Plant Physiol. (1994) 105: 681–689

Localization of Boron in Cell Walls of Squash and Tobacco and Its Association with Pectin[1]

Evidence for a Structural Role of Boron in the Cell Wall

Hening Hu and Patrick H. Brown*

Department of Pomology, University of California, Davis, California 95616

B deficiency results in a rapid inhibition of plant growth, and yet the form and function of B in plants remains unclear. In this paper we provide evidence that B is chemically localized and structurally important in the cell wall of plants. The localization and chemical fractionation of B was followed in squash plants (*Curcurbita pepo* L.) and cultured tobacco cells (*Nicotiana tabacum*) grown in B-replete or B-deficient medium. As squash plants and cultured tobacco cells became deficient, an increasingly large proportion of cellular B was found to be localized in the cell wall. Cytoplasmic B concentrations were reduced to essentially zero as plants became deficient, whereas cell wall B concentration remained at or above 10 μg B/g cell wall dry weight in all experiments. Chemical and enzymic fractionation studies suggest that the majority of cell B is associated with pectins within the cell wall. Physical analysis of B-deficient tissue indicates that cell wall plastic extensibility is greatly reduced under B deficiency, and anatomical observations indicate that B deficiency impairs normal cell elongation in growing plant tissue. In plants in which B deficiency had inhibited all plant growth, tissues remained green and did not show any additional visible symptoms for at least 1 week with no additional B. This occurred even though cytoplasmic B had been reduced to extremely low levels (<0.2 μg/g). This suggests that B in these species is largely associated with the cell wall and that any cytoplasmic role for B is satisfied by very low concentrations of B. The localization of B in the cell wall, its association with cell wall pectins, and the contingent effects of B on cell wall extensibility suggest that B plays a critical, although poorly defined, role in the cell wall structure of higher plants.

Seventy years have past since Warington (1923) presented the first evidence that B is an essential element for higher plants. Nevertheless, a definitive primary function for B in the plants is still unknown, making B the least well understood of all the essential plant nutrients. There is a remarkable uniformity of B deficiency symptoms among species, in that growing tissues are always affected first and in all cases root growth is rapidly impaired (Dugger, 1983). Disruption of growth results in varied symptoms depending on the type of tissue involved. In root and shoot tissue, apical meristems fail to extend, leading to a stumpy or dwarfed appearance (Kouchi and Kumazawa, 1976; Dugger, 1983). In expanding tissues B deficiency causes an uneven and disorganized cell expansion, resulting in a "bumpy" or misshapen leaf or malformed fruits and other storage organs (Lorenz, 1942; Spurr, 1957; Marschner, 1986). Each of these disorders suggests that B deficiency is associated with a disruption of normal cell division and/or expansion during periods of rapid organ growth. Inhibition of meristematic growth by B deficiency may, therefore, be the result of a decrease in cell division, cell elongation, or both of these processes. Although cell division is clearly impaired by B deficiency, evidence suggests that this occurs only after cell elongation has been reduced (Whittington, 1959; Slack and Whittington, 1964; Kouchi and Kumazawa, 1976).

A role for B in plant cell walls has long been hypothesized, and the effects of B deficiency on cell wall ultrastructure have been well documented. Thus, numerous reports have noted changes in cell wall structure as a result of B deficiency. B deficiency in beet (Lorenz, 1942) and celery (Spurr, 1957) results in thickening of the cell wall and the formation of small, irregularly shaped cells. Loomis and Durst (1992) interpreted these results to mean that the "outer pectic-rich regions (of the cell wall) are plasticized so that they no longer determine wall morphology and the shape of the cell." Evidence of a disruption of pectin metabolism was also provided by several authors who observed changes in Golgi apparatus size, density, and structure under B deficiency (Kouchi and Kumazawa, 1976; Hirsch and Torrey, 1980). In young cell walls, the Golgi apparatus is associated largely with the incorporation of pectins into the cell wall (Northcote, 1986). Several authors have examined B-deficient tissue by EM. Tissues from sunflower (Stark, 1963; Lee and Aronoff, 1966; Hirsch and Torrey, 1980) and tomato (Kouchi and Kumazawa, 1976) have been examined. In each case a thickening of the cell wall, coupled with an increasing degree of disorganization of the middle lamella (largely pectin), was observed. Disorganization of plant cell walls was associated with an accumulation of secretory vesicles at the wall-plasmalemma junction (Hirsch and Torrey, 1980). In sunflower shoot tips, Stark (1963) observed a reduction in microfibrillar structure so that only a scarce mesh of fibrils was present in

[1] This research was supported in part by a grant from U.S. Borax.

* Corresponding author; fax 1–916–752–8502.

Abbreviations: CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N′,N′*-tetraacetic acid; ICP-AES, inductively coupled plasma atomic emission spectroscopy.

B-deficient tissue. In tomato leaves, cell walls became thicker, and a disorganization of the middle lamellae was evident 8 h after transfer to B-deficient conditions. These changes are apparently not due to an alteration of synthesis of cell wall material (Goldbach and Amberger, 1986), since B deficiency does not consistently inhibit the incorporation of radiolabeled cell wall precursors into new cell walls (Augsten and Eichorn, 1976).

A function of B in plant cell wall expansion may occur as the result of an association of B with pectins in the cell wall. B forms complexes with polyols such as glycerol or mannitol with a specific *cis*-diol configuration (Duel and Neukom, 1949, cited by Loomis and Durst, 1992). These B complexes are in dynamic pH-dependent equilibrium. B also interacts with polyhydroxy polymers (notably pectin) to form borate-polymer cross-links. The addition of B to these pectic-polymers results in the formation of gels that are stable in aqueous alkaline or nonaqueous environments. Acidification of these B-pectic gels results in the rapid liquefaction of the gel. Large polysaccharide polymer molecules (such as pectin) have many B-binding sites and produce a multinet complex, cross-linked by the borate groups (Loomis and Durst, 1992). Matoh et al. (1992) recently presented evidence that B is localized in the cell wall, where it may be largely bound to cellular pectins (Yamauchi et al., 1986; Matoh et al., 1993).

