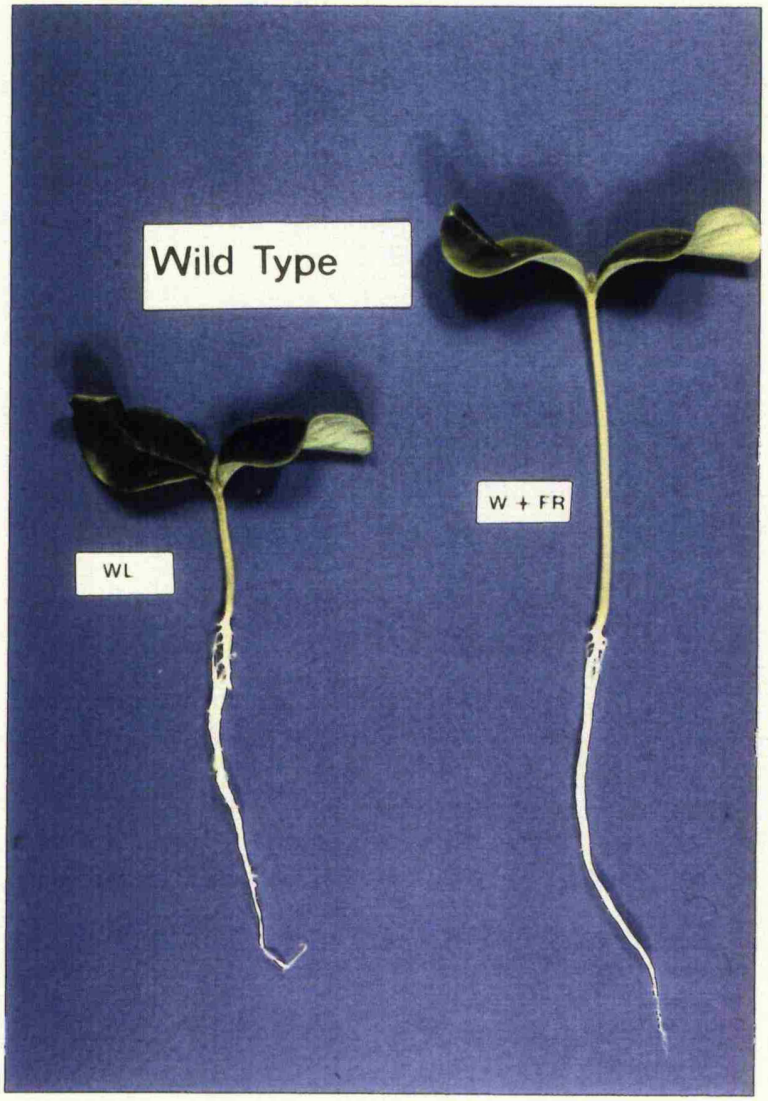


Fig 4.2: Phytochrome-regulation of stem extension of cucumber seedlings. Growth of 8-day old de-etiolated, wild type cucumber seedlings treated for 24 h in either a high (W) or low (W+FR) R:FR ratio.

In order to demonstrate that the hypocotyl extension in cucumbers is phytochrome mediated, placing seedlings growing at the faster rate of extension, those in a low R:FR , back into a high R:FR should reduce the growth rate in accordance with the reversible nature of phytochrome action. Following 2 days in a low R:FR , when seedlings growing at an increased rate were returned to a high R:FR , the rate of extension of the hypocotyl rapidly returned to that of the plants maintained in W light (Fig 4.3 and 4.4). Thus hypocotyl extension in cucumbers is seen to be reversibly controlled by phytochrome.

The long hypocotyl mutant was also tested for the regulation of hypocotyl growth by phytochrome. This mutant mimics the growth of the Wt in a low R:FR ratio Fig 4.5, and over a 24 h period the *lh*-mutant seedlings in a low R:FR showed no significant increase in hypocotyl extension when compared to the *lh*-mutants grown continuously in a high R:FR ratio Fig 4.5 and 4.6. The data shown here only represents 24 h of growth in an altered (lowered) R:FR ratio. The Wt seedlings although showing an acceleration of growth in comparison to the Wt seedlings maintained in a high R:FR ratio, have not yet reached the same height as the *lh* seedlings. With increasing time the Wt seedlings treated with supplementary FR light are almost indistinguishable from the *lh*-mutant.(Whitelam and Smith, 1991). As the *lh*-mutant is known to be deficient in phytochrome B and no response is observed with the addition of FR light, the shade avoidance responses in the Wt and any correlation with enzyme activity are likely to be attributable mainly to the action of phytochrome B.

4.2.2. The effect of blue light on hypocotyl extension in the Wt and *lh* seedlings

Cucumber seedling growth is well characterized as being inhibited by B light (Black and Shuttleworth, 1974, Cosgrove, 1981, Gaba and Black, 1983, Adamse et al., 1987). In order to examine the effect of B light without the effect of phytochrome regulation, *lh* seedlings, were grown under SOX lights which are deficient in the B range of the spectrum (Thomas and Dickinson, 1979). Wild type seedlings and SOX

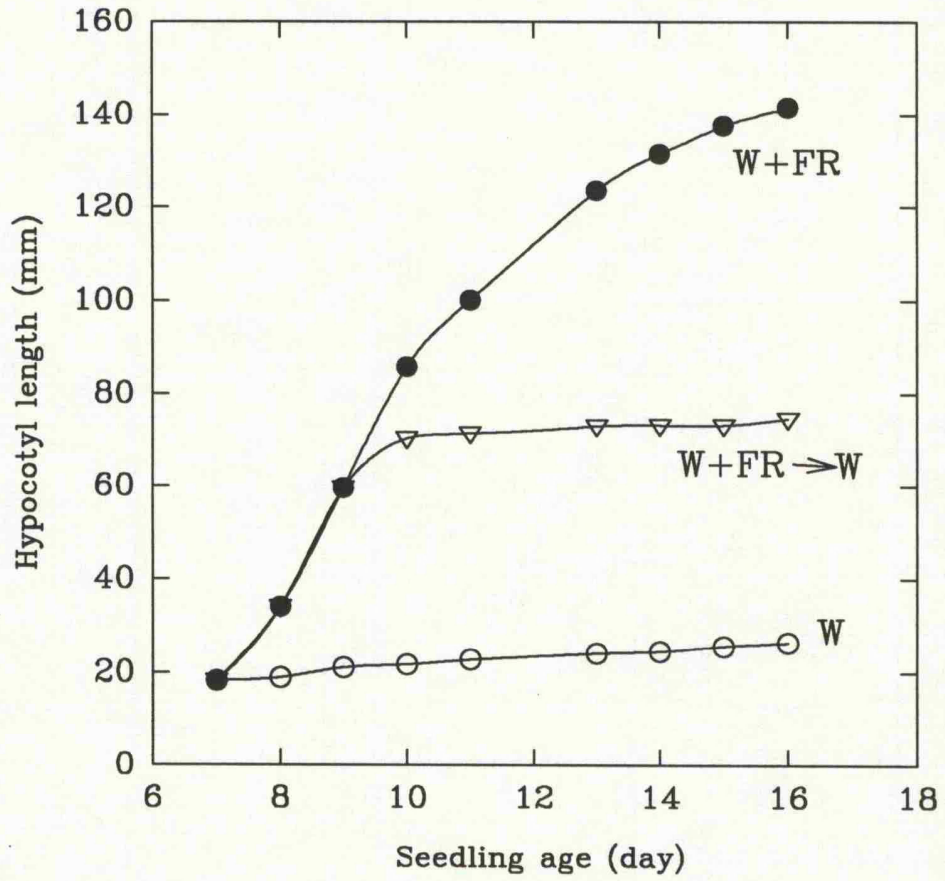


Fig 4.3: The reversible nature of the phytochrome increase in hypocotyl extension. Seven day old seedlings grown in continuous W were transferred to continuous illumination with additional FR (W+FR) for 2 days and then returned to continuous illumination minus additional FR (W+FR →W). Other seedlings from day 7 were maintained in continuous W illumination plus or minus FR. All values shown are the means of a minimum of ten plants and the SE <1.

Fig 4.4: Phytochrome-regulation of hypocotyl extension of wild-type cucumber seedlings. Seven day old seedlings grown in continuous W light were transferred to continuous illumination with additional FR light (W+FR) for two days and then returned to continuous illumination minus additional FR (W+FR →W). Other seedlings from day 7 were maintained in continuous W illumination plus or minus FR. All seedlings shown are 11-days old.

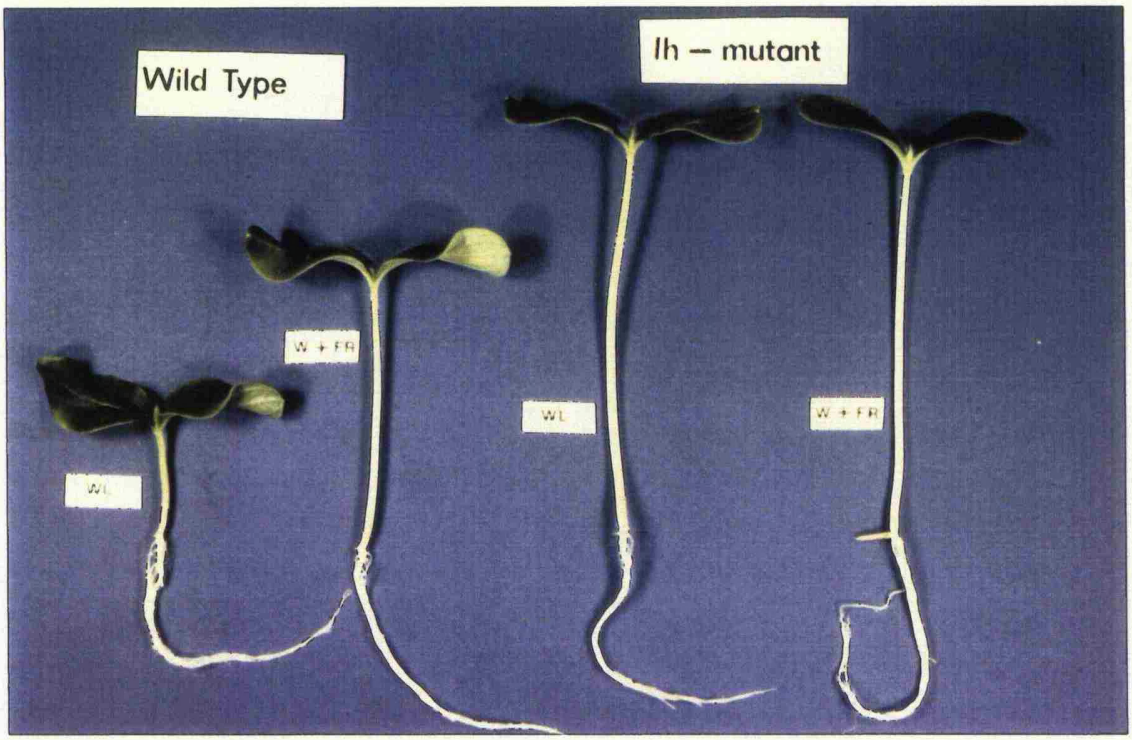


Fig 4.5: Phenotype of the *lh*-mutant compared with its isogenic Wt and the phytochrome-regulation of the seedlings growth. All seedlings were grown for 7 days in continuous W light and then either in a high (W) or low (W+FR) R:FR ratio for 24 h.

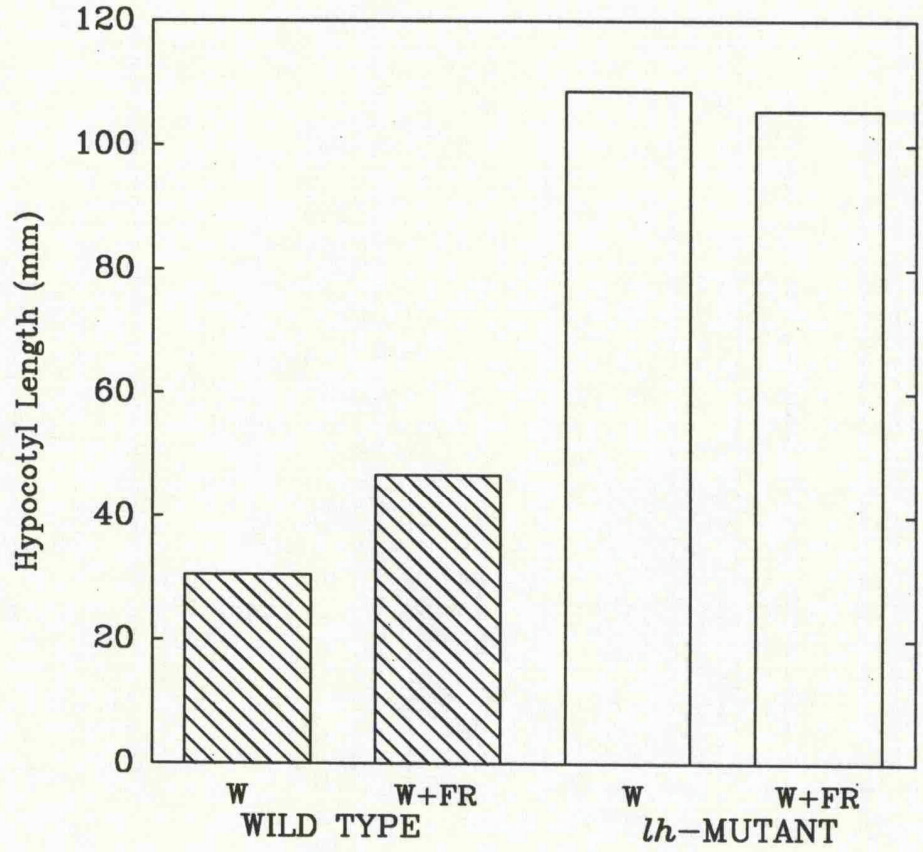


Fig 4.6: Phytochrome-regulation of hypocotyl extension of the Wt and *lh*-mutant cucumber seedlings. Hypocotyl length of 8-day old Wt and *lh*-mutant seedlings grown under continuous W light for 7 days followed by 1 day in either a high (W) or low (W+FR) R:FR ratio. All SE<1 and not therefore shown.

+ B light conditions were used as controls. Both the Wt and the *lh* seedlings showed a slight reduction in their rate of stem extension due to the presence of B light (SOX + B) compared with those seedlings grown in the absence of B light (SOX), Fig 4.7. The inhibition of stem extension observed here due to the presence of B light, although small, does constitute a significant result. The low fluence rate of B light used, 20-25 μmol m^{-2} s^{-1}, would not be high enough to elicit rapid or large growth responses (Gaba and Black, 1983, Cosgrove, 1981). Here, in accordance with Adamse et al. (1987), the Wt and *lh* show a similar reduction in stem extension rate by B light.

4.2.3. Region of extension growth

In order to localise the region of extension growth initial experiments were carried out by gently marking the hypocotyl with a fine felt tip pen. However after less than 24 h the tissue surrounding the pen mark started to form callus tissue. As peroxidase enzymes are known to be involved as a response to wounding and the development of callus tissue might in itself impede extension growth, an alternative method of marking the hypocotyl was tested. The seeds of tobacco were taken and Vaseline used to adhere them to the appropriate position along the hypocotyl. This method not only prevented the tissue from callusing but also made measuring the regions easier owing to the propensity of the ink markings to both spread and fade.

Sections down the hypocotyl were measured for their comparative rate of extension. Five day old cucumber seedlings were marked into 0.5 cm sections down the length of the hypocotyl and placed under continuous W light. Daily measurements of the sections were taken. Over a period of 7 days a gradient of growth was found from the apex to the base, the growth rate being highest at the apex (see Fig 4.8). This follows the pattern of extension usually expected in growing seedlings. To test whether the same growth pattern occurred when phytochrome-mediated an increase in extension growth, the same experiments were carried out on Wt seedlings grown in continuous W with supplementary FR light. The *lh*-mutant seedling was also tested to both observe its pattern of growth and to examine whether phytochrome B was

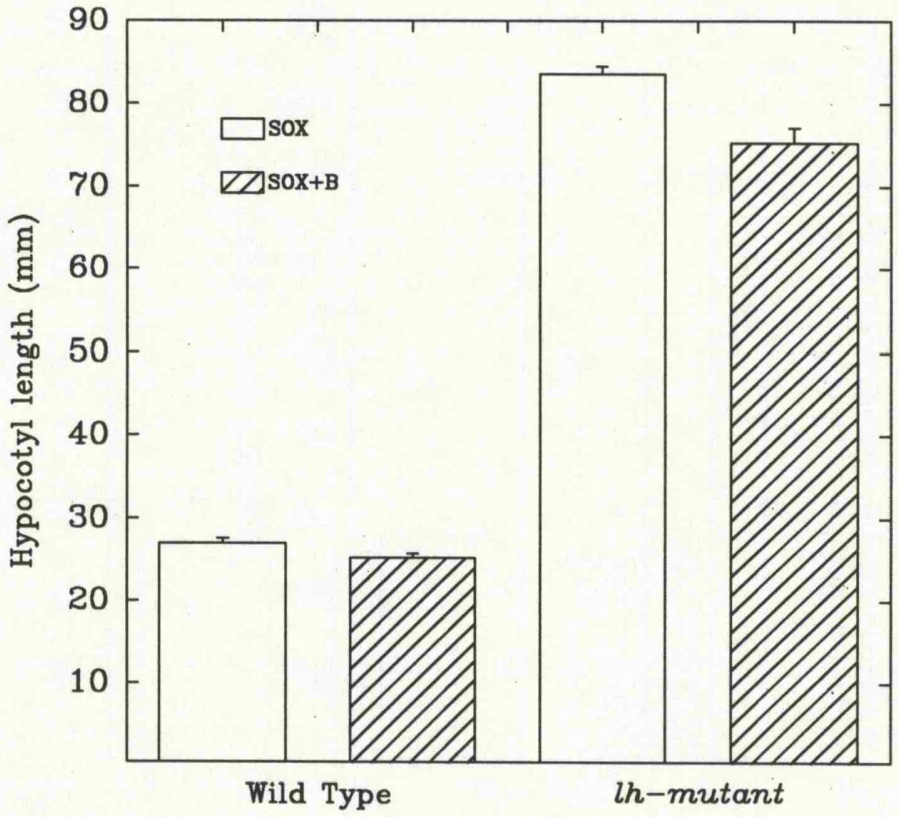


Fig 4.7: The effect of blue (B) light on the growth of cucumber hypocotyls. Length of the Wt and *lh*-mutant hypocotyls grown in a B light deficient environment (SOX). A control of seedlings grown under SOX+B light was used as a control. All seedlings measured were 8-days old and had been germinated and grown continuously under the respective light conditions indicated. All measurements are from a minimum of five seedlings.

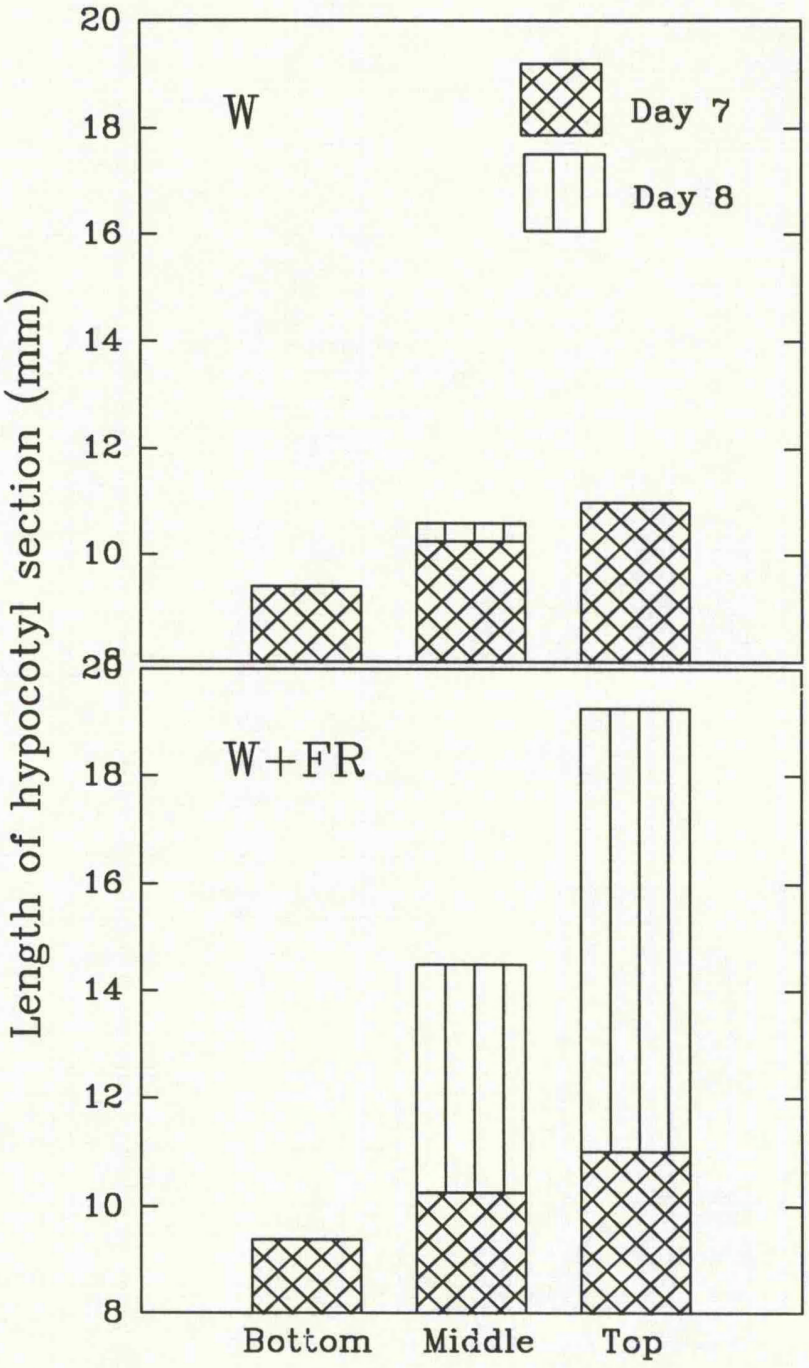


Fig 4.8: Phytochrome-regulation of growth of the bottom, middle and top thirds of Wt cucumber hypocotyls. Each section was marked by sticking a tobacco seed onto the hypocotyl with Vaseline. At day 6 the top and middle sections of W light grown seedlings were 10 mm in length, with the bottom section comprizing the remaining portion of the hypocotyl. The length of each section was measured on days 7 and 8 of seedlings grown in either a high (W) or low (W+FR) R:FR ratio. All values are the means of five plants and SE<1.

involved in the pattern of stem development. One centimetre rather than 0.5 cm sections were marked and measured, as this was found to increase accuracy of the individual measurements and was easier to carry out practically. Growth measurements were taken daily. A similar gradient of growth , decreasing from the apex to the base, was found in the Wt in a low R:FR ratio and in the *lh*-mutant seedlings Fig 4.8 and Fig 4.9. As the 0.5 cm growth section experiments gave the same pattern of growth and more detailed experiments were carried out using 1 cm sections, the growth of 0.5 cm sections has not been illustrated.

Another method used to compare the difference in growth was to directly compare the top, middle and bottom sections of the hypocotyls. At 6 days the Wt seedlings were approximately 30 mm long, the sections were taken as 10 mm for the top and middle, with the bottom section averaging 9.4 mm. These seedlings were grown for a further 24 h in continuous W light and then 24 h in either continuous W or W+FR light. Fig 4.8 represents the actual length of the hypocotyl sections in W and W+FR from day 7 to day 8. Whilst the distribution of growth in W light remains fairly constant through the growth period examined, in W+FR extension of the hypocotyl occurs mainly in the top region with the rate of extension decreasing down the mid region to the bottom section. Whilst this means of growth analysis is the same in a high R:FR ratio as the 1 cm sections, this method was used as it provides a convenient means of directly comparing equivalent areas in the distribution of peroxidase activity due to the phytochrome-regulation of growth.

