The Use of Gibberellin Mutants to Explore the Role of Microtubules in Stem Elongation

Anne Elizabeth Kitchener B.Sc. (Hons.) (Tas)

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Declaration of originality

The work presented in this thesis is, to the best of my knowledge and belief, original except where acknowledged in the text. I hereby declare that I have not submitted this material, either in whole or on part, for a degree at this or any other academic institution.

Anne E. Kitchener

AEK

ABSTRACT

Six single gene mutants of garden pea (*Pisum sativum* L.) were utilised to further examine the control of internode elongation. These short mutants could be divided into two types; GA-synthesis (*ls*, *le*), and GA-response (*lk, lka, lkb, lw*) types. They were compared with the parental wild-type cv. Torsdag in an analysis of the changes in orientation of cortical microtubules in expanding dark-grown internode tissue with distance from the apical hook. Immunofluorescence analysis of FITC-labelled cortical microtubules in tangential sections of epidermal cells and longitudinal sections of subepidermal cortical cells of the mutants and wild-type plants revealed a shift away from the wild-type microtubule arrangement toward less transverse microtubule arrays in the mutants. This lower average microtubule orientation (resulting from a reduced proportion of cells with predominantly transverse microtubule arrangement) correlated with decreases in the rate and distribution of extension growth along internode 3 of dark-grown mutants, providing additional circumstantial support for the role of transverse microtubule orientation in extension growth.

Analysis of microtubule arrangement and growth profiles of the GA-synthesis mutants, *le* and *ls*, revealed overall reductions in average microtubule orientation and rate and distribution of growth in comparison to wild-type plants. These changes correspond to the mutants' reduced endogenous GA_1 content and implicate GA_1 in the microtubule-mediated regulation of both rate and distribution of extension growth along the epicotyl. Exogenous application of GA_1 to GA-synthesis mutants resulted in a shift in average microtubule orientation toward the wild-type arrangement, and a corresponding increase in internode length, substantiating a role for GA_1 in microtubule-mediated extension growth.

Average microtubule orientation in *le* and *ls* was never as transverse as in the wild-type, and transverseness of the array decreased more rapidly than in the wild-type over the same length of epicotyl, implicating GA_1 in both the initial organization of microtubules and the maintenance of stability of transverse arrays.

Exogenous application of GA_1 to the GA-response mutants, *lk*, *lka* and *lkb*, did not result in any increase in average microtubule orientation or internode length, indicating that these mutants operate to reduce normal sensitivity to GA_1 at some point prior to microtubule arrangement. The reduced levels of IAA reported in *lkb*, and possibly *lk* and *lka* plants (McKay *et al.* 1994) may indicate that the pathways of auxin- and gibberellin-mediated extension may intersect prior to any effect on microtubule arrangement.

Application of colchicine to wild-type internodes resulted in a reduction in stem length and increase in stem diameter, relative to controls. The shorter, wider stems were a consequence of reduced cell length and increased cell width both in the epidermis and the cortex. Colchicine treatment approximated, but did not produce a phenocopy of the GA-response mutants, *lk*, *lka* and *lkb*, indicating that disruption of microtubule arrangement is not the only effect of these mutant alleles. It is suggested that the altered phenotype and growth characteristics of these mutants may also involve altered wall biophysical parameters.

Colchicine application resulted in a reduction in cell number (and hence cell division) in the cortex, and to a lesser extent in the epidermis of wild-type plants. The similarity of colchicine treatment to the reported effect of *lk* (JJ Ross and JB Reid 1986) and *lka* (JB Reid and JJ Ross 1989) upon cell number, coupled with the extremely reduced transverse microtubule component of *lk* and especially *lka* types, may suggest a more direct effect of these mutations on microtubules.

The *lw* allele resulted in an altered growth profile, which corresponded with a reduction in the component of cells with transversely oriented microtubules in the upper 6 mm of the epicotyl, in comparison to wild-type plants. Since the *lw* allele has been reported to modify phytochrome-mediated responses (Weller and Reid 1993) and an interaction between phytochrome function and the responsiveness of tissues to GA_1 has been suggested (Weller and Reid 1993), the present results may indicate that such an interaction is occurring prior to any influence of microtubule arrangement on extension growth. *lw* plants showed a significant increase in average microtubule orientation in response to applied GA_1, although average microtubule orientation did not reach wild-type levels and the response was considerably weaker than in the GA-synthesis mutants, consistent with the suggestion that *lw* reduces responsiveness to GA_1. Phenotypic dissimilarity to GA-deficient types may indicate that *lw* operates indirectly to reduce sensitivity to GA_1 at some point after reception of the GA_1 signal.

The *lv-1* allele was demonstrated to result in altered chloroplast ultrastructure. Transmission electron microscopy revealed a reduction in the number and organization of grana, and reduced thylakoid stacking in *lv-1* plants, consistent with suggestions that the ability to de-etiolate normally in response to white light is impaired in these mutants. Immunoflourescence analysis of microtubule arrangement revealed that *lv-1* plants grown under high-intensity white light had a higher average microtubule orientation than light-grown *Lv* plants, indicating that the greater internode lengths of light-grown *lv-1* plants may be mediated by the effect of the *lv-1* allele on microtubule arrangement. Since the reduced inhibition of elongation by white light may be due to a loss of normal biological activity of phyB conferred by the *lv-1* allele (Weller *et al.* 1995), it is suggested that the normal effect of phyB in elongation growth may be mediated by microtubules. Although average microtubule orientation was comparable in light- and dark-grown *lv-1* plants, light-grown *lv-1* types had shorter internodes and exhibited a greater degree of leaf expansion and chlorophyll development in comparison to dark-grown *lv-1* types, indicating that darkness may operate to alter phenotype via other receptors, such as phyA or blue light receptors, not affected by the *lv-1* mutation. The combined data for the *lw* and *lv-1* alleles supports an interaction of the transduction pathways for light and GA_1 prior to microtubule arrangement.

The use of mutants has allowed comment upon generally accepted ideas of microtubule-mediated elongation growth, and has provided a direction for future investigation of this process. A model of the possible sequence of events leading from the GA-biosynthetic pathway to extension growth is proposed, and the likely sites of action of the mutants used in this study are suggested.

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CHAPTER 1: THE CONTROL OF ELONGATION

1.1 The relationship between microfibril orientation and cell expansion

Elucidation of the control of stem elongation requires an understanding of the integration of the various contributing factors. Not least among these factors is the controlled expansion of the cell wall, upon which the final shape of a growing plant is dependent (Giddings and Staehelin 1991).

Cell shape and the cell wall

Cell growth occurs as a result of wall loosening and irreversible expansion driven by turgor pressure (Cosgrove 1993a, b). Although turgor pressure is non-directional, the stress pattern within a cell varies with the shape of the cell (Taiz 1984). The protoplast without a cell wall is unable to develop asymmetry and elongate in a particular direction, but tends toward an isodiametric shape. Taiz (1984) states that the mechanical anisotropy and the prevailing pattern of wall stresses govern the directionality of cell wall expansion. The stress pattern in a cylindrical cell with an isotropic wall is primarily transverse and favours transverse extension (Green 1963, 1980; Lang *et al.* 1982). The fact that such cells elongate can be explained only by some form of transverse reinforcement, without which the wall would extend isotropically (Green 1980). Studies involving the filamentous alga *Nitella* have been extremely useful in demonstrating the relationship between cell shape and the direction of turgor-driven cell extension. *Nitella* approximates a thin-walled cylinder (Taiz 1984), and its highly elongate internodes maintain a length to diameter relative growth rate (RGR) ratio of about 5:1 throughout development (Green 1969). Such a cylinder carries a 2:1 transverse to longitudinal stress ratio on the side walls (Castle 1937), and this unfavourable stress ratio would tend to promote growth in a transverse direction unless the wall was circumferentially reinforced (Taiz 1984). Green (1980) also concluded that the main reinforcing wall must be the side wall.

Cellulose microfibrils

The cell wall is a complex polymeric material (Cosgrove 1993a), in which crystalline cellulose microfibrils are embedded in a matrix of pectins, glucans, structural proteins and other materials (Cassab and Varner 1988; Talbott and Ray 1992a; Carpita and Gibeaut 1993).

In the primary cell wall of dicotyledonous plants xyloglucans are the major hemicellulosic component (Fry 1989b; Hayashi 1989; Hoson 1990; Edelmann and Fry 1992; McCann *et al.* 1992; Talbott and Ray 1992a; Carpita and Gibeaut 1993; Warneck and Seitz 1993), and are thought to perform a structural role in bonding adjacent microfibrils (Hayashi and Maclachlan 1984; Fry 1989a; Passioura and Fry 1992). It is the cellulose microfibrils in cell walls that provide the required reinforcement for cell expansion in higher plants (Green 1980; Hardham 1982). Only a crystalline material such as cellulose is strong enough when wet to withstand the high stress levels within the cell wall (Green 1980).

Microfibril arrangement and cell elongation

The capacity of microfibrils to withstand stresses in the wall appears to increase as their organization increases (see Seagull 1992). Microfibrils organized in parallel arrays exhibit strong hydrogen bonding between adjacent microfibrils, providing resistance to turgor pressure and cell expansion. Random organization reduces hydrogen bonding between adjacent microfibrils and reduces resistance to turgor pressure. Since no single microfibril reaches entirely around a cell, cross bridging between microfibrils by matrix components of the wall may also be necessary for reinforcement of the wall in the direction of the microfibrils (Lang *et al.* 1982).

Reviews on cell wall mechanics (Preston 1974; Wainright *et al.* 1976; Hettiaratchi and O'Callaghan 1978) have suggested that it is the orientation of organized microfibril arrays that determines the direction of greatest strength in the cell wall and therefore limits the direction of cell expansion. Cellulose microfibrils have high tensile strength and increase the rigidity of the matrix by introducing shear interactions, which are greatest when the wall stress is parallel to the microfibril orientation (Green 1969; Wainright *et al.* 1976).

The current concensus and long-standing hypothesis regarding the role of cellulose in directed cell expansion is that the deposition of transverse microfibrils in a cell favours elongation growth rather than increase in diameter (Shibaoka 1974; Green 1980; Hardham *et al.*1980; Hardham 1982; Taiz 1984; Hogetsu 1986a; Ishida and Katsumi 1991, 1992; Seagull 1992). However, agreement is not universal (Roland *et al.* 1982; Neville and Levy 1984). Numerous reports of the coincidence of transverse microfibril orientation and cell elongation have provided circumstantial evidence in favour of this hypothesis.

In *Pisum* root (Hogetsu 1986a; Hogetsu and Oshima 1986) and epicotyl (Iwata and Hogetsu 1989a), *Avena* coleoptile and mesocotyl (Iwata and Hogetsu 1989a), *Vinca* shoot apex (Sakaguchi *et al.* 1988), *Vigna* epicotyl (Takeda and Shibaoka 1981a, b), developing cotton fibres (Seagull 1986), and in maize coleoptile (Bergfeld *et al.* 1988), a correlation was observed between organ elongation and the transverse orientation of microfibrils in the cells of the particular organ. Work with cellulose synthesis inhibitors (e.g. coumarin, Hogetsu *et al.* 1974a) and anti-microtubule drugs (discussed in Section 1.2) has furnished additional support.

A variety of techniques have been employed to investigate the deployment of microfibrils and their relationship to cell elongation. Cellulose microfibril orientation has been assessed using polarized light (Preston 1974; Green 1980), freeze-etching (Preston 1974), ultrathin sections conventionally prepared for electron microscopy (Preston 1974; Sawhney and Srivastava 1975; Hardham *et al.* 1980; Lang *et al.* 1982), and sectioning and metal shadowing (see Preston 1974; Hogetsu and Shibaoka 1978a; Takeda and Shibaoka 1978). In addition, a replica method such as that described by Hogetsu (1986a) has been employed, and immunofluorescence microscopy following fluorescent staining of the cell walls with Calcofluor white (Falconer and Seagull 1985a) or Tinopal LPW (Galway and Hardham 1986) has proved successful. Recently, Sauter *et al.* (1993), using both immunofluorescence and electron microscopy, reported that circumferential (transverse) reinforcement by microfibrils in deepwater rice restricted growth in girth and promoted extension growth.

Microfibril polymerization

"Terminal complexes" localized in the plasma membrane have been visualized by freeze-fracture electron microscopy (see Gunning and Hardham 1982; Lloyd 1984; Giddings and Staehelin 1991). There is general agreement that these "terminal complexes" are responsible for the synthesis of cellulose and the secretion of microfibrils (Lloyd 1991). These "terminal complexes" have been interpreted as synthases (see references in Gunning and Hardham 1982; Lloyd 1984; Preston 1988) and for convenience will be herein referred to as synthases. The synthases fall into two broad categories according to their morphology (see Gunning and Hardham 1982; Brown 1985); those with a linear arrangement of particles, and those with particles forming rosettes. The linear type has been visualized in a number of algae (Brown 1985; Delmer 1987), while the rosette form has been described for a range of plants from algae to angiosperms (see Gunning and Hardham 1982; Brown 1985; Giddings and Staehelin 1991).

Work with *Micrasterias* cells (Giddings *et al.* 1980) has demonstrated a positive correlation between the number of rosettes at the terminus of a microfibril and the width of the microfibril, providing evidence for the involvement of rosettes in cellulose synthesis. Furthermore, the distribution of these putative synthesizing complexes is invariably correlated with the observed pattern of microfibril deposition (see Giddings and Staehelin 1991). Although the biochemistry of plant synthases is still uncertain (Delmer 1987), microfibrils are thought to form via a process of end-synthesis of ß-1,4 glucan polymers into crystalline fibrils (Preston 1974).

The multinet hypothesis

In her review, Hardham (1982) commented that "cellulose microfibrils are deposited at the extracellular surface of the plasma membrane so that the most recently deposited layers of microfibrils occur on the inner side of the cell wall adjacent to the plasma membrane, while the oldest layers lie next to the middle lamella or in the case of the outermost walls, on the surface of the plant". However, the method by which cellulose is deposited is still not fully understood. Roelofsen and Houwink (1953) proposed the multinet growth hypothesis whereby the microfibril network is developed by apposition of predominantly transversely oriented microfibrils on the inner surface of the wall. Subsequent cell expansion results in passive realignment of microfibrils in the direction of maximum strain (Green 1960; Preston 1974; Morikawa *et al.* 1978b), and the expansion and thinning of wall layers as they are displaced toward the outer surface by the continued apposition of new wall material (Taiz 1984).

The multinet hypothesis appears to hold true for various cell types (see reviews by Setterfield and Bayley 1961; Wardrop 1962; Roleofson 1965; Preston 1974; Taiz 1984). There is strong evidence for multinet growth in *Nitella* walls (Roelofsen and Houwink 1953; Green 1958a, 1960; Probine and Preston 1961; Roelofsen 1965; and references in Taiz 1984). In *Nitella*, microfibrils are transverse on the inner surface and longitudinal at the outer surface, in agreement with the predicted gradient in orientation. Apposition has been demonstrated by autoradiography (Green 1958b), by use of the cellulose synthesis inhibitor 2,6-dichlorobenzonitrile (DCB) (Hogetsu *et al.* 1974b) and by observations of sensitivity to pectinase incorporated into cell walls of pea epicotyls and oat coleoptiles (Ray 1967). Layers undergo thinning because of wall extension.

The hypothesis of passive realignment of microfibrils has also received support since Gertel and Green (1977) showed that physically clamping living cells to remove longitudinal strain prevented reorientation of microfibrils in the outer layers, and physically stretching living cells produces enhanced realignment of microfibrils in the outer layers. In contrast, inner microfibrils were not affected by either clamping or stretching, consistent with the generally accepted view that the deposition angle is controlled by microtubules in the cytoplasm (Gunning and Hardham 1982) (see Section 1.2).

Ordered fibril model

The multinet model does not appear to entirely account for a range of other cell types. For example, alternating layers of transverse and longitudinal microfibrils have been observed in cell walls of higher plant epidermis (Chafe and Wardrop 1972; Sawhney and Srivastava 1975; Deshpande 1976b; Takeda and Shibaoka 1978, 1981a), collenchyma (Wardrop 1969; Chafe 1970; Roland *et al.* 1975; Deshpande 1976a; Wardrop *et al.* 1979), and parenchyma (Roelofsen 1965; Chafe and Chauret 1974; Roland *et al.* 1975), and have been considered typical for many cells of higher plants (Chafe and Chauret 1974; Roland *et al.* 1975). To account for these cell types, Roland and colleagues (Roland *et al.* 1975, 1977, 1982; Roland and Vian 1979) proposed the "ordered fibril" model. They envisaged that the angle of microfibril deployment on the inner surface of the cell wall discontinuously changes with time to produce a crossed polylamellate wall architecture, selective loosening of layers of particular orientation enabling directed expansion. The crossed polylamellate architecture would suggest that no passive realignment occurs during elongation, apparently contradicting the multinet model (Roland and Vian 1979). Preston (1982) however, has shown mathematically that the angle of microfibril displacement during elongation of higher plant cells may be too small to detect. The ordered fibril model has been adapted to include passive realignment, bringing it closer to multinet growth (Sargent 1978; Roland *et al.* 1982).

Crossed polylamellate walls

There still remains uncertainty over the process of microfibril deposition in cells with crossed polylamellate walls. Iwata and Hogetsu (1989a), looking at wall microfibrils in *Avena* coleoptiles and mesocotyls and *Pisum* epicotyls reported that the majority of epidermal cells averaged a longitudinal orientation of microfibrils, although a considerable number of cells had transverse microfibrils on the inner surface of the cell wall. They found that it was not possible to deduce from their data the correct mechanism responsible for these observations. Whether the microfibrils in the cell wall were passively realigned by longitudinal expansion (in agreement with the

multinet theory), or whether the orientation of deposition changed periodically to favour longitudinal deposition was unclear.

Takeda and Shibaoka (1981a) reporting on the crossed polylamellate structure of the inner surface of the outer tangential walls of epidermal cells of *Vigna,* found that while transverse microfibrils deposited during earlier stages were reoriented, the transverse microfibrils deposited during later stages were not. They proposed that this difference was brought about passively by the distribution of strain throughout the wall, and concluded that as strain increases toward the outer portion of the wall, initial orientation followed by reorientation of microfibrils can produce the crossed polylamellate wall structure. Lang *et al.* (1982) commented that the orientation of cellulose microfibril deposition in the polylamellate walls of epidermal cells and some inner cells of pea epicotyl continually changes. They advocated that polylamellate walls can have a polarity based solely on the microfibril orientation, with the amount of longitudinal extension relative to transverse extension of cells with polylamellate walls influenced by the relative thicknesses of the longitudial and transverse lamellae. Thus, they explain directed cell expansion without having to propose a "selective loosener" (Roland and Vian 1979) which acts differently on differently oriented wall layers.

Relative importance of inner and outer wall layers

Some of the problems with hypotheses of directed cell expansion in cells with crossed polylamellate walls may be overcome if, as has been suggested by Green (1969, 1980), Richmond *et al.* (1980), Hardham (1982), and Taiz (1984), the inner wall layers are responsible for the control of cell expansion. Roelofsen (1965) originally proposed that the transverse stress is borne primarily by the inner wall layers containing transverse microfibrils, while the longitudinal stress is borne by the outer layers where longitudinal microfibrils are located. However, in a cell growing with a relative growth rate (RGR) of about 1.5-2.0%, the surface area is increased by around 25% with no apparent change in wall thickness, implying that the inner 25% of the wall has been replaced, while the outer 25% has been thinned to zero thickness (Taiz 1984). The thinned outer layers would thus be much less able to provide wall rigidity and it follows that the directionality of expansion would be determined by the properties of the inner 25% of the wall, which must therefore be the region bearing both transverse and longitudinal stress (Taiz 1984). The validity of this suggestion can be demonstrated by treating young *Nitella* cells with the cellulose synthesis inhibitor 2,6-dichlorobenzonitrile (DCB). Richmond (1983 in Taiz 1984) reported that after replacement of the inner portion of the wall with a cellulose-poor wall layer, the cells burst.

The general concensus is therefore that the inner wall layers determine the directionality of cell expansion. However, Taiz (1984) raised the question of how preferential growth in length could be maintained if the inner wall layer of a cell consists of longitudinally oriented microfibrils during part of its growth period. He suggested that interactions between epidermal cells and inner tissue cells may compensate for the lack of structural anisotropy in such cases. A study of microfibril orientation in the inner and outer tissues of pea epicotyl by Lang *et al.* (1982) appears to support this possibility. They reported that epidermal cell walls were polylamellate and deposited approximately half of their cellulose microfibrils longitudinally. Many cortical cells also had polylamellate walls and, although cortical tissue had cellulose oriented primarily transversely, more longitudinal microfibrils appeared to be deposited with age. This suggested that internal tissue would tend to expand more longitudinally than transversely, while the large amount of longitudinal cellulose in the epidermis would tend to limit longitudinal expansion, resulting in the internal tissue being under compression. Lang *et al.* (1982) concluded that such a tissue interaction may be responsible for the widening of the pea epicotyl below the apical hook. The hypothesis that internal tissue is under compression in the intact stem is supported by the fact that each half of a split pea segment curves outward (Went 1934), and by reports of elongating tension and contractile forces, respectively, in the inner tissue and epidermis of several elongating organs (Kutschera *et al.* 1987; Kutschera and Briggs 1988b; Iwata and Hogetsu 1989a; Kutschera 1989). Epidermal and cortical cells may also respond differently to endogenous hormones, increasing the complexity of tissue interaction. The differences between epidermal and internal tissue will be discussed in Chapter 2.

While there is still disagreement over some aspects of cell expansion, primarily related to the problem of directed expansion in cells with crossed polylamellate walls, the general concensus apears to be that "hoop" reinforcement of whole cylindrical organs, predominantly by orientation of cellulose microfibrils on the inner surface of outer tangential walls of epidermal cells, is the basis for directed turgor-driven growth.

1.2 The Cytoskeleton and Elongation

The microtubule/microfibril syndrome

There has been considerable speculation upon the relationship between cortical microtubules and cellulose microfibrils. In fact, ever since Ledbetter and Porter (1963) improved electron microscope visualization of cell ultrastructure by the incorporation of glutaraldehyde into fixation procedures, reports of the parallel alignment of microtubules and microfibrils in elongating cell systems have rapidly accumulated. Typically, the innermost layer of microfibrils parallels the interphase cortical microtubule array in both orientation and organization. For example, in differentiating protophloem sieve elements of *Triticum aestivum* L. root, where wall thickening is laid down in broad ridges which alternate with narrow, unthickened depressions (Eleftheriou 1987), microtubules were clustered directly opposite the depositing thickenings and appeared to parallel the adjacent microfibrils visualized in tangential thin sections. Immunofluorescence microscopy using anti-tubulin antibodies and Calcofluor have revealed a striking coincidence of the distribution of microtubules and the localized cellulose thickenings of the walls of wheat mesophyll cells (Jung and Wernicke 1990).

In general, articles dealing with this phenomenon (e.g. Green 1963; Newcombe 1969; Heath 1974; Palevitz and Hepler 1974; Gunning and Hardham 1982; Heath and Seagull 1982; Robinson and Quader 1982; Lloyd 1984, 1987; Roberts *et al.* 1985; Seagull 1989; Giddings and Staehelin 1991) have supported the hypothesis that cortical microtubules are involved in the orientation of cellulose microfibrils, as first suggested by Ledbetter and Porter (1963). The morphological observations of parallelism are often reinforced by a functional correlation between microtubule and microfibril orientations, such as is provided by the artificial alteration of microtubule stability and its effect upon microfibril deposition.

Microtubule-disrupting and -stabilizing agents

Elongating semicells of the unicellular green alga *Closterium* were shown to have a parallel transverse arrangement of cortical microtubules and cellulose microfibrils along the entire length of the cells. However, cells treated with colchicine lacked microtubules and deposited randomly arranged cellulose microfibrils, resulting in cells of a spherical, rather than the more usual cylindrical shape (Hogetsu and Shibaoka 1978b). Marchant and Hines (1979) demonstrated that treatment of regenerating protoplasts of the filamentous green alga, *Mougeotia*, with either colchicine or isopropyl-N-phenyl carbamate resulted in spherical protoplasts which developed cell walls in which the microfibrils were randomly oriented. Other anti-microtubule drugs

which support a role for microtubules in microfibril orientation and direction of cell expansion include amiprophosmethyl (APM) (*Avena* coleoptiles and mesocotyls and *Pisum* epicotyls, Iwata and Hogetsu 1989a) and isopropyl-phenylcarbamate (IPC) or trifluralin (*Nitella*, Richmond 1983 in Taiz 1984).

Microtubule-disrupting agents have resulted in similar disruption of microfibril orientation in other plant species (*Pinus* cells, Itoh 1976; *Vigna* epicotyls, Takeda and Shibaoka 1981b).

Application of taxol to protoplasts made from elongating pea epicotyls (Melan 1990) was reported to stabilize microtubule orientation, preventing their normal randomization during protoplast formation. Microfibril deposition was found to adopt this altered pattern rather than the pattern of orientation that would normally have formed.

Microtubule organization precedes changes in microfibril deposition

If microtubules are responsible for coordinated cellulose deposition it is expected that the microtubules must be organized in anticipation of any change in the pattern of cellulose deposition. The organization of microtubules prior to detectable changes in the angle of microfibril deposition was demonstrated by the analysis of leaf formation in *Graptopetalum paraguayense* E. Walther using polarized light microscopy (Hardham *et al.* 1980), and similar preceding changes are reported for radish root hairs (Newcombe and Bonnett 1965), and in the polylamellate cell walls of *Oocystis* (Robinson and Quader 1981). In addition, Hardham and Gunning (1979) report a correlation between the numbers of microtubules and the amount of wall deposition in root cells of *Azolla*.

Exceptions to microtubule/microfibril parallelism

The role of cortical microtubules in orienting the deposition of microfibrils has been disputed on the basis of observations of non-parallelism between microtubules and the most recently synthesized microfibrils (reviewed in Gunning and Hardham 1982; Robinson and Quader 1982; Lloyd 1984; Seagull 1989; Giddings and Staehelin 1991). Some reviewers (e.g. Lloyd 1984; Seagull 1989; Giddings and Staehelin 1991) believe that these apparent exceptions are generally attributable to the dynamic nature of the cortical arrays; microtubules were reoriented prior to the deposition of microfibrils in a corresponding new orientation. However, there are other instances where microtubules do not appear to control final microfibril orientation. An investigation of root-hair development (Emons 1982; Pluymaekers 1982; Emons and Wolters-Arts 1983; Traas *et al.* 1985; Emons and Derksen 1986; Emons and van

Maaren 1987) revealed that microtubules were axially aligned, while the cellulose microfibrils formed helical arrays. Traas and Derksen (1989) observed that in dry-cleaved preparations of *Equisetum* microfibrils were deposited at an angle adjacent to microtubules in the same root hair.

Seagull (1989) and Giddings and Staehelin (1991) conclude that the data presented by these authors cannot be simply dismissed as artifact or misinterpretation. They support the idea that, at least in some systems, microtubules may not be involved in the deposition of organized arrays of wall microfibrils, although it has been suggested that the helicoidal organization of cellulose microtubules might occur post-synthetically via self-assembly (see Giddings and Staehelin 1991).

Whilst the disruption of normal wall organization following loss of microtubules by microtubule-disrupting agents (e.g. colchicine) forms the basis of some of the most convincing arguments in support of microtubule involvement in microfibril deposition, the loss of microtubules does not result in the cessation of cellulose synthesis, or even necessarily in a loss of organized deposition. Despite disruption of microtubules by colchicine the alga, *Oocystis solitaria*, continued to deposit layers of parallel microfibrils although the normal shifts in orientation were inhibited, and resumed only after the colchicine was removed from the tissue by washing (Robinson and Quader 1981; Lloyd 1984). Further examples of organized cellulose deposition in the absence of ordered microtubules were found during differentiation of vessel members in *Coleus* (Hepler and Fosket 1971) and in the developing guard cells of *Allium* (Palevitz and Hepler 1976). The subject has been extensively reviewed by Gunning and Hardham (1982), Robinson and Quader (1982), Lloyd (1984), Seagull (1989), and Giddings and Staehelin (1991).

These findings do not, however, rule out a regulatory role for microtubules in microfibril orientation, since deposition of parallel microfibril arrays in the absence of microtubules is possible only if parallelism was initiated prior to microtubule depolymerization (Seagull 1989). This point is effectively illustrated by the colchicine-inhibition of GA-enhanced elongation of azuki bean epicotyl (Shibaoka 1972) and lettuce hypocotyl (Sawhney and Srivastava 1974). The inhibitory effect of colchicine was only apparent when colchicine was supplied prior to or early in the GA treatment, its effectiveness decreasing with delayed application times. The interpretation is that destruction of cortical microtubules by colchicine prevents GA_3-induced realignment of cellulose microfibril deposition but once a transverse microfibril orientation is initiated elongation can continue in the absence of microtubules. Deposition of ordered microfibrils following destruction of microtubules by colchicine, but not before

ordering has been established, is also documented for *Oocystis* (Robinson *et al.* 1976), *Closterium* (Hogetsu and Shibaoka 1978b), lettuce hypocotyl (Srivastava *et al.* 1977), and cells of azuki bean (Takeda and Shibaoka 1981a, b).

A similar situation has also been demonstrated using wound-induced tracheary element development (Brower and Hepler 1976). Thus it appears that although wall microfibrils can continue to be deposited in parallel with pre-existing microfibrils after cortical microtubules have been depolymerized by drugs, the presence of microtubules is required to change the orientation of microfibrils.

In elongating cells with polylamellate walls non-congruence between microtubules and the innermost microfibril layer has often been observed (e.g. Chafe and Wardrop 1970; Heyn 1972; Sawhney and Srivastava 1975), and has been cited as evidence against the involvement of microtubules in wall organization (see Hepler and Palevitz 1974; Robinson and Quader 1982; Seagull 1989; Giddings and Staehelin 1991). However, it is generally agreed that this type of system (reviewed by Roland and Vian 1979), in which microfibril alignment repeatedly changes during wall synthesis, involves dynamic microtubule arrays capable of changing orientation in response to biological stimuli such as endogenous levels of plant hormone (Seagull 1989). The apparent non-congruence between microtubules and microfibrils results from reorientation of microtubules prior to the deposition of microfibrils in a corresponding new orientation (Hardham 1982; Lloyd 1984; Seagull 1989; Giddings and Staehelin 1991). For example, in *Oocystis*, a unicellular green alga possessing a polylamellate cell wall, a shift in microfibril orientation is preceded by a shift in microtubule orientation (Robinson and Quader 1981). Polylamellate walls, in which each successive layer of microfibrils has a different orientation from the preceding one, are found in higher plants in collenchyma cells (Chafe and Wardrop 1970; Wardrop *et al.* 1979), epidermal cells of azuki bean epicotyl (Takeda and Shibaoka 1978), epidermal and cortical cells of pea epicotyls (Lang *et al.* 1982; Iwata and Hogetsu 1989a), and epidermal and cortical cells of lettuce hypocotyl (Sawhney and Srivastava 1975; Srivastava *et al.* 1977).

Reorientation of the microtubule array

If microtubules are responsible for the pattern of cellulose deposition in cells with cross-polylamellate walls, the entire cortical microtubule array must be altered in order to bring about alternating microfibril orientation. The mode by which reorientation of microtubules occurs is therefore of prime importance in gaining an understanding of the relationship. In *Oocystis* the regulation of shifts in microtubule pattern was

proposed to be by depolymerisation and subsequent repolymerization (Robinson and Quader 1980).

However, since the introduction of immunofluorescence techniques a range of microtubule orientations, from transverse to longitudinal within a single cell, have been reported (Lloyd 1983; Lloyd *et al.* 1985; Roberts *et al.* 1985; Hogetsu and Oshima 1986; Seagull 1986; Iwata and Hogetsu 1988, 1989b), in apparent conflict with suggestions of de- and repolymerization. During ethylene-induced reorientation of interphase cortical arrays in epidermal and cortical cells of pea epicotyl and mung bean hypocotyl, Roberts *et al.* (1985), found little or no evidence of gross microtubule turnover and suggested a wholesale reorientation of the cytoskeleton to account for the various intermediate microtubule orientations observed. The cortical arrays described by Roberts *et al.* (1985) are in keeping with the "helical" model of microtubule reorientation. Recent advances in cytoskeletal dynamics have been achieved by *in vivo* injection of fluorescent probes into plant cells. Microinjection of carboxyfluorescein-labelled brain tubulin into living cells, such as the stamen hair cells of *Tradescantia*, has allowed the visualization of microtubules, by confocal laser scanning microscopy, through various phases of the cell cycle (Zhang *et al.* 1990; Cleary *et al.* 1992; Cleary 1993; Hepler *et al.* 1993; Wasteneys *et al.* 1993; Zhang *et al.* 1993). It is hoped that these advances may eventually elucidate the dynamics of the interphase cortical microtubule array.

"Helical" and "hoop" models of microtubule reorientation

The introduction of immunofluorescence microscopy facilitated observations that suggested the interphase microtubules form helical arrays of variable pitch (Lloyd 1983, 1984). This model contrasts with the previously proposed "hoop" model suggested by electron microscopy (e.g. Hardham and Gunning 1978; Gunning and Hardham 1982), where relatively short, overlapping membrane-associated microtubules form hoops around the cell. Discussed initially by Lloyd (1984), and later extended (Traas *et al.* 1984, 1985; Lloyd and Seagull 1985) the dynamic helical model proposes that long elements, composed of shorter overlapping and interconnected microtubules which are able to slide relative to neighbours and to the plasma membrane, form helices of variable pitch. Such a helical conformation could be "unwound" from a flat-pitched (transverse) orientation to produce a steeply-pitched (oblique to longitudinal) array, thus accounting for the various orientations in which cortical microtubules have been observed. This model removed the requirement for large scale depolymerization followed by repolymerization of cortical arrays, although it was considered that some de- and repolymerization might be required to compensate for changes in length or width of the helix during winding or unwinding.

Subsequent research has provided considerable support for the dynamic helical model, with helical arrays reported in root hairs of *Allium* and *Urtica* (Traas *et al.* 1985), cortical cells of *Raphanus* (Traas *et al.* 1984), and in cotton fibres (Goosen-De Rue *et al.* 1984; Seagull 1986). Bergfeld *et al.* (1988) and Nick *et al.* (1990) reported reorientations of the entire microtubule array similar to the ethylene-induced switches in microtubule orientation observed by Roberts *et al.* (1985). They observed that in cells of maize coleoptiles, a reorientation of cortical microtubule arrays from a longitudinal to a transverse arrangement occurred in response to applied auxin. Ishida and Katsumi (1991) also reported an increase in transversely oriented microtubule arrays in the mesocotyl of the *d*5 mutant of *Zea mays* after GA application, and considered that an oblique microtubule orientation is a transition state between transverse and longitudinal arrays as elongation rate declines in normal plants. Lloyd and Seagull (1985) commented that such re-orientations may relate to endogenous changes in growth regulators able to influence the orientation of the cortical microtubule arrays.

Although the aforementioned studies provide strong evidence for the dynamic helical model, the mechanistic basis of the reorientation of whole cortical microtubule arrays is still unresolved, and some workers have suggested that their observations do not necessarily support the model. Wasteneys and Williamson (1987) in a study of developing internodal cells of *Nitella*, contend that at no time were microtubules observed in a helical conformation, as reported by Lloyd and Seagull (1985), preferring the transverse template proposed by Green and King (1966) for *Nitella*. Hogetsu (1987), reported from immunofluorescence observations of cortical microtubules in *Spirogyra*, that the microtubules occurred randomly at many sites over the cell. Hogetsu (1987) expressed some doubts that such a mode of ordering could bring about a helical conformation of wall microtubules. Lloyd (1987) cautioned that the dynamic helical model does not account for the way in which wall lamellae of one helical sign are sandwiched between lamellae of the opposite helical sign. Williamson (1990, 1991) directed some of the strongest criticism against the dynamic helical model, finding difficulties with its requirement for self-organization, amongst other aspects. Williamson (1991) contended that evidence for wholesale reorientation of arrays is ambiguous and suggests that a progressive and rapid replacement of transverse microtubules with microtubules newly assembled in the post-ethylene orientation (described by Roberts *et al.* 1985), should not be excluded.

Williamson (1991) suggested that microtubules could be organized by directional information, such information perhaps arising from gradients of chemical concentration (Nagorka 1989), from electrical gradients (Hush *et al.* 1990) or from mechanical forces in the cell walls (Green 1988; Hush *et al.* 1990; Williamson 1990). Preston (1988) has also discussed the orientation of cellulose by mechanical or electrical forces.

Although an approximately helical arrangement of microtubules is the generally favoured model, the number of concerns raised indicate that a true concensus is yet to be reached. A resolution of the subject has been hampered by difficulties in elucidating the mode(s) by which arrays of cortical microtubules are reinstated (post-cytokinesis), reassembled (after drug-induced repolymerization), or reoriented (during normal interphase cell expansion), and by gaps in our knowledge of microtubule nucleating sites and organizing centres. The following is a brief synopsis of the current knowledge of microtubule nucleation sites and organizing centres for interphase cortical microtubule arrays.

Microtubule nucleation and organization

Net microtubule assembly occurs during post-cytokinetic reinstatement of the interphase array, during reassembly after experimentally induced depolymerization, and through interpolation of additional microtubules during normal interphase cell expansion (Williamson 1991). During interphase, microtubules were observed to radiate from discrete sites at the edges of *Azolla* root cells (Gunning *et al.* 1978; Gunning 1980). However, Gunning *et al.* (1978) discounted a universal role for cell edges in microtubule assembly on the basis that many cells lack defined edges. They considered that edge sites might instead control abundance and orientation of microtubules on the different walls of the cell, with further refinement, perhaps by cross-bridges, leading to mature arrays. Seagull (1989) suggested that non-detection of such sites in other investigations does not negate their possible universality in plant cells, interspecific differences and technical difficulties contributing to the observed variability.

Immunofluorescence has implicated the perinuclear region in the postcytokinetic reinstatement of the interphase microtubule array (reviewed by Hepler and Wolniak 1984; Seagull 1989; Baskin and Cande 1990). Microtubules have been seen to radiate out from the nuclear surface (de Mey *et al.* 1982; Dickinson and Sheldon 1984; Clayton *et al.* 1985; Lloyd *et al.* 1985; Sheldon and Dickinson 1986; Cleary and Hardham 1988; Falconer *et al.* 1988).

Vantard *et al.* (1990) used *in vitro* microinjection of labeled *Paramecium* axonemal tubulin into lysed endosperm cells of the higher plant *Haemanthus*, to demonstrate a dense population of short, labelled microtubules around the nuclear envelope. The clustering around the nucleus of an auto-antibody to pericentriolar material has been taken as support for this suggestion (Seagull 1983; Clayton *et al.* 1985), and recently Chevrier *et al.* (1992) used monoclonal antibodies raised against mammalian centrosomes to identify a protein component which was permanently associated with pericentriolar material in a variety of cell types. Despite criticisms of the structural and immunological evidence for a perinuclear-related reinstatement of the interphase cortical array, perinuclear involvement cannot be discounted (see Williamson 1991).

A range of treatments, including microtubule-disrupting drugs and low temperature, have been utilized to investigate the reassembly of the interphase array (Hogetsu 1986b, 1987; Falconer and Seagull 1987; Cleary and Hardham 1988; Falconer *et al.* 1988; Galway and Hardham 1989; Wasteneys *et al.* 1993). Upon recovery from depolymerization cortical microtubules are reported to reassemble singly or in small groups at sites dispersed throughout the cortex of algae and flowering plants (Hogetsu 1986b, 1987; Falconer and Seagull 1987; Akashi *et al.* 1988; Cleary and Hardham 1988; Falconer *et al.* 1988; Galway and Hardham 1989; Wasteneys and Williamson 1989). Wasteneys and Williamson (1989) suggested that branching of the first formed microtubules, such as those seen during microtubule reassembly in *Nitella*, might be an important means of microtubule reassembly after depolymerization in plant cells. *In vivo* injection of fluorescently labelled brain tubulin into cells of *Tradescantia* and *Nitella* (Wasteneys *et al.* 1993) indicated that, following depolymerization by oryzalin, microtubules became re-established in transverse arrays similar to the pre-depolymerization arrays. Wasteneys *et al.* (1993) also reported an apparent bi-directional microtubule elongation during reassembly. In most instances, microtubules reassembling after depolymerization were initially poorly organized, and cells lacked microtubules radiating from the perinuclear region (see Cleary and Hardham 1988; Seagull 1989; Williamson 1991).

Work with *Azolla* (Hardham and Gunning 1979) and a range of flowering plants (Seagull 1983) demonstrated a continual interpolation of microtubules amongst the cortical array during normal interphase cell expansion. Hardham and Gunning (1979) proposed that the relatively reduced number of foci along *Azolla* root cell edges (Gunning *et al.* 1978) were sufficient to sustain microtubule assembly during cell expansion, and Seagull (1986) suggested that perinuclear sites would be spatially disadvantaged in highly elongated cells.

