The Mechanism of Boron Mobility in Wheat and Canola Phloem

James Stangoulis*, Max Tate, Robin Graham, Martin Bucknall, Lachlan Palmer, Berin Boughton, and Robert Reid

School of Biological Science, Flinders University, Bedford Park, South Australia 5042, Australia (J.S., R.G., L.P.); School of Food and Plant Science, University of Adelaide, Waite Campus, South Australia 5064, Australia (M.T.); Bioanalytical Mass Spectrometry Facility, University of New South Wales Analytical Centre, University of New South Wales, Sydney 2052, Australia (M.B.); Metabolomics Australia, School of Botany, University of Melbourne, Melbourne, Victoria 3010, Australia (B.B.); and School of Earth and Environmental Sciences, University of Adelaide, North Terrace Campus, Adelaide, South Australia 5005, Australia (R.R.)

Low-molecular-weight borate complexes were isolated from canola (Brassica napus) and wheat (Triticum aestivum) phloem exudates, as well as the cytoplasm of the fresh-water alga Chara corallina, and identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Phloem exudate was collected from field-grown canola inflorescence stalks by shallow incision, while wheat phloem exudate was collected by aphid styletomy. Chara cytoplasm was collected by careful manual separation of the cell wall, vacuole, and cytosolic compartments. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry showed the presence of isotopic borate complexes, at mass-to-charge ratio of 690.22/691.22 in the canola and wheat phloem and at 300.11/301.11 in canola phloem and Chara cytoplasm. Using reference compounds, the borate complexes with mass-to-charge ratio 690.22/691.22 was identified as a bis-sucrose (Suc) borate complex in which the 4,6-O-bis-hydroxyl pairs from the two α-glucopyranoside moieties formed an [L2B] complex. Further investigation using liquid chromatography electrospray ionization triple quadrupole mass spectrometry analysis confirmed the presence of the bis-Suc borate complex in wheat phloem with a concentration up to 220 μM. The 300.11/301.11 complex was putatively identified as a bis-N-acetyl-serine borate complex but its concentration was below the detection limits of the liquid chromatography electrospray ionization triple quadrupole mass spectrometer so could not be quantified. The presence of borate complexes in the phloem provides a mechanistic explanation for the observed phloem boron mobility in canola and wheat and other species that transport Suc as their primary photoassimilate.

Boron (B) is an essential element for plant growth (Warington, 1923) and it plays a structural role in the cell wall where it cross-links the pectic fractions of the dimeric polysaccharide complex known as RG-II (Matoh et al., 1993; Ishii and Matsunaga, 1996; Kobayashi et al., 1996; O’Neill et al., 1996) and more recently there is emerging evidence for a structural role for B in the cytoskeleton (Bassil et al., 2004). With the function of B becoming clearer over recent years, our knowledge of the mechanism of B uptake and transport within the plant has also improved. B exists in solution as boric acid that has a pKa of 9.2 and therefore is uncharged at physiological pH. As a consequence, it has a high membrane permeability (around 10⁻⁷ cm s⁻¹; Dordas et al., 2000; Stangoulis et al., 2001b) that would seem to negate the need for facilitated or active uptake, yet facilitated B transport has been observed at low B supply (Dannel et al., 2000; Stangoulis et al., 2001b). On entering the xylem, B moves apoplastically to the shoot via the transpiration stream. This dependence on transpiration leads to the observed patterns of accumulation where B concentrations are higher in older leaves compared to younger leaves, and under deficiency conditions, symptoms are first manifest within the growing points of roots and shoots (Stangoulis et al., 2001a). Such a pattern of distribution has lead to the belief that B is not mobile in the phloem. Oertli and Richardson (1970) reasoned that as B remains water soluble in plants, its immobility could not be due to chemical fixation. Instead, they suggested the apparent immobility was due either to the inability of B to enter the phloem, or to the absence of long-distance movement in phloem due to leakage back into the xylem as a result of its high membrane permeability. According to this hypothesis, once B reenters the xylem, it would simply travel to the ends of the transpiration stream. Measured gradients of B within
leaf tissue support this hypothesis (Oertli and Roth, 1969; Oertli, 1993; Reid et al., 2004), yet, there is also evidence that B can be translocated in phloem in species that use polyols (Brown and Hu, 1996) and Suc (Stangoulis et al., 2001a) as their primary photoassimilates, and that this may reduce the impact of deficiency. The mechanism for B mobility in polyol-transporting species has been linked to the presence of polyol-borate complexes (Hu et al., 1997), where polyols have cis-hydroxyl groups that favor complexation with B (Makkee et al., 1985). However, most species translocate Suc as the primary photoassimilate and complexation of B with Suc has not been reported. This study investigated the possibility that there are endogenous chelators for B that enable its retranslocation in the phloem of those species that translocate Suc as their primary photoassimilate. Here we investigated B mobility in canola (Brassica napus) and wheat (Triticum aestivum). A further investigation was initiated to determine whether other intracellular ligands also exist, and to do this, the fresh-water alga Chara corallina was used as it has very large cells enabling the separation of the cell wall, cytoplasm, and vacuole, thereby enabling the collection of pure cytosolic fluid. Due to its close evolutionary relationship to higher plants (Manhart and Palmer, 1990), the likelihood of similar complexes occurring in higher plants would be high.

