

Use of Phenylboronic Acids to Investigate Boron Function in Plants. Possible Role of Boron in Transvacuolar Cytoplasmic Strands and Cell-to-Wall Adhesion

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The only defined physiological role of boron in plants is as a cross-linking molecule involving reversible covalent bonds with cis-diols on either side of borate. Boronic acids, which form the same reversible bonds with cis-diols but cannot cross-link two molecules, were used to selectively disrupt boron function in plants. In cultured tobacco (*Nicotiana tabacum* cv BY-2) cells, addition of boronic acids caused the disruption of cytoplasmic strands and cell-to-cell wall detachment. The effect of the boronic acids could be relieved by the addition of boron-complexing sugars and was proportional to the boronic acid-binding strength of the sugar. Experiments with germinating petunia (*Petunia hybrida*) pollen and boronate-affinity chromatography showed that boronic acids and boron compete for the same binding sites. The boronic acids appear to specifically disrupt or prevent borate-dependent cross-links important for the structural integrity of the cell, including the organization of transvacuolar cytoplasmic strands. Boron likely plays a structural role in the plant cytoskeleton. We conclude that boronic acids can be used to rapidly and reversibly induce boron deficiency-like responses and therefore are useful tools for investigating boron function in plants.

Plant biologists have known since 1923 that boron is required for plant growth (Warington, 1923) and yet only recently has a definitive role for boron been identified in plant cell walls (Kobayashi et al., 1996; O'Neill et al., 1996, 2001; Matoh, 1997; Ishii et al., 1999). A sole role for boron in plant cell walls, however, is inadequate to explain all of the observed effects of boron deficiency seen in plants (Brown et al., 2002). The suggestion that boron plays a broader role in biology is primarily supported by the discovery that boron is essential for animals (Nielsen, 2000), where a pectin-rich cell wall is not present. Experimental data from plants and animals imply that boron may have a critical role in membranes and/or the extracellular matrix (for review, see Blevins and Lukaszewski, 1998; Brown et al., 2002).

Current understanding of boron physiology suggests that boron in plants likely functions as a cross-linking molecule (Loomis and Durst, 1992; Brown et al., 2002). Borate can cross-link molecules because it contains two pair of hydroxyl moieties that can form reversible diester bonds with molecules containing cis-diols in a favorable conformation. The importance of borate serving as a cross-linking molecule is highlighted by the discovery of several borate-dependent molecules, including rhamnogalacturonan II (RG-II) in plant cell walls (Ishii and Matsunaga, 1996; Kobayashi et al., 1996; O'Neill et al., 1996; Kaneko et al., 1997; Matoh, 1997), boron-polyhydric alcohol complexes

identified from phloem extracts (Hu et al., 1997), a bacterial signaling molecule and its sensor protein (Chen et al., 2002), as well as several antibiotics (Hunt, 2003).

Boronates (i.e. boronic acids) are a structurally similar but diverse class of molecules that can form reversible ester bonds with cis-diols in a manner functionally identical to borates (Bergold and Scouten, 1983; Springsteen and Wang, 2002). Boronates, however, contain only one pair of hydroxyl moieties and, unlike borate, cannot serve to cross-link two discrete molecules (Liu and Scouten, 2000). The stability of the boronate-diol complex is largely dependent on the ionization constants, the specific diol, and the concentrations of each (Bergold and Scouten, 1983; Springsteen and Wang, 2002). For a given pH, a lower pKa will favor ionization (i.e. more acidity) and result in a stronger complex (Power and Woods, 1997). Boronic acids with pKas lower than boric acid bind cis-diols more strongly and therefore competitively inhibit borate complexation to available cis-diol-binding sites, when both are present at equivalent concentrations under physiological pH (Winblade et al., 2000; Springsteen and Wang, 2002).

Strong binding of boronic acids has enabled their use in a diverse number of technological applications and is indicative of the broad range of molecules with which boronic acids can be expected to interact in vivo (Bouriotis et al., 1981; Singhal and DeSilva, 1992; Aoki et al., 1995; Westmark et al., 1996; Winblade et al., 2001; Wang et al., 2002). The diverse number and chemical characteristics of boronic acids suggest that they could be used to probe the biological function of boron in plants.

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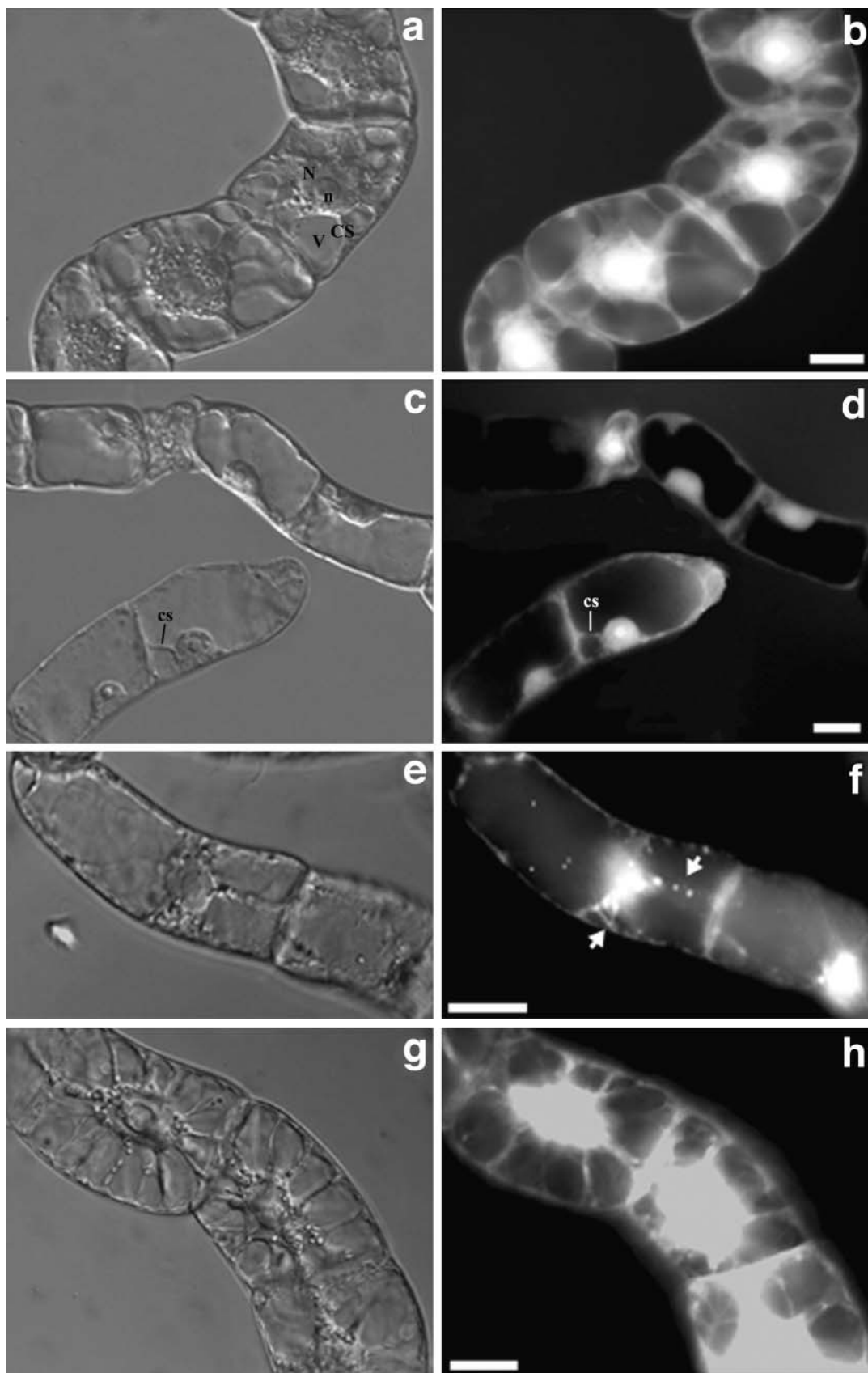


Figure 1. (Legend appears on following page.)

