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Microtubule Orientation in the Brassinosteroid Mutants *lk*, *lka* and *lkb* of Pea

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Abstract

Short brassinosteroid (BR) mutants *lk, lka* and *lkb* of pea (*Pisum sativum* L.) were investigated by immunofluorescence microscopy to elucidate the role of brassinosteroids in cell elongation via an effect on the microtubules (MTs). This study adds to our knowledge the fact that brassinolide (BL) can cause MT realignment in azuki bean and rescue the MT organization of BR mutants in *Arabidopsis*. It provides novel information on both cortical and epidermal cells and presents detailed information about the ratios of all MT orientations present, ranging from transverse (perpendicular to the elongating axis) to longitudinal (parallel to the elongating axis). Experiments were conducted *in vivo* using intact plants with direct application of a small amount of brassinolide (BL) to the internode. Employing a BR-receptor mutant, *lka*, and the BR-synthesis mutants, *lk* and *lkb*, allowed the identification and isolation of any BR-induced responses in the MT cytoskeleton following BL application. Increases in growth rate were noted in all pea lines including WT following BL application. These increases were strong in the BR-synthesis mutants, but weak in the BR-receptor mutant. Immunofluorescence revealed significant differences in the average MT orientation of cortical cells of mutants versus WTs. Importantly, these mutants possessed abundant MTs, unlike the BR-deficient *bul1-1* mutant in *Arabidopsis*. Following BL application, the epidermal and cortical cells of *lk* and *lkb* plants showed a large and significant shift in MT orientation towards more transverse, whereas *lka* plants showed a small and nonsignificant response in these cells. These results suggest that the BR response pathway is linked to the regulation of MT orientation.

Key words: Brassinosteroid; Cell elongation; Microtubules; Mutants; Pea; *Pisum sativum*

Introduction

The steroidal plant hormones, termed brassinosteroids (BRs), were discovered in the 1970 s and are essential for normal plant growth as well as other developmental processes (Clouse and Sasse 1998). Since then BR-insensitive (receptor) and BR-deficient (synthesis) mutants have been discovered in garden pea (*Pisum sativum* L.), *Arabidopsis* and tomato (Altmann 1998). Exogenously applied brassinolide (BL) has a marked effect on cell elongation, gene expression and cell physiology that is distinct from the effect of auxins, gibberellins (GAs) and

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cytokinins (Clouse 1996; Clouse and Sasse 1998). For example, the primary target tissue of BRs is thought to be the inner tissues, which is different from that of auxin, which primarily affects the outer tissues (Tominaga and others 1994). GAs have also been suggested to target outer tissues (Ishida and Katsumi 1992).

BR mutants in pea are characterized by an erectoides phenotype (reduced internode length, thickened stems and epinastic leaves) (Reid and Ross 1989; Nomura and others 1997). The extent of these features varies between mutants: *lk* is more severely dwarfed than *lkb* and *lka*. All these mutants have been characterized at the molecular level (Schultz and others 2001; Nomura and others 2003; Nomura and Jager unpublished data). These mutants are dwarfed relative to the WT because of reduced cell elongation both in the epidermis and cortex (Reid and Ross 1989). The *lka* and *lkb* mutants also have a significantly larger cell wall yield threshold and turgor pressure. However, the enhanced turgor pressure does not exceed the wall yield threshold, therefore cell elongation is reduced (Behringer and others 1990). If cell elongation is affected then it is likely that the cortical microtubules (MTs) are also affected because cell wall composition and structure, as well as the cytoskeleton, are vital in determining cell shape (Smith 2003). In fact, BRs have been shown to affect MT orientation and to alter mechanical properties of the cell wall (Wang and others 1993; Zurek and others 1994; Mayumi and Shibaoka 1995; Clouse 1996; Catterou and others 2001).

Cell elongation is driven by water uptake and by the irreversible yielding of the cell wall (Okamoto and others 1990). MT and cellulose microfibril (MF) orientation affect the direction of cell elongation in growing cells, mainly by influencing the deposition of wall materials, thus affecting wall extensibility (Smith 2003). It is widely accepted that the cortical MT array plays a major role in orienting cellulose MFs of the cell wall, yet they are not thought to do so directly and cannot be the only factor determining cellulose MF orientation (Emons and others 1990, 1992; Fisher and Cyr 1998). The way in which cortical MTs become aligned remains enigmatic but the MT/cellulose MF paradigm now includes the bi-directional flow of information between cellulose MFs and cortical MTs (Emons and others 1992; Fisher and Cyr 1998). The debate over the precise control of cortical MTs and cellulose MFs and their interaction is yet to be resolved.

Due to their dynamic nature, MTs can shift orientation; MTs in elongating cells are typically transverse to the elongation axis and oblique or parallel in non-elongating cells (Laskowski 1990). Changing rates of cell elongation alone do not trigger the reorganization of MT arrays, implying that other factors are involved (Laskowski 1990). For example, auxin is thought to be required for the reorientation of MTs from longitudinal to transverse in azuki bean epicotyls and the presence of gibberellin and auxin can suppress the reverse transition (Mayumi and Shibaoka 1996). BL can also cause a shift in MT orientation in epidermal cells, towards more transverse in segments of azuki bean epicotyls in an *in vitro* environment (Mayumi and Shibaoka 1995, 1996), although this presumably raised BL levels well above endogenous physiological levels. The BR-deficient *bul1,-1* mutant of *Arabidopsis* possesses fewer, shorter and dissociated MTs compared with WT plants (Catterou and others 2001), even though BL can restore MT organization to that of WT.