RESULTS

Borate Complexes in Canola and Wheat Phloem Exudates

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) identified two $[\text{L}_2\text{B}]^{2+}$ ions in canola phloem sap with mass-to-charge ratio ($m/z$) values of 300.11 and 301.11, and 690.22 and 691.22 with a natural abundance of $^{10}$B:$^{11}$B (20%:80%), confirming the existence of borate complexes (Figs. 1 and 2). Other identifiable compounds present in the spectra included the deprotonated Suc ($m/z$ 341.11), deprotonated 3-aminoquinoline (AQ; $m/z$ 143), and a deprotonated covalent 3-AQ dimer ($m/z$ 285). Putative borate complexes were assigned as bis-Suc borate at 690.22/691.22 (Fig. 3) and bis-N-acetyl-Ser borate at 300.11/301.11 (Fig. 4), respectively (Table I). Mixtures of Suc and boric acid (Fig. 5) and N-acetyl-Ser with boric acid (Fig. 6) verified the formation of the two complexes identified in canola phloem. The presence of borate complexes was confirmed in five exudate samples taken from five individual plants.

Figure 1. Negative mode MALDI-TOF-MS spectrum of canola phloem exudate cocrystallized in 3-AQ. [See online article for color version of this figure.]

Figure 2. Negative mode MALDI-TOF-MS spectrum of borate complexes isolated from canola phloem exudate cocrystallized in 3-AQ. [See online article for color version of this figure.]
In wheat phloem exudate, MALDI-TOF-MS identified one \([\mathrm{L}_2\mathrm{B}]^{-1}\) ion with \(m/z\) values of 690.22 and 691.22 with a natural abundance of \(^{10}\mathrm{B}:/^{11}\mathrm{B}(20\%:80\%), confirming the existence of a borate complex (trace not presented). This was confirmed in three individual exudate samples taken from three individual plants. Two samples of phloem exudates were sent to Metabolomics Australia to quantify the amount of borate complex present in the phloem. Both samples were from different plants that varied in age by up to 3 weeks, and using liquid chromatography electrospray ionization triple quadrupole mass spectrometry (LC-ESI-QQQ-MS), the borate complexes at 690.22 and 691.22 were present with a concentration of the borate dimer of 52 and 220 \(\mu\text{M}\) for the younger and older plant, respectively.

To investigate the binding position between Suc and borate, two standard mixtures were analyzed by MALDI-TOF-MS (Fig. 7). The first mix contained 400 \(\text{mM}\) 1-o-methyl-\(\alpha\)-D-glucopyranoside and trans-cyclohexane-1,2-diol and 1,6-anhydro-glucosan, with and without the addition of 0.3 \(\text{mM}\) boric acid (pH adjusted to 8.2 with KOH). In the absence of B no significant deprotonated ions were identified at \(m/z\) 115 for the cyclohexane diol or at 161 for the 1,6-anhydroglucosan, although a strong deprotonated ion at \(m/z\) 193 was observed for the 1-o-methyl-\(\alpha\)-D-glucopyranoside (data not presented). The analysis suggests that the hydroxyls in trans-cyclohexanediol and 1,6-anhydroglucosan have \(pK_s\)s that are too high to be ionized with the 3-AQ MALDI matrix. With the addition of boric acid to the standard mixture, there was formation of a \([\mathrm{L}_2\mathrm{B}]^{-1}\) ion with \(m/z\) 394.14 and a \([\mathrm{L}_2\mathrm{B}]^{-1}\) ion at 395.14 (Fig. 7, insert). The absence of a trans-cyclohexane-diol \([\mathrm{L}_2\mathrm{B}]^{-1}\) ion at \(m/z\) 238/239 and a 1,6-anhydroglucosan \([\mathrm{L}_2\mathrm{B}]^{-1}\) ion at \(m/z\) 330/331 further implicates the involvement of the 4,6-hydroxyl pairs from two \(\alpha\)-methyl-glucopyranoside molecules forming a \([\mathrm{L}_2\mathrm{B}]^{-1}\) borate complex. The 4,6-hydroxyl pairs on the \(\alpha\)-methyl-glucopyranoside molecule within Suc is therefore implicated as the most likely site for borate complexing.

