The Effects of the Phospholipase D-Antagonist 1-Butanol on Seedling Development and Microtubule Organisation in *Arabidopsis*

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The organisation of plant microtubules into distinct arrays during the cell cycle requires interactions with partner proteins. Having recently identified a 90-kDa phospholipase D (PLD) that associates with microtubules and the plasma membrane [Gardiner et al. (2001) *Plant Cell* 13: 2143], we exposed seeds and young seedlings of *Arabidopsis* to 1-butanol, a specific inhibitor of PLD-dependent production of the signalling molecule phosphatidic acid (PA). When added to agar growth media, 0.2% 1-butanol strongly inhibited the emergence of the radicle and cotyledons, while 0.4% 1-butanol effectively blocked germination. When normal seedlings were transferred onto media containing 0.2% and 0.4% 1-butanol, the inhibitor retarded root growth by about 40% and 90%, respectively, by reducing cell elongation. Inhibited plants showed significant swelling in the root elongation zone, bulbous or branched root hairs, and modified cotyledon morphology. Confocal immunofluorescence microscopy of root tips revealed that 1-butanol disrupted the organisation of interphase cortical microtubules. Butanol isomers that do not inhibit PLD-dependent PA production, 2- and 3-butanol, had no effect on seed germination, seedling growth, or microtubule organisation. We propose that production of PA by PLD may be required for normal microtubule organisation and hence normal growth in *Arabidopsis*.

**Keywords**: *Arabidopsis* — Development — Growth — Microtubule — Phospholipase D — Root.

Abbreviations: BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; MAP, microtubule-associated protein; PA, phosphatidic acid; PLD, phospholipase D.

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**Introduction**

Cortical microtubule arrays in elongating plant cells contribute to directional cellulose deposition and hence morphogenesis (Cyr 1994, Cyr and Palevitz 1995, Nick 1999, Baskin 2001). The organisation of these ordered microtubule arrays requires their interaction with other proteins, including structural microtubule-associated proteins (MAPs) that facilitate microtubule–microtubule interactions and linkages to the plasma membrane, as well as molecules involved in signalling to the microtubule cytoskeleton (Nick 1999). A 90-kDa microtubule-binding protein has been isolated from a membrane fraction of tobacco cells (Marc et al. 1996). Recently, we identified this protein as phospholipase D (PLD) (Gardiner et al. 2001). The PLD associates with the plasma membrane and colocalises with microtubule arrays throughout the cell cycle. The tobacco PLD is related to *Arabidopsis* PLDs by partial amino acid sequence and specifically related to PLD6 immunologically (Gardiner et al. 2001). The association of *Arabidopsis* PLD6 with the plasma membrane has been documented in detail (Wang and Wang 2001).

At least 12 PLD isoforms grouped into five functional groups are present in *Arabidopsis* (Elias et al. 2002, Qin and Wang 2002). PLD hydrolyses phospholipids to form phosphatidic acid (PA), which acts primarily as a second messenger in signalling cascades. PA can be generated either by PLD, or by phospholipase C in combination with diacylglycerol kinase (Munnik 2001). Known downstream targets of PA in plants include protein kinases (Lee et al. 2001) and potassium channels (Jacob et al. 1999). Generation of PA by PLD has been implicated in cellular processes such as seed germination (Ritchie and Gilroy 1998), stomatal movements (Jacob et al. 1999), responses to wounding and pathogen attack (Wang et al. 2000, van der Luit et al. 2000, den Hartog et al. 2001), responses to osmotic stress (Munnik et al. 2000, Katagiri et al. 2001, Sang et al. 2001), and senescence (Fan et al. 1997). Interestingly, stimuli that affect PLD expression, localisation, and activity often also cause microtubule reorganisation, indicating that PLD-dependent signalling may be involved in this process. For example, wounding induces a transient, systemic Ca2+-dependent increase in membrane-associated PLD as well as an increase in PA (Ryu and Wang 1996, Lee et al. 1997, Wang et al. 2000), and also leads to realignment of microtubules (Hush et al. 1990). Phytochrome activation increases PLD-dependent PA production, probably through a G-protein-mediated pathway (Park et al. 1996), and phytochrome also affects the orientation of microtubules (Fischer and Schopfer 1997). Similarly,
bacterial infection increases PLD levels and promotes its localisation to the plasma membrane (Young et al. 1996), while fungal pathogens cause microtubules to focus at the infection site (Kobayashi et al. 1994). Plant hormones that affect microtubule organisation (Shibaoka 1994) may also act through PLD-dependent signalling. For example, abscisic acid (ABA) signalling in stomatal guard cells, which leads to an efflux of K+ that facilitates stomatal closure, is mediated by PLD-dependent PA production (Jacob et al. 1999). ABA also causes the depolymerisation of microtubules in stomatal guard cells (Jiang et al. 1996) and microtubules are involved in guard cell opening (Zhou et al. 1999, Huang et al. 2000, Marcus et al. 2000). However, to date there is no direct evidence that PLD activity may be required for normal microtubule organisation in plants.