We know that exogenously applied BL can restore the phenotype of BR-synthesis pea mutants such as *lk* and *lkb* (Schultz and others 2001; Nomura and Jager unpublished data), but the effects at the cellular level have yet to be documented. This study examines in detail the orientation of MTs in both cortical and epidermal cells of BR pea mutants using immunofluorescence microscopy and investigates the link between BR-induced cell elongation and MT reorganization. The possession of the *lka* BR-receptor mutant in addition to the BR-synthesis mutants *lk* and *lkb* in pea provides a powerful tool for identifying potential effects of BL on the cytoskeleton. The information gained is maximized by the examination of both epidermal and cortical cells and permits the determination of whether BR-deficient mutants in pea are compromised in a similar way to *bul1-1* in *Arabidopsis*. Furthermore, it overcomes the use of potentially nonphysiological BL levels in previous investigations on segments of azuki beans *in vitro*.

Materials and Methods

Plant Material, Growing Conditions and Brassinolide Treatment

Three dwarf mutants were compared to their respective wild type (WT) lines: *lkb* (L5862) and *lka* (L5865) to WT L107, a selection from cv. Torsdag (Reid and Ross 1989), and *lk* ($L212^-$) to WT $L212^+$ (Symons and others 2002). Seeds were nicked and planted in 140-mm pots $^3/_4$ filled with a 1:1 mixture of dolerite chips and grade 3 vermiculite, topped with 5 cm of potting mix. Plants were grown at 20°C, with an 18-h photoperiod provided by fluo-

rescent (36 W cool white tubes; Osram, Munich, Germany) and incandescent (100 W bulbs; Thorn, Sydney, Australia) light (150 μmol photons m^{-2} s^{-1} at pot top). On day 11 after planting, the youngest internode (usually internode 5, 10–20% expanded) of each plant was either left untreated (control) or treated with 2 μl ethanol (ethanol control) or 200 ng of brassinolide (BL) dissolved in 2 μl of ethanol. Internodes were 10–20% expanded at time of treatment so that after 24 h they were all less than 40% expanded and still elongating rapidly. Plants were not watered following application. The growth rate of the expanding internodes was calculated for each line and both BL-treated and control plants from time of treatment to the time tissue were collected. Internodes were found to grow rapidly for 48 h after treatment. Results are presented for all groups in the Tables but only for BL-treated versus the ethanol control plants in the Figures as statistical analysis showed no significant differences between the two controls.

Immunofluorescence Microscopy

It was important to differentiate between epidermal and cortical cells, and to examine whole cells in a uniform orientation. Epidermal peels (which included epidermal and cortical cells) were taken from the third quarter (from the bottom) of elongating internodes and placed in fixative (4% paraformaldehyde and 0.2% dimethyl sulfoxide in PMEG [5 mM EGTA, 50 mM piperazine-N, N′-bis (2-ethanesulfonic acid) (PIPES) buffer containing 2 mM $MgSO_4$ and 4% glycerol] pH 6.8) for 1 h at room temperature, then washed several times in PMEG for 30 min (Harper and others 1996). Tissue was incubated in a detergent solution (2% IPEGAL CA630 in PMEG) for 1 h followed by several washes in PMEG for 30 min. Peels were transferred from glass dishes to welled slides and incubated for 8 min at 37°C in an enzyme solution containing 0.1% pectinase and 0.15% cellulase in phosphate-buffered saline (PBS: 0.137 M NaCl, 0.003 M KCl, 0.004 M Na_2HPO_4, 0.002 M KH_2PO_4) pH 7.2–7.6 to aid antibody penetration by partially breaking down cell wall components. This short incubation was optimal as cell walls were still intact and strong enough to survive further treatments. Longer incubations were disadvantageous, resulting in increased loss of wall integrity, fragility and hence disruption of tissue structure, which created problems in determining the long-axis of individual cells. Tissue was washed several times in PBS containing 50 mM glycine and 0.02% sodium azide for 30 min and incubated in primary antibody (monoclonal anti-α-tubulin [Sigma, Product no: T5168], 1:3000 dilution) for a minimum of 1 h at 37°C. Antibody incubations were carried out in a humid incubation chamber to prevent dehydration. Tissue was washed in PBS containing 50 mM glycine and 0.02% sodium azide then incubated in the fluorescently tagged secondary antibody (goat anti-mouse IgG FITC conjugate [Sigma, Product no: F0257], 1:40 dilution) for 1 h at 37°C. Tissue was given a final wash in several changes of PBS for 30 min before mounting in permafluor (Beckman Coulter, Gladesville, NSW, Australia) and stored in the dark at 4°C.