The remarkable effects of B deficiency on meristematic growth as well as B deficiency-induced changes in cell wall morphology suggest that B plays a role in cell wall ultrastructure that is critical to cell wall expansion. The present study was undertaken to determine the relationship between cellular B partitioning and B deficiency and to define the physical location and chemical form of B in the cell. These results are then interpreted in light of observed changes in cell wall characteristics, cell expansion, and plant growth.

MATERIALS AND METHODS

B Partitioning in the Cell Wall

Tobacco cells (*Nicotiana tabacum* L. Monsanto cell line TXD) were grown in liquid culture utilizing a modified Murashige and Skoog medium (Pech and Romani, 1979) with 2,4-D added at 1 μM. B (100 μM) was supplied as 99.51% ^{11}B-enriched (atom%) boric acid (Eagle-Picher, Inc., Quaqaw, OK). Cells were subcultured at weekly intervals, maintained in 250-mL Erlenmeyer flasks, and shaken at 110 rpm.

Cells in late logarithmic growth were filtered through a double layer of Miracloth (Calbiochem, La Jolla, CA) and washed thoroughly with "B-free" medium (see below). They were then cultured in the same medium as above, with or without ^{11}B, for 3 d. At the time of harvest, cells were washed for 1 min with double-deionized water (work in our laboratory indicates that desorption of extracellular B is completed within 30 s [Brown and Hu, 1994]). The cells were then divided into two equal aliquots. One aliquot was used for total cell B determination (see below). The other aliquot was used for wall extraction as follows.

The B-free medium was prepared as follows: all macronutrients and sugars were dissolved in double-deionized water and passed through an Amberlite IRA-743 (Sigma) column (0.5 × 20 cm) at a flow rate of 3 mL/min. Vitamins and micronutrients were then added to the B-free medium prior to autoclaving. This B-free medium contained 0.02 μg/g B when analyzed by ICP-AES (Thermo Jarrell Ash, Menlo Park, CA).

Two grams of fresh cells were rapidly frozen in liquid nitrogen, thawed, and then resuspended in 15 mL of 50 mM Hepes buffer (pH 7.0) containing 2 mM $K_2O_5S_2$. The suspension was then ultrasonicated at an output power of 40 W in a Branson model 450 sonifier (Branson Ultrasonics Corp., Danbury, CT). The temperature of the suspension was maintained below 10°C by submerging the sonication vessel in an ice bath. After several cycles of sonication for 6 min each, more than 99% of the cells were found to be disrupted. Previous work by Matoh et al. (1992) indicated that sonication does not result in significant release of cell wall B or result in the binding of cytoplasmic B to the cell wall.

After sonication, the cells were centrifuged at 1000*g* for 10 min. The precipitate was then resuspended and washed with cold water and centrifuged again. Finally, the precipitate was washed three times with 10 volumes of 80% ethanol and once with 10 volumes of methanol:chloroform mixture (1:1, v/v), followed by 10 volumes of acetone, dried, and then ashed. All procedures were carried out in polyethylene vessels or acid-washed glass test tubes.

Squash (*Cucurbita pepo* L.) cv Early Prolific Straight Neck was grown hydroponically for 2 weeks in one-quarter-strength Hoagland solution (Hoagland and Arnon, 1950) with 50 μM ^{11}B added as boric acid (99.51 atom%). Plants were grown in a growth chamber with a PPFD of 400 μmol m^{-2} s^{-1}, 12-h daylength, and day/night temperature of 25/20°C. At 2 weeks, plant roots were rinsed thoroughly with double-deionized water and transferred to new nutrient medium with or without ^{11}B for an additional 1 week and then harvested. Six plants were grown in each of three replicate tanks for a total of 18 plants. At this stage, plants had developed five leaves. Leaves from the same relative positions on each of the six plants were pooled and immediately frozen in liquid nitrogen. The leaves were numbered from the bottom to the top such that the oldest (lowest) leaf was designated 1, the second leaf as 2, etc. The major veins of the leaves were removed, and the remaining leaf blade was then cut into small pieces and divided into two parts, each of about 2 g fresh weight. One group was used to determine total B content, and the remaining half was used for wall extraction as follows.

The leaf pieces were suspended in double-deionized ice-cold water in a mortar and macerated with a tissue tearer (Biospec Products, Bartelsville, OK). Temperature was maintained close to 0°C in an ice bath. After all tissues were broken and no large fragments could be seen under the dissecting microscope, the macerate was centrifuged at 1000*g* for 10 min. The precipitate was then washed with 10 volumes of ice-cold water and recentrifuged. The precipitate was extracted with organic solvents as described above for tobacco cells and dry ashed.

For both tobacco cells and squash leaf, tissues were dried, weighed, placed in a porcelain crucible, and dry ashed at 500°C for 15 h. Ashed samples were then suspended in 1%

HNO_3 and analyzed for B content ICP-AES (Thermo Jarrell Ash).

Sequential Chemical Extraction

Cell wall preparation and chemical fractionation was modified from that given by Redgwell and Selvendran (1986) and McCann et al. (1990). Tobacco cells were desorbed for 3 min in double-deionized water to remove free B and then frozen immediately in liquid nitrogen. After the sample was thawed, 80 mL of 50 mM Hepes buffer, pH 7.0, containing 2 mM $K_2O_5S_2$ was added to 20 g of fresh cells. The suspension was ultrasonicated at an output power of 40 W. The temperature of the suspension was maintained below 10°C in an ice bath. After several cycles of sonication of 6 min each, the suspension was filtered and washed over Miracloth with cold Hepes buffer to remove cytoplasmic components from the broken cell preparation. The procedure was repeated until all the cells were broken and free of cytoplasmic contamination when checked under a light microscope.

