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Disruption of cellulose synthesis by isoxaben causes tip swelling and disorganizes cortical microtubules in elongating conifer pollen tubes

M. D. Lazzaro *, **J. M. Donohue**, and **F. M. Soodavar**

Department of Biology, College of Charleston, Charleston, South Carolina

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Summary. In elongating pollen tubes of the conifer *Picea abies* (Norway spruce), microtubules form a radial array beneath the plasma membrane only at the elongating tip and an array parallel with elongation throughout the tube. Tips specifically swell following microtubule disruption. Here we test whether these radial microtubules coordinate cell wall deposition and maintain tip integrity as tubes elongate. Control pollen tubes contain cellulose throughout the walls, including the tip. Pollen tubes grown in the presence of isoxaben, which disrupts cellulose synthesis, are significantly shorter with a decrease in cellulose throughout the walls. Isoxaben also significantly increases the frequency of tip swelling, with no effect on tube width outside of the swollen tip. The decrease in cellulose is more pronounced in pollen tubes with swollen tips. The effects of isoxaben are reversible. Following isoxaben treatment, the radial array of microtubules persists beneath the plasma membrane of nonswollen tips, while this array is specifically disrupted in swollen tips. Microtubules instead form a random network throughout the tip. Growth in these pollen tubes is turgor driven, but the morphological changes due to isoxaben are not just the result of weakened cell walls since pollen tubes grown in hypoosmotic media are not significantly shorter but do have swollen tips and tubes are wider along their entire length. We conclude that the radial microtubules in the tip do maintain tip integrity and that the specific inhibition of cellulose microfibril deposition leads to the disorganization of these microtubules. This supports the emerging model that there is bidirectional communication across the plasma membrane between cortical microtubules and cellulose microfibrils.

Keywords: Cell wall; Cytoskeleton; Isoxaben; Microtubule; *Picea abies*; Pollen tube.

Introduction

Pollen tubes are a tip-growing system in higher plants that delivers sperm cells for fertilization. Tube growth and sperm delivery in conifers is fundamentally different from angiosperms (reviewed in Singh 1978). Conifer pollen grains land in a pollination droplet, germinate, and tubes elongate through the megagametophyte. During tube growth, the generative cell remains within the pollen grain, and after the tube reaches the egg, the generative cell migrates to the pollen tube tip and forms two sperms, one of which fertilizes the egg. Our model system is Norway spruce (*Picea abies*, Pinaceae), where a network of microfilaments and microtubules extends out through the grain aperture forming a net axial array down the length of the tube (Lazzaro 1996, 1999). Microfilaments appear to be excluded from the actively growing tip region, which extends back about 25 μm from the tip (Anderhag et al. 2000). Within this tip, microtubules form a radial array beneath the plasma membrane that is perpendicular to the direction of tube growth. This array extends back 20 μm from the tip. There is an abrupt transition from a net radial to a net axial organization at the edge of the enriched region. In medial sections, microtubules are present in the center of the elongating tip (Lazzaro 1999). The cytoskeletal organization in this conifer model system differs from that seen in angiosperm pollen tubes, where microtubules are either excluded from the tip or found sparingly (reviewed in Pierson and Cresti 1992, Li et al. 1997). Microtubules are unusually important in this tip-growing system since their disruption significantly inhibits tube elongation and induces tube branching and tip swelling (Anderhag et al. 2000). Colchicine and propyzamide cause fragmentation of microtubules throughout the tube, leading to a decrease in tube

* Correspondence and reprints: Department of Biology, College of Charleston, 58 Coming Street, Charleston, SC 29424, U.S.A.
E-mail: lazzarom@cofc.edu

length and an increase in branching. Oryzalin and amiprophosmethyl cause a decrease in tube length and an increase in tube branching, but the morphology of microtubule disruption is different. Oryzalin and amiprophosmethyl cause a complete loss of microtubules from the tip back towards the tube midpoint but leave microtubules intact from the midpoint back to the grain. This loss of microtubules at the tip causes a significant increase in tip swelling and led us to the model that the radial array of microtubules strengthens the emerging tip and may organize cellulose synthesis (Anderhag et al. 2000).