4.2.4. Light perception by the cotyledons

A large proportion of the phytochrome control of de-etiolated cucumber stem extension is due to the perception of R light mainly in the cotyledons, the effect being transmitted to the hypocotyls, (Black and Shuttleworth, 1974). Covering the cotyledon leaves with foil to exclude light and monitoring the extension of the Wt and the *lh* hypocotyls in both low and high R:FR ratios, allows the location of involvement of phytochrome B to be examined. From Fig 4.10(a) it can be seen that in the Wt seedlings covering

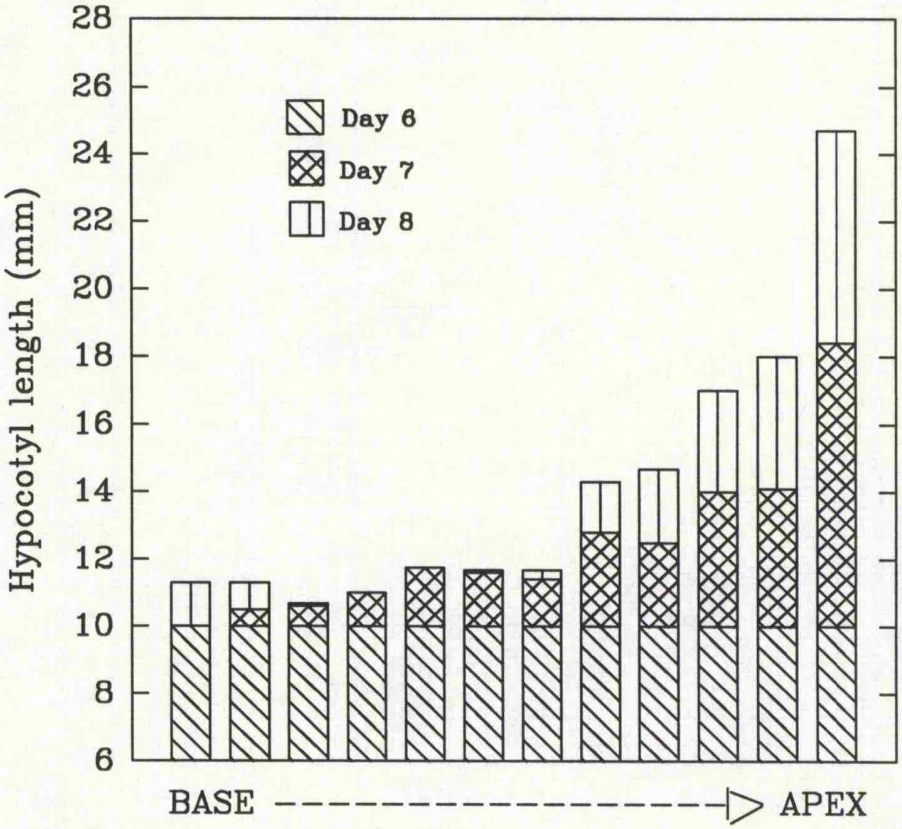


Fig 4.9: Gradient of growth from the apex to the base of the *lh*-mutant hypocotyl. The hypocotyls of 6-day old seedlings were marked into 1 cm sections and the length of each section measured on days 7 and 8. All seedlings were grown under continuous W light and results shown are an average of a minimum of 10 plants. SE<1 and not therefore shown.

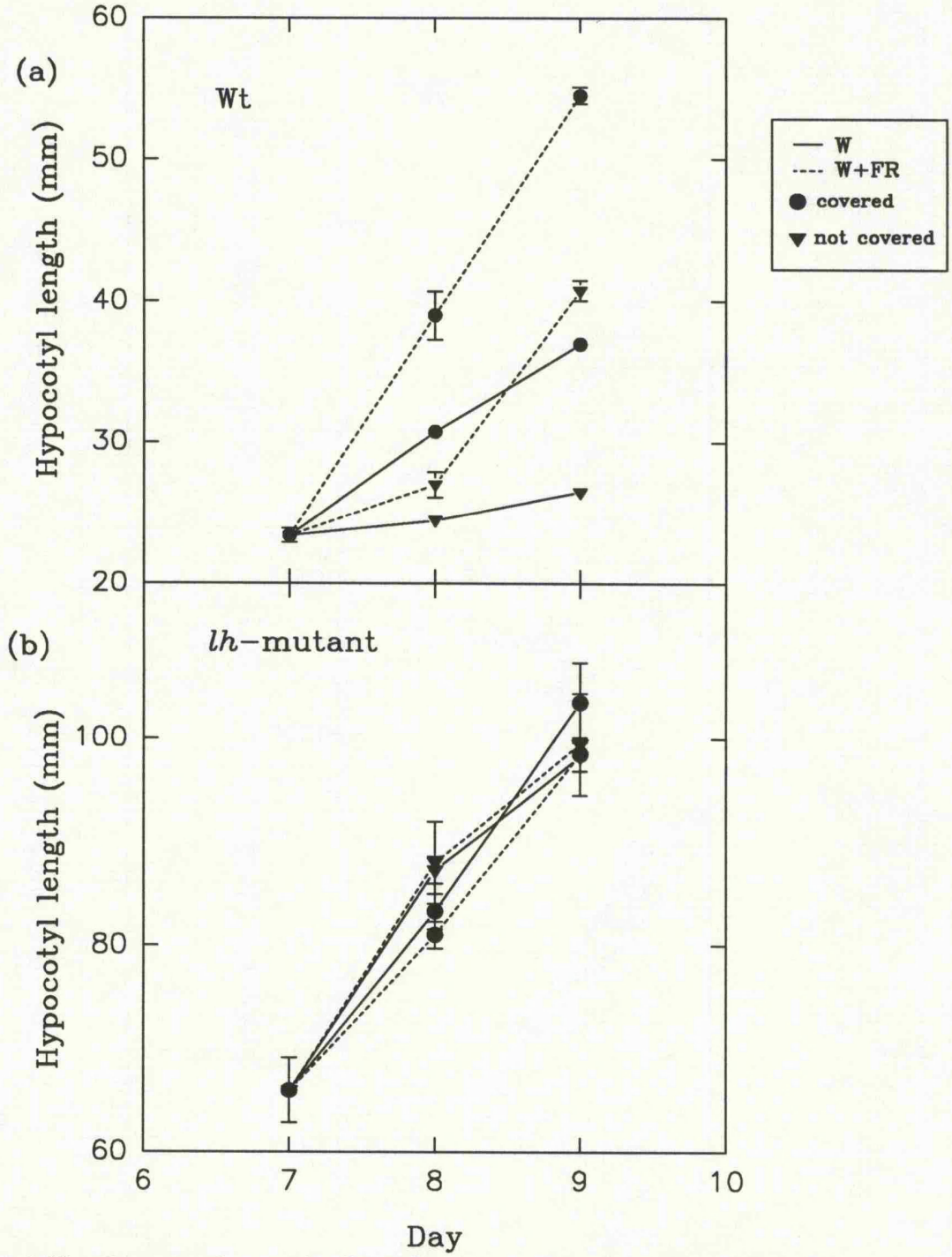


Fig 4.10: Hypocotyl growth of the Wt (a) and *lh* mutant seedlings (b) whose cotyledon leaves were covered with foil to examine for the location of light perception in the control of stem extension. The seedlings were grown under continuous W light for 7 days and then transferred to either a high (W) or low (W+FR) R:FR ratio. Uncovered seedlings were used as controls. All measurements are an average of a minimum of 10 seedlings, with the SE<1.

the cotyledons leads to an increase in stem extension in both a low and high R:FR ratio.

The seedlings with covered cotyledons in continuous W (a high R:FR ratio) showed an increase in stem extension compared to non-covered seedlings, but did not reach the same rate of extension as seedlings grown in W+FR (a low R:FR ratio). These differences in growth responses were present after 24 h, continuing the treatment for a further 24 h revealed the same pattern of growth. However, the *lh*-mutant showed no differences in growth when its cotyledon leaves were covered, in either W or W+FR light Fig 4.10(b). As the *lh*-mutant is known to be deficient in phytochrome B, the cotyledonary perception of R light appears to be involved specifically with this phytochrome species.

The experiment was repeated on the Wt seedlings with the addition of a SOX light treatment, which is deficient in B light, to examine what proportion of the growth differences might be attributable to a B light effect. The seeds used in this experiment were found to require vernalizing (see 2.1.2.1.) prior to germination, as described in 2.1.2. The Wt seedlings showed a similar, though much lower response when compared to the first experiment for the seedlings grown in W, an acceleration of growth apparent when the cotyledons were covered, although this response was not visible until 48 h after treatment began. However, the seedlings grown in W+FR light, either with or without covered cotyledons, remained indistinguishable after 24 h. After 48 h the seedlings with covered cotyledons remained at approximately two thirds of the height of the uncovered seedlings, the reverse of the growth pattern seen in the first experiment. The seedlings grown under SOX lights showed no differences in hypocotyl extension whether the cotyledons were covered or not Fig 4.11. Whilst it remains difficult to fully interpret these results and the differences found in comparison to the first experiment, it should be noted that as a consequence of vernalizing the seeds initial growth of the seedlings was more extensive than seeds germinated without vernalization. The height of the cotyledons averaged nearly 40 mm compared

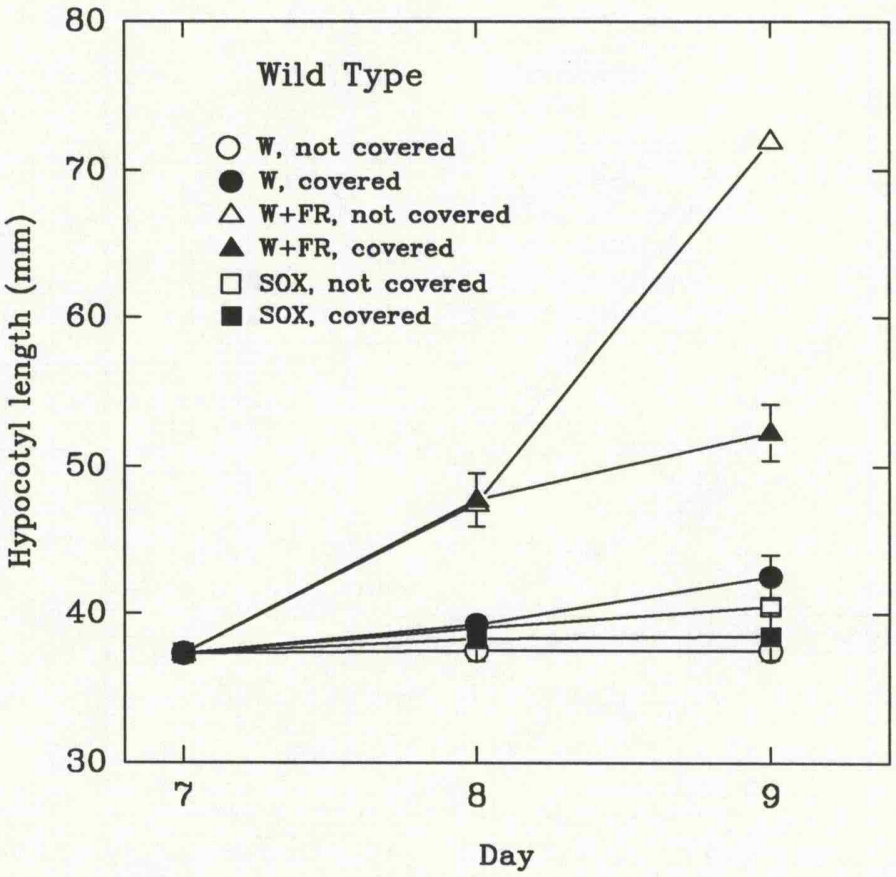


Fig 4.11: Hypocotyl growth of Wt seedlings with or without their cotyledon leaves covered with foil. Seven day old W light grown seedlings were placed under SOX lamp irradiation (SOX), a low R:FR ratio (W+FR) or a high R:FR ratio (W). Growth of the hypocotyl was measured over the next 2 days. All results are the means of a minimum of 10 plants +/- SE.

to an average height previously observed of approximately 20-25 mm. Owing to this increase in growth, the growth reserves from the cotyledons are likely to be exhausted. Cucumber seedlings are known to have a photosynthetic requirement from the cotyledons in order to grow and develop true leaves (Penny et al., 1976), by covering the foliar cotyledons their ability to carry out photosynthesis has been restricted, possibly resulting in the restriction of growth observed. This is most clearly demonstrated in the seedlings grown in W+FR (Fig 4.11).

4.2.5. Examination for a photosynthetic requirement for growth in the Wt and *lh*-mutant seedlings

The phytochrome regulation of 7 day old bleached Wt and *lh* seedlings was examined to test whether this regulation required a photosynthetic element. Seven day old de-etiolated Wt and *lh* seedlings that had been imbibed and then watered with 53 μM NF were grown for 24 h under a low or high R:FR ratio and the growth of the hypocotyl compared with non bleached seedlings grown under the same conditions (Fig 4.12 (a)). The NF treated Wt seedlings after 24 h in a low R:FR showed a restricted rate of hypocotyl extension compared to non-treated seedlings. This restriction of hypocotyl growth extension with NF treatment is not shown in the seedlings grown in a high R:FR ratio (Fig 4.13). This inhibition of stem extension in W+FR would seem to indicate that the regulation of growth by phytochrome has a requirement for photosynthesis. The *lh* mutant seedlings were also tested to examine for the specific involvement of phytochrome B. In both W and W+FR the NF treated *lh* seedlings were restricted in growth to the same extension rate as the Wt seedlings grown in continuous W+FR. These seedlings have therefore lost their constitutive faster growth rate irrespective of the R:FR ratio (Fig 4.13 and Fig 4.12 (b)). The inability of the cucumber seedlings to carry out photosynthesis is therefore seen to affect the rate of stem extension of the *lh*-mutant to a greater extent than the Wt seedlings. As the *lh*-mutant is deficient in phytochrome B (López-Juez et al., 1992), it therefore seems that the regulation of cucumber hypocotyl extension is affected not only by a

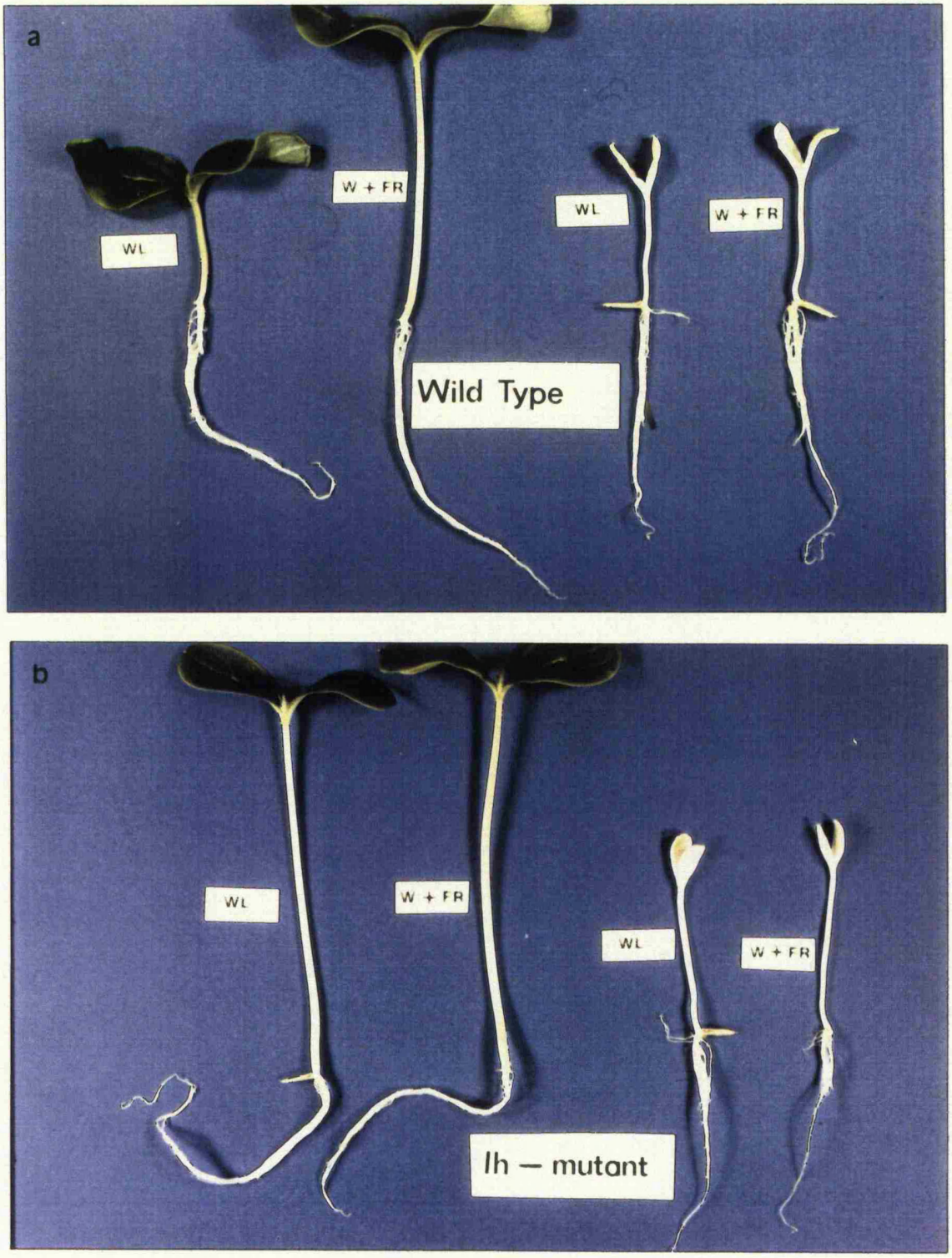


Fig 4.12: Phenotype of Wt (a) and *lh*-mutant seedlings (b) bleached by imbibing and growing them in a 53 µM Norflurazon solution, grown in a high (W) or low (W+FR) R:FR ratio. Non bleached seedlings were used as controls.

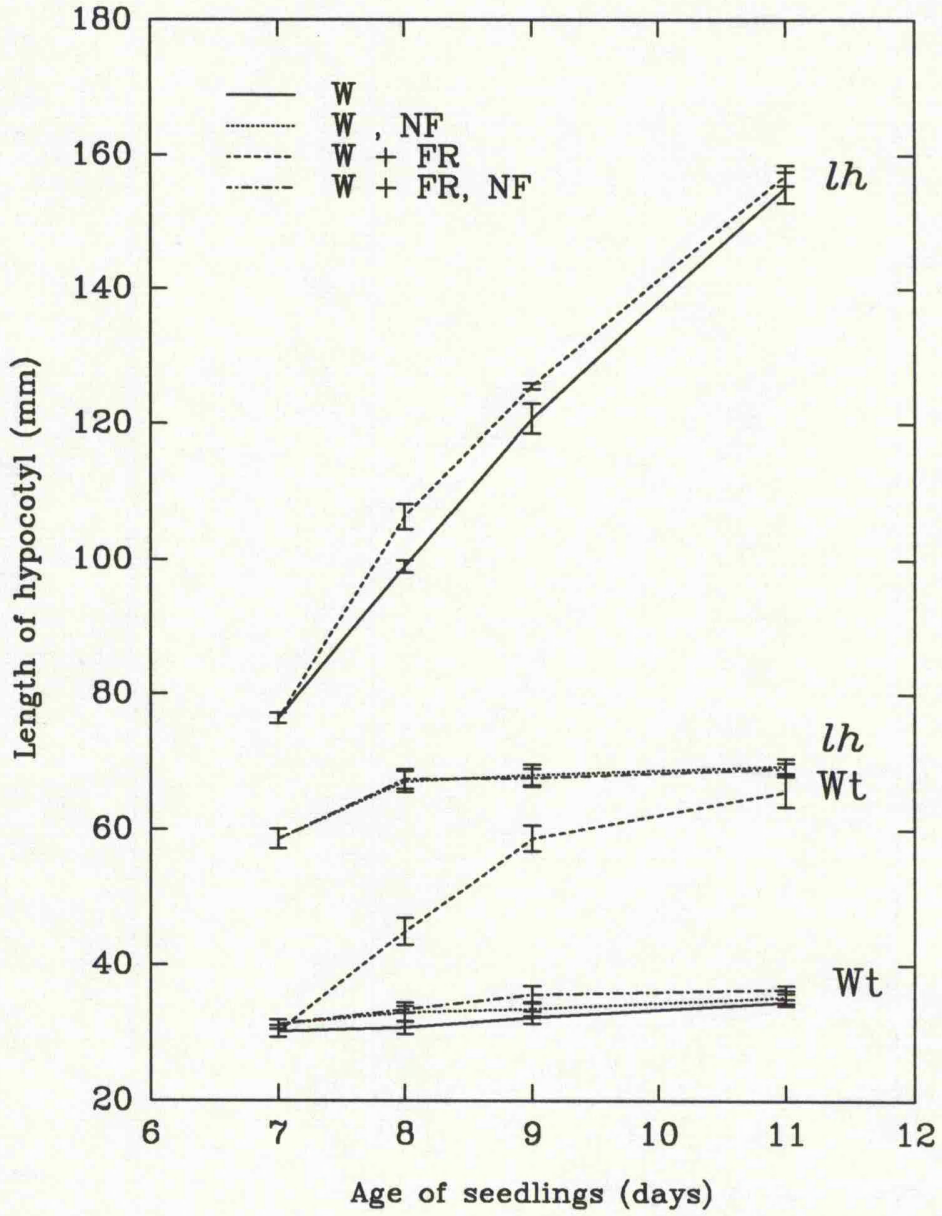


Fig 4.13: Photosynthetic requirement for the phytochrome-regulation of cucumber extension growth. Hypocotyl growth of 7-day old bleached (NF) and non-bleached Wt and *lh* seedlings. Seven day old, white light grown, treated and non treated seedlings were transferred to either a high (W) or low (W+FR) R:FR ratio for 4 days. The seedlings were bleached by imbibing and growing them in a 53 μM Norflurazon solution.

photosynthetic requirement but due to the phytochrome content of the plant as well. The use of NF to bleach other plant species e.g. *Sinapis alba* and *Chenopodium rubrum,* has not been found to cause any adverse effects on the seedlings growth and development (Holmes and Wagner, 1981, Holmes et al., 1982). However, after 7 days treatment with NF the cucumber plants were retarded in their development and it was found that even when additional sucrose was added the plants were unable to survive beyond day 8-9. This therefore raises a number of points that might question the validity of any experiments carried out on, and any results obtained from the use of cucumber seedlings that have been completely bleached by treatment with NF.

4.2.6. Rate of change of phytochrome-mediated extension growth

The rate of change of growth is an important factor to ascertain when the regulation of the processes indicated in these changes are being considered. In order to be considered of primary importance, any changes in enzyme activity must either precede or coincide with a change in the growth rate. Wild type seedlings were grown for 7 days in continuous W (a high R:FR ratio) and then transferred to continuous W plus supplementary FR or kept in continuous W. Growth measurements were taken at intervals to 24 h. After 2 h the seedlings given additional FR show an increase in stem extension when compared to those seedlings maintained in continuous W (Fig 4.14). These measurements were however taken using a millimetre ruler limiting the accuracy of finer measurements attempted at smaller time intervals. When the effect of FR light on cucumber hypocotyls was measured using a transducer, the rate of change of stem extension growth was found to occur after 16.2 min (Smith et al., 1992).

4.3. Etiolated cucumber hypocotyls

4.3.1. Effect of monochromatic R and FR light on cucumber hypocotyl extension

Five day old dark-grown Wt and *lh*-mutant seedlings were either maintained in the D or grown under continuous R or FR light and the length of the hypocotyl measured daily for a further 2 days. The change in hypocotyl length is represented on Fig

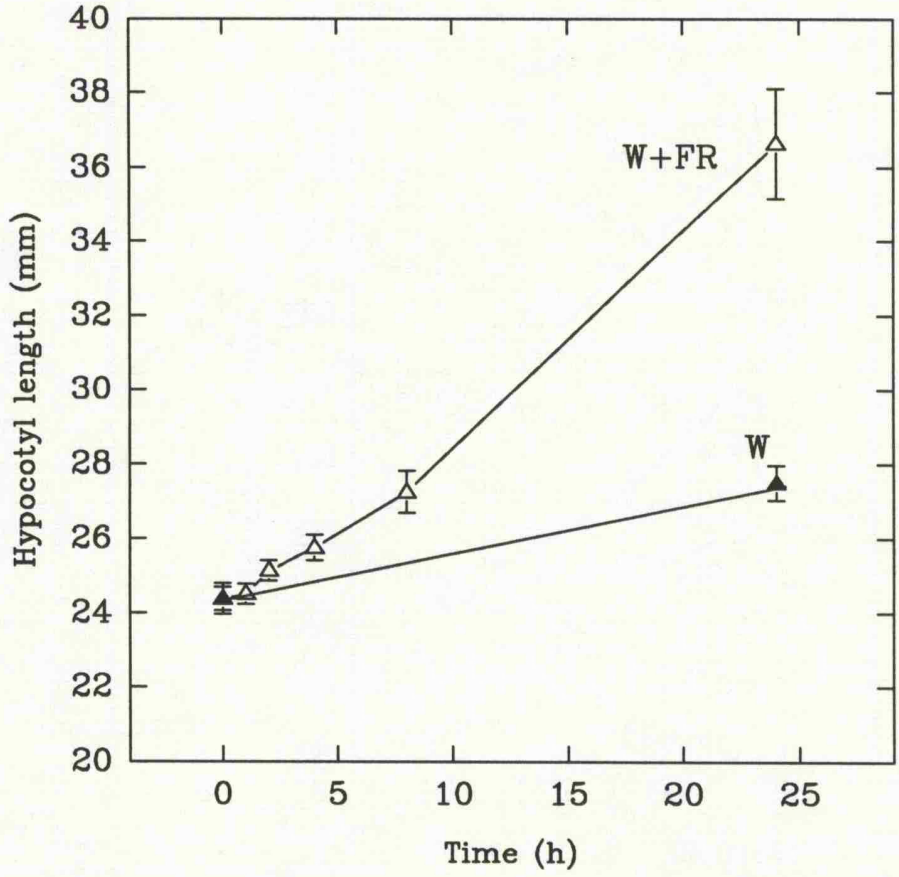


Fig 4.14: Rate of change of phytochrome-mediated increase in cucumber hypocotyl extension. Hypocotyl growth of 7 day old, W light grown, Wt cucumber plants transferred to a low R:FR ratio for a period of 24 h. The length of the hypocotyl was measured at 1, 2, 4, 8 and 24 h. A control of W grown plants were measured at time 0 and 24 h. Values are means +/- SE of a minimum of 6 plants.

