Boron (B) deprivation induces various responses in plant cells, some of which can be observed very early. However, it has been unknown what kind of signal is generated by the stress. We found that B deprivation induced the expression of stress-responsive genes within 1 h in suspension-cultured tobacco BY-2 cells. The induction was largely suppressed by withholding medium Ca²⁺ or by adding a Ca²⁺ channel blocker. Analysis using aequorin-expressing cells showed that B-deprived cells took up more Ca²⁺ than control cells. These results suggest that Ca²⁺ influx plays a role in B deprivation stress signaling.

**Keywords:** Boron deficiency • Calcium influx • Pectic polysaccharides • Stress signaling • Tobacco.

**Abbreviations:** AA, o-anisic acid; B, boron; DPI, diphenyleneiodonium; RG-II, rhamnogalacturonan II; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; RT–PCR, reverse transcription–PCR; SA, salicylic acid.

Boron (B) is an essential micronutrient for vascular plants. In cell walls, B as borate forms diester bonds with rhamnogalacturonan II (RG-II) to cross-link pectic polysaccharides (Kobayashi et al. 1999). The B–RG-II complex contributes significantly to the tensile strength of the cell wall (Ryden et al. 2003), and is essential for normal plant growth and development (O’Neill et al. 2001). Removal of B from the nutrient solution causes oxidative damage and cell death in plants (Dordas and Brown 2005, Kobayashi et al. 2009). Various cellular responses occur prior to cell death, some of which can be observed very early. A rapid and transient decrease of cell wall elasticity has been observed in squash roots within 10 min after B deprivation (Findeklee et al. 1997). Symplastic processes are also affected. For example, the amount of cytoskeletal proteins increases in Arabidopsis roots as early as 20 min after B deprivation (Goldbach et al. 2001). We previously reported that, in suspension-cultured tobacco BY-2 cells, B deprivation induces the expression of salicylate-inducible flavonoid glucosyltransferase (TOGT) within 30 min (Kobayashi et al. 2004). These observations indicate that cells detect the disappearance of B shortly after the deprivation. However, it is not known how plant cells sense the external B status, what kind of signal is generated upon B removal or via which pathway the signal is transmitted. Clarifying these processes is important to better understand the role of B in plants.

To gain insights into the mechanism for B deprivation responses of plants, we investigated immediate-early changes in gene expression in B-deprived tobacco BY-2 cells, with which we previously characterized B deprivation-induced cell death (Koshiba et al. 2009). Cells were washed with either a standard or B-free culture medium, and then cultured in the same medium (hereafter referred to as +B or –B cells, respectively). First, we carried out a PCR-based cDNA differential subtraction between +B and –B cells at 1 h, but could not identify any genes with a marked difference in expression between treatments. Next, using semi-quantitative reverse transcription–PCR (RT–PCR) analyses, we examined whether known immediate-early stress-responsive genes of tobacco were differentially expressed between +B and –B cells at 1 h after treatment. The results are shown in Fig. 1. TOGT was included in the analysis as a known −B-responsive gene (Kobayashi et al. 2004). Besides TOGT, the following genes were induced in −B cells: phenylalanine ammonia-lyase (PAL; Pellegrini et al. 1994), an NPR1/NIM1-interacting protein (NIMIN2a; Weigel et al. 2001), an ethylene-responsive element-binding protein (EREBP1; Horvath et al. 1998), mitochondrial alternative oxidase (AOX; Maxwell et al. 1999), wound-inducible protein kinase (WIPK; Seo et al. 1999), a wound-inducible gene with unknown function (KED; Hara et al. 2000a), a wound-responsive leucine zipper zinc finger protein (WIZZ; Hara et al. 2000b), a harpin-induced protein (HIN1; Gopalan et al. 1996) and a class IV chitinase (ChitIV; Shinya et al. 2007) (Fig. 1a, lanes +B and –B). The expression of PR1a, PR1b, PR2a and PR2b was not different between the treatments at this time point (data not shown).

TOGT, PAL, NIMIN2a, EREBP1 and AOX are salicylic acid (SA)-inducible genes (Pellegrini et al. 1994, Horvath and Chua 1996, Horvath et al. 1998, Ji and Ding 2001). Induction of these genes upon B deprivation prompted us to examine if SA was involved in the early response of tobacco cells to B deprivation. Unwashed control cells contained 8.3 ±0.9 ng S.A g FW⁻¹. The SA content of 0.5 h –B cells was slightly higher than this value, but was not
significantly different from that of 0.5 h +B cells (Table 1). Thus we could not ascribe the observed up-regulation of SA-responsive genes to a short-term SA accumulation. Induction of those genes may not be exclusive to SA, or there may be cross-talk between signaling pathways for SA and −B responses.