The deposition of cellulose microfibrils occurs close to the elongating tip in conifer pollen tubes. In *Pinus sylvestris*, microfibrils are found in the tip (Derksen et al. 1999) at a higher density than in *Nicotiana tabacum*, where cellulose microfibrils are first detected 5–15 μm away from the tip (Ferguson et al. 1998). The prevailing model for cellulose deposition in plant cells is that cortical microtubules direct the deposition of parallel cellulose microfibrils by guiding cellulose synthase complexes within the plasma membrane (reviewed in Carpita and Gibeaut 1993, Cyr 1994). The mechanism of this model suggests that a coordinating signal passes from the cytoplasm (microtubule alignment) to the extracellular matrix (the alignment of cellulose microfibrils). However, recent work indicates that the specific inhibition of cellulose synthesis by isoxaben (Heim et al. 1990) in newly formed *N. tabacum* protoplasts causes the disorganization of cortical microtubules (Fisher and Cyr 1998). This finding has led to the extension of the microtubule-microfibril paradigm to include bidirectional communication across the plasma membrane (Fisher and Cyr 1998, Baskin 2001).

The present study tests our hypothesis that the radial array of cortical microtubules helps strengthen the elongating tip and also examines the model that bidirectional communication exists between cellulose microfibrils and cortical microtubules in a polarized, tip-growing system. By growing pollen tubes in the presence or absence of isoxaben, we examine whether the inhibition of cellulose synthesis affects pollen tube elongation, tip morphology, and microtubule organization.

Material and methods

Pollen was field collected from Norway spruce trees (*Picea abies*) near Stockholm, Sweden, and stored at –20 °C. A 250 μM stock solution of isoxaben in 100% ethanol was diluted into cooling agar media (1% purified agar [Sigma A-7921], 500 mM sucrose, 1 mM $CaCl_2$, 1 mM H_3BO_3 in distilled water) for a final concentration of 2.5 μM and 1% ethanol. Agar media in controls also contained 1% ethanol, which has no effect on tube elongation or morphology (Anderhag et al. 2000). A thin layer of cooling agar media was prepared on glass slides. Pollen was dusted across cooled agar and the slides were then incubated for 24 h at 30 °C under high humidity. Images of germinated pollen were captured with a video camera on an inverted microscope and stored digitally for subsequent morphological analysis. To label microtubules, separate pollen aliquots were germinated on agar media (with or without 2.5 μM isoxaben) layered in the bottom of 1.5 cm diameter cylindrical sample wells (1 cm deep) glued onto glass slides. Pollen grains were sown on these sample wells and germinated for 24 h at 30 °C in humid chambers. Pollen tubes were then immunolabeled as described in detail in Lazzaro (1999). Briefly, cell walls were permeabilized after fixation by freeze shattering (Wasteneys et al. 1997). The resulting samples were immunolabeled with monoclonal antibodies to alpha tubulin (Cederlane Labs CLT9002) and Cy3-conjugated secondary antibodies (Sigma C2181). Controls omitting the primary antibody were also prepared. Pollen tubes were examined with an Olympus FluoView confocal laser-scanning microscope.

For recovery and lethality experiments, pollen grains were germinated in liquid culture media (500 mM sucrose, 1 mM $CaCl_2$, 1 mM H_3BO_3 in distilled water) containing 0, 2.5, or 2500 μM isoxaben and 1% ethanol. Pollen grains were sown on 2 ml of media in 6-well tissue culture plates (Falcon 3046). After incubation for 24 h at 30 °C, digital images of pollen aliquots were captured. The pollen grains and tubes were then rinsed by gravity sedimentation through two changes of culture media. This rinsing lowered the isoxaben concentrations from 2.5 and 2500 μM to approximately 0.1 μM and 100 μM, respectively. The remaining 2 ml of sedimented pollen was transferred back to culture wells for an additional incubation of 24 h at 30 °C and then aliquots were again examined.