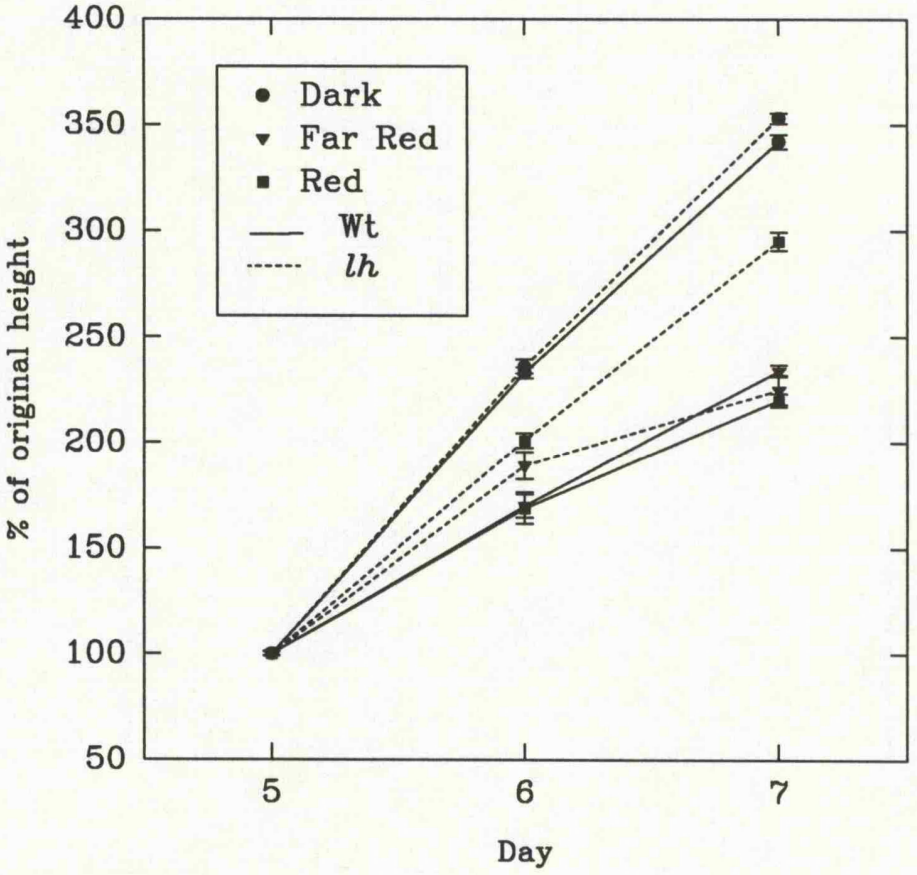


Fig 4.15: Phytochrome-regulation of etiolated cucumber extension growth. Relative growth of 5-day old etiolated Wt and *lh* -mutant cucumber hypocotyls transferred to monochromatic R, or FR, light or maintained in the dark (D) for 2 days. All measurements were taken with a millimetre ruler and are the means +/- SE of a minimum of 10 plants.

4.15 as a percentage of the original height prior to treatment. After 2 days treatment the Wt in both the R and FR and the *lh*-mutant in FR, approximately doubled their own hypocotyl lengths, all three having approximately equal heights. The *lh* grown in R shows a 50 % increase in hypocotyl length compared to the *lh* in FR light after 2 days but does not reach the extension rate seen in the dark-grown seedlings (approximately 87%). The dark grown Wt and *lh* seedlings were approximately three and a half times their original height and showed a similar rate of extension.

4.4. Discussion

4.4.1 Light-regulation of de-etiolated cucumber hypocotyl extension growth

Previous studies on the growth of cucumber seedlings have mainly been carried out using single band wavelengths. Both R and B light have been found to inhibit stem extension (Adamse et al., 1987, Gaba and Black, 1985, Cosgrove, 1981), with dual control indicated by the synergism found between phytochrome and a B light receptor (Attridge et al., 1984). A preliminary study on the phytochrome element of light-grown cucumber growth regulation was carried out by Whitelam and Smith (1991) who used continuous W growth conditions and lowered or increased the R:FR ratio accordingly. These workers found a rapid and marked increase in elongation growth in response to a reduced R:FR ratio. The growth experiments discussed here for de-etiolated seedlings also employ the use of continuous W or continuous W+FR, which more closely simulates natural light conditions than the use of monochromatic light.

The reversible nature of a response to changes in the R:FR ratio clearly indicates phytochrome as the regulating factor (Kronenberg and Kendrick, 1986). Demonstrating that the alteration in the rate of cucumber stem extension is regulated by phytochrome, is clearly shown by the reversible nature of this induction seen in the Wt cucumber seedlings (Fig 4.3 and 4.4). The decrease in the growth that could be induced by the addition of supplementary FR light with time (Fig 4.1), is most likely due to the decrease in growth potential of the hypocotyl itself. After day 9-10 the growth of the internode commences, as the plants development and extension growth is concentrated on the internodes rather than the hypocotyl, a lower rate of hypocotyl extension was seen.

As very clearly demonstrated phytochrome is seen to control the extension of Wt cucumber hypocotyls. The *lh*-mutant is a constitutively fast growing mutant which is known to be deficient in phytochrome B or a closely related light stable phytochrome

(López-Juez et al., 1992). The *lh* does not show any alteration in its growth rate due to the addition of FR light, whilst its isogenic Wt shows a rapid and large response to a low R:FR ratio (Fig 4.5 and 4.6). The lack of difference observed in the growth of the *lh*-mutant in low and high R:FR ratios (Fig. 4.6) is thought to be due to the deficiency of phytochrome B in these seedlings. Whitelam and Smith (1991) observed a small, but significant, acceleration of hypocotyl elongation in the *lh*-mutant when grown under a reduced R:FR ratio from day 6 to 20. The seedlings examined here were 8 days old and after 24 h in a reduced R:FR ratio showed no significant differences in growth. The recent findings by López-Juez et al. (1992) that the *lh*-mutant has < 1 % of light-stable phytochrome compared to the Wt, combined with the lack of responses to changes in the R:FR ratio, further suggests that the lack of growth response is due to the deficiency of this phytochrome. The shade avoidance response observed in the Wt cucumber seedlings is therefore considered to be due mainly to the action of phytochrome B. The use of the *lh*-mutant cucumber enabled the investigation as to whether changes in cell wall peroxidase activity were specifically regulated by phytochrome B or by other phytochromes.

The regulation of cucumber hypocotyl extension is also known to be inhibited by the action of B light (Shinkle and Jones, 1988, Gaba and Black, 1983, Cosgrove, 1981). It has also been postulated that the action of B light can be varied depending upon the activation of phytochrome (Attridge et al., 1984, Gaba et al., 1984, Drumm-Herrel and Mohr, 1991) and that the increase in the effectiveness of B light inhibition may be due to the activation of phytochrome in the cotyledons (Gaba et al., 1984). In the investigation of the regulation of cucumber growth by light it is important to ascertain whether the B light effect represents a comparable inhibition of hypocotyl extension rate in both the Wt and the *lh*-mutant. In accordance with the findings of Adamse et al. (1987), the *lh*-mutant and its isogenic Wt showed a similar degree of inhibition of stem extension (Fig 4.7). Any differences therefore observed between the Wt and *lh* seedlings is not due to differences in their regulation by B light, confirming that any

differences in growth and/or enzyme activity found, are most likely to be attributable to the lack of phytochrome B in the *lh*-mutant. Ballaré et al. (1991) also found that changes in B light levels evoked no elongation growth in the *lh*-mutant and postulated this to be due to the deficiency in phytochrome. The findings here suggest that if the induction of Pfr sensitizes the plant to an inhibition of stem extension by B light, then since the *lh* seedlings, which lack Pfr compared to the Wt, show a similar rate of growth inhibition, either a very low level of Pfr is required to elicit the response or another species of phytochrome (other than phytochrome B) might be involved in this phenomenon.

4.4.2 Region of extension growth

If growth rate changes reflect alterations in cell wall enzyme activity, it may be assumed that the region of the cucumber hypocotyl which is undergoing the most rapid growth rate changes should be where the maximum difference in cell wall enzyme activity occurs. This is important in the investigation of any changes since the effect would be diluted in areas where growth has ceased. The experiments revealed that whilst, as expected, the area of most rapid growth was immediately below the apex, a gradient of growth from the apex to the base of the hypocotyl was maintained (Fig. 4.8). This gradient of growth also being apparent in the *lh*-mutant cucumber (Fig. 4.9). A situation where this gradient of growth is not found is in cucumber hypocotyls grown under dim R light (Shinkle et al., 1992). Although the factor controlling the hypocotyl growth pattern is unknown, the fact that the phytochrome B mutant retains this growth gradient down the hypocotyl indicates that an alternative light receptor other than light-stable phytochrome, possibly another form of phytochrome or a B light receptor, might be controlling the pattern of hypocotyl extension.

4.4.3 The role of the cotyledons in the phytochrome-regulation of cucumber growth

Several experiments were undertaken to investigate the involvement of phytochrome B in the perception of light by the cotyledons. The induction of simulated phototropism was carried out by covering one cotyledon leaf and growing the plant under different light wavelengths. In R light the Wt seedlings showed a large degree of curvature towards the uncovered cotyledon, whilst this response was absent in the *lh* seedlings indicating a requirement for phytochrome B (Adamse et al., 1987). When both cotyledons of the Wt seedlings were covered and grown in a high R:FR ratio, the seedlings showed an increased rate of hypocotyl elongation when compared to the non covered seedlings (Fig 4.10a,b), indicating that R light perception by the cotyledons leads to an inhibition of hypocotyl extension. This effect was also found in Wt seedlings grown under a low R:FR ratio, the seedlings with covered cotyledons showing an increased rate of hypocotyl extension compared to the non-covered seedlings. The *lh* seedlings however showed no response to having the cotyledon leaves covered irrespective of the R:FR ratio. The perception of R light in de-etiolated tissue is thought to be mainly due to the action of phytochrome B. As covering the cotyledons of the Wt seedlings leads to an increase in stem extension in both low and high R:FR ratios and in the absence of phytochrome B (in the *lh* mutant), no response was found, these experiments show that phytochrome B is required to be present and active in the both the cotyledons and the hypocotyl.

An interesting observation was the seedlings ability to maintain extension growth despite the lack of photosynthate normally received from the cotyledonary leaves. It has been demonstrated by other workers that cucumber seedlings require photosynthates from the cotyledons in order to maintain growth (e.g. Penny et al., 1976) However 48 h after the cotyledons leaves had been covered, limiting their ability to carry out photosynthesis, the seedlings were able to continue growing. The requirement for photosynthesis in the phytochrome regulation of growth was therefore

examined using both the Wt and *lh* seedlings. Cucumber seedlings were 'bleached' to separate the effects of photosynthesis from the photomorphogenesis of the seedlings. The production of bleached seedlings should enable a range of experiments to be carried out to test for the action of phytochrome without the additional responses that may be seen due to photosynthesis and screening pigments. As the fluence rate increases so does the rate of photosynthesis making it difficult to ascribe an alteration in growth rate to any one mechanism. White seedlings therefore allow the action of phytochrome on the growth of the plant to be studied. The use of NF normally permits the production of white (non-photosynthetic) plants which are fully capable of photomorhogenic responses (Jabben and Dietzer, 1979). Other dicot species where NF has been used include *Sinapis* and *Chenopodium*, these seedlings were seen to grow and develop normally (Holmes and Wagner, 1981, Holmes et al., 1982). Cucumber seedlings that were treated with NF gave a different response in the phytochrome-mediation of growth when compared to *Sinapis* and *Chenopodium* seedlings, in that a large proportion of the R fluence rate dependant growth response was lost (Gaba et al., 1991). Here it was found that the NF treated Wt seedlings were unable to respond to a decrease in the R:FR ratio (Fig 4.12a and 4.13), indicating a photosynthetic requirement for the phytochrome-regulation of growth. The NF treated *lh* seedlings completely lost their normal elongated phenotype (Fig 4.12b and 4.13), further indicating a requirement for functional photosynthesis for cucumber seedling growth.

The Wt and *lh* seedlings treated with NF display a retarded and sickly development and were unable to survive beyond day 8-9. Cucumber seedlings that are grown and maintained in the dark for 8-9 days however, continue to grow without functional photosynthesis. This observation combined with their inability to grow with the addition of sucrose, suggests that NF is affecting processes besides carotenoid synthesis (which leaves the photosynthetic pigments unprotected to destruction by photobleaching, hence the production of white tissue). In light of these findings the

validity of any observed changes in the growth of NF treated cucumber seedlings remains doubtful. In the experiments carried out by Gaba et al. (1991) the NF treated seedlings being examined were only 5-6 days old and it is not apparent until day 8-9 that the bleached seedlings are in fact unable to survive due to the effects of the treatment. The findings of Gaba et al. (1991) and any other growth related experiments on NF treated cucumber seedlings should therefore be treated with caution.

4.4.4 Phytochrome-regulation on the extension growth in etiolated seedlings

Etiolated seedlings have a very high phytochrome content compared to de-etiolated seedlings, the majority of which is light-labile phytochrome or phytochrome A (Tokuhisha et al., 1985). Etiolated Wt and *lh* seedlings contain approximately equal levels of phytochrome (Adamse et al., 1987, 1988, Nagatani et al., 1989) and show similar responses to irradiation with both B and FR light. As recorded previously and found here (Fig 4.15), irradiation with R light fails to inhibit the extension growth of the *lh*-mutant compared to the Wt seedlings. Having established the changes in growth rate of the hypocotyl under various light conditions, the region in which extension growth is occurring and the role of the cotyledons in growth extension, these findings were used as a basis for testing whether the phytochrome-mediated growth rate changes correlated with changes in extracellular peroxidase activity. Chapter five looks at changes in the peroxidase activity in relation to these changes in the growth rate.

CHAPTER FIVE

CHAPTER FIVE - Phytochrome-regulation of extracellular peroxidase activity of cucumber hypocotyls.

5.1. Introduction

Many examples have been presented in support of the hypothesis that changes in extracellular peroxidase activity play an important role in the regulation of stem extension growth. These examples have been derived from studies on a variety of plants in which growth rates have been modified by a range of factors (Morrow and Jones, 1986, Goldberg et al. 1986, 1987, Kay and Basile, 1987, Valero et al., 1991, Zheng and van Huystee, 1992). The wide range of species that appear to show alterations in extracellular peroxidase activity in correlation with growth rate changes suggests that this might form part of a general mechanism in the control of growth. That phytochrome plays a fundamental role in the control of stem extension is well documented and therefore it is logical that altered cell wall peroxidase activity has been speculated to be linked with phytochrome regulated growth rate changes. As previously cited Kim *et al.* (1989), working with etiolated maize seedlings in which phytochrome induces rapid and opposite growth changes in different regions, found that an anionic peroxidase isolated from the cell wall, decreased in activity in the coleoptiles and increased in the mesocotyls. These changes in peroxidase activity were reported to precede the phytochrome regulated growth rate changes. Working with the first internode of mustard seedlings, Casal et al. (1990) showed that a phytochrome-regulated increase in growth rate correlated with a decrease in the activity of an anionic cell wall peroxidase that could be extracted by the infiltration/centrifugation method. However, the results obtained here on the efficiency of the infiltration/centrifugation method used and the lack of correlation in the major pool of the enzyme (see chapter three) cast doubt on this correlation between phytochrome-regulated growth rate changes and extracellular peroxidase activity. Nevertheless when the peroxidase activity of etiolated mustard hypocotyl cell walls was examined for regulation by light,

an increase in peroxidase activity was found when growth rate was reduced during de-etiolation.

A number of tall and dwarf genotypes/mutants of a range of species have been utilized in the study of growth-related changes in peroxidase activity. In tall fescue the ionically bound peroxidase activity through the leaf elongation zone of two genotypes was found to increase in the region where elongation growth declined (MacAdam et al., 1992a). In the slender pea phenotype, the enhanced stem elongation compared with the dwarf phenotype is due to an increase in internode length. The soluble and salt extractable peroxidase activities of the slender, compared with the dwarf phenotype were significantly lower giving further evidence for a peroxidase-mediated growth response of stem extension (Jupe and Scott, 1989). Plants deficient in a specific phytochrome provide powerful tools in the investigation on the action of that particular species. Plants deficient in phytochrome B are constitutively tall and so are useful tools for investigating the control of extension growth by particular phytochrome species. These mutants are therefore likely to be exceedingly useful in the study of phytochrome and cell-wall peroxidase activity.

The characterization of peroxidase-related changes in the elongation growth of cucumber has until now only been investigated in relation to the inhibition of stem extension by B light (Shinkle and Jones, 1988) and the changes occurring in the gradient of peroxidase distribution in the early stages of the de-etiolation process (Shinkle et al., 1992). Here a more detailed analysis has been undertaken, using the cucumber long hypocotyl (*lh*) mutant and its isogenic Wt. The *lh*-mutant has been shown to specifically lack a light-stable phytochrome species, most probably phytochrome B (López-Juez et al., 1992). The location of the peroxidase activity has been investigated and the changes in cell-wall peroxidase activity that coincide with the phytochrome-mediation of growth rate measured.

5.2. Extraction and assay conditions

5.2.1. Infiltration/centrifugation experimental parameters

A range of different ions have been used in the infiltration media used prior to centrifugation for the extraction of cell wall enzymes, e.g. Na^+, Li^+ and Ca^{2+} (Morrow and Jones, 1986, Shinkle et al., 1992, Li and McClure, 1989). To determine the most suitable salt for use in the extraction of cell wall peroxidase enzymes from the apical region of cucumber hypocotyls, 50 mM $CaCl_2$, LiCl and NaCl were used in the media and the peroxidase activity measured from the centrifugate obtained. The Li^+ and Ca^{2+} salts extracted approximately equal peroxidase activities in the first infiltration/centrifugation step, with Na^+ extracting a slightly lower activity. When the spun samples were homogenized in a 1M Na^+ salt solution to extract any enzymes that might be ionically bound to the cell wall, an equally high peroxidase activity was extracted from all the hypocotyl segments that had previously been infiltrated with any of the three salts (Fig. 5.1). Peroxidase activity that can only be extracted by a buffer of high ionic strength is generally accepted to be ionically-bound to the cell wall. Although both Ca^{2+} and Li^+ were equally efficient at extracting cell wall peroxidase activity, Ca^{2+} is generally considered to be less physiologically damaging to plant tissue than Li^+, therefore Ca^{2+} continued to be used in the infiltration extraction media.

5.2.2. pH of the assay for peroxidase activity

To ensure that the optimum conditions for the measurement of cucumber hypocotyl peroxidase activity were used, the pH of the assay medium was tested from pH 3-8. Fig 5.2 shows that for samples extracted by infiltration/centrifugation (C), homogenization in a weak buffer (H) or a high ionic strength buffer (IB), the optimum pH of the assay for peroxidase activity was pH 5. At all pH's the IB fraction had the highest peroxidase activity, with the homogenate fraction and the centrifugate fraction

	$CaCl_2$	NaCl	LiCl
C1	0.30	0.25	0.29
C2	0.09	0.02	0.05
IB	>2.5	>2.5	>2.5

Entries represent specific peroxidase activity(A450) per mg protein

Fig 5.1: Different salt ions used to extract the maximum possible amount of extracellular peroxidase activity from cucumber hypocotyls. Specific peroxidase activity (A450) per milligram protein of two sequential centrifugate extractions (C1 and C2), and an ionically bound (IB) extract from the top 1 cm section of 8-day old Wt cucumber hypocotyls. All seedlings were grown in continuous W light. Samples were extracted and tested for peroxidase activity in media containing either calcium, sodium or lithium salt ions.

pH	Centrifugate	Homogenate	Ionically Bound
3	0.05	0.08	0.11
3.5	0.10	0.30	0.38
4	0.09	0.70	1.0
5	0.12	0.93	>2.5
6	0.08	0.67	1.67
7	0.02	0.36	0.85
8		0.01	0.04

Entries represent specific peroxidase activity (A450) per mg protein

Fig 5.2: Optimum pH of the assay media for maximum peroxidase activity. Specific peroxidase activity (A450) as determined at a range of pH (3-8) of the assay buffer in centrifugate, homogenate and ionically bound extracts. All extracts were from the top 1 cm section of 8-day old Wt cucumber hypocotyls grown in continuous W light.

having lower activities respectively. Therefore all plate based assays were carried out in a medium of pH 5.

5.2.3. Comparison of the peroxidase isoforms eluted from different fractions of the hypocotyl

Having found a high ionically-bound peroxidase activity in the cucumber hypocotyls, the isoforms from both the centrifugate and the IB fraction were examined by separation on a NEPHGE gel (Fig 5.3). The ionically bound fraction was found to contain the same four cationic peroxidase isoforms as the isoforms that could be recovered from extracts obtained by centrifugation. It would therefore appear that these four cationic isoforms constitute the main cell wall isoforms from cucumber hypocotyls.

Whilst PMSF, a potent serine protease inhibitor, was used in the extraction medium and no problems were incurred, van Huystee (1976) found that the addition of BSA to the extraction medium for peanut cells reduced the number of peroxidase isoforms visualized on an IEF gel from five to one. The addition of BSA in the extraction medium for the ionically bound fraction of cucumber hypocotyls did not alter the number or pattern of bands seen on a high range NEPHGE gel (Fig 5.4).

5.3. Extracellular peroxidase activity in cucumber hypocotyls

5.3.1. Location of peroxidase activity

If peroxidase-mediated stiffening of the cell wall leads to changes in the growth rate, it might be expected that the responsible peroxidase activity should mainly be located in the epidermis, as this is the region most likely to regulate the extension of the hypocotyl (Masuda and Yamamoto, 1972). Ionically-bound peroxidase activities were extracted from epidermal peels and from the cortex region of 8-day old Wt cucumber seedlings grown in either a low or high R:FR ratio. Fig. 5.5 shows the peroxidase activity found within the epidermal and cortex region compared with the activity in the tissue as a whole. Within each treatment, approximately 65 % of the total peroxidase

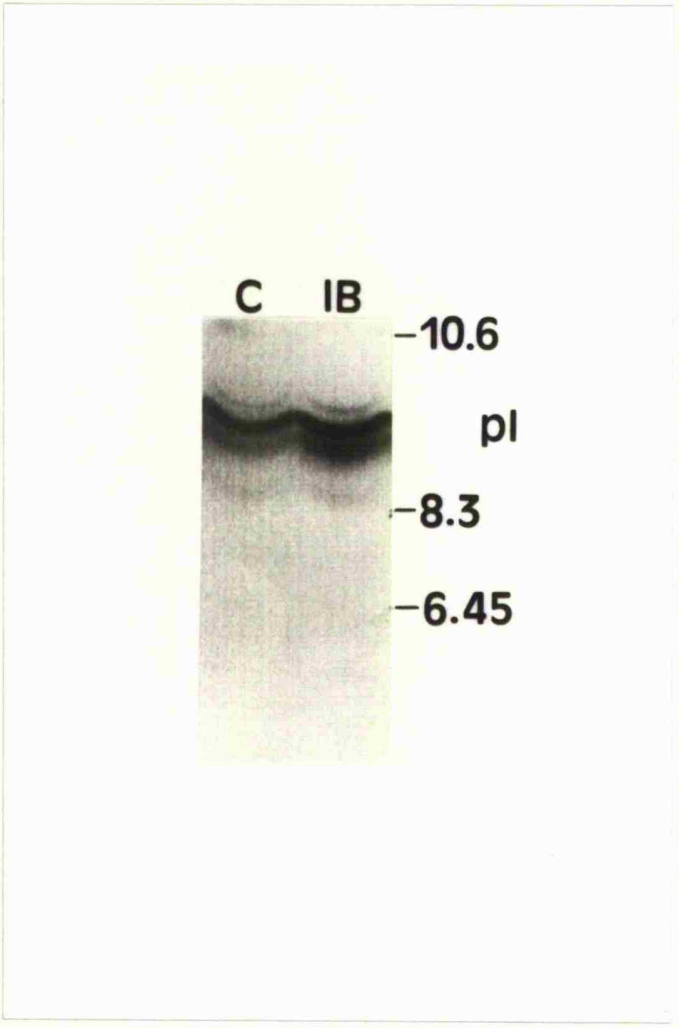


Fig 5.3: Peroxidase isoforms present in different fractions of the cucumber hypocotyl. Full range NEPHGE gel resolving centrifugate (C) and ionically-bound (IB) extracts from the top 1 cm section of 8-day old Wt cucumber hypocotyls grown in continuous W light. Samples were loaded on an equal protein basis and the gel stained for activity with 4CN.