One-tenth of the cell wall preparation was dry ashed directly to estimate total wall B. The remaining wall was then sequentially extracted with 1.5% SDS at 22°C for 2 h, 0.05 M CDTA (pH 6.5) at 22°C for 6 h; 0.05 M Na_2CO_3 at 1°C overnight and then 0.05 M Na_2CO_3 at 20°C for 3 h, 1 N KOH at 20°C for 2 h under N_2, 4 N KOH at 20°C for 2 h under N_2. The residue was further extracted with acidified sodium chlorite (pH 4) at 70°C for 2 h. The final residue was dry ashed. The 1 and 4 N KOH extracts were neutralized with 1 or 4 N HCl and then passed through an Amberlite IRA-743 column to trap the B and remove excess KCl. The column was then washed with double-deionized water and eluted with 1 N HCl, and B was determined in the eluant. In every step of the chemical extraction, the supernatant was collected by centrifugation. With every chemical change, double-deionized water was used to wash the residue; all wash solutions were added to the previous chemical extracts.

For squash, leaf 4 from the bottom of 3-week-old squash plants that had been grown in 50 μM ^{11}B was used (for details see above). This leaf was close to full expansion. After the major veins were removed, the leaves (about 15 g fresh weight) were suspended in 50 mL of 50 mM Hepes buffer and macerated with a tissue tearer until no large fragments could be seen under the light microscope. The macerate was then washed over Miracloth, ultrasonicated until all cells were broken, and thoroughly rewashed. Sequential chemical extraction was performed as described for tobacco cell wall.

All the chemical extracts were dry ashed and analyzed by ICP-AES. Matrix interference was corrected by the analysis of the chemical extractants in the absence of any plant tissue. Blank subtraction and standards of the National Institute of Standards and Technology (Gaithersburg, MD) were used throughout all analyses.

Enzymic Digestion

Tobacco cell walls were prepared as described in "Sequential Chemical Extraction." The walls were then incubated in 10 mM Mes buffer, pH 5.6, containing 1 mM $MgCl_2$ and either 1% hemicellulase (Sigma), 0.5% cellulysin (Calbiochem), or 0.1% pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., Tokyo, Japan). After 6 h of digestion at 22°C, the suspension was centrifuged at 1000*g* and the supernatant was collected. The residue was washed once with double-deionized water and centrifuged again. B content in the residue and supernatant was analyzed by ICP-AES.

To further investigate the chemical localization of cellular B and to assess the contamination activities of the enzymes used, an additional set of experiments was conducted. Fully mature Asian pear fruit (*Pyrus pyrifolia* [Burm.f.] Nakai) cv Shinseiki was collected from the pomology orchard in Davis, CA. After the fruit was peeled, it was cut into pieces (about 2 cm^3) and put into ice-cooled 25 mM Hepes buffer, pH 7.0, containing 2 mM $K_2O_5S_2$ and ground in a coffee grinder cooled in an ice bath. The macerate was then washed over Miracloth. The procedure was repeated. Finally the wall preparation was sonicated and thoroughly washed. This cell wall preparation was free of organelle and membrane fractions when checked under the light microscope. Enzymic digestion procedures were essentially identical with those used for tobacco cell wall with the exception that duration of digestion and the concentration of cellulysin and hemicellulase were varied (see "Results").

Anatomical Determinations

Leaf blade and petiole tissue from squash plants utilized in the proceeding experiment were examined under the light microscope to determine the effect of B deficiency on cell growth and leaf expansion. One week after transfer to B-deficient conditions a 1-cm^2 strip of leaf tissue was excised from the fourth leaf of each of eight individual plants from both B-deficient and control plants. In addition, a 1-cm length of leaf petiole immediately subtending leaf 4 was also sampled from each of the leaves used above. In control plants, leaf tissue samples were chosen from a region 5 cm from the leaf base and 1 cm from the main leaf vein, and strips were cut parallel to a secondary vein in each leaf. It was possible to locate the identical anatomical position in B-deficient plants based on leaf venation patterns; this typically occurred 4 cm from the leaf base and 0.8 cm from the midrib. Leaf samples were then thin-sectioned by hand microtome, stained with calcofluor to assist in cell wall identification, fixed, mounted on a glass microscope slide, and observed at ×40 or ×160 magnification. The number of cells in a fixed field width and the average cell size and width were determined in leaf cross-sections and longitudinal sections and petiole cross-sections with the aid of an optical micrometer. For both longitudinal sections and cross-sections the number of epidermal cells in a fixed distance was counted. The average length and width of 20 individual cells per sample were then determined.

Instron Analysis

Squash seed (cv Early Prolific Straight Neck) were germinated in Petri dishes and then grown in one-quarter-strength Hoagland solution (Hoagland and Arnon, 1950) in the presence or absence of 50 μM B for 7 d. At that time, the elongation of both the hypocotyl and cotyledon was observed to be

somewhat inhibited in the B-deficient treatment. The plants were then harvested and frozen in liquid nitrogen. After thawing, the hypocotyls were bisected longitudinally into equal parts with a home-made bisector. Sections were chosen for uniformity, and all sections were of the same length, width, and thickness. Squash hypocotyl tissue was chosen to ensure uniformity of samples; in this regard, it is superior to either leaf or petiole tissue.

Measurement of extensibility was performed as described by Cleland (1967). Half-sections were placed between the clamps of an Instron extensiometer and extended twice to a 45-g load at a rate of 1.22 mm min^{-1}. *Determination of cell* plasticity and elasticity were made on 15 samples for each treatment according to the methods of Cleland (1967).

RESULTS

B Partitioning

It was observed that cell growth stopped 3 d after transfer to B-deficient conditions (Fig. 1). At that time total B in tobacco cells was decreased by 70% in the B-deficient medium (Table I), although little change was detected in cell wall B. As a result, the proportion of wall B to total B increased from 64 to 97% as tobacco cells progressed into B deficiency. Concentrations of B in the cell wall varied from 23.6 (B deficient) to 30.4 μg/g (B sufficient) when expressed on a cell wall dry weight basis (Table I). In none of the experiments did we isolate cell wall material with less than 21 μg B/g cell wall dry weight. These results indicate that virtually all B is localized in the cell wall when tobacco cells are grown with an insufficient B supply.