We reason that the application of boronic acids with pKa values lower than borate should prevent or disrupt boron diester cross-links by forming mono-ester linkages at positions normally occupied by boron. Given the structural differences between borate and boronates, and the inability of boronates to act as bridging molecules, the resulting boronate-diol complex would not replace putative boron diester complexes important in biological processes. The addition of boronic acids should therefore induce a boron deficiency-like response (Milborrow, 1964) in a manner that is predictable, replicable, and proportional to the characteristics of the specific boronic acid used. This represents a degree of control over the induction of boron deficiency that is not currently possible and should allow for the resolution of primary from secondary effects of boron deficiency. Exactly which effects of boron deficiency are primary has been debated for some time (Brown et al., 2002).

From a series of experiments that utilize boronic acids to probe the function of boron, we conclude that boronic acids can be used to rapidly disrupt boron-type linkages and induce boron deficiency-like responses. The use of boronic acids suggests that boron is likely to be involved in the organization of transvacuolar cytoplasmic strands and/or participates in cell-to-cell wall attachment. If true, this would constitute another borate-dependent structural role in plants.

RESULTS

Boronic Acids Disrupt Transvacuolar Cytoplasmic Strands

When boronic acids are added to cultured tobacco (*Nicotiana tabacum*) cells dramatic morphological changes can be observed, including the disassembly of transvacuolar cytoplasmic strands and the collapse of the nucleus to the end wall (Fig. 1). Cytoplasmic strands, radiating from the nucleus to the cell periphery, can be visualized under both bright-field and fluorescence microscopy following fluorescein diacetate (FDA) staining (Fig. 1, A and B). The addition of boronic acids of differing binding strengths (pKa; see Table I for boronic acid characteristics) results in a disruption of cell structure that varies markedly and is directly proportional to the binding strength of the specific boronic acid used (Fig. 1, C–H). Treatment of cells with 0.1 mM 3-nitrophenylboronic acid (3-NBA; pKa 7.2) for 2 h severely disrupts cytoplasmic strands, causing the nucleus to lose its anchorage and collapse to the cell periphery (termed here as completely disrupted; Fig. 1, C and D). Cells treated with

Table I. Chemical and structural characteristics of boronic acids and their analogs

Boronate pKas were calculated from curves generated by titrating 5 mL of 10 mM boric acid or boronic acid with 0.1 N KOH. Boronic acid pKa was determined from the inflection point of the average of at least three titration curves (see Scheme 1 for structure).

Chemical Name	Abbreviation	Structure	pKa
3-Nitrophenylboronic acid	3-NBA	R ₁ = NO ₂	7.2
3-Nitrophenylacetic acid	3-NAA	R ₁ = NO ₂	3.9
3-Nitrophenol	3-NP	R ₁ = NO ₂	n.d. ^a
4-Nitrophenylboronic acid	4-NBA	R ₂ = NO ₂	7.3
3-Methoxyphenylboronic acid	3-MBA	R ₁ = CH ₃ O	8.6
3-Methoxyphenylacetic acid	3-MAA	R ₁ = CH ₃ O	n.d.
3-Methoxyphenol	3-MP	R ₁ = CH ₃ O	n.d.
4-Methoxyphenylboronic acid	4-MBA	R ₂ = CH ₃ O	9.4
Phenylboronic acid	PBA	No groups	8.8
Methylboronic acid	MeBA	CH ₃ B(OH) ₂	10.7
Butylboronic acid	BuBA	C ₄ H ₉ B(OH) ₂	10.4
Boric acid	B	H ₃ BO ₃	9.2

^an.d., Not determined.

the same concentration of 3-methoxyphenylboronic acid (3-MBA; Fig. 1, E and F; pKa 8.6) or phenylboronic acid (PBA; not shown; pKa 8.8) for 2 h remain in a transition state where most cytoplasmic strands disappear but the nucleus remains centered (termed here as partially disrupted). PBA-treated cells are not easily and consistently distinguished from 3-MBA-treated ones, except under some conditions where the former seem slightly less disrupted than the latter (data not shown). For both 3-MBA- and PBA-treated cells, disruption becomes progressively more severe with increasing concentration and time, and with sufficient time and concentration eventually resemble 3-NBA-treated cells. During the transition stage between control and disrupted cells, the nucleus becomes less visible and proportionally heavily stained particles appear around the nuclear periphery and in cytoplasmic strands. These particles stain similarly with 3,3'-dihexyloxa-carbocyanine iodide [DiOC₆(3); data not shown], suggesting that they are mitochondria associated with cytoplasmic strands (Van Gestel et al., 2002) and become apparent after disassembly of the strands following boronic acid treatment (Fig. 1F, arrows). Cells treated with methylboronic acid (MeBA; Fig. 1, G and H; pKa 10.7) were not distinguished from untreated or boron-treated cells (Fig. 1, A and B) and did not exhibit any visible cellular disruption even if treated for prolonged periods (1 d) and higher concentration (1 mM). While cells

Figure 1. Application of boronic acids causes disassembly of transvacuolar cytoplasmic strands and collapse of the nucleus in cultured tobacco cells. Cells were treated for 2 h with 0.1 mM boron (A and B), 3-NBA (C and D), 3-MBA (E and F), MeBA (G and H). A, C, E, and G, Differential interference contrast images of the same cells (shown in B, D, F, and H), which are fluorescent and result from esterase cleavage of FDA and are viewed using epifluorescence illumination. Arrows indicate particles that stain with DiOC₆(3) (F). V, Vacuoles; N, nuclei; n, nucleoli; CS, cytoplasmic strand. Scale bars, 50 μm.

with intact cytoplasmic strands fluoresce intensely when labeled with FDA, 3-NBA-, 3-MBA-, and PBA-treated cells fluoresce less intensely and fluorescence is limited to the collapsed nucleus, cytoplasmic strands, and cortical cytoplasm.

Semiquantitative analysis of the number and degree of cellular disruption caused by the various boronic acids indicates that 3-NBA and 4-NBA (pKa 7.3) consistently result in the most dramatic disruption of cytoplasmic strands and have the highest proportion of completely disrupted cells (Fig. 2). Within 4 h, 97% and 77% of cells treated with 3-NBA and 4-NBA were scored as either partially or completely disrupted, while only 29% of cells treated with 3-MBA were disrupted. Cells treated with 4-MBA (pKa 9.4) and MeBA could not be distinguished from controls.