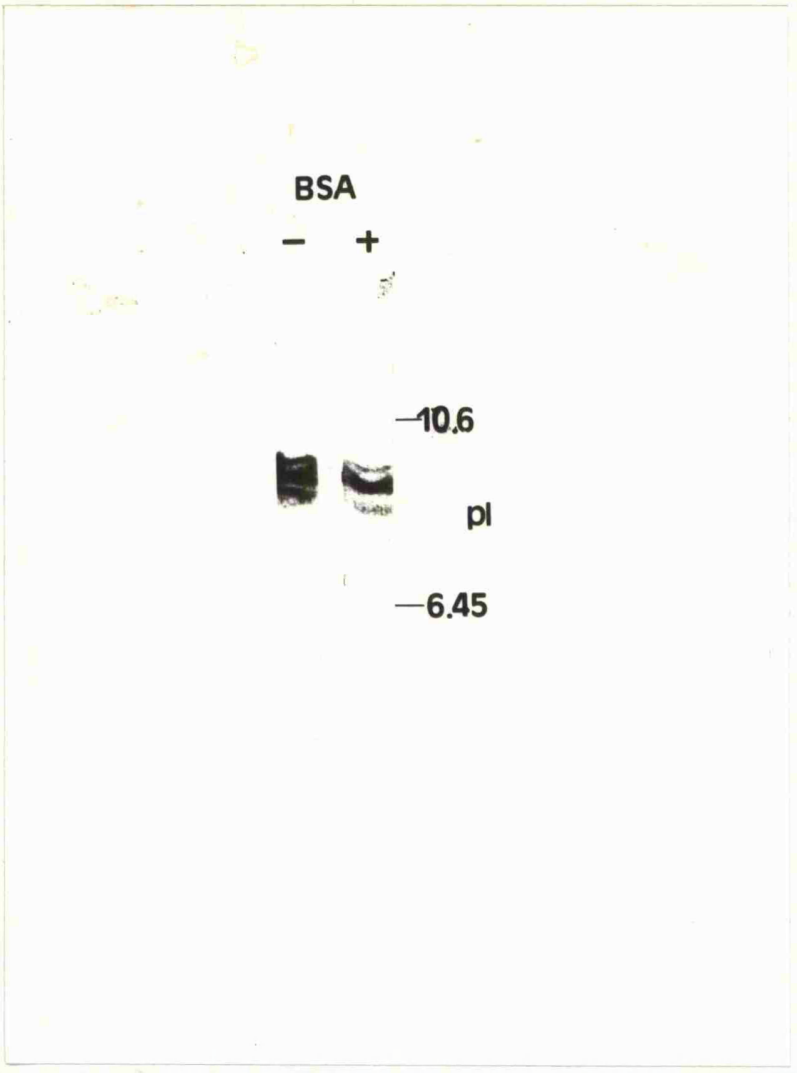


Fig 5.4: Peroxidase banding patten observed with the addition of bovine serum albumen (BSA) in the extraction medium. The standard extraction media contained Phenyylmethylsulphonyl fluride (PMSF) and 1 % BSA was added as indicated. Full range NEPHGE gel comparing the banding patten stained wiith 4CN of extracts taken with (+) or without (-) the addition of Bovine Serum Albumin. The extracts examined are from the top 1 cm section of 8-day old Wt cucumber hypocotyls grown in continuous W light.

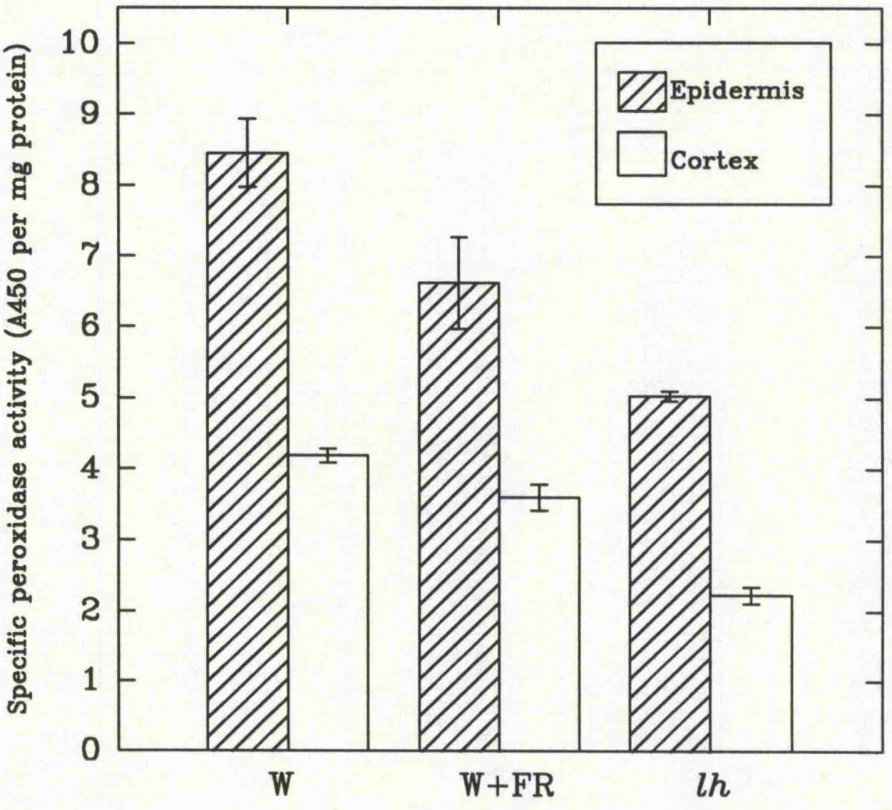


Fig 5.5: Distribution of the specific peroxidase activity (A450 per mg protein) +/- SE, between the epidermal and cortex regions in the top 1 cm section of 8-day old cucumber hypocotyls. All Wt seedlings were grown in continuous W light for 7 days and then either a high or low R:FR ratio (W or W+FR respectively) for 1 day. The *lh* -mutant seedlings were grown in continuous W light for 8-days.

activity was located in the epidermis. A similar distribution of peroxidase activity was observed in the *lh*-mutant (Fig 5.5). The similarity of peroxidase distribution between the different seedlings is consistent with the idea that extracellular peroxidase activity forms part of a general mechanism involved in the regulation of stem extension. Conditions which lead to increased hypocotyl extension i.e. Wt seedlings treated under low R:FR ratio and the *lh* mutation, also leads to decreased ionically-bound peroxidase activity extracted from epidermal cells. A proportionally similar decrease in ionically-bound peroxidase is observed for extracts of cortical cell walls (Fig 5.6). Thus, extraction of ionically-bound peroxidase from cell walls of whole hypocotyls will reflect the behaviour of peroxidase in the epidermis (and cortex). In all further extractions, whole hypocotyls were used as the enzyme source.

5.3.2. Phytochrome-regulation of extracellular peroxidase activity in light-grown cucumber hypocotyls

The peroxidase activities were examined in the C and IB fractions extracted from de-etiolated cucumber seedlings that had been grown under continuous W for 8 days or continuous W for 7 days and W+FR for a further 24 h. Comparable extracts from 8-day old light grown *lh* seedlings were also examined. Using a micro assay for peroxidase activity, with TMB as the substrate, a similar pattern of peroxidase activity was found in the C and IB fractions (Fig 5.7). The seedlings grown for 24 h in a low R:FR ratio (W+FR), which show an increased rate of stem extension (see 4.2.1), have a lower specific peroxidase activity in both the C and IB fractions compared with the seedlings maintained in a high R:FR ratio (W). In extracts from the *lh* seedlings, irrespective of the R:FR ratio, a lower peroxidase activity was found compared with the W light grown Wt seedlings. The level of specific peroxidase activity in extracts of the *lh* seedlings was found to be similar to the activity of extracts of Wt seedlings grown in a low R:FR ratio.

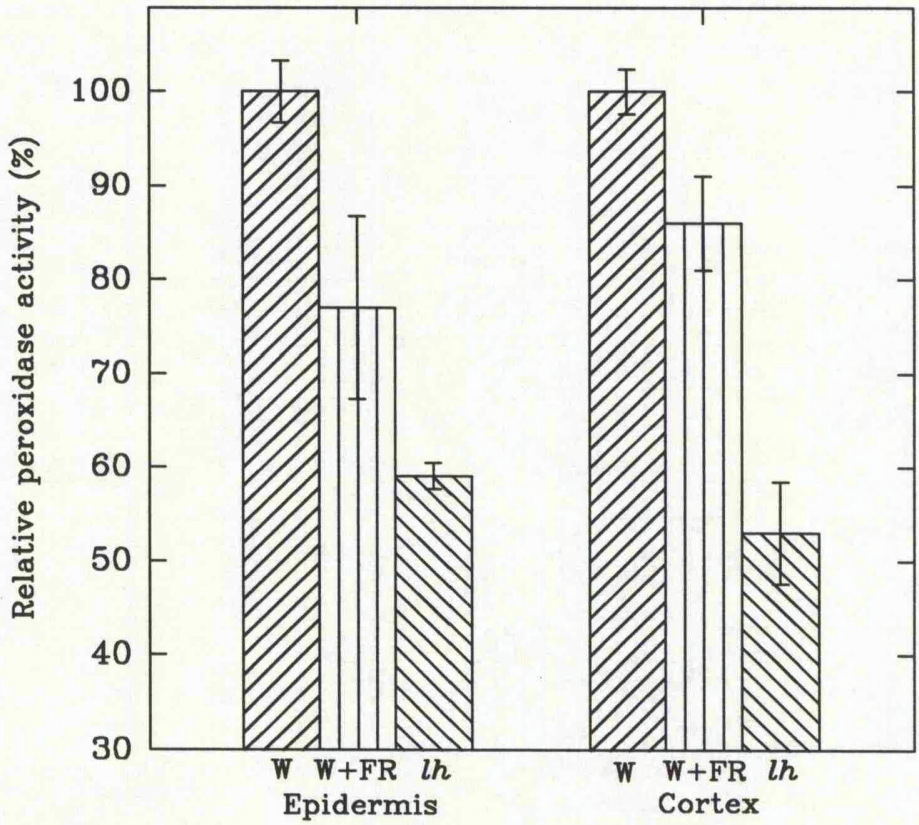


Fig 5.6: Phytochrome-regulation of the relative ionically bound peroxidase activity (+/- SE), distributed between the epidermal and cortex regions in the top 1 cm section of 8-day old cucumber hypocotyls. All Wt seedlings were grown in continuous W light for 7 days and then either a high or low R:FR ratio (W or W+FR respectively) for 1 day. The *lh* -mutant seedlings used to compare the distribution of peroxidase activity in a phytochrome B deficient mutant, were grown in continuous W light for 8-days.

Light treatment:	Specific peroxidase activity (A450 per mg protein)	
	W	W+FR
Centrifugate;		
Wt	0.53 (100 %)	0.36 (60 %)
lh	0.27 (57 %)	0.28 (58 %)
Ionically bound;		
Wt	6.05 (100 %)	3.70 (67 %)
lh	3.46 (62 %)	3.54 (60 %)

Fig 5.7: Examination of the phytochrome-regulation of peroxidase activities in different fractions extracted from cucumber seedling hypocotyls. Specific peroxidase activity (A450 per mg protein) of the centrifugate and ionically bound fractions extracted from de-etiolated cucumber seedlings that had been grown under continuous W for 8 days (W) or continuous W for 7 days and W+FR for a further 24 h (W+FR). Comparable extracts from 8-day old light grown *lh* -mutant seedlings were also examined. The percentage of peroxidase activity in the different treatments, relative to the Wt seedlings in W, for both the centrifugate and ionically bound extracts, is indicated in brackets.

The phytochrome-mediated change in IB peroxidase activity is not due to the specific regulation of the activity of any one isoform. In Wt seedlings grown in a low R:FR ratio the four isoforms, visualized by staining with 4CN on a NEPHGE gel, all showed reduced activity when compared with extracts from seedlings grown in a high R:FR ratio (Fig 5.8). Similarly, analysis of extracts of the *lh*-mutant reveal that the activity of all four isoforms are reduced when compared with extracts of Wt seedlings. Thus the low R:FR mediated increase in growth of Wt seedlings apparently correlates with a general down regulation of extracellular peroxidase activity in the cell wall fraction. A similar situation was also seen to occur in the *lh*-mutant. As the isoforms were the same in both the C and IB fractions (Fig 5.3) but a much higher activity was found in the IB fraction (Fig 5.1 and 5.2), this fraction was used in further analyses of the phytochrome-regulation of extracellular peroxidases.

5.3.3. Distribution of peroxidase activity and phytochrome-regulated growth

Experiments were carried out to investigate the relationship between the position along the hypocotyl and extractable IB peroxidase from cucumber seedlings extending at different rates. Whole hypocotyls were examined for total IB peroxidase activity which was expressed as the peroxidase activity per centimetre of the hypocotyl. The overall enzyme activity in the longer hypocotyls, those growing in a low R:FR ratio, is approximately 70 % of the activity found in cucumber hypocotyls grown for 8 days in a high R:FR ratio (Fig 5.9). Whilst this represents an overall view of the difference in peroxidase activity, the area of most rapid growth has been shown to be in the apical region with a gradient of growth in both the Wt and *lh* seedlings (see 4.2.3.). If growth rate changes do reflect alterations in cell wall enzyme activity, it may be assumed that the region of the cucumber hypocotyl which is undergoing the most marked growth rate changes should be where the maximum difference in cell wall enzyme activity occurs. This is important in the investigation of any changes since the effect could be diluted by extraction of peroxidase from areas where growth has ceased. Examining the activity of individual 1 cm sections along the hypocotyl from

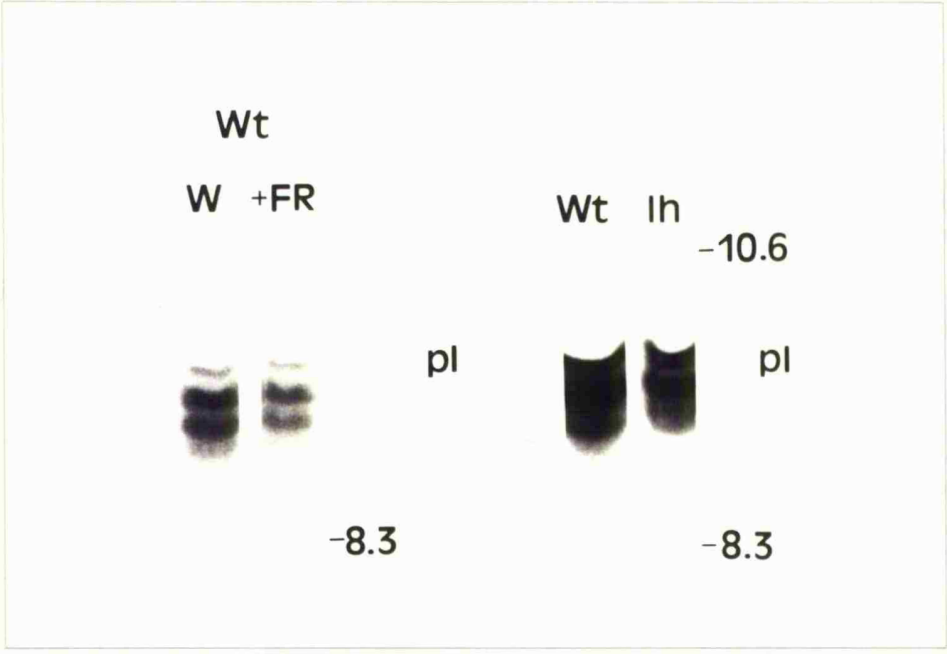


Fig 5.8: Phytochrome-regulation of the cationic peroxidase isoforms found in the top 1 cm section of cucumber hypocotyls. Left, a full range NEPHGE gel comparing the activity of the four ionically bound cationic peroxidase isoforms from 8-day old Wt seedlings treated for 1 day in either a high (W) or low (W+FR) R:FR ratio respectively. Right, the Wt cationic ionically bound peroxidase activity is also compared with extracts of the ionically bound activity from 8-day old *lh* -mutant seedlings.

Light treatment:	Specific peroxidase activity (A450 per mg protein)	Hypocotyl length (centimetres)	Specific peroxidase activity per centimetre length of hypocotyl
W	3.05 (+/- 0.28)	2.78 (+/- 0.11)	1.11 (+/- 0.12)
W+FR	3.82 (+/- 0.34)	5.05 (+/- 0.21)	0.77 (+/- 0.11)

Fig 5.9: Peroxidase activity found in the whole hypocotyl of Wt cucumber hypocotyls, grown in a low (W+FR) or high (W) R:FR ratio. The hypocotyls were measured and extracts made using the whole hypocotyl. Specific peroxidase activity (A450 per mg protein) is expressed as the activity per centimetre of hypocotyl. All seedlings used were 8-day old Wt cucumber seedlings and each value represents a minimum of five replicas.

seedlings grown in a high R:FR ratio, the IB peroxidase activity again showed an inverse correlation with growth, the highest activity occurring in the basal region and activity decreasing towards the apex. In the seedlings treated with a low R:FR ratio for 24 h, a lower IB peroxidase activity was found in the apical region compared with the activity in the seedlings grown in a high R:FR ratio. A more even distribution of peroxidase activity extending towards the base was also found (Fig 5.10).

Comparing equal one third sections of the final hypocotyl length enables more direct comparison of the gradients of peroxidase activity to be assessed. The peroxidase activity of extracts of hypocotyls from 8-day old Wt seedlings grown in a continuous high R:FR ratio throughout or for 7 days at a high R:FR ratio followed by 24 h in a low R:FR ratio, were examined. The ionically bound cell wall peroxidase activity of the hypocotyl is found to decrease from the apex to the base in both low and high R:FR ratios (Fig 5.11), thus showing an inverse correlation with the growth rate. The IB cell wall peroxidase activity of the *lh*-mutant also showed a gradient down the hypocotyl irrespective of the R:FR ratio (Fig 5.11).

5.3.4. Reversible nature of the phytochrome-regulation of peroxidase activity

The hypocotyl extension in cucumbers has been demonstrated to be reversibly controlled by phytochrome (4.2.1.). Following 2 days in a low R:FR ratio those seedlings growing at an increased rate, when returned to a high R:FR, showed a rapid deceleration of growth to approximately the same level of extension rate as the seedlings maintained continuously in W light. The specific peroxidase activity of the top 1 cm section of the hypocotyls, taken from each of the three growth conditions (W, W+FR and W+FR→W), was measured at day 11 of the experiment (Fig.5.12). The reduced level of peroxidase activity found in the seedlings growing in W+FR, a high R:FR ratio, increased when placed back into W (W+FR→W) i.e. when the growth rate was down regulated, back towards the level of those grown in W light. As such the IB peroxidase activity appears to be regulated in parallel with the

Sections of the hypocotyl (apex to base) in W light	Specific peroxidase activity	(% of the total peroxidase activity)	Sections of the hypocotyl (apex to base) with added FR	Specific peroxidase activity	(% of the total peroxidase activity)
W 1	7.49	16 %	FR 1	4.83	11 %
W 2	10.47	22 %	FR 2	3.55	8 %
W 3	10.87	23 %	FR 3	7.47	18 %
W 4	18.54	39 %	FR 4	8.53	20 %
			FR 5	9.02	21 %
			FR 6	9.44	22 %

Fig 5.10: Distribution of specific ionically bound peroxidase activity between 1 cm sections down the length of the hypocotyl of 8-day old Wt cucumber seedlings grown in a low (W+FR) or high (W) R:FR ratio.

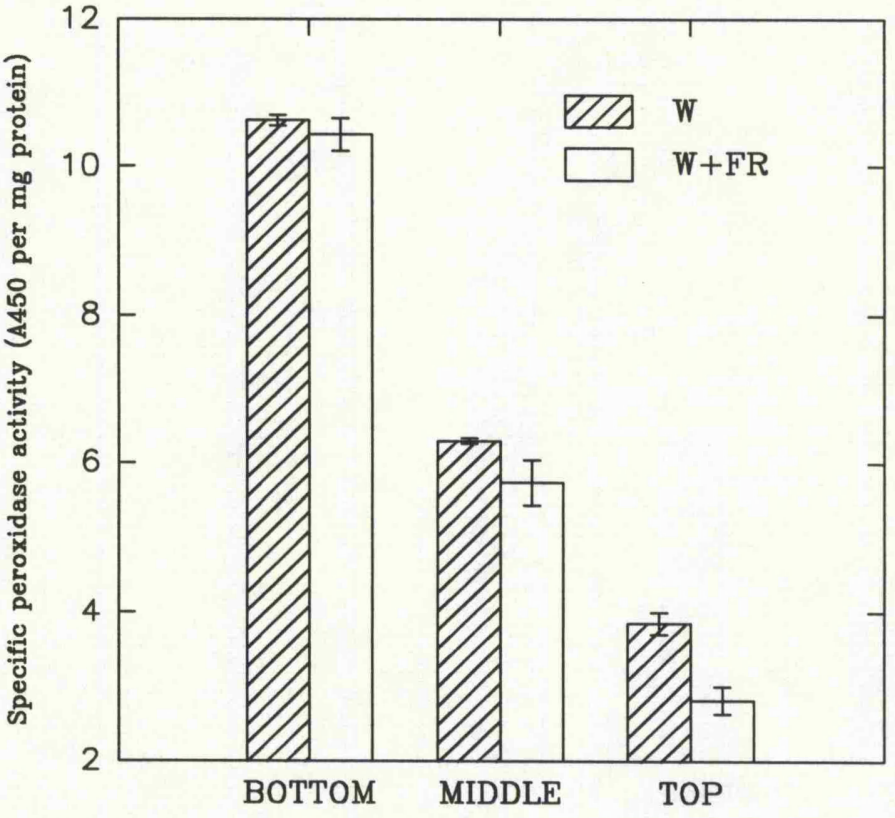


Fig 5.11: Specific peroxidase activity (A450 per mg protein) and phytochrome-mediation of, the bottom, middle and top third of 8-day old Wt cucumber seedlings grown in a high (W) or low (W+FR) R:FR ratio for 24 h. All peroxidase activity indicated is that of the ionically bound fraction and represents the mean of a minimum of five replicas +/- SE.

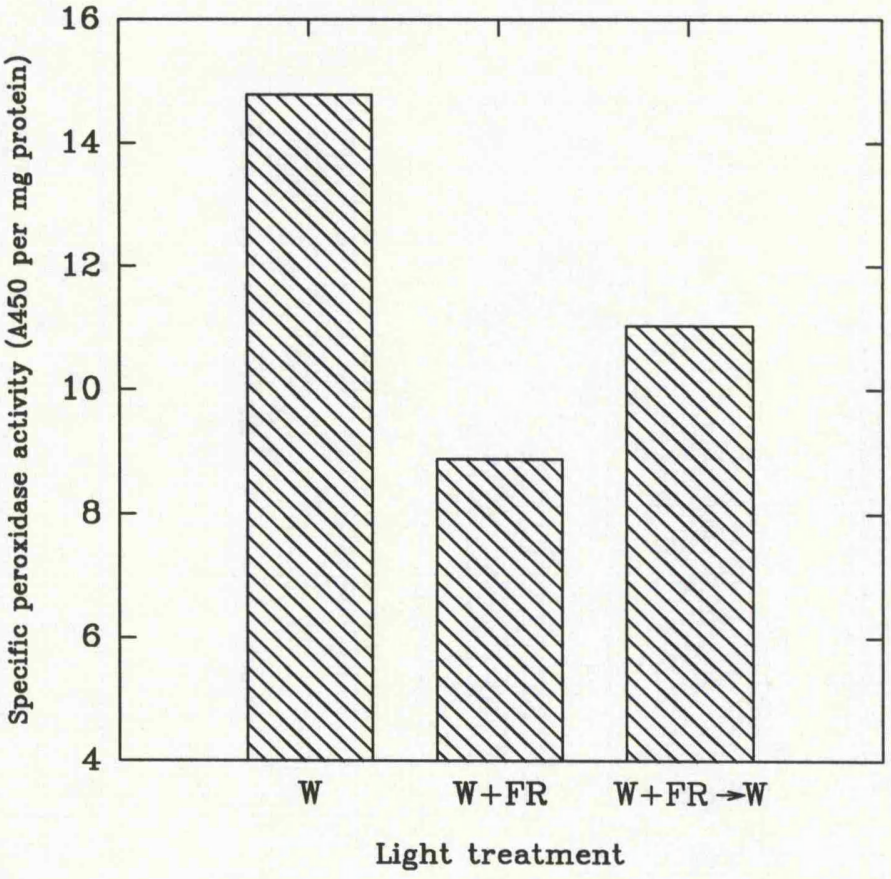


Fig 5.12: Reversible nature of the phytochrome-mediated changes in extracellular peroxidase activity. Specific peroxidase activity in the top 1 cm of Wt cucumber hypocotyls. The seedlings were grown under continuous W light for 7 days and then either maintained under the same conditions for four days, or placed into continuous W+FR for 4 days or 2 days in W+FR and 2 days in W (W+FR→W). All plants were 11 days old when extractions were made. The data shown here is a representative sample from one experiment due to the differences in the efficiency of extraction between experiments. The data does however represent the trend found within each experimental data set

phytochrome-mediated growth rate changes. Thus another example of an inverse correlation between peroxidase activity and phytochrome-mediated growth rate has been established.