A similar effect of B availability on B partitioning in the cell was observed in squash (Table II). When 2-week-old B-sufficient squash was transferred to B-deficient medium, the leaf 1 (oldest leaf) was already fully expanded, leaf 2 was close to full expansion, leaf 3 had intermediate development, and leaves 4 and 5 were in the early stage of development. Following transfer to B-free growth medium, leaves 3, 4, and 5 continued to develop for 2 d such that leaf 3 attained approximately 95% full size and leaves 4 and 5 were halted at 45 and 18% expansion, respectively. Because B is phloem immobile and does not retranslocate from older leaves to actively growing tissue, it can be concluded that leaves 4 and 5 were B deficient. Whereas in control plants, leaves 4 and 5 contained only 20 to 25% of cellular B in the cell wall, in B-deficient plants the cell walls of leaves 4 and 5 contained >95% of total cell B. The majority of this change in B distribution was the result of a decrease in the amount of B in non-cell wall fractions, which were reduced from 33.6 to 0.2 μg B/g dry cell weight (in leaf 4). At the same time, cell wall B content was reduced from 8.5 to 5.4 μg/g cell dry weight. Concentrations of B in the cell walls of squash leaves varied from 10.7 to 59.6 μg/g when expressed on a cell wall dry weight basis. In squash the lowest concentration of B ever observed in cell wall material was 10.7 μg B/g cell wall dry weight (Table II). Plants in which B deficiency was sufficiently severe to halt all further leaf and root development did not show any further signs of tissue death or significant visible damage and remained green for at least 1 week.

Sequential Chemical Extraction

Results of the sequential chemical extraction of tobacco cell walls are shown in Table III. Following a rinse in double-deionized water, tobacco cell walls were isolated as described earlier and were then exposed to a sequential extraction utilizing a variety of chemical extractants. SDS, which removes some residual cytoplasmic protein and membrane, extracted only a small amount of the cell wall B. Sequential extraction with CDTA, which removes chelate-soluble pectin, and Na_2CO_3 at 1°C and then 20°C, which removes other pectin, removed approximately 60% of all cell wall B. Subsequent extraction with 1 N KOH, 4 N KOH, and $NaClO_2$ removed a total of 31% of cell wall B. The final residue, which is mainly cellulose, contains little B. The removal of B by CDTA and two Na_2CO_3 extracts suggests possible association of B with cell wall pectin (Redgwell and Selvendran, 1986).

When the same experiment was repeated in squash, essentially the same results were obtained (Table III). Thus, the majority of cell B may be associated with pectin in the cell wall. The values for the $NaClO_2$ extracts presented here should be interpreted with caution, since there was considerable quenching of the plasma source during ICP-AES B determination. This resulted in the great deal of variability in estimated B content for this extract (Table III).

Enzymic Digestion

The possibility of B association with pectin was tested by enzymic digestion of tobacco cell wall (Table IV). Hemicellulase when used at 1% concentration did not release any detectable amount of B. Hence, B is probably not associated with hemicellulose. Cellulysin if used at 0.5% concentration released significant amounts of B. This may indicate that B

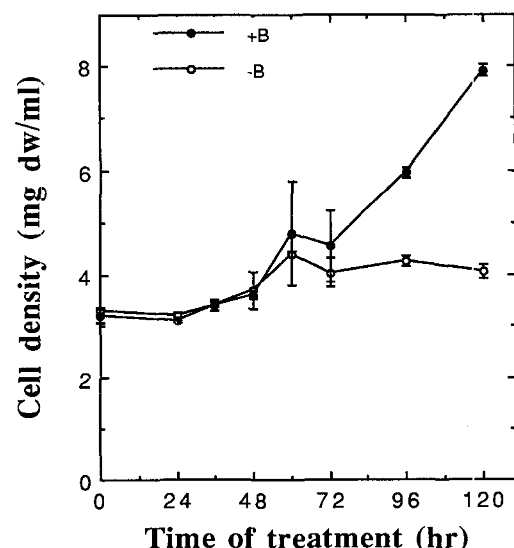

Figure 1. Tobacco cell growth under B treatment. Cultured cells were filtered through Miracloth and washed thoroughly with B-free medium. They were then cultured in the medium with or without B for the time intervals shown.

Table I. *Distribution of B in cultured tobacco cells*

Tobacco cells were precultured in 100 µM ^{11}B. Half of the cells were transferred to B-free medium for 3 d. Cell walls were then isolated as described in "Materials and Methods." Whole cells and cell walls were analyzed for B by ICP-AES. Shown are means ± SE of five replicates. dw, Dry weight.

Treatment	Total Cell B	Total Wall B	Wall B	Wall B
µM B added	*µg/g cell dw*	*µg/g cell dw*	*% of total*	*µg/g wall dw*
0	10.1 ± 0.1	9.7 ± 0.6	97	23.6 ± 0.1
100	17.2 ± 0.8	11.0 ± 1.7	64	30.4 ± 2.7

in the cell wall is associated with cellulose or, alternatively, that the release of B by cellulysin is due to contamination of the commercial cellulysin with a pectolyase-like enzyme(s). It is also possible that the degradation of cellulose resulted in the release of pectin and pectin-bound B. In all experiments, pectolyase, used at the lowest enzyme concentration (0.1%), released the greatest amount of B and was almost as effective as the three enzymes combined. It is possible that the release of B by pectolyase could be attributed to contamination activity with cellulyase in the commercial preparation of pectolyase. This is unlikely, however, since on a weight basis pectolyase used was at only one-fifth the concentration of cellulysin, and yet pectolyase released significantly more B than did cellulysin.