The degree to which cytoplasmic strands are disrupted is not only proportional to the binding strength of the boronic acid used, but also to its concentration, treatment duration, and cell age (data not shown). For example, treatment with 0.01 mM 3-NBA did not disrupt cells to the extent shown in Figure 1, C and D, until cells were treated for 12 h, whereas treatment with 0.5 mM 3-NBA caused complete disruption of cytoplasmic strands in just 0.5 to 1 h (data not shown). Washing tobacco cells to remove the boronic acids used to treat them and adding boron (up to 1 mM for 24 h) did not cause cytoplasmic strands to reestablish even though cells were still alive as assessed by Evans blue exclusion (data not shown).

Boronic Acids Cause Cell-to-Wall Detachment following Cytoplasmic Strand Disruption

In addition to disruption of cytoplasmic strands, boronic acids also disrupt cell anchorage to the cell wall. In untreated tobacco cells exposed to plasmolyzing conditions (0.3 M CaCl₂ or Suc), the reduction in the cytoplasmic volume causes the plasma membrane to

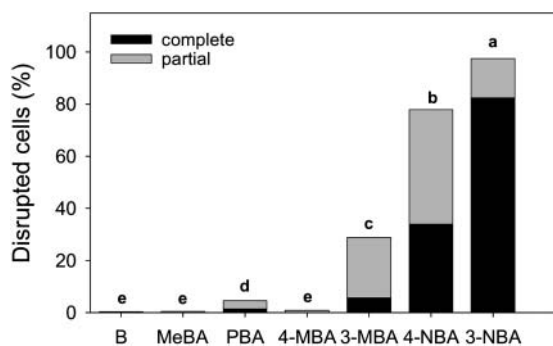


Figure 2. Treatment of cultured tobacco cells with boronic acids results in cellular disruption. Cells were treated by adding 0.25 mM of the indicated boronic acids for 4 h before scoring the degree of disruption. Same letters above the stacked bars indicate that disruption is not significantly different between treatments as determined by Tukey's studentized range test ($\alpha = 0.05$) using SAS (SAS Institute, Cary, NC). Data are from one representative experiment (repeated at least three times) having three replicates of each treatment.

pull back from the cell wall, except at distinct attachment points, resulting in concavely shaped regions along the plasma membrane between attachment points (Oparka et al., 1994). This response is clearly observed when boron is also included in the culture medium prior to plasmolyzing cells (Fig. 3A). However, when tobacco cells are treated with 0.5 mM 3-NBA, 3-MBA, or PBA for 1 h prior to plasmolysis, attachment points are rapidly lost following plasmolysis, resulting in spherically shaped protoplasts that have pulled away from the cell wall (Fig. 3, B and C). The change in the shape of plasmolysis suggests that cell-to-wall anchorage is lost. Incubation with 3-NBA caused the most dramatic cell-to-wall detachment (Fig. 3B). Complete detachment can be repeatedly observed after 1 h when 0.5 mM 3-NBA is used to treat tobacco cells before plasmolysis. Incubation with 0.5 mM 3-MBA caused moderate cell-to-wall-detachment, evidenced by some remaining attachment points and fewer concavely shaped regions along the plasma membrane, compared to the boron control, and therefore protoplasts that were not completely spherical (Fig. 3C). Incubation with PBA results in cells that are similar to cells treated with 3-MBA (data not shown; see Fig. 3C). Treatment with 4-MBA, MeBA, or butylboronic acid (BuBA; pKa 10.4), did not cause cell-to-wall detachment, and cells were morphologically identical to those treated with boron (see Fig. 3A). As was observed with cytoplasmic strands, the degree of cell-to-wall detachment is proportional to the boronic acid-binding strength, concentration, and duration of treatment (data not shown). Boronic acid-induced cell-to-wall detachment occurs in cells that have collapsed cytoplasmic strands (see below).

When tobacco cells were treated with 0.1 mM 3-NBA (1 h), as described for disruption of cytoplasmic strands (Fig. 1, C and D), spherical plasmolysis of the protoplast is not evident immediately. At this concentration and duration of 3-NBA treatment, cytoplasmic strands collapse (Fig. 3D), as indicated by the shape of plasmolysis (Fig. 3E), cell-to-wall attachments remained. To obtain cells resembling those shown in Figure 3B, longer exposure to 0.1 mM 3-NBA is required (approximately 4 h).

Boronic Acid Effects on Tobacco Cells Are Boron Specific

We wanted to verify that the observed effects of the boronic acids were specifically due to the disruption of boron cross-links and not of another general effect of the boronic acids. Tobacco cells were treated with the phenolic acids, termed here as boronic acid analogs, including 3-nitrophenylacetic acid (3-NAA), 3-nitrophenol (3-NP), 3-methoxyphenylacetic acid (3-MAA), and 3-methoxyphenol (3-MP) that are structurally similar to NBA and MBA but differ only in that they lack the putative borate functional group (Table I; Scheme 1). These analogs, even though they are much stronger acids (e.g. 3-NAA; pKa = 3.9) than NBA and MBA, when applied to tobacco cells at higher concen-