Cellulose microfibrils were labeled with calcafluor (Hughs and McCully 1975). Pollen grains were germinated in liquid culture media (with and without 2.5 μM isoxaben) and incubated for 24 h at 30 °C. A 10 mg/ml stock solution of calcafluor (Sigma F-3543) in distilled water was centrifuged at 14000 **g** for 5 min. The resulting supernatant was diluted 1 : 10 into an aliquot of pollen tubes in germination media. After 45 min of incubation in calcafluor, pollen tubes were transferred to glass slides and examined with differential interference contrast followed by epifluorescence (ultraviolet excitation). Digital images of epifluorescence were immediately captured with a charge-coupled-device camera (Micromax 1300-Y, Roper Scientific) at 10 ms, using IP Lab software (Scanalytics) for capture and analysis. To quantify the fluorescence due to calcafluor, captured images were first converted to 256 gray levels. Cell walls were then traced and the gray levels of pixels falling within the walls were measured. These gray-level values were displayed as a plot of pixel count versus gray level. The resulting bell curve describes the distribution of the intensity of calcafluor labeling across the entire cell wall of the pollen tube. The peak of each curve was taken as the median gray level for the pollen tube wall. Peak gray levels were calculated for isoxaben-treated pollen tubes with and without swollen tips, as well as control pollen tubes. Gray levels were also measured separately for pollen tube shanks and tips within each group. Differences in gray levels were statistically measured with Tukey's multiple comparison test at $P < 0.05$.

For osmotic experiments, pollen was germinated in liquid culture media. Control media contained 500 mM sucrose, 1 mM $CaCl_2$, and 1 mM H_3BO_3 in distilled water. To vary the osmotic concentration, mannitol was partially substituted for sucrose such that culture media contained 200, 400, or 600 mM mannitol plus 100 mM sucrose,

1 mM $CaCl_2$, and 1 mM H_3BO_3 in distilled water. Pollen grains were sown on 2 ml of culture media in culture plates, incubated for 24 h at 30 °C, and then examined.

Pollen tube morphology was analyzed with IP Lab, measuring tube length and width, and frequency of germination, tube branching, and tip swelling. Pollen tube length was measured as the distance from the aperture to the tube tip. Tube width was measured at the midpoint between the aperture and tip. Swollen tips were scored independently from tube width as tips whose diameter was at least 50% greater than the tube diameter near the grain aperture. All data were analyzed for statistical significance with Systat. Pollen tube length and width were analyzed with Tukey's multiple comparison test. The frequencies of germination and tip swelling were analyzed with Pearson's chi-square test (Zar 1984). Differences were significant at $P < 0.05$.

Results

Control pollen tubes are elongate with a uniform diameter. Amyloplasts are found throughout the tube but are excluded from the elongating tip (Fig. 1). Isoxaben changes the morphology of elongating *P. abies* pollen tubes (Figs. 3 and 5). When pollen grains germinate and tubes grow in the presence of 2.5 µM isoxaben (Fig. 3), they are significantly shorter (165 µm) than control tubes (183 µm). In addition, a significant percentage of pollen tubes (20%) have swollen tips (Fig. 5). Isoxaben does not significantly affect the width of tubes at the midpoint between the grain aperture and the tip. Finally, there is no significant change in germination frequency following isoxaben treatment (Table 1).

Tip swelling is reversible. When isoxaben is washed out of liquid media after 24 h and tubes are allowed to recover for an additional 24 h, the percentage of swollen tips drops from 34% to control levels (Table 2). Excessive amounts of isoxaben are also not lethal. Pollen grains in 2500 µM isoxaben germinate with the same frequency and grow with the same morphology and frequency of tip swelling as tubes in 2.5 µM isoxaben (Table 2).