The possibility of B association with pectin was further tested in pear cell wall, a tissue that contains a considerable quantity of pectin. Following 2 h of enzymic digestion with hemicellulase, no significant release of B was observed (Table IV), whereas cellulysin released 30% of cell wall B and pectolyase released >90% of the cell wall B. To test the relative effectiveness of the three enzymes at releasing cell wall B, each enzyme was then supplied at equal concentration. The results shown in Figure 2 clearly demonstrated that pectolyase was the most effective enzyme for solubilization of cell wall B. At these concentrations neither hemicellulase nor cellulysin released significant amounts of B.

The results of enzymic digestion are in agreement with the results of chemical fractionation, i.e. B is largely associated with pectin in the cell wall. Any release of B by cellulysin is probably due to contaminating activity of pectolyase. Significant pectin-degrading activity in the commercial cellulysin has been demonstrated in our laboratory (P.H. Brown, unpublished data).

Anatomical Observations

Removal of B from the growth medium resulted in an inhibition of longitudinal cell expansion. This is illustrated in Table V in which cell dimensions (Fig. 3) are clearly shown to be altered by B deficiency. B-deficient cells were significantly shorter in both the longitudinal and transverse directions than those supplied with B, by 60 and 18%, respectively (Table V). As a consequence, the number of cells per millimeter of epidermis was increased significantly in B-deficient tissue. This was expressed equally in both leaf blade and petiole tissue. Interestingly, in cross-sectional views, cell width (transverse direction) but not cell height (tangential direction) was reduced under B deficiency. Similar results were observed in petiole segments where B deficiency reduced longitudinal length by 60% and transverse length by 50% but did not affect tangential length. Thus, B deficiency reduces cell expansion in the longitudinal and transverse directions but does not appear to influence cell expansion in the tangential direction. Preliminary anatomical observations indicate that the cell walls of B-deficient leaf blade and petiole cells are thicker and more irregular than in B-sufficient cells. These results can be interpreted as a B deficiency-induced inhibition of normal cell wall expansion.

Table II. *Distribution of B in squash plants*

Plants were precultured for 14 d in 50 µM ^{11}B. Seven days prior to harvest half of the plants were transferred to B-free solution. Cell walls were isolated from each leaf (details in "Materials and Methods"), and B was determined in the leaf and cell wall samples by ICP-AES. Shown are means ± SE of three replicates.

Treatment	Leaf No.	Total Leaf B	Total Wall B	Wall B	Wall B	Growth Inhibition
		µg/g dry leaf	*µg/g dry leaf*	*% of total*	*µg/g wall dry wt*	
50 µM B	1	66.2 ± 22.3	28.6 ± 10.3	43	59.6 ± 33.8	None
	2	49.9 ± 10.7	27.9 ± 7.0	56	55.7 ± 23.0	None
	3	53.4 ± 0.9	17.7 ± 0.5	33	43.2 ± 1.8	None
	4	42.1 ± 2.4	8.5 ± 0.7	20	20.5 ± 3.3	None
	5	41.3 ± 2.5	10.3 ± 2.8	25	30.6 ± 11.4	None
0 µM B	1	46.0 ± 2.4	20.7 ± 0.6	45	40.5 ± 2.8	None
	2	18.9 ± 1.5	13.5 ± 0.8	71	27.7 ± 0.6	None
	3	5.6 ± 0.0	3.9 ± 0.1	70	10.7 ± 0.7	Mild
	4	5.6 ± 0.0	5.4 ± 0.4	96	12.2 ± 0.4	Severe
	5	6.3 ± 0.6	6.1 ± 0.7	95	12.9 ± 1.4	Severe

Table III. *Sequential chemical extraction of tobacco and squash cell walls*

A preparation of B-replete tobacco or squash cell wall was sequentially extracted with 1.5% SDS at 22°C for 2 h; 0.05 M CDTA (pH 6.5) at 22°C for 6 h; 0.05 M Na_2CO_3 at 1°C overnight and then 0.05 M Na_2CO_3 at 20°C for 3 h; 1 N KOH at 20°C for 2 h under N_2; 4 N KOH at 20°C for 2 h under N_2. The residue was further extracted with acidified sodium chlorite (pH 4) at 70°C for 2 h. In every step of the chemical extraction, the supernatant was collected by centrifugation and analyzed for B by ICP-AES.

Treatment	Fraction Solubilized	Tobacco Cell Wall[a]		Squash Cell Wall[b]	
		B content	Percentage of total B	B content	Percentage of total B
		μg		μg	
Untreated	Total wall	10.53 ± 0.64	100	13.04 ± 0.23	100
SDS	Protein, intracellular compounds	0.48 ± 0.11	5	0.19 ± 0.16	1
CDTA	Pectin (chelate soluble)	2.49 ± 0.54	24	3.40 ± 1.05	26
Na_2CO_3, 1°C	Pectin	1.53 ± 0.02	15	2.33 ± 0.04	18
Na_2CO_3, 20°C	Pectin	2.22 ± 0.21	21	1.99 ± 0.08	15
1 N KOH	Pectin and hemicellulose	0.86 ± 0.01	8	1.13 ± 0.23	9
4 N KOH	Hemicellulose	0.86 ± 0.12	8	0.34 ± 0.19	3
$NaClO_2$	Pectin and hemicellulose	1.55 ± 0.11	15	2.80 ± 4.0	22
Residue	α-Cellulose	0.32 ± 0.1	3	0.43 ± 0.1	3

[a] Means ± SE of three replicates. Each sample was prepared from 400 mg of dry cell wall. [b] Means ± SE of two replicates. Each sample was prepared from 270 mg of dry cell wall.