As yet, knowledge of reinstatement and reassembly processes is incomplete, and it remains possible that microtubule assembly is coordinated by a widespread capacity of the cortical cytoplasm, which would be difficult to detect with techniques presently available.

There have been many proposals put forward to explain the mechanism of re-orientation of cortical microtubule arrays. However, several authors (Lloyd and Seagull 1985; Traas 1990; Akashi and Shibaoka 1991; Giddings and Staehelin 1991) have suggested that the cross-bridges between microtubules and between microtubules and the plasma membrane may be of importance. Actin filaments have also been implicated (Kobayashi *et al.* 1988).

Intermicrotubule and microtubule/plasma membrane cross-bridges

Organization of microtubules into parallel arrays has been proposed to occur via cross-bridging with other microtubules, as has been reported in microtubule bundles underlying the secondary thickening of xylem elements (Falconer and Seagull 1985a, b; Roberts *et al.* 1985). Since microtubule organization appears to take place only after the microtubules nucleated in the cortex have contacted the plasma membrane (see Lloyd 1987), it has been suggested that organization of microtubules into arrays depends upon interactions between microtubules and between microtubules and the plasma membrane (Lloyd 1987; Galway and Hardham 1989). Intermicrotubule cross-bridges have been seen infrequently in conventionally fixed, thin sectioned cells (Hardham and Gunning 1978), possibly as an artefact of fixation, but have been more frequently observed in cells prepared by freeze-substitution (Tiwari *et al.* 1984; Lancelle *et al.* 1986). However, the importance of intermicrotubule cross-bridges is somewhat undermined by a study of microtubules in *Raphanus* root hairs in which Seagull and Heath (1980b) estimated that only approximately 20% of the microtubules lay close enough to another microtubule to allow bridging. Bridges between microtubules and the plasma membrane were reported as early as 1964 in *Juniperus* (Ledbetter and Porter 1964), and like intermicrotubule cross-bridges, may have been difficult to recognize, perhaps explaining the extremely low (3%) proportion of bridges observed in microtubules proximal to the plasma membrane (Seagull and Heath 1980b). Indeed, the attachment of cortical microtubules to plasma membrane isolated from burst protoplasts suggests bridging of microtubules to the plasma membrane during microtubule repolymerization, and has been demonstrated in a variety of plants (Hardham and Gunning 1978; Marchant 1978; Marchant and Hines 1979; Doohan and Palevitz 1980; Lloyd *et al.* 1980b; van der Valk *et al.* 1980; Hahne and Hoffman 1985; Kakimoto and Shibaoka 1986; Galway and Hardham 1989).

Microtubule-associated proteins

These cross-bridges visualized between adjacent microtubules and between microtubules and the plasma membrane are yet to be identified biochemically, although recent research has revealed the existence of putative plant microtubule-associated proteins (MAPs). Cyr *et al.* (1986) reported the association with plant microtubules of a protein derived from plasma membrane preparations, and Cyr and Palevitz (1989) identified from carrot suspension cells, proteins able to bind to microtubules with a periodicity suggestive of specific binding sites. Lancelle *et al.* (1986) have reported extensive and regularly spaced cross-bridges on microtubules.

Akashi and Shibaoka (1991) reported that application of proteases, trypsin and chymotrypsin, resulted in dissociation of cortical microtubules from the plasma membrane in tobacco BY-2 cells. However, non-disruption of microtubule arrays suggested that the proteases did not enter the protoplast to digest intermicrotubule or microtubule-plasma membrane cross-bridges, but rather digested the extracellular portion of an hypothesized transmembrane protein(s). These observations, together with findings that exogenous application of extensin or poly-L-lysine increased the cold-stability of cortical microtubules (Akashi *et al.* 1990), prompted Akashi and Shibaoka (1991) to speculate that cortical microtubules are cross-linked to the cell wall via a cross-bridge-transmembrane protein-extensin system. The possibility that intermicrotubule and microtubule-plasma membrane cross-bridges associate with extracellular molecules was previously suggested by Lloyd *et al.* (1980b), and Akashi and Shibaoka (1991) have considered the likelihood that intermicrotubule and microtubule-plasma membrane cross-bridges are identical to transmembrane protein(s).

Intermicrotubule and microtubule-plasma membrane cross-bridges have been proposed to affect the stability of the interphase array (Hepler and Palevitz 1974; Bajer and Mole-Bajer 1986b; Falconer and Seagull 1987; Lloyd 1987; Cleary and Hardham 1988), and more recently Akashi and Shibaoka (1991) have implicated transmembrane protein(s) in the stability of microtubule arrays. However, Galway and Hardham (1989) revealed in *Mougeotia* protoplasts an equal susceptibility to oryzalin of both ordered (presumably with relatively abundant cross-bridges) and unordered arrays of cortical microtubules. Plant hormones have been reported to variously affect the cold-stability of cortical microtubules (e.g. Mita and Shibaoka 1984b; Akashi and Shibaoka 1987; Sakiyama and Shibaoka 1990), possibly by influencing microtubule arrangement via the cross-bridge-transmembrane protein-extensin system (Akashi and Shibaoka 1991).

It seems likely that cross-bridges and/or transmembrane systems affect the arrangement and stability of interphase cortical arrays, and while many studies implicate plasma membrane bound components in the organization of arrays (e.g. Galway and Hardham 1989; Akashi and Shibaoka 1991), Bajer and Mole-Bajer (1986a) have reported self-ordering of microtubules in anucleate protoplasts of *Haemanthus* endosperm. It has also been suggested that microtubules in the cell cortex might be stabilized by bridging to other cytoskeletal elements.

F-actin and intermediate filaments

Microfilaments have been reported in association with and often parallel to cortical microtubules (Doohan and Palevitz 1980; Hardham *et al.* 1980; Seagull and Heath 1980a; Heath and Seagull 1982; Mueller and Brown 1982b; Tiwari *et al.* 1984; Traas 1984; Brown 1985; Traas *et al.* 1985; Lancelle *et al.* 1986; Seagull *et al.* 1987; Traas *et al.* 1987; Kobayashi *et al.* 1988). Lancelle *et al.* (1986) reported cross-bridging between microfilaments and adjacent microtubules, and microfilament arrays in parallel with microtubules have been shown to associate with the plasma membrane in a number of higher plant cells (Kakimoto and Shibaoka 1987b; Traas *et al.* 1987). The use of fluorescent probes, which specifically label cytoskeletal proteins (Lloyd 1987), has allowed the identification and localization of F-actin-like microfilaments. In particular, phallotoxins have demonstrated an extensive network of cables in a range of cell types (Wulf *et al.* 1979; Barak *et al.* 1980; Nothnagel *et al.* 1981; Parthasarathy *et al.* 1985; Derksen *et al.* 1986b; Kakimoto and Shibaoka 1987a; Kobayashi *et al.* 1987; Palevitz 1987; Seagull *et al.* 1987; Traas *et al.* 1987). Traas *et al.* (1987), reported extremely fine phalloidin stained elements in the cortex of carrot cells in patterns reminiscent of microtubule helices. Anti-actin antibodies, although not as successful as phallotoxins, have revealed subcortical bundles of microfilaments in isolated root-tip cells of wheat (McCurdy *et al.* 1988), and have demonstrated the co-occurrence of microfilaments and microtubules in differentiating *Zinnia* mesophyll (Kobayashi *et al.* 1988). The above findings strongly suggest that the cortical microfilaments are F-actin. It is therefore possible that the microfilaments previously observed by electron microscope techniques were also F-actin (Lloyd 1987), although no chemical evidence exists as proof and it is possible that the microfilaments were related to the intermediate filaments of animal cells. Using a monoclonal antibody to intermediate filament antigen, Dawson *et al.* (1985) demonstrated co-localization of an antigen related to intermediate filaments with the cortical microtubule array in higher plant cells.

Although more recent evidence (Kobayashi *et al.* 1988) argues for the involvement of F-actin rather than intermediate filaments in reorienting microtubule networks, the identity of the microfilaments visualized in co-incidence with microtubules by various techniques remains uncertain.

The elucidation of possible function(s) of microfilaments in plant cells has been partially achieved with the use of microtubule- and microfilament-disrupting agents. Anti-microtubule drugs were demonstrated to cause severe disruption of F-actin-like microfilament arrays, while disruption of F-actin-like microfilaments by cytochalasin B inhibited the arrangement of cortical microtubules in differentiating *Zinnia* mesophyll (Traas *et al.* 1987; Kobayashi *et al.* 1988). It seems possible that microfilaments, particularly F-actin-like microfilaments, could play an important role in reorienting the interphase cortical microtubule array, and thus indirectly affect cellulose microfibril deposition (Kobayashi *et al.* 1988; Traas 1990).

A role for either or both cross-bridges (intermicrotubule and microtubule-plasma membrane) and microfilaments in the organization of microtubule arrays appears to be supported by Hogetsu's proposal (1987) that a continuum of microtubule-orienting factors extends over the entire cell cortex, interacting with microtubules to determine their orientation. Upon recovery from amiprophosmethyl-induced disruption, Hogetsu (1987) observed in *Spirogyra* cells that ordering of microtubules occurred gradually over the cell without any obvious ordering centres. Hogetsu (1987) implicated fibrous skeletal components, such as microfilaments, in the mechanism of microtubule orientation. Giddings and Staehelin (1991) agreed that F-actin could play a significant role in reorienting microtubule arrays. The microtubule-orienting factors appeared to have greater resistance to sedimentation by centrifugation in the presence of microtubules and seemed likely to be in the vicinity of, and probably interacting with, the microtubules (Hogetsu 1987). In the light of the aforementioned observations of co-distribution of microtubules and microfilaments and drug-induced disruptions, Hogetsu's findings (1987) strongly support a role for microfilaments in microtubule orientation.

As discussed earlier (pp. 2), the direction of cell expansion is thought to be limited by the orientation of cellulose microfibrils in the cell wall. Most of the available evidence to date is consistent with the hypothesis that cortical microtubules (and possibly microfilaments) are involved in the organized deposition of wall microfibril. However, the exact mechanism by which the cytoskeletal elements orient microfibrils is unresolved.

Microtubule-directed cellulose deposition

As previously discussed (pp. 3), cellulose is thought to be synthesized by enzyme complexes located in the plasma membrane. Visualization of these putative synthases has facilitated progress in the understanding of microtubule-regulated microfibril deposition.

It has been hypothesized (Hepler and Palevitz 1974) that the direction of deposition of cellulose microfibrils is controlled by the interaction of the membrane-bound cellulose synthesizing complexes with cortical microtubules. Quader (1986) provided direct support for this hypothesis in a study of cell wall formation in *Oocystis*. A range of microtubule and microfibril disrupting agents resulted in randomization of synthases in the plasma membrane, which in turn resulted in random deposition of microfibrils. This observation suggests that microtubules exert their effect upon microfibril organization by influencing the alignment of terminal complexes in the plasma membrane.

The original hypothesis of cortical microtubule-directed cellulose deposition (Heath 1974) proposed that the synthases may be directly attached to microtubules by a dynein-like linkage that propels the complex within the plane of the membrane. Other models have subsequently been proposed (reviewed in Heath and Seagull 1982; Lloyd 1984; Seagull 1989; Giddings and Staehelin 1991). One of these, the "membrane channel" hypothesis proposed by Herth (1980), suggested that forces resulting from crystallization of microfibrils are sufficient to propel the synthases along channels delineated by cortical microtubules in the plane of the plasma membrane.

The two models conflict regarding whether or not mechanical linkage exists between the synthases and the microtubules. A study of *Closterium* by Giddings and Staehelin (1988) revealed a spatial separation of rosette synthases and microtubules which argued against direct mechanical linkage. The "membrane channel" hypothesis implies an interaction of microtubules with the plasma membrane such that synthases cannot cross the lanes created by underlying membrane-associated microtubules as they are propelled forward by the polymerizing and crystallizing cellulose microfibrils. In differentiating xylem, Herth and co-workers (Herth 1985; Schneider and Herth 1986) reported concentrations of rosette synthases in the plasma membrane over areas of thickening secondary wall. Since the synthases were not arranged in straight lines, as would be expected if they were in direct contact with the cortical microtubules, these observations were interpreted as supporting the "membrane channel" hypothesis (Giddings and Staehelin 1991).

In their review, Giddings and Staehelin (1991) concluded that microtubule associated proteins (MAPs), the most likely candidates for microtubules-plasma membrane cross-bridges, can be considered the "missing links" in the microtubule/microfibril parallelism phenomenon. Whether or not MAPs interact directly with the terminal complexes, it remains to be determined if their role in organized cellulose deposition is passive or active. A passive system whereby microtubule-plasma membrane cross-bridges form stationary barriers in the membrane which restrict travel of mobile terminal complexes has been proposed by Herth (1980). Alternatively, bulk flow of the plasma membrane produced by active, dynein-like molecules (Hepler and Fosket 1971; Hepler and Palevitz 1974; Brower and Hepler 1976; Mueller and Brown 1982a; Brown 1985) might orient microfibrils by shearing forces or by sweeping along synthesizing complexes. Robinson and Quader (1981) reported the deposition of cell wall lamellae composed of straight microtubules in *Oocystis* in the presence of microtubule-destabilizing agents, which Giddings and Staehelin (1991) have suggested argues against the need for an active mechanism.

Although still subject to a number of criticisms, Herth's (1980) passive, indirect "membrane channel" model of the control of microfibril orientation appears to have received the greatest amount of support from reviewers (e.g. Seagull 1989; Traas 1990; Giddings and Staehelin 1991). In essence, the model uses the force of microfibril formation to move the synthases along channels of altered membrane fluidity created by interaction of the microtubules with the plasma membrane, thus producing parallel alignment of microfibrils with microtubules, and allowing the maintenance of microfibril orientation once orientation has been established. Inconsistencies with this model, such as observations of non-alignment of microtubules and microfibrils (see p. 9) and the stability of microfibril orientation following colchicine-induced depolymerization of microtubules (see p. 10) are considered to result from the dynamic nature of microtubule arrays.

Thus, there has been general agreement that a causal relationship exists between interphase cortical microtubules and the orientation of cellulose microfibrils, with Herth's (1980) proposal the favoured model. However, the role of MTOCs, cross-bridges, F-actin and intermediate filaments, as well as processes of self assembly require further clarification, as do proposals such as that of Preston's (1988) which criticized models of microtubule-mediated organization of cellulose deposition and favoured the separate organization of microtubules and microfilaments.

If we accept a model such as that envisaged by Giddings and Staehelin (1991) where 'an integrated cytoskeletal network including microtubules, microfilaments, intermediate filaments and possibly other elements, could be assembled by various organizing centres, but then be subject to more global sensing and transducing systems', then we might expect that plant growth substances could set off a chain of events leading to a growth response.

The role of plant hormones

Plant hormones have been shown to regulate the arrangement of cortical microtubules in a range of plant species such as onion (Mita and Shibaoka 1984a, b), mung bean (Roberts *et al.* 1985), azuki bean (Shibaoka 1974), cucumber (Ishida and Katsumi 1992), lettuce (Sawhney and Srivastava 1975), maize (Mita and Katsumi 1986; Ishida and Katsumi 1991), and pea (Steen and Chadwick 1981; Lang *et al.* 1982; Roberts *et al.* 1985; Akashi and Shibaoka 1987; Sakiyama and Shibaoka 1990; Sakiyama-Sogo and Shibaoka 1993) and the effect of plant hormones on microtubules, cell wall organization, and organ growth is well documented (Gunning and Hardham 1982; Taiz 1984; Lloyd 1987; Seagull 1989; Traas 1990; Giddings and Staehelin 1991). The following section outlines the reported effects of the major groups of plant hormone upon various aspects of elongation growth, and primarily upon microtubule orientation.

Auxin:

Some studies have suggested that auxin treatments do not normally lead to a change in polarity unless incubation times are prolonged (Shibaoka 1972, 1974). However, Hall *et al.* (1985) demonstrated that addition of auxin to intact plants produced transient increases in elongation, although not the large and clear increases that are normally associated with the addition of gibberellin A_3 (GA_3) or gibberellin A_1 (GA_1) (Reid 1990). Elongation of coleoptile segments has also been reported to slow after excision (Bergfeld *et al.* 1988; Nick *et al.* 1990). Law and Davies (1990) and Behringer *et al.* (1992) have attributed changes in internode length to changes in indoleacetic acid (IAA) level. McKay *et al.* (1994) report reduced levels of IAA (compared with the wild-type) in the short mutants of the garden pea *lka* and *lkb*. They report that application to expanding tissue of the IAA transport inhibitors 2,3,5-triiodobenzoic acid (TIBA) (to *lka* and *lkb*) and 9-hydroxyfluorene-9-carboxylic acid (HFCA) (to *lkb*) resulted in an increase in IAA levels above the application site, and a corresponding promotion of elongation above the application site.

McKay *et al.* (1994) concluded that the level of IAA in wild-type internodes was necessary for normal elongation, and other studies (Bandurski *et al.* 1990; Ortúno *et al.* 1990) have indicated a quantitative relationship between IAA concentration and growth rate.

It is widely accepted that auxin brings about elongation of isolated stem and coleoptile segments by the promotion of cell wall extensibility (e.g.Taiz 1984; Kutschera 1989; Rayle and Cleland 1992; Cosgrove 1993a, b), although the exact nature of the wall loosening process is still largely unresolved. The subject of acid-growth was recently reassessed by Rayle and Cleland (1992), who contended that auxin-induced extension is initiated by an acidification of the cell wall which prompts the release of wall-lysing enzymes. The topic of acid-growth is controversial and not essential to the present discussion.

Microtubules have also been reported to be unaffected by IAA (Shibaoka 1974) or 2,4-dichlorophenoxyacetic acid (2,4 -D) (Lloyd *et al.* 1980a). However, IAA has been reported to have the ability to control the direction of cortical microtubules in the coleoptiles of *Zea mays* L. (Bergfeld *et al.* 1988; Nick *et al.* 1990), and in *Avena sativa* L. (Iwata and Hogetsu 1989b). Using maize coleoptiles, Bergfeld *et al.* (1988) demonstrated that auxin controlled the orientation of both microtubules and microfibrils, thus supporting a hormone-induced growth response mediated by microtubule reorientation and consequent change in microfibril deposition. Segments of maize coleoptiles from which the apices had been removed stopped elongating, apparently due to the absence of an endogenous source of auxin. Application of exogenous auxin to the segments restored elongation. In the absence of auxin, cortical microtubules and the most recently synthesized wall microfibrils were more longitudinally oriented than in intact, growing coleoptiles.

Nick *et al.* (1990) suggested that microtubule reorientation in maize and sunflower is a specific auxin-mediated response. Time course observations of flourescein antibody-labelled cortical microtubules in the outer epidermis of growing maize (Nick *et al.* 1990) revealed auxin-related movement of microtubules from a longitudinal to a transverse direction. This reorientation started after less than 15 min and was complete after 60 min. This rapidity of response seemed to suggest a relatively direct effect of auxin upon microtubule reorientation. However, the effects of colchicine on auxin-stimulated growth varied with the experimental system under consideration (Hardham 1982).

Ethylene:

Ethylene treatment of tissue segments results in the cessation of elongation and the promotion of lateral expansion which is mediated by a change in orientation of cellulose microfibrils from predominantly transverse to predominantly longitudinal (see Roberts *et al.* 1985). Following ethylene treatment, microfibril deposition in polylamellate walls of epidermal and cortical cells of pea epicotyls, and lettuce and azuki bean hypocotyls assumes a longitudinal direction (Apelbaum and Burg 1971; Ridge 1973). Veen (1970a, b in Eisinger 1983) found that longitudinally arranged microfibrils were deposited even when cell expansion was blocked by sucrose, indicating that longitudinal reorientation was not a passive result of cell expansion. Ridge (1973) confirmed that longitudinal microfibrils can occur independently of cell expansion, implicating microtubules in the orientation of the deposition of new cellulose microfibrils.

Several studies have shown that microtubules reorient in response to ethylene (Shibaoka 1974; Steen and Chadwick 1981; Lang *et al.* 1982; Roberts *et al.* 1985). The rapidity of microtubule reorientation by exposure to ethylene (15 min to 1 h) favours a shift in orientation of the microtubule array as opposed to reorientation by depolymerization and repolymerization (Roberts *et al.* 1985). This also suggests a short, or rapid chain of events between target and effector (Roberts *et al.* 1985).

In a review of the literature, Eisinger (1983) concluded that all proposed mechanisms of ethylene action involve the effect of this gas on the mechanical properties of the cell wall as a result of altered cellulose microfibril orientation (presumably via cortical microtubules), cross-linking within the wall, or enrichment of the pectic fraction. The effect of ethylene is reversed by low temperature and colchicine, both known microtubule depolymerization agents, suggesting that some of the ethylene responses may have been due to a stabilizing effect on microtubules, perhaps by influencing inter-microtubule and microtubule-plasma membrane connections (Steen and Chadwick 1981).

ABA:

Abscisic acid (ABA) has been reported to change the orientation of microtubules from transverse to longitudinal in dwarf pea epicotyls (Sakiyama and Shibaoka 1990) and in cucumber hypocotyls (Ishida and Katsumi 1992). However, Sakiyama and Shibaoka (1990) demonstrated that ABA-induced reduction in the rate of cell elongation occurred prior to the ABA-induced changes in the orientation of microtubules in dwarf pea epicotyls.

Hogetsu and colleagues (Hogetsu and Oshima 1986; Iwata and Hogetsu 1988, 1989b) also observed that cessation of cell elongation preceded the appearance of longitudinal microtubules for a variety of plant organs. These results appear to suggest that ABA might alter the orientation of microtubules by supressing cell elongation. However, using decapitated epicotyl cuttings from dwarf pea seedlings, Sakiyama-Sogo and Shibaoka (1993) examined the effects of ABA on the arrangement of cortical microtubules under conditions where this hormone does not change the rate of cell elongation. Results showed that ABA brought about a predominance of longitudinal microtubules in decapitated epicotyl cuttings without an effect on epicotyl expansion, indicating that ABA changed the orientation of microtubules in dwarf pea epicotyls by a mechanism other than one involving an ABA-induced decrease in the rate of cell elongation. That orientation of cortical microtubules can be changed by a mechanism that does not involve changes in the rate of cell elongation has previously been suggested by Laskowski (1990) and Williamson (1991).

Wakabayashi *et al.* (1989) reported that ABA caused partial inhibition of cellulose synthesis in squash hypocotyls. The finding that GA_3 did not promote stem elongation while cellulose synthesis was inhibited by coumarin (Hogetsu *et al.* 1974a) or by 2,6-dichlorobenzonitrile (Hogetsu *et al.* 1974b), suggested that the ABA reversal of GA_3-induced epicotyl elongation observed by Sakiyama and Shibaoka (1990) might also be via the inhibition of cellulose synthesis. Sakiyama and Shibaoka (1990) suggested that ABA might stabilize cortical microtubules by modification of the cell wall composition.

Cytokinin:

Cytokinins have been reported to inhibit elongation and stimulate lateral expansion of cells (Sommer 1961; Katsumi 1962). In kinetin-treated epicotyl cells of azuki bean (Shibaoka 1974; Takeda and Shibaoka 1978), and pea (Steen and Chadwick 1981; Lang *et al.* 1982; Roberts *et al.* 1985) and in mung bean hypocotyls (Roberts *et al.* 1985), cortical microtubules were oriented in a direction parallel to the cell axis. Kinetin-induced inhibition of IAA-induced elongation has been reported in azuki bean, and its inhibitory effect of kinetin upon GA-induced elongation has been shown to be repressed by colchicine (Shibaoka 1974).

Gibberellin:

Gibberellin (GA)-induced axial extension, arising from a promotion of longitudinal expansion and supression of lateral expansion of cells and increased cell division, has been reported in a range of plants including azuki bean (Shibaoka 1972, 1974; Takeda and Shibaoka 1981a, b), lettuce (Sawhney and Srivastava 1974, 1975), tomato (Jupe *et al.* 1988), cucumber (Taylor and Cosgrove 1989; Ishida and Katsumi 1992), onion (Mita and Shibaoka 1984b), sweet pea (Ross *et al.* 1990c), pea (Ingram *et al.* 1986; Akashi and Shibaoka 1987; Chory *et al.* 1987; Sakiyama and Shibaoka 1990), rice (Nick and Furuya 1993), and maize (Mita and Katsumi 1986; Chory *et al.* 1987). Ingram *et al.* (1986) and Ross *et al.* (1989) have reported a quantitative relationship between the level of GA_1 and internode growth in *Pisum sativum*, GA_1 being the only active GA *per se* in maize, rice, and peas (Phinney 1984; Ingram *et al.* 1986).

The relationship between GA and transverse microfibril arrangement has been reported for a range of plant species. In the polylamellate walls of epidermal cells of azuki bean (Takeda and Shibaoka 1981a, b), a GA-induced increase in longitudinal extension was associated with an increase in the amount of time cells spent depositing transverse microfibrils. When hypocotyls of lettuce (Sawhney and Srivastava 1975) were induced to elongate several-fold by GA_3, the cortical cells developed a predominantly transverse arrangement of microfibrils as opposed to the alternating transverse and longitudinal layers in untreated cells. In a study of deepwater rice, Sauter *et al.* (1993) found that GA_3-treatment of rapidly growing internodes resulted in a more gradual reorientation of microfibrils from a transverse to oblique arrangement along the zone of elongation than evident in controls.

In addition to the promotion of a transverse microfibril arrangement, GA has been demonstrated to increase the number of cells with microtubules oriented transversely to the axis of cell elongation in the epicotyl of pea (Akashi and Shibaoka 1987; Sakiyama and Shibaoka 1990; Sakiyama-Sogo and Shibaoka 1993), the epidermis of azuki bean epicotyl (Shibaoka 1974; Takeda and Shibaoka 1981b), the epidermis of maize mesocotyl (Mita and Katsumi 1986; Ishida and Katsumi 1991), the epidermis of onion leaf sheath (Mita and Shibaoka 1984a, b), the epidermis of cucumber hypocotyl (Ishida and Katsumi 1992), the epidermis of rice mesocotyl (Nick and Furuya 1993), and in lettuce (Sawhney and Srivastava 1975).

The use of systems that do not alter the rate of cell elongation have provided valuable insight into the effect of GA on microtubule orientation by eliminating any possible effect that cell elongation *per se* may have upon microtubule orientation. Mita and Shibaoka (1984b) reported a GA_3-induced predominance of transverse microtubules in onion leaf sheaths in which it did not promote cell elongation, indicating that microtubules were reoriented by a mechanism that did not involve GA-induced changes in cell elongation. Application of GA_3 to decapitated epicotyls of dwarf pea seedlings induced a transverse orientation of microtubules without stimulating elongation (Sakiyama-Sogo and Shibaoka 1993). These results from pea are in agreement with previous findings of Laskowski (1990).

The lack of GA-promotion of elongation in decapitated dwarf pea cuttings was suggested to result from the elimination of endogenous auxin by removal of apices (Sakiyama-Sogo and Shibaoka 1993), since it has been reported that GA does not produce elongation of stems in the absence of auxin (Shibaoka 1972). In addition, Sakiyama-Sogo and Shibaoka (1993) suggested that ABA might negate the effect of GA_3 on the orientation of microtubules in dwarf pea epicotyls, thus eliminating the GA_3-induced predominance of microtubules, even though GA_3 was still present in the cells. However, GA_3 was unable to counter the ABA-induced predominance of longitudinal microtubules while ABA was still in the cell. These results hint at the interactive nature of the relationship between plant hormones and microtubule orientation, the consequences of which will be discussed later in relation to the present study.

GA-biosynthesis inhibitors, such as S-3307, have been used to investigate the relationship between GA and cortical microtubules. Application of S-3307 to onion leaf sheath cells (Mita and Shibaoka 1984a) caused swelling of leaf sheaths, prior to which the normally transverse arrangement of cortical microtubules changed to longitudinal or oblique, indicating that endogenous GA plays an important role in promoting the transverse arrangement of microtubules.

The availability of GA biosynthesis mutants has provided a second possibility for investigating the effect of GA deficiency upon microtubule orientation. Mita and Katsumi (1986) reported that GA_3 controls the arrangement of microtubules in the mesocotyl epidermal cells of the *dwarf*-5 (*d5*) mutant of dark-grown *Zea mays*, and proposed that an insufficient supply of endogenous GA in the *d5* mutant is responsible for the reduced population of cells with high elongating potential associated with predominantly transverse microtubule orientation.

The *d5* gene controls the formation of ent-kaurene, a precursor of GA_1 (Hedden and Phinney 1979). However, *d5* is a leaky mutant which produces a very low level of GA_1. If the transverse arrangement of microtubules is normally controlled by endogenous GA, then in dwarf plants such as the *d5* mutant of maize, which has a lesion in GA biosynthesis (Phinney 1984), a reduction in transversely orientated microtubules would be expected. Ishida and Katsumi (1991) demonstrated that the frequency of the occurrence of cells with transversely arranged microtubules is higher in normal plants than in *d5* plants, especially in the tangential wall of the epidermis.

Dwarfing in the *d5* mutant of maize (Mita and Katsumi 1986) and in the "Little Marvel" mutant of *Pisum sativum* L. (Akashi and Shibaoka 1987) is reduced by GA_3 treatment. Mita and Katsumi (1986) proposed that exogenously applied GA_3 induced growth of the dwarf mutants by stabilizing microtubule orientation in a transverse direction. However, they noted that some transverse microtubules were observed in a region of the mesocotyl where rate of elongation was very low, and debated whether GA-induced promotion of cell elongation can be exclusively explained by the transverse orientation of microtubules.

Studies which utilize the effects of GA and colchicine in tandem have provided strong evidence for microtubule-mediated GA-induced cell elongation. Cell elongation induced by GA_3 can be reduced or eliminated by treatment with colchicine (Shibaoka 1972; Sawhney and Srivastava 1975; Takeda and Shibaoka 1981b), and colchicine-induced cell swelling can be reduced by subsequent treatment with GA_3 (Mita and Shibaoka 1984b).

Takeda and Shibaoka (1981b) reported that GA promoted epicotyl elongation and altered the direction of newly depositing microfibrils in azuki bean. They reported that GA-induced elongation of azuki bean epicotyl cells is reversed by microtubule-disrupting agents such as colchicine (Shibaoka 1972). Simultaneous application of GA and colchicine reversed the GA effect on microfibril orientation, and resulted in a slight predominance of longitudinal microfibrils, and loss of cross-polylamellate wall structure (Takeda and Shibaoka 1981a, b). Colchicine inhibited the GA_3-induced elongation of lettuce hypocotyls (Sawhney and Srivastava 1974), and induced lateral swelling. However, colchicine was only effective during the initial period of GA treatment. The ability of colchicine to inhibit growth decreased with later application times, and was lost altogether 6 h after GA_3 application in azuki bean (Shibaoka 1972) and 36 h after GA_3 application in lettuce (Sawhney and Srivastava 1974).

Seagull (1989) suggested that the observed antagonism between GA_3 and colchicine with respect to cell elongation indicated that the two agents may have targets in common, i.e. microtubules. Colchicine, which functions by binding to tubulin dimers and inhibiting the addition of subunits to the polymerizing end of the microtubule (Margolis and Wilson 1977), was reported to destroy microtubules in lettuce hypocotyl cells within 4 h (Srivastava *et al.* 1977). GA has been reported to increase the resistance of cortical microtubules to disruption by colchicine. In onion leaf sheath cells GA increased the number of transverse microtubules, and prevented the depolymerization of microtubules by colchicine, cremart and low temperature (Mita and Shibaoka 1984b). However, variability in the colchicine response has been reported for many systems (Gunning and Hardham 1982), and can perhaps be accounted for by poor binding between plant tubulin and colchicine (Morejohn *et al.* 1987a). Since colchicine binds only to tubulin dimers in solution, the implication is that colchicine is effective only when microtubules are in a state of constant turnover, such that stable microtubules would be less susceptible to disruption (Seagull 1989). This might account for some of the variability in the colchicine response and appears to support the hypothesis that GA increases resistance to colchicine by stabilizing microtubules.

In a study of the mechanism of onion bulb formation, Mita and Shibaoka (1984b) found that GA increased the number of transverse microtubules, and stabilized them against disruption by the anti-microtubular drugs colchicine and cremart, and against low temperature effects. Since disruption of microtubules by anti-microtubular drugs was found to decrease the resistance to chilling of cotton seedlings (Rikin *et al.* 1980), suggesting that cortical microtubules provide resistance to chilling in plant cells, it follows that GA might increase chilling resistance by stabilizing microtubules. However, GA has been reported to decrease chilling resistance in *Acer* (Irwing and Lanphear 1968) and in *Medicago* (Rikin *et al.* 1975), arguing against GA stabilization of the cortical microtubules. Akashi and Shibaoka (1987) reported that GA_3 also decreased the cold stability of microtubules in epidermal cells of pea internodes; transversely oriented microtubules in rapidly elongating cells were cold labile, while longitudinal arrangements were cold stable. The conflicting results for onion leaf sheath (Mita and Shibaoka 1984b) and pea internode (Akashi and Shibaoka 1987) might result from their different growth responses to GA_3, which promoted elongation in pea internode but did not promote growth in onion leaf sheath (Akashi and Shibaoka 1987). These results led Akashi and Shibaoka (1987) to hypothesize that GA stabilizes cortical microtubules in a direct manner, but destabilizes them indirectly by enhancing cell elongation.

Thus, there is considerable support for the hypothesis that GA-induced elongation of plant organs is mediated by its organizational effect on microfibrils via microtubules (e.g. Shibaoka 1974; Mita and Katsumi 1986; Katsumi and Ishida 1990; Sakiyama-Sogo and Shibaoka 1993). Some of the strongest support for this hypothesis has come from demonstrations of the directness of GA effect on microtubule orientation in systems where the rate of cell elongation is not altered by GA application, from studies that make use of GA biosynthesis inhibitors, from the use of mutants with lesions in GA biosynthesis, and from studies which observed the effect of microtubule-disrupting agents on hormone-induced cell expansion. There is also evidence that GA-induced elongation may involve cell wall loosening.

Whilst Cleland *et al.* (1968) and Katsumi and Kazama (1978) contended that GA increased elongation rates in cucumber hypocotyls by increasing turgor, Taylor and Cosgrove (1989) have reported contradictory findings. Yoda and Ashida (1960) reported that GA stiffened the wall in pea stems. However, GA application has more recently been reported to increase wall extensibility, as measured by mechanical methods such as the Instron technique, in oat internodes (Adams *et al.* 1975), cucumber hypocotyls (Taylor and Cosgrove 1989), lettuce hypocotyls (Kamisaka *et al.* 1972; Kawamura *et al.* 1976; Stuart and Jones 1977), and pea epicotyls (Lockhart 1960; Nakamura *et al.* 1975). *In vivo* stress relaxation techniques such as the pressure-block technique (Cosgrove 1987) have also demonstrated a GA-related increase in wall extensibility in peas (Cosgrove and Sovonick-Dunford 1989; Behringer *et al.* 1990a) and cucumber (Taylor and Cosgrove 1989). Cosgrove and Sovonick-Dunford (1989) and Behringer *et al.* (1990a) have favoured a primarily chemorheological, rather than a biophysical basis for GA-induced wall expansion in peas, and Taylor and Cosgrove (1989) have reported similarly for cucumber.

1.3 Conclusions

The above evidence, taken as a whole, suggests that cortical microtubules play an important role in stem elongation. There is little doubt that the arrangement of cellulose microfibrils limits the direction of expansion of the cell wall, and there is strong evidence for a causal relationship between cortical microtubules and microfibril orientation.

Plant hormones have been demonstrated to influence cell expansion, and the ability to orient cortical microtubules has been attributed to all principal plant hormones. In intact plants GA causes increased stem elongation, as does auxin, although to a lesser extent than GA. Both GA and auxin have been demonstrated to promote transversely oriented cellulose microfibrils and microtubules. The rapidity of the auxin-induced reorientation of cortical microtubules (Nick *et al.* 1990) suggests a rather direct effect of auxin, and systems where GA has been shown to produce a transverse reorientation of microtubules in the absence of cell elongation (Sakiyama-Sogo and Shibaoka 1993) point toward a direct role for this hormone in microtubule reorientation. However, the use of colchicine has disclosed variability in different systems. Both GA and auxin have also been implicated in the alteration of wall properties during hormone-induced extension growth, although they may affect different aspects of wall loosening.

Ethylene, ABA and cytokinins cause cessation of elongation and increased lateral expansion in stems, and a predominantly longitudinal arrangement of microfibrils and microtubules, which, for ethylene and ABA at least, is not prevented by blocking cell elongation. This, and the rapidity of ethylene-induced microtubule reorientation, may suggest a relatively direct effect upon microtubules, although modifications of the wall are also likely.

This study exploits the value of stem-length mutants to further the understanding of stem elongation, focussing upon microtubule orientation in a range of GA-synthesis and GA-response mutants of the garden pea, *Pisum sativum* L.

CHAPTER 2: THE RELATIONSHIP BETWEEN RATE AND DISTRIBUTION OF EXTENSION GROWTH AND MICROTUBULE ORIENTATION IN INTERNODE LENGTH MUTANTS OF *PISUM*

2.1 Introduction

The use of mutants in plant development

Reid and Ross (1993) have recently discussed the merits of a mutant-based approach to understanding plant growth. Indeed, mutants have proven successful as tools for investigating a range of developmental processes in plants, in particular where the mutations affect genes involved in the hormonal regulation of growth.

An understanding of the physiology and biochemistry of the flowering process has benefited from the isolation of flowering mutants. These mutants may influence the level of, or responsiveness to "flowering hormone(s)", or may indirectly affect flowering by altering growth rate (Murfet 1985; Murfet and Reid 1985; Reid 1987).

The control of plant water status has been partially illuminated by the availability of mutants which alter the level of plant hormones. The wilty mutants of tomato, flacca (*flc*), sitiens (*sit*), and notabilis (*not*) (Tal and Nevo 1973; Tal 1966), exhibited decreased resistance to water stress as a result of abnormal stomatal control and increased root resistance to water uptake (Tal and Nevo 1973). Wilty types have been shown to possess reduced levels of endogenous ABA (Neill and Hogan 1985). Treatment with ABA produced a phenocopy of the wild-types (Tal and Nevo 1973), implicating ABA in water stress. In addition, *flc* appeared to increase auxin and cytokinin activity (Tal *et al.* 1970; Tal and Imber 1970) and to increase ethylene evolution in the shoots (Tal and Nevo 1973; Tal *et al.* 1979). The *sit* and *flc* mutants in tomato both appear to block the step from ABA-aldehyde to ABA (Taylor *et al.* 1988; Parry and Hogan 1992), whereas *not* blocks prior to cleavage of the 9'-cis-neoxanthin (Parry and Hogan 1992). All three mutants appear to be leaky since the double homozygous types, *not flc* and *not sit*, led to a more extreme wilty phenotype than single homozygous mutant types *flc* and *sit* (Taylor and Tarr 1984). Wilty mutants have also been reported in potato (Simmonds 1965), corn (Postlethwaite and Nelson 1957), capsicum (Tal *et al.* 1976), *Arabidopsis thaliana* (Korneef *et al.* 1982), and pea (Marx 1976; Wang *et al.* 1984). The inferences contributed by each mutant are discussed in Reid (1987).

The characterization of mutants which influence aspects of stem elongation, has proven especially useful in furthering the understanding of the mechanism of stem elongation. Mutations affecting internode length have been reported for a variety of species including maize (see Phinney and Spray 1982), sweet pea (see Reid and Ross 1993) and pea (see Reid and Ross 1993).

In particular, the mechanism of GA-dependent stem elongation has benefited from the range of internode length mutants which alter either the level of, or the responsiveness to, GA. In *Pisum* (Ingram *et al.* 1986; Ross *et al.* 1989), and in other species possessing an early-13-hydroxylation pathway for GA biosynthesis (Phinney 1984; Fujioka *et al.* 1988), use of mutants with impaired GA synthesis has demonstrated a positive correlation between the concentration of endogenous GA_1 and increased stem elongation. These mutants, which block the biosynthesis and metabolism of GA at specific sites in the early 13-hydroxylation pathway, have helped elucidate the various steps in this pathway.

Phinney (1961) initially demonstrated that nonallelic dwarf mutants of maize, *d*1, *d*2, *d*3 and *d*5, had reduced GA-like activity when compared to wild-types, and became phenocopies of wild-types after exogenous application of GA_3. It was concluded that the mutations each impair a different step in the early-13-hydroxylation pathway of GA biosynthesis (see Phinney *et al.* 1990). For example,the *d*1 gene controls the conversion of GA_{20} to GA_1, although the mechanism by which it does this is still undetermined (Phinney *et al.* 1990).