Epidermal peels were examined with a Zeiss Axioskop 2 plus (Carl Zeiss Inc., Germany) using a 12 V mercury vapour 100 W lamp and the Zeiss filter set no. 09 (BP 450–490,FT 510, LP 515) to visualize the FITC-labeled MTs. Images were taken with a digital Zeiss AxioCam HRc camera using AxioVision 3.1 software. Epidermal tissue could be easily differentiated from cortical tissue by cell size and shape in WT and by the presence of stomata in the mutants because epidermal and cortical cells were less distinct.

Determination of Microtubule Orientation

Six categories of MT orientation were established: (i) transverse (perpendicular to the elongating axis) (0–18°), (ii) transverse-oblique (19–36°), (iii) oblique (37–54°), (iv) longitudinal-oblique (55–72°), (v) longitudinal (parallel to the elongating axis) (73–90°) and (vi) mixed (MTs not unidirectional) (see Figures 1a–f). These angles were established by measurements prior to the experiments and used as a reference during sampling. Digital images were taken to aid in accurate angle measurements. Cortical MTs of epidermal and cortical cells were examined separately and individual cells were placed into one of these categories according to the dominant (>80%) MT orientation. All cells observed fit into one of these categories. Most cells possessed a dominant MT orientation, but when no dominant orientation could be determined, MTs were classed as mixed (multiple orientations present). All cells in a field of view were categorized and each field of view (usually 30–80) was selected randomly to eliminate bias.

Data Analysis

Mixed model analysis of MT orientation was performed using the SAS statistical software program