5.3.5. Correlation between rate of phytochrome-mediated growth rate changes and changes in peroxidase activity

In order for changes in the activity of extracellular peroxidases of cucumber to be considered as a primary mechanism in altering the rate of stem extension, the change in activity must either correlate with, or precede, any phytochrome regulated growth rate changes. A change in the rate of hypocotyl extension was seen to occur within 2 h following a decrease in the R:FR ratio, as detectable by measurements using a ruler (Fig 4.14). The level of IB peroxidase activity extracted from the apical 1 cm of cucumber hypocotyls in a low R:FR ratio is seen to be dramatically reduced within 2 h compared with those seedlings maintained in a high R:FR ratio. The IB peroxidase activity continuing to decline, but more gradually, over the following 22 h (Fig 5.13). The slight reduction in peroxidase activity seen over the 24 h period for extracts of within the seedlings grown in a high R:FR ratio, is not thought to be of significance. Thus, within 2 h of a reduction in R:FR ratio there is a substantial reduction in IB cell wall peroxidase activity and a clearly measurable increase in hypocotyl extension.

5.4. Peroxidase activity of a *Brassica rapa* phytochrome B-deficient mutant compared to its wild type

The *Brassica rapa ein* mutant shows a elongated hypocotyl compared with its Wt (Normal) in a high R:FR ratio (Fig 5.14). The IB peroxidase activity of the phytochrome B deficient *B. rapa ein* mutant (Devlin et al., 1992) was compared with its Wt to see if a similar response between the cucumber *lh* mutant and its Wt was apparent. Analysis of extracts of cell walls of the *ein* mutant showed a reduction

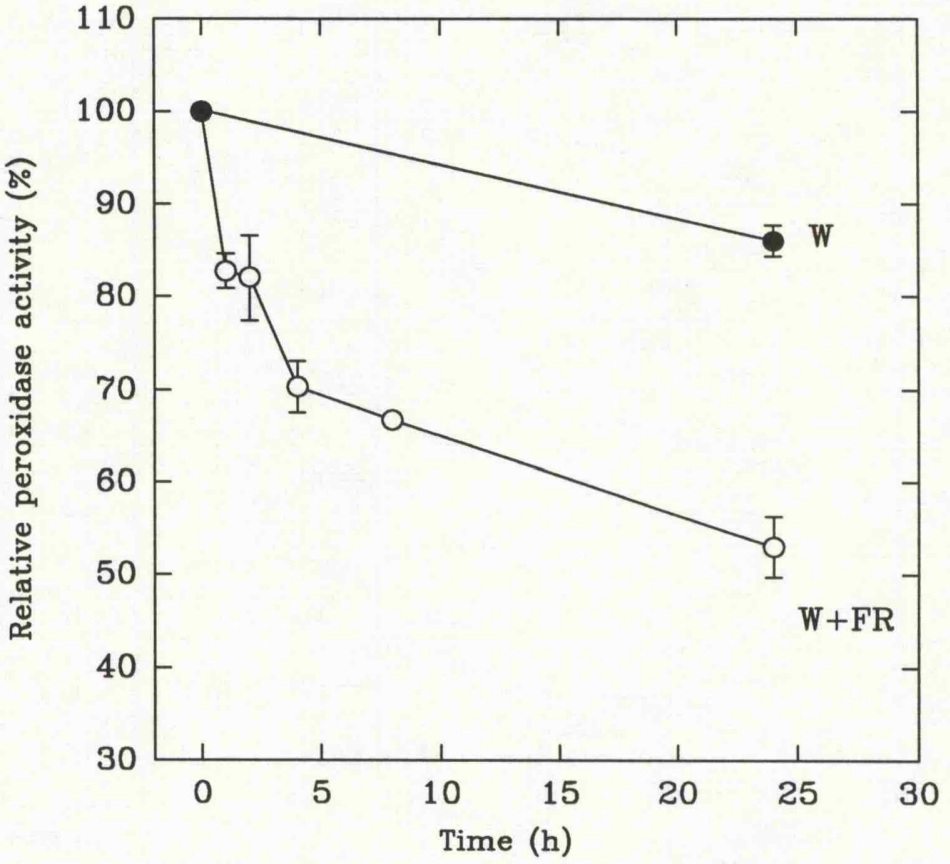


Fig 5.13: Change in extracellular peroxidase activity in light-grown cucumber hypocotyls following exposure to light. Relative peroxidase activity (%) of the ionically bound fraction from the top 1 cm of Wt cucumber hypocotyls grown in a high R:FR ratio (continuous W), compared with extracts from seedlings transferred to a low R:FR ratio (W+FR) over 24 h. All seedlings were 7-days old at the beginning of the experiment. Comparative activities of seedlings maintained in continuous W light were taken at times 0 and 24 h. The slight difference seen between the specific peroxidase activity at these two time points is relatively small compared with the difference observed in the seedlings growing in W+FR.

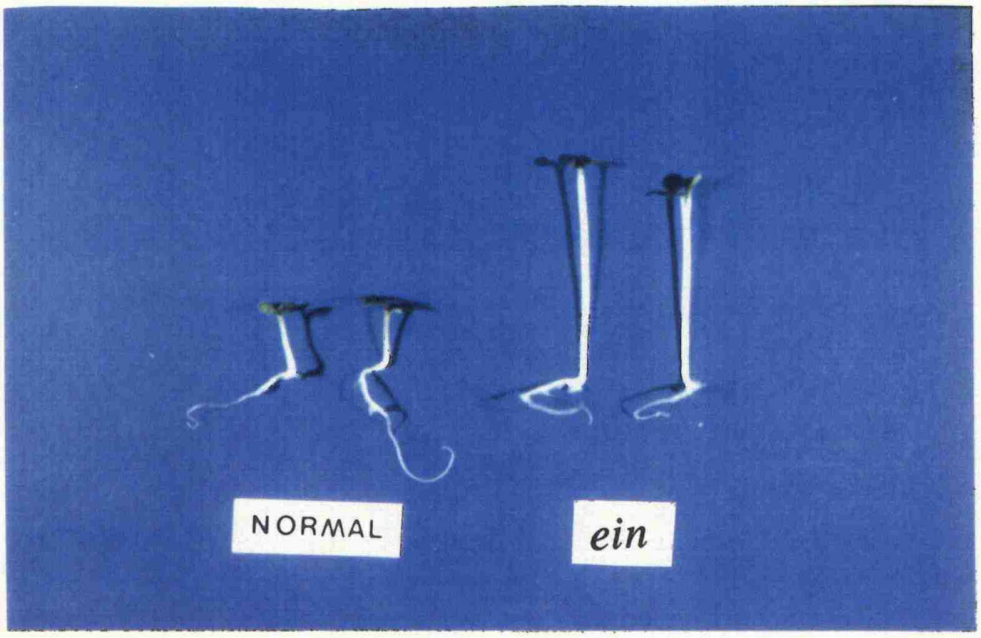


Fig 5.14: The *Brassica rapa* plant, illustrating the elongated hypocotyl of the *ein* mutant compared with the wild type (normal) when grown in a high R:FR ratio. One-day-old, dark germinated seedlings were transferred to, and grown under continuous white light (W) for 3 days.

in peroxidase activity of approximately 30 % compared with similar extracts from Wt seedlings (data not shown). This provides another example that phytochrome B appears to be the main regulator of growth related changes in extracellular peroxidase activity.

5.5. Regulation of peroxidase activity in etiolated cucumber seedlings

5.5.1. Phytochrome-regulation of de-etiolation and extracellular peroxidase activity

Extracellular peroxidase activity has been shown to inversely correlate with the growth rate of de-etiolated cucumber hypocotyls. In order to examine whether regulation of cationic cell wall peroxidase may provide a general mechanism by which growth of cucumber hypocotyls is regulated, peroxidases were analyzed in extracts of cell walls from etiolated cucumber seedlings. This was done by examining whether the phytochrome-regulated inhibition of stem extension during the process of de-etiolation, correlated with an increased activity of the same phytochrome-regulated isoforms apparent in the shade avoidance mechanism.

Cucumber seedlings were grown for 5 days in the dark followed by 3 days in R, FR or D. The IB peroxidase isoforms from the hypocotyls were extracted by the same method as used for the de-etiolated tissue and separated by gel electrophoresis on a full range NEPHGE gel. A full range pI gel would enable other peroxidase isoforms that might be present in the etiolated cucumber seedlings to be visualized. The gel (**Fig 5.15**) shows the same cationic isoforms that are seen in de-etiolated tissue as present in etiolated tissue. However no difference could be observed in the activity of the cationic peroxidase enzymes extracted from Wt seedlings that were etiolated (D), or exposed to R or FR for 24 h (**Fig 5.15**). Thus for etiolated seedlings, conditions that lead to a substantial alteration in hypocotyl extension growth, i.e. exposure to R or FR light, caused no detectable change in extractable ionically bound peroxidase

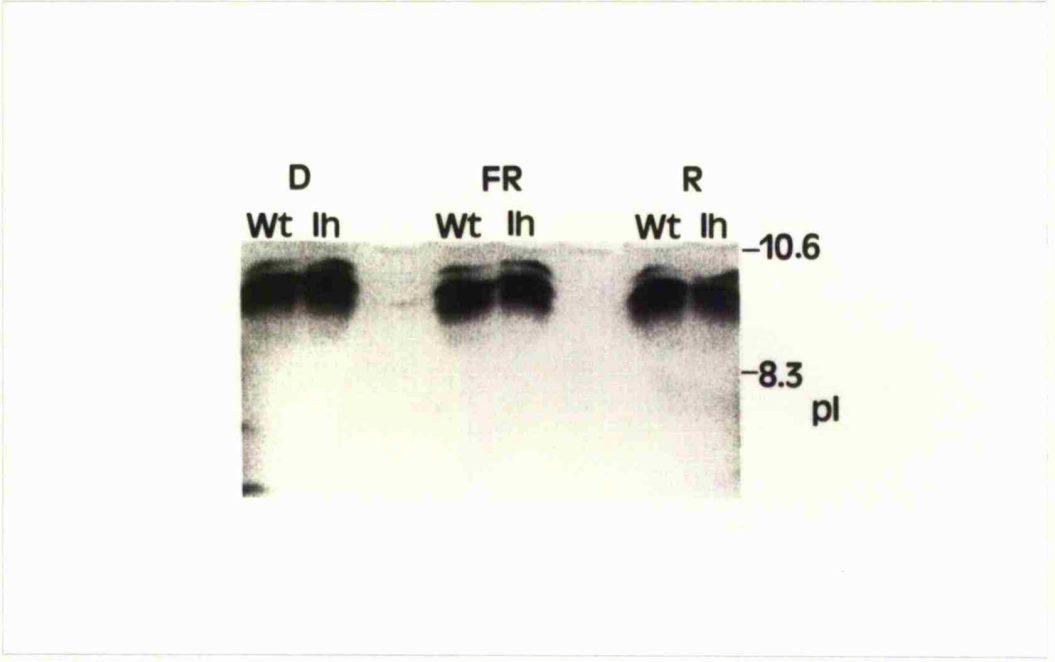


Fig 5.15: Effect of 24 h monochromatic red (R) or far-red (FR) light on the cationic peroxidase activity in etiolated cucumber hypocotyls. Full range NEPHGE gel of the ionically bound peroxidase isoforms present in etiolated seedlings (D) compared with de-etiolated seedlings (R) or partially de-etiolated seedlings (FR). Comparable extracts were taken from 8-day old Wt and *lh* seedlings, and stained with 4CN.

activity. In the extracts from etiolated *lh* seedlings, again no regulation of the cationic peroxidase isoforms was observed (Fig 5.15), despite large differences in the growth rates.

5.5.2. Developmental aspect of phytochrome-regulation of extracellular peroxidase activity

Etiolated seedlings given different light treatments (R, FR or D) show substantial differences in their hypocotyl lengths. Despite this large difference in growth no apparent phytochrome-regulation of the cationic peroxidase isoforms could be found 24 h after exposure to light (Fig 5.15). The seedlings examined were the same chronological age as the de-etiolated seedlings examined previously, in which phytochrome-mediated growth rate changes showed a clear correlation with changes in the IB peroxidase activity. This may suggest that some developmental point needs to be reached before phytochrome-regulated changes in activity of the IB cationic peroxidase isoforms takes place. In the light-grown (de-etiolated) seedlings, the *lh* mutation is characterized by a reduced peroxidase activity in the extracts from hypocotyl cell walls compared to its Wt. However, for etiolated *lh* seedlings exposed to R, where an elongated phenotype is displayed, IB cell wall peroxidase activity is not different from the Wt seedlings (Fig 5.15). To test whether a certain period of de-etiolation is necessary before any coupling between phytochrome and peroxidase activity takes place, total peroxidase activity was initially examined in extracts of D grown 8-day old Wt seedlings or similar extracts from seedlings grown for 4 days in D followed by transfer to W light. Total activity decreased in the de-etiolated Wt seedlings. The *lh*-mutant showing similar activity in etiolated or de-etiolated tissue of the same age (Fig. 5.16). However, at least 2 days exposure to W light is required before differences in IB cell wall peroxidase can be detected relative to extracts of D-grown seedlings (Fig 5.17). After 3 days of W light treatment IB peroxidase activity in whole hypocotyl extracts was found to be approximately 140-160 % of the activity of similar extracts of D-grown hypocotyls (Fig 5.17). This difference in

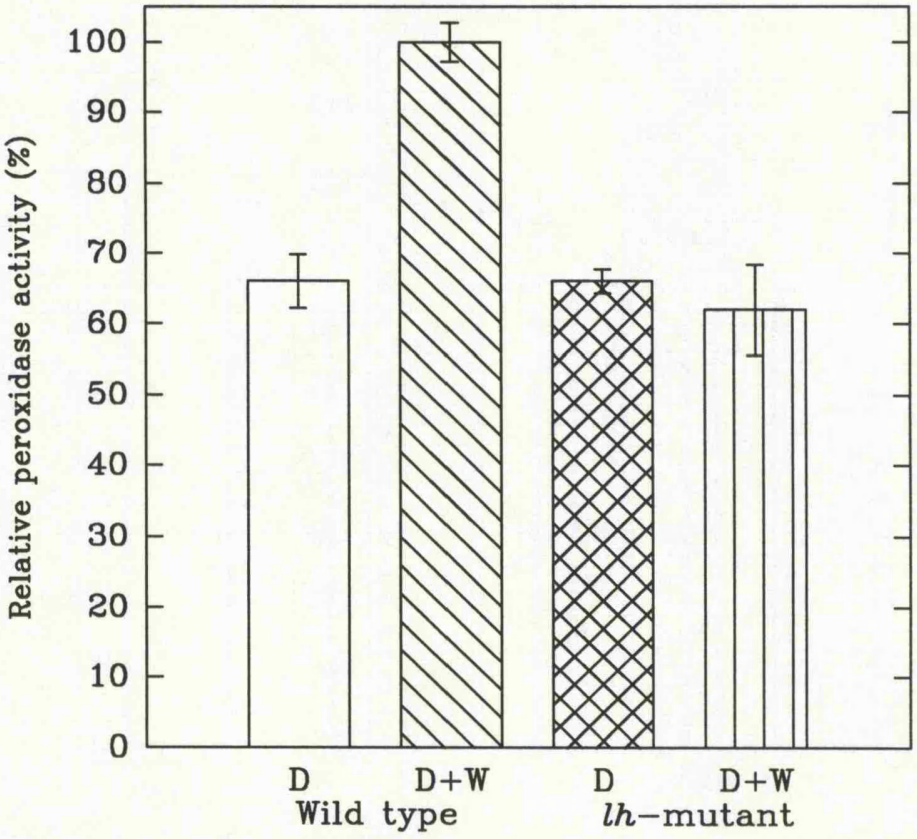


Fig. 5.16: Light regulation of extracellular, ionically-bound, peroxidase activity from etiolated cucumber seedlings. Relative specific peroxidase activity from the top 1 cm section of 8-day old, etiolated wild type (Wt) and *lh* -mutant cucumber hypocotyls, grown for 4 days in the dark and then either maintained in the dark (D) for a further 3 days , or transferred to white light (W) for 3 days.

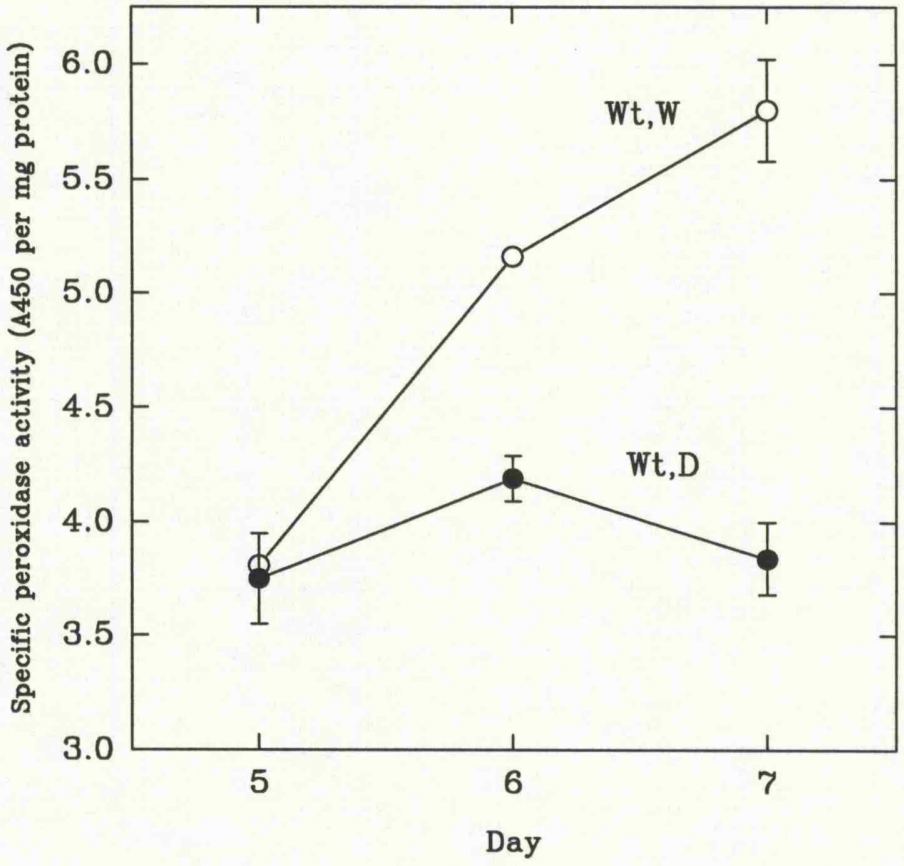


Fig 5.17: Light-regulation of the extracellular peroxidase activity of etiolated cucumber hypocotyls. Four day old dark-grown Wt cucumber seedlings were either maintained in the dark (D) or transferred to white light (W). The specific peroxidase activity was measured at 24 h intervals using TMB as a substrate.

peroxidase activity is of the range observed when comparing light-grown Wt and *lh* seedlings (see 5.3.2).

Since exposure of etiolated cucumber seedlings to W light leads to a very marked reduction in extension growth rate, very easily discernible by eye within 12 h of light treatment, yet it does affect the IB cell wall peroxidase activity This is a situation where a correlation between growth rate and extracellular peroxidase activity does not therefore exist. Thus there appears to be limitations to the situations in which a decrease in the rate of stem extension can be attributed to an increase in extracellular peroxidase activity.

5.6. Discussion

5.6.1 Location and distribution of ionically bound peroxidase in de-etiolated cucumber hypocotyls

To be considered as part of a primary mechanism in the phytochrome-regulation of stem extension, it is important that the location of the extracellular peroxidase activity enables the peroxidase enzymes to act within the short time that a change in phytochrome status takes to alter the growth rate of the stem. Growth rate changes of cucumber hypocotyls have been shown in chapter four to occur within 2 h of a reduction in the R:FR ratio, although Smith et al. (1992) found the rate of change of hypocotyl extension to increase within approximately 16 min after the onset of supplementary FR illumination to a W light background.

The epidermis is believed to be the region of the cell wall most directly involved in the control of extension growth, the orientation of the microfibrils within this region further supporting this idea (Taiz, 1984). This zone also possesses abundant phenolics which may be tightened by the formation of peroxidase catalyzed cross links (Fry, 1988). In this study, the major pool of IB peroxidase in cucumber hypocotyls (approximately 65 % of the total extractable IB activity, Fig 5.5) was found to be located in the epidermal region of the hypocotyl. In mung bean hypocotyls several isoperoxidases were found to be firmly bound to the epidermis and in the epidermal cells peroxidases were localized in the same cell wall areas as hydrogen peroxide which is required for the peroxidase to be active (Goldberg *et al.*, 1987). These peroxidases and the associated hydrogen peroxide were found to be mainly located in the junction zones and part of the radial walls between two neighbouring cells. Whilst experiments to detect the location of hydrogen peroxide in the epidermis of cucumber hypocotyls have not been carried out here, the high level of peroxidase in the epidermis itself infers the importance of these peroxidases in the control of stem extension. The same four ionically bound, cationic peroxidase isoforms that are

observed in the extracts from whole hypocotyl sections were found to be present in both the cortex and in the epidermal regions (data not shown). The peroxidase activity found within the cortex might suggest that its presence there is part of a mechanism by which rapid limitations to growth occurring in the epidermis, are then followed by further peroxidase catalyzed cross-linking of cell wall macromolecules in order to strengthen the cortical cell walls. When the rate of hypocotyl growth was altered by changes in the R:FR ratio, the relative changes in the whole hypocotyl were reflected in the epidermis and cortex regions (Fig 5.6). Thus the IB peroxidases of cucumber satisfy a necessary criteria for their involvement in controlling hypocotyl elongation; namely a predominantly epidermal location.

5.6.2 Phytochrome-mediated changes of the ionically-bound peroxidase activity of de-etiolated cucumber hypocotyls

In all de-etiolated seedlings growing at a faster rate (Wt in a low R:FR ratio and the *lh*-mutant), the extracellular peroxidase activity was lower than the slower growing Wt seedlings in a high R:FR ratio (Fig 5.7). This infers that the altered activity of peroxidase enzymes might be involved in the control of the stem extension of cucumber hypocotyls. Alternatively the reciprocal of this idea might also apply, that is changes in the rate of extension might lead to an alteration in the activity of extracellular peroxidase. To distinguish between these, the rate of change of peroxidase activity, inorder to be considered as part of the primary mechanism in the phytochrome control of stem extension, would have to either precede or coincide with the phytochrome-mediated changes in growth rate. The *lh* -mutant shows an absence of growth rate changes irrespective of alterations in the R:FR ratio, in parallel with these findings, no alteration in the extracellular peroxidase activity was observed between *lh* seedlings growing in altered R:FR ratios. As the *lh*-mutant lacks phytochrome B it would appear that not only is the shade avoidance response controlled primarily by phytochrome B but that extracellular peroxidase activity may also regulated by phytochrome B. This regulation could be direct or indirect.

Phytochrome B could directly regulate peroxidase activity and this could control elongation growth. Alternatively phytochrome B could regulate extension growth and this in turn could lead to changes in the extracellular peroxidase activity. The similar finding of a reduction of peroxidase activity in the hypocotyls of a phytochrome B deficient *Brassica ein* mutant supports a role for phytochrome B in the control of cell wall peroxidase (either directly or indirectly). However, the original/direct hypothesis is questioned by the findings from etiolated tissue where changes in growth rate do not correlate with an alteration in the IB peroxidase activity (see later).

Changes in peroxidase activity, due either to growth rate changes or wounding, have often been found to correspond to a change in activity of one or two specific isoforms. In cucumber seedlings a 33 cationic and a 60 kDa anionic peroxidase was found to be induced by ethylene (Abeles et al, 1988) and with B light the inhibition of stem extension correlated with an increase in the anionic peroxidase fraction (Shinkle et al., 1992). In the phytochrome control of de-etiolated cucumber hypocotyl extension growth, no one peroxidase isoform activity was found to be specifically mediated by these changes. The increase in peroxidase activity associated with the decrease of growth rate in a high R:FR ratio, correlates with a general increase of the cationic isoforms visualized on a NEPHGE gel (Fig 5.8). Whether the experimental procedures used might have caused the peroxidase enzymes to become fragmented, as possibly reflected by the seemingly uniform regulation of the isozymes of close but different pI values, seems unlikely as neither the addition of PMSF or BSA altered the pattern of the peroxidase isoforms observed (Fig 5.4).