Isoxaben does inhibit cellulose synthesis in these pollen tubes. In controls, calcafluor labeling indicates that cellulose microfibrils are present throughout the tube wall, including the elongating tip (Fig. 2). Isoxaben-treated pollen tubes have less dense cellulose microfibril networks. There is a significant quantitative decline in fluorescently labeled cellulose in the walls of isoxaben-treated pollen tubes (Figs. 4 and 6). The average fluorescence gray level in the walls of controls was 143 (n = 13 pollen tubes) compared to 109 (n = 18 pollen tubes) in isoxaben-treated tubes with normal tips. Cellulose microfibril density is further reduced throughout the walls of isoxaben-treated tubes with swollen tips (Fig. 6), where the average gray level was 78 (n = 19 pollen tubes). However, fluorescently labeled cellulose microfibrils are still detected even in extremely swollen tips (Fig. 6). Fluorescence was also measured separately between tips and shanks of all pollen tubes. There was no significant difference between microfibril density in the growing tip (gray level, 142) compared to the pollen tube shank (gray level, 142) in controls. There was also no significant difference between normal tips (gray level, 123) and shanks (gray level, 92) in isoxaben-treated tubes. Finally, there was no significant difference between swollen tips (gray level, 77) and shanks (gray level, 79).

Table 1. Effects of isoxaben and external osmoticum on pollen tube dimensions and frequency of tip swelling and germination

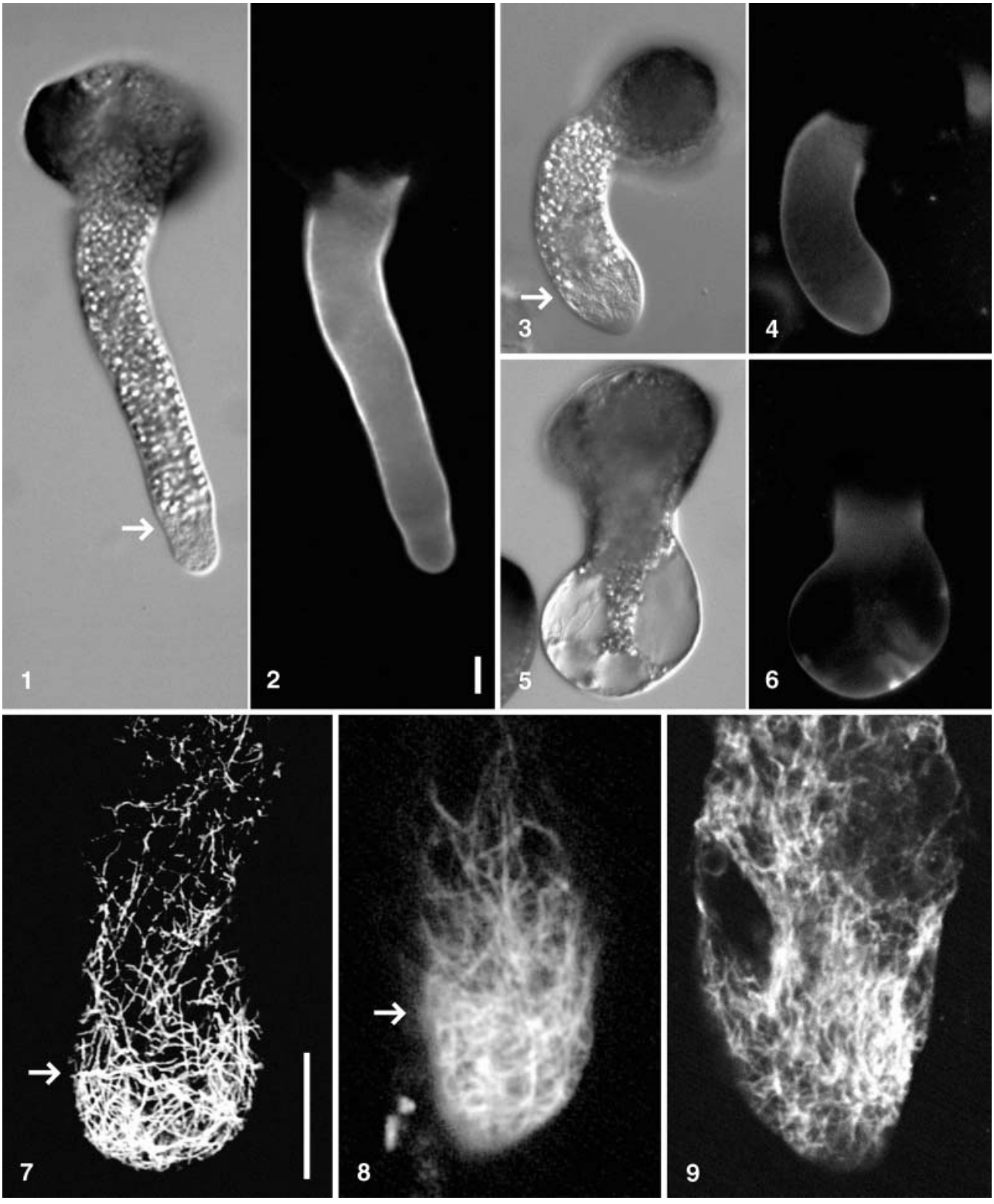
Treatment	Mean dimension (µm) of tubes		% Germinating pollen grains	% Tubes with swollen tips
	length	width		
Control	183 (138)[a]	39 (242)	86 (338)	2 (338)
Isoxaben (2.5 µM)	165[b] (109)	37 (156)	83 (318)	20[b] (318)
Osmoticum				
Low	183 (194)	53[c] (224)	85 (384)	20[c] (277)
Normal	163 (159)	42[c] (162)	89 (278)	9[c] (218)
High	101[d] (168)	37[c] (127)	80[d] (307)	1[c] (220)