For experimental purposes, PLD activity can be manipulated by applying the specific inhibitor 1-butanol (Munnik et al. 1995). Generally, PLD-catalysed hydrolysis removes the head group of structural phospholipids, forming a short-lived phosphatidyl-PLD intermediate (Munnik et al. 1998). Under normal conditions the PLD then transfers the phosphatidyl moiety to H2O, forming PA. In the presence of 1-butanol, however, the alcohol is used as an alternative trans-phosphatidylation substrate, resulting in the formation of phosphatidylbutanol (Munnik et al. 1995). Because phosphatidylbutanol has no known biological activity, 1-butanol is a useful inhibitor of formation of PA by PLD. Two controls are available: 2-butanol, which activates PLD through a G-protein signalling pathway but is not trans-phosphatidylated, thus acting as a control for G-protein-dependent activation of PLD by 1-butanol; 3-butanol, which neither activates PLD nor acts as a substrate for trans-phosphatidylation and therefore is a control for any non-specific butanol effect (Munnik et al. 1995). 1-butanol has been useful in studies of PLD-dependent signalling in plants. For example, 1-butanol antagonises ABA and gibberellic acid signalling in barley aleurone (Ritchie and Gilroy 1998), blocking the ABA-mediated rise in PA and inhibiting ABA-induced closure of stomatal guard cells (Jacob et al. 1999).

If PLD-dependent PA production is required for normal microtubule organisation, application of a specific inhibitor of this process to a plant organ should affect its pattern of growth. To test this hypothesis, we applied 1-butanol to Arabidopsis seeds and seedlings, and examined its effects on microtubule organisation and seedling morphology. Our results show that 1-butanol dramatically suppresses seed germination and root elongation, and also produces striking disturbances in the morphology of root hairs. Immunofluorescence microscopy shows that 1-butanol also disrupts cortical microtubule arrays. These results are therefore consistent with a role for one or more PLDs, possibly including PLDδ, in microtubule organisation and morphogenesis in plants.

**Results**

1-butanol inhibits the emergence of radicles and cotyledons during germination, and reduces growth of seedlings transplanted from butanol-free media

Arabidopsis seeds germinated under continuous light on agar plates containing 1-, 2- or 3-butanol demonstrated that the
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Specific inhibitor of PLD-dependent PA production, 1-butanol, delayed the emergence of the radicle and cotyledons (Fig. 1). In control agar plates without any butanol, about 93% of radicles emerged by 3 d after sowing (Fig. 1A). Inhibition of radicle emergence by 1-butanol became obvious 2 d after sowing, even at the low concentration of 0.05%, but particularly at concentrations of 0.1% and above. Even after 4 d, only about 40% of the radicles emerged on agar plates containing 0.2% 1-butanol and only 10% of the radicles on 0.4% 1-butanol, representing 60 and 90% inhibition, respectively. The inactive isomers 2- and 3-butanol initially delayed radicle emergence at concentrations of 0.1% and above, but this delay disappeared after 3 d (Fig. 1A).