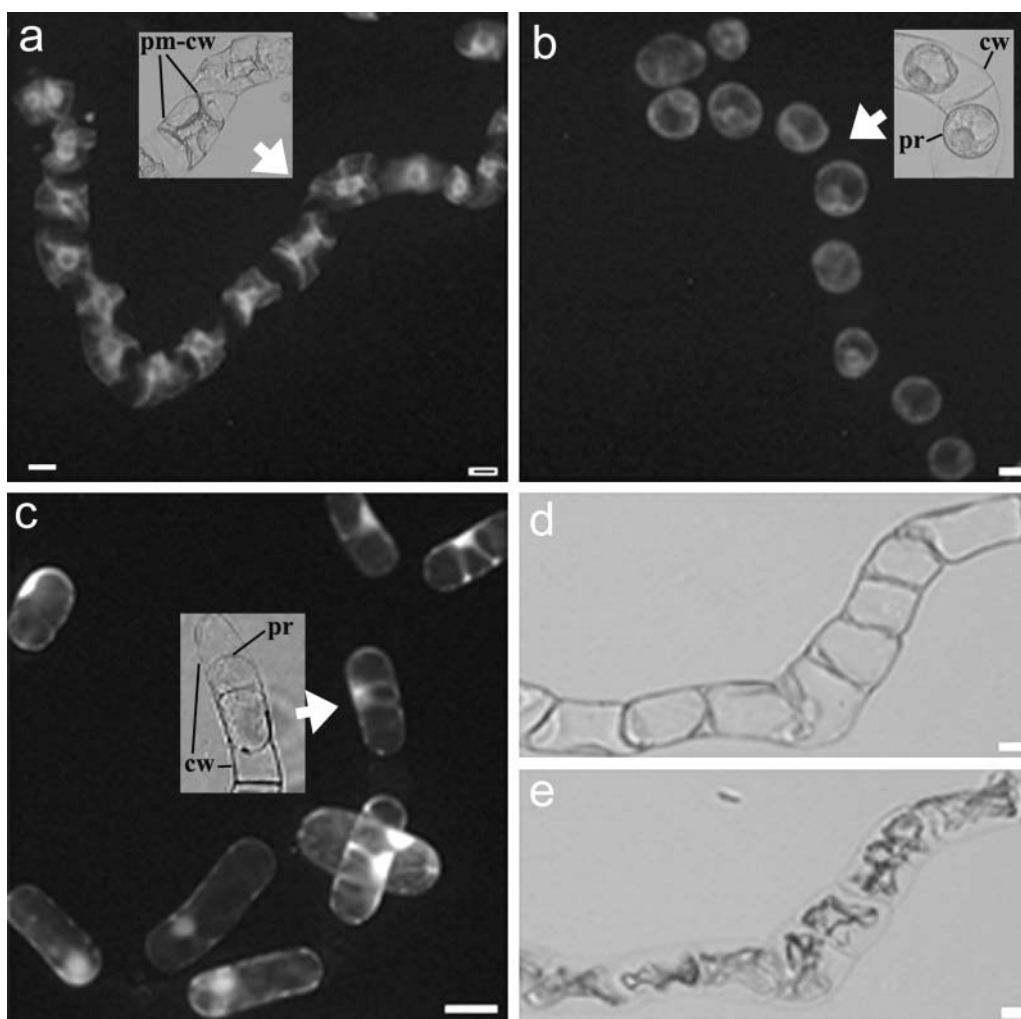


Figure 3. Boronic acids cause plasma membrane-to-cell wall detachment in cultured tobacco cells. Cells were treated with 0.5 mM boron (A), 3-NBA (B), 3-MBA (C), or 0.1 mM 3-NBA (D and E) for 1 h. Fluorescent images result from Nile red staining of the membrane, viewed using epifluorescence illumination (A–C). For reference, light images of cells pointed by arrows (A–C) are included as insets. D, Light image of nonplasmolyzed cells; E, cells from D that are plasmolyzed. Plasmolysis was induced by adding a 0.3 M CaCl_2 solution to one corner of the coverslip while blotting the other end with filter paper. cw, Cell wall; pm-cw, plasma membrane-cell wall contact; pr, protoplast. Scale bars, 50 μm .

tration (0.5 mM) and for longer periods (up to 2 d) than the corresponding boronic acids, repeatedly caused no discernible effects on tobacco cells, including disruption of cytoplasmic strands and cell-to-wall detachment. Whereas 24 h of 3-NBA treatment resulted in 85% cell death, treatment with 3-NAA and 3-NP resulted in only 2% and 1% cell death, respectively. These results suggest that the effects of the boronic acids are a direct result of their ability to form cis-diol complexes in cells.

The specificity of boronic acids for putative cellular cis-diols was further examined by including a variety of cis-diol-containing sugars and sugar alcohols in the reaction mixture. If boronic acids disrupt cells by binding to cis-diol-containing molecules, then the presence of cis-diol-containing sugars or sugar alcohols should competitively reduce the capacity of

boronic acids to cause cytoplasmic disruption. Results confirm that the addition of various sugars and sugar alcohols to 3-MBA-treated tobacco cells reduces disruption of cytoplasmic strands proportionally to the affinity of 3-MBA for the sugar (Fig. 4). Among the five sugars used, sorbitol was consistently the most effective at alleviating 3-MBA-induced cytoplasmic strand disruption, followed by Fru, while Glc, glycerol, and Suc did not significantly differ from no addition of sugar (Fig. 4).

Boronic Acids Can Compete with Boron in Germinating *Petunia* Pollen

Pollen requires a high concentration of boron to germinate and maintain tube elongation (Blevins and Lukaszewski, 1998; Brown et al., 2002), making it

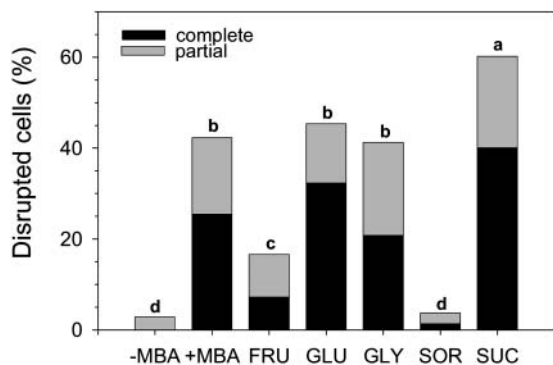


Figure 4. Sugars that can bind to boronic acids reduce 3-MBA-induced cellular disruption of cultured tobacco cells. Cells were treated with 0.5 mM 3-MBA for 4 h, except in -MBA where no 3-MBA was added. +MBA indicates treatment with 3-MBA and no sugar added. Fru, Glc, glycerol (GLY), sorbitol (SOR), or Suc was added at 100 mM together with 3-MBA. Cells were scored for degree of disruption as described in "Materials and Methods." Same letters above the stacked bars indicate that disruption was not significantly different between treatments ($\alpha = 0.05$). Data are from one representative experiment repeated three times having three replicates of each treatment.

a good system to determine the competitiveness of boronic acids for boron-binding sites. Under constant boron supply (0.5 mM), the presence of PBA (0.005–5 mM) reduced the percentage of germinated pollen in a concentration-dependent manner (Fig. 5). Above 2 mM PBA, no pollen germination occurred.

In a second experiment, PBA concentration was kept constant (0.25 mM) while boron concentration was varied (Fig. 6). In the presence of 0.25 mM PBA, germination was inhibited regardless of boron supply (Fig. 6A). At (low) 0.01 and 0.025 mM boron, maximum germination was 12% and 39% in the presence of PBA, while it was 25% and 66% without PBA, respectively. Therefore, higher boron concentration was required to achieve the same percent germination when PBA was added to the germination medium. It was repeatedly observed that the degree to which PBA inhibited pollen germination was not uniform across the boron concentration range. Detailed examination of the data revealed that the inhibition of pollen germination by PBA has two characteristics. First, in the presence of PBA, maximum pollen germination was obtained at about 1 mM boron, while in the absence of PBA, maximum pollen germination occurred at about 0.25 mM boron. Second, the effect of PBA inhibition on pollen germination was proportionally much greater at lower boron concentrations than at higher boron concentrations. This means that if percentage germination of +PBA is compared with that of -PBA at the same boron supply, the ratio was small when boron concentration was low and increased as boron concentration increased (Fig. 6C). The ratio gradually approached saturation when boron concentration was higher than 0.5 mM. These data indicate that PBA is less inhibiting at higher boron concentrations because boron can more effectively compete with PBA.

Over the boron concentration range used in our experiments, 0.25 mM MeBA did not cause significant reduction of pollen germination (data not shown) likely because MeBA is a much weaker acid than PBA (Table I). At boron concentrations higher than 0.5 mM, the presence of 1 mM MeBA did not significantly reduce pollen germination. However, under low boron supply (<0.05 mM), 1 mM MeBA did inhibit pollen germination (Fig. 6B).

Boronates Can Disrupt Borate-Glycoprotein Linkages in Vitro

We employed an *m*-aminophenylboronic acid affinity column (m-PBA) to determine whether boronic acids can specifically disrupt a known glycoprotein-borate ester linkage. At pH 8.7, and given the pKa of boron and 3-NBA (Table I), a solution of 100 mM boron contains approximately 24 mM borate, while a solution of 25 mM 3-NBA would be almost completely ionized. This makes 100 mM boron and 25 mM 3-NBA comparable in terms of percentage of ionized species. Similarly, 80 mM boron and 20 mM 3-NBA, as well as 60 mM boron and 15 mM 3-NBA, can also be compared. The concentration of ionized PBA in the m-PBA column was calculated to be near 25 mM (Maestas et al., 1980; Bouriotis et al., 1981).