[a] Numbers in parentheses are the total numbers of pollen tubes or grains
[b] Significantly different from control values ($P < 0.05$)
[c] Significantly different from values obtained by either of the other osmoticum treatments
[d] Significantly different from values obtained with both hypo- and isosmotic media

Table 2. Pollen grain germination and tube tip swelling at different concentrations of isoxaben and after washout

Isoxaben (µM)	% Germinating pollen grains	% Tubes with swollen tips	% Tubes with swollen tips after dilution[a]
0 (control)	76 (356)[b]	4 (170)	10 (120)
2.5	80 (326)	34[c] (181)	14 (113)
2500	78 (348)	31[c] (195)	37[c] (89)

[a] By gravity sedimentation, isoxaben concentrations were lowered from 2.5 and 2500 µM to 0.1 and 100 µM, respectively, after 24 h and then the tubes were allowed to recover for another 24 h
[b] Numbers in parentheses are the total numbers of pollen grains or tubes
[c] Significantly different from control values ($P < 0.05$)



Figs. 1–9. Control pollen tubes are filled with amyloplasts (Fig. 1) but have a distinct zone (arrow) at the terminal 20–30 μm that lacks plastids and contains vesicles. Isoxaben-treated tubes are shorter (Fig. 3), but the tip zone is preserved (arrow). This tip zone swells (Fig. 5) in a significant percentage of isoxaben-treated tubes. In controls, cellulose microfibril density is uniform throughout pollen tube walls (Fig. 2) and microfibrils are detected within the tip. Cellulose is still present following isoxaben treatment (Fig. 4), but microfibril density is reduced. Microfibril density is further reduced in pollen tubes with swollen tips (Fig. 6), but cellulose is still detected within the tip wall. Cortical microtubules are parallel with elongation through tubes but form a radial array (arrow) beneath the plasma membrane at the tip (Fig. 7). This radial array remains (arrow) in isoxaben-treated tubes with normal tips (Fig. 8) but is lost in swollen tips (Fig. 9) where microtubules form random bundles. Bars: in Fig. 2 for Figs. 1–6, 25 μm; in Fig. 7 for Figs. 7–9, 25 μm