Instron Analysis

Further evidence for an effect of B deficiency on cell wall structural characteristics is provided by analysis of cell wall physical properties. Under B deficiency, the total extensibility of squash hypocotyls was reduced to only half that of hypocotyls supplied with adequate B (Table VI). However, further analysis of the data reveals that the reduced extensibility was mainly due to a reduction in the plastic extension component, which was reduced by 60% under B deficiency to a value of 20×10^{-8} cm/dyne for B deficiency compared to 49×10^{-8} cm/dyne for B-sufficient tissue. Elastic extension was also reduced by B deficiency, although the reduction was much less striking. These results suggest that B-deficient cell walls are more rigid and less able to undergo cell wall expansion than B-sufficient cell walls; this agrees with the anatomical observation described above.

DISCUSSION

It has been repeatedly observed in our laboratory both in squash and sunflower, and is well documented in the literature (Dugger, 1983; Loomis and Durst, 1992), that B deficiency results in a rapid inhibition of both root and shoot meristems. These results clearly suggest that B is required primarily, if not exclusively, by growing tissue. The most distinctive feature of growing plant tissue that is not present in more mature tissue is the synthesis and expansive growth of primary cell walls. The localization of B in plant cell walls may indicate that B has a structural role in the cell wall matrix or, alternatively, that B is required for the synthesis of new cell wall material. This latter possibility has been studied extensively, but a consistent effect of B deficiency on new cell wall synthesis has not been shown, even in tissue that exhibited marked alteration in cell wall ultrastructure (Slack

Table IV. *B release from tobacco or pear fruit cell wall after enzymic digestion*

Cell wall preparations were suspended in 10 mM Mes buffer, pH 5.6, containing the various enzymes shown. After 6 h (tobacco) or 2 h (pear fruit) of digestion at 22°C, the suspension was centrifuged. The supernatant and residue were separated, and, after dry ashing, B was analyzed by ICP-AES.

Treatment	B Distribution			
	Tobacco cell wall[a]		Pear fruit cell wall[b]	
	Residue	Supernatant	Residue	Supernatant
	μg		μg	
No enzyme	1.13 ± 0.02	0.14 ± 0.06	3.27 ± 0.12	0.44 ± 0.02
1% hemicellulase	1.14 ± 0.05	0.22 ± 0.08	3.29 ± 0.06	0.46 ± 0.06
0.5% cellulysin	0.49 ± 0.08	0.80 ± 0.08	2.46 ± 0.07	1.12 ± 0.08
0.1% pectolyase	0.37 ± 0.10	0.89 ± 0.14	0.34 ± 0.02	3.16 ± 0.22
All enzymes together	0.38 ± 0.07	0.91 ± 0.15	—	—

[a] Means ± SE of two replicates. Each sample was prepared from 2.5 g of fresh wall paste. [b] Means ± SE of two replicates. Each sample was prepared from 4 g of fresh wall paste.

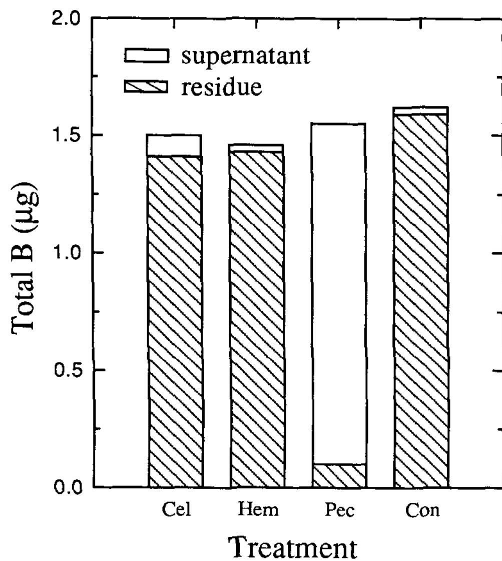

Figure 2. B release from Asian pear fruit after enzymic digestion. Two grams of cell wall paste were prepared from Asian pear fruit cv Shinseiki and placed in Mes buffer, pH 5.6, containing 0.1% of each enzyme. Cel, Cellulysin; Hem, hemicellulase; Pec, pectolyase; Con, no enzyme. After 2 h of digestion, samples were centrifuged and separated into residue and supernatant. B was analyzed by ICP-AES after dry ashing.

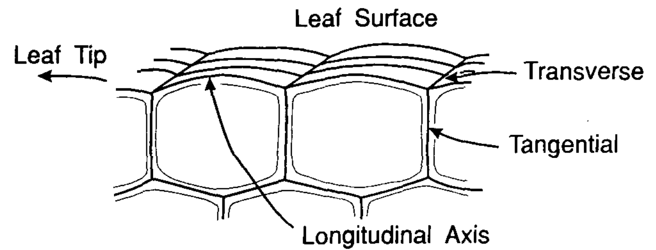

Figure 3. Schematic representation of cell walls referred to in Table V.

and Whittington, 1964; Augsten and Eichorn, 1976; Timashov 1977; Goldbach and Amberger, 1986). The accumulated evidence suggests, therefore, that changes in cell wall polysaccharide composition and synthesis are not the primary cause of growth inhibition (Goldbach and Amberger, 1986). Thus, we hypothesize, as did Loomis and Durst (1992), that B is an essential structural component of plant cell walls.

If B is an important structural component of the cell wall, then one might expect a relatively large proportion of cellular B to be associated with the cell wall. This is indicated in the results presented here in that the extent of B partitioning was strongly dependent on cellular B status. Under B-limiting conditions, cell wall B represented from 96 to 97% of cellular B. These results agree with those of Matoh et al. (1992), who found that more than 98% of cell B was present in the wall in the cultured tobacco cell. Increased B partitioning into the cell wall as plants progressed into B deficiency was also reported by Smith (cited by Loomis and Durst, 1992). Evidence presented here that B is physically located and structurally important in the cell wall is in agreement with the hypothesis of Skok (1957) that B is involved in the formation of structural units or building blocks rather than in metabolic reactions. Growth will not occur until enough B is obtained to form the proper cell wall structure. That B functions as a structural component of the cell wall or building blocks can explain the well-known observation that B is not reutilizable and must be supplied at all times during plant growth (Skok, 1957; Loomis and Durst, 1992).

