Running Head: Isolation of boron complexes in plant phloem and Chara

All correspondence should be sent to:

Associate Professor James Stangoulis
Flinders University, School of Biological Sciences, GPO Box 2100, Adelaide 5001, South Australia.

Phone: +61 8 82012610
Fax: +61 8 82013015
james.stangoulis@flinders.edu.au

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Title: The mechanism of boron mobility in wheat and canola phloem

Authors: James Stangoulis¹, Max Tate², Robin Graham¹, Martin Bucknall³, Lachlan Palmer¹, Berin Boughton⁴ and Robert Reid⁵

Institution Address:
¹ Flinders University, School of Biological Science, Bedford Park, South Australia 5042, Australia.
² The University of Adelaide, School of Food and Plant Science, Waite Campus, South Australia 5064, Australia.
³ Bioanalytical Mass Spectrometry Facility, UNSW Analytical Centre, University of New South Wales, Sydney 2052, Australia.
⁴ Metabolomics Australia, The University of Melbourne, School of Botany, Melbourne, Victoria 3010, Australia.
⁵ The University of Adelaide, School of Earth and Environmental Sciences, North Terrace Campus, Adelaide, South Australia 5005, Australia.
Footnotes:
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Corresponding author;
Associate Professor James Stangoulis
james.stangoulis@flinders.edu.au
Fax: +61 8 82013015
Abstract

Low molecular weight borate complexes were isolated from canola (Brassica napus L.) and wheat (Triticum aestivum L) phloem exudates, as well as the cytoplasm of the fresh-water algae, Chara corallina (Klein ex Will. Esk. R.D. Wood) and identified using matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS). Phloem exudate was collected from field-grown canola inflorescence stalks by shallow incision, while wheat phloem exudate was collected by aphid stylectomy. Chara cytoplasm was collected by careful manual separation of the cell wall, vacuole and cytosolic compartments. MALDI-TOF-MS showed the presence of isotopic borate complexes, at m/z 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 m/z 690.22/691.22 was identified as a bis-sucrose borate complex in which the 4,6 hydroxyl pairs from the two α-glucopyranoside moieties formed an [L₂B]⁻¹ complex. Further investigation using LC-ESI-QQQ analysis confirmed the presence of the bis-sucrose 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 LC-ESI-QQQ 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 sucrose as their primary photo-assimilates.
Introduction

Boron (B) is an essential element for plant growth (Warrington, 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 (Elias 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 which 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., 2001a) which 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., 2001a). 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., 2001b)). 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 re-enters 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 (Hu et al., 1997) and sucrose (Stangoulis et al., 2001b) 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 favour complexation with B (Makkee et al., 1985). However, most species translocate sucrose as the primary photoassimilate and complexation of B with sucrose has not been reported. The current study investigated the possibility that there are endogenous chelators for B that enable its re-translocation in the phloem of those species that translocate sucrose as their primary photoassimilate. Here we investigated B mobility in canola (Brassica napus L.) and wheat (Triticum aestivum L.). A further investigation was initiated to determine whether other intracellular ligands also exist, and to do this, the fresh-water algae 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

MALDI-TOF-MS identified two \([L_2B]^-\) ions in canola phloem sap with \(m/z\) values of 300.11 and 301.11, and 690.22 and 691.22 with a natural abundance of \(^{10}\text{B}:^{11}\text{B} (20:80\ \%)\) confirming the existence of borate complexes (Fig 1 and Fig 2). Other identifiable compounds present in the spectra included the deprotonated sucrose (\(m/z\) 341.11), deprotonated 3-AQ (\(m/z\) 143) and a deprotonated covalent 3-AQ dimer (\(m/z\) 285). Putative borate complexes were assigned as bis-sucrose borate at 690.22/691.22 (Fig 3) and bis-N-acetyl-serine borate at 300.11/301.11 (Fig 4) respectively (Table 1). Mixtures of sucrose and boric acid (Fig 5) and N-acetyl-serine 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.

In wheat phloem exudate, MALDI-TOF-MS identified one \([L_2B]^-\) ion with \(m/z\) values of 690.22 and 691.22 with a natural abundance of \(^{10}\text{B}:^{11}\text{B} (20:80\ \%)\) 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 the 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 three weeks and using LC-ESI-QQQ, the borate complexes at 690.22 and 691.22 were present with a concentration of the borate-dimer of 52 \(\mu\text{M}\) and 220 \(\mu\text{M}\) for the younger and older plant respectively.