In the garden pea (*Pisum sativum* L.), Reid and colleagues (see Reid and Ross 1993) have identified mutations at 15 loci that influence stem growth. The mutations have been divided into two major categories; GA-synthesis types, and GA-response types.

The mutant alleles *le*, *na*, *ls*, *lh* and *sln*, have been reported to block the biosynthesis and metabolism of GA at specific sites in the early-13-hydroxylation pathway (see Fig. 1, Reid and Ross 1993), altering the endogenous levels of GA_1 and resulting in altered phenotypes (Ingram *et al.* 1983, 1984; Potts and Reid 1983; Ross *et al.* 1989; Ingram and Reid 1987a; Reid *et al.* 1992; Swain and Reid 1992; Ross *et al.* 1993; SM Swain and Y Kamiya, unpublished results). These GA-deficient genotypes are therefore of extreme value in elucidating the role of endogenous GAs in the control of various plant growth responses. To this end they have been used to propose that GA_1 may be the only endogenous GA controlling stem elongation *per se* in plants possessing the early 13-hydroxylation GA biosynthetic pathway (Phinney 1984).

Other mutant alleles, *lk*, *lka*, *lkb*, *lkc* and *lkd* (Reid 1986; Reid and Potts 1986; Reid and Ross 1989; Behringer *et al.* 1990a; Reid *et al.* 1991; Cramp and Reid 1992; Lawrence *et al.* 1992; Reid and Davies 1992), *la cry*^{s} (Potts *et al.* 1985), *lm* (Reid and Ross 1988b), *lv* (Reid and Ross 1988a; Nagatani *et al.* 1990; Weller and Reid 1993) and *lw* (Jolly *et al.* 1987; Weller and Reid 1993) have been reported to result in an altered response to applied GA_1, and have allowed some resolution of the steps leading to shoot elongation after reception of the GA_1 signal. Of these mutants, some (e.g. *lk*, *lka*, *lkb*, *lkc*, *lkd*) may block steps in a pathway between reception and elongation, while others (*lv*, *lw*) may act indirectly by overriding the normal response to GA_1 (Reid and Ross 1993).

Such processes as the transduction chain from phytochrome, and the interaction between phytochrome function and the responsiveness of tissues to GA_1 (Nagatani *et al.* 1990; Weller and Reid 1993) as well as the chemorheological modification of the cell wall (Behringer *et al.* 1990a), have benefited from the availability of mutant alleles which affect responsiveness to GA_1, although such processes are still largely unresolved. Equally unknown is the effect of the GA-response mutations upon the arrangement of microtubules or microfibrils.

It is the primary purpose of this chapter to utilize a selection of these internode length mutants from the range of GA-synthesis (*le*, *ls*) and GA-response (*lk*, *lka*, *lkb*, *lw*) types, as tools to explore the relationship between endogenous plant hormones (particularly GA and auxin), extension growth and the arrangement of cortical microtubules in *Pisum sativum* L.

Characterization of the mutants

GA-synthesis mutants

Gene *le:*

The *le* mutation causes a reduction in the 3ß-hydroxylation of GA_{20} to GA_1 (Ingram *et al.* 1983, 1984; Ross *et al.* 1989). Support for this suggested site of action for *le* has come from three different approaches. Metabolism studies (Ingram *et al.* 1984) have demonstrated that after feeds of [^{13}C, ^{3}H]GA_{20}, *le* plants produced only the biologically inactive [^{13}C, ^{3}H]GA_{29}, whereas comparable *Le* plants produced three major metabolites, [^{13}C, ^{3}H]GA_1, [^{13}C, ^{3}H]GA_8, and [^{13}C, ^{3}H]GA_{29}. However, *le* is thought to be a leaky mutant with only a partial block in the 3ß-hydroxylation of GA_{20} to GA_1, since large-scale feeds of [^{13}C, ^{3}H]GA_{20} to *le* plants (Ingram *et al.* 1986) have resulted in the detection of small quantities of [^{13}C, ^{3}H]GA_1 and [^{13}C, ^{3}H]GA_8.

Measurements of endogenous levels of GA in the apical portions of *Le* and *le* plants were assessed by bioassay (Potts *et al.* 1982), by gas chromatography-mass spectrometry (GC-MS) (Ingram *et al.* 1984; Ross *et al.* 1989), and by gas chromatography-selected ion monitoring (GC-SIM) (Ross *et al.* 1992a; Smith *et al.* 1992). Reid and Ross (1993) have indicated that the combined information gained by use of GC-SIM and from isolines most accurately reflects endogenous GA content. This method has estimated that GA_1 levels are up to 20 times higher in *Le* than in *le* plants, and that GA_8 is also elevated in *Le* plants, while GA_{20} (Ross *et al.* 1992a) and GA_{29} (Smith *et al.* 1992) levels are lowered. GA application experiments have demonstrated reduced responsiveness of *le* plants to applied GA_{20}, while both *le* and *Le* plants respond similarly to GA_1 (Ingram *et al.* 1984). *le* plants have a dwarf phenotype and become true phenocopies of wild-type plants after treatment with appropriate quantities of GA_1.

Gene *ls:*

The *ls* mutation results in plants with very low levels of GA-like activity (Reid and Potts 1986). Application studies have shown that *ent*-kaurene causes a significant promotion of elongation in *ls* plants, similar to its effect upon comparable wild-type plants (i.e. those dwarfed with the GA-synthesis inhibitor AMO1618), suggesting that *ls* blocks GA-biosynthesis prior to *ent*-kaurene (Ingram and Reid 1987a). Since *ls* did not appear to interfere with carotenoid production, it has been suggested that the mutation has its effect after production of geranylgeranylpyrophosphate (GGPP) (Ingram and Reid 1987a). *In vitro* metabolism studies using GGPP and copalyl pyrophosphate (CPP) have indicated that *ls* blocks metabolism between these two compounds (S. M. Swain and Y. Kamiya, unpublished results). Like *le*, *ls* appears to be leaky, since double recessive types (e.g. *na ls*) are shorter than the single recessive *ls* types (Reid 1986) and low levels of GA_1 (or other GA intermediates after the blocks) have been found in the mutants (Ross *et al.* 1989). *ls* plants have an extreme dwarf phenotype and become true phenocopies of wild-type plants after treatment with appropriate quantities of GA_1. The GA_1-deficient *ls* plants show an elevated wall yield threshold (2.7 bars) and reduced wall yield coefficient when compared with the wild-type (Behringer *et al.* 1990a), and since all normal GA-mediated processes are altered, turgor and osmotic pressures are similar to the wild-type.

GA-response mutants

Gene *lw:*

In comparison with wild-type plants, the *lw* mutation produces plants with reduced internode lengths, slightly darker green foliage, an enhanced flowering response to photoperiod, increased symptoms of water congestion, and a reduced response to applied GA_1 (Jolly *et al.* 1987; Weller and Reid 1993).

Unlike genes *le* and *ls*, gene *lw* does not appear to directly influence the biosynthesis of active gibberellins, since the shoots of *lw* plants were reported to contain similar levels of extractable GA-like substances to comparable wild-type plants, and application of GA_1, GA_{20} or GA_{19} did not produce a phenocopy of the wild-type (Jolly *et al.* 1987). However, *lw* plants with genetically (genes *na* and *le*) and chemically (AMO 1618 and PP333) reduced internode lengths showed marked stem elongation in response to applied GA_1 (Jolly *et al.* 1987).

Weller and Reid (1993) have indicated that *lw* appears to modify phytochrome-mediated responses, since the extent of dwarfing of *lw* plants was dependent on light quality and quantity. Dwarfing was greater in plants grown under red light, than those supplied with far-red light, blue light or grown in darkness. In comparison with isogenic wild-type plants, *lw* plants also possessed a lower fluence requirement for inhibition by white fluorescent light and an enhanced end-of-day response to far-red light.

These results suggest that gene *lw* probably does not reduce elongation by influencing GA-synthesis, but acts via a mechanism that partially overrides the response to GA_1. The possibility of an interaction between phytochrome function and the responsiveness of tissues to GA_1 is supported by the decreased response of the mutant *lw* to GA_1 (Jolly *et al.* 1987). The *lw* mutation therefore provides a possibility for exploring the transduction pathway leading from phytochrome.

Genes *lk*, *lka* and *lkb:*

Genes *lk* (erectoides) and *lka* and *lkb* (semi-erectoides) result in plants which are substantially shorter than the wild-type, have ridged stems and petioles which are swollen and brittle, and reduced leaflet size, peduncle length and basal branching (Ross and Reid 1986; Reid and Ross 1989). They possess essentially normal levels of endogenous GA_1 (Reid 1986; Reid and Potts 1986; Reid and Ross 1989; Behringer *et al.* 1990a; Reid and Davies 1992), and exhibit a reduced response to applied GA_1 in comparison with wild-type plants of a similar stature (Reid and Potts 1986; Reid and Ross 1989).

GC-SIM and ^{13}C- or $^{2}H_2$-labelled internal standards were used to determine the levels of GA_1, GA_8, GA_{19}, GA_{20}, GA_{29} and the biologically active GA_1 in the apical portions of *lk*, *lka* and *lkb* plants (Reid *et al.* 1990). They reported only small differences between levels of GA in the mutants and levels in essentially isogenic wild-type lines, with GA_1 content slightly reduced in *lk* and *lkb* plants, and slightly increased in *lka* plants.

These small differences in GA_1 levels cannot account for the up to 7-fold reduction in internode length in these mutants, since comparable internode length reductions are produced by upwards of a 10-fold reduction in GA_1 levels in the GA-deficient plants *le* (Ross *et al.* 1992a) and *na* (S. M. Swain, pers. comm.). In addition, Steane *et al.* (1989) have shown that the metabolism of $[^{3}H]GA_1$ is similar in *lk*, *lka*, and wild-type plants.

The differences between genotypes *lk*, *lka*, and *lkb* were maintained in both the light and the dark (Reid and Potts 1986; Reid and Ross 1989), and the response to various light regimes was largely comparable to wild-type plants (Reid and Davies 1992). However, the time to maximal growth inhibition on transfer of plants from darkness to red light, was slightly greater in the mutants (Behringer *et al.* 1990b), and minor changes in the relative stem lengths have been observed under different light regimes (J. L. Weller, pers. comm.). It is unlikely, however, that these mutations act by altering photomorphogenic responses (Reid and Davies 1992).

The physical basis of reduced elongation in the mutants was examined by Behringer *et al.* (1990a). They showed that *lka* and *lkb* plants had substantially lower growth rates than wild-type plants at all points of measurement below the apex, and a zone of elongation which extended down the stem only two-thirds the distance of wild-type plants. Behringer *et al.* (1990a) reported significant differences in *in vivo* measurements of wall relaxation between the genotypes, as determined by the pressure-block technique (Cosgrove 1987; Cosgrove and Sovonic-Dunford 1989), which correlated with differences in extension rate. The wall yield threshold for *lka* and *lkb* plants was respectively 4.5 and 5.7 bars, compared with 0.4 bars in the wild-type. The wall yield coefficient also appeared to be reduced in *lka*.

Behringer *et al.* (1990a) also employed the pressure probe technique to measure the turgor pressure of cortical cells in the zone of maximum extension. They found substantially greater turgor pressures in *lka* and *lkb* plants than in comparable wild-type plants or GA-deficient *ls* plants of similar stature, and since the osmotic pressures of *lka* and *lkb* plants were also elevated, diminished solute concentrations or reduced turgor were not seen as likely causes of the reduced growth. The increased turgor pressure may be responsible for the swelling and horizontal splitting observed in dark-grown *lka* and *lkb* plants (Fig. 5, Reid and Davies 1992), which is absent in GA-deficient mutants of comparable stature but is also present in *lk* plants.

The mutations do not appear to affect the reception of the GA_1 signal, since *lk*, *lka*, and *lkb* plants are phenotypically dissimilar to the GA-deficient mutants. Rather, they appear to modify the steps that are normally required for this signal to be expressed at the phenotypic level.

These mutants possess a reduced response to applied GA_1 (Reid and Potts 1986; Reid and Ross 1989) and, in contrast to the GA-synthesis mutants, phenocopies of wild-type plants are not produced by this treatment. It is possible that *lka* and *lkb* specifically block one or more GA-stimulated processes that affect wall yielding and thus elongation, without disrupting other aspects of the normal GA response such as solute translocation, since turgor pressure was raised in these genotypes but not in dwarf, GA-deficient *ls* plants.

lk, *lka*, and *lkb* plants have been referred to as GA-response mutants, although it is possible that the mutant allele at the *lkb* locus, and perhaps those at the *lka* and *lk* loci, may act by reducing the level of IAA. Reid and Davies (1992) have reported a consistent two- to threefold reduction in the IAA content of *lkb* plants compared with wild-types. McKay *et al.* (1994) have recently shown that application of the IAA transport inhibitors, 2,3,5-triiodobenzoic acid (TIBA) and 9-hydroxyfluorene-9-carboxylic acid (HFCA), to expanding internodes resulted in promotion of elongation and increased IAA levels above the treatment site in *lkb* plants, but not in *Lkb* plants, although TIBA reduced both the level of IAA and elongation in wild-type plants below the site of application. *lka* plants responded similarly to *lkb* plants. Davies and associates (P. J Davies; unpublished results) have recently shown that IAA application to expanding internodes of *lkb* plants promotes internode elongation, by increasing length of both cortical and epidermal cells, although phenocopies of the wild-type were not produced.

Epidermal peels from *lkb* plants contained normal IAA levels, indicating that IAA deficiency is confined to the inner tissues (McKay *et al.* 1994). These results have led McKay *et al.* (1994) to propose that the banding observed in light-grown *lk*, *lka* and *lkb* plants as a result of "zig-zagging" of the inner pith and vessels (Ross and Reid 1986) is, at least in *lkb* plants, a result of IAA deficiency in the inner tissues. They have further suggested that the ridging *per se* may be responsible for elevated ethylene levels in the tissues which may further inhibit elongation. Significantly, use of the ethylene inhibitor, AVG, in combination with the auxin transport inhibitor, HFCA, restored the wild-type internode length to *lkb* plants, whereas AVG or HFCA alone did not.

In all three mutants the epidermis consists of cells that are shorter (*lk*, *lka*, *lkb*) and sometimes fewer (*lk*, *lka*) than those of the wild-type (Ross and Reid 1986; Reid and Ross 1989). In view of the ability of ethylene to inhibit elongation growth (see Chapter 1), the conclusions of McKay *et al.* (1994) seem plausible.

2.2 Materials and Methods

Plant material

The pure lines of *Pisum sativum* L. used to assess growth profile along the epicotyl and for analyses of microtubule orientation are held in the collection at Hobart (Department of Plant Science, University of Tasmania). The short mutant lines used are essentially isogenic, all (except L212) having been produced from single gene mutations of cv. Torsdag (Wild-type tall, *Le Ls Lk Lka Lkb Lw*) and include NGB5865 (semi-erectoides, *lka*), NGB5862 (semi-erectoides, *lkb*), K29 (short, *lw*), L181 (dwarf, *ls*) and NGB5839 (dwarf, *le*). L212 (erectoides, *lk*) was produced from a cross between Torsdag and JI1420 (erectiodes, *lk*). Further details about the genotypes and phenotypes of these lines may be found in Reid and Ross (1989).

Growing conditions

Plants were grown in 140-mm-diameter black Slimline pots at 2 plants per pot in a 50:50 mixture of vermiculite and dolerite chips topped with 20 to 30 mm of potting mix, as specified by Reid (1986). Plants were watered on the day of sowing (day 0), and then daily until seedlings could be seen emerging from the substrate.

Subsequent watering was every 2 to 3 days as required. Plants were grown in darkness at 22° C from the time of sowing until the plants were harvested, with the use of a safe green light for watering, observations and measurements. Node counting began with the cotyledonary node as zero.

Initially, all 7 lines were allowed to maximally expand internode 3 in order to establish final lengths for this internode under these growing conditions (Table 1).

2.2.1 Determination of extension growth

Growth analysis

The growth profile along the epicotyl was measured (after the general method of Behringer *et al.* 1990) by applying India ink at 2 mm intervals over approximately the upper 30 mm of the epicotyl with a fine cotton-thread stamp. Plants were stamped when internode 3 was 25%-40% expanded (Table 1). After 6-8 h, plants were severed at the cotyledonary node using a Gillette Blue razor blade and immediately photocopied onto paper. Distance between the ink markings was measured under a dissecting microscope, and the percent increase in length per hour for each 2 mm interval was calculated to provide a growth profile for each of the lines (Fig. 1). Sample size and standard error values are presented in Table 3 to facilitate interpretation of growth profiles. Overall extension growth was also measured and the average rate of extension calculated for each of the lines (Table 2).

The choice of dark-grown plants provided material comparable to that used in the analysis of microtubule orientation. In addition, the investigation of a growth profile along the epicotyl for each of the mutant lines was facilitated by the increased internode lengths of the dark-grown plants. The ink stamp was more easily applied to plants with sufficiently elongated internodes, and in comparison with light-grown plants, the increased extension rate of dark-grown plants (Reid 1983, 1988) magnified the displacement of stem markings.

2.2.2 Immunofluorescence

Plants were harvested for immunofluorescence work when internode 3 reached 25-40% expansion (Table 1), which, at 22° C, was 7-9 d after sowing.

Harvesting procedure

At 25-40% expansion of internode 3, darkgrown seedlings were decapitated just above the cotyledonary node and immediately placed into a shallow pool of fixative. Using a fine (Gillette Blue) razor blade 1 mm length segments were cut from 2 mm zones at intervals [0-2 (position A), 4-6 (B), 8-10 (C), 12-14 (D), 20-22 (E) and 30-32 (D) mm] down the epicotyl beginning directly beneath the apical hook at the point at which the epicotyl straightens and begins to swell.

Tissue fixation

Epicotyl segments were placed into vials of fresh fixative [3% formaldehyde (Ajax; Sydney, Australia) in microtubule stabilizing buffer (MSB): 50 mM 1, 4-piperazinediethanesulphonic acid (Pipes) buffer (Sigma; St. Louis. USA), 2 mM $MgSO_4$, 5 mM ethyleneglycol-bis-(ß-aminoethylether)-N, N, N, N'-tetraacetic acid (EGTA) (Fluka; Buchs, Switzerland), 10% NaN_3, adjusted to pH 6.8 with 10 M NaOH], to which 10% dimethylsulphoxide (DMSO) (Sigma) was added to improve microtubule preservation (see Hush *et al.* 1990) and intensity of the fluorescence image (Iwata and Hogetsu 1988), and 0.1% Nonidet P 40 (Fluka) to improve fixation (Iwata and Hogetsu 1988). Tissue was vacuum-infiltrated with the fixative for several minutes, after which the vacuum was slowly released. After fixation for 1 h at room temperature, the tissue was rinsed twice with MSB.

Sectioning

Tissue segments were frozen in Tissue-Tek (Miles Laboratory; Melbourne, Australia) over liquid nitrogen. Tangential sections (8-12 µm) of epidermal cells and axially oriented sections (8-10 µm) of sub-epidermal cortical cells were cut using a Bright 5030 rotary rocking cryostat/microtome (Bright Instrument Co.; Huntingdon, England) at approximately -35° C. Cryosections were picked up by touching onto the surface of a multiwell fluorescent antibody slide (Cell Line, cat. no. 5638-01940, Imbros; Moonah, Tasmania) coated with a gelatin (1 $g \cdot l^{-1}$) and chromium potassium sulphate (0.1 $g \cdot l^{-1}$) solution. Slides stored in the dark at 4°C with self indicating silica gel (Ajax) in a sealed container retained their immunoreactivity for several weeks.

Tubulin immunolabelling

Welled slides were flooded with phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.0 mM Na_2HPO_4) pH 7.3 for 30 min. then blotted with filter paper. The primary antibody (Ascites fluid, cat. no. N.357, Amersham International; Buckinghamshire, England), a mouse monoclonal antibody raised against the ß-tubulin subunit of chick brain tubulin, was used at a dilution of 1:500 in PBS pH 7.3 with 1% bovine serum albumen (BSA) and 0.02% NaN_3, and applied in

20 µl aliquots to each section for 1 h at 37°C under conditions of high humidity. Excess antibody was blotted with filter paper and the slides rinsed in PBS pH 7.3 (2 x 10 min.) before blotting. The secondary antibody, fluorescein isothiocyanate (FITC)-labelled sheep anti-mouse IgG (code df, Silenus Laboratories; Hawthorn, Australia) was used at 1:30 dilution in the PBS/BSA/NaN_3 solution, and applied in 20 µl aliquots to each section for 1 h at 37°C under conditions of high humidity.

Excess FITC was blotted with filter paper and the slides rinsed in PBS pH 7.3 (3 x 10 min.) and distilled water (1 x 10 min.) before blotting. Cryosections were mounted in a Mowiol 4-88 medium (Hoechst; Frankfurt, West Germany) (pH 8.5) (Osborn and Weber 1982) to which 0.1% paraphenylenediamine was added to retard quenching of the fluorescence (Johnson and De C. Nogueira Araujo 1981).

Microscopy

Sections were examined with a Zeiss Axiovert 35 (Carl Zeiss Inc.; West Germany) inverted photomicroscope equipped with incident light fluorescence and fitted with a 12 v mercury vapour 100 w lamp. FITC-labelled microtubules were observed using the Zeiss filter set no. 09 containing the following filter combination: exciter filter BP 450-490, LP 520, chromatic beam splitter F 510. Photographs were taken on Kodak Ektachrome (400 ASA).

Analysis of microtubule orientation

Measurements were made from photographs which were interpreted with the assistance of direct observation by fluorescence microscopy. From cells in which the orientation of microtubules was principally unidirectional, an average microtubule orientation was calculated and expressed in terms of degrees of divergence from the longitudinal axis of the cell. This averaged microtubule orientation was then used to designate a single orientation value to each cell. To facilitate discussion, three microtubule orientation categories (after the method of Laskowski 1990) were specified: longitudinal (occupying a 30° sector on either side of the longitudinal axis of the cell), transverse (occupying a 30° sector on either side of the transverse axis of the cell), and oblique (occupying a sector between 30° and 60° from either axis). Cells which were apparently undisturbed but which lacked a single primary microtubule orientation were termed "random". Cells that were obviously deformed, or in which microtubules were fragmented or lacking, were omitted from the analysis.

Histograms showing the percentages of microtubules falling into the three orientation categories (transverse, oblique and longitudinal), at specified distances from the apical hook, were constructed (Fig.2a, b). Scatter graphs (Fig 3a, b) were also constructed to show the average microtubule orientation for the wild-type and stem-length mutants at each distance from the apical hook. Obtaining a standard sample size was made difficult by certain aspects of the tissue preparation procedure detailed above. Tissue loss during preparation, tissue disturbance during cryosectioning, as well as variability of fixation or quality of staining, all contributed to the variability of sample size. A range between 9 and 310 cells were measured for each combination of line, tissue type, and internode position.
However, for any one histogram, 47 to 490 cells were examined, and scatter graphs used between 1307 and 1328 cells for their construction. Sample size and standard errors for average microtubule orientation are given in table form (Table 4a, b) to facilitate interpretation of the orientation data.

Scatter graphs showing the relationship between growth rate of internode 3 and average microtubule orientation for wild-type and stem-length mutants were constructed (Fig.7a, b).

2.3 Results

2.3.1 Rate and distribution of extension growth

The effect of the mutant recessive alleles, *ls, le, lw, lk, lka,* and *lkb*, upon the overall rate of extension growth in dark-grown seedlings is presented in Table 2. The reduction in the rate of extension growth by the dwarfing alleles is approximately 25% to 90%. The GA-synthesis mutants, *le* and *ls*, produced reductions in rate of extension of 33% and 58% respectively. The GA-response mutants resulted in extension rates which ranged from between 25% to 92% lower than in wild-type plants. *lka* and *lkb* plants had extension rates of 0.4 mm h^{-1}, compared with 1.2 mm h^{-1} in the wild-type. Extension rate in *lk* plants was even further reduced to 0.1 mm h^{-1}. Contrasting with the other GA-response types, *lw* plants recorded an average extension rate of 0.9 mm h^{-1}, a reduction of only 25% from wild-type values.

The distribution of growth along the stem of the different genotypes was obtained by plotting growth as a function of position along the epicotyl (Fig. 1). Wild-type plants were observed to have a growth zone extending approximately 30-32 mm below the apical hook. In the wild-types, the most rapid elongation occurred in the upper 4-8 mm of the epicotyl, with a maximum extension rate of over 7.3% per h at 4-6 mm below the apical hook.

In the GA-synthesis types *le* and *ls*, as in wild-type plants, most rapid elongation occurred in the upper 4-8 mm of the epicotyl. However, in both *le* and *ls*, maximum extension rate was decreased in comparison to wild-type plants. Maximum extension rate was 5.8% per h for *le* and 4.9% per h for *ls* at 2-4 mm below the apical hook. In addition, the growth zone of *ls* plants extended only 24 mm below the hook, having decreased to 1.5% per h by 14-16 mm. The growth zone of *le* plants, while extending as far as 30 mm, had decreased to 1.8% per h by 14-16 mm, compared with a growth rate of 4.1% per h for wild-type plants at a comparable distance from the apical hook.

The zone of most rapid elongation in the GA-response mutants, *lk, lka,* and *lkb*, occurred in the upper 4-6 mm of the epicotyl, a slightly smaller zone than in the GA-synthesis and wild-type plants. Similarly, the growth zones of the GA-response types were also reduced in comparison to the wild-types. Extension growth had ceased altogether by 22-24 mm in *lka* and *lkb* plants, and by 18-20 mm in *lk* plants. Maximum extension rates for the GA-response types were reduced in comparison to wild-type plants, and were lower than that of the GA-synthesis types. Maximum extension was reduced by more than 40% in *lka* and *lkb* plants, and occurred at 2-4 mm below the apical hook. In *lk* plants, maximum extension occurred at 4-6 mm and was reduced by almost 80% in comparison to wild-type plants.

The shape of the growth profile for the putative photomorphogenic mutant, *lw* (Fig. 1), was noticeably different from wild-type plants and from the other mutant types. The zone of most rapid elongation occurred in the upper 8-14 mm of *lw* plants, which were maximally elongating at a rate of 5.6% per h at 8-10 mm below the apical hook. Epicotyl elongation extended 30-32 mm below the apical hook, epicotyl extension decreasing at a rate comparable to that of wild-type plants in the 12-32 mm interval. Extension rate in *lw* plants was initially low, beginning at 2.3% per h at 0-2 mm below the apical hook and increasing rather steeply to 5.4% per h by 6-8 mm.

Table 1. Expanded length of third internodes, and length at 25% and 40% expansion of etiolated seedlings of stem length mutants of *Pisum sativum* L.

Line	Genotype Phenotype	Total Length Internode 3 (mm)	Length at 25% Expansion (mm)	Length at 40% Expansion (mm)
Torsdag	*Le Ls Lk Lka Lkb Lw* Tall	161.53 +/- 3.31 (15)	40.38	64.61
NGB5839	*le Ls Lk Lka Lkb Lw* Dwarf	100.00 +/- 3.18 (7)	25.00	40.00
L181	*Le ls Lk Lka Lkb Lw* Dwarf	49.24 +/- 0.84 (17)	12.31	19.69
K29	*Le Ls Lk Lka Lkb lw* Short	138.25 +/- 4.21 (8)	34.56	55.30
L212	*Le Ls lk Lka Lkb Lw* Erectoides	34.25 +/- 1.71 (8)	8.56	13.70
NGB5865	*Le Ls Lk lka Lkb Lw* Semi-erectoides	52.75 +/- 1.76 (8)	13.19	21.10
NGB6862	*Le Ls Lk Lka lkb Lw* Semi-erectoides	50.00 +/- 1.21 (19)	12.50	20.00

Total internode lengths are means +/- standard errors. Sample sizes are included in parentheses. 25% and 40 % expansion refers to length of internode 3 at 25% and 40%, respectively, of total length.

Table 2 Elongation rates for expanding third internodes of etiolated seedlings of *Pisum sativum* L.

Line	Genotype Phenotype	Elongation Rate Av. mm h^{-1}
Torsdag	*Le Ls Lk Lka Lkb Lw* Tall	1.2 +/- 0.10 (7)
NGB5839	*le Ls Lk Lka Lkb Lw* Dwarf	0.8 +/- 0.03 (8)
L181	*Le ls Lk Lka Lkb Lw* Dwarf	0.5 +/- 0.03 (13)
K29	*Le Ls Lk Lka Lkb lw* Short	0.9 +/- 0.07 (13)
L212	*Le Ls lk Lka Lkb Lw* Erectoides	0.1 +/- 0.01 (15)
NGB5865	*Le Ls Lk lka Lkb Lw* Semi-erectoides	0.4 +/- 0.04 (9)
NGB6862	*Le Ls Lk Lka lkb Lw* Semi-erectoides	0.4 +/- 0.07 (7)

Elongation rates are means +/- standard errors, calculated as the average rate of extension over a 6-8 h period. Sample sizes are included in parentheses.

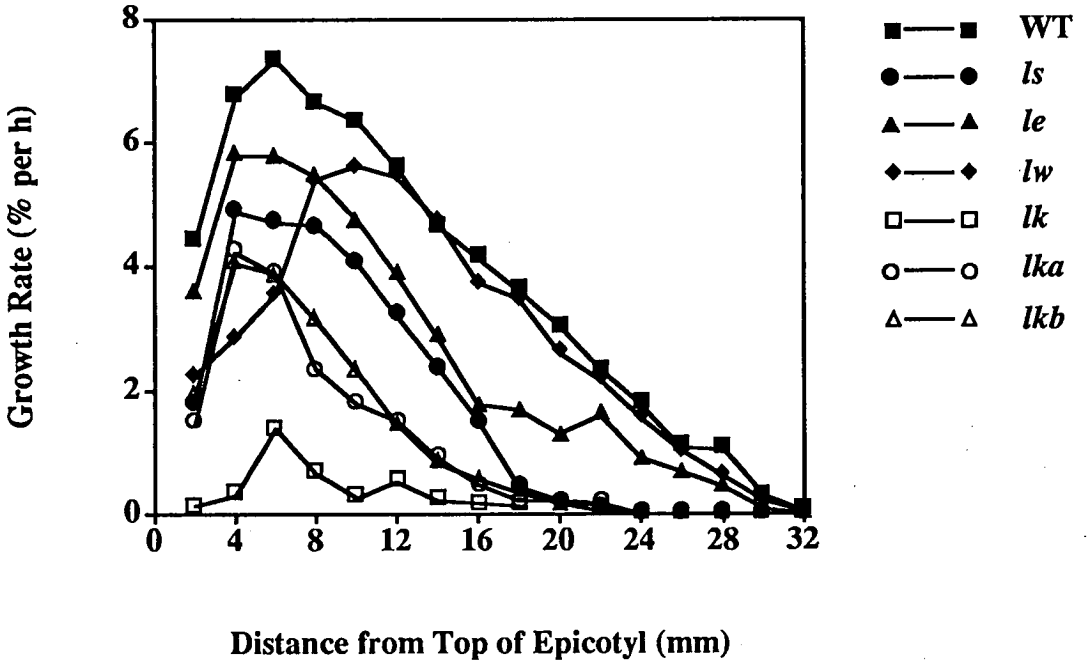


Fig. 1 Distribution of growth along the epicotyl of etiolated seedlings of a range of stem length mutants of *Pisum sativum* L. Growth was determined by measuring the displacement of ink marks over 6 to 8 h. Data points are the averages of 6 to 15 plants.

Table 3 Elongation rates at distance from the apical hook for third internodes of etiolated seedlings of *Pisum sativum* L.

Distance From Apex (mm)	Line Genotype Phenotype	Torsdag *Le Ls Lk Lka Lkb Lw* Tall	NGB5839 *le Ls Lk Lka Lkb Lw* Dwarf	L181 *Le ls Lk Lka Lkb Lw* Extreme Dwarf	L212 *Le Ls lk Lka Lkb Lw* Erectoides	NGB5865 *Le Ls Lk lka Lkb Lw* Semi-erectoides	NGB5862 *Le Ls Lk Lka lkb Lw* Semi-erectoides	K29 *Le Ls Lk Lka Lkb lw* Short
0-2		4.39 +/- 1.00 (7)	3.59 +/- 0.34 (8)	1.79 +/- 0.41 (13)	0.11 +/- 0.06 (15)	1.48 +/- 0.45 (9)	1.95 +/- 0.39	2.25 +/- 0.49 (13)
2-4		6.73 +/- 0.91 (7)	5.78 +/- 0.44 (8)	4.88 +/- 0.55 (13)	0.32 +/- 0.10 (15)	4.23 +/- 0.77 (9)	4.04 +/- 0.49	2.86 +/- 0.42 (13)
4-6		7.32 +/- 0.81 (7)	5.76 +/- 0.28 (8)	4.70 +/- 0.42 (13)	1.38 +/- 0.44 (15)	3.88 +/- 0.41 (9)	3.85 +/- 0.37	3.55 +/- 0.30 (13)
6-8		6.62 +/- 0.51 (7)	5.43 +/- 0.29 (8)	4.63 +/- 0.45 (13)	0.67 +/- 0.22 (15)	2.34 +/- 0.43 (9)	3.13 +/- 0.53	5.37 +/- 0.30 (13)
8-10		6.32 +/- 0.39 (7)	4.73 +/- 0.28 (8)	4.05 +/- 0.34 (13)	0.28 +/- 0.11 (15)	1.18 +/- 0.42 (9)	2.31 +/- 0.57	5.58 +/- 0.30 (13)
10-12		5.59 +/- 0.45 (7)	3.87 +/- 0.30 (8)	3.23 +/- 0.35 (13)	0.54 +/- 0.25 (13)	1.51 +/- 0.32 (9)	1.46 +/- 0.40	5.44 +/- 0.35 (13)
12-14		4.63 +/- 0.38 (7)	2.87 +/- 0.31 (8)	2.37 +/- 0.36 (13)	0.22 +/- 0.14 (13)	0.93 +/- 0.32 (8)	0.83 +/- 0.21	4.71 +/- 0.33 (13)
14-16		4.14 +/- 0.36 (7)	1.77 +/- 0.24 (8)	1.50 +/- 0.25 (13)	0.17 +/- 0.08 (11)	0.47 +/- 0.18 (8)	0.55 +/- 0.19	3.73 +/- 0.24 (13)
16-18		3.62 +/- 0.30 (7)	1.69 +/- 0.17 (8)	0.45 +/- 0.14 (13)	0.13 +/- 0.05 (7)	0.20 +/- 0.11 (7)	0.34 +/- 0.19	3.46 +/- 0.26 (13)
18-20		3.02 +/- 0.34 (7)	1.29 +/- 0.21 (8)	0.21 +/- 0.01 (13)	*	0.20 +/- 0.11 (7)	0.17 +/- 0.13	2.61 +/- 0.23 (13)
20-22		2.33 +/- 0.31 (7)	1.61 +/- 0.10 (8)	0.12 +/- 0.07 (13)	*	0.18 +/- 0.10 (7)	0.05 +/- 0.05	2.17 +/- 0.30 (13)
22-24		1.78 +/- 0.25 (6)	0.90 +/- 0.16 (8)	0.00 +/- 0.00 (13)	*	0.00 +/- 0.00 (7)	0.00 +/- 0.00	1.58 +/- 0.26 (13)
24-26		1.10 +/- 0.33 (6)	0.69 +/- 0.08 (8)	0.00 +/- 0.00 (13)	*	0.00 +/- 0.00 (7)	0.00 +/- 0.00	1.02 +/- 0.24 (13)
26-28		1.06 +/- 0.40 (6)	0.45 +/- 0.13 (8)	0.00 +/- 0.00 (13)	*	0.00 +/- 0.00 (7)	0.00 +/- 0.00	0.62 +/- 0.17 (13)
28-30		0.29 +/- 0.15 (7)	0.09 +/- 0.07 (8)	0.00 +/- 0.00 (13)	*	0.00 +/- 0.00 (7)	0.00 +/- 0.00	0.22 +/- 0.16 (13)
30-32		0.06 +/- 0.03 (6)	0.02 +/- 0.02 (8)	0.00 +/- 0.00 (13)	*	0.00 +/- 0.00 (7)	0.00 +/- 0.00	0.04 +/- 0.04 (13)

Elongation rates are means +/- standard errors, calculated as the average percentage rate of extension per 2 mm interval over a 6-8 h period. Sample sizes are included in parentheses.

A feature common to the growth profiles of wild-type and mutant lines is that growth rate is slower immediately below the apical hook (Fig. 1). In all except *lk* plants, extension rates at 0-2 mm below the apical hook were approximately 40-65% lower than the maximum expansion rates (i.e. at 2-6 mm below the apical hook). In *lk* plants initial extension rate was reduced by greater than 90% from maximal extension rate. Maximum growth rate was reached between 2-6 mm from the apical hook, except for *lw* plants which were maximally expanding at 10-12 mm below the apical hook. An appreciation of microtubule orientation in the 0-2 mm zone might help to elucidate the action of the mutant alleles. Such an analysis was attempted, but a combination of repeatedly poor fixation of this region and pro-chlorophyll autofluorescence made interpretation of microtubule orientation unreliable.

Growth profiles of the WT and mutant lines indicated that, with the exception of *lw*, maximum expansion rates of darkgrown internode 3 were between 2-6 mm from the apical hook. On this basis, internode position B (4-6 mm) was chosen for immunoflourescent analyisis of microtubule orientation. By 12-14 mm (internode position D) from the apical hook, expansion rates were reduced in all lines: expansion rates were approximately 50% of maximum expansion rate in the GA-synthesis mutants *ls* and *le*, and were further reduced in the GA-response mutants, *lk*, *lka* and *lkb*. WT and *lw* plants had similar elongation rates at this position. On the basis of these reduced, and distinctively different, expansion rates in the WT, GA-synthesis and GA-response mutants, internode position D (12-14 mm) was also selected for immunoflourescent analysis of microtubule orientation. The somewhat distinctive growth profile of the *lw* mutant, which recorded a maximum expansion rate at 8-10 mm form the apical hook, prompted an additional analysis of microtubule orientation at internode position C (8-10 mm, results not shown) for this mutant.

Wild-type plants were observed to have growth zones which extended along the epicotyl for approximately 30 mm beneath the apical hook. In contrast, the growth zones of the mutant lines, with the exception of *lw*, were attenuated to approximately 24 mm along the epicotyl. In *le* plants, although elongation continued for about the same distance of epicotyl as wild-type plants, by 14-16 mm growth rate was considerably lower than wild-type levels, and continued at a reduced rate for the remaining length of epicotyl. The distance to which the growth zone of *lw* plants extended was comparable to that of wild-type plants.

2.3.2 Microtubule orientation in the epidermis and cortex

Measurement of microtubule orientation in the epidermis and the outer cortical tissue for each of the seven lines examined revealed a substantial difference in microtubule orientation in these two tissues, and prompted a separate analysis of microtubule orientation for the two tissue types. The two measures of microtubule orientation, average microtubule orientation and microtubule orientation category, produced somewhat different estimates of microtubule arrangement. The categorization of microtubule orientation obscured the spread of orientations within each orientation range. However, a simple measure of average orientation did not illustrate the observed range of microtubule orientations. Therefore, both measures were employed in the assessment of microtubule orientation.

Epidermis

Microtubule orientation in epidermal cells from wild-type L107 plants (Fig. 1) was exclusively transverse at 4-6 mm (position B), and almost exclusively so at 12-14 mm (position D), where 94.6% of the cells fell into the transverse microtubule orientation category and 5.4% were recorded as having an oblique orientation. At no time was a longitudinal orientation observed.

In contrast, the stem-length mutants covered a greater range of microtubule orientations (Fig. 2a). The average microtubule orientation of the stem-length mutants (Fig. 3a, Table 4a) was significantly different ($P < 0.01$) from the wild-type, the stem-length mutants having a smaller component of cells with a transverse microtubule orientation, and higher frequencies of cells with obliquely and longitudinally oriented microtubules than the wild-type at both B and D stem positions. Figs. 4, 5 and 6 illustrate the difference in pattern of microtubule arrangement between the wild-type (L107) and the stem-length mutants NGB5839 (*le*) and L212 (*lk*) in the epidermis at 12-14 mm beneath the apical hook. In all lines, average microtubule orientation in the cells of the epidermis was significantly lower ($P < 0.01$) at stem position D than at stem position B, corresponding to decreases in the frequency of the transverse microtubule orientation class, and increases in the oblique and longitudinal classes. The relationship between microtubule orientation and the change in rate of stem elongation with distance from the apical hook in the wild-type and the stem length mutants will be discussed later in this chapter.