Cotyledon emergence lagged behind radicle emergence, as expected, so that only 70% of cotyledons emerged after 3 d and 90% after 4 d (Fig. 1B). Compared with radicle emergence, the emergence of cotyledons was more strongly inhibited by 0.05 and 0.1% 1-butanol and almost completely blocked by 0.2 and 0.4% 1-butanol. Again, the inactive isomers 2- and 3-butanol initially delayed cotyledon emergence, although this delay gradually diminished with time. Clearly, a striking contrast exists between the effects of 1-butanol and the two inactive isomers on the inhibition of germination.

Four-day-old Arabidopsis seedlings that had been germinated on control media showed significant reduction in growth rate when transplanted onto agar plates containing 1-butanol, but not 2- or 3-butanol (Fig. 2). In control seedlings maintained on inhibitor-free media, the roots grew at a consistent rate of around 5 mm per day over 3 d [Fig. 2; compare the 0% data points after day 1 (A), day 2 (B) and day 3 (C)]. While 0.1% 1-butanol had no noticeable effect, higher concentrations dramatically inhibited root growth. After 3 d, root elongation was inhibited 40% by 0.2% 1-butanol and almost completely blocked by 0.4% 1-butanol when compared to controls on inhibitor-free media. The inactive isomers 2- and 3-butanol had no effect at the low concentration (0.1%), and even at 0.4% concentration the two inactive isomers had little effect, reducing root elongation by only about 10% relative to controls on inhibitor-free media.

1-butanol alters seedling morphology

Under control conditions, cells in Arabidopsis roots show anisotropic growth, with increases in cell volume occurring solely through cell elongation and not through any increase in cell diameter (Wasteneys and Collings 2003). Cell elongation commences in the distal elongation zone immediately behind the meristem, but reaches a maximum in the elongation zone which lies around 0.3–1.0 mm from the root tip in roots of this age (Beemster and Baskin 1998). In our experiments, although roots treated with 2- or 3-butanol appeared normal, seedlings treated with 1-butanol had altered morphology (Fig. 3A–F). In the presence of 0.2 or 0.4% 1-butanol for 3 d, cells in the elongation zone became swollen, leading to a bulge forming in the root (Fig. 3A; arrows). This effect did not occur with the inactive isomers 2- and 3-butanol at the same concentrations.

We quantified root swelling by staining cell walls with the fluorescent probe propidium iodide, then reconstructing confo-
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Fig. 3 1-butanol distorts root shape, root hair morphology, and cotyledon morphology. Three-day-old Arabidopsis seedlings were transplanted onto agar plates containing 1-, 2- or 3-butanol at varying concentrations, and after 3 d growth the effects on root morphology were observed. (A) Seedlings grown on 1-butanol (1-B) at 0.2 and 0.4% (v/v) concentration developed a distinct swelling in the root elongation zone (arrows), whereas seedlings exposed to the inactive isomers (2-B, 2-butanol; 3-B, 3-butanol) showed no changes in root morphology, and were similar to seedlings grown on inhibitor-free media (not shown). Some images are composed of montages of multiple photographs. (B–D) While seedlings grown on inhibitor-free media had long, straight root hairs (B), the root hairs of seedlings grown on 0.2% (v/v) 1-butanol were often shorter and swollen (D) or branched (C). (E, F) Compared with the cotyledons in control seedlings (E), 1-butanol [0.2% (v/v)] inhibited the expansion of cotyledons and the cotyledons had a more rounded shape (F). Scale bars = 200 μm (A), 150 μm (B, D), 700 μm (E, F).