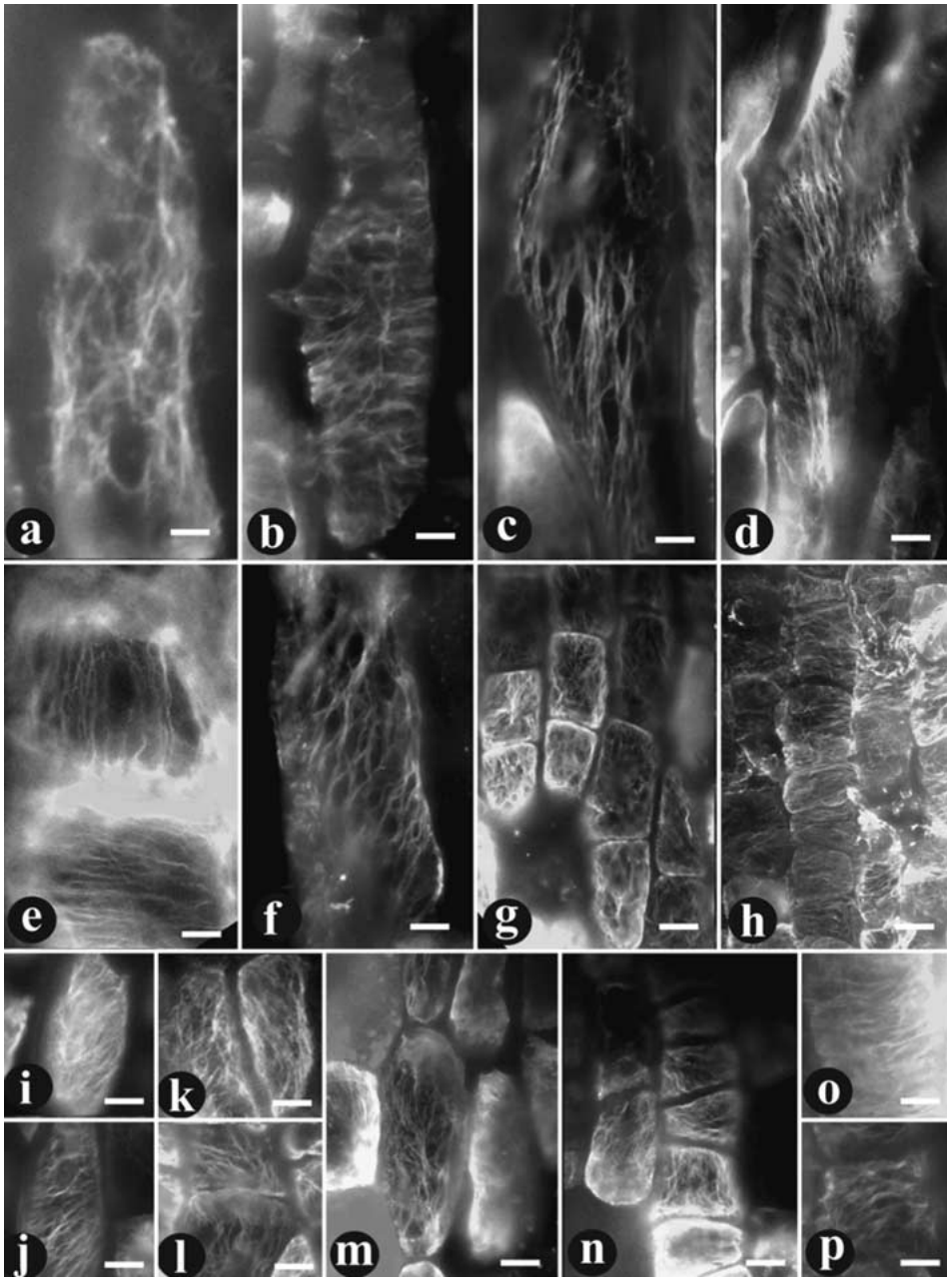

Figure 1. Immunofluorescent images of representative cells showing labeled cortical microtubules (MTs) in elongating internodes of WT and brassinosteroid pea mutants. All cells are arranged such that their longitudinal and elongation axis are vertical. (**a–f**) Examples of cells in each category of MT arrangement/orientation: (**a**) *lk*-mixed (multiple MT angles), (**b**) WT (L212^{+}) – transverse-oblique (19–36°), (**c**) WT (L212^{+}) – longitudinal (73–90°), (**d**) WT (L107) – longitudinal-oblique (55–72°), (**e**) *lkb* – upper cell has longitudinal MTs (73–90°), lower cell has transverse MTs (0–18°), (**f**) L107 – oblique (37–54°), (**g–p**) MT organization in the mutants before and after brassinolide (BL) application, (**g**) *lkb* control cortical cells showing mixed and longitudinal MTs, (**h**) *lkb* BL-treated cortical cells showing predominantly transverse MTs, (**i**) *lka* control epidermal cell showing oblique MTs, (**j**) *lka* BL-treated epidermal cell showing oblique MTs, (**k**) *lka* control cortical cells showing longitudinal and oblique MTs, (**l**) *lka* BL-treated cortical cells showing longitudinal and oblique MTs, (**m–n**) *lk* control cortical cells showing predominantly longitudinal and oblique MTs, (**o–p**) *lk* BL-treated cortical cells showing transverse MTs. Scale bars: **a** = 3.5 μm, **b**,**c**,**d**,**f**,**k**,**m**,**n** = 7 μm, **e** = 2 μm, **g–j** = 5 μm, **l**,**o**,**p** = 4 μm.

ver. 8.12 (SAS Institute Inc. 2000) based on two independent experiments employing 6–12 plants per pea line and 2–4 peels from each internode. Data were collected from 220–654 cells for each line and treatment and were based on pseudoreplication. Standard error values have been shown for the

average MT orientation for each treatment and control. To perform statistical analysis, a degree value was given to each category of MT orientation: transverse (0°), transverse-oblique (22.5°), oblique (45°), longitudinal-oblique (67.5°) and longitudinal (90°). Student's two-tailed *T*-tests were performed on growth rate data to determine whether applied BL had a significant effect on stem growth. A χ^2_1 2 × 2 contingency test was performed on *lka* data comparing the number of cortical cells with transverse and all other categories of MT orientation, in control and treated plants.

Table 1. Microtubule Differences between the Mutants and their Respective Wild Type

	Average microtubule angle (°)*	
	Epidermal cells	Cortical cells
WT	53.27 ± 3.44 a	18.37 ± 3.45 a
lka	48.41 ± 4.51 a	25.72 ± 3.48 ab
lkb	54.16 ± 4.00 a	28.27 ± 4.08 b
WT	64.90 ± 3.50 a	17.68 ± 3.47 a
lk	54.75 ± 3.52 a	49.00 ± 3.47 b

**0° is transverse and 90° is longitudinal to the elongation axis of the cell. lka and lkb are compared to WT (L107); lk is compared to WT (L212⁺). Values with different letters within the same cell type and compared to their respective WT are significantly different (P < 0.05).*

Results

Cellular Phenotype of WTs and Brassinosteroid Mutants

A variety of MT orientations existed in WT and mutant cells under control conditions at any one time (Figures 1a–f depict an example of each of the MT orientation categories employed in this study). This is highlighted in Figure 1e, which shows two adjacent cortical cells from an *lkb* plant, one with longitudinal and the other with transverse MT alignment. All of these orientations were observed in each of the tissues examined, but the percentage of cells falling into each orientation category varied depending on cell type and genotype. Cortical and epidermal cells showed distinct differences in the proportion of cells falling into each of the MT orientation categories and were examined separately. Under control conditions, the average MT angle of the epidermal cells was not significantly different between the mutants and their respective WT (Table 1). However, in the cortical cells, the BR-synthesis mutants *lk* and *lkb* differed significantly from their respective WTs. The difference for the BR-receptor mutant *lka* in this analysis was not significant (Table 1), but when a χ^2_1 2 × 2 contingency test was performed comparing the number of cells with transverse and all other categories of MT orientation, the difference between *lka* and WT was highly significant ($P < 0.001$).