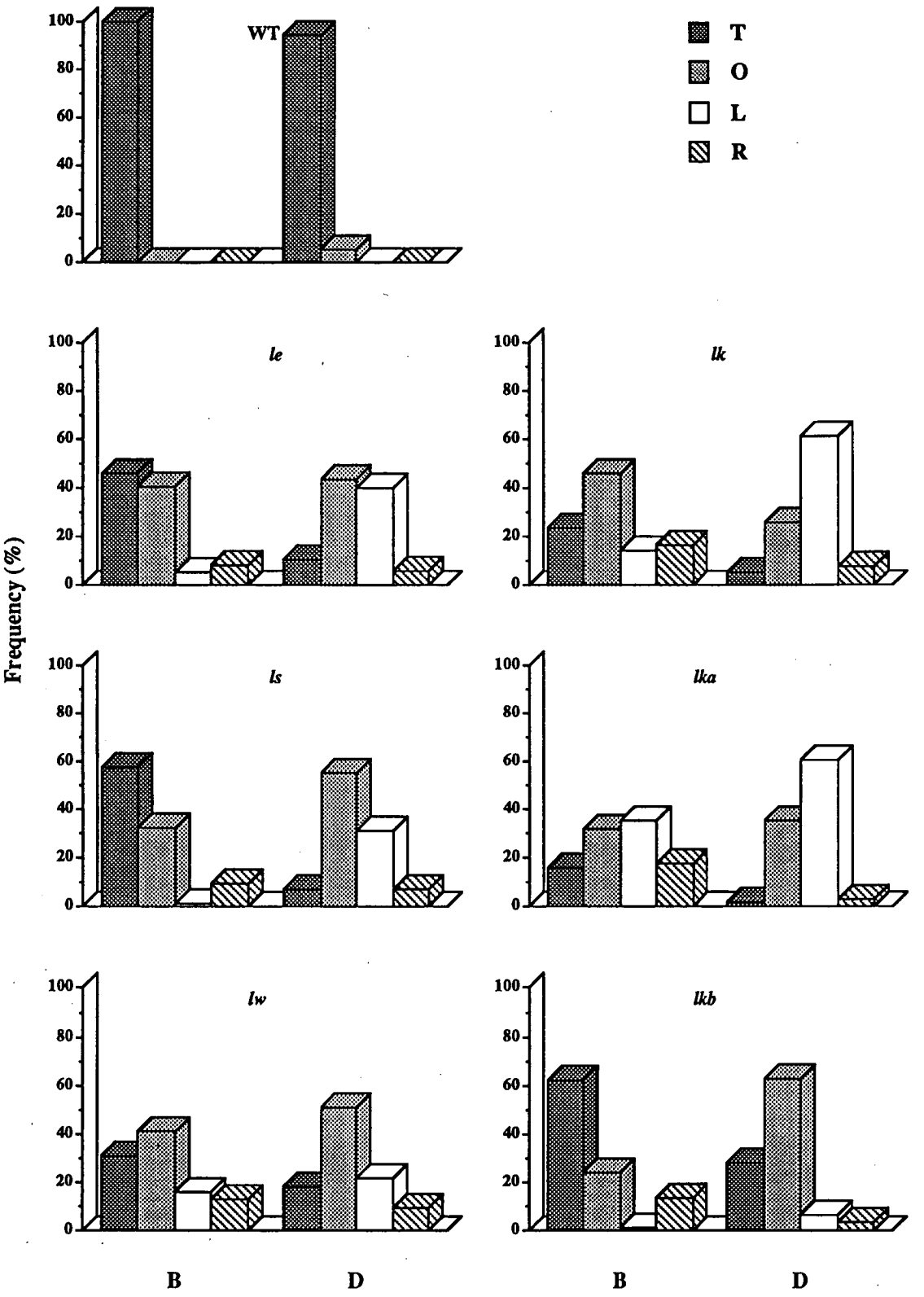


Fig. 2a Microtubule orientation in the tangential surface of epidermal cells in the 4-6 mm (B) and 12-14 mm (D) regions below the apical hook in internode 3 of a range of stem length mutants of *Pisum sativum* L. epicotyls. Observations were made when the internode was between 20-40 % expanded. The histograms show the percentage distribution of cells having microtubules arranged within the angular ranges of 0°-30° (longitudinal = *L*), 30°-60° (oblique = *O*), and 60°-90° (transverse = *T*) to the direction of the cell axis. Cells with a random (*R*) microtubule orientation are also recorded.

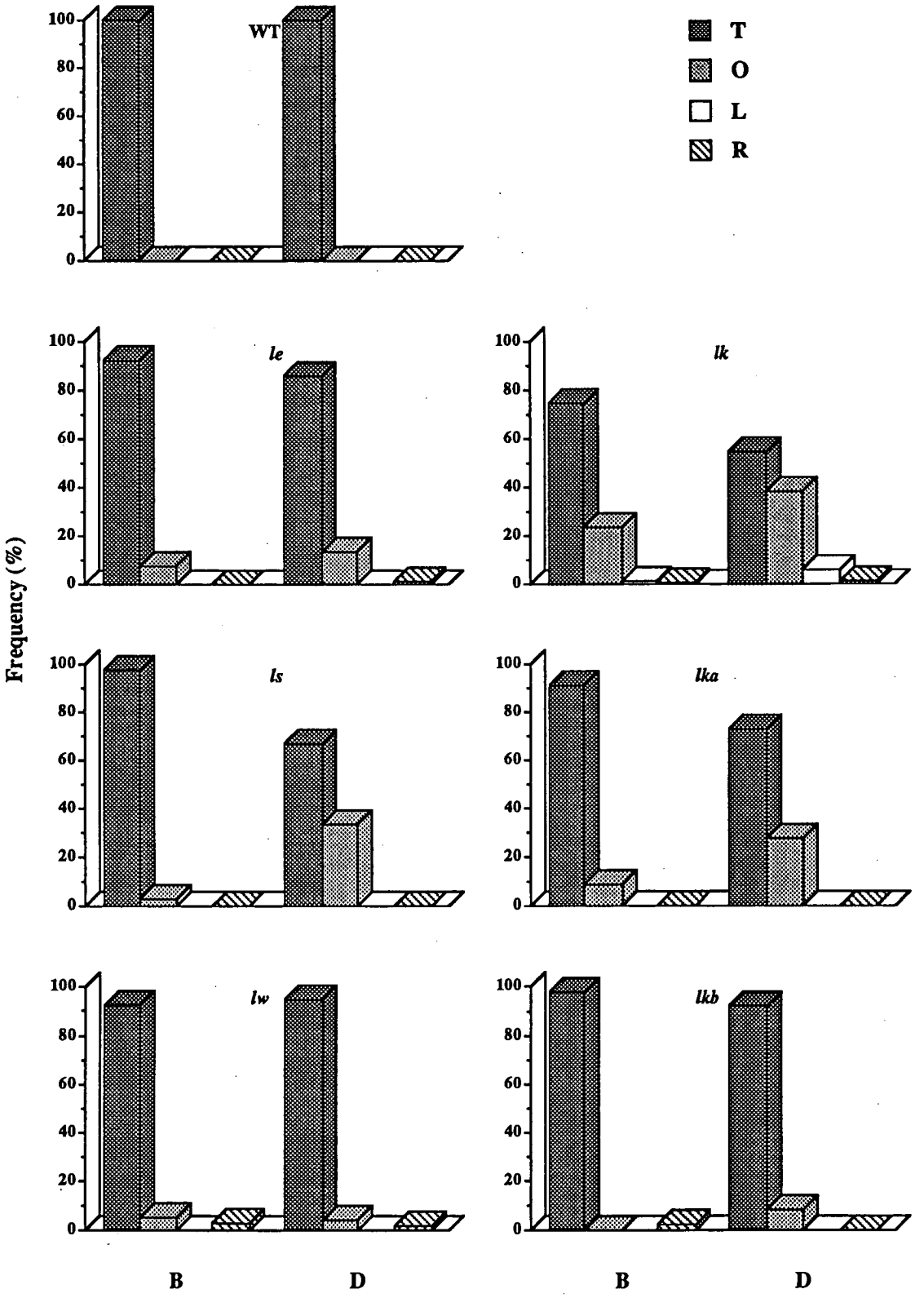


Fig. 2b Microtubule orientation in longitudinal sections of cortical cells in the 4-6 mm (B) and 12-14 mm (D) regions below the apical hook in internode 3 of a range of stem length mutants of *Pisum sativum* L. epicotyls. Observations were made when the internode was between 20-40 % expanded. The histograms show the percentage distribution of cells having microtubules arranged within the angular ranges of 0°-30° (longitudinal = *L*), 30°-60° (oblique = *O*), and 60°-90° (transverse = *T*) to the direction of the cell axis. Cells with a random (*R*) microtubule orientation are also recorded.

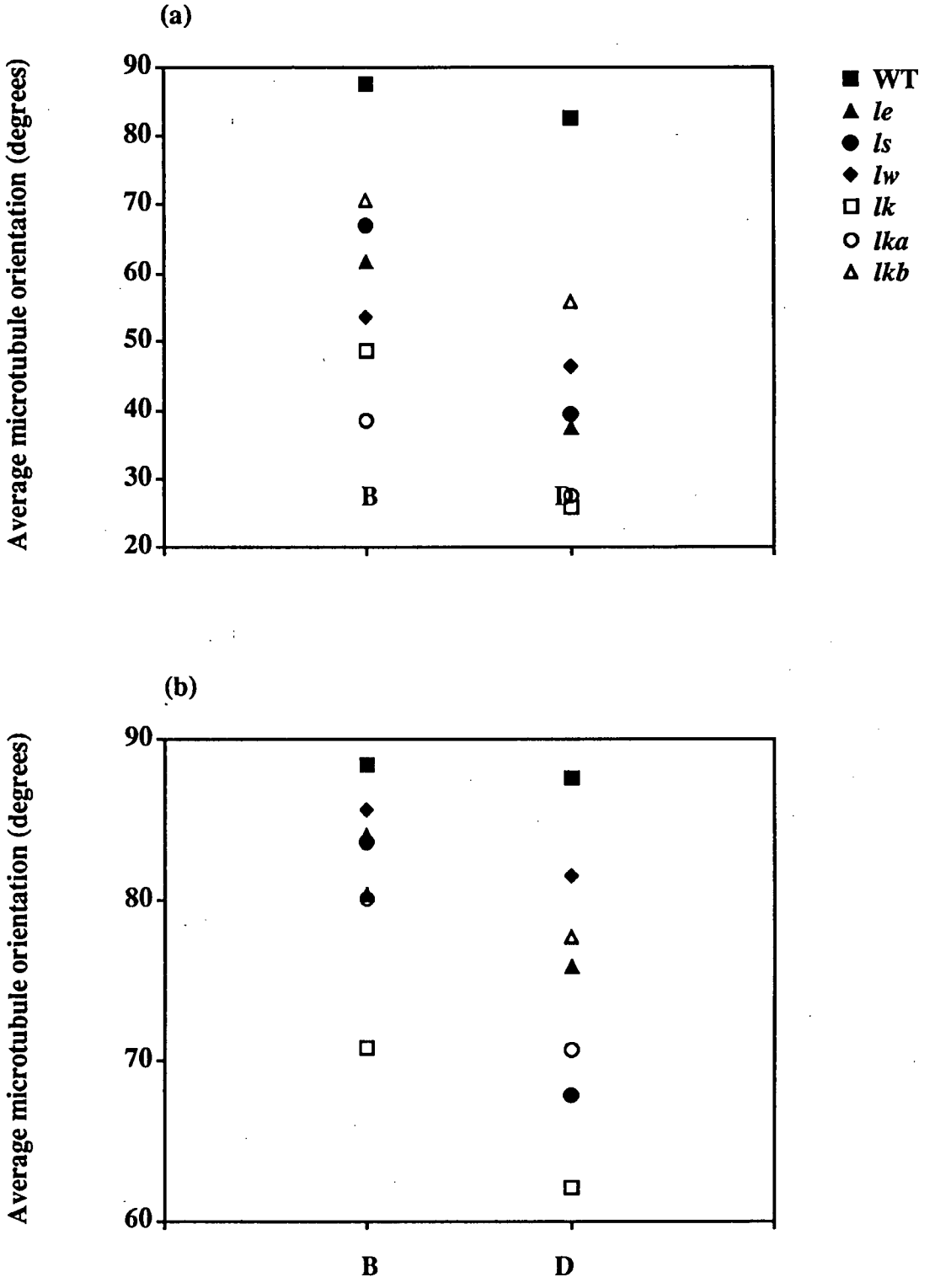


Fig. 3 Average microtubule orientation (degrees from cell axis) in **a**) the tangential surface of epidermal cells and **b**) longitudinal sections of cortical cells in the 4-6 mm (B) and 12-14 mm (D) regions below the apical hook in internode 3 of a range of stem length mutants of *Pisum sativum* L.

Table 4 Microtubule orientation at distance from the apical hook in (a) the epidermis, and (b) the subepidermal cortical cells of WT and stem-length mutants of pea

(a)		WT		*le*		*ls*		*lw*		*lk*		*lka*		*lkb*	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Internode Position B	n=	87.33 45	0.51	61.41* 172	1.47	66.66* 79	1.88	53.47* 62	2.69	48.34* 71	2.37	38.06* 47	3.31	70.48* 102	1.59
Internode Position D	n=	82.39 74	1.28	37.25* 170	1.55	39.26* 27	2.54	46.06* 180	1.46	25.52* 165	1.42	27.19* 105	1.31	55.52* 31	2.55

(b)		WT		*le*		*ls*		*lw*		*lk*		*lka*		*lkb*	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Internode Position B	n=	88.36 31	0.44	80.28* 310	0.62	83.47 38	1.12	85.5 40	1.35	70.67* 174	1.03	80.02* 56	1.51	83.91 45	1.01
Internode Position D	n=	87.44 34	0.61	75.76* 178	0.86	67.78* 9	3.66	81.48^ 153	0.69	61.94* 188	1.23	70.53* 40	1.95	77.62^ 13	3.03

Average microtubule orientation (degrees from cell axis) in a) the tangential surface of epidermal cells and b) longitudinal sections of cortical cells in the 4-6 mm (B) and 12-14 mm (D) regions below the apical hook in internode 3 of a range of stem length mutants of *Pisum sativum* L. Data are means, standard error (SE), and sample size (n). The stem-length mutants were tested for differences from the wild-type (WT) using the Fisher PLSD test. ^ $P < 0.05$; * $P < 0.01$.

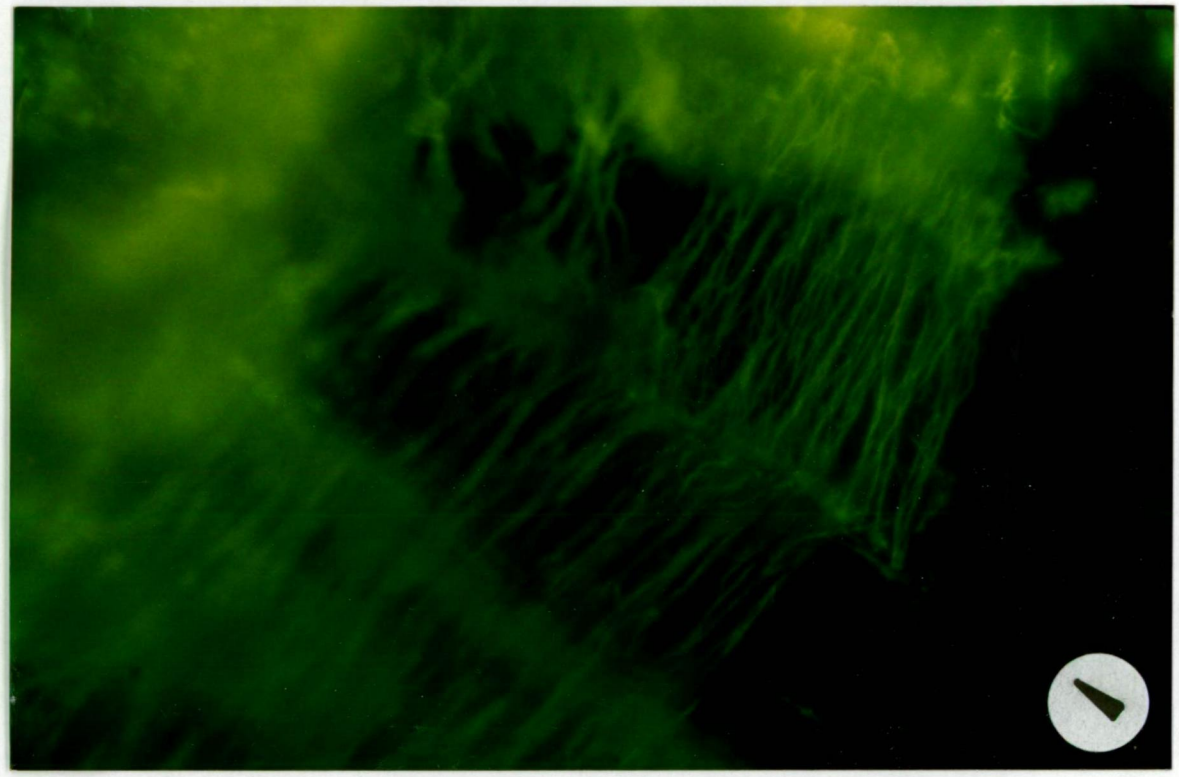


Fig. 4 Immunofluorescence micrograph of a transverse pattern of microtubule arrangement in the tangential surface of epidermal cells in the 12-14 mm (D) region below the apical hook of expanding 3rd internode of wild-type tall cv. Torsdag (L107). Direction of arrow indicates longitudinal cell axis. Magnification at x100.

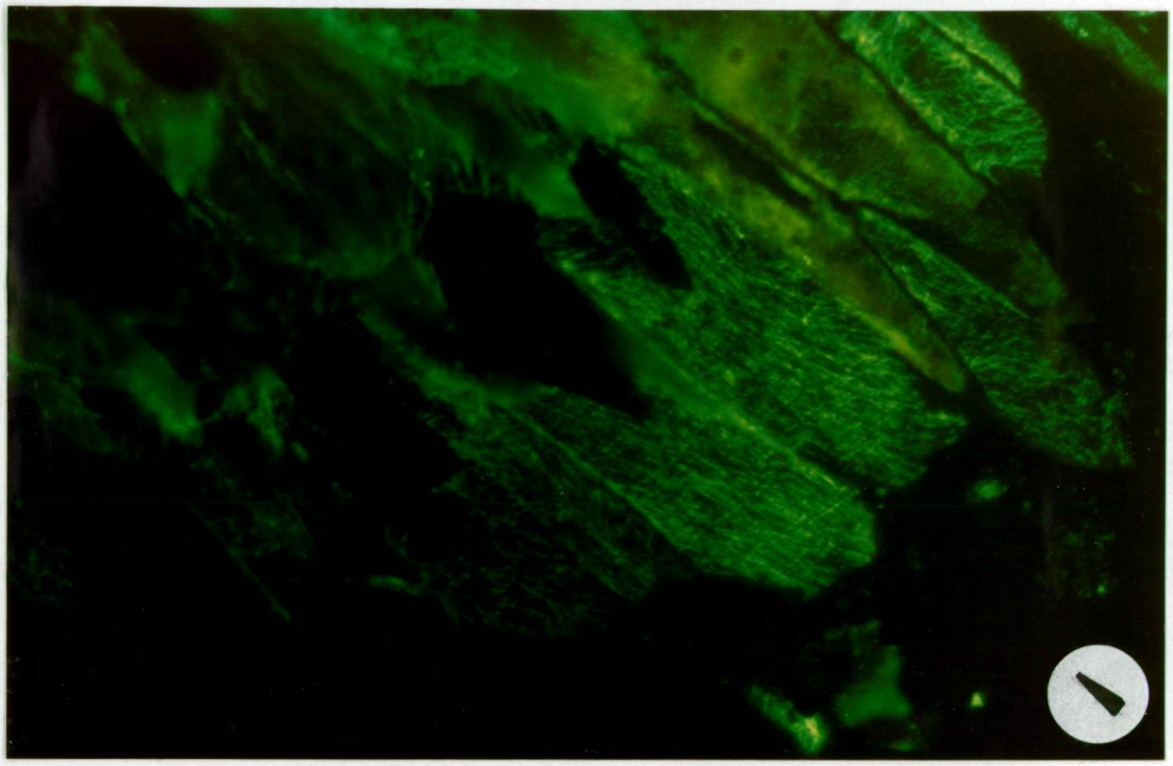


Fig. 5 Immunofluorescence micrograph of an oblique pattern of microtubule arrangement in the tangential surface of epidermal cells in the 12-14 mm (D) region below the apical hook of expanding 3rd internode of GA-synthesis mutant NGB5839 (*le*, dwarf). Direction of arrow indicates longitudinal cell axis. Magnification at x63.

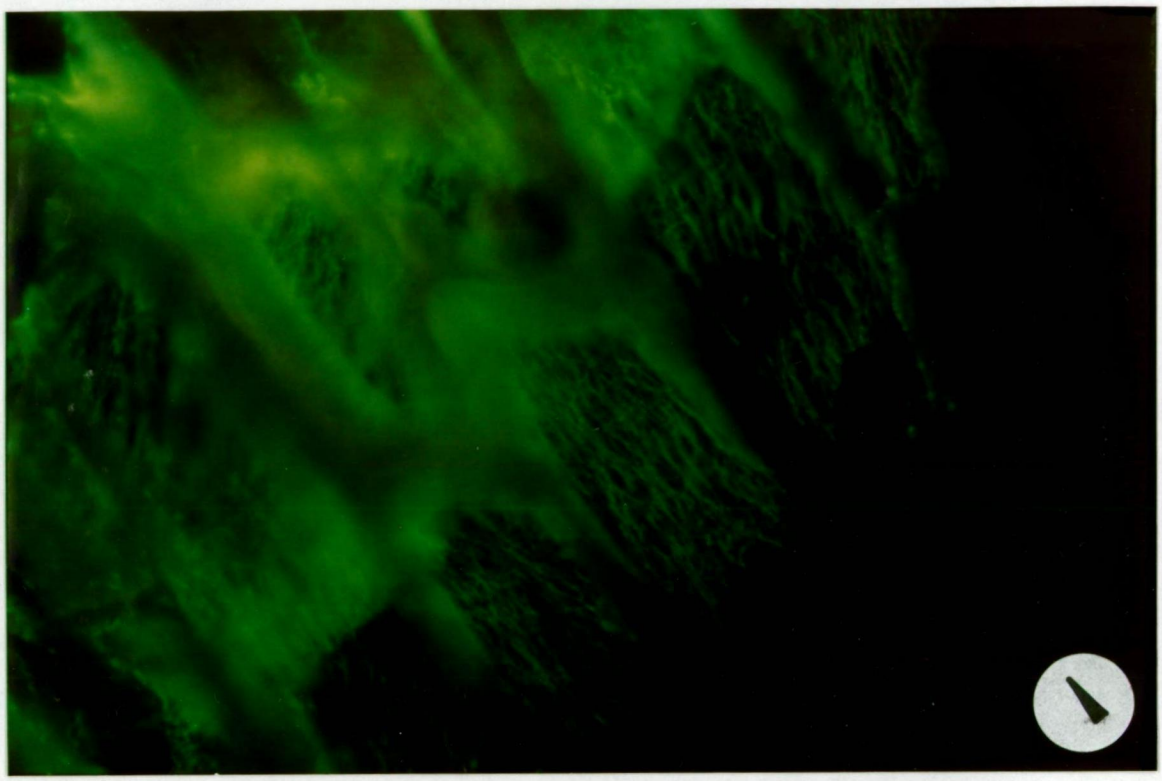


Fig. 6 Immunofluorescence micrograph of a longitudinal pattern of microtubule arrangement in the tangential surface of epidermal cells in the 12-14 mm (D) region below the apical hook of expanding 3rd internode of GA-response mutant L212 (*lk*, erectoides). Direction of arrow indicates longitudinal cell axis. Magnification at x63.

Although the more severely dwarfed of the GA-synthesis mutants, *ls* plants were observed to have a significantly higher ($P < 0.05$) average microtubule orientation at stem position B than *le* plants. At position D average microtubule orientation for these two lines was more comparable (Fig. 3a, Table 4a). Analysis of the frequency of cells within the various orientation classes (Fig. 2a) revealed that *ls* plants have a larger proportion of cells with transversely oriented microtubules at stem position B than *le* plants. At B, 57.5% of *ls* cells fell into the transverse orientation category, compared with 45.7% in *le* plants.

The GA-response mutants also differed significantly ($P < 0.01$), with respect to average microtubule orientation, from one another at both B and D internode positions, except for *lk* and *lw* at B, where average microtubule orientation, although higher in *lw*, was not significantly different from *lk*, and *lk* and *lka* at D, at which position these mutants had similarly oriented microtubules (Fig. 3a, Table 4a).

Of the GA-response mutants, *lkb* had the highest average microtubule orientation with the highest frequency of cells with transversely oriented microtubules at both stem positions. At B, 61.9% of *lkb* cells fell into the transverse orientation category, compared with 31%, 23.5%, and 15.8% in *lw*, *lk*, and *lka* plants, respectively (Fig. 2a). With the exception of *lkb* at B, the GA-response mutants had significantly different ($P < 0.01$) average microtubule orientations to the GA-synthesis mutants. At B, *lkb* and *ls* had similar average microtubule orientations (Table 4a), and the frequency of cells in the orientation classes were also comparable (Fig. 2a). Although *lka* and *lkb* are morphologically very similar plants, microtubule orientation in *lka* is more comparable to *lk* and *lw*, than to *lkb*.

Cortex

Microtubule orientation in subepidermal cortical cells from wild-type plants was exclusively transverse at both stem positions, B and D (Fig. 2b), average microtubule orientation not differing between the two stem positions (Fig. 3b, Table 4b).

Of the stem-length mutants, only *le*, *lk*, and *lka* differed significantly ($P < 0.01$) from the wild-type average microtubule orientation at B (Fig. 3b, Table 4b), having a lower frequency of cells falling into the transverse orientation category (Fig. 2b). However, at D, average microtubule orientation of all mutant lines was significantly different from the wild-type, having a comparatively reduced component of cells with transverse orientation, and an increase in the oblique component. All lines differed with respect to average microtubule orientation from the wild-type at the $P < 0.01$ level, except *lw* and *lkb* which differed at the $P < 0.05$ level. In the cortical cells of all mutant lines, as in

the epidermis, average microtubule orientation was significantly lower ($P < 0.01$) at stem position D than at stem position B (Table 4b), corresponding to decreases in the frequency of the transverse microtubule orientation class, and an increase in the oblique class (Fig. 2b).

The GA-synthesis mutants *le* and *ls*, had comparable average microtubule orientation at both B and D stem positions (Fig. 3b, Table 4b), although at D the frequency of the transverse orientation class was substantially higher for *le* (85.6%) than for *ls* (66.7%) (Fig. 2b).

The relationship between the GA-response mutants appeared more variable. These types differed significantly ($P < 0.01$), with respect to average microtubule orientation, from one another at both B and D internode positions, except for *lka* and *lkb*, and *lkb* and *lw* at B and D, at which positions they had similarly oriented microtubules (Fig. 3b, Table 4b).

Of the GA-response mutants, the tallest, *lw*, had the highest average microtubule orientation at both stem positions. Indeed, frequency of cells in the transverse orientation category increased slightly from 92.6% at B, to 94.8% at D (Fig. 2b), although average microtubule orientation was still significantly ($P < 0.01$) lower at D (Fig. 3b, Table 4b).

lkb, although of considerably shorter stature than *lw*, had an average microtubule orientation which closely approached that of *lw* (Fig. 3b), and at B the size of the *lkb* transverse orientation category exceeded that of *lw* (Fig. 2b).

In *lkb* plants average microtubule orientation was not significantly different from *lka* plants (to which *lkb* is morphologically most similar) at either stem position in the cortex (Fig. 3b, Table 4b), although the frequencies of cells in the different orientation categories was quite dissimilar at D. At this stem position, *lka* plants had a lower component of cells with a transverse microtubule orientation (72.5%), compared with 92.3% of cells with transverse microtubule orientation in *lkb* (Fig 2b).

lk plants were recorded to have the lowest average microtubule orientation values for both stem positions (Fig. 3b, Table 4b). Similarly, *lk* plants had the smallest transverse cell component at both B and D (Fig. 2b). Interestingly, plants with the *lk* allele, the most severe of the GA-response mutations, also recorded a component of cells with longitudinally oriented microtubules, 1.1% at B, and 5.8% at D.

2.3.3 Microtubule orientation and growth rate

Scattergraphs showing the relationship bewteen growth rate and microtubule orientation (Fig. 7a, b) revealed a generally positive correlation between higher rates of elongation and a more transverse microtubule arrangement. Higher growth rate and transverse microtubule orientation was less strongly correlated in the epidermis than in the cortex. It is suggested that the inherent variability of microtubule orientation in epidermal cells may have, to some extent, contributed to the weakness of the correlation.

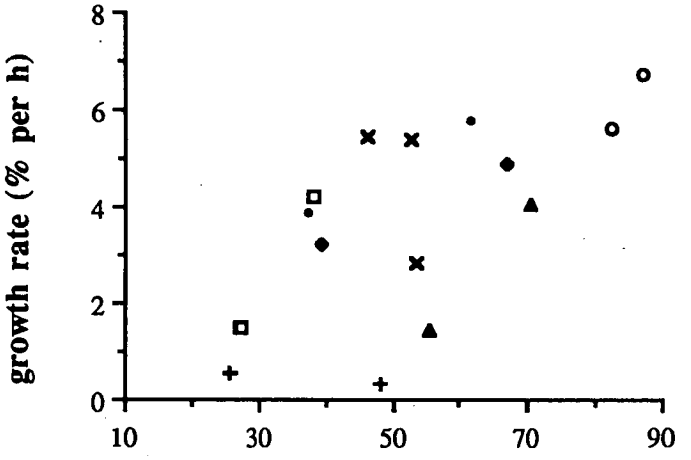
In the epidermis (Fig. 7a) the correlation between growth rate and microtubule orientation had an R^2 value of 0.415. A positive correlation between higher growth rate and a more transverse microtubule orientation is exhibited by the wild-type and by the mutants *le*, *ls*, *lka* and *lkb*. Wild-type plants exhibited both the highest growth rates and the most transversely arranged microtubules. The GA-synthesis mutants *le* and *ls* displayed a similar trend, although elongation rates were lower, and microtubule orientation less transverse, than in the wild-type. GA-response types *lka* and *lkb*, had very similar growth rates at both positions B and D, although at similar growth rates average microtubule orientation was considerably less transverse in *lka* than in *lkb*.

A positive correlation between higher growth rate and transverse microtubule orientation was not displayed by *lk* plants. However, the extreme reduction of *lk* internode length made application of the ink stamp difficult, and possibly resulted in the zone of most transversely oriented microtubules being missed.

In contrast to the other lines, *lw* types did not exhibit a positive correlation between elongation rate and transverse microtubule arrangement. Although average microtubule orientation was comparable at stem positions B and C (data not shown), elongation rate was substantially lower at B. However, this anomalous pattern may be explained if is accepted that organization of the microtubule array into a relatively transverse arrangement may precede the onset of elongation growth.

In the cortex (Fig. 7b) the correlation between growth rate and microtubule orientation had an R^2 value of 0.600. The pattern of correlation was generally similar to that observed for the epidermis. However, the change in transversness of microtubule orientation with decreasing growth rate appeared more gradual than in the epidermis.

a)



b)

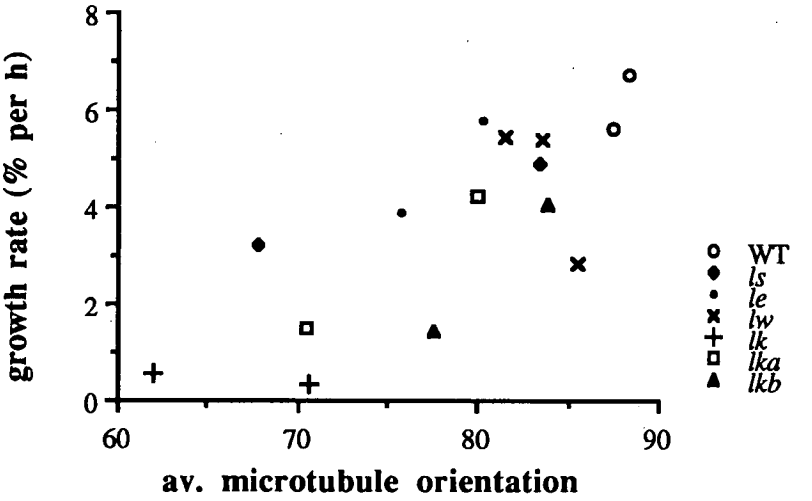


Fig. 7a, b The correlation between average microtubule orientation (degrees from longitudinal cell axis) and growth rate (% per h) for a) epidermal and b) cortical cells. r^2 = 0.42 and 0.60 respectively. Growth rate and microtubule orientations were obtained at 4-6 mm (B), 6-8 mm (C) and 12-14 mm (D) from the apical hook (see Tables 3 and 4a, b).

2.4 Discussion

The valid assessment of the relative importance of various parameters related to extension growth requires that these parameters are precisely related to rate of extension growth. A detailed growth profile, showing rate and distribution of growth along the stem, is therefore essential to meeting these requirements.

Behringer *et al.* (1990a) measured the growth profile along the epicotyl of a range of dark-grown stem length mutants of *Pisum*, both GA-synthesis and GA-response types, in order to investigate the relationship between certain biophysical parameters (osmotic presure, plastic compliance, plastic extensibility, wall yield threshold, and wall yield coefficient) and GA-related internode extension. They found a positive correlation between wall yield threshold and rate of epicotyl extension in the five genotypes examined. Since the altered rates of stem elongation in these genotypes result from altered GA levels or responsiveness, Behringer *et al.* (1990a) concluded that GA regulates growth, at least in part, by influencing the yield threshold of the wall.

The present study has extended the work of Behringer *et al.* (1990a) by determining the growth profiles of additional stem length mutants within the GA-synthesis and GA-response categories of *Pisum*.

It is considered that the growth profiles from these mutants have further clarified the role of endogenous GA upon extension growth by demonstrating the effects of the various impairments to GA action on rate and distribution of extension growth, and by providing the basis for interpretation of the relationship between microtubule orientation and extension growth.

Growth profiles of GA-synthesis and GA-response mutants

The construction of growth profiles (Fig. 1) for the range of internode-length mutants in this study revealed that the mutant alleles, *le*, *ls*, *lw*, *lk*, *lka*, and *lkb*, resulted in both a decreased rate of extension and an attenuated region of expansion in comparison to wild-type plants. It appears therefore, that at the morphological level, dwarfism in both the GA-synthesis and GA-response mutants is the result of both a decreased rate of extension and an attenuated region of expansion. Excluding *lw*, the stem length mutations appear to cause such a reduction in the dimensions of the wild-type growth curve without markedly altering its overall shape (Fig. 1).

Relationship between elongation rate and microtubule orientation

Microtubule orientation in the expanding dark-grown epicotyls of a range of GA-synthesis and GA-response mutants of *Pisum* was investigated. Comparison of wild-type microtubule orientation with the elongation profile for wild-type plants revealed a positive correlation between transverse microtubule orientation in epidermal cells and elongation rate (Fig. 7a). Average microtubule orientation in the wild-type was more transverse at 4-6 mm than at 12-14 mm below the apical hook (Fig. 3a) corresponding to a decrease in growth rate over this distance (Fig. 1). These results are consistent with proposals that a transverse microtubule orientation promotes extension growth.

Comparison of microtubule orientation and extension rates in the GA-synthesis and GA-response mutants also support a causal relationship between a transverse microtubule arrangement and elongation. In both the epidermis and the cortex of *le* and *ls* plants, a more transverse microtubule arrangement appears to correlate with higher rates of extension growth. However, the relationship between microtubule orientation and elongation rate may be subject to modification by other factors. *ls* plants were observed to have a slightly higher transverse microtubule component than *le* plants at position D, even though extension rates in *le* internodes were higher at this position.

The effect of the stem length mutations on rate and distribution of extension growth and microtubule orientation

Genes *le* and *ls:*

The mutant alleles *le* and *ls* have been reported to block the biosynthesis and metabolism of GA at specific sites in the early-13-hydroxylation pathway (see Fig. 1, Reid and Ross 1993). The resultant reductions in endogenous levels of GA_1 produce plants which are phenotypically dissimilar to the wild-type (Ingram *et al.* 1983, 1984; Ingram and Reid 1987b; Ross *et al.* 1992a; S. M. Swain and Y. Kamiya, unpublished results). The dwarfing effect of the *le* mutation is completely removed by application of GA_1 to *le* plants.

The substantially reduced extension rates of the GA-synthesis mutants, *le* and *ls*, correspond to their reduced levels of GA_1 (Ingram and Reid 1987a; Ross *et al.* 1989), and support a quantitative relationship between the level of GA_1 and internode growth. Ingram *et al.* (1986), Ross *et al.* (1989) and Swain and Reid (1992) have previously reported such a relationship in *Pisum.*

Average microtubule orientation in *le* plants was shown to differ significantly from the wild-type arrangement in the epidermis (Fig. 3a, Table 4a), and, to a lesser extent, in the subepidermal cells of the cortex (Fig. 3b, Table 4b), indicating that the *le* mutation affects microtubule orientation, primarily in the epidermis.

It would appear that the *le* mutation has its effect upon microtubule orientation through the reduction of endogenous GA_1 caused by the disturbance of the 3ß-hydroxylation of GA_{20} to GA_1. The putative involvement of GA receives support from previous reports, outlined in Chapter 1, of a positive relationship between level of GA_1 and transverse microtubule orientation in a number of plant species. The severely reduced endogenous level of GA_1 in the *d*5 mutant of maize (Phinney 1984) is thought to be responsible for the relatively small population of cells with transversely arranged microtubules (Ishida and Katsumi 1991). Exogenous GA treatment has been shown to produce an increase in the number of cells with transversely oriented microtubules in the epicotyl of pea (Akashi and Shibaoka 1987; Sakiyama and Shibaoka 1990; Sakiyama-Sogo and Shibaoka 1993) and azuki bean (Shibaoka 1974; Takeda and Shibaoka 1981b), the mesocotyl of maize (Mita and Katsumi 1986; Ishida and Katsumi 1991) and rice (Nick and Furuya 1993), the hypocotyl of cucumber (Ishida and Katsumi 1992), the leaf sheath of onion (Mita and Shibaoka 1984a, b), and in lettuce (Sawheney and Srivastava 1975). The use of GA-biosynthesis inhibitors, such as S-3307, has also yielded results which support the involvement GA in the altered microtubule arrangement of *le* plants.

Mita and Shibaoka (1984a) reported a shift from a normally transverse arrangement of microtubules to an oblique to longitudinal arrangement in onion leaf sheath cells following application of S-3307.

The situation in *le* appears to be comparable with that found in certain dwarf mutants of maize. The nonallelic dwarf mutants of maize, *d*1, *d*2, *d*3 and *d*5, have reduced GA-like activity when compared to wild-types, and become phenocopies of wild-types after exogenous application of GA_3 (Phinney 1961; Fujioka *et al.* 1988). The mutations each impair a different step in the early-13-hydroxylation pathway of GA biosynthesis (see Phinney *et al.* 1990). Like *le*, the *d*1 gene controls the conversion of GA_{20} to GA_1 (Phinney *et al.* 1990).

Katsumi and Ishida (1990), using immunofluorescence microscopy, observed a distinct difference in the distribution of microtubule orientation patterns between *d5* and wild-type seedlings. The frequency of transversely oriented microtubules in the tangential surfaces of epidermal cells and in the cells of the cortex of *d5* plants was much lower than in wild-type plants. Katsumi and Ishida (1990) have proposed that the dwarf habit of *d5* is attributable to a disturbance to microtubule orientation resulting from an insufficient supply of endogenous GA.

Although the relationship between GA and transverse microtubule orientation appears indisputable, it is unclear whether GA is responsible for the initial orientation of microtubules into transverse arrays, the stabilization microtubules already in a transverse arrangement, or a combination of the two. Some clarification of this problem has been provided. Mita and Katsumi (1986) reduced dwarfing in the *d5* mutant of maize by exogenous application of GA_3. They proposed that exogenously applied GA_3 induced growth of *d5* by stabilizing microtubule orientation in a transverse direction. This conclusion is supported by the findings of Katsumi and Ishida (1990), who reported that exogenous application of GA_3 resulted in an increase in the number of cells with transversely oriented microtubules.