Table 1 Root morphology is altered by treatments with 1-butanol

<table>
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<tr>
<th>Treatment</th>
<th>N</th>
<th>Root diameter (μm)</th>
<th>Cell widths (μm)</th>
<th>Cell lengths (μm)</th>
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<td>Trichoblasts</td>
<td>Atrichoblasts</td>
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<tr>
<td></td>
<td></td>
<td>Cortex</td>
<td>Trichoblasts</td>
<td>Atrichoblasts</td>
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<td>9</td>
<td>149± 7</td>
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<td>1-butanol</td>
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<tr>
<td>0.1% (v/v)</td>
<td>11</td>
<td>150± 6</td>
<td>36.2±1.8</td>
<td>19.4±0.7</td>
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<tr>
<td>0.2% (v/v)</td>
<td>7</td>
<td>222± 7**</td>
<td>54.0±3.0**</td>
<td>26.5±1.2**</td>
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<tr>
<td>0.4% (v/v)</td>
<td>5</td>
<td>223±10**</td>
<td>49.5±4.7*</td>
<td>30.8±2.0**</td>
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<td>2-butanol</td>
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<tr>
<td>0.1% (v/v)</td>
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<td>138± 8</td>
<td>31.7±1.9</td>
<td>17.8±0.4</td>
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<td>0.2% (v/v)</td>
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<td>149± 6</td>
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<td>10</td>
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<td>161± 5</td>
<td>33.8±2.2</td>
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<td>0.2% (v/v)</td>
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<td>35.6±0.9</td>
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<td>0.4% (v/v)</td>
<td>9</td>
<td>148± 5</td>
<td>34.2±0.8</td>
<td>18.6±0.3</td>
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</table>

* Number of replicate roots.

** Data are means ± standard errors, with the value for each plant generally being an average of four or more cells.

** For cell length measurements, identification of cells belonging to the root cortex, or as trichoblasts or atrichoblasts within the epidermis, was possible through the analysis of reconstructed transverse sections.

** T-test; significant at P < 0.001 when compared to the control.

* T-test; significant at P < 0.02 when compared to the control. Although the cell widths for trichoblasts in 0.2% 3-butanol-treated roots is significantly higher than untreated controls, the number measurements made of the different 2- and 3-butanol treatments (42) is such that a false positive would not be unexpected at P = 0.02. Further, unlike the consistent effects shown by 1-butanol at 0.2 and 0.4%, the cell widths for trichoblasts in 0.2% 3-butanol-treated roots are not replicated by a significant change at the higher 3-butanol concentration.
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From these images, we calculated average root diameters, cell diameters, and cell lengths (Table 1). These data show that 1-butanol at either 0.2 or 0.4% caused a significant increase of 50–70% in root diameter and in the diameters of cells in the epidermis and cortex, compared with butanol-free controls. Significant reductions in cell length of approximately 80% also occurred with these treatments. The inactive isomers 2- and 3-butanol showed no significant differences in root and cell diameters or cell lengths when compared with the controls.

Further morphological changes also occurred following 1-butanol treatments. The root hairs of seedlings treated with 1-butanol were short and often bulbous or branched (Fig. 3C, D), in contrast to the long and straight root hairs of butanol-free seedlings (Fig. 3B), or seedlings grown on agar plates containing the inactive butanol isomers (data not shown). The cotyledons of seedlings treated with 0.2 or 0.4% 1-butanol also showed signs of retarded growth and altered morphology (Fig. 3E, F).

The morphological defects and inhibition of growth by 1-butanol are reversible

To determine whether the effects of 1-butanol were reversible, seedlings were first maintained on 0.2% 1-butanol for 3 d and then transplanted back onto inhibitor-free agar plates. The 1-butanol-treated seedlings showed the usual reduction in the rate of root elongation (Fig. 4A), but after transplanting onto inhibitor-free agar plates, the rate of root elongation increased and the morphology of the newly formed part of the roots returned to normal (Fig. 4A). Unlike the stunted and bulbous root hairs in the 1-butanol-inhibited part of the root (Fig. 4B, open arrowheads; also Fig. 3C, D), newly formed root hairs were long and straight (Fig. 4B, filled arrowheads) and were similar to those seen in controls (Fig. 3B). The newly formed root tip also regained its normal appearance (Fig. 4C), without the bulge in the elongation zone that is typical of 1-butanol-treated roots (Fig. 4B, arrow).