To investigate the binding position between sucrose and borate, two standard mixtures were analysed by MALDI-TOF-MS (Fig 7). The first mix contained 400 mM 1-\(o\)-methyl-alpha-D-glucopyranoside and \(\text{trans}\)-cyclohexane-1,2-diol and 1,6-anhydro-glucosan, with and without the addition of 0.3 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 \(\text{trans}\)-cyclohexanediol and 1,6-ahydroglucosan have pKa’s which 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 \([L_2^{10}\text{B}]^-\) ion with a \(m/z\) 394.14 and a \([L_2^{11}\text{B}]^-\) ion at
395.14 (Fig 7 insert). The absence of a trans-cyclohexane-diol [L2B]-1 ion at m/z 238/239 and a 1,6-anhydroglucosan [L2B]-1 ion at m/z 330/331 further implicates the involvement of the 4,6 hydroxyl pairs from two α-methyl-glucopyranoside molecules forming a [L2B]-1 borate complex. The 4,6 hydroxyl pairs on the α-methyl-glucopyranoside molecule within sucrose is therefore implicated as the most likely site for borate complexing.

Borate complexes in Chara cytoplasm

The MALDI-TOF-MS spectrum of Chara cytoplasm is quite complex with many low-molecular weight compounds present (Fig 8). One [L2B]-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-serine complex. The predicted isotopic abundance of [10B]:[11B] clearly identifies a common [L2B]-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, 2001a). Due to the inherently high permeability of boric acid across cellular membranes (10^-7 cm s^-1; Dordas et al., 2000; Stangoulis et al., 2001a), 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 species (i.e. B. napus) that translocate sucrose as their primary photoassimilate, the mechanism of B mobility has until now not been identified. Results presented in this study provide 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 Brassica napus and most likely the related species, B. 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 borate complexes at m/z of 300/301 and 690/691, which we infer to be due to the presence of a bis-N-acetyl-borate and bis-sucrose 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) and the concentration of the borate dimer quantified at up to 220 µM. These results provide further evidence to support mobility of boron in the phloem of this species which translocates sucrose as its primary photoassimilate.

In this study, phloem sucrose was quantified with concentrations around 300 mM while boron
concentration was determined by ICP-OES at 0.3 mM, hence the sucrose:borate ratio is very high. This, together with the high pH of the phloem exudate (measured at close to pH 8.5) favours the formation of a bis-sucrose borate complex. The complex is weak with stability constants of borate-sucrose complexes having Log $\beta_1$ and Log $\beta_2$ values for [LB]$^{-1}$ and [L$_2$B]$^{-1}$ of 0.86 and 0.70 respectively (Verchere and Hlaibi, 1987). This is in contrast to the high stability constants for fructose-borate complexes of 2.82 (Log $\beta_1$) and 4.97 (Log $\beta_2$) for the [LB]$^{-1}$ and [L$_2$B]$^{-1}$ complexes respectively (Verchere and Hlaibi, 1987). The low stability constants between sucrose and borate are due to the complexation site not being on the fructose moiety, but on the 4,6 glucose hydroxyls of the sucrose molecule. Results presented in this study also support this observation; that borate complexes to sucrose via the 4,6 hydroxyl pairs on the $\alpha$-methyl-glucopyranoside molecule.

In the phloem, the bis-sucrose borate complex will be in a dynamic equilibrium with uncomplexed sucrose and will therefore obey source-sink rules. It is likely that during phloem loading of sucrose in a leaf, free B in the leaf cell cytoplasm would equilibrate with the phloem because of its high membrane permeability. The sucrose would act as a trap which would effectively result in an accumulation of B in the phloem. As the sucrose is unloaded, B would be released and would diffuse into the sink cells. Given the high requirement for boron during the reproductive phase of plant growth (Rerkasem and Jamjod, 2004), the B delivered to the florets via the bis-sucrose borate complex could be very significant in maintaining reproductive success. The absence of a bis-sucrose borate complex in Chara cytoplasm, where the sucrose concentration is significantly lower (around 20 mM (Ding et al., 1992)) to the level of sucrose in plant phloem, may also indicate that on unloading from the phloem, the bis-sucrose 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 serine in canola phloem or Chara cytoplasm was not quantified in this study due to inadequate amounts of phloem sample for analysis. However, un-acetylated serine is known to be one of the major amino acids in the phloem and has been reported to total 14 % (approx. 62 mM) of the total amino acids present in B. napus (var. Duplo) phloem exudate (Lohaus and Moellers, 2000) so one would also expect a similar content in the phloem sap of the Barossa variety which was used in this study. Likewise, serine makes up around 3 % (approx. 2.9 mM) of total amino acids in the cytoplasm of Chara (Sakano et al., 1984). B concentration in the cytoplasm was measured by ICP-OES at 13 $\mu$M (Stangoulis et al., 2001a), 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 et al., 2001). 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-serine 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 serine-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 references therein) and therefore a similar effect of the serine-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 serine borate complex.