**Figure 3.** Putative bis-Suc borate complex isolated from canola and wheat phloem.

**Borate Complexes in Chara Cytoplasm**

The MALDI-TOF-MS spectrum of *Chara* cytoplasm is quite complex with many low-\(M_r\) compounds present (Fig. 8). One \([\mathrm{L}_3\mathrm{B}]^{-1}\) ion was observed from the spectra with two distinct peaks at \(m/z\) 300.11 and 301.11 with putative identification as a bis-N-acetyl-Ser complex. The predicted isotopic abundance of \(^{10}\mathrm{B}:/^{11}\mathrm{B}\) clearly identifies a common \([\mathrm{L}_3\mathrm{B}]^{-1}\) ion in both higher plants and the fresh-water algae *Chara*.

**DISCUSSION**

Knowledge surrounding the mechanisms of long-distance B transport within plants has increased substantially over recent years, with strong evidence for B mobility in the phloem (Hu et al., 1997; Penn et al., 1997; Stangoulis et al., 2001b). Due to the inherently high permeability of boric acid across cellular membranes (10\(^{-7}\) cm s\(^{-1}\); Dordas et al., 2000; Stangoulis et al., 2001b), the presence of a phloem-mobile chelator is required to reduce phloem-xylem leakage and subsequent translocation to the ends of the transpiration stream. In those species that translocate polyols as their primary photoassimilate, the presence of cis-diols such as mannitol, sorbitol, and dulcitol provide stable borate polyol complexes and subsequent mobility within the phloem (Hu et al., 1997). In those

**Figure 4.** Putative bis-N-acetyl-L-Ser borate complex isolated from canola phloem and *Chara* cytoplasm.
species (i.e. canola) that translocate Suc as their primary photoassimilate, the mechanism of B mobility has until now not been identified. Results presented in this study provide, to our knowledge, the first direct evidence for an endogenous chelator for borate in the phloem of wheat and canola and provide a mechanistic explanation for the observed phloem mobility in canola and most likely the related species, *Brassica oleracea* where B mobility has also been reported (Shelp, 1988; Liu et al., 1993). The significance of the results are also heightened by the identification of two (Shelp, 1988; Liu et al., 1993). The significance of the results are also heightened by the identification of two borate complexes at m/z of 300/301 and 690/691, which we infer to be due to the presence of bis-N-acetyl-borate and bis-Suc borate complexes, respectively. The presence of the borate complex in wheat phloem was also confirmed by two different analytical methods (MALDI-TOF-MS and LC-ESI-QQQ-MS) and the concentration of the borate dimer quantified at up to 220 μM. These results provide further evidence to support mobility of B in the phloem of this species that translocates Suc as its primary photoassimilate.

In this study, phloem Suc was quantified with concentrations around 300 mM while B concentration was determined by inductively coupled plasma-optical emission spectrometry at 0.3 mM, hence the Suc-to-Borate ratio is very high. This, together with the high pH of the phloem exudate (measured at close to pH 8.5), favors the formation of a bis-Suc borate complex. The complex is weak with stability constants of borate-Suc complexes having log β1 and log β2 values for [LB]^{-1} and [L₂B]^{-1} of 0.86 and 0.70, respectively (Verchere and Hlaibi, 1987). This is in contrast to the high-stability constants for Fructoborate complexes of 2.82 (log β1) and 4.97 (log β2) for the [LB]^{-1} and [L₂B]^{-1} complexes, respectively (Verchere and Hlaibi, 1987). The low-stability constants between Suc and borate are due to the complexation site not being on the Fru moiety, but on the 4,6-Glc hydroxyls of the Suc molecule. Results presented in this study also support this observation; that borate complexes to Suc via the 4,6-hydroxyl pairs on the α-methyl-glucopyranoside molecule.