Horseradish peroxidase (HRP) has a high affinity for the m-PBA column but can be eluted with buffer containing 50 mM sorbitol, because sorbitol at this concentration effectively competes with HRP for m-PBA-binding sites. Elution with either 3-NAA or taurine buffer alone did not wash out any significant amounts of HRP (Fig. 7). Including the indicated concentrations of boron or 3-NBA in the running buffer caused HRP to elute from the column. Decreasing boron or 3-NBA concentration caused less protein

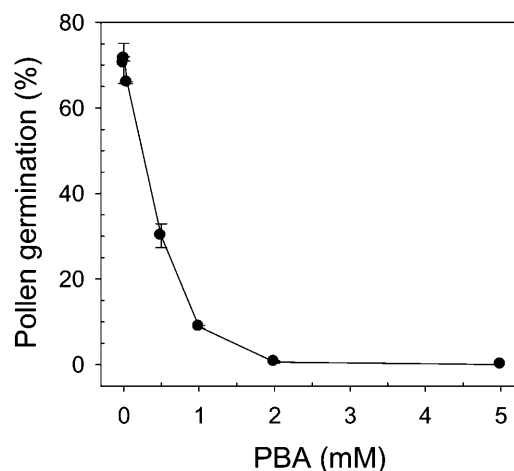


Figure 5. Phenylboronic acid reduces petunia pollen germination. Pollen was considered as germinated if pollen tube length was greater than pollen grain diameter after 1 h of treatment. For each treatment, 400 to 600 pollen grains were counted. Error bars indicate the SE of three independent experiments and are not visible when smaller than symbols.

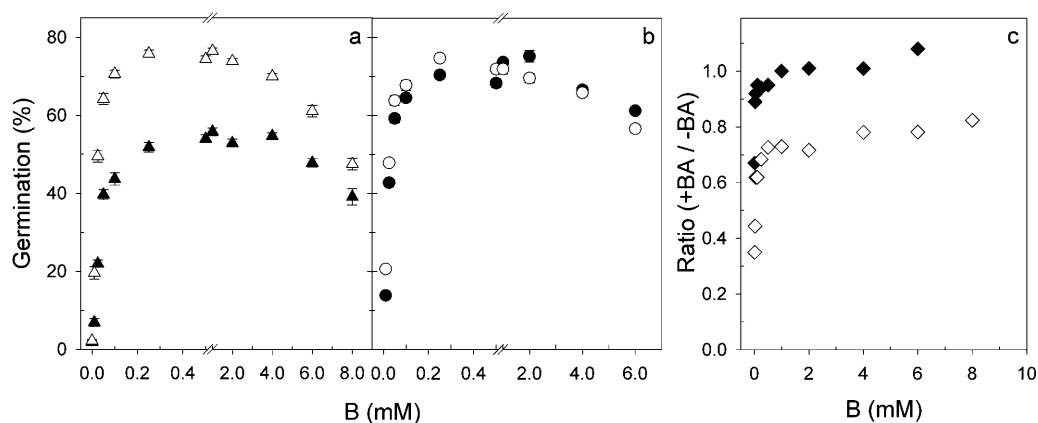


Figure 6. Boronic acids compete with boron for boron-binding sites but do not substitute for boron in petunia pollen germination. Percentage pollen germination with (A) no PBA added (Δ) or 0.25 mM PBA added (\blacktriangle), or (B) no MeBA added (\circ) or 1 mM MeBA added (\bullet). C, Pollen germination ratio, defined as germination in presence/germination in absence of PBA (\diamond) or MeBA (\blacklozenge) of data in A and B. BA indicates the boronic acids PBA or MeBA. Pollen was considered as germinated if pollen tube length was greater than pollen grain diameter after 1 h of treatment. Note break in x axis. Data are the average of six experiments. Error bars indicate the SE and are not visible when smaller than symbols.

to elute during the boron or 3-NBA wash and proportionally more to elute with buffer containing sorbitol, such that the total amount of protein washed out by boron or 3-NBA and sorbitol was very consistent. Furthermore, the elution profiles between 100 mM boron and 25 mM 3-NBA are similar as are those between 80 mM boron and 20 mM 3-NBA and those between 60 mM boron and 15 mM 3-NBA. This is in agreement with the similar proportion of ionized species in those comparable solutions. At concentrations of 40 mM boron and 10 mM 3-NBA, no significant amount of HRP protein was washed out (data not shown). At these concentrations of boron and 3-NBA, the concentration of ionized species would be approximately 10 mM and would not be expected to compete with m-PBA for HRP.

The presence of Mg^{2+} influenced significantly the extent of HRP retention in the m-PBA column. When only 1 mM Mg^{2+} was included in the running buffer, proportionally lower concentrations of boron or 3-NBA were needed to wash out bound HRP (data not shown). However, a comparison of the elution profiles between boron- and 3-NBA-containing buffers did not change at several different Mg^{2+} concentrations in preliminary experiments (data not shown).

DISCUSSION

Since the imposition of boron deficiency simultaneously disrupts plant growth and results in a host of secondary effects that obscure the identification of primary functions (Brown et al., 2002), additional methods are required to investigate boron function in plants. Because of their structural and ester-bonding similarities with boron, and the availability of boronic acids with well-defined pKas, boronic acids may offer the possibility to quickly and specifically displace boron bound to biologically important molecules.

Since one boron function involves its acting as a cross-linking molecule (Loomis and Durst, 1992; Brown et al., 2002), displacing boron from relevant cross-linked molecules should also perturb the structural function of boron and therefore aid in the identification of additional putative functions in plants.

Early work using boronic acids suggested that the biological activity of boronic acids is related to their ability to complex with cis-diols (Torssell, 1956, 1963; Odhnoff, 1961; Wildes and Neales, 1969). Application of PBA to bean caused root elongation even in the presence of boron and was used to propose that boron acting as a diester cross-linker has two effects on plant growth, both promoting root elongation and simultaneously stabilizing the cell wall and thereby inhibiting excessive elongation (Odhnoff, 1961). The latter is supported by Fleischer et al. (1998), who showed that omission of boron from cultured *Chenopodium* cells in the stationary phase caused the cells to continue to expand and rupture. These data suggest that the stabilizing function of boron could be disrupted by boronic acids, which form monoester linkages and cannot replace the requirement for borate diester cross-links. These experiments, however, do not focus on cellular processes that may be boron dependent.