Microtubule Orientation in the BR-Synthesis Mutants *lk* and *lkb* is Restored After Application of BL

An effect of ethanol, used to solubilize the BL, was eliminated because there were no significant differences between the average MT orientation angle of the two controls (ethanol and no treatment) in any of the pea lines (Table 2). Applied BL had a significant effect on the MTs in cortical cells of BR-synthesis mutants, *lk* and *lkb*, resulting in a shift towards more cells with transverse MTs (that is, a decrease in the average MT orientation angle) (Table 2, Figures 2a, 3a). In terms of average MT angle, there was a complete recovery of *lkb* cortical cells following BL application and a partial recovery for the more severely affected *lk* mutant (Table 2). This restoration of MT orientation can be seen clearly by comparing Figures 1 g with Figure 1 h; Figure 1 g shows cortical cells from control *lkb* tissue possessing MTs with predominantly mixed and longitudinal-oblique orientation, whereas Figure 1 h shows BL-treated *lkb* tissue with a predominance of cells with transverse MT alignment. The same BL response was observed for *lk* (compare Figures 1 m–n with Figure 1 o-p). Recovery was correlated with an increase in growth rate of the expanding internode of three-fold in *lk* and almost three-fold in *lkb* (Table 3). The BR-synthesis mutants, *lk* and *lkb*, also showed a significant drop in the average MT angle in epidermal cells (Table 2). This was attributed to a decrease in the proportion of cells with longitudinal MT alignment and an increase in cells with transverse MTs (Figures 2b, 3b). A small, significant ($P < 0.05$) decrease in average MT angle was also observed in the epidermal cells of one WT line (L212⁺) (Table 2) and was attributed to a drop in cells with longitudinal MTs and an increase in cells with oblique and transverse MTs (Figure 3b).

In addition to having an elevated average MT angle (more longitudinal), the *lk* and *lkb* mutants had a higher percentage of cells with mixed or non-uniform MT organization (Figure 1a). Although the MT orientation in the mutants was affected and the cells were often smaller, there was no qualitative difference (based on visual inspection) in MT density between the mutants and the WT (Figure 1).

Table 2. Microtubule Differences between the Mutants and their Respective Wild Type after Exogenously Applied Brassinolide

	Average microtubule angle (°)*					
	Epidermal cells			Cortical cells		
Treatment	WT	*lka*	*lkb*	WT	*lka*	*lkb*
None	52.67 ± 5.97 a	52.31 ± 6.04 a	62.23 ± 8.27 a	18.97 ± 5.95 a	28.22 ± 6.05 a	41.92 ± 8.74 a
Ethanol	52.73 ± 5.93 a	44.27 ± 8.52 a	63.31 ± 6.25 a	20.16 ± 6.00 a	25.47 ± 6.10 a	32.95 ± 6.17 a
Brassinolide	54.40 ± 5.95 a	48.67 ± 8.42 a	36.94 ± 6.01 b	15.99 ± 6.00 a	23.48 ± 5.94 a	9.93 ± 5.95 b
Treatment	**WT**	***lk***		**WT**	***lk***	
None	70.48 ± 6.05 a	62.00 ± 6.11 a		14.55 ± 6.01 a	56.06 ± 6.03 a	
Ethanol	71.73 ± 6.06 a	61.08 ± 6.10 a		24.55 ± 5.99 a	55.91 ± 6.03 a	
Brassinolide	52.47 ± 6.05 b	41.18 ± 6.05 b		13.94 ± 6.01 a	35.01 ± 5.96 b	

**0° is transverse and 90° is longitudinal to the elongation axis of the cell.*
lka and lkb are compared to WT (L107); lk is compared to WT (L212^{+}). Values with different letters within the same cell type and same genotype are significantly different ($P < 0.05$).