In the phloem, the bis-Suc borate complex will be in a dynamic equilibrium with uncomplexed Suc and will therefore obey source-sink rules. It is likely that during phloem loading of Suc in a leaf, free B in the leaf cell cytoplasm would equilibrate with the phloem because of its high membrane permeability. The Suc would act as a trap that would effectively result in an accumulation of B in the phloem. As the Suc is unloaded, B would be released and would diffuse into the sink cells. Given the high requirement for B during the reproductive phase of plant growth (Rerkasem and Jamjod, 2004), the B delivered to the florets via the bis-Suc borate complex could be very significant in maintaining reproductive success. The absence of a bis-Suc borate complex in *Chara* cytoplasm, where the Suc concentration is significantly lower (around 20 mM; Ding et al., 1992) to the level of Suc in plant phloem, may also indicate that on unloading from the phloem, the bis-Suc borate complex does not remain intact. The lower pH in the cytoplasm would also support cleavage of the complex and formation of boric acid instead of the borate anion.

N-acetyl-Ser in canola phloem or *Chara* cytoplasm was not quantified in this study due to inadequate amounts of phloem sample for analysis. However, unacetylated Ser is known to be one of the major amino acids in the phloem and has been reported to total 14% (approximately 62 mM) of the total amino acids present in canola (var Duplo) phloem exudate (Lohaus and Moellers, 2000) so one would also expect a similar content in the phloem sap of the Barossa variety that was used in this study. Likewise, Ser makes up around 3% (approximately 2.9 mM) of total amino acids in the cytoplasm of *Chara* (Sakano et al., 1984). B concentration in the cytoplasm was measured by inductively coupled plasma-optical emission spectrometry at 13 μM (Stangoulis et al., 2001b), although this level of B may not exist in a natural environment where B levels may fluctuate and where we expect for rapid equilibration of cytosolic B with the B concentration in the external growth medium (Stangoulis

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**Table I.** m/z of borate complexes determined by MALDI-TOF-MS

| Formula                  | Molecular Weight | Description                                      |
|--------------------------|------------------|--------------------------------------------------|
| [(C₁₀H₁₄N₂O₈)₁B]        | 300.11           | Borate with 2 N-acetyl-L-Ser                     |
| [(C₁₀H₁₄N₂O₈)₁B]        | 301.11           | Borate with 2 N-acetyl-L-Ser                     |
| [(C₂⁴H₄⁰O₂₂)₁B]         | 690.22           | Borate complex with 2 Suc                       |
| [(C₂⁴H₄⁰O₂₂)₁B]         | 691.22           | Borate complex with 2 Suc                       |

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**Figure 5.** Negative mode MALDI-TOF-MS of 300 mM Suc + 0.3 mM boric acid (pH adjusted to 9.0 with KOH) cocrystallized in 3-AQ. [See online article for color version of this figure.]
et al., 2001b). It should also be noted that essentiality for B in *Chara* has not yet been proven, even though recent evidence suggests a facilitated B uptake system at low B supply indicates that a B requirement could exist in this species. Also, the occurrence of the putative bis-N-acetyl-Ser borate complex at 300.11/301.11 in *Chara* cytoplasm requires an explanation for its existence. While further research is required in this area, one might hypothesize a role similar to that of the Ser-borate complex in animal systems where it acts as a transition-state inhibitor of γ-glutamyl transpeptidase (Tate and Meister, 1978), with the membrane-bound enzyme functioning in the degradation of glutathione (Leustek et al., 2000). γ-Glutamyl transpeptidase activity is also widely distributed in plant species (Leustek et al., 2000, and refs. therein) and therefore a similar effect of the Ser-borate inhibition on γ-glutamyl transpeptidase activity may exist. Further research utilizing the benefits of working with *Chara* where one can extract pure cytosolic and vacuolar fluids, would improve our understanding of the role of B in the bis-N-acetyl-Ser borate complex.

### MATERIALS AND METHODS

**Canola Phloem Exudate Collection**

Phloem exudate was collected from more than 10 field-grown canola (*Brassica napus*) plants (cv Dunkeld) that were grown in close proximity to the laboratory at around 5 h after the beginning of the photoperiod. Shallow incisions were made into the attached inflorescence and the site was immediately rinsed with high-purity water (≥18 M resistivity). The initial droplet of phloem exudate formed was discarded to minimize contamination. Exudate was collected over the following 20 to 30 min into small Eppendorf tubes placed on ice. After collection, samples were immediately frozen at −80°C until analysis by MALDI-TOF-MS.