Loomis and Durst (1992) pointed out that B-deficiency symptoms occur within hours in the root tips and within minutes or seconds in pollen tube tips and are characterized by cell wall abnormalities. Kouchi and Kumazawa (1976) reported that in B-deficient tomato root tips the inhibition of root elongation in the advanced stage of B deficiency was not associated with any necrotic symptoms at the subcellular level such as a degradation of the membranous system. However, they found that cell walls suffered an irregular thickening with a ragged or serrated structure. These cell wall alterations appeared most distinctly in the longitudinal walls. This was in agreement with the suggestion made by Slack and Whittington (1964), who reported that the longitudinal expansion of newly formed cells is particularly affected in B-deficient tissue. These results are in good agreement with those presented here.

Evidence from anatomical observations and characterization of plant cell wall attributes presented here are consistent with a B deficiency-induced inhibition of cell wall expansion and a subsequent thickening of plant cell walls. This inhibition of cell expansion is most pronounced in the longitudinal

Table V. *Influence of B deficiency on the cell size of squash leaf and petiole epidermal cells*

Measurements were made in three directions and are labeled according to Figure 3. Cell size was estimated by measuring 20 cells in each section with an ocular micrometer. Average number of cells per 1 mm in the longitudinal axis was also determined. Shown are means ± SE of eight samples.

Plant Tissue	Treatment	Wall Length			Cells/1 mm (longitudinal)
		Longitudinal	Transverse	Tangential	
	μM B added		*μm*		
Leaf blade	50	16.5 ± 1.0	13.0 ± 1.2	12.0 ± 1.0	62
	0	10.2 ± 0.2	11.0 ± 0.5	11.7 ± 0.7	100
Petiole	50	14.3 ± 0.3	12.0 ± 0.7	11.4 ± 0.9	70
	0	9.0 ± 0.3	8.0 ± 0.6	11.7 ± 1.0	112

Table VI. *Influence of B treatment on squash hypocotyl physical parameters*

Squash seeds were germinated and then grown in 0 or 50 μM B for 7 d. The hypocotyls were then collected and extensibility was measured by Instron. Half-sections were placed between the clamps of an Instron extensiometer and extended twice to a 45-g load at a rate of 1.22 mm/min. Shown are means ± SE of 15 replicates.

Treatment	Total Extension	Elastic Extension	Plastic Extension
μM B added	*mm*	*10^{-8} cm/dyne*	*10^{-8} cm/dyne*
0	0.76 ± 0.1	82 ± 12	20 ± 9
50	1.48 ± 0.3	107 ± 18	49 ± 14

and transverse directions. Coincident with this decrease in cell elongation is a reduction in the plastic extensibility of B-deficient tissues. In addition, B-deficient tissue is characteristically brittle and more rigid than B-sufficient tissue. Thus, B-deficient meristematic tissue is easily broken, whereas B-replete tissue remains far more pliable. The production of a stiff, inelastic cell wall that cannot undergo normal cell-stretching growth as a consequence of B deficiency would result in the rapid inhibition of meristematic growth, a decrease in cell plasticity, and the contingent reduction in cell expansion that is observed under B deficiency. Determination of the chemical component with which B is associated in the cell wall may provide information concerning the exact role of B in plant cell walls and help define the role of given wall components in growth.

Growing walls consist of elastic elements in series with plastic elements (Cosgrove, 1987). The elastic elements return to their original shape when tension is relieved, whereas plastic elements shear or distend irreversibly when placed under tension. Both elements bear the mechanical wall stress produced by cell turgor pressure and are critical components of cell wall expansive growth. It is widely accepted that pectin plays a key role in determining the plastic extensibility of plant cell walls (Jarvis, 1984). Northcote (1986) pointed out that pectin is deposited in the wall during primary growth only and as such may play a critical role in determining the physical characteristics of the primary cell wall. In the experiments reported here, we found that the majority of cell wall B was associated with pectin. It is suggested, therefore, that B influences cell wall structural characteristics through its interactions with cell wall pectin. Since the initial symptom of B deficiency occurs in growing tissue, where it results in a rigidification of the cell wall, and B is found to be associated with pectin, thought to be important in cell wall expansive growth, we propose (in agreement with Loomis and Durst, 1992), that B plays a fundamental role in the physical structure of expanding cell walls. It is significant that the chemical reactivity of B makes it likely that it will form bonds with pectin, a characteristic that has been demonstrated in vitro (Deuel and Nuekom, 1949, cited by Loomis and Durst, 1992).

B association with pectin has been suggested by Yamauchi et al. (1986). Recently, Matoh et al. (1993) isolated a B-polysaccharide complex from mature radish roots and demonstrated that >60% of cell wall B was associated with this pectin-like polysaccharide. Their elegant work, however, considered only mature (tuberous) tissue of one species and did not demonstrate the nature of B localization and complexation in young tissues, where the effects of B deficiency are first expressed. In the experiments presented here, we have provided verification that the association of B with pectic fractions occurs in three additional species and three different tissues. Significantly, we have demonstrated that this B-pectin association is present in growing tissues and that B deficiency in these tissues results in alterations to cell wall physical characteristics and expansive growth that are consistent with a role of B in cell wall structure. We are currently determining whether the B-containing fractions we have isolated are similar to those identified by Matoh et al. (1993) in radish root.

The mechanism by which B influences cell wall expansion is poorly understood, although a useful theoretical model has been proposed by Loomis and Durst (1992), who suggested that B may play a fundamental role in the physical structure of expanding cell walls through the formation of borate-ester cross-links with pectin in the cell wall. The rapid breaking and reformation of these acid-labile, borate-ester bonds might facilitate the controlled creep of expanding cell walls while maintaining the cell wall strength necessary for continued cell growth. Since many B esters are dissociated at pH values in the range of 3.5 to 5.0 (in vitro evidence, Loomis and Durst, 1992), it is feasible that dissociation and reformation of B-ester linkages are integrally involved in cell wall expansion. The results presented here are not inconsistent with this hypothesis.