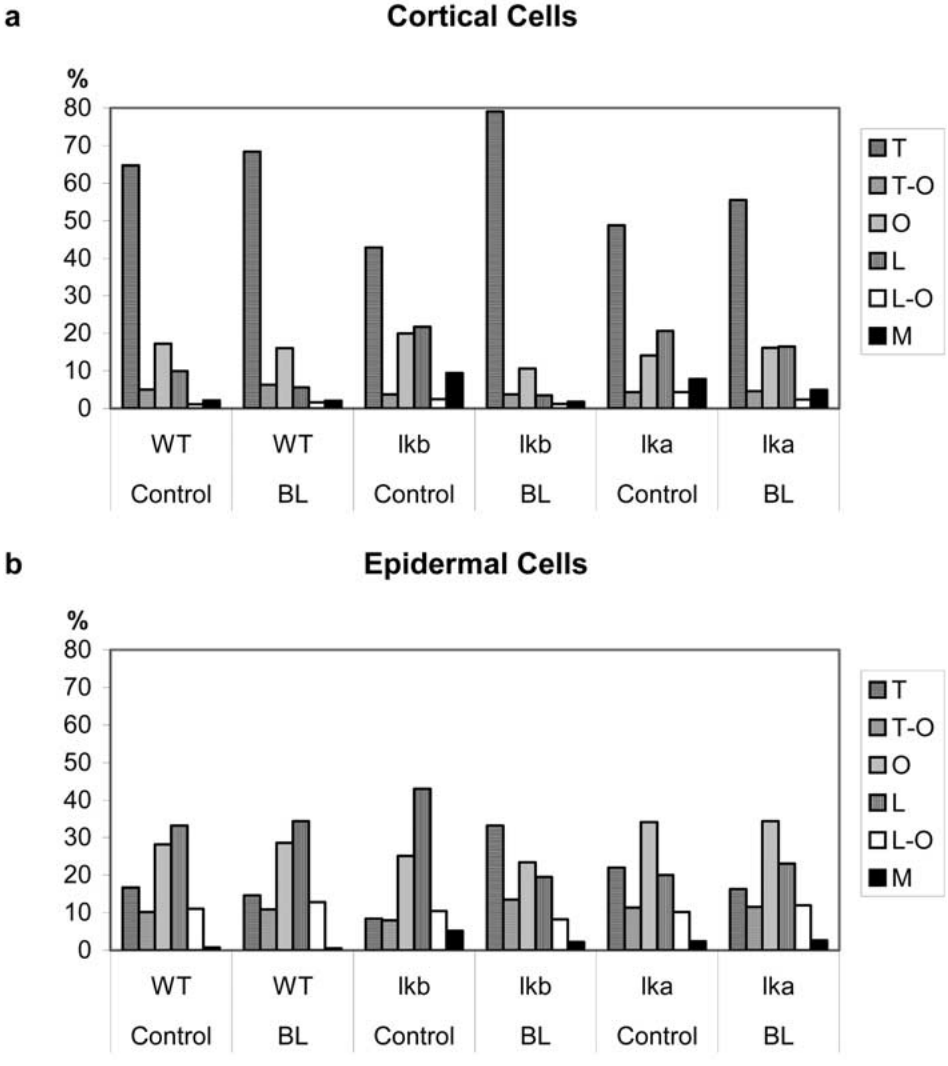

Figure 2. Microtubule (MT) organization in cortical cells (**a**) and epidermal cells (**b**) in wild-type (L107) and the brassinosteroid mutants *lka* and *lkb* with and without applied brassinolide (BL). MT organization categories: T = transverse (0–18°); T–O = transverse-oblique (19–36°); O = oblique (37–54°); L–O = longitudinal-oblique (55–72°); L = longitudinal (73–90°); M = mixed (multiple MT angles). Data were collected from 220–654 cells from 2–4 peels each of 6–12 plants per pea line.

