Vascular tissue-specific expression of \textit{BnaC4.BOR1;1c}, an efflux boron transporter gene, is regulated in response to boron availability for efficient boron acquisition in \textit{Brassica napus}

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Abstract

Aims \textit{BnaC4.BOR1;1c} is required for B acquisition in \textit{Brassica napus} (\textit{B. napus}) under low B stress. This study aimed to reveal the B regulatory mechanism of \textit{BnaC4.BOR1;1c} and its physiological roles in B translocation from roots to shoots and B distribution in shoots.

Methods Transgenic \textit{Arabidopsis} plants expressing GUS (β-glucuronidase) under different promoters were generated and the mRNA, and GUS activity was quantitatively measured. The in-situ PCR and immunohisto-chemistry in \textit{B. napus} were performed to investigate \textit{BnaC4.BOR1;1c} expression pattern and localization. Furthermore, assays of B transport and distribution in wild type \textit{B. napus} and \textit{BnaC4.BOR1;1c} RNAi lines were carried out to elucidate its physiological roles.

Results Results showed that \textit{BnaC4.BOR1;1c} mRNA abundance is negatively correlated with B availability, which was mediated by the 29 nt sequence in the 5′ terminal region of 5′-UTR. Besides, the 5′-UTR simultaneously regulates protein expression level, most probably depending on the translation efficiency. \textit{BnaC4.BOR1;1c} mainly localizes on the plasma membrane of vascular bundle cells in roots and shoots with a polar localization manner that is probably beneficial to B xylem loading in roots and B unloading from xylem to phloem in vascular bundle of shoots. Short-term 10\textsuperscript{B} uptake analysis demonstrates that \textit{BnaC4.BOR1;1c} preferentially distributes B to developing leaves and flowers under B deficiency.

Conclusion This study reveals combined regulatory action of mRNA abundance and translation efficiency mediated by the 5′-UTR in \textit{BnaC4.BOR1;1c} in response to B availability and its physiological role in preferential B acquisition in developing tissues of \textit{B. napus}.

Keywords \textit{Brassica napus} · \textit{BnaC4.BOR1;1c} · Boron response · Boron acquisition

Introduction

Boron (B) is one of the essential micronutrients for plant growth, but B excess is toxic. B plays an essential structural role in maintaining the cell wall integrity by crosslinking two rhamnogalacturonan II (RG-II) molecules to form RG-II-B complexes (O’Neill et al. 2001; Funakawa and Miwa 2015). B is relatively immobile in plant cells; thus, B deficiency defects are early occurred in the developing tissues, such as necrosis and
elongation cessation of the root tip, curl and reduced expansion of young leaves, and low fertility (Broadley et al. 2012; Zhang et al. 2017). Boron deficiency is a worldwide agricultural problem. The continuous B acquisition of plants from the environment mainly depends on two type B transporters. AtNIP5;1 (nodulin 26-like intrinsic protein 5;1) was identified as a boric acid influx transporter, and AtNIP5;1 protein mainly localizes on the plasma membrane (PM) toward the soil-side in the outmost cell layer and endodermis of a root tip in Arabidopsis (Takano et al. 2006, 2010). Loss of AtNIP5;1 function significantly reduces root B uptake capacity (Takano et al. 2006). AtBOR1 functions as a boric acid/borate exporter on the PM of root epidermis and endodermis although expression in the cotyledon tip also reported (Takano et al. 2002; Yoshinari et al. 2016). In contrast to AtNIP5;1, AtBOR1 localizes on the PM toward the stele-side (Takano et al. 2010). Furthermore, many homologs of Arabidopsis NIP5;1 and BOR1 have been functionally characterized in succession in various species such as rice (Nakagawa et al. 2007; Hanaoka et al. 2014; Shao et al. 2021), barley (Sutton et al. 2007), wheat (Leanuhtitikananchana et al. 2013), maize (Chatterjee et al. 2014; Durbak et al. 2014; Leonard et al. 2014) and oilseed rape (Zhang et al. 2017).