Cortical microtubules become disorganised in 1-butanol-treated roots

We used confocal immunofluorescence microscopy of whole roots of *Arabidopsis* to examine the effects of 1-butanol on the integrity of microtubule arrays in the distal elongation zone, the site where pronounced elongation of cells first begins (Fig. 5). In *Arabidopsis* roots treated with 0.4% 2-butanol for 12 h, the cortical microtubules formed highly parallel arrays that were transverse to the direction of cell elongation in all cell layers, including epidermal cells (Fig. 5D) and cells of the cortex and developing stele (Fig. 5E, F). Similarly, highly ordered arrays of microtubules also occurred in roots treated with 0.4% 3-butanol for 12 h (data not shown) and in control roots (Fig. 5G–I). In contrast, cells at a similar location in roots treated with 0.4% 1-butanol for 12 h showed different microtubule pat-
Role of phospholipase D and microtubules in growth patterns (Fig. 5A–C). While some microtubules in the epidermis continued in transverse arrays (Fig. 5A, asterisk), many cells showed either disorganised microtubules (arrowhead), or ordered arrays of non-transverse microtubules whose orientation varied between adjacent cells (arrow). Such microtubule disorganisation was observed to varying degrees in all nine Arabidopsis roots analysed in detail, but was never seen in controls or treatments with the inactive butanol isomers.

Discussion

Effects of 1-butanol on seed germination, root elongation, and seedling morphology

Experiments with the inhibitor of PLD-dependent PA production, 1-butanol, showed that both seedling germination and root elongation were inhibited by concentrations as low as 0.2% 1-butanol, and that these treatments changed the morphology of roots and root hairs. Control treatments with the inactive isomers 2- and 3-butanol confirmed that the effects of 1-butanol were specific. The effects of 1-butanol on seedling growth were not due to necrosis, as seedlings transplanted back onto inhibitor-free agar plates recovered and resumed normal growth patterns. Therefore, PLD-dependent PA production is likely to be important for seed germination as well as for subsequent growth and development of roots, root hairs and cotyledons.

The growth inhibition and disturbances in seedling morphology seen in the presence of 1-butanol are consistent with early developmental processes in Arabidopsis relying on PLD-dependent signalling. Three different PLD isoforms have been detected in castor bean endosperm (Wang et al. 1993, Dyer et al. 1994). One isoform disappears 3 d post-imbibition, another isoform remains over 5 d, and a third isoform appears 3 d post-imbibition (Dyer et al. 1994). Interestingly, PLDδ is expressed particularly strongly in the elongation zone of Arabidopsis roots (Katagiri et al. 2001), consistent with the location of the effects of 1-butanol in our experiments. The presence of multiple PLD isoforms in plants and functional redundancy between the isoforms during development (Wang et al. 1993, Dyer et al. 1994, Qin and Wang 2002) may also explain why no PLD mutants or anti-sense plants have shown aberrant phenotypes to date (Fan et al. 1997, Wang et al. 2000, Sang et al. 2001, Katagiri et al. 2001, Munnik and Musgrave 2001).