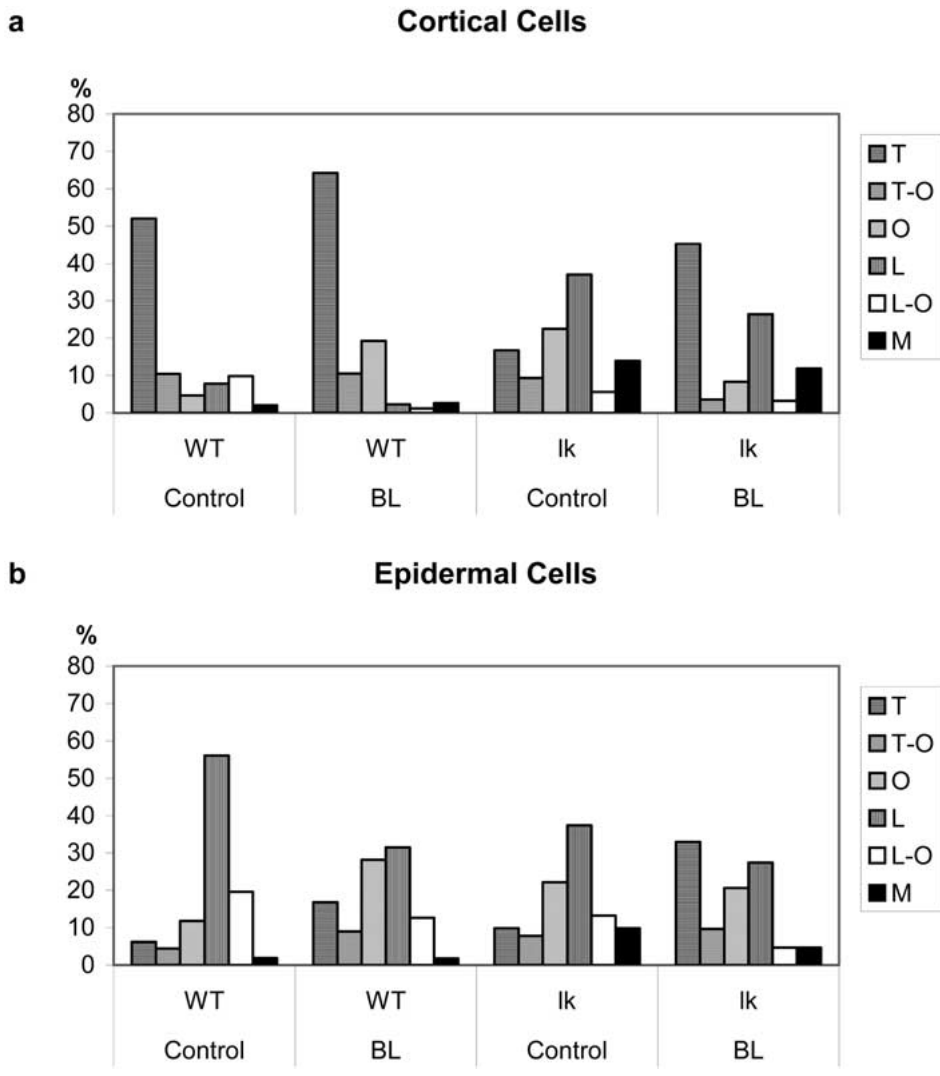

Figure 3. Microtubule (MT) organization in cortical cells (**a**) and epidermal cells (**b**) in wild-type ($L212^+$) and the brassinosteroid mutant *lk* with and without applied brassinolide (BL). MT organization categories: T = transverse (0–18°); T–O = transverse-oblique (19–36°); O = oblique (37–54°); L–O = longitudinal-oblique (55–72°); L = longitudinal (73–90°); M = mixed (multiple MT angles). Data were collected from 220–654 cells from 2–4 peels each of 6–12 plants per pea line.

Similarly, the length of the MTs in cells of the BR mutants was not obviously compromised if cell size is taken into account, except possibly in cells with mixed MT orientation (Figure 1).

Microtubule Orientation of the Receptor Mutant *lka* is not Restored After Application of BL

The BR-receptor mutant, *lka*, and the corresponding WT responded significantly to applied BL ($P < 0.001$) in terms of increased growth rate, but the magnitude of the response was far less than it was for the BR-synthesis mutants *lk* and *lkb* (Table 3). Applied BL had a small but non-significant effect on the average MT orientation angle in both the epidermal and cortical cells of *lka* (Table 2) and on the relative proportion of cortical cells in each of the six MT orientation categories (Figure 2a). Immunofluorescence images visually depict this lack of a significant BL restoration for *lka* in both epidermal (Figures 1i–j) and cortical (Figures 1 k-l) cells.

Table 3. Average Growth Rate of Youngest Elongating Internode of 11-day-old Pea Seedlings ($n \geq 16$) Showing the Effect of Applied Brassinolide

	Average growth rate (mm h^{-1})	
	Control	Brassinolide
WT	0.40 ± 0.15 a	0.61 ± 0.15 b
lkb	0.09 ± 0.03 a	0.25 ± 0.07 b
lka	0.10 ± 0.03 a	0.15 ± 0.05 b
WT	0.48 ± 0.14 a	0.77 ± 0.14 b
lk	0.09 ± 0.02 a	0.27 ± 0.09 b

lka and lkb are compared to WT (L107); lk is compared to WT ($L212^+$). Values with different letters within the same row are significantly different ($P < 0.001$).

Discussion

Cell Elongation and MT Organization are Affected in BR-Synthesis Mutants

The BR mutants of pea employed in this study were compromised to varying extents in terms of growth and development, exhibiting reduced internode and cell length (Reid and Ross 1989; Nomura and others 2003; Nomura and Jager unpublished data). It is known that exogenously applied BL can restore the morphological phenotype of BR-deficient mutants in pea (Yokota 1997; Schultz and others 2001; Nomura and Jager unpublished data), but it is not known how this impacts on the cell cytoskeleton. We employed BR-synthesis mutants and a BR-receptor mutant to examine the effect of BR deficiency on the cortical MTs of epidermal and cortical cells and found the MTs of the BR-synthesis mutants *lk* and *lkb* to be significantly affected. These mutants possessed an elevated average MT angle (that is, more longitudinal relative to the long axis of the cell) that was reduced (that is, became more transverse) by exogenously applied BL. This corresponded with a marked increase in growth rate of *lk* and *lkb*, which supports an effect of BL on plant growth that stimulates cell elongation, probably via an effect on MTs. This result is strengthened by employment of the *lka* BR-receptor mutant, which shows a weak growth response to applied BL and coordinately little or no response of MTs. The level of growth response was presumably not great enough for the response to be mirrored in a significant MT reorientation.

The angles of MTs in *lk* and *lkb* cortical cells were restored fully by treatment with BL, yet growth rates of *lk* and *lkb*, although increased dramatically with applied BL, did not reach the WT growth rate after 24 h (Table 3). This is due to the fact that BR biosynthesis mutants of pea begin to show a growth response to applied BL in 12–24 h but the full response is not attained until approximately 48 h (personal observation). Thus, a complete growth response was not expected. However, the aim of the experiment was to obtain cells at a particular developmental stage where BL can exert its influence.

MT Orientation Classification and Discordant MTs

This study found that the cells of elongating pea stem tissue were not uniform in their cortical MT orientation. A dominant orientation could be identified, but at any one time a variety of orientations were found to co-exist in both epidermal and cortical cells. The ratios of these categories shifted among cell type, genotype and treatment. This meant employing a method that permitted cells to be categorized as possessing a particular MT orientation while still obtaining maximum information. Employing six different categories of MT orientation was the best way to achieve this as it allowed intermediate stages between transverse and longitudinal orientation to be identified. The mixed category was also important for identifying cells that could not be classed as having a dominant MT orientation. These mixed or discordant MTs, such as the increased percentage found in the BR-synthesis mutants *lk* and *lkb*, have been shown to appear when the cell is undergoing a transition from one dominant alignment to another (Lloyd and others 1996). The tissue examined was taken from young elongating internodes and should, in theory, be composed of cells with a predominantly transverse MT orientation that favors cell elongation as seen in the cortical cells of both WTs (Figures 2a, 3a). Although the mixed category forms a minor group in the mutants, it is clear that there is some confusion in the MT orientation signal of these plants. The BL signal appears to be able to lower the number of cells with mixed MTs, especially in *lkb* cortical cells, suggesting that BL may play an important role in the pathway that regulates MT organization.