The reversible nature of the phytochrome-mediated growth rate changes in cucumber hypocotyls coincides with an up and down alteration of peroxidase activity, the activity inversely correlating with the growth rate (Fig 5.12). If the mechanism of peroxidase action involves binding to the cell wall then this reversible activity may be due either to the peroxidase being released from its site of action or the activity of the peroxidase enzyme itself might have been altered. Zheng and van Huystee (1992) found the

addition of meta-flurotyrosine to the growth medium of peanut hypocotyl segments led to an inhibition of elongation and a decrease in protein concentration. However the activity of the cationic ionically bound cell wall peroxidase was seen to increase, enhancing the idea that a change in the peroxidase activity is not due to newly synthesised proteins but is more readily explained by a change to the existing pool. Although the protein concentration of the IB peroxidase fraction extracted from the apical section of cucumber hypocotyls showed little variation between samples, the protein content itself was very low, as such any alteration in the concentration may not have been within the limits of detection.

All the data presented here for de-etiolated cucumber hypocotyl growth, support the hypothesis that phytochrome is involved in the regulation of cell wall peroxidase activity as a means of controlling stem extension. In order to further this idea, as mentioned earlier, the alteration in peroxidase activity must either precede or coincide with any phytochrome-mediated growth rate changes. When examined the IB peroxidase activity increased in activity within 2 h of the addition of supplementary FR light (a low R:FR ratio, Fig 5.12), correlating with a change in the rate of growth that could be detected using a millimetre scale ruler. Whilst this indicates that a correlation does exist between phytochrome regulated growth rate changes and extracellular peroxidase activity, the rate of change of stem extension of the growing apex of cucumber hypocotyls is known to occur with a lag phase of 16.2 min after the addition of FR light (Smith et al., 1992). Experimental constraints, together with the dynamic nature of the change in peroxidase activity, makes accuracy within this 2 h time period difficult. Extracts made of IB cell wall peroxidase from Wt cucumber seedlings, 30 min after transfer from a high to a low R:FR ratio, were highly variable showing no specific pattern in the change in peroxidase activity. Therefore although a correlation exists between the phytochrome-regulated change in growth rate and extracellular peroxidase activity, it appears unlikely that the alteration in peroxidase activity is fast enough to be the main contributory factor in the initiation of these changes.

5.6.3 Regulation of ionically-bound peroxidase activity in etiolated cucumber hypocotyls

The phytochrome-mediated growth rate changes in the hypocotyls of etiolated cucumber seedlings do not correlate with changes in extracellular peroxidase activity. The establishment of inverse correlations between growth and peroxidase activity appears therefore to be linked to the development of the seedlings themselves. Five day old dark grown seedlings treated with R or FR light for 3 days did not show any regulation of the ionically bound peroxidase isoforms despite the differences observed in their growth rate. Seedlings of the same age that had been D grown and then treated with W for 3 days, exhibit an increase in total peroxidase activity compared to D grown R, FR or D treated tissue (Fig 5.16). However no change in the IB peroxidase activity could be detected in the Wt seedlings during the early stages of de-etiolation. Only after 2 days W light treatment i.e. the commencement of de-etiolation, was a change in the peroxidase activity observed (Fig 5.17). Whilst speculating that the extracellular peroxidase activity is regulated by phytochrome B, it must be remembered that there is evidence that when etiolated plants are first transferred to light it is the action of phytochrome B that initiates at least some of the de-etiolation processes, including the inhibition of hypocotyl extension. This is supported by analysis of the *lh*, *hy3* and *ein* mutants which all lack a light-stable phytochrome B-like phytochrome (López-Juez et al., 1992, Sommers et al., 1991, Devlin et al., 1992). These mutants show a normal inhibition of hypocotyl elongation under prolonged FR, reflecting the HIR which is thought to be mediated by phytochrome A, but a reduced inhibition under prolonged R, which is, in part, mediated by a light-stable phytochrome (possibly phytochrome B). Thus, from the cucumber hypocotyl growth data it seems likely that phytochrome B is active during the early stages of the de-etiolation process and as such the lack of change observed in the IB peroxidase activity questions whether IB peroxidase activity constitutes a general mechanism in the phytochrome-regulation of stem growth.

The B light inhibition of cucumber hypocotyl extension has been shown to inversely correlate with peroxidase activity (Shinkle and Jones, 1988). The findings of Shinkle et al. (1992), that the inhibitory effect of B light on hypocotyl elongation was faster than the observed change in total cell wall peroxidase activity, questioned the causal relationship between growth and peroxidase activity. In this study, the lack of change in the IB peroxidase activity of etiolated seedlings placed into W light, i.e. the early stages of de-etiolation, further questions the role of peroxidase activity as a general mechanism in the control of stem extension. The B light component of the W light treatment initiating the de-etiolation process, does not appear to be altering the total extracellular peroxidase activity. Whilst the cationic extracellular isoforms showed no alteration in activity to B light, a qualitative change in the two anionic isoforms was observed without a concomitant alteration in total peroxidase activity (Shinkle et al., 1992). Thus these isoforms were postulated to play an important role in the regulation of stem extension. However the lack of change in total peroxidase activity found here through the first 24 h of de-etiolation, combined with no observable change in the peroxidase isoforms that could be visualized on a full range NEPHGE gel after 72 h de-etiolation, casts doubt on the original direct hypothesis. The indirect hypothesis, that phytochrome-mediated changes in growth rate leads to an alteration in the extracellular peroxidase activity, also fails in the situation of the etiolated seedlings. Thus in de-etiolated seedlings, the phytochrome-regulated inhibition of cucumber stem extension is seen to lead to an increase in extracellular peroxidase activity, whereas the phytochrome regulated inhibition of etiolated cucumber stem extension does not show any change in peroxidase activity. The earlier evidence put forward here from the de-etiolated cucumber seedlings, that infers the role of phytochrome B in the regulation of extracellular peroxidase activity as a means of controlling stem extension (including those of cucumber), is not the case when looking at the etiolated seedlings. In etiolated seedlings phytochrome has been shown to lead to changes in growth that are detectable within a few hours of the onset of irradiation (e.g. Adamse et al., 1987). However, the extracellular peroxidase activity in cucumber hypocotyls remains

unaltered until 2 days after the commencement of de-etiolation. The lack of correlation seen in the etiolated cucumber hypocotyls is the reverse of the situation found in the mustard hypocotyls, where the extracellular peroxidase activity of etiolated hypocotyls clearly increases within 1 h of light treatment. Thus there appears to be two separate mechanisms in cucumber hypocotyls by which phytochrome-mediated growth rate changes and associated changes in cell wall extensibility are regulated. These will be discussed in the main discussion (Chapter seven).

CHAPTER SIX

CHAPTER SIX - Characterization of the strongly basic cell wall peroxidase associated with the regulation of cucumber hypocotyl growth.

6.1 INTRODUCTION

The work presented in chapter five suggests that although phytochrome-mediated growth rate changes in light-grown cucumber seedlings are correlated with extracellular peroxidase activity, this is not the case for etiolated seedlings. Thus, although changes in extracellular peroxidase activity cannot represent a universal mechanism underlying the regulation of extension growth, it could contribute to phytochrome-mediated growth effects under some circumstances. This being the case, the availability of antisera raised against cucumber peroxidases afforded the opportunity to further characterize the phytochrome-responsive peroxidases from light-grown cucumber seedlings.

6.1.1 Immunochemical studies

Antibodies act as sensitive, specific probes and can be used to quantify changes in specific proteins within a given system. The use of antibodies to specific enzymes allows the inactive as well as the active enzyme content to be measured. Whilst it is important when studying the mechanism of enzyme action to know what proportion of the enzyme is active and by how much that activity changes under different conditions, measuring the whole pool of the enzyme protein should indicate whether changes in activity are due to changes in the number of enzyme molecules, or whether some modification of pre-existing enzyme molecules e.g. glycosylation are involved.

Kim et al. (1988) produced monoclonal antibodies to an anionic cell wall peroxidase from maize seedlings. An increase in immunodetectable levels of this anionic peroxidase was observed within a few minutes following a R light pulse. The increase in level of this anionic peroxidase was found to be opposite to the change in the overall

peroxidase activity which was seen to decrease when measured by an enzyme linked immunosorbent assay (ELISA). This shows that not all peroxidases are equally modulated by light and supports the hypothesis that specific peroxidases may help to regulate cell wall extensibility.

In a study of cucumber peroxidases, Abeles et al. (1988) produced two antisera raised to two immunochemically-distinct peroxidases from cucumber cotyledons; a 33 and 60 kDa peroxidase. It was found that the 33 kDa peroxidase was cationic and that its appearance could be induced by ethylene but not by a range of other hormones. The 60 kDa peroxidase was found to be acidic and to have a different amino acid composition compared with the 33 kDa protein. Any changes in these peroxidase enzymes were studied by using immunoprecipitation techniques. Prior to ethylene treatment the 60 kDa protein was more prevalent than the 33 kDa protein, treatment with ethylene led to a 4 fold and 20 fold increase in their respective abundance. This indicates that although both of these proteins were induced by ethylene, the increase in the 33 kDa protein was far greater.

Following the observation that the activity of the IB cell wall peroxidase from cucumber hypocotyls increases with the phytochrome-regulated decrease in stem extension, further studies were carried out utilizing the antisera kindly made available by Dr. F. B. Abeles. In the study of the extracellular cationic light-responsive peroxidases, antisera that are specific to these isoforms would be of considerable valuable in their characterization. The use of cell suspension cultures would then be of value in isolating the appropriate enzyme in the production of antisera.

6.1.2 Cell suspension culture

It is well documented that cell suspension cultures release various macromolecules into the surrounding culture medium (Olson et al., 1969). Cell suspension cultures have been used for the study of extracellular enzymes in many different species e.g. spinach (Fry, 1979, Sticher et al., 1981), peanut (Kossatz and van Huystee, 1976) and

Nicotiana tabacum L. (Mader and Walter, 1986). The benefits of studying extracellular enzymes in cell suspension cultures stems from the difficulty in separating the soluble cell wall components of growing plant organs and maintaining the tissue free from cytoplasmic contamination. Cells that are growing in a liquid medium effectively use the medium as a continuation of the cell wall, providing a bulk source of extracellular compounds. This should therefore provide an indication of the concentration and activity of any enzymes that would be present in the cell wall matrix. This also provides a source of cell wall enzymes for possibly purification and characterization.

There are many enzymes that have been investigated by the use of cell suspension cultures. Amongst these ascorbate oxidase activity was found to rapidly increase during callus formation and the activity in both the cells and the medium increased soon after the transfer to fresh medium, reaching a maximum at about 5 days (Esaka et al. (1988a). The addition of copper, a prosthetic metal of the enzyme, into the liquid medium also increased the activity of the enzyme (Esaka et al., 1988b). Peroxidase activity has been found to increase in the medium of many plant cell suspension cultures as the culture increases in age. Kossatz and van Huystee (1976), investigating the growth cycle of peanut suspension cultures, found that whilst the protein content decreased, the specific peroxidase activity increased with increasing age of the culture. This supports the idea that an increased level of peroxidase activity might lead to a decrease in the growth rate of plant cells. The addition or chelation of calcium has been found to regulate the cationic peroxidase activity of spinach and peanut cell suspension cultures (Sticher, 1981, Hu et al., 1987). Low concentrations of Ca^{2+} ions (mM) added to a spinach cell suspension culture also led to an increase in the secretion of peroxidase activity (Sticher, 1981). Hu et al., (1987) also found that a 5 % increase in Ca^{2+} ion concentration in the medium of cultured peanut cells led to a two fold increase in the peroxidase activity but concluded this increase to be primarily due to newly synthesized enzyme. From the study of suspension cultures of spinach

cells it has been postulated that Ca^{2+} regulates cationic peroxidase activity via three mechanisms; direct activation, binding to membranes and secretion (Penel, 1986).

The work here was undertaken to set up a cell suspension culture from cucumber hypocotyls to provide a large source of extracellular enzymes in the culture medium, for use in the further characterization of the phytochrome-regulated cationic peroxidase isoforms and as a source of enzyme for purification and antiserum.

6.2 Immunochemical studies

6.2.1 SDS Western blots

Abeles et al., (pers. comm.), carried out their investigations on the ethylene induced 33 and 60 kDa peroxidase isoforms by using radial immunodiffusion and an immunoprecipitation assay system. Ideally, by first resolving the peroxidases and then probing with the antisera using an immunoblot method, it should be possible to examine any changes that might be occurring in the abundance of the peroxidase enzymes, in this case due to differences in the growth rate. Here a Western blotting technique was used to investigate whether the IB cationic isoforms found in the extracts of Wt and *lh* cucumber hypocotyl extracts were stained by either of the antisera. F. B. Abeles provided two antisera raised against; (a) a 33 kDa peroxidase from cucumber cotyledons and (b) a 60 kDa peroxidase from cucumber cotyledons. For convenience these two antisera are referred to as anti-33 kDa and anti-60 kDa antibodies. To test whether the two antisera could be used with Western blotting, cotyledon extracts were prepared following the same procedure as Abeles et al. (1988). Ionically-bound peroxidase samples from the hypocotyls were prepared following the standard method described earlier. The cotyledon samples were separated according to their molecular weight by a 12 % SDS PAGE gel and transferred to nitrocellulose membranes by semi-dry electroblotting. All samples analyzed by western blotting were loaded on the SDS gel on an equal protein basis. The initial loadings and washing procedures led to a large number of protein bands

being stained by both the anti-33 and anti-60 kDa antibodies (Fig 6.1a). The blots were repeated and developed using a more stringent washing protocol in an effort to enhance the specificity of staining by the individual antibodies. A composite of three sugars was added to the washing buffer, to "mop up" any sugar-reactive antibodies from the serum, thus preventing those antibodies from binding to glycans in proteins on the blot. However, varying the amount of extracts loaded and following the more stringent washing procedures continued to yield blots where a large number of proteins were stained (Fig 6.1b). The initial procedure used both primary antibodies at a dilution of 1:5,000. Diluting the anti-33 kDa antibody to 1:10,000 continued to yield blots in which multiple bands were stained. However the same dilution of the anti-60 kDa antibody stained one main protein band at approximately 54 kDa, although other very feint bands were also stained (Fig 6.2). The anti-60 kDa antibody was therefore used to study whether any changes that occurred in peroxidase activity following phytochrome-mediated growth rate changes, might be due to changes in the amount of the polypeptide stained by this antibody.

6.2.2 Phytochrome-regulation of the polypeptides stained by the anti-60 kDa antibody

Initial experiments were carried out by probing IB extracts from the apical region of 8-day old cucumber hypocotyls of Wt seedlings grown in different R:FR ratios. Fig 6.3 illustrates that two major bands at approximately 54 and 42 kDa are stained in immunoblots of extracts taken from seedlings growing in a high R:FR ratio. Two feinter bands at higher molecular weight were also visualized. Immunoblots of extracts from seedlings growing in a low R:FR ratio also led to staining of the same four protein bands. The two major bands at 54 and 42 kDa were however stained less intensely than the same bands seen in the comparative extracts from seedlings growing at a high R:FR ratio (Fig 6.3). In contrast the two fainter bands did not appear to be regulated by changes in the light quality as the intensity of staining of these bands was similar for both extracts. It therefore appears likely that the abundance of the polypeptides that comprise the two major bands are phytochrome-regulated.

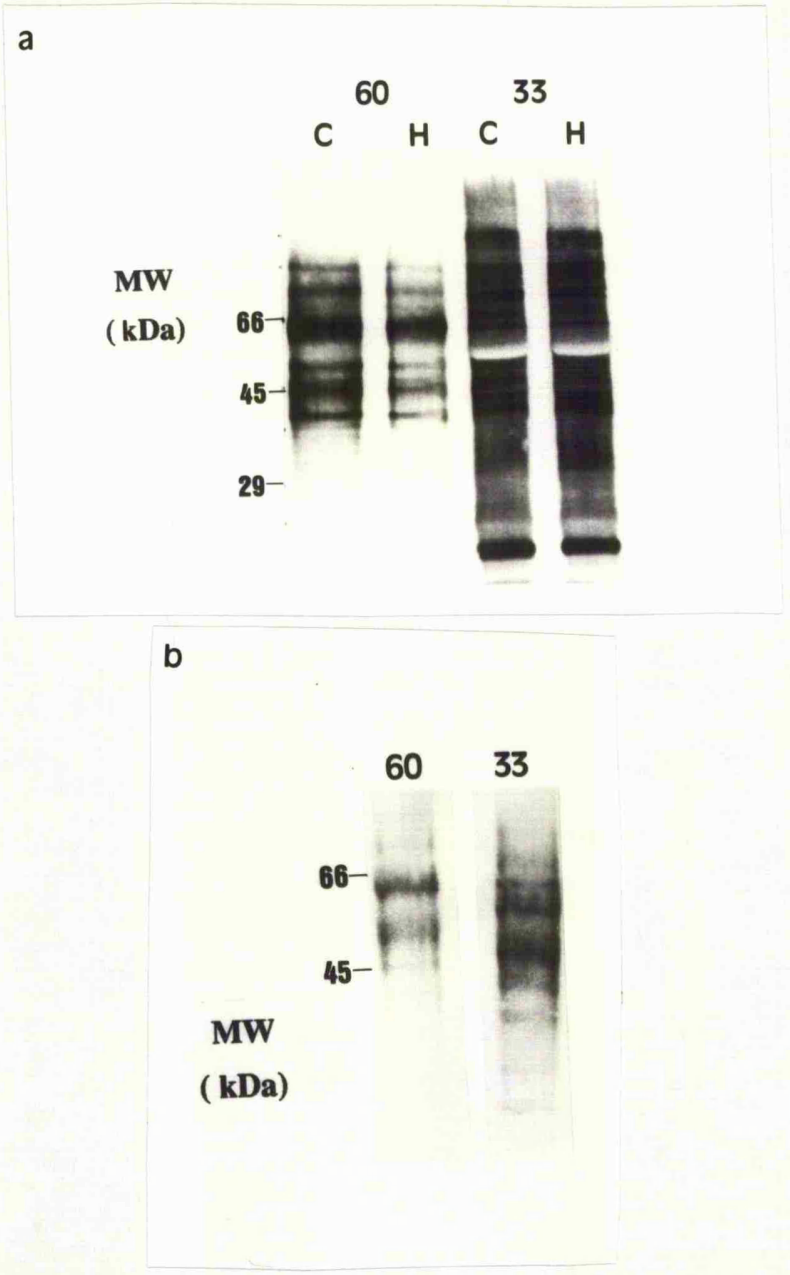


Fig 6.1: Western blot analysis of cotyledon (C) and hypocotyl (H) extracts from 8-day old wild type cucumber seedlings. Samples were probed with either a 60 kDa antidody (60) or a 33 kDa antibody (33), at a 1:5000 dilution. The antibodies were supplied by Dr. F. B. Abeles. **(a)** Staining pattern observed in cotyledon and hypocotyl extracts, probed with the 60 and 33 kDa antibodies. The washing buffer containing TBS-Tween only. **(b)** Staining pattern observed in ionically bound cell wall extracts from cucumber hypocotyls probed with the 60 and 33 kDa antibodies. The washing buffer contained an additional composite of three sugars (50 mM xylose, manose and hexose). MW refers to the migration of Sigma MW-SDS molecular weight marker proteins and the figures indicate their relative molecular mass.

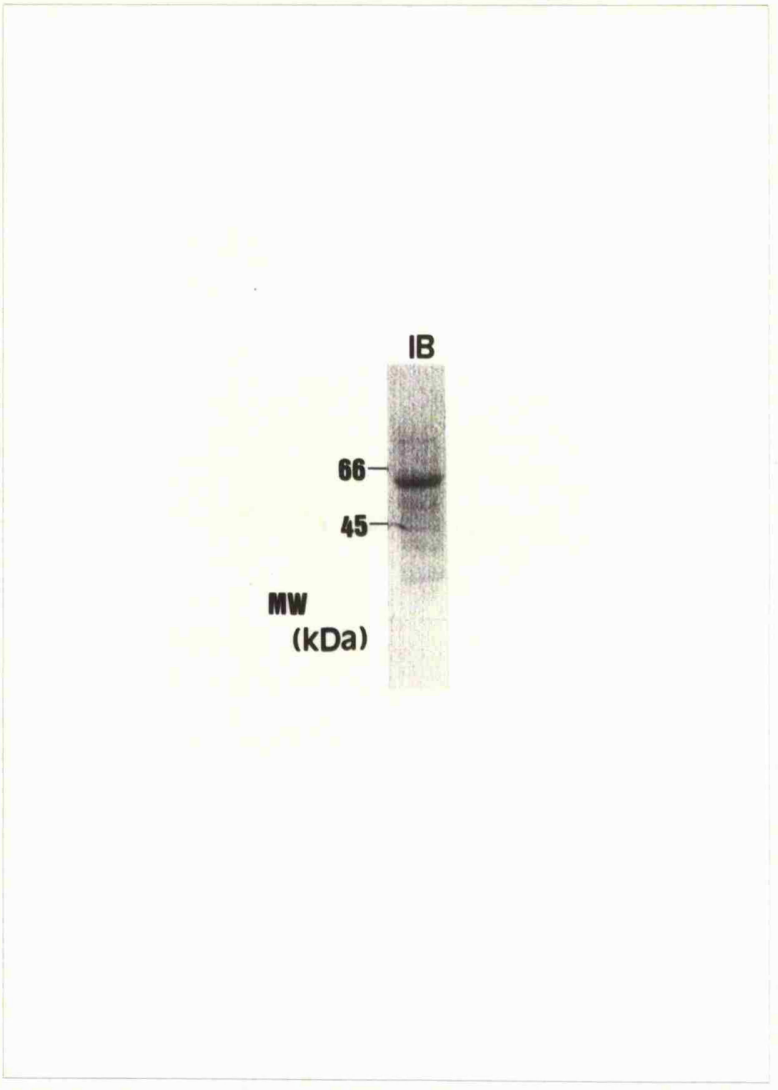


Fig. 6.2: Western blot analysis of an ionically bound extract from 8-day old white light-grown, wild-type cucumber hypocotyl seedlings, probed with a 60 kDa antibody raised to a 60 kDa peroxidase from cucumber cotyledons. This primary antibody was used at a dilution of 1:10,000. MW refers to the migration of Sigma MW-SDS molecular weight marker proteins and the figures indicate their relative molecular mass.

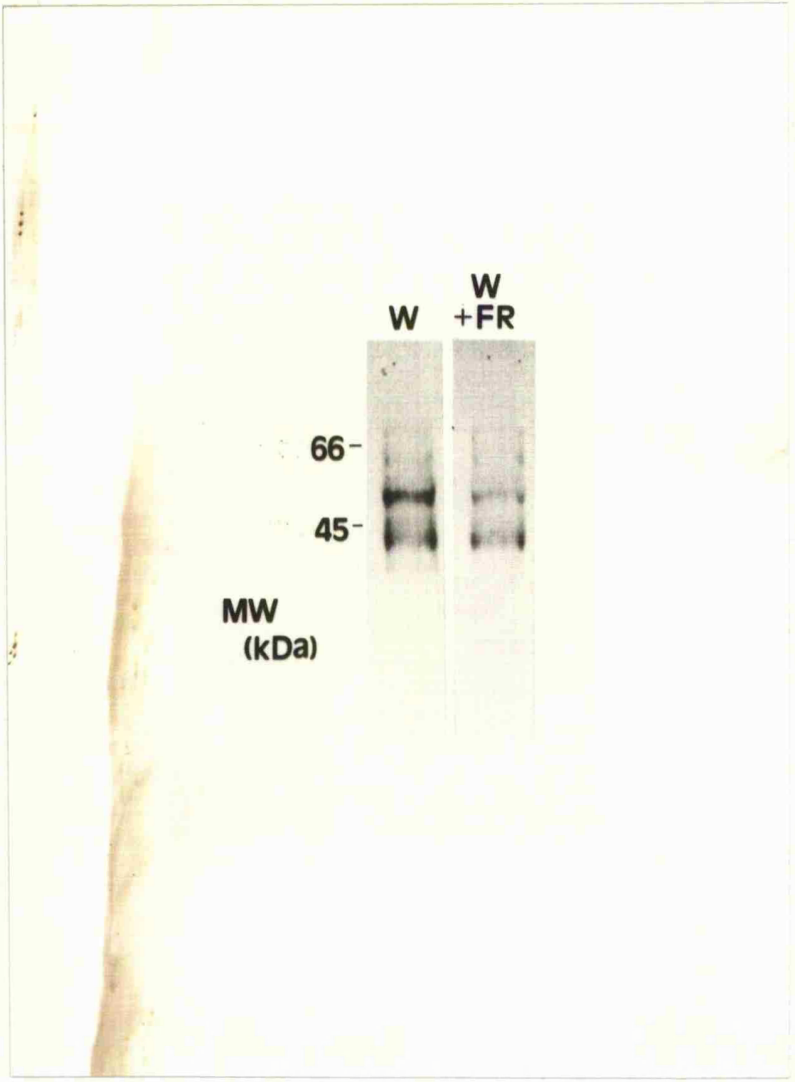


Fig. 6.3: Phytochrome-regulation of the polypeptides stained by the anti-60 kDa antibody. Western blot analysis of ionically bound extracts from 8-day old wild type cucumber hypocotyls, grown for either 8 days in a high R:FR ratio (W) or 7 days in a high R:FR ratio followed by 24 h in a low R:FR ratio (W+FR). The 60 kDa antibody was used at a 1:10,000 dilution. MW refers to the migration of Sigma MW-SDS molecular weight marker proteins and the figures indicate their relative molecular mass.