Application of Boronic Acids Perturbs Boron Function

When boronic acids, differing in their affinity to form borate-type diester linkages with cis-diols, were applied to cultured tobacco cells, dramatic morphological alterations were observed. Of the boronic acids used, those with the greatest binding strength (i.e. low pKa) caused the most dramatic disruption, and those with a weaker binding strength than boric acid (i.e. MeBA, BuBA, and 4-MBA), did not disrupt cells. Disruptions followed the order 3-NBA > 4-NBA > 3-MBA > PBA > Boron > 4-MBA > MeBA, and BuBA

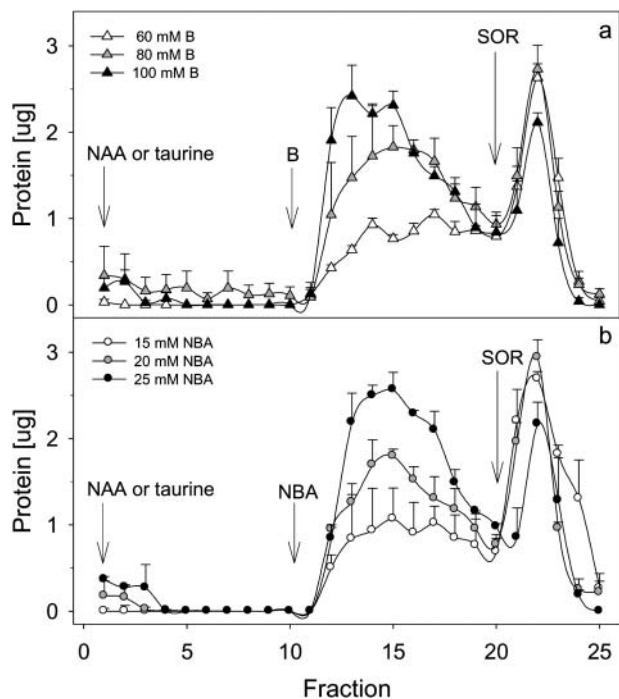


Figure 7. Phenylboronic acid affinity chromatography of HRP indicates that boronate can disrupt glycoprotein-borate ester linkages. Thirty micrograms of HRP type VI-A in an m-PBA acid affinity column was eluted with 50 mM taurine buffer containing 5 mM Mg^{2+} (pH 8.7) or taurine buffer containing 3-NAA. Arrows indicate a switch of elution buffer containing boron (A) or 3-NBA (B). SOR indicates a switch to buffer containing 50 mM sorbitol. The concentration of ionized borate in 60, 80, and 100 mM boron was 14.4, 19.2, and 24 mM, respectively. The concentration of ionized boronate in 15, 20, and 25 mM 3-NBA was 14.6, 19.4, and 24.3 mM, respectively. Error bars indicate the SE of three individual experiments.

and support the idea that formation of borate ester linkages is a prerequisite for cellular disruption. Boronic acid esterification to cis-diols was previously suggested to inhibit bacterial sporulation (Davis-Mancini et al., 1978).

The fact that cellular disruption not only occurs in accordance with boronic acid-binding strength, but also can be rescued with the addition of cis-diol-rich sugars in a manner proportional to the affinity of the boronate for the sugar, indicates that sugars are able to compete for boronate-binding sites in cells. Our results are in close agreement with older studies where such sugars have relieved boron-induced inhibition of enzyme activity (Roush and Gowdy, 1961), and to well-established binding constants of sugar-borate and sugar-boronate complexes (Makkee et al., 1985; Westmark et al., 1996; Springsteen and Wang, 2002). The data also imply that the effect of boronic acids is likely a direct consequence of their affinity for and esterification with cellular cis-diols containing molecules that may, under physiological conditions, have boron cross-links important for normal cellular morphology (and function). It is unlikely that sugar relief is a consequence of the inability of the boronate-sugar

complex to cross the plasma membrane (Westmark et al. 1996), but probably due to competition for boronic acids between boronic acid-complexing sugars and cis-diol-containing sites inside cells. The specificity of the boronic acid effect is further indicated by the observation that phenol analogs of the boronic acids used here had no observable effect on intercellular structure, supporting the conclusion that this is a boron-type specific response.

Pollen tubes are rich in pectin, especially at the tip, and have a high boron requirement (Li et al., 1997). Pollen competition experiments reported here indicate that PBA and MeBA cannot substitute for the boron requirement, presumably due to their inability to serve as cross-linking molecules. Pollen experiments also indicate that boronic acid(s) compete with boron for boron-binding sites. If boronic acid reduced pollen germination through a process not related to boron, then the degree of inhibition, due to boronic acid treatment, would be expected to be uniform across all levels of boron supply. This was not observed. Instead, inhibition of germination by PBA was much higher at lower boron supply and decreased with increasing boron supply. Early studies also showed that PBA does not substitute for boron and is inhibitory to pollen germination at high (>0.04 mM) concentration (Stanley and Lichtenberg, 1963; Torsell, 1963). The affinity of boronic acids for boron-binding sites was suggested previously when a fluorescein boronic acid conjugate was used as a marker for borate-binding sites in roots (Glusenkamp et al., 1997).

To determine whether boronic acids can specifically displace a known glycoprotein-borate ester linkage, we used the m-PBA column, commonly used for separation of glycosylated molecules, including glycoproteins, because of the strong boron-ester binding that occurs with certain glycosylated molecules (Maestas et al., 1980; Bouriotis et al., 1981; Myohanen et al., 1981; Liu and Scouten, 2000). Our data indicate that boronates can indeed disrupt a boron-protein linkage because the covalently bound HRP can be eluted from the m-PBA column with boronates included in the elution buffer. This finding supports the possibility that boronic acids applied to BY-2 cells disrupted boron normally bound to glycomolecules *in vivo* and therefore caused cellular disruption of the type described in this article.

A comparison of the protein elution profiles of boron and 3-NBA at several different concentrations indicated that the amount of protein washed was similar between concentrations of boron and 3-NBA that had comparable concentrations of ionized borate and boronate (i.e. 100 mM boron and 25 mM 3-NBA, etc). Such data support the idea that it is the concentration of ionized boronic acid (i.e. boronate) that determines its ability to disrupt boron-dependent linkages. The importance of boronate ionization is further illustrated by the fact that cellular disruption in BY-2 cells occurred according to the concentration of boronate as determined by pKa.

Cis-diol-containing molecules of biological interest react predominantly with borate, but not boric acid (Zittle, 1951), because of the greater stability of borate linkages (Gerrard, 1961) and easy hydrolysis of trigonal boron esters (Otsuka et al., 2003). Borate diester complexes and free boric acid, but not monoester complexes, have been found in a number of plant species (Matsunaga and Nagata, 1995; O'Neill et al., 1996; Chuda et al., 1997; Hu et al., 1997; Ishii et al., 1999).

Taken together, this evidence strongly suggests that boron in plants likely exists as borate diester complexes and free boric acid, with insignificant amounts of boron monoester. Therefore, we propose that any biological function of boron is the result of its role as a cross-linking molecule.

Does Boron Function in Transvacuolar Cytoplasmic Strands and/or Cell-to-Wall Adhesion?

Boronic acids caused transvacuolar cytoplasmic strand disassembly, nucleus collapse, and breakage of cell-to-wall linkages. The close occurrence of cytoplasmic strand collapse and plasma membrane-to-cell wall detachment following the addition of a high concentration of boronic acid (0.25 mM) makes it difficult to discern whether the two events occur independently or are linked processes, especially since the cytoskeleton-plasma membrane-cell wall is currently considered a continuous structural assembly (Wyatt and Carpita, 1993; Miller et al., 1997). Nevertheless, treatment of tobacco cells with a lower concentration (0.1 mM) of 3-NBA did not cause significant plasma membrane-to-cell wall detachment in cells with disrupted cytoplasmic strands, suggesting that cytoplasmic disruption may precede the detachment of cell-to-wall linkages. The results suggest a role for boron in cytoskeleton structure and associated processes and are supported by a host of theoretical and experimental data discussed below.