The observed microtubule orientation in *le* plants may indicate that GA functions in these plants in the stabilization of arrays in a transverse arrangement. Although significantly less frequent than in the wild-type, *le* plants were observed to have a population of transversely oriented microtubules at position B, 4-6 mm below the apical hook in internode 3, suggesting that wild-type levels of GA_1 are not necessary for an initially transverse microtubule arrangement. However, the reduced component of cells with transversely oriented microtubules may indicate that wild-type levels of GA_1 are required to produce the exclusively transverse arrays visualized at B in wild-type plants. Hence, the involvement of GA in the initial organization of microtubules into transverse arrays cannot be excluded.
In addition, *le* is a leaky mutant in which small quantities of GA_1 and GA_8 are quantifiable. These low levels of endogenous GA may be sufficient to account for the observed transverse microtubule component.

The size of the transverse orientation category in the epidermis of *le* plants became further reduced with distance down the epicotyl, from 61.4% at B to 37.3% at D, compared with a reduction of less than 5% in the wild-type over the same distance (Table 4a). These results indicate that microtubules remain in a transverse orientation over a shorter distance of epicotyl in *le* plants than in wild-type plants. It is also possible that the *le* mutation reduces the duration of time microtubule arrays remain

transversely oriented, although this cannot be concluded from the present results. Alternatively, it could be argued that the shorter distance over which the array remains transversely oriented is a direct consequence of the reduced cell lengths of *le* internodes. However, GA-induced microtubule reorientations in non-elongating systems (Mita and Shibaoka 1984b; Laskowski 1990; Sakiyama-Sogo and Shibaoka 1993) do not support passive, elongation-dependent, orientation changes. It is possible that GA has a dual role in the maintenance and initial assembly of transverse arrays.

The present results support the conclusions reached by Katsumi and colleagues (Mita and Katsumi 1986; Katsumi and Ishida 1990) and others (see Chapter 1) that an insufficient supply of endogenous biologically active GA can limit cell elongation by altering microtubule arrangement, and thus reducing the population of cells with a high growth potential.

The *ls* mutation results in very low levels of GA-like activity which corresponds to the extreme-dwarf phenotype exhibited by *ls* plants (Reid and Potts 1986). Although endogenous levels of GA_1 are severely reduced in *ls* plants (Swain 1993), *ls* plants appear to be leaky (Reid 1986; Ross *et al.* 1989). Internode length in *ls* plants is even shorter than in *le* plants, as a direct consequence of reduced cell length and number (Reid and Potts 1986). However, despite even lower GA_1 content, microtubule orientation in *ls* plants at times diverged less from the wild-type arrangement than in *le* plants. This anomaly was most noticeable in the epidermis, where average microtubule orientation in *ls* was more transverse at B than in *le* plants. At D, however, average microtubule orientation in *le* and *ls* plants was comparable. Average microtubule orientation was also comparable in the cortex, although, at D, *ls* had a smaller component of cells with transversely oriented microtubules.

As for *le*, it would appear that altered microtubule arrangement in *ls* is a consequence of reduced levels of endogenous GA_1 in the tissue, although average microtubule orientation does not appear to correlate exactly with endogenous levels of active GA_1 (Ross *et al.* 1989; Swain 1993). It is possible that a threshold exists, below which levels of GA are insufficient to maintain microtubule arrays in a transverse orientation. Thus, the difference in endogenous GA_1 levels in *le* and *ls* plants may be masked as a result of the extremely low GA concentrations. That is, at such low levels, the stability of microtubules or factors which affect their stability, may be insensitive to differences in the level of GA. It may be possible to test this proposal by exogenous application of a range of GA_1 concentrations to wild-type plants treated with inhibitors of GA-biosynthesis.

The proposal that microtubule orientation may not be affected by changes in GA_1 levels at very low concentrations, does not exclude the possibility that the extensibility of the cell wall itself is susceptible to even small differences in endogenous GA levels. Behringer *et al.* (1990a) reported that the GA_1-deficient *ls* plants have an elevated wall yield threshold (2.7 bars) and reduced wall yield coefficient when compared with the wild-type. Differences in GA_1 levels and their effect upon wall extensibility may contribute toward the comparatively greater cell lengths in *le* plants than *ls* plants, despite the sometimes more severely altered microtubule arrangement in *le* plants, although at some point, microtubule orientation could become limiting. The effect of GA upon wall extensibility will be discussed further in this chapter.

Gene *lw:*

The gene *lw* produces a phenotype which, in comparison with wild-type plants, exhibits reduced internode length when grown in darkness, although not to the same extent as *le* and *ls* (Jolly *et al.* 1987; Weller and Reid 1993).

In comparison with wild-type plants, *lw* was found to produce a decrease in maximum growth rate. However, unlike *le* and *ls*, *lw* does not appear to directly influence the biosynthesis of active gibberellins, since the shoots of *lw* plants have been reported to contain similar levels of extractable GA-like substances to comparable wild-type plants (Jolly *et al.* 1987). Therefore, the reduced growth rates exhibited by *lw* plants cannot be explained by decreased endogenous levels of active GA_1.

Neither can the significantly ($P < 0.01$) lower average microtubule orientation in the epidermis of *lw* plants, when compared with wild-type plants, be explained by a decrease in endogenous GA_1. However, the alteration of microtubule orientation from the wild-type pattern by the *lw* allele is consistent with some impairment to GA_1 action.

A reduced response to exogenously applied GA has been demonstrated in *lw* plants, application of GA_1, GA_{20} or GA_{19} to *lw* plants failing to produce a phenocopy of the wild-type (Jolly *et al.* 1987). It is therefore possible that normal microtubule orientation (and therefore extension growth) in *lw* plants is impaired by the decreased responsiveness to endogenous GA of some developmental process. However, it seems probable that the *lw* allele acts via a mechanism that only partially overrides the response to GA_1, since the further reduction of *lw* internode length by genetic or chemical means has been shown to be markedly reversed by applied GA_1 (Jolly *et al.* 1987).

Although *lw* plants possess longer internodes than the GA-synthesis mutants (Jolly *et al.* 1987), average microtubule orientation in the epidermis of *lw* plants was significantly ($P < 0.01$) lower than the GA-synthesis types *le* and *ls* at B, but generally higher at D. Average microtubule orientation at a point intermediate between B and D along the epicotyl (position C, 6-8 mm; data not shown) was not significantly different from B, indicating that the state of microtubule orientation at B was maintained for at least 6 mm along the epicotyl. In this respect, average microtubule orientation in *lw* differed from the typical pattern of decrease from B to D seen in *le* and *ls* plants, and from GA-response types where average microtubule orientation was observed to become gradually and continuously less transverse from B to D.

Correlating with this pattern of microtubule orientation, *lw* also appeared to shift maximum growth rate along the epicotyl further from the apical hook in comparison to the wild-type, although below 12-14 mm, growth rate of *lw* plants was parallel to that measured for wild-type plants.

The growth profile of *lw* mutants is also dissimilar to the GA-synthesis mutants and the GA-response mutants, *lk*,*lka* and *lkb*. Initially growth rate is comparable with *lka* and *lkb*, but in comparison to these mutants, and with *le* and *ls*, increase in rate of growth is only gradual. Maximum growth rate of *lw* is comparable to that of the GA-synthesis types, *le* and *ls*, however, the position at which maximum growth rate occurs in *lw* is noticeably further down the epicotyl. As a consequence, *lw* types have an elongation zone that extends over a greater distance of epicotyl in comparison to both mutant categories.

This departure from the more typical pattern of microtubule orientation and growth rate and distribution is consistent with the suggestion that in *lw* a different mechanism is operating from that found in either the GA-synthesis types or the other GA-response mutants in this study. Weller and Reid (1993) have indicated that *lw* appears to modify phytochrome-mediated responses, since the extent of dwarfing of *lw* plants was dependent on light quality and fluence rate.
Weller and Reid (1993) reported that the *Lw/lw* difference in internode length was expressed to a much greater extent in red (41% reduction in length relative to the wild-type) and in white (51% reduction) light, than in darkness. In darkness, *lw* plants were only 13% shorter than wild-type plants. The dwarfing effect of the *lw* mutation was also found to be expressed most clearly at low irradiances (73% reduction at 6 $\mu mol \cdot m^{2} \cdot s^{-1}$) than at higher irradiances (54% reduction at 25 $\mu mol \cdot m^{2} \cdot s^{-1}$). It is possible that the *Lw/lw* differences in microtubule orientation would be more clearly expressed in *lw* plants grown under conditions of low fluence white light-irradiation.

However, the masking effect of chlorophyll autofluorescence was minimized in dark-grown seedlings, greatly facilitating immunofluorescence observation of the FITC-stained microtubules.

Since average microtubule orientation became only gradually less transverse with distance along the epicotyl, changing only marginally between B and C, it seems unlikely for a large decrease in transverseness to have ocurred prior to B. It is therefore suggested that *lw* might affect the orientation into which microtubules are initially assembled, rather than their maintenance in a transverse orientation. Such alteration of initial microtubule orientation might translate into a supression of initial elongation rate, resulting in the flattened peak of maximum growth observed in the *lw* growth profile.

The involvement of phytochrome argues in favour of an indirect role for the *lw* allele in the impairment of normal microtubule organization, and, given the decreased response of the mutant *lw* to GA_1 (Jolly *et al.* 1987), it is possible that the gene *lw* acts to reduce internode length via a mechanism that partially overides the response to GA_1. However, the nature of the relationship between phytochrome function, responsiveness to GA_1, microtubule orientation and elongation is, at present, unknown, as is the effect of the *lw* allele upon the biophysical properties of the cell wall.

Genes *lk*, *lka*, and *lkb:*
The putative GA-response types are characterized by the erectoides (*lk*) and semi-erectoides (*lka, lkb*) phenotypes. Plants with these phenotypes have substantially reduced internode lengths when compared with the wild-type (Ross and Reid 1986; Reid and Ross 1989).

The GA-response mutants, *lk*, *lka*, and *lkb*, exhibited more severely reduced growth rates than the GA-synthesis mutants. *lk*, *lka*, and *lkb* had lower maximum growth rates and maintained maximum growth over a shorter length of epicotyl than the GA-synthesis mutants, although length of growth zones in GA-synthesis and GA-response types were approximately comparable.
The differences in growth rates of the GA-synthesis and GA-response types are consistent with the impairment of separate processes leading to elongation growth.

Average microtubule orientation in the epidermis of all three lines was significantly ($P < 0.01$) less transverse than in wild-type plants. Microtubule orientation in the cortex was also less transverse, although not always significantly, and never to the extent reached in the epidermis.

Since endogenous levels of GA_1 are essentially normal in the three mutant lines (Reid and Davies 1992; Reid 1986; Reid and Potts 1986; Reid and Ross 1989; Behringer *et al.* 1990a; Lawrence *et al.* 1992), it is unlikely that reduced levels of endogenous GA_1 are responsible for either the differences in rate and distribution growth or the disturbance to wild-type microtubule orientation in these lines, as is apparently the case with *le* and *ls*.

The *lk*, *lka*, and *lkb* alleles result in plants which fail to become phenocopies of wild-type plants in response to applied GA_1 (Reid and Potts 1986; Reid and Ross 1989). In this respect these types are similar to *lw*. In addition, like *lw*, the mutations do not appear to affect the reception of the GA_1 signal, since *lk*, *lka*, and *lkb* plants are phenotypically dissimilar to the GA-deficient mutants. However, *lk*, *lka*, and *lkb* plants are substantially dissimilar to *lw* in a number of other respects. Unlike *lw*, the response to various light regimes has been shown to be largely comparable to wild-type plants (Reid and Davies 1992), and it is unlikely that these mutations act on microtubule arrangement via altered photomorphogenic responses.

Although *lk*, *lka*, and *lkb* plants have been referred to as GA-response mutants, it is unlikely that these mutant alleles operate entirely upon microtubule orientation via a reduced sensitivity to the biologically active GA_1. It is suggested that the mutant allele at the *lkb* locus, and perhaps those at the *lka* and *lk* loci, may exert their effect upon microtubule arrangement, and hence extension growth, by reducing the endogenous level of IAA.

A relationship between IAA and extension growth in the mutants *lk*, *lka* and *lkb* of *Pisum* is supported by the following observations. Bandurski *et al.* (1990) and Ortúno *et al.* (1990) have indicated that a quantitative relationship exists between IAA concentration and growth rate. Law and Davies (1990) and Behringer *et al.* (1992) have attributed changes in internode length of *Pisum* to changes in IAA level, and Reid and Davies (1992) have reported a 2 to 3-fold reduction of IAA levels in *lkb* plants in comparison with wild-type plants.

In addition, McKay *et al.* (1994) have recently used IAA transport inhibitors (TIBA and HFCA) to promote elongation and increase IAA levels above the treatment site in expanding internodes of *lka* and *lkb* plants. Davies and colleagues (P. J. Davies; unpublished results) have recently demonstrated an IAA-induced promotion of internode length in *lkb* plants, which resulted from an increase in both cortical and epidermal cell length, although phenocopies of the wild-type were not produced.

In addition to its proposed effects on extension growth, the involvement of IAA in the disturbed microtubule arrangment in the mutants is also suggested. IAA has been reported to promote a transverse microtubule orientation (Bergfeld *et al.* 1988; Iwata and Hogetsu 1989b; Nick *et al.* 1990). Iwata and Hogetsu (1989b) showed that exogenous IAA application retarded the change from a transverse to an oblique microtubule orientation in *Avena* mesocotyls, induced by white light irradiation.

The IAA deficiency in *lkb* has been observed to be restricted to the inner tissues, since epidermal peels contained normal IAA levels (McKay *et al.* 1994). McKay *et al.* (1994) have proposed that this inner tissue deficiency is responsible for the "zig-zagging" of the inner pith and vessels observed in light-grown *lk*, *lka* and *lkb* plants (Ross and Reid 1986). It is also possible that the reduced level of endogenous IAA in *lk*, *lka*, and *lkb* is directly responsible for the disturbed microtubule orientation in the cortex of these plants, despite the proposals that the epidermis is the primary site of auxin action in elongation growth (Masuda and Yamamoto 1972; Mentze *et al.* 1977; Brummel and Hall 1980; Kutschera and Briggs 1988b), a proposal which has been disputed by Rayle *et al.* (1991). Furthermore, the reduced IAA levels in the cortex may also be indirectly responsible for the loss of transverse microtubule arrangement in the epidermis.

McKay *et al.* (1994) have suggested that increased stress caused by "zig-zagging" of the inner tissues might cause the production of elevated ethylene levels in the tissues. That tissue stress is increased in these mutants is supported by the measurements of wall yield threshold, wall yield coefficient, and turgor pressure reported by Behringer *et al.* (1990). In addition, Reid and Davies (1992) have postulated that the swelling and horizontal splitting observed in the stems of dark-grown *lk*, *lka* and *lkb* plants, which is absent in GA-deficient mutants of comparable stature, may result from increased turgor pressure in these mutants.

The available literature has indicated that ethylene favours longitudinal microtubule orientation (Shibaoka 1974; Steen and Chadwick 1981; Lang *et al.* 1982; Roberts *et al.* 1985).and that ethylene treatment of tissue segments results in the cessation of elongation and the promotion of lateral expansion (see Roberts *et al.* 1985).
Since the rapidity of microtubule reorientation by exposure to ethylene is suggestive of a short, or rapid chain of events between target and effector (Roberts *et al.* 1985), it is conceivable that this proposed increase in ethylene production, induced by stresses in the cortex, could bring about the observed microtubule arrangement in the epidermis over a relatively short length of epicotyl.

Ross and Reid (1986) found that ethylene production was higher in *lk* segregates than in *Lk* segregates, and demonstrated that application of the ethylene synthesis inhibitor, AVG, resulted in increased internode elongation in *lk*, but not *Lk* plants. The ethylene-releasing compound, ethephon, had the reverse effect when applied to *Lk* plants. However, AVG did not result in a phenocopy of the wild-type, and ethephon did not produce the erectoides phenotype.

The shoot anatomy of *lk*, *lka*, and *lkb* plants is also consistent with higher ethylene levels. In all three mutants the epidermis consists of cells that are shorter (*lk*, *lka*, *lkb*) and sometimes fewer (*lk*, *lka*) than those of the wild-type (Ross and Reid 1986; Reid and Ross 1989). Ethylene has been previously reported to promote isodiametric growth (Burg and Burg 1965) and to inhibit cell division (Apelbaum and Burg 1972) in pea internodes.

Ethylene-induced reorientation of microtubules away from a transverse arrangement may at least partially account for the reduced transverse microtubule component in *lk*, *lka*, and *lkb*, at a distance of only 4-6 mm beneath the apical hook. Alternatively, the *lk*, *lka*, and *lkb* alleles may prevent the initial assembly of microtubules into transverse arrays. Complications with staining microtubules in this region, and masking of FITC fluorescence by pro-chlorophyll autofluorescence, made quantification of microtubule orientation difficult. There is some evidence that the former situation is operating in *lkb*, since immunofluorescence observation of microtubules in the 0-2 mm region below the apical hook of *lkb* plants provided some evidence of transversely oriented arrays, and in any case a transiently transverse array might easily remain undetected. However, the low frequency of cells with transversely arranged microtubules, even in rapidly elongating epicotyl, is consistent with the latter proposal.

It is possible that a combination of reduced IAA level and increased ethylene production are operating to reduce extension rate, at least in *lkb* plants, since the use of the ethylene inhibitor, AVG, in conjunction with the auxin transport inhibitor, HFCA, has been reported to restore the wild-type internode length to *lkb* plants, whereas AVG or HFCA alone did not produce a phenocopy (McKay *et al.* 1994).

Although it is likely that mutants *lk*, *lka*, and *lkb* act to reduce IAA levels, a reduced sensitivity to GA_1 may also operate. *lk*, *lka*, and *lkb* may exert their effect well down the transduction pathway which leads from GA_1 reception to stem elongation. An interactive relationship between GA and IAA may exist, since Shibaoka (1972) has reported the inability of GA_3 to promote elongation in azuki bean epicotyl sections in the absence of auxin. Sakiyama-Sogo and Shibaoka (1993) have also proposed that the inability of GA_3 to promote the elongation of stems of decapitated dwarf pea cuttings results from the elimination of endogenous auxin caused by the removal of apices.

The situation in *lka* (and perhaps also in *lk*) may differ from that in *lkb* since, although growth profiles of *lka* and *lkb* were comparable, the frequency of cells with transversely oriented microtubules was considerably lower in these two mutants in the cortex, and particularly in the epidermis (Figs 2a, b). In addition, *lka* plants also exhibited a reduction in cell number, in contrast to *lkb* in which cell number did not differ significantly from wild-type plants (Reid and Ross 1989). This impairment to cell division in *lka*, may imply a more direct affect upon initial microtubule arrngement than is suggested to operate in *lkb* plants. The nature of this effect is unknown, and warrants further investigation using more sensitive techniques.

The relative importance of microtubule orientation in the epidermis and the cortex in elongation growth

A limiting role for the epidermis in elongation growth has received general support in the available literature. Ishida and Katsumi (1992) have observed the pattern of microtubule orientation in both the epidermis and the cortex of wild-type cucumber hypocotyl. In the tangential surface of epidermal cells (Ep-t), microtubule orientation ranged from oblique, at 0-1 mm below the cotyledonary node, to oblique/longitudinal at 3-4 mm below the cotyledonary node, while microtubule arrangement was primarily transverse in the radial surfaces of epidermal cells (Ep-r) and entirely transverse in the radial surfaces of cortical cells (C-r) at both positions. A similar pattern of microtubule orientation has been reported for maize mesocotyls (Ishida and Katsumi 1991) and coleoptiles (Nick *et al.* 1990). Ishida and Katsumi (1992) found that the frequency of the occurrence of transverse microtubules in Ep-t was highly correlated to the growth rate of the epicotyl, while microtubule orientation in the Ep-r and C-r was not. Ishida

and Katsumi (1992) have suggested that microtubule orientation in Ep-r and C-r may not be critical to axial expansion, and that Ep-t may be limiting for elongation growth, as previously discussed by Ishida and Katsumi (1991).

Iwata and Hogetsu (1989b) have reported that upon white light-induced cessation of elongation, microtubule orientation changed toward a less transverse arrangement in the epidermis of *Avena* mesocotyl and coleoptile. The change in microtubule orientation in the epidermis preceded that in parenchymal cells. These results may be interpreted as supporting a primary role for the epidermis in limiting elongation growth. However, in *Pisum* epicotyl, no marked change in microtubule orientation was detected in the epidermis following light irradiation. Iwata and Hogetsu (1989b) have suggested that light-induced cessation of growth in *Pisum* epicotyls is not mediated by a change in microtubule arrangement in the epidermis, and since microtubule orientation changed from transverse to oblique in parenchymal tissue after cell elongation ceased, microtubule orientation in the cortex cannot be responsible for the observed growth cessation. It is, however, possible that cessation of elongation following light irradiation operates via a different mechanism in *Pisum*, and the results do not necessarily conflict with the proposal of a limiting role for the epidermis.

For the stem-length mutants in the present study, higher elongation rates corresponded to a more transverse microtubule arrangement in both the epidermis and the cortex. Although microtubule orientation in the cortex appeared to be more closely correlated with elongation rate, microtubule orientation in the epidermis may be limiting for elongation growth, since epidermal arrays appear to be shifted more rapidly from a more transverse (facilitating elongation) to a less transverse (limiting elongation) arrangement. In addition, microtubule orientation in the epidermis does not reach the degree of transversness seen in the cortex. These results are consistent with proposals that microtubule orientation in the epidermis, specifically in the outer tangential wall, is limiting for elongation growth. It is also possible that variability of microtubule orientation in the epidermis may have reduced the strength of the correlation between extension rate and transverseness of the microtubule array, and that the separate characteristics of the mutations themselves may act to obscure the relationship between microtubule orientation and elongation rate.

The role of microtubule orientation in stem elongation

Although average microtubule orientation of *lka* and *lkb* plants was not comparable (Fig. 3a, b), growth profiles for these two lines were extremely similar (Fig. 1). Such inconsistencies between growth rate and microtubule orientation might be explained by sampling error due to an uneven distribution of cells with a particular microtubule orientation (Laskowski 1990), or by a more rapid shift away from a transverse microtubule orientation after direction of growth is determined. However, the size of the difference in microtubule orientation between these two lines suggests that microtubule orientation may not be the sole determinant elongation rate.

The literature has suggested that microtubule orientation plays an important role in stem elongation by directing the deposition of cellulose microfibrils, the orientation of which controls the direction of expansion of the cell wall. However, the shorter internodes in the mutants may result from modification of other components of wall extensibility by the mutant alleles, as well as from altered microtubule arrangement.

A quantitative relationship between the level of GA_1 and internode growth in *Pisum* has been reported by Ingram *et al.* (1986) and Ross *et al.* (1989). It has been suggested that GA affects both the viscoelasticity and chemorheology of the cell wall. Viscoelastic modification of the wall depends upon changes to the overall structure and bonding between wall polymers at any given instant in time, whereas chemorheological modifications involve yielding, slippage, and perhaps reformation of load bearing polymers in the wall (Behringer *et al.* 1990a; Cosgrove 1993b). Behringer *et al.* (1990a) reported changes in both viscoelastic and chemorheological properties of the cell walls of the GA-deficient *ls* mutant of *Pisum*. Taylor and Cosgrove (1989) reported concomitant changes in both viscoelastic and chemorheological properties of cell walls during GA_3-induced growth of light-grown cucumber seedlings. Cosgrove and Sovonick-Dunford (1989) showed that in *Pisum* internodes the GA-biosynthesis inhibitor uniconozol resulted in reduced wall relaxation as a result of chemorheological modification of the wall.

Auxin-stimulated growth has been reported to produce only subtle changes in wall viscoelasticity which typically lag behind changes in growth rate (Cleland 1984; Masuda 1990). In addition, treatment with excessive auxin produced walls which, although viscoelastically weaker, did not result in enhanced cell expansion. Cosgrove (1993b) has argued against a viscoelastic mechanism for auxin-induced wall relaxation, favouring auxin-induced wall relaxation via changes to chemorheological properties.

Thus, the reduced internode expansion in the stem-length mutants could result from alteration of microtubule orientation, altered chemorheological and perhaps viscoelastic properties of the cell walls, or some combination of these.

The shorter internodes of the GA-deficient synthesis mutants, *le* and *ls*, might then result primarily from a reduced stability of transverse microtubule arrays coupled with decreased wall relaxation resulting from altered chemorheological and viscoelastic properties of the cell walls.

Reduced internode lengths in the GA-response mutants, *lk*, *lka*, and *lkb*, may stem from aberrant initial microtubule assembly and/or reduced stability of transverse microtubule arrays as a result of reduced GA sensitivity and/or reduced levels of IAA. In addition, reduced sensitivity to GA and/or reduced levels of IAA may result in decreased wall relaxation as a consequence of altered chemorheological properties of the cell walls. Behringer *et al.* (1990a) have reported that *lka* and *lkb* appear to affect primarily the chemorheological properties of the cell walls.

The reduction in internode length of the GA-response mutant *lw* may also result from an initially reduced extension rate possibly resulting from a disturbance of initial microtubule arrangement as a consequence of the mutant's decreased responsiveness to GA_1. Although an interaction between phytochrome and responsiveness to GA_1 seems likely (Weller and Reid 1993), the nature of the relationship between phytochrome function, responsiveness to GA_1 and microtubule orientation is uncertain.

CHAPTER 3: THE EFFECT OF GA_1 UPON MICROTUBULE ARRANGEMENT AND THE CONTRIBUTION OF ALTERED MICROTUBULE ARRANGEMENT TO MUTANT PHENOTYPES

3.1 Introduction

The conclusions reached in the Chapter 2 implicate microtubules, and in particular the hormonal control of microtubule orientation in the altered phenotypes of the GA-synthesis (*le, ls*) and GA-response (*lw, lk, lka, lkb*) mutants of *Pisum* L.

The GA-synthesis mutants, *le* and *ls*, exhibited both a reduced maximum growth rate and distribution of growth along the epicotyl, as well as a reduced component of cells with a transverse microtubule arrangement, in comparison to wild-type plants. Lower endogenous levels of GA_1, resulting from a reduction in the 3ß hydroxylation of GA_{20} to GA_1 in *le* plants (Ingram *et al.* 1983, 1984; Ross *et al.* 1989), and a probable block in the metabolism of GA between geranylgeranylpyrophosphate and copalyl pyrophosphate in *ls* plants (S. M. Swain and Y. Kamiya, unpublished results), are thought to be responsible for the altered growth profiles and microtubule arrangements measured in these mutants.

Since endogenous levels of GA_1 are essentially normal in the GA-response mutants, *lw* (Jolly *et al.* 1987), *lk, lka* and *lkb* (Reid and Davies 1992; Reid 1986; Reid and Potts 1986; Reid and Ross 1989; Behringer *et al.* 1990a; Lawrence *et al.* 1992), it is considered unlikely that reduced levels of endogenous GA_1 are responsible for the altered growth profiles and microtubule arrangement in the GA-response mutants. However, a reduced responsiveness of these mutants to GA_1 is hypothesised to explain the altered growth profiles and disturbed microtubule arrangements documented.

The present chapter examines further the proposal that low endogenous levels of GA_1 are reponsible for the altered microtubule arrangements of the mutants, *le* and *ls*, and attempts to restore the wild-type microtubule arrangement by exogenous application of GA_1. Further,the effect of exogenous application of GA_1 to the GA-response mutants is examined in order to determine whether loss of GA-responsiveness occurs prior to, or after microtubule arrangement.

Since growth profiles of the GA-response mutants *lka* and *lkb* were extremely similar (Fig. 7), but average microtubule arrangement was substantially different, it was suggested that the elongation rate in these two lines may not be solely determined by microtubule orientation, but may also result from altered chemorheological and viscoelastic properties of the cell walls. Therefore, in addition to the role of GA_1 in microtubule arrangement and extension growth, the relative contribution of microtubule arrangement to stem extension is examined.

The role of microtubules in shaping cells has been effectively illustrated by the use of a range of microtubule disrupting agents (see Gunning and Hardham 1982; Seagull 1989). For example, the normally cylindrically shaped elongating cells of the green alga *Closterium*, have been demonstrated to assume a more isodiametric form after treatment with colchicine (Hogetsu and Shibaoka 1978b). This loss of polarity was shown to correspond with a shift from a parallel transverse to a random arrangement of cellulose microfibrils, and a concurrent loss of cortical microtubules (Hogetsu and Shibaoka 1978b). Colchicine treatment of regenerating protoplasts of the alga *Mougeotia* resulted in loss of microtubules and the development of sperical cells, the walls of which contained randomly oriented cellulose microfibrils (Marchant and Hines 1979). Mueller and Brown (1982b) reported that colchicine treatment of maize seeds resulted in swelling of root cells and a concurrent loss of cortical microtubules.

The effect of microtubule disruption on cell shape is also expressed at the level of organ development. Mita and Shibaoka (1984b) have recorded bulb-like swelling in onion leaf sheath in response to application of the microtubule disrupting agents, colchicine and cremart.

Of the microtubule disrupting agents available, the effect of colchicine is perhaps the best documented. The disruptive effect of colchicine upon microtubules has been reported to result from the ability of colchicine molecules to bind to tubulin subunits in solution. Margolis and Wilson (1977) have reported that the addition of this altered tubulin complex to the polymerizing ends of the microtubule renders microtubules unavailable for subsequent addition of tubulin subunits.

Although the arrangement of cellulose microfibrils in the cell wall is also disturbed by application of colchicine, this disturbance is presumably a direct result of the destruction of microtubules by colchicine, rather than an impairment of cell wall synthesis (Chrispeels 1972; Robinson *et al.* 1976; Srivastava *et al.* 1977). Srivastava *et al.* (1977) recorded the destruction of cortical microtubules in the cells of colchicine-treated lettuce hypocotyl as early as 4 h, but detected no change in polysaccharide synthesis.

An interactive relationship between colchicine and a number of plant-growth hormones has been demonstrated. GA_3-induced cell elongation has been shown to be reduced or eliminated by treatment with colchicine. Pretreatment with colchicine resulted in inhibition of the normally promotive effect of GA_3 upon stem elongation in lettuce hypocotyl (Srivastava *et al.* 1977) and pea epicotyl (Steen and Chadwick 1981). In addition, Mita and Shibaoka (1984b) reported the supression of colchicine-induced swelling of onion leaf sheath cells by GA_3. Seagull (1989) has commented that the observed antagonism between GA_3 and colchicine indicates that the two agents may have targets in common. Steen and Chadwick (1981) reported that application of colchicine reversed the effect of ethylene in straight growth tests of pea stem. Kinetin and benzimidazole-induced cell swelling in azuki bean were also prevented by colchicine (Shibaoka 1974).

The GA-response mutants, *lk*, *lka* and *lkb*, phenotypically resemble the gross changes in stem morphology which result from colchicine-induced microtubule disruption. These mutants all exhibit substantially reduced internode length and increased internode diameter (Reid 1986; Ross and Reid 1986; Reid and Ross 1989). Reduced length of internode 7 appears to be primarily attributable to a reduction in cell length in *lkb* (Ross and Reid 1986; Reid and Ross 1989). A reduction in both cell length and cell number is responsible for the reduced internode lengths of *lk* and *lka* types in the epidermis and cortex (Ross and Reid 1986; Reid and Ross 1989).

Immunofluorescence microscopy did not reveal any obvious depolymerization of microtubules in the cells of the GA-response mutants, *lk*, *lka* and *lkb*, in comparison to wild-type plants (Chapter 2). However, the nature of the immunofluorescence technique has the potential to obscure any subtle differences in the microtubule complement within a cell. Williamson (1991) contended that immunofluorescence exaggerates the area of plasma membrane occupied by the microtubules. In addition, immunofluorescence microscopy may not be sufficiently sensitive to disclose any possible perturbations of the structure of individual microtubules in the GA-response mutants.

Given the overall morphology of the GA-response mutants, coupled with the reduction in cell length, it is possible that the mutant alleles *lk*, *lka*, and *lkb*, as well as disturbing microtubule orientation, may exert a more direct effect upon microtubule structure. This seems more likely in *lk* and *lka* types, which exhibit, in addition to reduced cell length, an impairment of cell division, manifest in their reduced cell number in both the epidermis and the cortex (Ross and Reid 1986; Reid and Ross 1989).

The aim of this chapter is to assess the relative contribution of microtubules to the altered morpology exhibited by the GA-response mutants. Application of colchicine, a known microtubule disrupting agent, was applied to the internodes of wild-type plants in an attempt to produce a phenocopy of a GA-response mutant.

3.2 Materials and Methods

3.2.1 Application of GA_1 to GA-synthesis and GA-responese mutants

Plant material

GA_1 application work utilized the tall wild-type cv. Torsdag and the mutant lines NGB5865 (semi-erectoides, *lka*), NGB5862 (semi-erectoides, *lkb*), K29 (short, *lw*), L181 (dwarf, *ls*), NGB5839 (dwarf, *le*) and L212 (erectoides, *lk*). Details about the genotypes and phenotypes of these lines may be found in Chapter 2 and in Reid and Ross (1989).

GA_1 application to seeds

The seed testa was nicked with a razor blade above the cotyledon and GA_1 was applied to the cotyledon at 2µg GA1 / 2µl ethanol with a microcapillary pipette. Control seeds received 2µl ethanol.

Growing conditions

Plants were grown in 140-mm-diameter black Slimline pots at 4 plants per pot in a 50:50 mixture of vermiculite and dolerite chips topped with 20 to 30 mm of potting mix, as specified by Reid (1986). Prior to sowing the seeds, the potting mixture was saturated with water. Seeds were dropped into holes 1 cm in depth and covered over without watering in (day 0). Pots were placed immediately in darkness. Pots were watered gently on day 2, and then every 2 to 3 days as required. Plants were grown in darkness at 22° C from the time of sowing until harvested, with the use of a safe green light for watering, observations and measurements. Node counting began with the cotyledonary node as zero.

Plants were harvested for immunofluorescence work when internode 3 reached 25-40% expansion (Table 1), which, at 22° C, was 7-9 d after sowing. It should be noted that application of GA_1 to the GA-synthesis mutants *le* and *ls*, produced internode lengths approaching that of the wild-type, and therefore *le* and *ls* plants were harvested when their third internode reached lengths equivalent to 25-40% of the final length of internode 3 of wild-type plants.

Harvesting procedure

At 25-40% expansion of internode 3, darkgrown seedlings were decapitated just above the cotyledonary node and immediately placed into a shallow pool of fixative. Using a fine (Gillette Blue) razor blade 1 mm length transverse segments were cut from stem position B, 4-6 mm below the apical hook.

Tissue fixation, sectioning, tubulin immunolabelling, microscopy and analysis of microtubule orientation followed the methods detailed in Chapter 2.

Histograms showing the percentages of microtubules falling into the three orientation categories (transverse, oblique and longitudinal), at specified distances from the apical hook, were constructed (Fig. 9a, b). Scatter graphs (Fig. 10a, b) were also constructed to show the average microtubule orientation for the wild-type and stem-length mutants at each distance from the apical hook. Sample size and standard errors for average microtubule orientation are given in table form (Table 5a, b) to facilitate interpretation of the orientation data.

A model of the possible sequence of events leading from the GA-biosynthetic pathway to extension growth was constructed using the combined data from Chapters 2 and 3, and the available literature (Fig. 11).

3.2.2 Application of colchicine to the wild-type Torsdag

Plant material

Colchicine application experiments utilized the tall, wild-type cv. Torsdag (L107) and the semi-erectoides L5865 (*lka*); further information about the genotypes and phenotypes of these lines may be found in Chapter 2 and in Reid and Ross (1989).

Growing conditions

For all experiments, seeds were sown in 140-mm-diameter black Slimline pots in the potting mix specified in Chapter 2. Seeds were sown at 2 seeds per pot, except for the soil watering experiment (1 seed per pot). Plants were watered on the day of sowing (day 0), and then daily until seedlings could be seen emerging from the substrate. Subsequent watering was every 2 to 3 days as required. Growth was in the controlled environment of a growth cabinet which remained at 17.5°C. Plants received an 18 h photoperiod and received, at the pot surface, 250 $\mu mol \cdot m^{2} \cdot sec^{-1}$ of photosynthetically active radiation (P.A.R.) supplied by a mixture of incandescent and fluorescent lights. Node counting began with the cotyledonary node as zero. Initially, L107 plants were allowed to maximally expand internode 7 in order to establish final lengths for internodes 3-7 under these growing conditions (Table 6).

Colchicine application

For the soil watering experiment, a 1.25 mM colchicine solution was watered into the soil of 6 pots. 6 controls received water only. Soil was watered to saturation daily.

For application of colchicine to the leaves of L107 plants, 10 µl of 25 mM colchicine solution was applied with a microcapillary pipette to the expanding third leaf of L107 plants. 6 plants received colchicine plus ethanol (C+E), 6 plants received colchicine plus water (C+W), and 6 plants received alcohol only (C). All treatments contained 0.05% Tween-20 to facilitate leaf wetting.

For the initial colchicine applications to internodes of L107 plants, colchicine was applied in the following doses to rapidly expanding internodes (i.e. between 20% and 50% expanded): 10 µl of 12.5 mM colchicine to alternate internodes, 10 µl of 12.5 mM solution per internode, 10 µl of 2.5 mM solution per internode, and 10 µl of a 1.25 mM solution per internode. Control plants received water only. All treatments contained 0.05% Tween-20 to facilitate internode wetting. L5865 plants were grown as a comparison. Length of internodes 1-9 and maximum diameter of internodes 3-9 were measured (Table 7).

In an attempt to produce a phenocopy of a stem-length mutant, 10 µl of 1.25 mM colchicine/0.5% Tween 20 solution was applied with a micro-pipette to internodes 3-7 of L107 plants when each internode had (a) reached 5 mm in length (14 plants), or (b) expanded to 20% of its final length (14 plants). 12 control plants received 10 µl of 0.5% Tween 20 solution under either condition (a) or (b).

Epidermal strips (to which were attached a layer of sub-epidermal cortical cells) were removed from internode 7 after elongation growth had ceased. Epidermal strips were obtained by making a shallow horizontal incision 1-2 mm below the node and peeling the epidermis back with a fine forceps. Each epidermal/cortical strip was divided into 3 sections: the uppermost 30% (a), the middle 30% (b), and the bottom 30% (c) of the internode and temporarily mounted in glycerol. For each of the 3 sections, 10 cells were measured for both length (parallel to the stem axis) and width (perpendicular to the cell axis) using a calibrated light microscope. Scattergraphs showing average cell length and width for each of the treatments were constructed (Fig. 13a, b). Average length of sections (a), (b), and (c) were recorded for each of the colchicine treatments, and average epidermal and cortical cell numbers for these sections were calculated by dividing the average cortical or epidermal cell length of each section into the average length of that section (Table 8a, b).

3.3 Results

3.3.1 Application of GA_1 to GA-synthesis and GA-responese mutants

Exogenous application of GA_1 to GA-synthesis mutants resulted in an increase in length of internode 3 to lengths approaching that of the wild-type, Torsdag (Fig. 8). Exogenous application of GA_1 to GA-response mutants resulted in a somewhat increased length of internode 3 of *lw* plants, but no noticeable increase in length of internode 3 in *lk*, *lka* and *lkb* plants (Fig. 8).

Measurement of microtubule orientation in the epidermis and the outer cortical tissue of wild-type plants at 4-6 mm from the apical hook indicated that exogenous application of GA_1 had no significant effect on microtubule orientation in either epidermis or cortical cells, as measured by percentage orientation category (Fig. 9a, b) and average microtubule orientation (Fig. 10a, b; Table 5a, b).

Exogenous application of GA_1 to the GA-synthesis mutants, *le* and *ls*, resulted in significant increases in average microtubule orientation in both epidermal and cortical cells (Fig. 10a, b; Table 5a, b), resulting from an increased proportion of cells falling into the transverse microtubule orientation category (Fig. 9a, b).