The lack of change in staining intensity of the two fainter bands indicates that any changes occurring in the staining intensity of the two bands is unlikely to be a consequence of irregular extractions and as such the feint-staining polypeptides act as internal controls.

To examine whether these phytochrome-regulated polypeptides were more specifically regulated by phytochrome B, IB extracts from Wt cucumber hypocotyls were compared with equivalent extracts from the phytochrome B deficient *lh*-mutant. After resolving and immunoblotting these samples, the blot was probed with the anti-60 kDa antibody. A different banding pattern was seen (Fig 6.4) in comparison with the Wt binding pattern seen previously (Fig 6.3). In Fig 6.4 four major bands were stained in extracts taken from both the Wt and *lh* seedlings, importantly these bands were at different molecular weights to those previously seen. Whilst the same banding pattern was highlighted in both the Wt and *lh* extracts, the *lh* bands all showed a reduced intensity when compared with those present in the Wt (Fig 6.4). This general reduction in staining intensity of all the bands in the extracts from the *lh*-mutant, is different than the situation found with Wt seedlings growing in different R:FR ratios where only the two major bands were seen to have a reduced intensity of staining. This indicates that not surprizingly, the *lh* mutation does not exactly mimic Wt seedlings grown under a low R:FR ratio.

Whilst no one specific polypeptide that was immunostained with the anti-60 kDa antibody was seen to be regulated by phytochrome, a decrease in staining was found in either two or all of the polypeptides visualized (Fig 6.3 and 6.4). Earlier work from chapter five indicated that the phytochrome-mediated growth rate changes alter the activity of all of the IB cationic cell wall peroxidases. The results here, whilst differing in the number of bands that show reduced staining, illustrate that the phytochrome-regulated growth rate changes are accompanied by a decrease in the total protein content of bands that could be stained with an anti-60 kDa antibody.

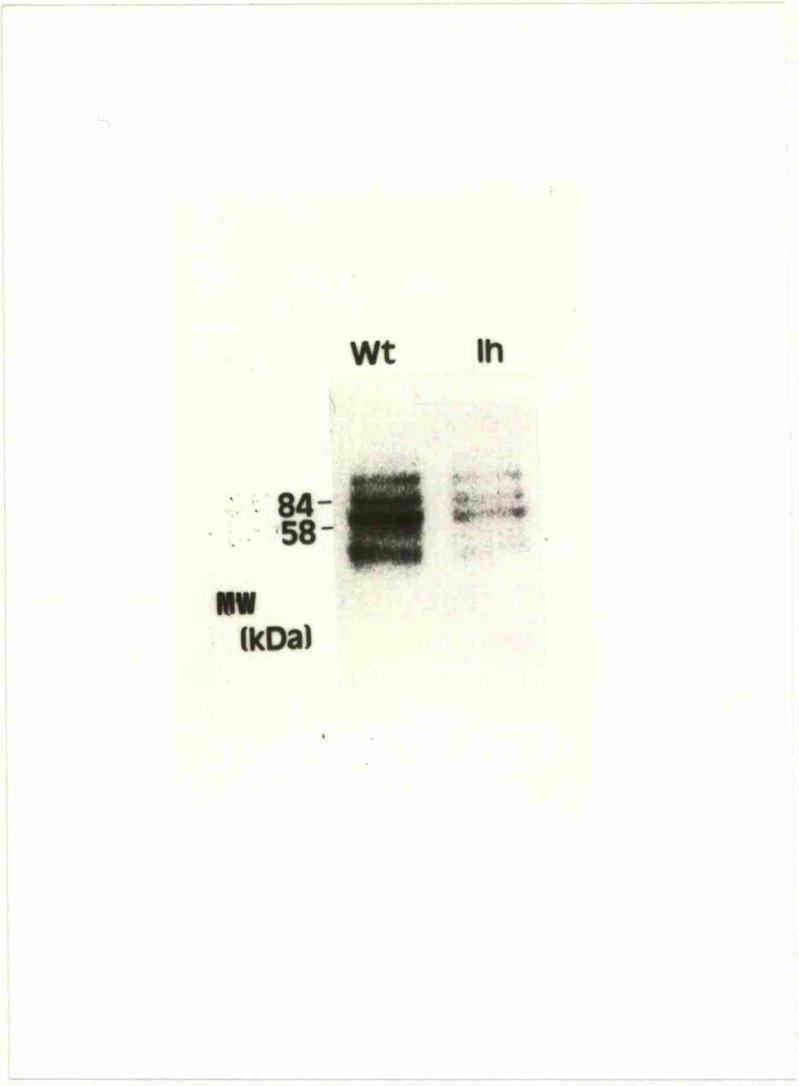


Fig. 6.4: Phytochrome-regulation of the polypeptides stained by the anti-60 kDa antibody, using a phytochrome B deficient mutant. Western blot analysis of ionically bound extracts from 8-day old white light grown wild type (Wt) or *lh*-mutant (lh) cucumber hypocotyls. The 60 kDa antibody was used at a 1:10,000 dilution. MW refers to the migration of Sigma MW-SDS molecular weight marker proteins and the figures indicate their relative molecular mass.

6.2.3 NEPHGE contact blots (Eastern blotting)

The lack of specificity found with the antibodies limited their usefulness in the characterization of the peroxidase isoforms being examined. Whether any of these phytochrome-regulated peroxidase isoforms that had previously been identified by charge, corresponded to the immunostained polypeptides was tested. The IB samples from de-etiolated Wt and *lh* hypocotyl sections were separated on a full range NEPHGE gel and transferred to nitrocellulose by contact blotting. Both the anti-33 and the anti-60 kDa antibodies stained extra bands not usually seen with 4CN staining. These additional bands were distributed throughout the pI range used (pI 3- 10) (Fig. 6.5). The extract from the *lh*-mutant showed a lower intensity of staining of all bands compared with the Wt, again illustrating a general increase in peroxidase activity when growth rate is reduced. Whilst the bands that were stained were not very sharp only one diffuse band could be seen in approximately the same pI range that the four bands stained with 4CN on a NEPHGE gel, are usually found. The extra bands seen on the blot are possibly peroxidase fragments that do not include the active haem moiety. Alternatively they may be other cell wall proteins that were stained by the antisera. The lack of specific staining of the samples normally seen with 4CN on a NEPHGE gel blot means that whether the phytochrome-regulated 54 and 42 kDa bands stained with the anti-60 kDa antibody are peroxidase enzymes is unable to be resolved from these experiments. The use of 2-D gel techniques may provide a means of addressing this question.

6.3 Cell suspension culture

6.3.1 Growth of callus and selection of hormonal balance in the growth media

Sterilized explants were placed on solid MS medium containing different hormone concentrations, in order to induce callus growth. Whilst all combinations of hormones tested 1) 0.5 mg^{-1} 2,4-D 2) 0.1 mg^{-1} BAP & 0.5 mg^{-1} 2,4-D 3) 0.5 mg^{-1}

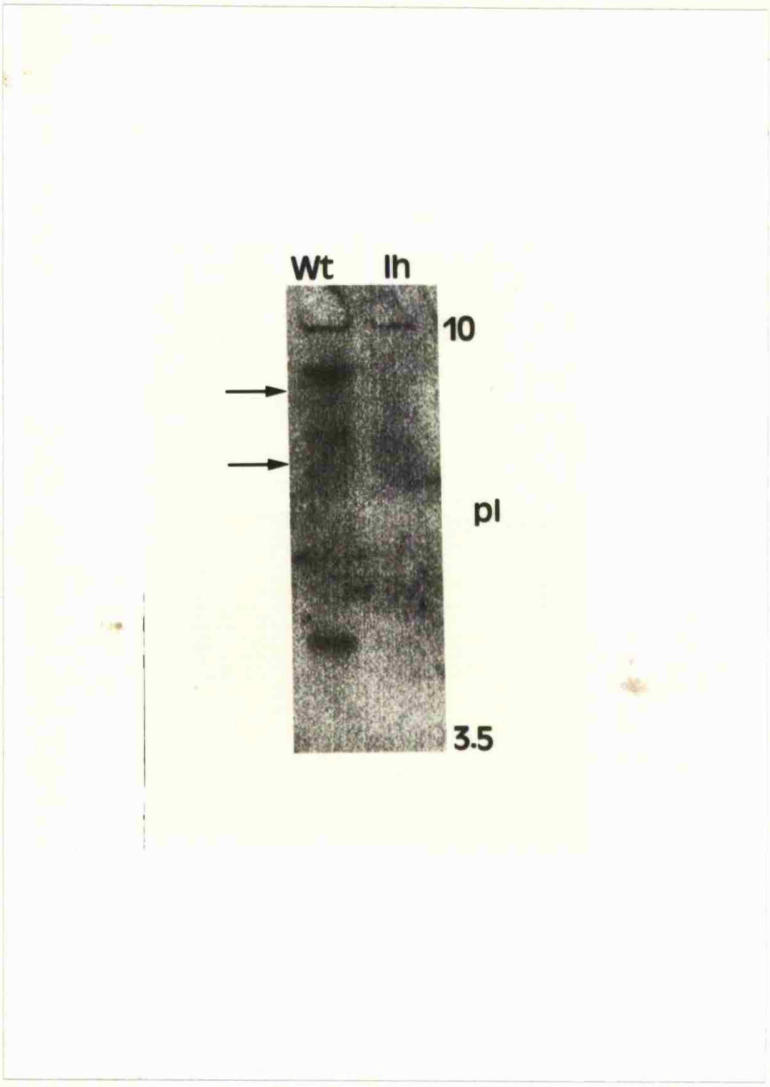


Fig. 6.5: Phytochrome-regulation of the peroxidase isoforms from 8-day old cucumber hypocotyls separated by charge. Ionically bound cell wall extracts from the top 1 cm section of cucumber hypocotyls separated on a full range NEPHGE gel, transferred to nitrocelluose by Eastern blotting and stained with the 60 kDa antibody. The 60 kDa antibody was used at a 1:10,000 dilution. The 2 arrows indicate the area between which the 4 cationic peroxidase isoforms are usually seen when stained with 4CN on a full range NEPGHE gel.

NAA and 4) 0.1 mg^{-1} BAP & 0.5 mg^{-1} NAA, initiated cell division, 0.5 mg^{-1} 2,4-D produced the highest rate of uniform callus growth. The most prolific and the least differentiated callus was produced by media containing 0.5 mg^{-1} 2,4-D (1) (Fig 6.6). Callus grown on this media with 0.5 mg^{-1} 2,4-D, was therefore added to a liquid MS medium of equal composition to maintain favourable growth conditions. Three independent lines were set up and all results presented are the mean from several replicates of each line.

6.3.2 Fresh weight, protein content and peroxidase activity of a cucumber cell suspension culture over a 14 day cycle

After leaving the suspension cultures for 14 days to become established, each line was sub-cultured in a 14 day cycle by a 1:10 dilution in fresh MS medium + 0.5 mg^{-1} 2,4-D. The growth of the culture was examined over the 14 day cycle. The fresh weight of the cells showed an initial spurt of growth to day 4 followed by a gradual, but continued, increase until the end of the cycle (Fig 6.7).

Extracts of the free (those present in the culture medium), soluble (those present in the supernatent of an homogenate of the cultured cells) and ionically bound peroxidases were taken and examined at day 0, 4, 7, 11 and 14. The peroxidase activity of the medium and soluble fractions showed a steady rise throughout the 14 day cycle. The IB fraction however, steadily increased in peroxidase activity until day 7 after which a rapid increase occurred to day 11, the activity was then seen to decline rapidly until the end of the 14 day cycle (Fig 6.8).

The protein content of the samples was also analyzed in order to compare the change in protein concentration with the observed changes in peroxidase activity. The medium and soluble fractions showed a slow increase in protein content, comparable to the peroxidase activity. The protein content of the IB fraction dropped to day 4 and then maintained a steady increase throughout the remainder of the growth cycle (Fig 6.9). Repeated analysis of the protein and peroxidase levels within the culture following subsequent sub-cultures were made. However even when the protein

Hormone balance	Day 3	Day 7	Day 14
(1) 0.5 mg^{-1} 2,4-D	++	+++	+++++
(2) 0.1 mg^{-1} BAP, 0.5 mg^{-1} 2,4-D	+	++	++ S
(3) 0.5 mg^{-1} NAA	++	++ R	+++ R S
(4) 0.1 mg^{-1} BAP, 0.5 mg^{-1} NAA	+	+	++ R

+ = relative amount of non differentiated callus, R = root differentiation, S = shoot differentiation

Fig. 6.6: Growth of callus with different hormonal concentrations and balance in the growth media. Relative growth of sterile explants of cucumber hypocotyls on solid MS media containing 1) 0.5 mg^{-1} 2,4-D, 2) 0.1 mg^{-1} BAP & 0.5 mg^{-1} 2,4-D, 3) 0.5 mg^{-1} NAA or 4) 0.1 mg^{-1} BAP & 0.5 mg^{-1} NAA. The growth of callus and root/shoot differentiation was monitored over 14 days.

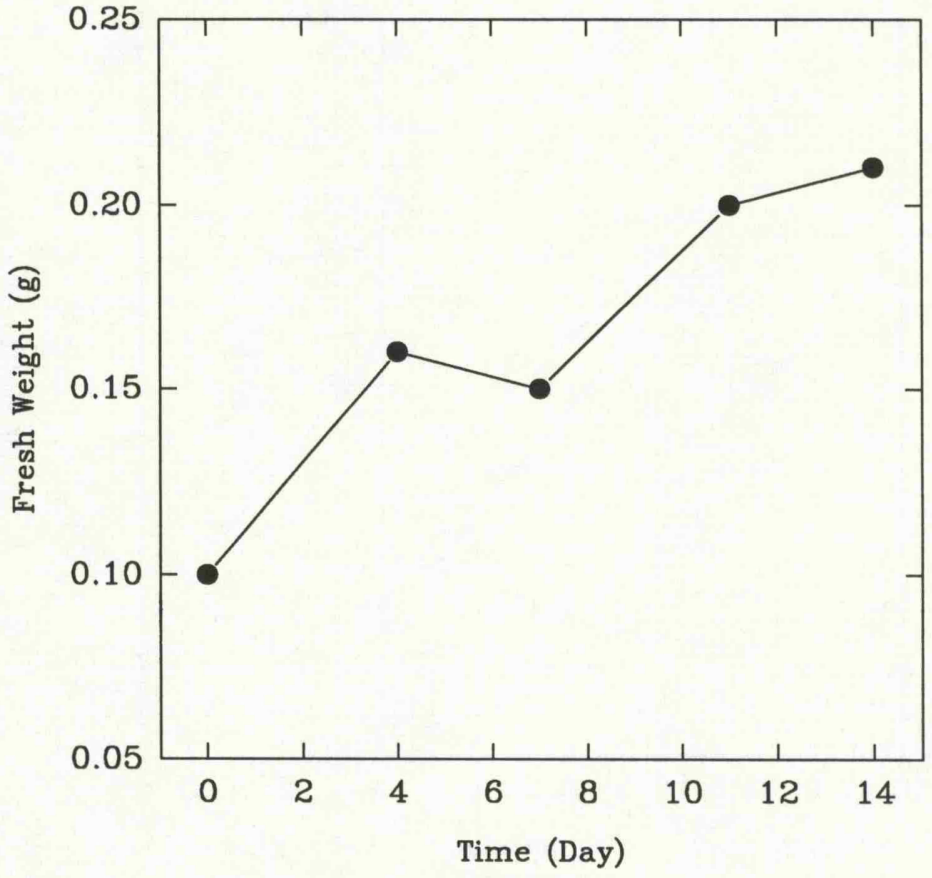


Fig. 6.7: Growth of a cucumber cell suspension culture over a 14 day cycle. The cell susupension culture was subbed by a 1:10 dilution into fresh MS medium + 0.5 mg^{-1} 2,4-D and fresh weight (g) of the cells measured over a period of 14 days. A 1ml aliquot of the culture was taken and gently centrifuged, the supernatent removed and the weight of the cell pellet taken as the fresh weight of the cells.

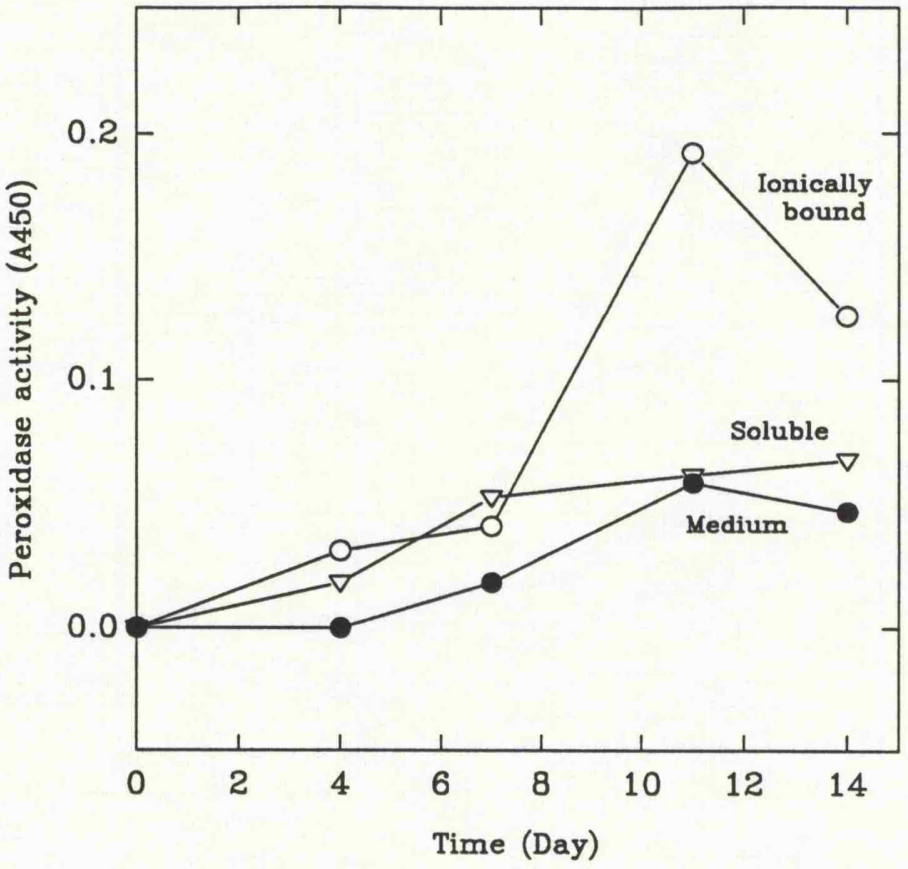


Fig. 6.8: Peroxidase activity a cucumber cell suspension culture over a 14 day cycle. Extracts were taken of peroxidases that were, present in the medium (those free within the culture medium), soluble (those present in the supernatent of an homogenate of the cultured cells) and ionically bound (the peroxidase activity that could be extracted by a 1 M salt solution from the cultured cells). All activity was measured using TMB as a substrate. The peroxidase activity was measured at day 0, 4, 7, 11 and 14.

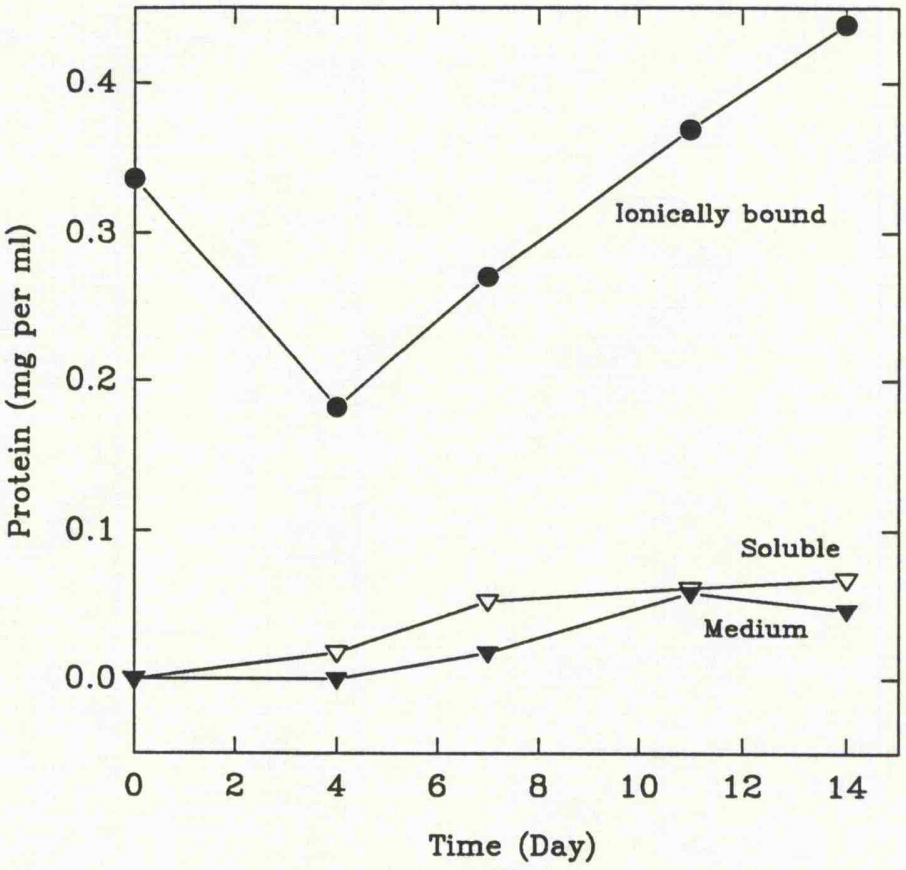


Fig. 6.9: Protein content of a cucumber cell suspension culture over a 14 day cycle. Protein content (mg ml^{-1}) of the extracts taken of the medium, soluble and ionically bound peroxidases. Protein concentraion was measured using Bicinchoninic acid.

content remained at approximately the same level as that observed in the relatively juvenile cultures, with sequential sub-cultures, decreasing levels of peroxidase activity were observed in all three fractions. Eventually 5-7 subcultures after the culture had been set up, no peroxidase activity could be found in the culture at any stage of the growth cycle.

6.3.3 The addition of calcium to the liquid growth medium

The addition of calcium to the culture medium has been found to increase the level of peroxidase activity in suspension cultures of spinach and peanut (Sticher et al., 1981, Hu et al., 1987). In an attempt to increase the level of peroxidase activity in the cucumber suspension culture, 1 mM of calcium chloride was added to the medium 24 h after subbing had taken place. In the spinach and peanut suspension cultures, the addition of calcium led to rapid increases in the activity of the extracellular peroxidase activity (Sticher et al., 1981, Hu et al., 1987). However following the time course 0, 1, 2, 4 h no increase in peroxidase activity in the cucumber suspension culture was observed. Even at day eleven of the growth cycle, following the addition of calcium, no increase in peroxidase activity could be found. In fact no peroxidase activity could be found at any stage in the growth cycle. The loss of any measurable peroxidase activity within the cucumber cell suspension negated the potential use of the culture as a source of extracellular peroxidase enzymes for further characterization.

6.4 Discussion

6.4.1 Antibodies

The IB peroxidase isoforms being examined here from cucumber hypocotyls have been shown to be cationic by separation on a NEPHGE gel (see 5.3.2). The extracellular peroxidase isoforms isolated from cucumber hypocotyls were separated by SDS page electrophoresis and probed with the anti-33 kDa and anti-60 kDa antibodies. Following the lack of definition with the anti-33 kDa antibody only the anti-60 kDa was used. This antibody stained two major bands at approximately 54 and 42 kDa (Fig 6.4). Even this antibody proved to be exceedingly sensitive and therefore had to be used at a very high dilution and a stringent washing procedure followed. If the bands stained on the SDS blot were the same isoforms as those seen to be regulated by phytochrome on a NEPHGE gel, then a lower degree of staining with the antibody would be expected with samples extracted from Wt seedlings growing in a low R:FR ratio (W+FR) in comparison to those growing in a high R:FR ratio (W). When these samples were probed with the anti-60 kDa antibody, a lower intensity of staining was seen in bands from the extract of seedlings grown in a low compared with a high R:FR ratio (Fig 6.5). Two fainter bands of higher molecular weight, showed a similar staining intensity irrespective of the R:FR ratio. It would therefore appear that the two major bands seen by probing with the 60 kDa antibody are regulated by phytochrome. The down regulation of the bands stained with the anti-60 kDa antibody seen in the extracts from the *lh*-mutant, when compared with its Wt (Fig 6.6), further suggests that the regulation of these isoforms might involve phytochrome B. It is however inconclusive as to whether these phytochrome-regulated polypeptides are in fact peroxidase isomers.