Fig. 5 Disturbances of microtubule arrays in roots by 1-butanol. Four-day-old Arabidopsis seedlings were transplanted onto agar plates containing 0.4% (v/v) butanol, and after 12 h were fixed, immunolabelled and microtubule organisation determined by confocal microscopy. Treatments with 1-butanol (A–C) randomised microtubules, compared with the highly transverse organisation of microtubules in roots in 2-butanol (D–F) or in butanol-free controls (G–I). The images show cells in the distal elongation zone where rapid cell elongation commences, and have the root tip oriented downwards. Moreover, the images are maximum projections of between 8 and 10 optical sections, with the approximate depths from the root surface covered by each projection shown in micrometres. The projected planes have been selected to show the organisation of the cortical microtubule arrays at the outer surface of the epidermal cells (A, D and G), at the junction between the epidermal and cortex cell layers (B, E and H) and in cells of the endodermis and pericycle (C, F and I), although the tangential nature of these deeper sections means that the outer edges of the images still show cortical microtubules from epidermal cells. The asterisk in (A) indicates a cell that has retained transverse cortical microtubules, while the arrowhead and arrow indicate random, and locally parallel but oblique microtubules respectively. Scale bar in G = 20 μm for all images.
The alignment of cellulose microfibrils in tip-growing cells, and microtubule motors in vesicular transport and cellulose deposition, both in diffuse- and tip-growing cells. PLD and PA interactions with microtubules are consistent with the swelling measured in the elongation zone of treated roots and the reduction in cell elongation. Microtubule-depolymerising drugs such as oryzalin, and the microtubule-stabilising drug taxol, generate a similar root swelling phenotype in Arabidopsis roots (Baskin et al. 1994, Baskin and Bivens 1995). Similar swelling also occurs in Arabidopsis mutants that show disrupted microtubules. For example, in the mor1 mutant, which is defective in a 217-kDa MAP, transverse arrays of microtubules in elongating cells become disorganised at the restrictive temperature (Whittington et al. 2001). Root swelling is also seen in botero1 and fra2 mutants, which are defective in the p60 subunit of the microtubule-severing protein katanin. In botero1, the swelling is likely due to faulty microtubule organisation, as interphase microtubules fail to form normal transverse arrays in elongating cells from the more random arrays present in postmitotic cells (Bichet et al. 2001). In fra2, perinuclear microtubules present after division are retained longer and the organisation of microtubules into transverse cortical arrays is delayed (Burk et al. 2001).

The swelling seen in Arabidopsis roots treated with 1-butanol is apparently less severe than that seen in roots treated with microtubule-depolymerising agents such as oryzalin (Baskin et al. 1994, Baskin and Bivens 1995). Nonetheless, trichoblasts, atrichoblasts, and cortical cells in treated roots all showed significantly reduced elongation as well as significant radial swelling (Table 1). The relatively less severe swelling of the root in the presence of 1-butanol may reflect inhibition of other cellular functions, such as cellulose synthesis (Arioli et al. 1998, Sugimoto et al. 2001) or vesicular transport (Baskin and Bivens 1995). Significantly, however, the majority of inhibitors of root elongation tested by Baskin and Bivens (1995) failed to produce the swelling phenotype. Thus, the root swelling measured after 1-butanol treatments in our experiments is consistent with the hypothesis that PLD signalling through PA is involved, either directly or indirectly, in the organisation of the microtubule cytoskeleton.

Other morphological changes induced by 1-butanol are also consistent with cell expansion defects and interaction with the microtubule cytoskeletons. The reduced size of cotyledons in 1-butanol-treated samples is similar to the effect seen in the rsw1 mutant at the restrictive temperature (see Fig. 1, Williamson et al. 2001). PLD-dependent PA production is probably also important for the normal tip-growth of root hairs, as shown by their bulged and branched morphology in the presence of 1-butanol. Although microtubules do not contribute to the alignment of cellulose microfibrils in tip-growing cells, they remain important in root hair morphology. For example, branched root hairs are seen in Arabidopsis lines with reduced α-tubulin (Bao et al. 2001). Furthermore, microtubule polymerisation with oryzalin, or stabilisation with taxol, induces root hair waving and branching in Arabidopsis (Bibikova et al. 1999).

Interactions between PLD, microtubules, and the plasma membrane

A 90-kDa MAP isolated from the membrane fraction of tobacco cells (Marc et al. 1996) is related immunologically to PLDδ in Arabidopsis (Gardiner et al. 2001). Since the 90-kDa MAP associates with both plasma membrane and microtubules (Gardiner et al. 2001), and since cortical microtubules associate with the plasma membrane, a PLD may act as a structural or signalling link between the plasma membrane and the microtubule cytoskeleton in tobacco and Arabidopsis.