This hypothesis also satisfactorily explains the limited essentiality of B to vascular plants and provides an explanation for the differing requirements for B within higher plants (Tanaka, 1967; Loomis and Durst, 1992). Pectin represents about one-third of the primary cell wall of dicots but only a small fraction of the cell wall of graminaceous monocots (Carpita, 1989) and is of negligible importance in nonvascular plants. The relative abundance of pectin in cell walls corresponds well with the differential requirement for B in these same species. Further work to define the role of B in the cell wall expansive process is underway.

ACKNOWLEDGMENTS

We greatly appreciate the help of Dr. J. Labavitch with cell wall extraction techniques and his critical review of this manuscript. We wish also to acknowledge the assistance of Dr. Henrik Stolz.

Received November 16, 1993; accepted February 24, 1994.
Copyright Clearance Center: 0032-0889/94/105/0681/09.

LITERATURE CITED

Augsten H, Eichorn B (1976) Biochemistry and physiology of the effect of boron in plants. Biol Rundsch **14:** 268–285

Brown P, Hu H (1994) Boron uptake by sunflower, squash and cultured tobacco cells. Physiol Plant (in press)

Carpita NC (1989) Pectic polysaccharides of maize coleoptiles and proso millet cells in liquid culture. Phytochemistry **28:** 121–125

Cleland R (1967) Extensibility of isolated cell walls: measurement and changes during cell elongation. Planta **74:** 197–209

Cosgrove DJ (1987) Wall relaxation and the driving forces for cell expansive growth. Plant Physiol **84:** 561–564

Dugger WM (1983) Boron in plant metabolism. *In* A Läuchli, RL

Bieleski, eds, Encyclopedia of Plant Physiology, Vol 15B. Inorganic Plant Nutrition. Springer-Verlag, New York, pp 626–650

Goldbach H, Amberger A (1986) Influence of boron nutrition on cell wall polysaccharides in cell cultures of *Daucus carota* L. J Plant Physiol **123:** 263–269

Hirsch AM, Torrey JG (1980) Ultrastructural changes in sunflower root cell in relation to boron deficiency and added auxin. Can J Bot **58:** 856–866

Hoagland DR, Arnon DI (1950) The Water-Culture Method for Growing Plants without Soil. *In* California Agricultural Experiment Station circular 347. The College of Agriculture, University of California, Berkeley, CA

Jarvis MC (1984) Structure and properties of pectin gels in plant cell wall. Plant Cell Environ **7:** 153–164

Kouchi H, Kumazawa K (1976) Anatomical responses of root tips to boron deficiency. III. Effect of boron deficiency on sub-cellular structure of root tips, particularly on morphology of cell wall and its related organelles. Soil Sci Plant Nutr **22:** 53–71

Lee SG, Aronoff S (1966) Investigations on the role of boron in plants. III. Anatomical observations. Plant Physiol **41:** 1570–1577

Loomis WD, Durst RW (1992) Chemistry and biology of boron. Biofactors **3:** 229–239

Lorenz OA (1942) Internal breakdown of table beets. Cornell University Agricultural Experiment Station, memoir 246. Cornell University, Ithaca, NY

Marschner H (1986) Mineral nutrition in higher plants. Academic Press, Orlando, FL, pp 321–334

Matoh T, Ishigaki K-i, Kaori O, Azuma J-i (1993) Isolation and characterization of a boron-polysaccharide complex from radish roots. Plant Cell Physiol **34:** 639–642

Matoh T, Ishigaki K-i, Mizutani M, Matsunaga W, Takabe K (1992) Boron nutrition of cultured tobacco BY-2 cells. I. Requirement for and intracellular localization of boron and selection of cells that tolerate low levels of boron. Plant Cell Physiol **33:** 1135–1141

McCann MC, Wells B, Roberts K (1990) Direct visualization of cross-links in the primary cell wall. J Cell Sci **96:** 323–334

Northcote DH (1986) Control of pectin synthesis and deposition during plant cell wall growth. *In* ML Fishman, JJ Jen, eds, Chemistry and Function of Pectin, American Chemical Society Symposium series 310. American Chemical Society, Washington, DC, pp 134–140

Pech JC, Romani RJ (1979) Senescence of pear fruit cells cultured in a continuous renewed auxin-deprived medium. Plant Physiol **64:** 814–817

Redgwell RJ, Selvendran RR (1986) Structural features of cell-wall polysaccharides of onion *Allium cepa*. Carbohydr Res **157:** 183–199

Skok J (1957) The substitution of complexing substances for boron in plant growth. Plant Physiol **32:** 308–312

Slack CR, Whittington WJ (1964) The role of boron in plant growth. III. The effects of differentiation and deficiency on radicle metabolism. J Exp Bot **15:** 495–514

Spurr AR (1957) The effect of boron on cell-wall structure in celery. Am J Bot **44:** 637–650

Stark JR (1963) Effect of boron on the cell wall structure in sunflower. Acta Soc Bot Pol **32:** 619–623

Tanaka H (1967) Boron adsorption by plant roots. Plant Soil **27:** 300–302

Timashov ND (1977) Effect of a boron deficiency on the incorporation of glucose-^{14}C into fractions of polysaccharides of sunflower organ cell walls. Vestn Khar'k Univ **158:** 36–38

Warington K (1923) The effect of boric acid and borax on the broad bean and certain other plants. Ann Bot **37:** 629–672

Whittington WJ (1959) The role of boron in plant growth. II. The effect on growth of the radicle. J Exp Bot **10:** 93–103

Yamauchi T, Hara T, Sonoda Y (1986) Distribution of calcium and boron in the pectin fraction of tomato leaf cell wall. Plant Cell Physiol **27:** 729–732