Table 1 SA content of the cells with +B or −B treatment

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Salicylic acid (ng g FW⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+B</td>
</tr>
<tr>
<td>0.5</td>
<td>13.6±2.6</td>
</tr>
<tr>
<td>1</td>
<td>9.7±1.8</td>
</tr>
</tbody>
</table>

Values represent the mean±SD (n=3).

To obtain further evidence for the Ca²⁺ involvement in −B stress signaling, we examined Ca²⁺ influx into B-deprived cells using aequorin-expressing tobacco BY-2 cells (Takahashi et al. 1997). If B deprivation lets Ca²⁺ channels open, −B cells would take up more Ca²⁺, and therefore emit stronger aequorin luminescence than +B cells. The standard culture medium for tobacco BY-2 cells contains 3 mM CaCl₂. For this experiment, cells containing reconstituted aequorin were washed with and resuspended in a medium containing a minimal concentration of CaCl₂ (20 µM) with or without B. This was to maintain the integrity of the cell wall and membrane, but not to allow Ca²⁺ influx during the treatment. The external Ca²⁺ concentration was then raised to 3 mM to trigger Ca²⁺ influx through the opened channels. As shown in Fig. 2a, −B cells emitted a luminescence twice as strong as that emitted by +B cells. No change in luminescence was observed when water instead of CaCl₂ was added to the suspension (data not shown). Luminescence emission from −B cells was completely suppressed by 1.5 mM of either La³⁺ (Fig. 2b) or Gd³⁺ (data not shown). These results collectively suggest that the observed luminescence represents a Ca²⁺ influx through channels. Since +B cells also emitted

Fig. 1 Boron deprivation-induced expression of stress-responsive genes of tobacco. Cells were washed with and resuspended in (a) standard (+B), B-free (−B) or B- and Ca-free (−B−Ca) culture medium, or (b) standard (+B), B-free (−B) culture medium or B-free culture medium supplemented with 1.5 mM La³⁺ (−B+La). (c) Cells were treated as in (a) (+B and −B) or pre-incubated for 1 h in a medium containing 30 mM CaCl₂ prior to B deprivation (high Ca −B). Cells were harvested at 1 h after resuspension and analyzed for the expression of known stress-responsive genes by RT–PCR (see text). C, unwashed control cells; ACT, actin.
Ca²⁺ involvement in −B stress signaling

significant luminescence (Fig. 2a), the luminescence from −B cells could be due in part to a mechanical stress-induced Ca²⁺ influx. Nonetheless, −B cells emitted reproducibly more intense luminescence than +B cells (Fig. 2a), suggesting that the difference between treatments represents the effect of B deprivation. Taken together, we conclude that B deprivation induces an opening of a certain type of Ca²⁺ channel. The change occurred within minutes after disappearance of B from the external medium, since in this experiment Ca²⁺ uptake was measured as early as 7 min after the onset of the washing procedure.

As shown in Fig. 2b, diphenyleneiodonium (DPI), a plasma membrane NAD(P)H oxidase inhibitor, suppressed 80% of luminescence emission from −B cells. This result suggests that reactive oxygen species (ROS) produced by NAD(P)H oxidase are necessary to activate −B-induced Ca²⁺ influx. Mitogen-activated protein kinase (MAPK) seems to work downstream of Ca²⁺ influx, since B deprivation induced WIPK expression in a Ca²⁺-dependent manner (Fig. 1a). Thus we identified Ca²⁺, ROS and MAPK as the possible components of the −B stress signaling pathway. To our knowledge, this is the first report to show that Ca²⁺ can act in plant responses to nutrient deprivation.