Table 5 The effect of GA_1 on microtubule orientation 4-6 mm from the apical hook in (a) the epidermis, and (b) subepidermal cortical cells of WT and stem-length mutants of pea

(a)	WT		*le*		*ls*		*lw*		*lk*		*lka*		*lkb*	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	85.50 (61)	0.85	54.68 (28)	2.24	49.21 (19)	1.91	38.10 (31)	1.84	45.65 (17)	4.249	42.75 (60)	1.86	44.17 (46)	2.11
GA_1	81.67 (61)	1.57	75.45 (64)	1.9	57.86 (43)	2.35	46.63 (38)	2.36	41.292 (24)	3.65	32.519 (61)	1.55	37.28 (18)	4.36
Control vs GA_1	NS		***		*		**		NS		***		NS	

(b)	WT		*le*		*ls*		*lw*		*lk*		*lka*		*lkb*	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	87.16 (38)	0.60	79.24 (66)	0.90	72.28 (51)	1.44	77.22 (51)	1.46	69.73 (52)	1.03	76.21 (125)	0.82	69.43 (81)	1.54
GA_1	87.60 (82)	0.55	86.56 (86)	0.57	82.33 (42)	1.35	78.09 (54)	1.05	65.58 (24)	1.76	69.09 (87)	1.68	63.12 (51)	2.51
Control vs GA_1	NS		***		***		NS		*		***		*	

Average microtubule orientation (degrees from cell axis) in a) the tangential surface of epidermal cells and b) longitudinal sections of cortical cells in the 4-6 mm (B) region below the apical hook in internode 3 of a range of stem length mutants of *Pisum sativum* L. Data are means, standard error (SE), and sample size (n). Controls and GA-treated plants were tested for differences in MT orientation using the Fisher PLSD test. * $P < 0.05$; ** $P < 0.01$ and ***$P < 0.001$, NS = not significant

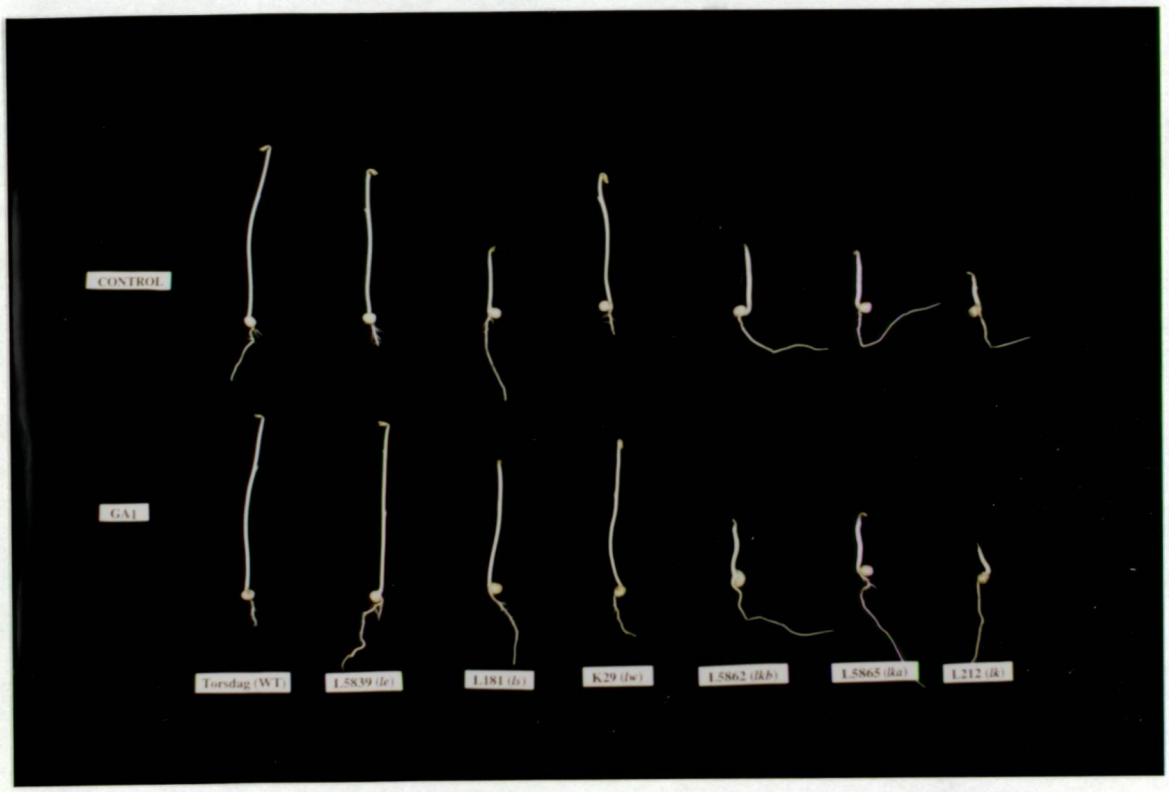


Fig. 8 Photograph showing the effect of exogenously applied GA_1 on internode length of wild-type and mutant lines of pea.

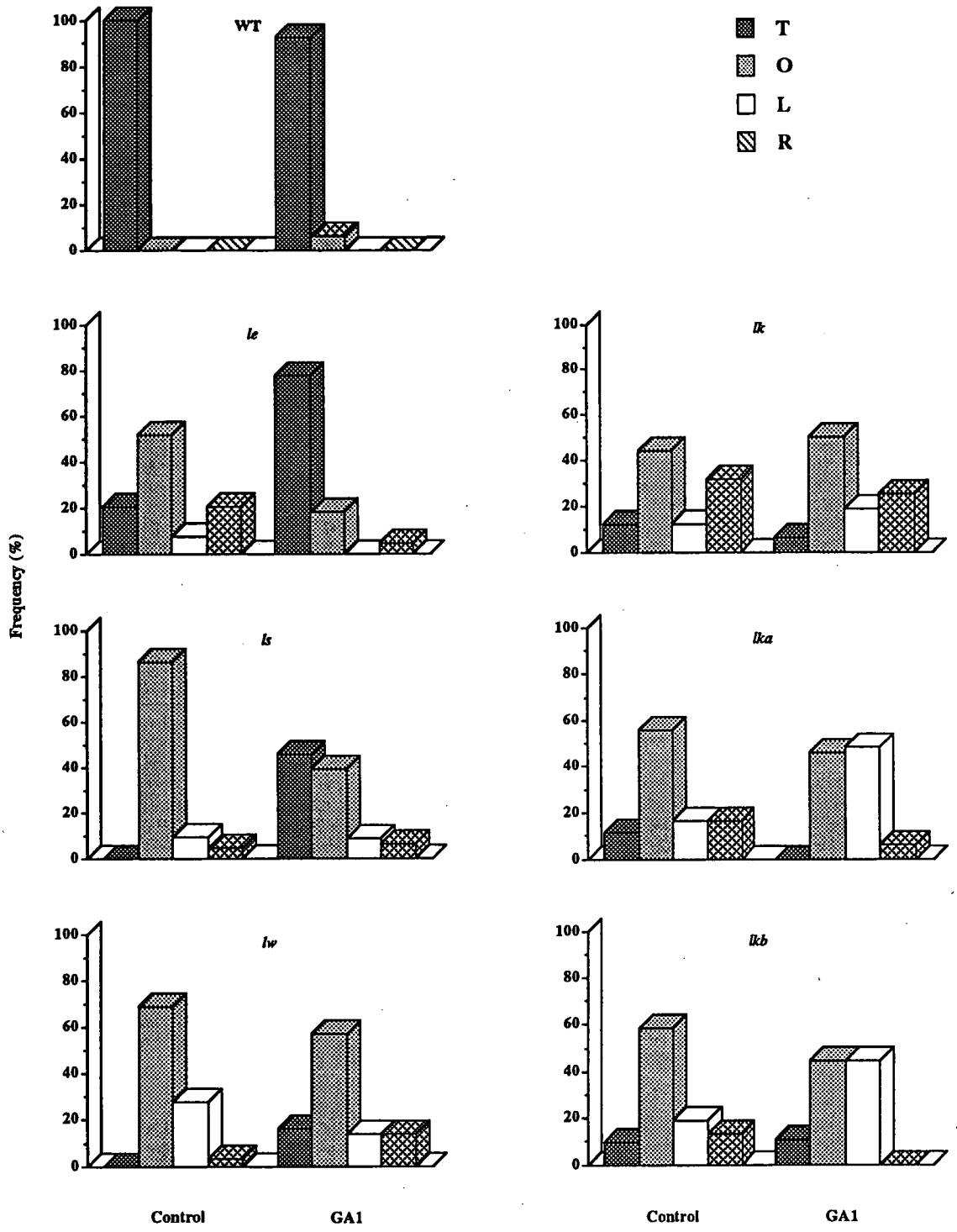


Fig. 9a Microtubule orientation in the tangential surface of epidermal cells in the 4-6 mm (B) region below the apical hook of internode 3 of a range of stem length mutants of *Pisum sativum* L epicotyls. Control plants received 2 µl of ethanol. GA1-treated plants received 2µg of GA1 in 2µl ethanol. Observations were made when the internode was 25-40% expanded. The histograms show the percentage distribution of cells having microtubules arranged within the angular ranges of 0°-30° (longitudinal =*L*), 30°-60° (oblique =*O*), and 60°-90° (transverse = *T*) to the direction of the cell axis. Cells with a random (*R*) microtubule orientation are also recorded.

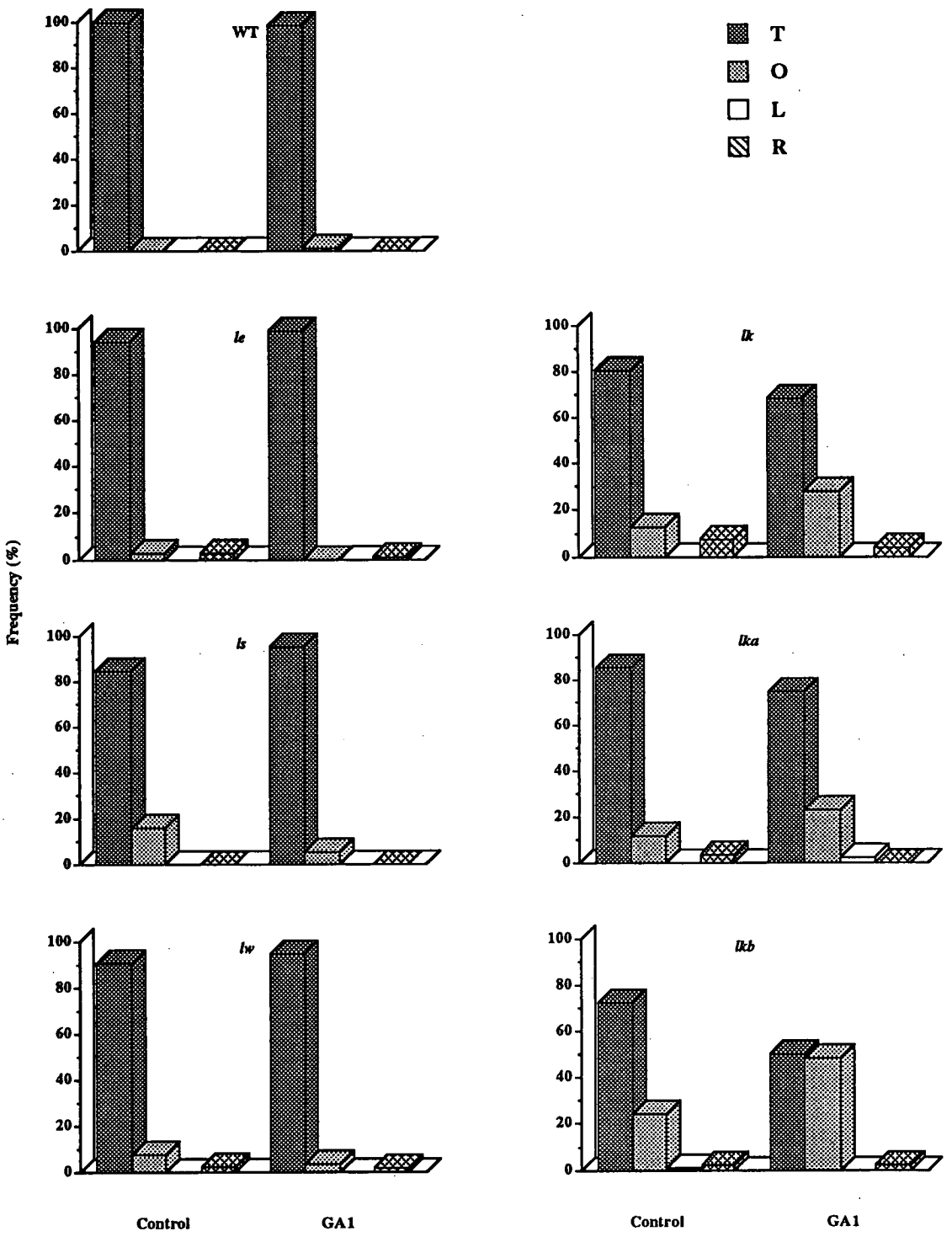


Fig. 9b Microtubule orientation in longitudinal sections of subepidermal cortical cells in the 4-6 mm (B) region below the apical hook of internode 3 of a range of stem length mutants of *Pisum sativum* L epicotyls. Control plants received 2 µl of ethanol. GA1-treated plants received 2µg of GA1 in 2µl ethanol. Observations were made when the internode was 25-40% expanded. The histograms show the percentage distribution of cells having microtubules arranged within the angular ranges of 0°-0°o (longitudinal =*L*), 30°-60° (oblique =*O*), and 60°-90° (transverse = *T*) to the direction of the cell axis. Cells with a random (*R*) microtubule orientation are also recorded.

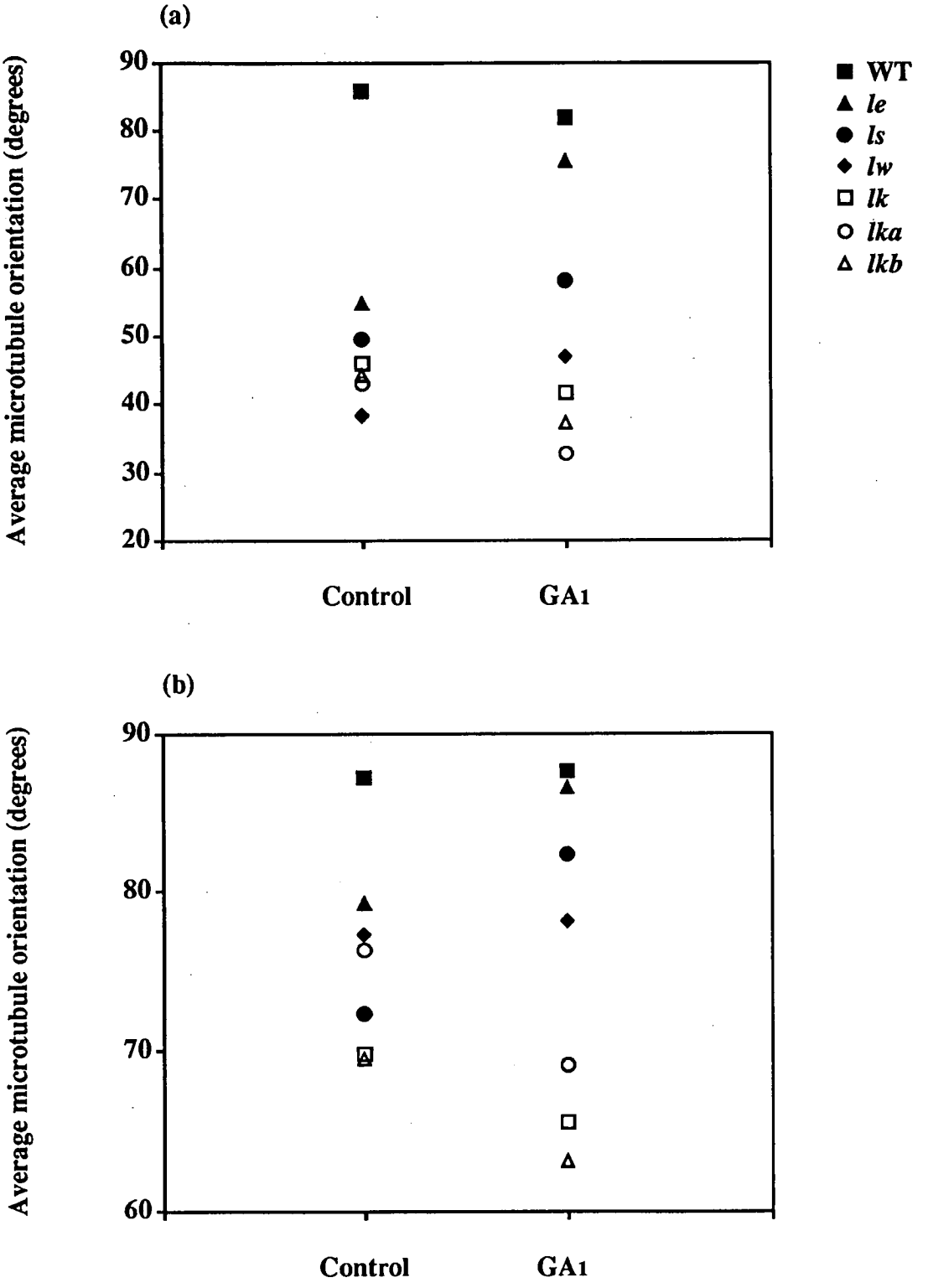


Fig. 10a, b Average microtubule orientation (degrees from cell axis) in **a)** the tangential surface of epidermal cells and **b)** longitudinal sections of subepidermal cortical cells in the 4-6 mm region below the apical hook of internode 3 of a range of stem length mutants of *Pisum sativum* L. epicotyls. Control plants received 2 µl of ethanol. GA1-treated plants received 2 µg of GA1 in 2 µl of ethanol. Observations were made when the internode was 25-40% expanded.

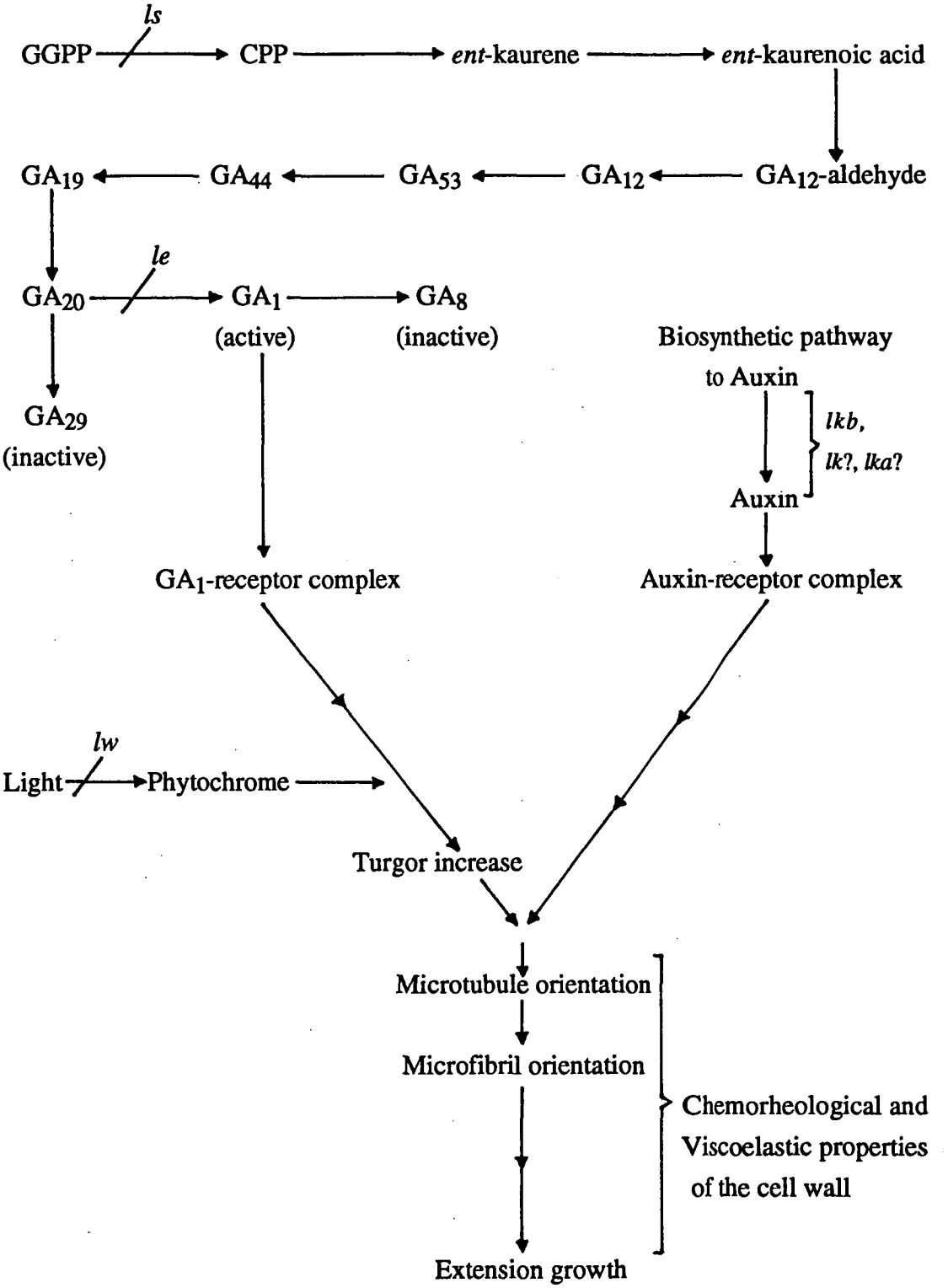


Fig. 11 Model of the possible sequence of events leading from the GA-biosynthetic pathway to extension growth in *Pisum*, including the proposed sites of action of the GA-synthesis and GA-response genes. (Modified from Reid 1990, 1993).

Application of GA_1 to seeds of *lw* plants also resulted in a significant increase in average microtubule orientation in the cells of the epidermis, corresponding with a slight, although non-significant increase in the cortex (Fig. 10a, b; Table 5a, b). This trend toward a higher average microtubule orientation resulted from an increase in the proportion cells with transversely orientated microtubules in GA_1-treated plants (Fig. 9a, b).

Exogenous application of GA_1 to the GA-response mutants, *lk*, *lka* and *lkb*, did not result in an increase in either average microtubule arrangement (Fig. 10a, b; Table 5a, b) or the proportion of cells with transversely orientated microtubules (Fig. 9a, b). Instead, average microtubule orientation was somewhat lower in all three lines, significantly so in the epidermis of *lka* plants, and in the cortex of *lk*, *lka* and *lkb* plants. The lower average microtubule arrangement resulted from a relatively reduced proportion of cells of GA_1-treated plants falling into the transverse microtubule orientation category.

3.3.2 Application of colchicine to the wild-type Torsdag

Watering colchicine into the soil of pots where seeds of L107 were sown resulted in the unsuccessful germination of seeds, while control seeds germinated normally. Some of the treated seeds put out a radicle but failed to elongate sufficiently to break through the soil surface. These seeds eventually rotted. A few plants developed extremely short shoots, and a very limited root system. The remaining seeds showed no sign of germination.

Colchicine applied to leaves in either water or ethanol, did not result in reduced internode lengths. It seemed likely that colchicine was not transported from the leaves into the stem. As a result, treatments involving the direct application of colchicine to internodes were implemented.

Initial colchicine applications to internodes of L107 indicated that a 1.25 mM solution of colchicine, to which 0.5% Tween 20 had been added, was most effective in 10 µl applications to each internode (Table 7). Although other treatments often resulted in reductions in internode length and maximum diameter of L107 plants more closely approximating L5865 stem morphology, less variability was produced by application of the lower dose to each internode.

Application of 10 µl of 1.25 mM colchicine solution directly to internodes 3-7 of wild-type tall plants of L107 resulted in an altered phenotype, in comparison to controls. Colchicine applications produced a noticeable decrease in stem length and a concomitant increase in stem width (Fig. 12). Colchicine treatment at 20% expansion resulted in an approximately 50% reduction in length of internode 7, while treatment at 5 mm expansion produced an average reduction in length of over 80% (Table 8a,b). However, this shortening and widening effect was not consistently exhibited over internodes 3-7, and was frequently variable within an internode.

Measurements of cell length and cell width from internode 7 (Fig. 13a, b) revealed a concomitant decrease in cell length and increase in cell width for colchicine-treated tissue. The shortening and widening effect of colchicine upon cells was observed for both epidermal (Fig. 13a) and sub-cortical cells (Fig. 13b). Colchicine treatment at both 5 mm and 20% expansion resulted in altered cell shape, with the application of colchicine to internodes at 5 mm expansion producing a more isodiametric shape in the cells of the epidermis, than did colchicine application at 20% expansion.

Table 6 Length of internodes 3-7 of light-grown tall L107 plants

	Length of internode (mm)	
Internode	100 % expanded	20 % expanded
3	24.0	4.8
4	30.0	6.0
5	41.5	8.3
6	51.5	10.3
7	61.5	12.3

Epidermal cell measurements from the top, middle and bottom 30% of internode 7 for control plants were comparable (Fig. 13a). Conversely, colchicine treatments resulted in differences in length and width of epidermal cells at the different internode positions. These differences were not consistent for the two colchicine treatments.

Table 7. Internode length and diameter (mm) in the semi-erectoides mutant L5865. Varying colchicine treatments to wild-type L107 (Torsdag) internodes attempts to simulate L5865 internode morphology.

Treatment	Total length	Internode 1		Internode 2		Internode 3		Internode 4		Internode 5		Internode 6		Internode 7		Internode 8		Internode 9		Nodes expanded
		a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	
L5865 (*lka*)	191.3 ± 5.2 (10)	-	-	-	-	13.3 ± 1.0 (10)	2.7 ± 0.1 (10)	7.5 ± 0.6 (10)	2.5 ± 0.1 (10)	16.7 ± 0.7 (10)	2.5 ± 0.1 (10)	18.9 ± 0.7 (10)	2.5 ± 0.1 (10)	24.4 ± 1.4 (10)	2.7 ± 0.1 (10)	25.9 ± 1.0 (10)	3.0 ± 0.1 (10)	35.6 ± 0.9 (10)	3.2 ± 0.1 (10)	9.7 ± 0.1 (10)
L107 12.50 mM colchicine per internode	164.6 ± 32.2 (9)	17.4 ± 0.7 (9)	-	2.6 ± 0.2 (9)	-	24.3 ± 2.5 (9)	2.1 ± 0.1 (9)	20.8 ± 1.6 (9)	2.9 ± 0.1 (9)	19.9 ± 3.8 (9)	3.5 ± 0.2 (9)	25.6 ± 6.9 (9)	2.9 ± 0.2 (9)	25.4 ± 6.5 (7)	3.1 ± 0.2 (7)	19.2 ± 7.8 (6)	3.3 ± 0.2 (5)	21.6 ± 14.8 (5)	2.4 ± 0.3 (5)	7.8 ± 0.04 (9)
L107 12.50 mM colchicine alternate internodes	191.9 ± 34.1 (8)	17.8 ± 0.5 (8)	-	3.4 ± 0.6 (8)	-	27.8 ± 3.8 (8)	2.1 ± 0.04 (8)	18.0 ± 2.9 (8)	3.0± 0.3 (8)	29.6 ± 6.8 (8)	3.1± 0.3 (8)	26.7 ± 5.3 (7)	3.0 ± 0.3 (7)	40.7 ± 9.5 (6)	3.2 ± 0.3 (6)	21.0 ± 6.0 (5)	3.9 ± 0.2 (5)	19.8 ± 12.1 (5)	2.8 ± 0.3 (5)	8.1 ± 0.5 (8)
L107 2.50 mM colchicine per internode	165.6 ± 23.8 (9)	18.1 ± 0.5 (9)	-	3.6 ± 0.6 (9)	-	26.9 ± 2.9 (9)	2.0 ± 0.04 (9)	19.1 ± 2.0 (9)	2.7 ± 0.2 (9)	25.3 ± 4.0 (8)	3.2 ± 0.2 (8)	27.8 ± 5.9 (8)	2.8 ± 0.2 (7)	23.4 ± 3.4 (7)	3.3 ± 0.1 (7)	16.7 ± 5.3 (7)	2.8 ± 0.2 (7)	14.7 ± 7.8 (6)	2.3 ± 0.1 (5)	7.8 ± 0.6 (9)
L107 1.25 mM colchicine per internode	154.6 ± 15.4 (9)	15.2 ± 0.8 (9)	-	3.4 ± 0.4 (9)	-	26.8 ± 2.2 (9)	2.0 ± 0.1 (9)	19.9 ± 1.8 (9)	2.7 ± 0.2 (9)	18.2 ± 1.5 (9)	3.4 ± 0.1 (9)	21.6 ± 4.2 (9)	2.7 ± 0.3 (9)	20.8 ± 3.5 (8)	2.5 ± 0.2 (8)	16.3 ± 4.8 (8)	2.1 ± 0.2 (8)	12.6 ± 2.0 (5)	1.7 ± 0.1 (5)	7.9 ± 0.3 (9)
L107 control	316.5 ± 10.6 (8)	19.0 ± 0.7 (8)	-	2.9 ± 0.3 (8)	-	28.8 ± 3.1 (8)	1.9 ± 0.03 (8)	28.9 ± 1.1 (8)	1.9 ± 0.1 (8)	45.6 ± 1.5 (8)	2.1 ± 0.04 (8)	48.1 ± 2.0 (8)	2.3 ± 0.1 (8)	57.4 ± 3.2 (8)	2.5 ± 0.1 (8)	47.9 ± 5.1 (8)	2.5 ± 0.2 (8)	22.1 ± 4.7 (7)	2.0 ± 0.2 (7)	8.4 ± 0.1 (8)

"a" values represent internode lengths. "b" values represent maximum internode diameters. Lengths and diameters are given as averages ± standard errors. Sample sizes are included in parantheses.

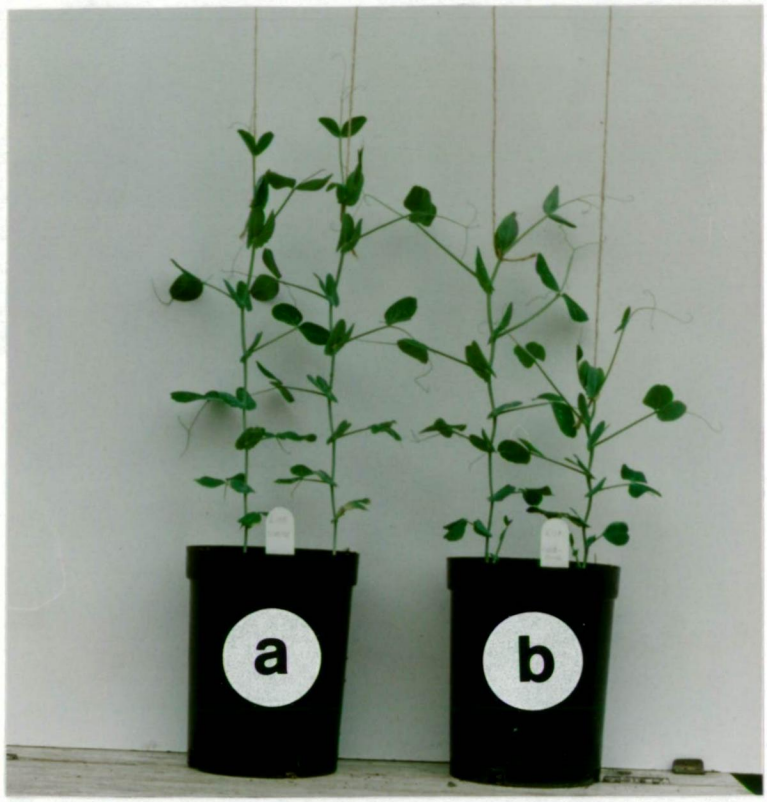


Fig. 12. Application of colchicine to internodes of the wild-type tall L107 plants. Plants received (a) 10µl of 0.05% Tween-20 (controls) and (b) 10 µl of 1.25 mM colchicine in 0.05% Tween-20 per internode.

Table 8 Total length of internode 7, cell length and cell number for (A) epidermal and (B) cortical cells of wild-type (L107) plants of *Pisum*

(A)

Colchicine Treatment	Av. Total Length Internode 7 (mm)	Internode Position	Av. Cell Length (μm)	Av. Cell Number
Control		a	341.6 +/- 10.2 (110)	50.4 +/- 2.7
12 plants	56.7 +/- 2.13	b	344.3 +/- 8.9 (100)	48.9 +/- 1.5
		c	325.0 +/- 12.8 (110)	52.9 +/- 2.4
20 %		a	166.9 +/- 13.6 (130)	51.7 +/- 5.9
14 plants	28.7 +/- 3.12	b	168.6 +/- 15.3 (126)	50.2 +/- 4.5
		c	135.0 +/- 17.1 (130)	76.3 +/- 10.7
5 mm		a	100.2 +/- 15.2 (140)	33.1 +/- 3.6
14 plants	10.3 +/- 1.59	b	86.1 +/- 13.7 (139)	36.9 +/- 2.8
		c	59.2 +/- 5.6 (120)	49.5 +/- 7.3

(B)

Colchicine Treatment	Av. Total Length Internode 7 (mm)	Internode Position	Cell Length (μm)	Cell Number
Control		a	91.8 +/- 2.0 (100)	187.1 +/- 10.6
12 plants	56.7 +/- 2.13	b	80.6 +/- 2.4 (60)	195.0 +/- 7.1
		c	82.3 +/- 1.7 (77)	184.7 +/- 4.2
20 %		a	63.4 +/- 2.1 (130)	195.9 +/- 16.9
14 plants	28.7 +/- 3.12	b	55.1 +/- 1.7 (128)	219.1 +/- 17.2
		c	60.1 +/- 1.9 (130)	204.7 +/- 16.4
5 mm		a	62.2 +/- 2.0 (135)	40.4 +/- 3.2
14 plants	10.3 +/- 1.59	b	56.3 +/- 1.8 (120)	70.8 +/- 13.7
		c	53.3 +/- 1.5 (109)	34.3 +/- 2.6

Total internode lengths are averages of 12-14 plants. Internode positions are (a) top, (b) middle, and (c) bottom 30% of internode 7. Cell lengths are averages +/- standard error. Sample size is in parentheses. Cell numbers are averages +/- standard error of number of cells per section of internode, calculated by dividing average cortical cell length into length of internode section.

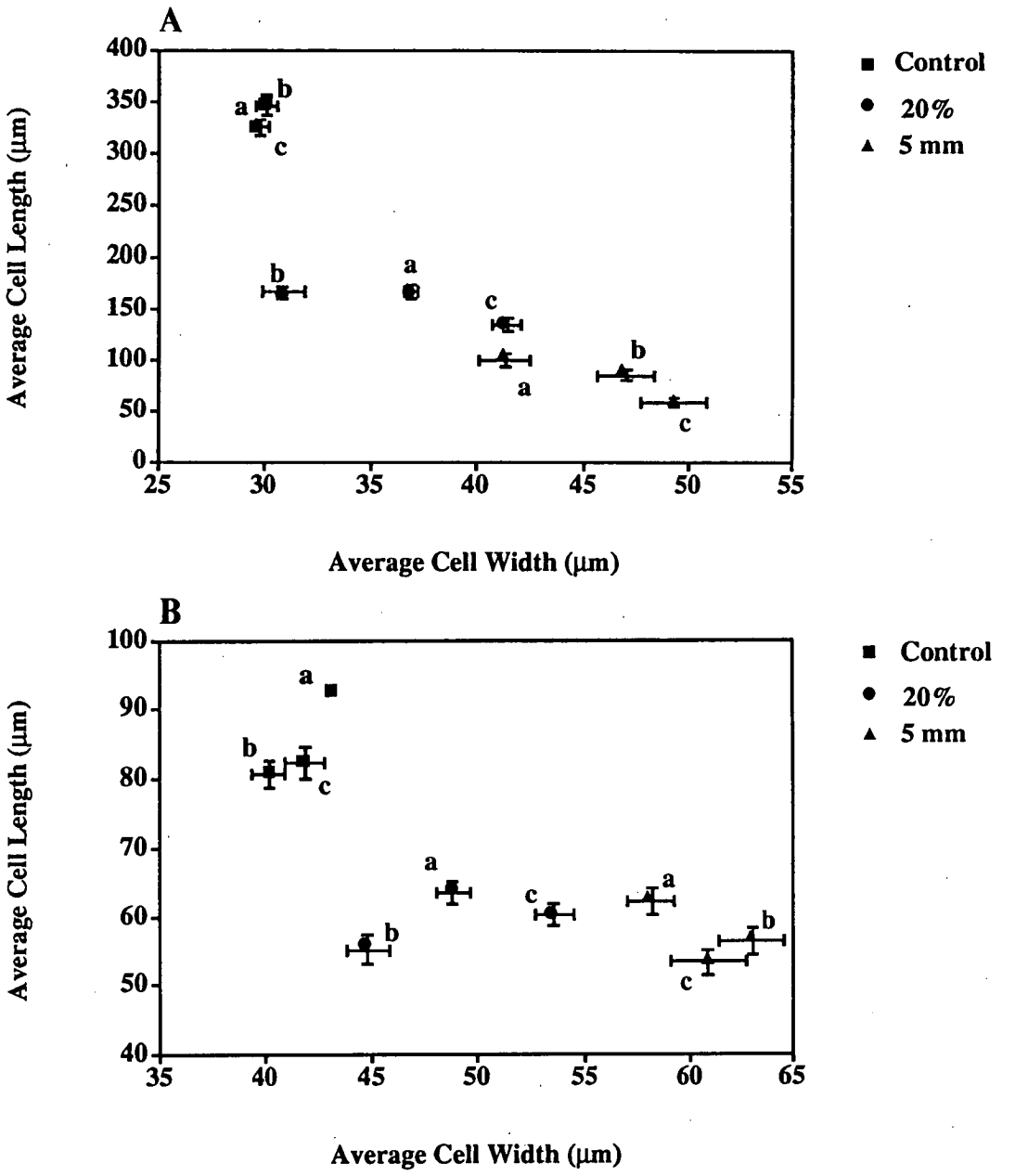


Fig. 13A, B Effects of colchicine on the average length and width of A) epidermal cells and B) cortical cells from internode 7 of wild-type plants at different stages of internode expansion. Colchicine (10 µl per plant of 1.25 mM colchicine in 0.05% Tween 20) was applied to internodes which had reached 5mm in length (5 mm) or which had expanded to 20% of their final length (20%). Control plants received only the detergent solution at either 5 mm or 20% expansion. Cell measurements were obtained from the top (a), middle (b) and bottom (c) one thirds of internode 7.

Cortical cell measurements (Fig. 13b) disclosed slightly more variation in control cell shape between the different internode positions. Colchicine treatments resulted in cortical cell shape changes similar to those observed in the epidermis.

Estimates of cell number (Table 8a, b), from average cell lengths, revealed a decrease in the numbers of epidermal and cortical cells of internode 7 after colchicine application at 5 mm expansion. Epidermal and cortical cell numbers for control plants and plants treated with colchicine at 20% expansion were largely comparable.

3.4 Discussion

3.4.1 Effect of exogenous GA_1 application on microtubule arrangement in the wild-type and mutant lines.

An analysis of microtubule arrangement in GA_1-treated mutants and their wild-type progenitor supports the numerous reports in the literature that GA results in a more transverse orientation of microtubules (see Chapter 1).

In addition, the present findings further clarify the mechanism by which the various stem length mutations in this study operate to alter the wild-type phenotype.

In the GA-synthesis mutants, *le* and *ls*, application of GA_1 to the seeds resulted in a shift in average microtubule orientation toward the wild-type arrangement, and a concomitant increase in internode length. Although the wild-type phenotype was not completely restored by GA_1 treatment, the results support the suggestion that the mutant alleles, *le* and *ls*, by blocking the production of active GA_1, are operating to reduce the proportion of cells with a transverse microtubule arrangement, thus limiting the potential for extension growth. Taken together with the findings of the previous chapter (2), these results indicate that *le* and *ls* are operating to reduce internode length prior to any involvement of microtubules.

Exogenous application of GA_1 to *lw* plants also produced an increase in average microtubule orientation in the epidermis of these plants, although microtubules remained significantly less transverse than in the GA-synthesis mutants. This decreased responsiveness to GA_1 is consistent with the findings of Jolly *et al.* (1987) who report that exogenously applied GA_1 substantially promoted stem elongation in *lw*, but produced only an approximate phenocopy of the wild-type cv Torsdag, and with the proposal that *lw* operates via a mechanism that partially overrides the response to GA_1 (Jolly *et al.* 1987).

Application of GA_1 to the GA-response mutants *lk*, *lka* and *lkb*, failed to result in any increase in average microtubule orientation, consistent with previous suggestions that these mutants possess a reduced responsiveness to GA_1. In combination with the previous chapter (2), these results indicate that the *lk*, *lka*, and *lkb* mutations block the progression toward extension growth prior to microtubule arrangement.

The apparent reduction in average microtubule arrangement resulting from GA_1 application to the mutants *lk*, *lka* and *lkb*, may be an artifact of an increased rate of internode development caused by GA_1 application (without any increase in final internode length). In the extremely short internodes of the GA-response mutants, even a slight increase in rate of internode development could potentially shift GA_1-treated plants ahead of control plants, introducing the possibility that microtubule orientation in GA_1-treated plants may have already begun to move toward a less transverse orientation. Alternatively, exogenously applied GA_1 may have resulted in some form of "toxicity". In support of the latter proposal, there is some evidence (although inconsistent and not presented here) of increased splitting in stems of GA_1-treated-plants.

A model of the early 13-hydroxylation pathway of GA biosynthesis and the possible sites of action of certain GA-synthesis mutants has been summarised by Reid (Fig. 1, Reid 1993). While the *le* mutation is believed to produce a partial block in the 3ß-hydroxylation of GA_{20} to GA_1 (Ingram *et al.* 1983, 1984; Ross *et al.* 1989), *ls* is believed to exert its effect much earlier in the pathway, resulting in a partial block between geranylgeranylpyrophosphate (GGPP) and copalyl pyrophosphate (CPP).