In one scheme, the PLD-microtubule complex may detach from the plasma membrane through the calcium-dependent lipid-binding domain (C2 domain) found in most plant PLDs (Wang and Wang 2001, Elias et al. 2002). This in turn may affect the organisation of the microtubule cytoskeleton. Calcium ions are known to influence the stability of microtubules and their attachment to the membrane through calmodulin (Cyr 1991, Fisher et al. 1996), and movements of PLDs between membrane and cytoplasmic compartments are common (Wang 1999, Wang 2000). Production of PA may also modify the physical properties of the plasma membrane (Ahn and Yun 1998), thus affecting its interaction with the PLD-microtubule complex or other potential links between the membrane and microtubules (Yamauchi and Purich 1987). It is unclear whether the effects of 1-butanol on microtubule organisation in Arabidopsis involve PLDδ or other PLD isoforms.

PA production by PLD may also affect the dynamics and stability of microtubules through other proteins. For example, RAFT1 (rapamycin and FKBP12 target 1 protein) is activated by PLD-generated PA in yeast and mammals (Fang et al. 2001). In yeast, inhibition of RAFT1 by rapamycin alters the assembly, elongation and stability of microtubules, causing defects in spindle orientation, nuclear movement and positioning, karyogamy, and chromosomal stability (Choi et al. 2000). RAFT1 interacts with microtubules through the protein BIK1 (bilateral defect in karyogamy 1), which binds to both RAFT1 and microtubules (Choi et al. 2000). Thus the 1-butanol-induced disruption of microtubule organisation in Arabidopsis may involve a PA-dependent signalling pathway mediated by an Arabidopsis homologue of RAFT1, and effected through MAPs that also bind to RAFT1. Indeed, the Arabidopsis homologue of RAFT1 is crucial for early embryo development, and is expressed specifically in undifferentiated, dividing cells (Menand et al. 2002).

Another possibility is the involvement of microtubules and microtubule motors in vesicular transport and cellulose deposition, both in diffuse- and tip-growing cells. PLD and PA.
are involved in vesicular transport in mammals (Jones et al. 1999), and the yeast sporeulation mutant spo14 lacking PLD is unable to form a new spore membrane (Rudge et al. 1998). Thus it has been suggested that wall deposition in plants, which relies upon the transport of Golgi vesicles to appropriate locations along the wall, may also depend on generation of PA by PLD (Munnik and Musgrave 2001).

Conclusions

Experiments with 1-butanol indicate that PLD-dependent PA production is likely to be crucial for germination and morphogenesis in Arabidopsis, possibly by maintaining the correct transverse alignment of microtubules in elongating root cells. The mechanism by which this occurs is unclear, but studies in other organisms indicate that it is likely to involve interactions between the plasma membrane, PLD, other proteins, PA, and perhaps PA-dependent RAFT1 signalling pathway. It is not known whether PLD6b is the isoform responsible for the regulation of microtubule orientation, but since its tobacco homologue associates with microtubules throughout the cell cycle and with the plasma membrane (Gardiner et al. 2001, Wang and Wang 2001), it is a leading candidate.

Materials and Methods

Germination and growth experiments with Arabidopsis seedlings

Agar plates for seed germination were prepared using modified Hoagland solutions containing agar (1.2% w/v; Bactoagar, Difco, Detroit, MI, U.S.A.) and sucrose (3% w/v) (Baskin and Wilson 1997). 1-, 2- and 3-butanol were added to the agar at concentrations up to 0.4% (v/v) just prior to the pouring of 55-mm diameter plates. For germination trials, Arabidopsis thaliana var. Columbia seeds were surface-sterilised using 1/3 (v/v) household bleach (sodium hypochlorite solution, 1% available chlorine; 10 min), and washed in sterile distilled water (3×10 min). Twenty-five seeds were sown per agar plate, and these were incubated vertically at 25°C under continuous overhead illumination from cool white fluorescent lights (100 μmol m⁻² s⁻¹ phototaxonomically active radiation). Seeds were examined with a dissecting microscope for the emergence of cotyledons and radicles after 1, 2, 3 and 4 d.