The actin cytoskeleton is an important component of cytoplasmic strands because strands are thought to be stabilized by F-actin. Latrunculin, which depolymerizes F-actin (van Gestel et al., 2002) and profilin, a G-actin-binding protein (Staiger et al., 1994; Valster et al., 1997), disassemble the F-actin network, causing the breakdown of most transvacuolar cytoplasmic strands and the collapse of the nucleus (Staiger et al., 1994; Valster et al., 1997) in a manner similar to that caused by the boronic acids reported here. The boronic acid-induced destruction of cytoplasmic strands and the concomitant collapse of the nucleus therefore suggest that boron may be involved in cytoskeleton function, probably by participating in the cytoskeleton-dependent structural organization of the cell. In Arabidopsis roots and maize (*Zea mays*) root apices, boron deprivation caused a rapid increase in actin and tubulin proteins and also altered their polymerization pattern (Yu et al., 2001, 2003). These cytoskeletal changes were thought to be the result of cytoskeletal

adjustments necessary to compensate for a weakened cell wall observed under boron deficiency (Findekle and Goldbach, 1996; Goldbach et al., 2001).

The cytoskeletal function of boron could either be direct, by affecting the organization or stability of cytoskeletal components, or indirect, through its interaction with other cytoskeletal binding or anchoring molecules such as glycoproteins and/or glycolipids. In animal cells, for example, membrane-to-cytoskeleton adhesion occurs through bonds between cytoskeletal proteins and glycoproteins/lipids in the membrane (Sheetz, 2001). Glycoproteins are also likely to be involved in membrane-to-wall linkages in plants (Pont-Lezica et al., 1993; Canut et al., 1998). The exact nature of these anchoring points remains to be identified (Kohorn, 2000), but is likely to involve complex molecules localized along the cytoskeleton-plasma membrane-cell wall continuum. Glycoproteins and glycolipids, including those that are glycosylphosphatidylinositol anchored, contain several boron-binding sugars such as Man and Gal, among others (Simons and Ikonen, 1997), and can theoretically, along with boron, structurally cross-link cellular components (Loomis and Durst, 1992; Brown et al., 2002). One such group of suitable anchoring molecules is the glycosylphosphatidylinositol-anchored arabinogalactan proteins (AGPs), which exist in the apoplastic side of the plasma membrane (Oxley and Bacic, 1999) in Hechtian strands (Zhao et al., 2002) and can interact with pectins or other cell wall-localized proteins (Baldwin et al., 1993; Kohorn, 2000; Showalter, 2001). In the *reb1* mutant of Arabidopsis, AGPs are also required for anisotropic expansion and orientation of microtubules (Andeme-Onzighi et al., 2002); therefore, defects in AGPs could thus lead to cytoskeletal disorganization (Roberts, 1989; Ding and Zhu, 1997).

The work of Bonilla and coworkers provides strong support for a structural role of boron involving glycoproteins. Bonilla et al. (1997) convincingly showed that boron is required for the incorporation and presumably the cross-linking of hydroxy Pro-rich structural glycoproteins into the cell wall. Whether boron is directly involved in cross-linking glycoproteins into the wall or its requirement determines cell wall pore size (Fleischer et al., 1999) or other cell wall perturbations, which in turn affect protein incorporation into the wall, is currently not known. Bolaños et al. (1996) have also shown that boron is important in cell-surface interactions that occur during infection thread development and symbiosome establishment, where glycoproteins are thought to play a critical role in the complex interactions that exist between *Rhizobium* and the peribacteroid membrane (Bolaños et al., 2001). Interestingly, a glycoprotein that cross-reacted with the same antibody used by Bonilla et al. (1997) was proposed as a possible adhesion molecule between the plasma membrane and the cell wall (Pont-Lezica et al., 1993). In animal cells, a molecule containing two boronic acid residues [*N,N'*-bis-3-(dihydroxylborylbenzene)-adipamide] caused the agglutination of red

blood cells in suspension (Burnett et al.,1980), suggesting that borate linkages can function in cell-to-cell adhesion.

Our results also support a structural cross-linking role for boron because boronic acids may have caused the collapse of cytoplasmic strands (followed by cell-to-wall detachment) by preventing or displacing boron functioning in diester borate cross-links present at critical points along the cytoskeleton-plasma membrane-cell wall assembly, necessary for the structural integrity of the cell. We hypothesize that boronates likely target structural glycoproteins located along the cytoskeleton-plasma membrane-cell wall assembly that are normally cross-linked by boron, especially since results confirm that boronates can indeed disrupt a boron-glycoprotein linkage.

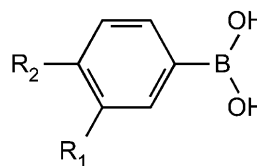
One alternative explanation for the boronic acid-induced cellular disruption could be that boronic acids primarily target the cell wall. O'Neill et al. (2001) have shown that a primary effect of boron deficiency is reduced cross-linking of RG-II by borate. Since we consider treatment by boronic acids to be analogous to boron deficiency, and if we accept the cytoskeleton-plasma membrane-cell wall to be a continuous structural assembly, then the collapse of cytoplasmic strands and cell-to-wall detachment could be a consequence of RG-II-borate dimer disruption. Interestingly, Yu et al. (2002) have demonstrated that boron deprivation completely blocked cytoplasmic internalization of cell wall pectins in maize and wheat. Efforts are under way to determine whether the cell wall and, more specifically, RG-II are the primary target sites of the boronic acids.

The possibility that the cellular disruption reported here is unique to boronates forming ester linkages with molecules that might not ordinarily interact with boron cannot be excluded. Nevertheless, taken together, the data as a whole suggest that boronates specifically compete with borate by disrupting or preventing the formation of important borate-specific cross-links. The experiments conducted here, in addition to the earlier boron research, suggest that boronic acids can be used to probe boron function. Also provided is evidence for a likely role of boron in processes not previously recognized as boron dependent, such as the stability/organization of transvacuolar strands. Experiments to further investigate these possible functions of boron, including an effort to identify boron-binding glycoproteins, continue.

MATERIALS AND METHODS

Chemicals Used

Boronic acids and boronic acid analogs were purchased from Sigma-Aldrich (St. Louis) or Ryscor Science (Raleigh, NC) and described in Scheme 1 and Table I. Boronic acid analogs include the two 3-MBA analogs, 3-MAA and 3-MP, and the two 3-NBA analogs, 3-NAA and 3-NP. The only difference between 3-MBA (or 3-NBA) and 3-MAA (or 3-NAA) is that the borate group of



Scheme 1.

3-MBA (or 3-NBA) is replaced by an acetic acid group in 3-MAA (or 3-NAA) at the same position. Boronic acids were selected based on their structure and pKas, determined by their functional groups and position on the phenyl ring. For example, the N_2O group is more electron-withdrawing than the CH_2O group, especially when in the 3 (instead of 4) position. Consequently, 3-NBA has a much lower pKa and also forms ester linkages with cis-diols more strongly and stably. All stock solutions were made in water, except 4-NBA, which was made in 50% methanol.