Microtubule organization is disrupted in the swollen tips of pollen tubes exposed to isoxaben. Control pollen tubes contain longitudinal microtubules running the length of the tube into the tip and a radial layer of microtubules beneath the plasma membrane in the elongating tip (Fig. 7) (Lazzaro 1999). This radial array persists in isoxaben-treated pollen tubes that are shorter but with normal tips (Fig. 8). However, this cortical array is disrupted in swollen tips. Instead, microtubules throughout the tip are in random bundles (Fig. 9). The longitudinal array of microtubules is essentially unchanged by isoxaben treatment; only the radial layer of cortical microtubules is lost in swollen tips.

Isoxaben inhibits cellulose synthesis (Heim et al. 1991) and we find this inhibition is greater in pollen tubes with swollen tips compared to normal tips (Figs. 4 and 6). Since it is possible that the weakened cell walls are directly causing tip swelling, we need to differentiate between morphological changes caused by weak walls versus changes caused by isoxaben-induced microtubule disorganization. The driving force behind pollen tube elongation in angiosperms is regulated turgor pressure (Benkert et al. 1997). If the morphological changes observed following isoxaben treatment were due only to weakened cell walls exposed to normal turgor pressure, then these changes should be manifested in pollen tubes grown in hypoosmotic solutions, irrespective of the presence of isoxaben. To test this, we germinated pollen tubes in hypo- (302 mM), iso- (502 mM), and hyperosmotic (702 mM) solutions. The morphology of pollen tubes grown in hypoosmotic media was different from tubes grown in the presence of isoxaben. There was no statistically significant decrease in tube length compared to tubes grown in isosmotic media (Table 1). There was a significant increase in tube diameter at the midpoint between the grain aperture and tip in pollen tubes grown in hypoosmotic media (53 μm wide) compared to isosmotic (42 μm) and also to hyperosmotic media (37 μm). Similar to isoxaben treatment, a significant percentage of pollen tubes had swollen tips (21%) when grown in hypoosmotic media (Table 1). When pollen tubes were grown in hyperosmotic solution, they were significantly shorter (101 μm) than tubes grown in iso- and hypoosmotic solutions. In addition, the frequencies of tip swelling (1%) and germination (80%) were both significantly reduced in hyperosmotic media (Table 1).

In conclusion, cellulose is synthesized within the tip of *P. abies* pollen tubes. Isoxaben inhibits this synthesis and this inhibition leads to a decrease in tube elongation and an increase in tip swelling. Specifically within these swollen tips, the radial array of cortical microtubules is lost.

Discussion

The microtubule disorganization following isoxaben is limited to the tip, while the microtubule array throughout the tube parallel with elongation is unchanged. The radial microtubule array at the tip is also specifically disrupted by oryzalin and amiprophos-methyl, causing tip swelling (Anderhag et al. 2000). The relationship between cortical microtubules and cellulose microfibrils is similar to that seen in other tip-growing plant cells, excluding angiosperm pollen tubes. In root hairs, microtubule disruption causes tip swelling and cortical microtubules are coincident with cellulose microfibrils in the root hair tip (reviewed in Miller et al. 1997). In fern protonema, microtubules form a radial array within the tip (Kadota and Wada 1999) which is coaligned with cellulose microfibrils (Murata et al. 1987). In fucoid algae, isoxaben disruption of cellulose synthesis weakens tips. However, microtubule disruption with oryzalin has no effect on tip strength, while microfilament disruption with latrunculin also weakens tips (Bisgrove and Kropf 2001). This is in contrast to conifer pollen tubes, where microfilament disruption does not increase tip swelling (Anderhag et al. 2000).