For root growth experiments, Arabidopsis seedlings were grown for 4 d on butanol-free agar plates and then transplanted onto agar plates containing up to 0.4% of 1-, 2- or 3- butanol. Plates were scanned on a flat-bed scanner, with lengths determined in Adobe Photoshop, immediately following transplantation and after 1, 2, 3 and 4 d of growth. For recovery experiments, 4-day-old seedlings were grown on 0.2% 1-butanol plates for 3 d and then transplanted back onto agar plates without butanol for another 3 d.

Assessment of seedling morphology

Control and butanol-treated plants were examined under transmitted light on an Olympus Provis AX70 microscope (Olympus, Tokyo, Japan) equipped with a CCD camera (Photometrics, Tucson AZ, U.S.A.) and image acquisition system (Scanalytics, Fairfax, VA, U.S.A.). Images of roots, root hairs and cotyledons were recorded for at least ten plants, and representative examples were recorded for presentation.

In order to quantify the effects of butanol treatments, 4-day-old Arabidopsis seedlings were transferred to butanol-containing agar plates for 3 d. Seedlings were then stained for 1–2 h with propidium iodide (Molecular Probes, Eugene, OR, U.S.A.) (20 μg ml⁻¹ in water), a cell-impermeant stain that marks the location of cell walls. Optical stacks were recorded through the living roots at 1-μm steps with a confocal laser scanning microscope (Leica SP2, Wetzlar, Germany) with excitation at 488 nm, and with emission recorded with a 63 × NA 0.70 water-immersion lens from 500 to 600 nm. Leica’s software was used to generate transverse slices through the roots, and cell width and length measurements were made from these images.

Confocal immunofluorescence microscopy of microtubules in Arabidopsis roots

Arabidopsis roots were processed for immunofluorescence microscopy using methods modified from Sugimoto et al. (2000). Whole seedlings were fixed for 30 min in PME buffer [50 mM PIPES pH 7.2 (K⁺), 2 mM EGTA, 2 mM MgSO₄ and 0.1% Triton X-100] containing 4% (v/v) formaldehyde and 1% glutaraldehyde (Sigma). Seedlings were then washed in PME (3×10 min), extracted for 1 h in PME buffer in which the Triton X-100 concentration was raised to 1.0%, and washed again in PME buffer. Cell walls were digested with 1.0% cellulase Y6 and 0.1% pectolyase Y23 (ICN, Seven Hills, NSW, Australia) dissolved in PME buffer containing 1% BSA (Fraction V, Sigma, Sydney, NSW, Australia) and 0.4 M mannitol (20 min), and washed in PME buffer (2×10 min). Material was then permeabilised in methanol (–20°C, 10 min), rehydrated in PBS (131 mM NaCl, 5.1 mM Na₂HPO₄, 1.56 mM KH₂PO₄, pH 7.2), and free aldehyde groups reduced with freshly bubbling sodium borohydride (5 mg ml⁻¹) in PBS (30 min). After several rinses in PBS, non-specific antibody-binding sites in seedlings were blocked using incubation buffer (PBS containing 1% BSA and 50 mM glycine, 20 min), and roots were then incubated for 2 h at 30°C in monoclonal anti-α-tubulin (clone B512, Sigma) diluted 1/1,000 in incubation buffer. After removing primary antibody, root tips were washed in PBS containing 50 mM glycine (4×15 min), then incubated in goat anti-mouse IgG coupled to Alexa-488 (Molecular Probes) diluted 1/200 in incubation buffer (2 h at 30°C). After the removal of secondary antibodies, root tips were washed in PBS containing 50 mM glycine (3×15 min) then stained for 15 min in 1 μg ml⁻¹ 4’,6-diamidino-2-phenylindole hydrochloride (DAPI, Sigma) diluted in PBS. Roots were mounted whole in Citifluor (Citifluor, London, U.K.), and coverslips sealed with nail polish. Samples were viewed with the confocal microscope Leica SP2 and a 63 × NA 1.32 oil-immersion lens. DAPI and Alexa-488 were excited using laser lines at 361 and 488 nm, and fluorescence emissions were collected from 400 to 480 and 500 to 600 nm respectively.

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References


Role of phospholipase D and microtubules in growth

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