Which Tissues Do BRs Affect?

The differences in MT orientation between epidermal and cortical cells indicated that these cells should be examined separately. It also shows that there are more cortical cells with transverse MT orientation than epidermal cells and although they respond similarly to applied BL, the cortical cells are responding more strongly than epidermal cells. Many papers investigating MT orientation have only examined epidermal cells (Roberts and others 1985; Laskowski 1990; Sakiyama-Sogo and Shibaoka 1993; Yuan and others 1994; Mayumi and Shibaoka 1995; Lloyd and others 1996; Whittington and others 2001). Our findings suggest that it may be more informative to study both cell types. This is particularly the case because Tominaga and others (1994) found that BRs probably do not target the epidermis of elongating stems, which is the proposed primary target tissue of GAs (Ishida and Katsumi 1992) and auxin (Tanimoto and Masuda 1971).

The phenotype of many hormone mutants that are dwarfed can be restored to that of WT by

application of the deficient hormone such as GA or BL, but how do the processes differ? BRs (Mayumi and Shibaoka 1995; Catterou and others 2001) and GAs (Shibaoka 1974; Ishida and Katsumi 1991;1992; Lloyd and others 1996) both promote cell elongation and influence the angle of the MTs; hence they appear to operate, at least in part, via a common pathway. However, there are clear differences between the modes of action of BRs and GAs early in the developmental process. For example, the yield coefficient and turgor pressure are affected in BR pea mutants but not in GA mutants (Behringer and others 1990). Both types of mutant have reduced cell elongation, but GA mutants have a greater effect on cell division (Reid and Ross 1989). The phenotypes of the two mutant types also differ. BR mutants have characteristic swollen, ridged stems (Reid and Ross 1989), a feature not exhibited in GA mutants. Therefore, BRs and GAs have unique modes of action early in the pathway that regulates cell elongation, but overlap or merge in the latter stages that regulate MT orientation. This is supported by Tanaka and others (2003) who show that BRs act on light-grown hypocotyl elongation independent of, but cooperatively with, GAs and auxin. It is clear that multiple hormones affect the elongation of cells. However, further work is required to dissect the interaction and specific roles of these hormones and to determine the cell types they affect.

MTs Are Compromised But Not Deficient in Pea BR-Synthesis Mutants

Catterou and others (2001) report that the MTs of the BR-deficient *bul1-1* mutant in *Arabidopsis* were few, short and dissociated. This was not the case in the BR mutants *lk*, *lkb* and *lka* in pea, in which the MTs were clearly visible, often formed unidirectional arrays and did not appear to be reduced in length if cell size was taken into consideration. However, the MTs of the BR-synthesis mutants *lk* and *lkb* were still compromised relative to the WT, there being fewer cells with transverse MT alignment and an increased proportion of discordant MTs. The ability of applied BL to restore the predominant MT orientation, shifting it towards more transverse in the BR-synthesis mutants *lk* and *lkb*, but not the BR-receptor mutant *lka*, supports the theory that BL may be directly involved in MT organization. This result is in accordance with findings by Catterou and others (2001) who discovered that applied BL could restore the MT orientation in the *Arabidopsis* BR deficient *bul1-1* mutant. It also confirms results of Mayumi and Shiboaka (1996) who showed that applied BL could increase the percentage of transverse MTs in epidermal cells of azuki bean epicotyls.

Phytohormone Response in the WT

Applied BL has the most significant effect on the internode growth and MT organization of the BR-synthesis mutants but it also affected both WT lines. The significant effect of BL on the epidermal cells of the WT L212^{+} was not surprising because, for a phytohormone to be functional, its level needs to be below saturation and hence able to regulate plant growth. Therefore, adding exogenous phytohormone would be expected to have some effect on the plant depending on endogenous levels and the stage of its development. In theory, the BR- receptor mutant, *lka*, should not be able to detect the presence of BL and therefore not respond to it. However, the small effect of BL on the growth rate of *lka* is probably because *lka* does not possess a null BL phenotype (Nomura and others 2003) and hence a reduced ability to detect and respond to exogenously applied BL. This small response in *lka* was not strong enough to be reflected in a significant effect on MT orientation.

In summary, it is evident that the BL response pathway is able to influence the orientation of cortical MTs in both epidermal and cortical cells and is thus linked in some way to MT regulation. This result indicates that a deficiency in BL or its perception in pea, although affecting MTs, still permits formation of organized MT arrays, whereas in *Arabidopsis* MTs are few, short and dissociated (Catterou and others 2001). The integration of these pathways and the interaction between MTs and cellulose MFs is an area that needs future investigations.

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