**Wheat Phloem Exudate Collection**

Aphid stylectomy procedures were adapted from Fisher and Frame (1984) and Pritchard (1996). Aphids (*Rhopalosiphum padi*) were secured to wheat (*Triticum aestivum*) plants overnight using specially prepared cages. The cages were removed and stylectomy was performed using high-frequency microcautery under a microscope (Leica MZ16) using a chemically sharpened tungsten needle. Exudate was collected using glass micropipettes and exudate volume quantified using an attached Leica DFC280 digital camera in combination with the Leica application suite software (V2.8.1 with multitime and interactive measurement modules). The exudation rate was measured at 30 to 40 nL per hour. Phloem exudates were expelled from the capillaries into the lid of a 0.2-ml PCR tube containing a small volume of high-purity MilliQ water for ease of removing the exudate from the capillary. A quick rinse of the microcapillary also ensured total removal of exudate and the total volume of MilliQ water used was 10 μL. Samples were then freeze dried and stored at −80°C until analysis by MALDI-TOF-MS and I.C.-ESI-QQQ-MS. In total, eight wheat plants were sampled and individual phloem exudates kept separate for method development and for quantification of the borate complex.

**Collection of Chara Cytosolic Contents**

Procedures for isolation of *Chara* cytoplasm, described in Stangoulis et al. (2001b) were utilized. *Chara* was cultured indoors in plastic tanks filled with tap water on a substrate of garden soil and river sand, under fluorescent lamps with a 16/8-h light/dark cycle at room temperature (around 22°C). Individual internodal cells were excised from shoots and cells were blotted dry, allowed to wilt slightly, and ends excised. Vacular contents were carefully removed with a small amount of the cytosolic contents to reduce vacuolar contamination of cytosolic fluid. The remaining cytosolic contents were collected in small Eppendorf tubes placed on ice. After collection, samples were immediately frozen at −80°C until analysis by MALDI-TOF-MS.

**MALDI-TOF-MS Analysis**

Aliquots of canola and barley (*Hordeum vulgare*) phloem exudate and *Chara* cytoplasm (0.5 μL) were mixed directly on the MALDI target with 0.7 μL of a saturated solution of 3-AQ (Aldrich) in ethanol (approximately 0.4 μL, used as MALDI matrix). The samples were allowed to dry under ambient laboratory...
conditions (Hu et al., 1997; Penn et al., 1997). Samples were analyzed using a Perseptive Biosystems DE-STR TOF MS (Applied Biosystems) equipped with a nitrogen laser (337 nm, 3 ns pulse). All spectra were obtained in reflectron-delayed extraction-negative ion mode with each spectrum produced by summing data from multiple laser shots. Spectra were internally m/z calibrated using the 3-AQ monoisotopic [M-H]⁻ ion at m/z 143.0604, and monoisotopic dimeric [2M-H]⁻ ion at m/z 285.1135. Mass accuracy using this protocol is estimated to be ±0.002%.

**LC-ESI-QQQ-MS Analysis**

Samples of phloem exudate were redissolved in 10 μL of 50% MeCN NH₄HCO₃ (1 mM, pH = 8.02) buffer. Five-microliter sample volumes were injected into an Agilent 6410 LC-ESI-QQQ-MS equipped with an Agilent 1200 series LC system and a Cogent Diamond Hydride guard column (100A 4 μm, 2.0 mm × 20 mm). Running buffers included 100% NH₄HCO₃ (1.5 mM, pH = 8.05; buffer A) and 90% acetoni-trile with 10% NH₄HCO₃ (1.5 mM, pH = 8.05; buffer B), while the gradient was used 100% buffer B for 5 min, followed by 75% buffer B for a further 5 min and then followed by reequilibration with 100% buffer B over 10 min. QQQ parameters included a selected ion monitoring scanning mode that was run with negative polarity. Gas temperature of 300°C with a gas flow rate of 10 L min⁻¹, a nebulizer pressure of 45 psi, and a capillary voltage of 4,000. Sucrose concentrations in the phloem were quantified using a standard calibration curve of known Suc-borate concentrations. Planta 211: 833–840

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