Cell Culture

Tobacco (*Nicotiana tabacum* L. cv BY-2) cell suspension was provided by B. Liu (University of California, Davis, CA). BY-2 cells were subcultured weekly in 50 mL of medium (pH 5.7) containing 4.3 g L^{-1} Murashige and Skoog salt, 0.37 g L^{-1} KH_2PO_4 , 1 mg L^{-1} thiamine, 0.2 mg L^{-1} 2,4-D, 100 mg L^{-1} inositol, and 30 g L^{-1} Suc by transferring 0.5 mL of stationary culture to fresh medium. Cultures were kept at 27°C on a rotary shaker (125 rpm).

Plant and Pollen Growth

Petunia (*Petunia hybrida* cv ultrawhite) was obtained from a nursery and grown in a growth chamber under a 12-h-dark/12-h-light cycle, a day/night temperature of 25°C/20°C, relative humidity of 70%, and fertilized weekly with one-half-strength Hoagland solution (Hoagland and Arnon, 1950). Pollen was collected on a weekly basis by shaking anthers into a vial and air drying for 6 h before storage at -20°C or use. Pollen was hydrated for 1 h before germinating in petri dishes (35 × 10 mm) at a concentration of 2 mg mL^{-1} of medium. Germination medium was modified from Jahnen et al. (1989) to obtain the highest and most uniform pollen germination for our conditions, and contained 0.07% $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.02% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01% KNO_3 , 15% polyethylene glycol 3350, and 2% Suc in 20 mM MES buffer, pH 6.0. Boron was added as boric acid at concentrations specified for each experiment.

Boronic Acid, Boronic Acid Analog, and Sugar Treatment of Cultured Tobacco Cells

Tobacco cells were treated by directly transferring 4-d-old cell suspension to MES buffer, pH 6.0, containing various boronic acids or their analogs in 1-mL total volume and cultured in 35 × 10-mm petri dishes. Cell density was maintained approximately equal between experiments. To induce a quick response to boronic acids and avoid secondary effects due to boron deficiency, a high concentration (0.25–0.5 mM) of boronic acid(s) was intentionally chosen. An equimolar concentration of boron was also added to reduce the possibility that boronic acid effects on cells are not a consequence of toxicity. Repeated experiments demonstrated that up to 0.5 mM boron did not cause any visual effects on BY-2 cells when treated for up to 4 d as well as when cells were treated with boronic acids in the absence of added boron. Cells were treated for 2 to 4 h, then stored at 5°C for 20 min before estimating cellular disruption. We found that storage at 5°C was necessary to slow down disruption until measurements and observations were completed, otherwise more reactive boronic acids continued to disrupt cells and therefore confounded results.

For each treatment, cells were scored into three categories. Unaffected (undisrupted) cells had clearly visible cytoplasmic strands in which the nucleus is suspended near the cell center. These cells, when plasmolyzed with 0.3 M CaCl_2 or Suc for 10 min, also maintain adhesion points between the plasma membrane and cell wall that result in concave regions along the

plasma membrane. Completely disrupted cells have no defined cytoplasmic strands and the nuclei have collapsed to the cell periphery, but are still viable according to Evans blue exclusion (0.1%). Partially disrupted cells are cells in transition to becoming completely disrupted and are defined here as having a nucleus that is centered, but with no discernible cytoplasmic strands. Often the nucleus is dotted with circular particles around its periphery. For each treatment, more than 500 cells were counted in triplicate for 6 to 10 repeated experiments.

For the sugar relief experiments, tobacco cells were treated with 0.5 mM 3-MBA for 4 h as described above, except that 100 mM Fru, Glc, glycerol, sorbitol, and Suc were also included. 3-MBA was used instead of 3-NBA, because 3-NBA caused severe disruption of cells and application of sugars to treated cells did not allow for a good separation of the effects of different sugar applications. Tobacco cells were scored for disruption as noted above.

Boronic Acid Treatment of Germinating Pollen

To determine whether boronic acids can compete with boron in germinating petunia pollen, two experiments were conducted. In the first experiment, PBA or MeBA was applied to germinating pollen (as described above) at concentrations of 0, 0.01, 0.05, 0.5, 1, 2, and 5 mM and 0, 0.05, 1, 2.5, and 5 mM, respectively. Here boron was kept constant at 0.5 mM. Preliminary experiments established that petunia pollen requires high boron concentration for germination and tube growth and that 0.25 to 0.5 mM was considered as optimum. In the second experiment, boron concentration was varied at concentrations of 0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 2, 4, 6, and 8 mM, either in the presence or absence of 0.25 mM PBA or 0.25 or 1 mM MeBA at each boron concentration. Following 1 h of germination, a 15- μ L sample from each treatment was pipetted onto a slide, and paired samples (i.e. with or without PBA/MeBA at the same boron concentration) were stored at 5°C (15 min) to stop pollen germination or further tube elongation before percent germination was determined. Pollen was considered as germinated when the pollen tube was longer than the pollen diameter. For each treatment, 400 to 600 pollen grains were counted.

Phenylboronate Affinity Chromatography

m-PBA acid immobilized on beaded agarose (Sigma) was packed in a plastic column to obtain a packing volume of 0.25 mL (8 [diameter] \times 5 [height] mm). Column void volume was 90 μ L and flow rate was 2 mL h⁻¹. The gel was preequilibrated with 50 mM taurine buffer (Sigma), pH 8.7, containing 10 mM MgCl₂ and regenerated following each run as described by the manufacturer. Thirty microliters (30 μ g) of HRP type VI-A (Sigma) followed by 20 μ L of 50 mM taurine containing 10 mM Mg²⁺ were loaded into the column and allowed to react for 30 min. HRP was eluted with buffer (50 mM taurine, pH 8.7, with 5 mM Mg²⁺) alone or with the buffer containing 10 mM NAA followed by the buffer containing boron or 3-NBA (see "Results" for concentrations). Finally, the buffer containing 50 mM sorbitol was used to elute the remaining HRP. Fractions of 0.35 mL were collected and assayed for protein according to Bradford (1976), using bovine serum albumin as standard made in solutions containing the same buffer used for each elution. Previous experiments determined that NAA concentration as well as running the column at 4°C compared to room temperature did not change the protein elution profile significantly.

Fluorescence Staining and Microscopy

Differential interference contrast images were recorded with a Photometrics CoolSnap digital camera (Roper Scientific, Trenton, NJ) mounted on a Leica DM RE microscope using Metamorph version 5.0 software (Universal Imaging, West Chester, PA). The deconvolution feature of the software (0.55 scaling factor, 0.75 N.A.) was used to enhance optical sections of the images. Fluorescent images were visualized using a 11001 Chroma filter set (Chroma Technology, Rockingham, VT). Tobacco cells were stained with FDA according to Heslop-Harrison and Heslop-Harrison (1970). Cell membranes were stained with Nile red (1 ng mL⁻¹) and mitochondria with 3,3'-dihexyloxa-carbocyanine iodide (DiOC₆(3); Molecular Probes, Eugene, OR) as described in Van Gestel et al. (2002). Cell viability was determined by Evans blue exclusion (0.1%).

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