Reid (1990) has also produced a model which includes the possible sequence of events leading to elongation after reception of the GA_1 signal, and the likely sites of action of mutations conferring reduced responsiveness to GA_1. Since the GA-response mutants, *lk*, *lka* and *lkb*, possess essentially normal levels of endogenous GA_1 (Reid 1986; Reid and Potts 1986; Reid and Ross 1989; Behringer *et al.* 1990a; Reid and Davies 1992), it is unlikely that they exert their effect prior to metabolism of the active GA_1. Neither do these mutations appear to affect reception of the GA_1 signal, since *lk, lka* and *lkb* plants are phenotypically dissimilar to the GA-deficient mutants. Rather, these mutations appear to modify responsiveness to GA_1, possibly operating to block one or more GA-stimulated processes leading to wall yielding.

Similarly, the GA-response mutant *lw* does not appear to directly influence the biosynthesis of active gibberellins (Jolly *et al.* 1987), but may act to reduce elongation via a mechanism that partially overrides the resonse to GA_1.

Using the available literature and the results obtained in the present study, a revised model of the presumed pathway leading to extension growth, particularly the sequence of events leading from the reception of the GA_1 signal, is constructed.(Fig. 11).

GA-synthesis mutants

The *ls* mutation operates early in the sequence of events, prior to CPP. Behringer (1990a) has reported both an elevated wall yield threshold and reduced wall yield coefficient in *ls* plants, in comparison to wild-type plants. However, since the positioning of the mutation results in the alteration of all normal GA-mediated processes, turgor and osmotic pressures remain similar to wild-type values, in keeping with the proposed model.

The *le* allele operates later in the sequence bewteen GA_{20} and GA_1. However, like *ls*, the resultant deficiency of active GA_1 means that all normal GA-mediated processes are altered. Although turgor and osmotic pressure have not been evaluated for this mutant, it is expected that these parameters would remain similar to the wild-type.

GA-response mutants

The *lw* allele has been reported to modify phytochrome-mediated responses (Weller and Reid 1993) and the possibility of an interaction between phytochrome function and the responsiveness of tissues to GA_1 has been suggested (Weller and Reid 1993). The present results indicate that such an interaction is occurring prior to any influence of microtubule arrangement on extension growth. Although *lw* showed a significant increase in average microtubule orientation in response to applied GA_1, average microtubule orientation did not reach wild-type levels, consistent with the suggestion that *lw* reduces responsiveness to GA_1. It is likely that *lw* operates indirectly to reduce sensitivity to GA_1 at some point after reception of the GA_1 signal, since *lw* plants are phenotypically dissimilar to GA-deficient types. However, since the effect of the *lw* allele on the biophysical properties of the cell wall is not known, the point of such an interaction remains uncertain. The effect of the *lw* allele on osmotic pressure and turgor is also unknown. However, since *lw* does not result in the extremely swollen and brittle stems or stem splitting characteristic of *lk, lka* and *lkb* plants, it is considered likely that *lw* operates at some point prior to normal turgor increase.

The *lk, lka* and *lkb* mutants have essentially normal levels of endogenous GA_1 (Reid and Davies 1992; Reid 1986; Reid and Potts 1986; Reid and Ross 1989; Behringer *et al.* 1990a; Lawrence *et al.* 1992), discounting the possibility that reduced levels of GA_1 are responsible for the altered phenotype of these mutants. In addition, like *lw*, the mutations do not appear to affect reception of the GA_1 signal. Behringer (1990a) has reported an increased wall yield threshold in *lka* and *lkb* plants, and a reduced wall yield coefficient in *lka* plants relative to the wild-type. This may indicate that the mutations operate in the sequence of events leading to elongation prior to biophysical relaxation of the cell wall. In addition, substantially elevated turgor pressure and osmotic pressure has been measured in *lka* and *lkb* mutants in comparison with wild-type plants and GA-deficient mutants of a similar stature (Behringer 1990a), suggesting that *lka, lkb* and possibly *lk* plants are operating after normal turgor increase has been initiated.

The possible involvement of IAA in the disturbed microtubule arrangements in mutants *lk, lka* and *lkb* is also considered. Reduced endogenous levels of IAA in *lkb* and possibly *lk* and *lka* plants (Reid and Davies 1992; McKay *et al.* 1994) may contribute to the reduced stature of these mutants by directly influencing arrangement of microtubules, or by interacting with GA_1 to produce an altered microtubule arrangement. It is also possible that reduced endogenous IAA could result in altered wall biophysical properties, although Cleland (1984) and Masuda (1990) have reported only subtle changes in wall viscoelasticity in response to GA, which typically lag behind changes in growth rate. Cosgrove (1993b) has also argued against a viscoelastic mechanism for auxin-induced wall relaxation, favouring auxin-induced changes in wall relaxation via changes to the chemorheolohical properties of the cell wall.

The position in the sequence at which wall physical properties take effect is not clarified by the present findings, and it is unclear whether chemorheological and viscoelastic responses occur prior to, or after microtubule and microfibril orientation has been initiated.

The mechanism of operation of *lka*, and perhaps also *lk*, may differ from that in *lkb*. *lka* plants exhibited a reduction in cell number, in contrast to *lkb* in which cell number did not differ significantly form wild-type plants (Reid and Ross 1989), perhaps implying a more direct effect upon microtubules in *lka* plants. The present results, however, do not provide a resolution for this debate.

3.4.2 Effect of colchicine application on the wild-type, Torsdag.

Application of colchicine solution to internodes 3-7 of wild-type plants resulted in decreased stem length and increased stem width (Fig. 12). However, this shortening and widening effect was not consistently exhibited over internodes 3-7, and was frequently variable within an internode. A large component of the variability resulted from difficulties with application of the colchicine solution. The wetting agent, Tween-20, reduced but did not completely eliminate the beading effect of the solution. The tendency for these droplets of solution to collect at the base of each internode resulted in the uneven distribution of colchicine over the expanding internode. The application of colchicine in a lanolin paste to internodes may provide a solution to these difficulties.

Average lengths of 8 +/- 0.06 mm, 24.0 +/- 1.1 mm and 22.2 +/- 1.0 mm for internode 7 have been recorded for *lk*, *lka* and *lkb* plants, respectively (Ross and Reid 1986; Reid and Ross 1989). These plants were grown under different photoperiod and temperature conditions than those in the present study. However, proportional reductions in wild-type internode length by the *lk*, *lka* and *lkb* alleles, are approximately comparable to reductions in internode length caused by colchicine treatments. Length of internode 7 was reduced by approximately 85% in *lk* plants, and almost 70% in *lka* and *lkb* types. These values are largely within the range of internode length reductions produced by the two colchicine treatments (Table 8a, b).

Although decreases in internode length and increases in stem diameter are consistent with dimensions recorded for the mutants, colchicine application did not produce a phenocopy of *lk*, *lka* or *lkb* types. The characteristic stem banding exhibited by all 3 mutants (Ross and Reid 1986; Reid and Ross 1989) was not evident in colchicine-treated stems.

Average cell length in the epidermis of internode 7 of colchicine-treated wild-type plants (Fig. 13a) was reduced between about 54% (20% treatment) and 76% (5 mm treatment) in comparison to controls. These results are comparable to epidermal cell lengths of *lk*, *lka* and *lkb* plants (Ross and Reid 1986; Reid and Ross 1989), which were reduced in comparison to the wild-type epidermal cells lengths by approximately 60%, 48% and 73%, respectively.

Average cell length in the cortex of internode 7 of colchicine-treated wild-type plants (Fig. 13b) was reduced between about one third by both treatments in comparison to controls. Once again, these results compare favourably with cortical cell lengths of *lka*, *lka* and *lkb* plants (Ross and Reid 1986; Reid and Ross 1989) which were reduced in comparison to wild-type cortical cells by approximately 47%, 34% and 51%, respectively.

Average cell width in the epidermis of internode 7 of colchicine-treated wild-type plants (Fig. 13a) was increased between approximately 21% (20% treatment) and 53% (5 mm treatment) in comparison to controls. In contrast, epidermal cell widths of *lk* types were only slightly increased in comparison to *Lk* types (Ross and Reid 1986).

Average cortical cell width of internode 7 was increased approximately 45% by the 5 mm colchicine treatment, in comparison to average cell width in the cortex of *lk* plants (Fig. 13b) which was only slightly increased relative to *Lk*. Epidermal and cortical cell widths were only slightly increased by colchicine treatment at 20% expansion, which better approximated *lk* types.

Reductions in length of internode 7 by the mutant alleles *lk*, *lka* and *lkb* (Ross and Reid 1986; Reid and Ross 1989) were most closely approximated by colchicine application to the stem of wild-type plants when internodes had reached 5 mm in length. However, colchicine-treatment of internodes which had expanded to 20% of their final length resulted in reductions in epidermal and cortical cell length which were comparable to those produced by the *lk* and *lka* alleles. Only epidermal cell length of *lkb* types was better approximated by 5 mm colchicine treatment.

Differences between colchicine treatments may stem from the reduced inability of colchicine to inhibit elongation growth at progressively later application times. Application of colchicine has been reported to become ineffective in the inhibition of GA_3-induced elongation after 6 h in azuki bean (Shibaoka 1972) and after 36 h in lettuce (Sawhney and Srivastava 1974). The reduced effectiveness of colchicine in the inhibition of elongation growth is presumably because the pattern of cellulose deposition has already been determined by the cortical microtubule template.

It seems reasonable to assume that the destruction of the microtubule array after this stage will have no effect on cellulose deposition and thus cell shaping. As a result, plants which received colchicine at 5 mm expansion exhibited a more severe reduction of cell length than those which received colchicine at 20% expansion. At 20% expansion, internode 7 of L107 had reached 12.3 mm in length, and therefore were given a relatively longer interval prior to microtubule depolymerization by colchicine in which to establish the direction of cellulose deposition.

Since it is generally accepted that the orientation of the preprophase band (PPB) of microtubules determines both the site and plane of cell division (see Tiwari *et al.* 1984; Hush *et al.* 1990; Cleary *et al.* 1992; Apostolakos and Galatis 1993), the disruption of this category of microtubules should therefore result in perturbation of cell division.

Colchicine application to L107 resulted in a reduction of cell number (Table 8a, b), and hence cell division in both the epidermis and cortex. Colchicine treatment at 5 mm expansion resulted in approximately a 21% reduction in cell number in the epidermis and a 74% reduction in the cortex, relative to controls. Colchicine treatment at 20% expansion did not greatly influence cell number. Ross and Reid (1986) reported decreases in cell number of about 60% in the epidermis, and about 70% in the cortex of *lk* plants. Reid and Ross (1989) recorded reductions in cell number of the order of 35% in the epidermis and 52% in the cortex for *lka* types, while *lkb* types exhibited a significant reduction (40%) in cortical cell number only, in comparison to wild-type L107 plants. These values are within the range of reduction in cell numbers produced by colchicine treatment at 5 mm expansion (Table 8a, b).

Therefore, in addition to disturbing the orientation of cortical microtubule arrays, it is suggested that the mutants (particularly *lk* and *lka*) may also act to impair the functioning of microtubules in the PPB.

Cell division is apparently less disturbed in the epidermis than in the cortex by the application of colchicine to internodes. This is despite the fact that the solution was directly applied to the epidermis. The possibility that cell division in the epidermis and the cortex is differentially sensitive to colchicine cannot be excluded.

Once again, differences in cell number produced by the two application treatments may be explained by the relatively later stage at which plants in the 20% application category were supplied with colchicine.

In conclusion, application of colchicine to the stems of wild-type 107 plants produced an approximation of the gross stem morphology of the GA-response mutants *lk*, *lka* and *lkb*. The shortened internodes produced by the application of colchicine were primarily the result of the reduced cell length in the epidermis, and reduced cell number and cell length in the cortex. The increased diameter of colchicine-treated internodes resulted from the increased width of both epidermal and cortical cells.

The depolymerization of microtubules by colchicine (and therefore the loss of normal microtubule arrangement) is well documented. Similarities exist between the effects of the GA-response mutants, *lk*, *lka* and *lkb*, and colchicine application upon the number and dimension of cells in the epidermis and the cortex of wild-type plants. These results indicate that the contribution of microtubule arrangement to the normal development of stem extension in these mutants is relatively great.

However, the failure of the colchicine applications to produce a phenocopy of any of the mutants suggests that disruption of microtubule arrangement is not the only effect of the mutants alleles, *lk*, *lka* and *lkb*, and suggests that an effect on wall biophysical parameters is also likely.

CHAPTER 4: THE RELATIONSHIP BETWEEN GA_1, LIGHT AND ELONGATION IN *PISUM*: EFFECT OF THE *Lv* MUTATION ON CHLOROPLAST STRUCTURE AND MICTOTUBULE ARRANGEMENT

4.1 Introduction

Mutants which alter responsiveness to gibberellin A_1 (GA_1) have been described for a range of plants (e.g. maize, Phinney 1961; wheat, Gale and Marshall 1973; and peas, Reid and Potts 1986). In the garden pea, *Pisum sativum* L., the majority of these mutants (i.e. *lw*, *lk*, *lka*, *lkb*, and *lkd*) exhibit a reduced stem elongation response to GA_1 (Reid 1986; Reid and Potts 1986; Jolly *et al.* 1987; Reid and Ross 1989; Cramp and Reid 1993; Weller and Reid 1993). These dwarf GA-response mutants have been valuable in exploring the steps between reception of the GA_1 signal and stem elongation.

Mutants that result in an enhanced elongation response to GA_1 are rare in comparison. Such a mutant, *lv-1*, has been identified in the garden pea (Reid and Ross 1988a). In contrast to elongated mutants which appear to cause GA overproduction (e.g. *sln*, Reid *et al.* 1992; Ross *et al.* 1993; Swain and Reid 1993), the *lv-1* gene does not appear to be involved with GA_1 synthesis or breakdown. Reid and Ross (1988a) reported that the metabolism of $[^3H]$-GA_{20} was qualitatively similar in *lv* and *Lv* plants. Weller *et al.* (1994) reported comparable levels of GA_1 in the apical portions of both *lv-1* and wild-type plants, despite a two-fold increase in length compared to wild-type plants.

Neither do *lv-1* plants behave as if saturated with gibberellins (e.g. *la cry*^{s}, Potts *et al.* 1985). Plants with the *lv-1* allele showed a greater elongation response to all levels of exogenously applied biologically active GA_1 (including a saturating dose), compared with *Lv* plants (Reid and Ross 1988a; Weller *et al.* 1994). The *lv-1* allele does not appear to confer enhanced sensitivity to GAs prior to GA_1 in the biosynthetic pathway (e.g. GA_{20}), since the relative response to GA_1 and GA_{20} was comparable in both *lv-1* and *Lv* plants (Reid and Ross 1988a).

In addition to this hypersensitivity to GA, plants containing the *lv-1* allele have been shown to exhibit an altered response to light (Nagatani *et al.* 1990). Under red, blue and white light, *lv-1* plants elongated to a greater extent than isogenic *Lv* plants. At the cellular level, the epidermal cells of red-, blue- or white-light-grown *lv-1* plants were comparable with cell lengths of *Lv* plants grown in darkness. In contrast, comparable *Lv* plants showed a marked reduction in cell length after exposure to red or white light

(Nagatani *et al.* 1990). However, under far-red light and darkness no significant difference between the mutant and the wild-type was apparent (Nagatani *et al.* 1990). In addition, quantification of GA_1 from the apical portion of dark- and light-grown wild-type pea seedlings revealed no substantial difference in the level of GA_1, despite a three-fold difference in length (Weller *et al.* 1994). Since the *Lv/lv-1* difference is apparent under only certain light regimes, *lv-1* can be considered, at least in a general sense, to be a photomorphogenic mutant (Nagatani *et al.* 1990).

Photomorphogenic mutants have been reported in several other species, including *Arabidopsis*, tomato and cucumber. In tomato, the long hypocotyl phenotype is conferred upon plants by the gene *au* (Koorneef *et al.* 1985). *au* plants have markedly reduced chlorophyll levels, and exhibit strongly reduced chloroplast development, anthocyanin synthesis and seed development. The gene *au* confers a clear end-of-day stem elongation response to far-red light treatment (Adamse *et al.* 1988b; López-Juez *et al.* 1990b). Spectrophotometric (Koorneef *et al.* 1985) and immunological (Parks *et al.* 1987) analyses have shown that the phytochrome content of dark-grown *au* plants is reduced to below 5% of the wild-type. Light-grown *au* plants exhibited a reduction in phytochrome content of approximately 50% (Adamse *et al.* 1988b). These results may suggest that the *au* mutation prevents the accumulation of light-labile phytochrome (phyA) (Parks *et al.* 1987) but does not influence the accumulation of light-stable phytochrome (phyB) (López-Juez *et al.* 1990b). However, over-expression of transgenic phyA in *au* plants does not restore a wild-type phenotype (van Tuinen *et al.* 1993), suggesting that the *au* phenotype may result from deficiency in the phytochrome chromophore (Sharma *et al.* 1993).

In *Arabidopsis*, a number of long-hypocotyl mutants with altered phytochrome responses have also been isolated (e.g. Koorneef *et al.* 1980). The mutants *hy1*, *hy2* and *hy6* are deficient in spectrophotometrically detectable phytochrome in etiolated seedlings, although they accumulate the phyA apoprotein (Koorneef *et al.* 1980; Chory *et al.* 1989a; Parks *et al.* 1989). Their effect is due to impaired synthesis of the tetrapyrrole chromophore (Parks and Quail 1991; Quail 1991).

By comparison, mutants of *hy3* and *hy5* have been demonstrated to possess normal levels of spectrophotometrically detectable phytochrome in etiolated seedlings (Koorneef *et al.* 1980; Chory *et al.* 1989a; Parks *et al.* 1989). However, the *hy3* mutation results in reduced phy B levels (Nagatani *et al.* 1991a; Somers *et al.* 1991) due to a mutation in the *phyB* gene (Reed *et al.* 1993). Plants possessing *hy3* are not strongly inhibited by continuous red light (Koorneef *et al.* 1980) and lack normal end-of-day far-red response (Nagatani *et al.* 1991a; Whitelam and Smith 1991).

The *lh* mutant in cucumber, and the *ein* mutant in *Brassica*, are phenotypically similar to *hy3*. Both *lh* and *ein* appear to be deficient in phyB (López-Juez *et al.* 1990a, 1992; Devlin *et al.* 1992). These combined results may suggest a role for phyB in red-light perception and red: far-red responses (McCormac *et al.* 1992).

The *lv-1* mutant in pea (Nagatani *et al.* 1990) is phenotypically similar to the *lh* mutant in cucumber and *hy3* in *Arabidopsis*. All three mutants exhibit an elevated growth response to white or red light in comparison to wild-type plants, and all possess slightly lower chlorophyll levels and lack the normal end-of-day far-red response (e.g. Adamse *et al.* 1987, 1988a; Nagatani *et al.* 1990; Reed *et al.* 1993). In addition, Weller and Reid (1993) have reported a small but significant decrease in elongation in response to end-of-day far-red light in *lv-1* plants in comparison to *Lv* plants. A similar decrease in elongation in response to end-of-day far-red has also been reported for the *lh* mutant of cucumber (López-Juez *et al.* 1990a).

Comparison of the response of *lv-1* and *Lv* to changing red:far-red ratio (Weller and Reid 1993) revealed a 34% decrease in stem elongation of *Lv* types with decreasing far-red content of the incident light. Plants with the *lv-1* allele were longer than comparable *Lv* plants at all red:far-red ratios and, in contrast to *Lv* plants, the maximum inhibition of elongation occurred with the highest far-red content.

The red/far-red reversibility of the *Lv/lv-1* difference demonstrated by Nagatani *et al.* (1990), indicated the involvement of phytochrome in the *Lv/lv-1* difference. Several different phytochrome molecules have been demonstrated to exist in higher plants (Abe *et al.* 1985; Konomi *et al.* 1987; Sharrock and Quail 1989; Somers *et al.* 1991; Dehesh *et al.* 1991; Wang *et al.* 1991). The existence of "labile" and "stable" pools of phytochrome, distinguished by differential persistence of far-red light reversibility, has been indicated by physiological studies involving light control of some developmental processes (Furuya 1989; Smith and Whitelam 1990; Tomizawa *et al.* 1990). In dark-grown tissue light-labile phyA is abundant, but is rapidly depleted by light, whereas light-stable phyB is much less abundant in etiolated tissue and not substantially altered by light. A recent study of phyA-deficient mutants (Parks and Quail 1993) has confirmed that the "labile" pool is phyA.

Analysis of the original allele (*lv-1*) indicated similar levels of spectrophotometrically and immunologically detectable phytochrome between the mutant and its wild-type progenitor cv. Sparkle in either light or darkness (Nagatani *et al.* 1990). These results prompted Nagatani *et al.* (1990) and Weller and Reid (1993) to suggest that *lv-1* plants possess a modified response to phyB resulting from a block in the transduction

pathway leading from phyB. More recently, Weller *et al.* (1995) have used immunological methods to confirm the normal presence of phyB-like apoprotein in *lv-1* mutants. However, other alleles at the *lv* locus, *lv-2* (R83), *lv-3* (Wt10895) and *lv-4* (L80m) have recently been shown to be deficient in phyB apoprotein (Weller *et al.* 1994, 1995). The existence of allelic mutants with and without phyB apoprotien has led Weller *et al.* (1995) to suggest that *Lv* may be a structural gene for a B-type phytochrome, the *lv-1* mutation resulting specifically in the loss of normal biological activity of this phytochrome.

Nagatani *et al.* (1990) measured the total level of chlorophyll in mature expanded leaves of *lv-1* and *Lv* plants. They found that chlorophyll level in *lv-1* was significantly lower than in comparable *Lv* plants. Visually, this decrease in chlorophyll content was detectable as a slight yellowing of the leaves. The ratio of chlorophyll a/b was apparently unchanged by *lv-1*, since levels of chlorophyll a and b were similar to those measured in wild-type plants (Nagatani *et al.* 1990). In tomato, genes *au* and *yg*-2 result in pronounced yellowing of leaves (Koorneef *et al.* 1985), and, in primary leaves of the *lh* mutant of cucumber, a small reduction in the level of chlorophyll has been reported (Adamse *et al.* 1987).

The above observations suggest that *lv-1* plants behave as if unable to completely de-etiolate in response to red or white light. In light of this observation, an investigation of the effect of *lv-1* on chloroplast organization was undertaken. In addition, since the altered elongation response to light exhibited by *lv-1* plants under certain light regimes suggested the possible involvement of microtubules, an analysis of microtubule orientation in *Lv* and *lv-1* plants grown under white light or in darkness was completed.

4.2 Materials and Methods

4 2.1 Analysis of chloroplast structure

Plant material

The pure lines of pea used for this work are held in the collection at Hobart (Department of Plant Science, University of Tasmania). The elongated mutant NEU3 (*lv-1 le La cry*^{c}) was derived from cv. Sparkle (*Lv le La cry*^{c}) by Drs. T. A. La Rue and B. E. Kneen of the Boyce Thompson Institute, Ithaca, N. Y. Both lines are dominant for the internode length genes *Lm Ls Lh Lw Lk Lka Lkb Lkc Lkd and Na.* Further details about the genotypes and phenotypes of these lines can be found in Reid (1987) and Reid and Ross (1988a).

Growing conditions

Plants were grown in 140-mm-diameter black Slimline pots in a 50:50 mixture of vermiculite and dolerite chips topped with 30-30 mm of potting mix, as specified by Reid (1986). From two weeks after sowing, plants received twice weekly application of nutrient solution (Aquasol). Plants were grown in the controlled environment of a growth cabinet which was held at 17.5 °C. Plants received a 24 h photoperiod and 200 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ of photosynthetically active radiation (P.A.R.) at the pot surface, supplied by a mixture of incandescent and fluorescent light.

Harvesting procedure

Leaf sections (1 mm^2) were cut using a fine razor blade from the mid lamina of a leaflet at node 6, and immediately placed into a drop of fixative (4% glutaraldehyde in cacodylate buffer). Leaf sections were further sliced into 5 to 6 strips to facilitate penetration of the fixative.

Tissue fixation

Tissue remained in vials of fixative on a rotator for 48 h. After rinsing in distilled water (2 x 30 min), tissue was transferred into 2% osmium tetroxide and fixed for a further 1 h. Following rinsing in distilled water (2 x 30 min), tissue was placed in 2% uranyl acetate for 1 h, after which time an equal volume of 100% ethanol was added to the tissue. After 2 h in 50:50 uranyl acetate /ethanol, tissue was transferred to a 30:70 mix and left overnight. Over 2 h, the proportion of ethanol was slowly increased to 100%, after which the tissue was transferred to 100% Epon for embedding, via a propylene oxide/Epon series.

Tissue embedding

Leaf tissue was placed in unlidded vials on a rotator for 4 to 5 h at 37 °C to allow the evaporation of any propylene oxide residue. While Epon remained liquid, tissue was transferred to fresh 100% Epon in flat plastic paraffin moulds. Embedding was at 37 °C for 24 h, then 60 °C for a further 48 h. The hard Epon mix used is as follows: 11.66 g Spi-pon 812, 3.3 g DDSA, 8.41 g NMA, 0.3-0.4 g DMP-30.

Transmission electron microscopy

Tissue was sectioned using an LKB III ultramicrotome (Sweden) using either hand-cut glass knives or a diamond knife (2 mm DDK, Delaware, USA). Sections were picked up on a combination of 1 mm/2 mm slot or 200-300 mesh Formvar-coated transmission electron microscopy grids. Grids were placed in drops of 4% uranyl acetate on Parafilm and heated at 40-50 °C for 45-60 min. Grids were rinsed in a gentle stream of distilled water for 30-40 s and then dried over a gentle heat. After drying,

grids were placed into drops of lead citrate stain (0.12 g lead citrate plus 0.4 g NaOH in 100 ml freshly boiled distilled water) on Parafilm for 10-20 min. Lead staining was in a covered dish, to which NaOH pellets had been added to maintain a dehydrated atmosphere. The sections were examined under a Hitachi H-300 electron microscope at 30, 000 magnification. From 4 plants of cv. Sparkle (*Lv*) and 3 plants of NEU3 (*lv-1*), 10 chloroplasts from the upper half of palisade mesophyll cells situated directly below the upper epidermis were examined and their characteristics measured (Table 9).

4.2.2 Analysis of microtubule orientation

Plant material

This work utilized the mutant NEU3 (*lv-1*) and its wild-type Sparkle (*Lv*) (both described above), as well as the essentially isogenic lines 232- (*lv-1*) and 232+ (*Lv*), derived from a common F6 ancestor of the cross cultivar (cv.) Torsdag (WT) x NEU3 (*le cry^c / lv-1*) (Reid and Ross 1988a, Reid 1989).

Growing conditions

Plants were grown (6 plants / pot) in 140-mm-diameter black Slimline pots in a 50:50 mixture of vermiculite and dolerite chips topped with 30 mm of potting mix, as specified by Reid (1986). Light-grown plants were grow in a controlled-environment cabinet held at a constant temperature of 22°C. These plants received a 24 h photoperiod and 150-200 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ of photosynthetically active radiation (P.A.R.) at the pot surface, supplied by cool-white fluorescent tubes (Thorn). Dark-grown plants were grown in complete darkness at a constant temperature of 22°C. A weak safe-green light was used for monitoring growth. Light and darkgrown plants were watered on the day of sowing (day 0) and and then daily until seedlings could be seen emerging from the substrate. Subsequent watering was every 2 to 3 days as required.

Initially, all lines were allowed to maximally expand internode 3 in order to establish final lengths for this internode under these growing conditions (Table 10a, b). Plants were harvested for immunofluorescence work when internode 3 reached 25-40% expansion, which, at 22° C, was 5-6 d after sowing for light-grown plants, and 8 d after sowing for plants grown in darkness. Fig. 15a, b shows the approximate lengths of a) light- and b) dark-grown *Lv* and *lv* plants at time of harvest.

Harvesting procedure

At 25-40% expansion of internode 3, seedlings were decapitated just above the cotyledonary node and immediately placed into a shallow pool of fixative. Using a fine (Gillette Blue) razor blade 1 mm length segments were cut from the 2 mm zone at internode position B, 4-6 mm from the apical hook.

Tissue fixation, sectioning, tubulin immunolabelling, microscopy and analysis of microtubule orientation followed the methods detailed in Chapter 2.

Histograms showing the percentages of microtubules falling into the three orientation categories (transverse, oblique and longitudinal), at specified distances from the apical hook, were constructed (Fig. 16a, b). Scatter graphs (Fig. 17a, b) were also constructed to show the average microtubule orientation for the wild-type and stem-length mutants at each distance from the apical hook. Sample size and standard errors for average microtubule orientation are given in table form (Table 11a, b) to facilitate interpretation of the orientation data.

4.3 Results

4.3.1 Analysis of chloroplast structure

Transmission electron microscopic observation of the chloroplasts from mature leaves of *Lv* and *lv-1* plants revealed a difference in the overall appearance of chloroplasts of these types. Analysis of chloroplast structure revealed a reduction in the number of grana per chloroplast in *lv-1* types (Table 9). Number of grana were reduced in *lv-1* types by approximately 50%, in comparison with *Lv*. In addition to a reduction their number, grana were less well organized in *lv-1* than in *Lv* (Fig. 14a, b). The form of individual grana were less discrete in *lv-1* than in *Lv*, their constituent thylakoids appearing to merge to a certain extent with those of neighbouring grana. In addition to a reduction in the number and organization of grana, the number of thylakoids per granum was reduced in *lv-1*. In comparison to *Lv*, thylakoid number in *lv-1* was reduced by about 50%.

Starch grains within the chloroplasts of *lv-1* plants were more numerous and larger than in *Lv* types (Table 9). In *lv-1*, starch grains were 3-4 times more numerous and in the order of about 3 times as large as in chloroplasts from comparable *Lv* plants.

Table 9 Mean +/- SE number of grana and starch grains per chloroplast, thylakoids per granum and the length and breadth of starch grains from *Lv* and *lv-1* plants. n ≥ 27.

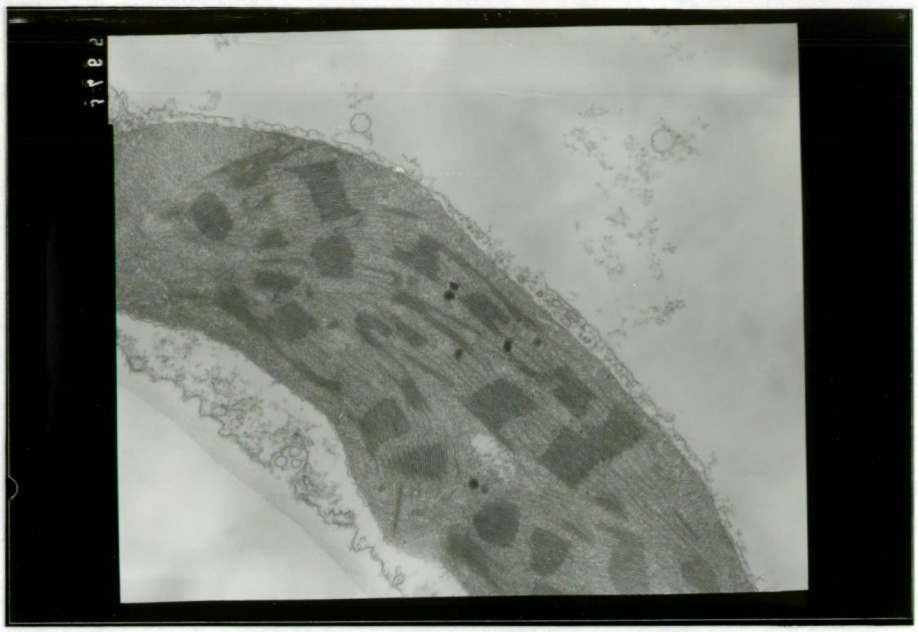
Genotype	Number of grana	Number of starch grains	Thylakoids per granum	Starch grain Length (μm)	Starch grain Breadth (μm)
Lv	30.9 +/- 1.5	1.4 +/- 0.2	20.4 +/- 0.5	0.71 +/- 0.05	0.39 +/- 0.03
lv-1	14.2 +/- 1.1	5.2 +/- 0.3	11.8 +/- 0.4	1.19 +/- 0.05	0.81 +/- 0.05

4.3.2 Analysis of microtubule orientation

Immunofluorescence analysis of microtubule orientation in wild-types (*Lv*) and mutant (*lv-1*) plants revealed that, when grown under high intensity cool-white light, average microtubule orientation in the mutants was significantly higher than in wild-type plants. However, when grown in complete darkness, average microtubule orientation of *Lv* and *lv-1* plants was not significantly different (Table 11a; Fig. 17a). This trend was also evident in the subepidermal cells of the cortex (Table 11b; Fig. 17b).

Increased average microtubule orientation is a result of a relatively larger proportion of both epidermal and sub-epidermal cortical cells with a predominantly transverse microtubule orientation (Fig. 16a, b). The similar average microtubule orientation of the dark-grown mutants and wild-type plants results from the relative increase in average microtubule orientation in dark-grown *Lv* in comparison to dark-grown *lv*.

(a)



(b)

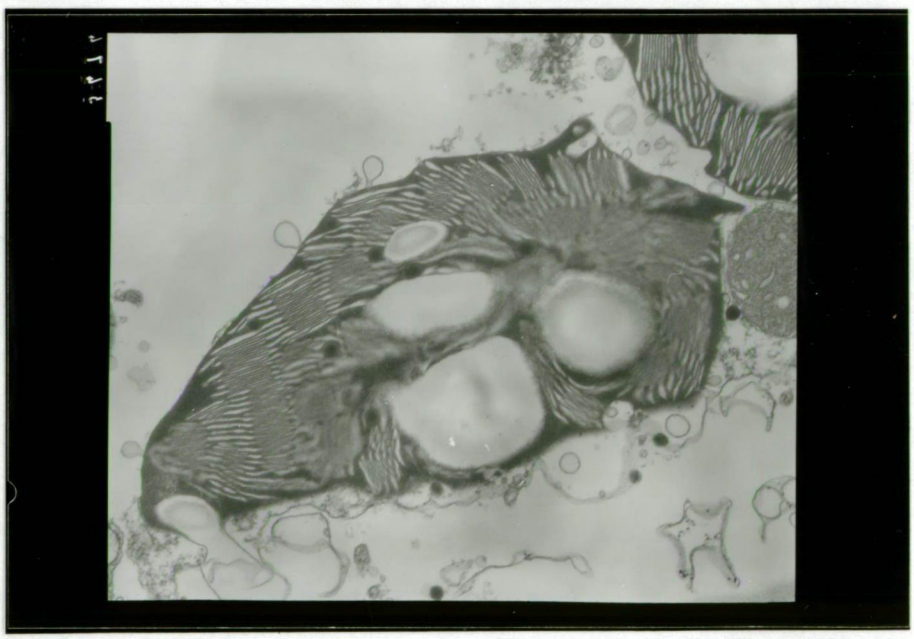


Fig.14a, b Transmission electron micrographs of chloroplasts from the upper half of palisade mesophyll cells of (a) cv. Sparkle (*Lv*) and (b) NEU3 (*lv-1*). Magnification at x30, 000.

a)

b)

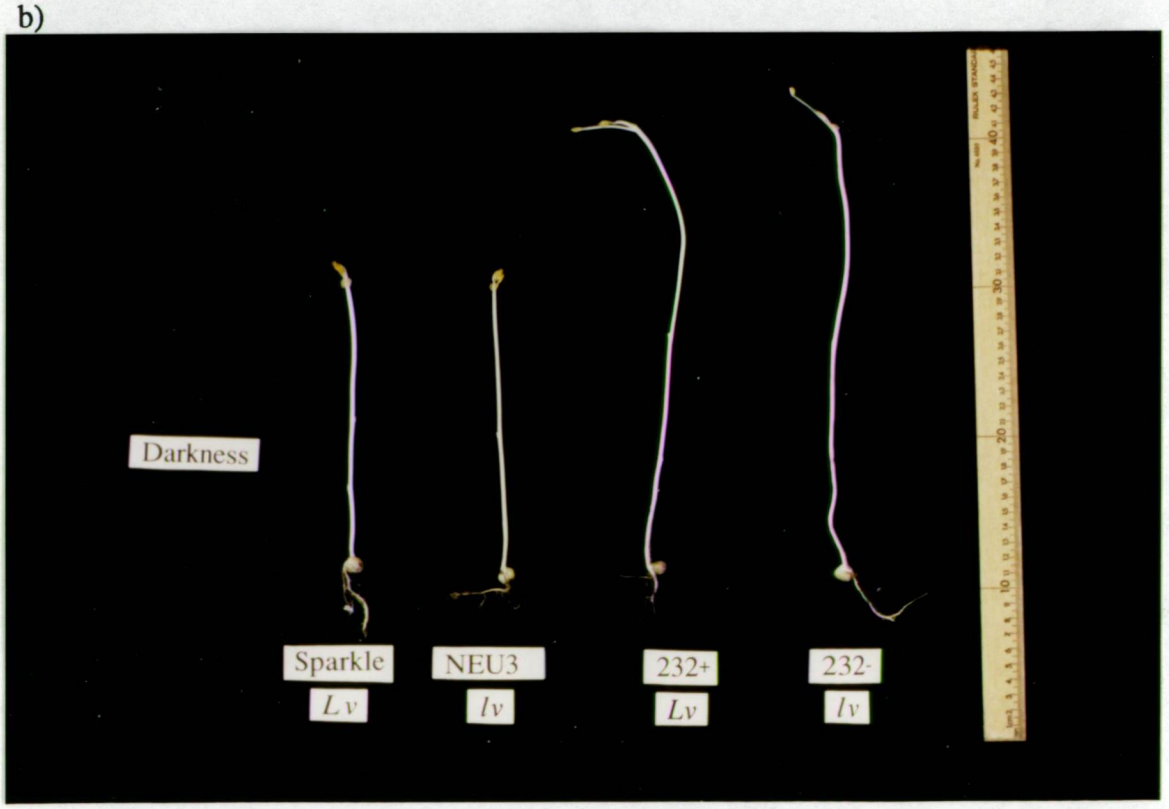


Fig. 15a, b Photographs of wild-type (*Lv*) and mutants NEU3 (*lv-1*) and 232- (*lv-1*) plants grown under a) white light and b) in complete darkness.

Table 10a, b Expanded lengths of third internodes, and length at 25% and 40% expansion of a) light grown and b) dark grown seedlings of wild type (*Lv*) and mutants (*lv*) of *Pisum sativum* L.

Line	Genotype	Total Length Internode 3	Length at 25% Expansion (mm)	Length at 40% Expansion (mm)
Sparkle	*Lv*	11.78+/- 0.24 (23)	2.95	4.71
NEU3	*lv-1*	30.33 +/- 0.87 (21)	7.58	12.13
232+	*Lv*	24.83 +/- 4.31 (23)	6.21	9.93
232-	*lv-1*	67.36 +/- 2.12 (22)	16.84	26.95

(a)

Sparkle	*Lv*	88.86 +/- 1.86 (22)	22.22	35.55
NEU3	*lv-1*	99.48 +/- 3.12 (21)	24.87	39.79
232+	*Lv*	137.44 +/- 3.16 (23)	34.36	54.97
232-	*lv-1*	147.75 +/- 5.64 (20)	36.94	59.1

(b)

Total internode lengths are means +/- standard errors. Sample sizes are included in parentheses. 25% and 40% expansion refers to length of internode 3 at 25% and 40%, respectively, of total length.