The molecular mechanism by which cells immediately sense the disappearance of B from the external medium has yet to be identified. The mechanical strength of the squash cell wall decreases rapidly upon B deprivation (Findeklee et al. 1997). The change may be due to insufficient cross-linking of pectic polysaccharides at RG-II regions, as the B−RG-II complex contributes significantly to the tensile strength of cell walls (Ryden et al. 2003). B−RG-II complexes are highly stable in vitro (Kobayashi et al. 1999) and it is unclear at this time whether the B−RG-II complex in the cell wall decomposes immediately after B deprivation. However, it is possible that the B−RG-II bonding in vivo is less stable than in vitro, or that a failure in cross-linking newly secreted polysaccharides is critical. As plant cell walls enclose protoplasm and counteract its osmotic pressure, a decrease in tensile strength of the cell wall could lead to an expansion of the protoplasm. The change would impose stretch stress on plasma membranes to trigger the cell’s signaling cascades. Consistent with this speculation, we found that a high dose of Ca²⁺, which may stabilize the pectic network structure, abolished the −B response of some genes (Fig. 1c). Hence we propose that plasma membrane mechanosensitive Ca²⁺ channel(s) are involved in the B deprivation response of tobacco BY-2 cells. In yeast, a similar mechanism involving stretch-activated Ca²⁺ channels and MAPK senses and maintains cell wall integrity (Levin 2005). In this study, −B-induced Ca²⁺ influx was suppressed by DPI, a plasma membrane NAD(P)H oxidase inhibitor (Fig. 2b). Several types of Ca²⁺-permeable channels are activated by ROS, while mechanosensitive Ca²⁺ channels are not. Therefore, a plausible mechanism is that a mechanosensitive Ca²⁺ channel mediates an initial Ca²⁺ uptake, which in turn activates ROS production by NAD(P)H oxidase and secondary Ca²⁺ influx through separate ROS-responsive channels. RNAi (RNA interference) suppression of mechanosensitive channels such as MCA1 (Nakagawa et al. 2007) would be necessary to examine this hypothesis.

Materials and Methods

Tobacco BY-2 cells (Nicotiana tabacum L. cv. Bright Yellow-2) were cultured and deprived of B as described (Koshiba et al. 2009). For La²⁺ treatment, phosphate was omitted from the medium to avoid precipitation.

Semi-quantitative RT−PCR analysis was performed as described (Koshiba et al. 2009). The primer sets used are listed in Supplementary Table S1.

Aequorin was reconstituted by incubating 4-day-old apoaequorin-expressing tobacco cells with 2.5 mM native coelenterazine in the culture medium in darkness overnight. Luminescence was measured with GloMax 96 Microplate Luminometer (Promega, Madison, WI, USA). A 100 µl aliquot of cell suspension was transferred to a 96-well microplate, and the medium was pipetted off. Cells were washed twice with low Ca (20 µM) medium with or without B, and resuspended in 300 µl of the same medium. The microplate containing the cell suspension was set in the luminometer chamber, and left at 25°C for 5 min. A 25 µl aliquot of 39 mM CaCl₂ was then injected into the suspension. Luminescence measurement was started 1 min before Ca²⁺ injection and done at 1 s intervals.

Cells were ground in liquid nitrogen with a mortar and pestle. A 1 g aliquot was vigorously mixed with 2 ml of 90% (v/v) methanol containing 1 µg of o-anisic acid (AA) as an internal standard, and centrifuged at 12,000 × g for 5 min at 4°C.
The pellet was re-extracted with 2 ml of methanol. Combined extracts were added with 2 µl of 1 M NaOH and evaporated to near dryness. The condensed extract and washings of the flask using 1 mM NaOH were pooled, and cleaned by passing through a Sep-Pak Plus C18 cartridge (Waters, Milford, MA, USA) pre-conditioned with 1 mM NaOH. The flow-through and the column washing were pooled, acidified with 1 M HCl, and left for 30 min at 25°C. The solution was applied to a fresh Sep-Pak cartridge to collect SA. Absorbed SA was eluted with methanol, evaporated to dryness, and reconstituted in 100 µl of 80% methanol.

The SA and AA concentrations were quantified using liquid chromatography–mass spectrometry with an API3000 LC/MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization interface used to generate negative ions. The SA and AA were separated on a Mightsyl RP-18GP column (3 µm, 2.0 × 100 mm, Kanto Kagaku, Tokyo, Japan) at 40°C with a linear gradient of 2–35% (v/v) acetonitrile in 0.1% (v/v) formic acid in 10 min at a flow rate of 0.2 ml min⁻¹ for SA, or 15–50% (v/v) acetonitrile in 0.1% (v/v) formic acid in 10 min for AA. Quantification was performed by multiple reaction monitoring of the deprotonated precursor ion and the related product ion for SA or AA. The mass transitions (Q1/Q3) used for SA and AA were m/z 137/93 and 151/107, respectively.

**Supplementary data**

Supplementary data are available at PCP online.

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