**Materials and Methods**

*Canola Phloem Exudate Collection*

Phloem exudate was collected from more than 10 field grown canola plants (cultivar 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 MΩ resistivity). The initial droplet of phloem exudate formed was discarded to minimize contamination. Exudate was collected over the following 20-30 min into small Eppendorf® tubes placed on ice. Samples were then 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 plants overnight using specially prepared cages. The cages were removed and stylectomy was performed using high-frequency microcautery under a microscope (Lecia MZ16) using a chemically sharpened tungsten needle. Exudate was collected using glass micro-capillaries and exudate volume quantified using an attached Lecia DFC280 digital camera in combination with the Lecia application suite software (V2.8.1 with multi-time 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 micro-capillary 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 LC-ESI-QQQ. 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. (2001) 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. Vacuolar 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 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-aminoquinoline (3-AQ, Aldrich) in ethanol (approx 0.4 M, used as MALDI matrix). The samples were allowed to dry under ambient laboratory conditions (Hu et al., 1997; Penn et al., 1997). Samples were analysed using a Perseptive Biosystems DE-STR time-of-flight mass spectrometer (Applied Biosystems, Framingham, Ma, USA) 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 ± 20 ppm.

**LC-ESI-QQQ analysis**

Samples of phloem exudate were re-dissolved in 10 µL of 50% MeCN NH₄HCO₃ (1 mM, pH = 8.02) buffer. 5 µL sample volumes were injected into an Agilent 6410 LC-ESI-QQQ equipped with an Agilent 1200 Series LC system and a Cogent Diamond Hydride Guard Column (100A 4µm, 2.0mm x 20mm). Running buffers included 100% NH₄HCO₃ (1.5 mM, pH = 8.05) (Buffer A) and 90% Acetonitrile with 10% NH₄HCO₃ (1.5 mM, pH = 8.05) (Buffer B), while the gradient used was 100% Buffer B for 5 minutes, followed by 75% Buffer B for a further 5 minutes and then followed by re-equilibration with 100% Buffer B over 10 minutes. QQQ parameters included a selected ion monitoring (SIM) 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 4000. Sucrose-borate concentrations in the phloem were quantified using a standard calibration curve of known sucrose-borate concentrations.

References


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Fig 1. Negative mode MALDI-TOF-MS full spectrum of canola phloem exudate co-crystallized in 3-aminoquinoline.

Fig 2. Negative mode MALDI-TOF-MS spectrum of borate complexes isolated from canola phloem exudate co-crystallized in 3-aminoquinoline.

Fig 3. Putitive bis-sucrose borate complex isolated from canola and wheat phloem.

Fig 4. Putitive bis-N-acetyl-L-serine borate complex isolated from canola phloem and Chara cytoplasm.

Fig 5. Negative mode MALDI-TOF-MS of 300 mM sucrose + 0.3 mM boric acid (pH adjusted to 9.0 with KOH) co-crystallized in 3-aminoquinoline.

Fig 6. Negative mode MALDI-TOF-MS full and expanded spectrum of 50 mM N-acetyl-DL-serine + 10 mM boric acid co-crystallized in 3-aminoquinoline.

Fig 7. Negative mode MALDI-TOF-MS full (A) and expanded (B) spectrum of 400 mM 1-σ-methyl-α-D-glucopyranoside + 400 mM 1-transcyclohexane1,2-diol + 40 mM 1,6 anhydro-B-D-glucose + 0.3 mM boric acid (pH adjusted to 9 with NH3OH) co-crystallized in 3-aminoquinoline.

Fig 8. Negative mode MALDI-TOF-MS of Chara cytoplasmic fluid co-crystallized in 3-aminoquinoline.
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<th>Formula (all $-1$ charge)</th>
<th>Molecular weight</th>
<th>Description</th>
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<td>$(C_{10}H_{14}N_{2}O_{8})^{11}B$</td>
<td>301.11</td>
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<td>$(C_{24}H_{40}O_{22})^{10}B$</td>
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<tr>
<td>$(C_{24}H_{40}O_{22})^{11}B$</td>
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<td>Borate complex with 2 Suc</td>
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