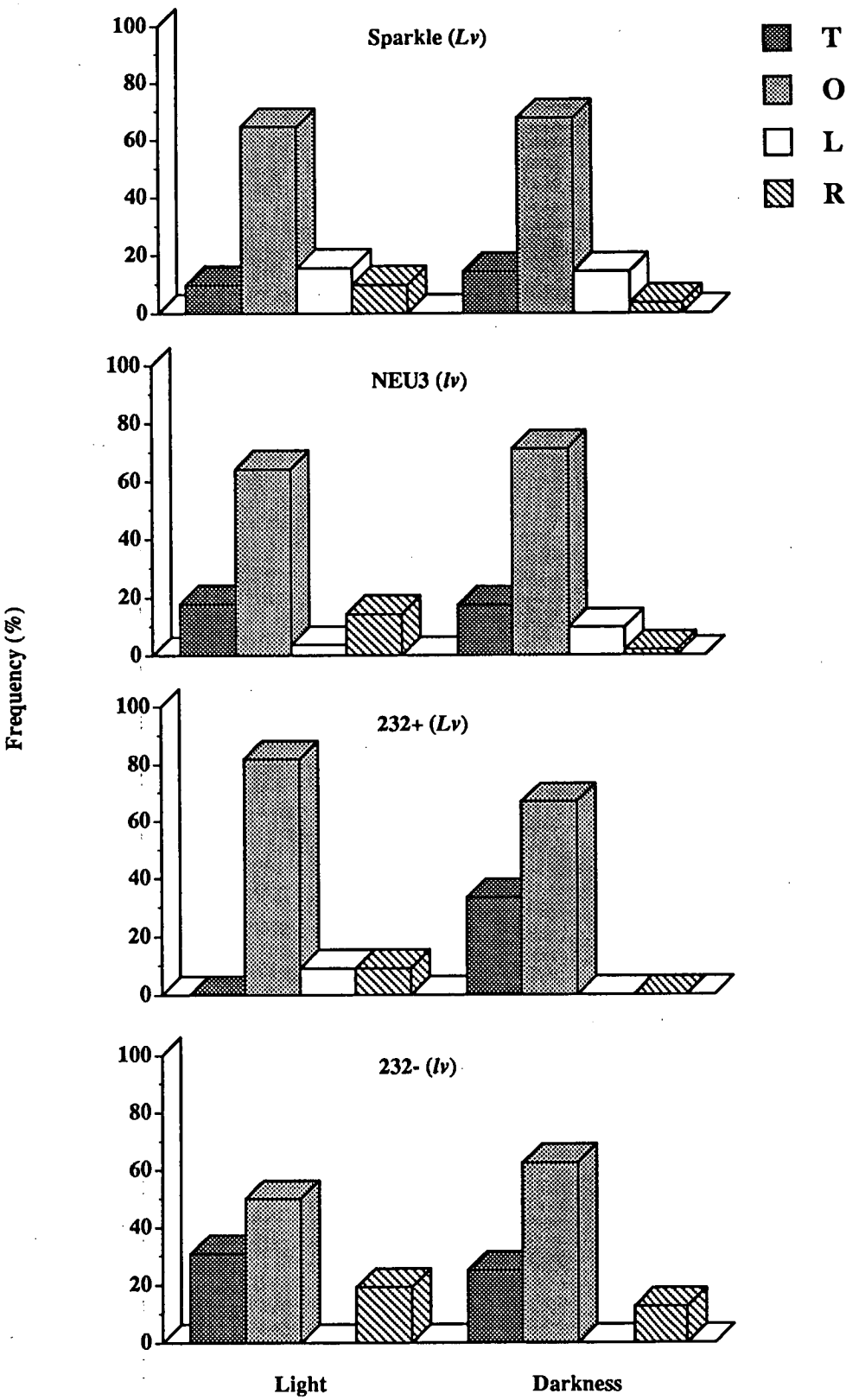


Fig. 16a Microtubule orientation in the tangential surface of epidermal cells in the 4-6 mm (B) region below the apical hook in internode 3 of wild-type (WT) (*Lv*) and mutant (*lv*) lines of *Pisum sativum* L. epicotyls grown in the light and in darkness. Observations were made when the internode was 25-40% expanded. Histograms show the percentage distribution of cells having microtubules arranged within the angular ranges of 0°-30° (Longitudinal = *L*), 30°-60° (oblique = *O*), and 60°-90° (transverse = *T*) to the direction of the cell axis. Cells with a random (*R*) microtubule orientation are also recorded.

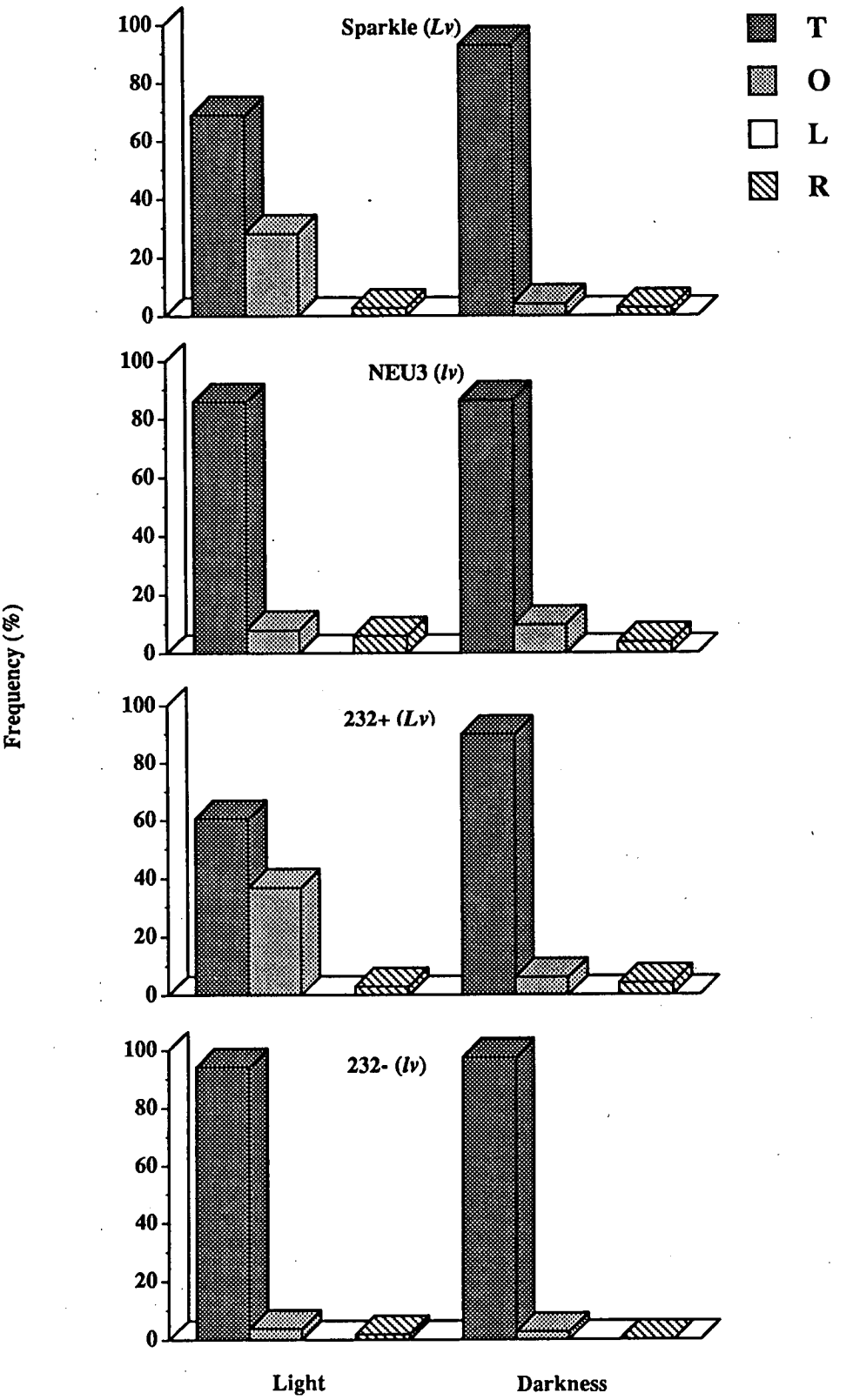


Fig. 16b Microtubule orientation in longitudinal sections of cortical cells in the 4-6 mm (B) region below the apical hook in internode 3 of wild-type (WT) (*Lv*) and mutant (*lv*) lines of *Pisum sativum* L. epicotyls grown in the light and in darkness. Observations were made when the internode was 25-40% expanded. Histograms show the percentage distribution of cells having microtubules arranged within the angular ranges of 0°-30° (Longitudinal = *L*), 30°-60° (oblique = *O*), and 60°-90° (transverse = *T*) to the direction of the cell axis. Cells with a random (*R*) microtubule orientation are also recorded.

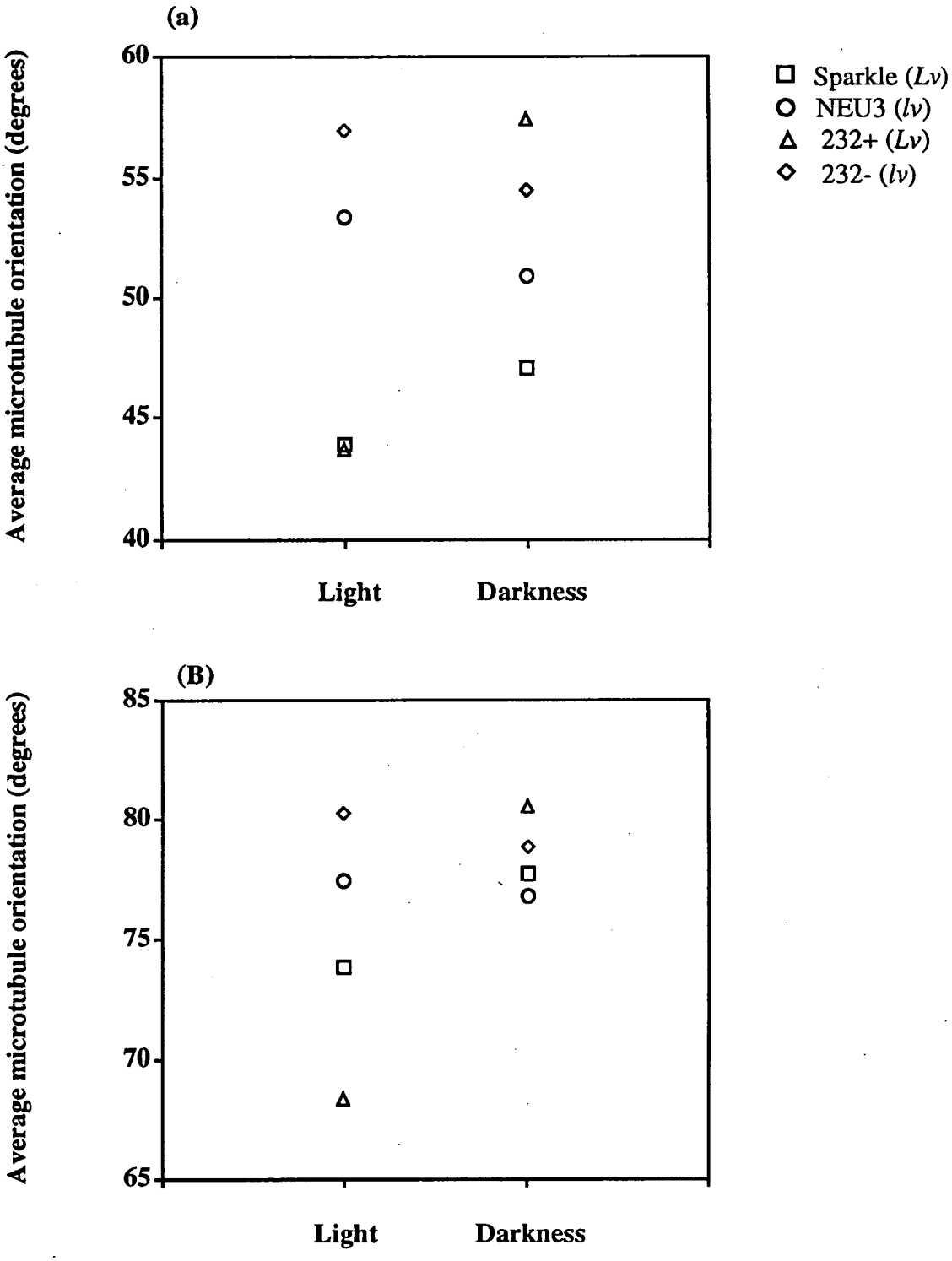


Fig. 17a, b Average microtubule orientation (degrees from cell axix) in **a**) the tangential surface of epidermal cells and **b**) longitudinal sections of cortical cells in the 4-6 mm (B) region below the apical hook in internode 3 of wild-type (WT) (*Lv*) and photomorphogenic mutants (*lv*) of *Pisum sativum* L. grown in light and in darkness.

Table 11 Microtubule orientation 4-6 mm from the apical hook in (a) the epidermis and (b) subepidermal cortical cells of WT (*Lv*) plants and photomorphogenic mutants (*lv*) of pea grown in light and in darkness

(a)	Light		Darkness		
	Mean	SE	Mean	SE	Light vs Darkness
Sparkle (*Lv*)	43.78 (46)	1.97	47.0 (27)	2.96	NS
NEU3 (*lv*)	53.29 (24)	2.14	50.78 (51)	1.73	NS
Sparkle vs NEU3	**		NS		
232^+ (*Lv*)	43.6 (20)	1.82	57.27 (15)	3.17	***
232^- (*lv*)	56.81 (21)	2.94	54.38 (21)	2.66	NS
232^+ vs 232^-	***		NS		

(b)	Light		Darkness		
	Mean	SE	Mean	SE	Light vs Darkness
Sparkle (*Lv*)	73.78 (50)	0.96	77.57 (70)	0.85	**
NEU3 (*lv*)	77.30 (47)	1.83	76.64 (50)	1.39	NS
Sparkle vs NEU3	*		NS		
232^+ (*Lv*)	68.34 (35)	1.87	80.43 (47)	1.316	***
232^- (*lv*)	80.14 (51)	1.09	78.71 (38)	1.44	NS
232^+ vs 232^-	***		NS		

Average microtubule orientation (degrees from cell axis) in a) the tangential surface of epidermal cells and b) longitudinal sections of subepidermal cortical cells in the 4-6 mm (B) region below the apical hook in internode 3 of wild type (WT) (*Lv*) and photomorphogenic mutants (*lv*) of *Pisum sativum* L. Data are means, standard error (SE), and sample size (n). WT plants and photomorphogenic mutants were tested for differences in MT orientation in light and in darkness, and photomorphogenic mutants were tested for differences from their respective WT using the Fisher PLSD test. *$p<0.05$, 88$P<0.01$, ***$P<0.001$, NS = not significant at $P<0.05$.

4.4 Discussion

The mutant allele *lv-1* has been shown to cause a modification of the wild-type chloroplast ultrastructure. *lv-1* plants exhibited both a reduced number and organization of grana, and grana were observed to have fewer constituent thylakoids.

Chlorophyll levels, measured as total level of chlorophyll in mature expanded leaves, have also been shown to be significantly reduced in *lv-1* plants in comparison to *Lv* plants (Nagatani *et al.* 1990), although the ratio of chlorophyll a/b was apparently unchanged by *lv-1* (Nagatani *et al.* 1990).

These observations suggest that the *lv-1* allele appears to result in some impairment of the de-etiolation process. Weller *et al.* (1994) have reported that etiolation and the *lv-1* mutation produce a shift in the dose-response curve for GA_1 (Fig. 8a, Weller *et al.* 1994). Both *lv-1* and de-etiolation result in a increase in elongation, relative to light-grown wild-type plants, at comparable concentrations of GA_1 (either endogenous or applied).

However, Weller *et al.* (1994) have reported differences between the effects of the *lv-1* mutation and GA_1 application on the rate of node development, cell extension and division, and leaf development of seedlings. Weller *et al.* (1994) have suggested that these differences indicate that, in pea, the control of stem elongation by light in general, and by phyB in particular is not mediated by changes in GA_1 content. This is consistent with the evidence that *lv-1* plants and the GA_1-overproducing mutant *sln* are not true phenocopies (Weller *et al.* 1994).

Instead, Weller *et al.* (1994) have proposed that the increased elongation of dark-grown and *lv-1* plants appears to result from an increased responsiveness of the plant to its endogenous levels of GA_1. However, the use of *Pisum* mutants deficient in GA_1 (i.e. *na*, *ls*, *le*) and a range of double mutants (i.e. *na lv*, *ls lv*, *le lv*) enabled Weller *et al.* (1994) to demonstrate that the relative magnitude of this change in responsiveness is dependent on GA_1 level.

Weller *et al.* (1994) have suggested that differences in the pleiotropic effect of GA_1 application and the *lv* mutation might indicate that light and GA_1 interact relatively late in their respective transduction pathways. Similarities between the effects of GA_1 and etiolation on cell wall biophysical parameters (Behringer *et al.* 1990) and microtubule orientation (Nick and Furuya 1993) have prompted Weller *et al.* (1994) to suggest that the two pathways may intersect prior to microtubule arrangement, consistent with the

suggestion of Nick and Furuya (1993) that light may act, in part, to constrain transduction of the GA_1 signal at a relatively late stage.

The present findings provide support for an interaction of the transduction pathways for light and GA_1 prior to microtubule arrangement. Under high-intensity white fluorescent light, the *lv-1* allele resulted in a disturbance of wild-type microtubule orientation, *lv-1* types possessing higher average microtubule orientation than wild-type plants (Table 11a, b; Fig. 17a, b).

These results are consistent with the model of the possible sequence of events leading to stem extension (Fig. 11) outlined in Chapter 3. When grown in the light, *lv-1* plants had a larger transverse microtubule component, and consequently greater stem length than comparable *Lv* plants.

It has been suggested that the reduced inhibition of elongation by white light is due to a loss of normal biological activity of phyB conferred by the *lv-1* allele (Weller *et al.* 1995). Therefore it is also suggested that the normal effect of phyB in elongation growth is mediated by microtubules. Average microtubule orientation was considerably increased in dark-grown *Lv* plants in comparison with light-grown *Lv* plants. Darkness did not produce a similar effect in *lv-1* plants.

Darkness has been shown to incease responsiveness to GA_1 (Reid 1988; Reid and Ross 1982), raising the question of whether darkness and the gene *lv-1* act via a similar mechanism. However, while darkness and *lv-1* result in a similar increase in internode length and transverse microtubule component, it is clear that dark-grown *Lv* plants are phenotypically different to *lv-1* plants grown in the light, indicating that darkness may also influence developmental processes other than those controlled by phyB. Indeed, *lv-1*plants grown in darkness are phenotypically dissimilar to those grown in light. While microtubule arrangement was largely comparable in light- and dark-grown *lv-1*plants, light-grown *lv-1* types had shorter internodes and exhibited a greater degree of leaf expansion and chlorophyll development in comparison to dark-grown *lv* plants. It is possible that darkness may operate to alter phenotype via other receptors, such as phyA or blue light receptors, not affected by the *lv-1* mutation.

CHAPTER 5: GENERAL DISCUSSION

The regulation of internode elongation involves interaction of the range of partial processes that contribute to elongation growth. Insight into the nature of a number of these processes has been facilitated by the availability of mutants which primarily affect stem elongation. In particular, mutants which affect the synthesis of, or the responsiveness to GA have been of considerable value in defining the role of GA in the various components of stem elongation (e.g. Phinney 1984; Reid and Ross 1993).

With this application in mind, the present study utilized a range of stem length mutants, both GA-synthesis and GA-response types, to investigate the role of GA in microtubule-mediated stem elongation.

Microtubule orientation and extension growth

It is generally accepted that the orientation of the cortical microtubule array determines the direction in which cellulose microfibrils are deposited, and that the oriented deposition of cellulose microfibrils determines the directionality of cell expansion (see Chapter 1).

An analysis of microtubule orientation in GA-synthesis mutants and their wild-type revealed a positive correlation between transverse microtubule orientation and elongation rate, consistent with a causal relationship between a transverse microtubule arrangement and stem extension.

The regulation of shifts in microtubule pattern has been suggested to occur via de- and re-polymerization (Robinson and Quader 1980). However, in the present study, observation of microtubule orientation in the wild-type and the stem length mutants suggests that the reorientation of microtubules from a more to a less transverse arrangement does not involve gross de- and re-polymerization of arrays. Instead, reorientation is suggested to be achieved by a wholesale shift from a transverse orientation to a more longitudinal one. The increasing frequency of obliquely, to longitudinally oriented microtubules in conjunction with decreasing elongation rate supports this proposal, as does the majority of available literature (e.g. Lloyd 1983; Lloyd *et al.* 1985; Roberts *et al.* 1985; Hogetsu and Oshima 1986; Seagull 1986; Iwata and Hogetsu 1988, 1989b).

The role of GA_1 in microtubule-mediated elongation growth

GA-induced axial extension has been reported in a range of plants including azuki bean (Shibaoka 1972, 1974; Takeda and Shibaoka 1981a, b), lettuce (Sawhney and Srivastava 1974, 1975), tomato (Jupe *et al.* 1988), cucumber (Taylor and Cosgrove 1989; Ishida and Katsumi 1992), onion (mita and Shhibaoka 1984b), sweet pea (Ross *et al.* 1990c), pea (Ingram *et al.* 1986; Akashi and Shibaoka 1987; Chory *et al.* 1987; Sakiyama and Shibaoka 1990), rice (Nick and Furuya 1993), and maize (Mita and Katsumi 1986; Chory *et al.* 1987).

Ingram *et al.* (1986), Ross *et al.* (1989) and Swain and Reid (1993) have previously reported a quantitative relationship between the level of GA_1 and internode elongation in peas. The present study provides additional support for such a relationship. Reid and Ross (1986) and Ross *et al.* (1989) have demonstrated greatly reduced endogenous levels of GA_1 in *le* and *ls* plants, resulting from impairment of the early-13-hydroxylation pathway of GA biosynthesis. The construction of growth profiles revealed that, at the morphological level, reduced internode lengths of the GA-synthesis mutants used in this study resulted from both a decreased rate of extension and an attenuated region of expansion. The substantially altered growth profiles of *le* and *ls* support a quantitive relationship for GA_1 and internode elongation, and suggest that GA is responsible for regulating both rate of extension and the length of epicotyl over which extension growth is maintained. Since both *le* and *ls* are leaky, possessing low levels of GA_1 (Reid 1986; Ross *et al.* 1989), it appears that wild-type levels of GA_1 are required for the wild-type growth profile.

In addition, GA has been demonstrated to increase the number of cells with transversely oriented microtubules in a variety of plants including pea (Akashi and Shibaoka 1987; Sakiyama and Shibaoka 1990; Sakiyama-Sogo and Shibaoka 1993).

In agreement with the available literature, analysis of microtubule orientation in the GA-synthesis mutants, *le* and *ls* and their wild-type revealed a substantial reduction in average microtubule orientation in both *le* and *ls* plants, corresponding to their reduced endogenous GA_1 levels. Application of GA_1 to seeds of these mutants resulted in a shift in average microtubule orientation toward the wild-type arrangement, and a concomitant increase in internode length, although the wild-type phenotype was not completely restored.

Taken together these findings indicate that the GA-synthesis mutants, *le* and *ls*, by blocking the production of GA_1, are operating to reduce the proportion of cells with a transverse microtubule arrangement, thus limiting the potential for extension growth. It would appear that by blocking the sequence of events leading to elongation prior to synthesis of the active GA_1, the mutations disable all of the normal GA-mediated processes, since an elevated wall yield threshold and reduced wall yield coefficient has been demonstrated in *ls* plants, while turgor and osmotic pressure remained comparable to the wild-type (Behringer *et al.* 1990a).

Average microtubule orientation in *le* and *ls* was never as transverse as in the wild-type, and since transverseness of the array decreased more rapidly in the mutants than in the wild-type over the same length of epicotyl, GA may be implicated in both the initial organization of transverse arrays, and the stabilization of transverse arrays.

GA has been previously reported to stabilize arrays in a transverse orientation. Mita and Shibaoka (1984b) found that GA stabilized microtubule orientation against disruption by the anti-microtubule drugs colchicine and cremart. In contrast, GA has also been reported to reduce cold-induced microtubule stability in *Acer* (Irwing and Lanphear 1968), *Medicago* (Rikin *et al.* 1975) and pea (Akashi and Shibaoka 1987) The present results, however, support a role for stabilization of microtubules by GA_1 in pea.

The growth profiles of the GA-response mutants, *lk*, *lka* and *lkb*, were similar to those of the GA-synthesis mutants, although rate and distribution of growth and the transverse microtubule population of *lk, lka* and *lkb* plants were even further reduced. However, the essentially normal levels of endogenous GA_1 in these mutants (Reid 1986; Reid and Potts 1986; Reid and Ross 1989; Behringer *et al.* 1990a; Lawrence *et al.* 1992) discount the possibility that reduced levels of GA_1 are responsible for the altered phenotype in these mutants. Similarities with the GA-synthesis types might suggest that *lk*, *lka* and *lkb* operate to reduce sensitivity to GA_1. Indeed, application of GA_1 to the seeds of these mutants did not result in any increase in transverse microtubule component or internode length.

In addition, only some aspects of the normal response to GA_1 appear to be impaired, since these mutants are not phenocopies of the GA-synthesis mutants. Behringer *et al.* (1990a) have reported an increase in wall yield threshold in *lka* and *lkb* plants and a reduced wall yield coefficient in *lka* plants, relative to their wild-type. This coupled with higher osmotic and turgor pressures in *lka* and *lkb* plants, in comparison with

wild-type and *ls* plants, may indicate that *lka* and *lkb* result in a lesion in the wall relaxation process without a concomitant effect on the process of solute transport.

A possible role for auxin in alteration of microtubule arrangement and growth profiles of *lkb*, and possibly *lk* and *lka* from the wild-type pattern should not be overlooked. Reid and Davies (1992) have reported a consistent two- to three-fold reduction in IAA content of *lkb* plants in comparison to wild-types. McKay *et al.* (1994) have demonstrated that application of the IAA transport inhibitors TIBA and HFCA to expanding internodes of *lkb* plants, both promoted elongation and increased IAA levels above the application site. A similar response was not measured in *lkb* types. In addition Davies and colleagues (P. J. Davies; unpublished results) have promoted internode elongation in *lkb* plants by IAA applications to expanding internodes. A quantitative relationship between IAA concentration and growth rate has previously been reported by Bandurski *et al.* (1990) and Ortúno *et al.* (1990). Auxin-induced reorientations of cortical microtubules have been reported in *Zea mays* (Bergfeld *et al.* 1988; Nick *et al.* 1990), and *Avena sativa* (Iwata and Hogetsu 1989b). Thus, the altered phenotype of *lkb* and possibly *lk* and *lka* plants may result from a reduction in endogenous level of IAA.

IAA deficiency in *lkb* plants was reported to be restricted to inner tissues, IAA level in the epidermis closely approximating wild-type levels (McKay *et al.* 1994). In contrast, in *lk, lka* and *lkb* plants microtubule orientation was less severely affected in the cells of the cortex, the greatest deviation from the wild-type arrangement being observed in the epidermis. In order for reduced IAA level in the cortex to primarily affect microtubule orientation in the epidermis it may be necessary to propose a differential sensitivity of epidermal and cortical tissue to auxin.

It has been previously suggested for a range of species including maize and pea, that the epidermis is an unique target tissue for auxin-induced growth (Kutschera and Schopfer 1986a; Kutschera *et al.* 1987; Kutschera and Briggs 1988a, Kutschera 1989; b; Hohl and Schopfer 1992c). Kutschera and Briggs (1988b) demonstrated an almost 20-fold increase in sensitivity to IAA of intact segments of pea internode, in comparison to segments in which the epidermis had been removed by peeling. However, Rayle *et al.* (1991) and Rayle and Cleland (1992) have provided arguments against the uniqueness of the epidermis in auxin-induced growth. They have reported the ability of both epidermal and cortical tissue of *Avena* and *Zea* coleoptile and pea epicotyl to respond to auxin, and suggest that the previously reported lack of response of peeled pea sections to auxin may have resulted from acid extension of these sections due to the use of distilled water. Peters *et al.* (1992) have reported that H^+ ion

secretion is induced by auxin in peeled segments, in agreement with suggestions of non-uniqueness of epidermal tissue to auxin by Rayle and colleagues.

Although microtubule orientation in *lk*, *lka* and *lkb* plants was not altered from the wild-type arrangement to the extent visualized in the epidermis, microtubule orientation in the cortex was nevertheless changed by these mutations. If a reduced endogenous level of IAA is responsible for the altered microtubule arrangement in the cells of the cortex, the present results may be interpreted as providing further evidence against the uniqueness of epidermal tissue with respect to the action of IAA.

However, the normal levels of IAA in the epidermis of *lkb* plants suggests that reduced IAA *per se* is not responsible for the altered microtubule arrangement in the epidermis. The findings of Ross and Reid (1986) that the ethylene synthesis inhibitor, AVG, resulted in internode elongation in *lk*, but not *Lk* plants, has suggested the involvement of ethylene in the *lk* phenotype. McKay *et al.* (1994) have suggested that tissue stresses in the cortex resulting from IAA deficiency might be responsible for an increase in ethylene evolution.

Ethylene has been reported to result in rapid reorientation of the microtubule array toward a more longitudinal arrangement (Steen and Chadwick 1981; Lang *et al.* 1982; Roberts *et al.* 1985). Given this effect, an increase in ethylene production could account for the shift away from the more transverse wild-type microtubule arrangement in the epidermis. However, a differential sensitivity of the epidermal microtubule array to increased ethylene levels might be required.

The putative involvement of IAA (and possibly ethylene) in the altered phenotype of the GA-response mutants does not, however, exclude the possibility of reduced responsiveness to GA. Shibaoka (1972), and Sakiyama-Sogo and Shibaoka (1993) have suggested an interactive relationship between GA and IAA, on the basis that GA_3 failed to promote stem elongation in decapitated dwarf pea cuttings in the absence of IAA. These findings may indicate that IAA and GA influence common target(s). It is widely accepted that both IAA and GA act to promote the extensibility of the cell wall (e.g. Taiz 1984; Cosgrove 1987; Cosgrove and Sovonick-Dunford 1989; Kutschera 1989; Taylor and Cosgrove 1989; Behringer *et al.* 1990a; Rayle and Cleland 1992; Cosgrove 1993a, b), and both have been implicated in the reorientation of cortical microtubule array (e.g. Shibaoka 1974; Sawhney and Srivastava 1975; Takeda and Shibaoka 1981b; Mita and Shibaoka 1984a, b; Mita and Katsumi 1986; Akashi and Shibaoka 1987; Bergfeld *et al.* 1988; Iwata and Hogetsu 19849b; Nick *et al.* 1990; Sakiyama and Shibaoka 1990; Ishida and Katsumi 1991, 1992; Nick and Furuya 1993;

Sakiyama-Sogo and Shibaoka 1993). It is possible, therefore, that reduced endogenous IAA levels may contribute to the reduced stature of the mutants directly by influencing microtubule arrangement, or indirectly by interacting with GA_1 to produce some decrease in GA-sensitivity.

The relative importance of the eidermis and cortex in extension growth

In both the wild-type plants and the GA-synthesis and -response mutants, a higher proportion of cells with transversely oriented microtubules was observed in the cells of the cortex, in comparison with the epidermis. Change in microtubule orientation towards a less transverse arrangement along a gradient of decreasing growth rate was also more rapid in the epidermal tissue. A more transverse arrangement of microtubules is thought to confer to cells the capacity for elongation via the ordered deposition of transverse cellulose microfibrils in the cell wall. Therefore, although the correlation between transverse microtubule orientation and growth rate was stronger in the cortex ($R^2 = 0.60$) than in the epidermis ($R^2 = 0.42$), it is suggested that the epidermis in peas, due to its generally lower component of transversely oriented microtubules, is the limiting organ in elongation growth. This conclusion is consistent with previous suggestions that the epidermis, in particular the outer epidermal cell wall, has a limiting role in extension growth (Kutschera *et al.* 1987; Kutschera and Briggs 1987, 1988b; Kutschera 1989; Hohl and Schopfer 1992a).

Relative contribution of microtubules to elongation growth

Disruption of microtubules by colchicine application to the internode of wild-type plants was used to assess the relative contribution of microtubules to the altered phenotypes of the GA-response mutants *lk*, *lka* and *lkb*. Application of colchicine to wild-type plants resulted in a reduction in stem length and an increase in stem diameter, relative to controls. These shorter, wider stems were a consequence of reduced cell length and increased cell width in both the epidermis and the cortex. The colchicine-induced changes to cell shape approximated the reduction in cell size and increase in cell width measured for the mutant lines (Ross and Reid 1986; Reid and Ross 1989). However, since colchicine treatment failed to produce a phenocopy of any of the three mutants, it is suggested that altered microtubule arrangement in *lk*, *lka* and *lkb* plants is not the sole effect of the mutations. Consequently, the altered phenotype and growth characteristics of the mutants do not result entirely from the disturbance to their microtubule array. It is suggested that reduced responsiveness to GA and/or reduced levels of endogenous IAA, in addition to their putative effect on microtubule orientation, might also exert a more direct effect on extensibility of the cell wall.

Auxin has been reported to result in promotion of elongation by cell wall loosening (e.g. Taiz 1984; Kutschera 1989; Rayle and Cleland 1992; Cosgrove 1993a, b), however the process by which this occurs is largely unresolved. A recent and comprehensive discussion of the subject of acid-growth-induced wall extension is provided by Rayle and Cleland (1992). The promotive effect of gibberellin upon cell wall loosening has also been reported (e.g. Kamisaka *et al.* 1972; Adams *et al.* 1975; Kawamura *et al.* 1976; Stuart and Jones 1977; Cosgrove 1987; Cosgrove and Sovonick-Dunford 1989; Taylor and Cosgrove 1989; Behringer *et al.* 1990a; Cosgrove 1993b).

Colchicine application to wild-type internodes also resulted in a reduction in cell number (and hence cell division) in the cortex, and to a lesser extent in the epidermis. In this respect, the effect of colchicine most closely resembles the effect of the *lk* and *lka* alleles upon cell division. Ross and Reid (1986) and Reid and Ross (1989) reported significant decreases in cell number, particularly in the cortex, of *lk* and *lka* plants. These results, coupled with the extremely reduced proportion of transverse microtubules in *lk* and especially *lka* types, may suggest a more direct effect of the mutant alleles upon microtubules. The nature of this impairment is, as yet, unknown.

The relationship between altered light perception, GA and elongation growth

The GA-response mutants *lw* and *lv* have contributed to an understanding of the role of light perception, via phytochrome, in the process of elongation. In addition, these mutants have established a relationship between light perception and responsiveness to GA_1 (Nagatani *et al.* 1990; Weller and Reid 1993; Weller *et al.* 1994, 1995).

The elongated mutant *lv* (Nagatani *et al.* 1990; Weller *et al.* 1994, 1995) and the short mutant *lw* (Weller and Reid 1992; Weller *et al.* 1994), both appear to modify phytochrome-mediated processes. The gene *lv* results in plants which are substantially elongated in comparison with wild-type plants when grown under white or red light. However, under far-red light or in darkness *lv* plants remain phenotypically comparable to wild-types (Nagatani *et al.* 1990). In *lv* plants the normal end-of-day far-red and shade avoidance responses are lacking, and these plants possess reduced chlorophyll levels (Nagatani *et al.* 1990; Weller and Reid 1993).

lv was shown to result in a disturbance of chloroplast structure. Transmission electron microscopic observation of chloroplasts from the palisade mesophyll of leaves revealed a reduction in number and organization of grana, a reduction of thylakoid stacking, and an increase in size and number of starch grains in *lv* plants, relative to the parental cv. Sparkle (*Lv*). These results are consistent with suggestions that the ability to de-etiolate normally in response to white light is impaired in *lv* types.

The phenotype of *lv-1* plants has close similarities with the phytochrome deficient mutants *lh* in cucumber (López-Juez *et al.* 1992) and *hy3* in *Arabidopsis* (Nagatani *et al.* 1991). However, normal levels of spectrally active phyB have been measured in *lv-1* mutants (Nagatani *et al* 1990; Weller *et al.* 1995), and Weller *et al.* (1995) have recently confirmed that the *lv-1* mutant is deficient in responses regulated by phyB. Since *lv-1* plants exhibit an enhanced responsiveness to exogenous GA_1 application (Reid and Ross 1988a), it is possible that the loss of normal biological activity of phyB conferred by the *lv-1* allele may exert its effect upon stem elongation via altered responsiveness to endogenous GA_1 levels.

lw plants, although apparently phenotypically opposite to *lv* plants, are also thought to result from impaired light perception resulting from hypersensitivity to phytochrome (Weller and Reid 1993). Like *lv*, *lw* exhibits an altered responsiveness to GA_1, but unlike *lv*, responsiveness to GA_1 is decreased (Jolly *et al.* 1987). The decreased response of the mutant *lw* to GA_1 lends support to the suggestion that the interaction between phytochrome function and elongation might involve altered responsiveness of the mutant to endogenous levels of GA_1.

Since the *lv-1* and *lw* alleles resulted in a shift in cortical microtubule orientation away from the wild-type arrangement, an interaction of the transduction pathways of light and GA_1 prior to any influence of microtubule arrangement upon extension growth is suggested.

The *lw* allele resulted in a decreased transverse microtubule component which, although significantly inceased by application of GA_1, did not attain the degree of tranversness exhibited by wild-type plants or GA_1-treated GA-synthesis mutants. These results are consistent with suggestions that *lw* results in reduced responsiveness to GA_1, and indicate that decreased responsiveness is mediated, at least in part, by altered microtubule arrangement.

The pattern of microtubule arrangement and the shape of the growth profile of *lw* types may suggest that *lw* is acting to impair the initial organization of microtubules into transverse arrays, and is thus responsible for the reduction in initial rate of elongation evidenced by the growth profile of this mutant.

In light-grown plants the *lv-1* allele resulted in an increase in average microtubule orientation concomitant with an incease in internode length, suggesting that the normal effect of phyB in elongation growth is mediated by microtubule arrangement. In support of this suggestion, it was observed that average microtubule orientation in dark-grown *Lv* plants was significantly higher than in light-grown *Lv* plants.

The effect of darkness upon microtubule orientation in *lv-1* mutants and wild-type *Lv* plants was examined. Although darkness and *lv* resulted in comparable increases in internode length and transverse microtubule component, darkgrown *Lv* plants were phenotypically dissimilar to *lv* plants grown in the light, indicating that darkness may operate to alter phenotype via other receptors not affected by the *lv-1* allele.

Summary

In summary, the use of mutants with impaired synthesis of or responsiveness to GA_1 demonstrated the promotive effect of GA_1 upon rate and distribution of growth in *Pisum*. Fluorescence microscopy of these mutants and their wild-type (L107) revealed a positive correlation between transverse microtubule arrangement and growth rate with distance from the apical hook. The decreased transverseness of microtubule orientation in the mutant lines, implicated GA_1 in both the initial organization and the maintenance of transverse microtubule arrays. GA_1-induced restoration of transverse microtubule arrangement in GA-synthesis mutants toward a wild-type arrangement confirmed that the altered microtubule arrangement in these mutants results from reduced levels of endogenous GA_1. The failure of exogenous GA_1 application to produce an increase in average microtubule orientation in the GA-response mutants *lk, lka* and *lkb*, is consistent with a decreased responsiveness to GA_1 operating in these mutants. The more pronounced decrease in the transverseness of microtubules in epidermal cells, in comparison to cells of the cortex, favours a role for the epidermis in limiting internode elongation.

Decreased IAA levels in *lka* and *lkb* mutants, may indicate the involvement of IAA in microtubule orientation. The promotive effect of combined IAA and AVG upon elongation in *lkb* internodes may also suggest the involvement of ethylene in the production of the semi-erectoides (and possibly erectoides) phenotype.

The altered shape of the growth profile for *lw* corresponded with a substantial reduction in the component of cells with transversely oriented microtubules in the upper 6 mm of epicotyl, in comparison to wild-type plants. The dissimilarity between the shape of the *lw* growth profile and those of the other mutants examined in this study, is consistent with the suggestion that *lw* operates via a different mechanism (i.e. altered phytochrome perception) to reduce internode length.

Analysis of chloroplast structure in *lv* revealed a reduction in both number and organization of grana, and a reduction in the number of constituent thylakoids. The promotive effect of *lv* on the size and number of starch grains was also observed. These results support suggestions that *lv* results in impaired de-etiolation of plants in response to white light. Analysis of microtubule orientation in *Lv* and *lv-1* plants indicated that *lv-1* operates to disturb wild-type microtubule arrangement, suggesting that phyB effects are normally mediated by microtubule arrangement.

Application of colchicine to expanding wild-type internodes clarified the contribution of microtubule arrangement to the GA-response mutant phenotype, and suggested that disturbance to the microtubule array may not be the only effect of the *lka* allele. The similarity of the effect of *lka* and colchicine application on cell shape and number suggests that the *lka* allele may have a more direct effect on microtubules.

Future directions

It is suggested that a clarification of the *lka/lkb* difference might be gained by the use of more sensitive techniques, perhaps similar to those recently employed by Duckett and Lloyd (1994), who have used immunoblotting and gel electrophoresis to demonstrate changes in tubulin isotypes in response to GA_3-induced elongation pea stem cells.

The putative involvement of IAA and ethylene in the disturbed microtubule orientation in *lkb*, and perhaps *lk* and *lka* types, might be clarified by observing the combined effect of IAA and AVG on microtubule arrangement in these mutants.

Analysis of cell wall physical properties as well as turgor and osmotic potential of the photomorphogenic mutants, *lw* and *lv-1*, might further narrow the range of partial processes over which the pathways of light and GA_1 might intersect.

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