Abeles et al. (1988) found that the 33 kDa peroxidase from cucumber cotyledons was cationic, whereas the 60 kDa peroxidase was anionic. The IB polypeptides from cucumber hypocotyls were more specifically stained by the 60 kDa antibody.

However, the staining of multiple bands seen here questions the proposed specificity of the antibody being used. Also, the phytochrome-regulated peroxidase isoforms from cucumber hypocotyls are cationic, further questioning its usefulness in the work carried out here. Whether the phytochrome-regulated bands stained with the anti-60 kDa antibody on the SDS blot, were the same bands as those peroxidase isozymes that had previously been identified as being phytochrome-regulated on a NEPHGE gel was examined. Contact blots were made from NEPHGE gels and probed with the anti-60 kDa and anti-33 kDa antibodies. Both antibodies stained bands not previously seen on the NEPHGE gels when stained with 4CN. These additional bands may represent inactive peroxidase isoforms or fragments lacking the active haem moiety that would not be stained with 4CN. Alternatively these bands may be proteins other than peroxidase.

The phytochrome-mediated inhibition of stem extension and the associated increase in the activity of the peroxidase isoforms, also coincides with an increase in the amount of polypeptides that can be stained with a anti-60 kDa antibody. However, specific antibodies to the isoforms found in the IB extracts of cucumber hypocotyls would be required for further immunological studies of this particular system.

6.4.2 Cell suspension culture

A cucumber cell suspension culture was set up as a means of producing a bulk source of cell wall enzymes for use in their characterization. A 14-day growth cycle was chosen for examination of the suspension culture following a general method used by other workers examining the growth and enzyme activities of other cell suspension cultures (e.g. van Huystee, 1976). The cucumber culture set up here appeared to have more or less ceased growth by day 14 after sub-culturing and therefore the changes in the protein concentration and peroxidase activity were monitored over this time period. The initial high levels of peroxidase activity found in the medium, soluble and IB fractions were not maintained in subsequent growth cycles. Thus the initial peroxidase activity appears possibly to be an artefact of the callus going into a liquid medium.

Kevers et al. (1981) observed that in habituated sugar beet callus, there was a decrease in peroxidase activity. This was not found to be due to an increase in the amount of active peroxidase inhibitors. Whilst the actual reason for the loss of peroxidase activity in the cucumber cell suspension culture is unclear, the initial high levels of peroxidase activity found might be due to a wound type response following the introduction of the callus tissue to the liquid culture medium. The cucumber cell culture was however sub-cultured at 14 day intervals and growth was occurring over that period as indicated by the fresh weight (Fig 6.8). It is therefore surprizing that no peroxidase activity could be detected within any of the fractions examined. Although the sub-culturing cultured cells of both peanut and *Nicotiana tabacum* L. leads to an increase in peroxidase synthesis (Kossatz and van Huystee, 1976, Mader and Walter, 1986) no evidence of this was apparent in the cucumber cultures even with the addition of low concentrations of calcium. The proposed use of the suspension culture as a bulk source of extracellular enzymes was therefore not possible.

CHAPTER SEVEN

CHAPTER SEVEN - General Discussion

This study set out to investigate the relationship between any phytochrome-mediated growth rate changes and changes in the extracellular peroxidase activity. The initial work undertaken set out to further characterize a phytochrome-mediated change in the activity of an anionic apoplastic peroxidase isoform, A4, previously found by Casal et al. (1990). These workers observed that in the first internodes of mustard seedlings given a 20 min end-of-day R or FR light treatment, phytochrome appeared to mediate a 50 % decrease in the extractable activity of A4. Since the end-of-day light treatments led to differences in growth, it was suggested therefore, that this isoform was specifically involved in the mediation of the observed growth regulation. This idea was supported by the finding that the activity of another, more abundant, anionic peroxidase isoform, A3, remained unaltered by the light treatments. The activity of A3 was observed to increase in response to wounding. The results in the Casal et al. study were obtained by assaying peroxidase activity from the apoplastic fluid that could be extracted by vacuum infiltration and gentle centrifugation of internode segments. In this study it has been established that this represents only about 6 % of the total A4 pool present in the upper half of the first internode of light-grown mustard seedlings. Experiments to identify the location of A4 revealed this enzyme to be more or less exclusively extracellular. Clearly, the extraction procedure employed by Casal et al. (1990) is very inefficient in releasing A4. When the major pool of A4, that which could be extracted by homogenization of the tissue, was assayed, no light-mediation of activity in the internodes was apparent irrespective of any phytochrome-regulated growth rate changes occuring. The anomaly between the results found with different fractions of the A4 enzyme raises some interesting questions as to the reasons behind the discrepancy. That the majority of the A4 peroxidase enzyme activity remains unaltered by light treatments known to induce phytochrome-regulated growth rate changes is not in question. Thus the potential significance of a 50 % decrease in the activity of A4 in a centrifugate fration; which represents only a 3 % change in the

total A4 activity needs to be considered. Although this change in activity comprises only a small fraction of the overall pool, it remains plausible that it could be of primary importance in regulating growth. It is possible that this 3 % change in the activity of A4 may represent a critical change in activity that occurs for example within a limited number of critical epidermal cells. These critical cells may inhibit the elasticity of the epidermis so imposing a physical constraint on the extensibility of the cortical tissue. The observed change in A4 peroxidase activity in the centrifugate could be due to either a change in the extractability of a fraction of the enzyme or due to an alteration of the activity of a small fraction of apoplastic enzyme. In relation to growth rate changes, the small decrease in A4 activity was found to precede the second, but not the first, phase of the phytochrome-mediated increase in mustard internode extension (Casal et al., 1990). The delay in changes in A4 peroxidase activity, behind the growth rate changes, may reflect either a secondary mechanism by which growth extension is inhibited or it could constitute a separate mechanism utilized to control the second growth phase. Alternatively, the phytochrome-mediated changes in growth rate may lead to changes in cell wall peroxidase activity.

Changes in cell wall pH, for example by an IAA mediated acidification, can modify the activity of the extracellular peroxidases, leading to alterations in the extensibility of the cell wall and hence growth rate. A low wall pH slows the peroxidase conversion of extensin monomer to multimer. This retardation of the formation of inter- or intramolecular ionic bonds, leads to a more open extensin network, which would favour wall loosening (Ray, 1991, Biggs and Fry, 1987). As peroxidase reaches its pH optimum, it shifts the balance from monomer to network extensin thereby inhibiting wall extensibility and slowing growth (Gasper et al., 1991). The light regulation of IAA, and corresponding changes in cell wall pH, may therefore alter the activity of cell wall peroxidase. This IAA induced acidification of the cell wall can be very rapid, possibly accounting for initial changes in growth rate when no changes in cell wall peroxidase activity are apparent. If changes in pH are the major regulating

factor of modifications in cell wall extensibility, then any delay between the phytochrome-mediation of growth seen in the mustard internodes and alterations in the extracellular peroxidase activity, may be due to changes in peroxidase activity being an indirect manifestation of the regulation of IAA activity.

In 2 day old mustard hypocotyls a clear light regulated increase in peroxidase activity occurs within 1 h after the onset of de-etiolation. The extracellular peroxidase activity in the light treated tissue was two and a half times that of the dark treated tissue. Although this clearly indicated that a change in the peroxidase activity maybe involved in the growth rate changes of mustard hypocotyls, the impracticality of working with mustard hypocotyls and the potential use of cucumber seedlings led to an alternative system being used.

Cucumber seedlings as well as being larger than mustard seedlings, are very responsive to changes in the light environment. Correlations between growth and peroxidase activity had already been the subject of investigation in the B light regulation of cucumber hypocotyl growth (Shinkle and Jones, 1988, Shinkle et al., 1992). These factors, together with the availability of a mutant deficient in a phytochrome B-like phytochrome (López-Juez et al., 1992), made cucumber plants an ideal choice for experimentation.

Seven days after germination in W light, the extension growth of cucumber hypocotyls ceases and the hypocotyl maintains a stationary growth phase. The addition of FR light to the fluorescent W light background, a lowering of the R:FR ratio, leads to a rapid acceleration of growth at any point up until approximately 20 days after germination. The maximum growth potential of the cucumber hypocotyl, when not being inhibited by R light (i.e. when the seedlings are grown in a low R:FR ratio), is seen here to be approximately 130 mm. In comparison typical hypocotyl growth in W light ceases at approximately 20 mm in length. The capacity of the hypocotyl to elongate is also seen to decrease with increasing age of the seedling. Thus

mechanisms must exist that not only reversibly inhibit the growth of the hypocotyl but also that are temporal in irreversibly lowering the growth capacity of the hypocotyl. It may be that these two mechanisms of growth regulation are manifestations of the same phenomenon. For instance, if the peroxidase-catalysed accumulation of cross-links between cell wall components increases with increasing age of the seedlings, a reduced potential for growth will exist. Similarly, any remaining peroxidase activity, the amount possibly decreasing with time, might be reversibly controlled in order to regulate the remaining growth capacity. Cell wall turgor and the metabolic activity of the cell are also important criteria involved within the growth mechanism (see chapter one) and would therefore greatly affect the reversible growth capacity of the cell itself.

The notion that phytochrome mediates changes in peroxidase activity as a means of controlling stem extension appears to be supported by a number of experimental findings. The reversible nature of the photo-regulation of cell wall peroxidase activity in cucumber hypocotyls correlates with a reversible phytochrome-regulation of extension growth. Thus, seedlings growing at a high rate of stem extension, in a low R:FR ratio, have relatively low extracellular peroxidase activity. In response to an increase in the R:FR ratio, this high growth rate ceases and the extracellular peroxidase activity is seen to increase. The changes in extension growth of light-grown cucumber hypocotyls, measurable with a millimetre ruler, and changes in peroxidase activity were both seen to occur within 2 h. Measurement of the peroxidase activity within this 2 h period gave inconsistent results, possibly due to the dynamic nature of the peroxidase enzyme itself. Phytochrome-mediated growth rate changes of cucumber hypocotyls are however known to occur within approximately 16 min after the onset of FR illumination (Smith et al., 1992). Whether changes in peroxidase activity precede these growth rate changes was not therefore able to be determined. Although no pattern could be discerned on the early changes in peroxidase activity, small critical changes may be occurring that were hidden by the bulk activity. A number of factors need to be considered within this idea. There are multiple extracellular cationic

isoperoxidases from cucumber hypocotyls showing the same behaviour, as such any slight differences between these may not have been discernible within the system used. One means by which the peroxidase isoforms may have different activities could be due to different binding properties. Barcelo et al. (1989) suggested that binding may inactivate the enzyme, which in the cell walls of lupin is compartmentalized into an immobile phase of the cell wall. It has been shown however, that the IB cationic enzymes of cucumber hypocotyls examined are all freely secreted into the medium of cultured cells. There therefore appears to be no differential binding properties between any specific isozyme examined. Although the experiments here concentrated on the ionically bound isoforms that could be extracted with 1M salt, the same cationic isoforms, although in lower abundance, were found in the centrifugate extract. If in cucumber hypocotyls the binding properties of this small fraction released by centrifugation were differentially regulated, a mechanism not dissimilar to that discussed in relation to the findings from mustard internodes might be operating. However in the case of cucumber hypocotyls, no differences were observed in the phytochrme-regulation of the extracellular peroxidase isoforms extracted by the centrifugation technique. Thus, the argument appears to remain unfounded.

It may be the case that peroxidase activity is specifically regulated in different regions of the stem. However any modifications in activity within a specific location might have remained unobserved if the overall activity of the peroxidase within the section of hypocotyl tested remained unchanged. Following growth in a low R:FR ratio, in comparison with extracts from the same regions growing in a high R:FR ratio, the peroxidase activity within the epidermis was approximately 9 % lower than in the cortex. Although differences in the observed peroxidase activity are thought to reflect the changes in overall activity, smaller discrete changes within these individual regions might have been masked. As the epidermal region is considered to be the region that imposes the greatest physical constraint on cell extension (Taiz, 1984), slight changes in peroxidase may lead to large physiological alterations in the cell wall and hence

extension growth. This is further highlighted by the possibility that specific changes in peroxidase activity occurring in the growing tip, may be masked by incorporating regions of less active growth within the 1 cm hypocotyl section being examined.

The lack of response to a lowering of the R:FR ratio by phytochrome B-deficient mutants, has led to the suggestion that phytochrome-B is largely responsible for shade avoidance phenomenon. The cucumber phytochrome B-deficient (*lh*) mutant constitutively shows an elongated phenotype that compares with the phenotype of Wt seedlings exhibiting the shade avoidance response. Comparable observations have been made with mutants of other species deficient in phytochrome B such as the *Arabidopsis hy3* and the *Brassica rapa ein* mutants. Thus the shade avoidance response is generally considered to be mediated by phytochrome B. If biochemical changes that occur in the cell wall environment, due to the phytochrome B mediated increase in growth rate, are phenocopied by the phytochrome B deficient mutants, then these phenomena are most likely to be regulated by phytochrome B. The possibility that phytochrome B regulates biochemical changes in the cell wall during the shade avoidance response cannot however be taken unequivocally as the functions of phytochromes C-E remain unknown. A further possibility might be that phytochrome B indirectly regulates the action of these other phytochromes by allowing or enhancing their responsivity. However it is thought most likely to be phytochrome B regulating the shade avoidance response and as such any growth related biochemical changes. This is supported by the finding that the *lh* -mutant phenocopies the reduced level of peroxidase activity of its isogenic Wt grown in a low R:FR ratio. This mutant remains insensitive to changes in the light conditions and the extracellular peroxidase activity also remained unaltered. The lack of change in the peroxidase activity and the deficiency of phytochrome B in the mutant indicates that this is the most likely phytochrome species to be influencing the level of cell wall peroxidase activity in Wt seedlings. To date, extracellular peroxidase activity in cucumber hypocotyls is the only enzyme that has been shown to be regulated specifically by phytochrome B.

The alteration of peroxidase activity that occurs with phytochrome-mediated growth rate changes, may either be regulated by phytochrome as a means of altering the extensibility of the cell, or may change as a secondary mechanism due to the phytochrome-mediated growth rate changes. To test whether alterations in the growth rate of cucumber hypocotyls leads to a change in cell wall peroxidase activity or vice versa, growth and peroxidase activity would have to be uncoupled. To achieve this peroxidase activity can either be inhibited or promoted and any corresponding changes in the growth rate examined.

In a study on B light-induced changes in the growth rate of cucumber hypocotyls, 1 mM ascorbate was used to inhibit peroxidase activity (Shinkle and Jones, 1988). An increase in growth rate was observed when peroxidase activity was inhibited in the absence of alterations in the light environment. The seedlings used were 4 days old and had been grown in dim R light. In experiments carried out here, when fully de-etiolated seedlings were cut or damaged by pen marks, callus tissue rapidly appeared. This callusing would not only interfere with the application of any exogenous inhibitor or enzyme, but is also likely to induce any peroxidase activity involved in the lignification processes of callusing. Whilst any changes in the overall peroxidase activity may therefore occur, these activities would not be specifically attributable to growth rate changes. An alternative method used to study in vivo affects of high levels of peroxidase activity utilized a cDNA clone to a primary isozyme form of peroxidase (Lagrimini et al., 1990). These workers produced transgenic tobacco plants that showed an increase in total peroxidase activity. The transformed plants that overproduced peroxidase by at least two fold, showed chronic wilting at the time of flower bud initiation. Whether this method could provide a practical system by which the cause and effect relationship of growth and cell wall peroxidase activity can be separated in light grown cucumbers has not yet been tested.

The relationship between phytochrome-regulated alterations of light-grown cucumber hypocotyl extension and changes in the extracellular peroxidase activity is therefore

difficult to fully clarify. However it is clear that changes in extracellular peroxidase activity do not appear to occur in relation to light-mediated inhibition of hypocotyl elongation in etiolated cucumber seedlings. In fact, no alteration of extracellular peroxidase activity was detectable until 2 days after dark grown seedlings were placed into R, FR or W light. However, extension growth of the Wt and *lh* cucumber hypocotyl is inhibited by light within this time period. Hypocotyl elongation in Wt seedlings is inhibited by R, FR and W light, whilst *lh* hypocotyls are only responsive to FR and W. Despite these large alterations in growth rate, extracellular peroxidase activity remains unaltered, showing neither a cause or effect relationship. There are a number of possible mechanisms not involving peroxidases by which alterations in the extensibility of the cell wall might be regulated, e.g. changes in the activities of other cell wall enzymes or a reduction in the synthesis of cell wall macromolecules. However the lack of change in peroxidase activity is somewhat surprising. Blue light treatment has also been shown to increase the level of cell wall peroxidase activity (Shinkle and Jones, 1988). However the B light component of W light here, failed to cause a detectable change in the extractable peroxidase activity of the etiolated cucumber seedlings. So again no relationship between peroxidase activity and growth exists.

In etiolated *Arabidopsis* seedlings, the inhibition of hypocotyl elongation by continuous FR and R light is thought to be mediated by phytochrome A and phytochrome B respectively. In etiolated cucumber hypocotyls no change in extracellular peroxidase activity was observed during de-etiolation of the hypocotyl. Phytochrome A or B does not therefore appear to be regulating extracellular peroxidase activity as a mechanism to inhibit stem extension in etiolated cucumber seedlings. In support of this, the *lh*-mutant which is deficient in phytochrome B but shows normal levels of phytochrome A, also failed to exhibit any change in peroxidase activity irrespective of the growth rate changes during de-etiolation. Therefore, although phytochrome B does appear to be regulating peroxidase activity in the control of the

shade avoidance response in light-grown seedlings, during the phytochrome B mediated inhibtion of stem extension in etiolated seedlings a separate mechanism to decrease cell wall extensibility must operate.

The mechanisms which control the rate of extension growth, if not directly then indirectly, are likely to exert a strong influence on the formation of cross-links between cell wall macromolecules, in order to alter the extensibility of the cell. If phytochrome does control extracellular peroxidase activity then it would appear that there is a requirement for a developmental threshold to be reached in the seedlings growth prior to this mechanism coming into operation. As the seedlings de-etiolate the photosynthetic apparatus develops and becomes functional. It would not therefore be unreasonable to postulate that the control of cell wall peroxidase activity, as a means of regulating extension growth, requires a photosynthetic element. Norflurazon has been used to produce photobleached seedlings to separate photosynthesis from photomorphogenic affects (e.g. Gaba et al., 1991). Norflurazon inhibits the synthesis of the carotenoid pigments which protect the plastids from photo-degradation. This therefore disrupts the production of haem within the plastids. For the peroxidase enzyme to be active it also requires a haem moiety. However the testing to see whether active peroxidase was present and then for a regulation by phytochrome was not carried out as the cucumber seedlings treated with NF suffered severe retardation in their normal development. These findings also question the findings of Gaba et al. (1991), that the fluence rate dependent inhibition of cucumber hypocotyl elongation has a photosynthetic requirement, which is different to that of *Sinapis* and *Chenopodium* . It is uncertain whether these apparent differences in the phytochrome-regulation of growth are in fact due to the cucumber seedlings loss of normal growth due to treatment with NF. In the experiments carried out by Gaba et al. (1991) the NF treated seedlings being examined were only 5-6 days old and it is not apparent until day 8-9 that the bleached seedlings are in fact unable to survive due to the effects of the treatment. The findings of Gaba et al. (1991) and any other growth

related experiments on NF treated cucumber seedlings should therefore be treated with caution. To investigate the relationship between phytochrome-regulation of growth, extracellular peroxidase activity and any photosynthetic requirement, it would be judicious to examine an alternative species whose development is not thought to be affected by NF treatment. A possible candidate for this experiment would be mustard seedlings as they have been shown here to exibit phytochrome-regulation of cell wall peroxidase activity and their development is not thought to be affected by NF treatment (Jabben and Holmes, 1983).

A second species appears to differentially regulate cell wall peroxidase activity in the control of stem extension. In pea seedlings, differences in growth rate of light-grown plants inversely correlate with changes in peroxidase activity (Jupe and Scott, 1989), the ionically bound isoforms rapidly increasing in activity during the later stages of cell expansion. However in etiolated pea cell walls the ionically bound proteins are not involved in alterations in the extension growth (Melan and Cosgrove, 1988). Thus changes in the activity of cell wall peroxidase are only being regulated to alter the rate of stem extension in light-grown plant tissue. This again suggests that two mechanisms are operating by which the rate of stem extension is controlled.

Although the peroxidase-catalysation of cell wall cross links may lead to an inhibition of extension growth, there are a number of points that have to be considered when hypothesising about any mechanisms of control. This is bourne out further when it appears that two separate mechanisms are clearly operational in the inhibition of stem extension, in etiolated and light-grown cucumber tissue. Different light conditions lead to alterations in the growth rate, operated via different phytochrome species and a B light receptor. These changes have in some instances been found to correlate with changes in either a specific or group of peroxidase isoforms. As an alteration of peroxidase activity does not accompany all changes in the growth rate, a mechanism of tight control must be being exerted over its activity and an alternative mechanism utilized by which stem extension is inhibited.

Any changes in extracellular peroxidase activity, that are able to initiate major changes on the extensibility of the cell wall, would require tight regulation in order that the developmental and adaptational morphology of the plant can be controlled. Although here the phytochrome-mediation of growth rate changes was utilized to study related changes in extracellular peroxidase activity, corresponding changes that occur in peroxidase activity are also induced by a number of different factors that are able to alter the growth rate e.g. IAA and GA (Fry, 1986). Therefore changes in peroxidase activity appears to constitute a general mechanism that can be utilized under certain conditions in the alteration of extension growth. Whether this relationship is cause or effect remains unclear. It is suggested that the interaction between the many factors governing growth rate form a complex interaction of events that in turn regulate peroxidase activity.

If the changes in growth rate lead to changes in the peroxidase activity, as opposed to changes in peroxidase activity leading to changes in growth rate, than this phenomenon may still form a general mechanism in the alteration of growth. This may form part of a secondary mechanism in which initial or rapid changes in growth catalysed by other mechanisms e.g. XET, diamine oxidase, pectin methyl esterase, ascorbate acid oxidase or chamges in cell wall pH (see above), is then followed by the peroxidase catalized cross-linking of cell wall macromolecules further inhibiting extension growth. Here it has been established that growth rate changes can correlate with changes in extracellular peroxidase activity but not always. Whilst in light-grown cucumber plants and pea seedlings, peroxidase activity is seen to increase in response to a decrease in etiolation growth, the activity is not altered in response to an inhibition of growth in etiolated seedlings of these species. Thus the mechanism of increased peroxidase activity is not operational in neither a cause nor effect capacity in etiolated tissue. In this study alterations in the extracellular peroxidase activity have not been found to occur without a concomitant change in growth rate in either mustard or cucumber seedlings.

The main points that have been established here are that;

1) The anionic apoplastic peroxidase isoform, A4, from the first internode of mustard seedlings, is more or less exclusively extracellular.

2) Phytochrome regulates a small change (equivalent to 3 % of total activity) in extractable A4 peroxidase activity extractable from the upper half of the first internode of mustard seedlings by centrifugation.

3) The infiltration/centrifugation technique is an inefficient means of extracting apoplastic fluid from certain tissue and should therefore be used with caution.

4) The major pool of A4, that which could be extracted by homogenization of the upper half of the first internode of mustard seedlings, is not regulated by phytochrome.

5) Anionic peroxidase activity from mustard hypocotyls is clearly light-regulated within 1 h after the onset of de-etiolation.

6) The ionically bound cationic peroxidase isoforms from the top 1 cm section of 14-day old light-grown cucumber hypocotyls, decreases in activity when growth rate increases following a lowering of the R:FR ratio. However the phytochrome-mediated increase in growth rate occurs approximately 16 min after the addition of FR illumination. Correlations with peroxidase activity could not be accurately measured within this time period and therefore the cause and effect relationship remains unresolved.

7) Phytochrome-regulated growth rate changes in etiolated cucumber seedlings do not correlate with any detectable change in the ionically bound peroxidase activity. Changes in peroxidase activity only occur 2 days after the onset of de-etiolation. Therefore not all growth rate changes utilize changes in peroxidase activity as a means of altering the rate of extension growth.

8) The cucumber *lh*-mutant has a constitutively low level of cell wall peroxidase activity irrespective of the R:FR ratio. Therefore, in light-grown cucumber hypocotyls, phytochrome B is thought to regulate the ionically bound, cationic peroxidase activity.

From these findings, changes in peroxidase activity are not seen to be a prerequisite in the phytochrome-mediation of growth rate changes. In specific situations where the growth rate is altered, changes in peroxidase activity are also observed. It is therefore suggested that peroxidase activity is important in the control of specific stages in a plants development, whilst at other times alternative mechanisms to alter cell extensibility are used. Whether changes in peroxidase activity are infact due to alterations in the cell wall, controlled via other mechanisms, remains difficult to define. Until cause and effect phenomena are able to be separated, or accurate measurements are able to be made of the rate of change of peroxidase activity, it is difficult to ascribe the relative importance of the role played by extracellular peroxidase activity in the control of growth rate changes.

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