Calcafluor labeling indicates that cellulose microfibrils are found at the very tip of these conifer pollen tubes, confirming the finding that cell walls at the tips of *Pinus sylvestris* pollen tubes contain cellulose (Derksen et al. 1999). This differs from flowering plants, where microfibrils were not detected in walls until 5–15 μm away from the tips of *N. tabacum* pollen tubes (Ferguson et al. 1998). Conifer pollen tubes grow almost 2 orders of magnitude slower than angiosperms. The average growth rate for *P. abies* pollen tubes in culture is 20 μm/h (Anderhag et al. 2000), while it is 5–25 μm/min in *Lilium longiflorum* (Parton et al. 2001). This slower growth rate might allow for insertion and activation of cellulose synthase complexes within the tip region rather than massive vesicle fusion at the tip and insertion of synthase complexes outside of the growing tip seen in angiosperms (Ferguson et al. 1998).

Isoxaben specifically inhibits cellulose biosynthesis (Heim et al. 1990) and we found that microfibril density declined in isoxaben-treated tubes and de-

clined further in tubes with swollen tips. The driving force behind pollen tube elongation in angiosperms is regulated turgor pressure (Benkert et al. 1997) and turgor pressure also drives elongation in *P. abies* pollen tubes since tubes were shorter in external hyperosmotic solution. If the morphological changes observed following isoxaben treatment were due only to weak cell walls exposed to normal turgor pressure, then these changes should be manifested in pollen tubes grown in hypoosmotic solutions, irrespective of the presence of isoxaben. This was not the case. There was no significant decrease in tube length compared to isosmotic media, but there was a significant increase in tube diameter. This indicates that the effects of high turgor are manifested along the length of the tube. This was not seen in isoxaben treatment, where tips swelled but tubes recovered to a normal diameter outside the tip region. The swelling was coincident with the disruption of microtubules in the tip, while a normal, longitudinal microtubule array was seen in the tube. It is puzzling that the entire pollen tube is not swollen following isoxaben treatment, especially since there was no significant difference in microfibril density between walls in the tip versus the pollen tube shank in isoxaben-treated tubes. We conclude that this transient swelling is due to the specific loss of the radial array of microtubules at the tip (Lazzaro 1999, Anderhag et al. 2000). If this array specifically strengthens the tip, then its loss due to isoxaben disruption of cellulose synthesis would cause swelling. Pinaceous tips secrete hydrolases, esterases, and acid phosphatases (Pettitt 1985) and Picea tubes elongate through degenerating gametophyte tissue to reach the egg cell (Dawkins and Owens 1993, Runions and Owens 1999). The radial microtubules may contribute to tip integrity in vivo as the tubes elongate through a harsh environment of hydrolytic enzymes and degraded cells.

The dominant paradigm for cellulose deposition in plant cells is that cortical microtubules direct the deposition of parallel cellulose microfibrils by guiding cellulose synthase complexes within the plasma membrane (Carpita and Gibeaut 1993, Cyr 1994). The mechanism of this model suggests that a coordinating signal passes from the cytoplasm (microtubule alignment) to the extracellular matrix (the alignment of cellulose microfibrils). However, recent work indicates that the specific inhibition of cellulose synthesis by isoxaben (Heim et al. 1990) in newly formed *N. tabacum* protoplasts causes the disorganization of cortical microtubules (Fisher and Cyr 1998). This finding has led to the extension of the microtubule-microfibril paradigm to include bidirectional communication across the plasma membrane (Fisher and Cyr 1998, Baskin 2001). However, microtubules may only sense the presence of a wall, not specifically cellulose microfibrils. Cells grown for extended periods on the cellulose biosynthesis inhibitor dichlobenil replace their cellulose walls with a pectin-enriched wall, and in these, the microtubule array is not disorganized (Sabba et al. 1999). Our results support the concept of bidirectional communication. Microtubule disorganization and tip swelling only occurred in treated pollen tubes with the lowest microfibril density. In these elongating cells where cellulose synthesis occurs at the tip, the disruption of this synthesis leads to the disorganization of cortical microtubules.

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