Efficient acquisition and avoiding B toxicity need a fine-tuning of the B homeostasis due to the narrow window of physiological B concentration between deficiency and toxicity (Warington 1923; Nable et al. 1997). B concentration in vivo is negatively correlated with the window of physiological B concentration between fine-tuning of the B homeostasis due to the narrow B concentration in vivo is negatively correlated with the B efficiency and toxicity (Warington 1923; Nable et al. 1997). The distinct B-dependent regulatory mechanisms are found in the AtBOR1 gene expression regulation (Takano et al. 2010; Aibara et al. 2018). Protein translation efficiency of AtBOR1 is inhibited upon the 5'-UTR, and its protein suffers degradation in the vacuole under high B stress (Kasai et al. 2011; Takano et al. 2010; Aibara et al. 2018). Besides, overexpression of AtBOR4 (paralog of AtBOR1) can exclude B from the root resulting in enhanced tolerance to B toxicity (Miwa et al. 2007). A similar role of Bot1 (AtBOR1 homolog) in barley was characterized, and a high copy number of Bot1 increases barley tolerance to B toxicity (Reid 2007; Sutton et al. 2007).

Developing tissue with low transpiration preferentially acquires B from xylem sap in many plants suggesting that B transporter might contribute to B translocation (Marentes et al. 1997; Huang et al. 2001; Takano et al. 2001; Matoh and Ochiai 2005). To date, AtNIP6;1 and AtNIP7;1 were functionally characterized to facilitate B acquisition in young leaves and flowers (Tanaka et al. 2008; Li et al. 2011; Routray et al. 2018). In rice, OsNIP3;1 acts as a boric acid channel and preferentially distributes B to the developing tissues by unloading B from the xylem in nodes (Hanaoka et al. 2014; Shao et al. 2018). Besides, mineral element distribution in shoot tissues mediated by their transporters in the nodes has been well established in rice (Yamaji and Ma 2014, 2017). However, little is known about the exact role of members of the BORs family in distributing B in shoots. We previously identified a boric acid/borate transporter gene BnaC4.BOR1;1c in Brassica napus (B. napus) with low B inducible expression pattern (Sun et al. 2012). BnaC4.BOR1;1c localizes in the stele of root, nodes in shoots, and base of the floral organ (Zhang et al. 2017). RNA interference of BnaC4.BOR1;1c significantly reduced B concentrations in shoots and flowers accompanied by the B defective phenotype of B. napus (Zhang et al. 2017), probably due to the reduced B xylem loading in roots. However, the B-dependent regulatory mechanism of BnaC4.BOR1;1c and its physiological role in B distribution in shoot tissues remains unclear. Here, we found that B not only regulates mRNA abundance but also adjusts BnaC4.BOR1;1c protein level in a 5'-UTR-dependent manner. Furthermore, tracer B experiments indicate that BnaC4.BOR1;1c plays a vital role in preferentially distributing B from xylem to phloem in nodes for developing tissue growth under B deficiency.

Results

The 5'-UTR is required for the B-dependent regulation of BnaC4.BOR1;1c

It has been reported that low B upregulates BnaC4.BOR1;1c expression in a B-efficient B. napus cv. ‘QY10’ (Sun et al. 2012). To investigate the mechanism of B-dependent mRNA regulation, the mRNA abundance in B. napus root was quantified by quantitative real-time PCR (qRT-PCR) in the presence of low B (0.25 μM), medium B (25 μM), and high B (250 μM).
Low B enhanced \textit{BnaC4.BOR1;1c} expression relative to medium B condition, while high B significantly inhibited mRNA accumulation compared to medium B condition (Fig. 1a). This regulation pattern of \textit{BnaC4.BOR1;1c} by B supply is similar to the B-dependent mRNA regulatory mechanism of \textit{NIP5;1} in \textit{Arabidopsis} (Tanaka et al. 2011, 2016). The 5′-UTR sequence of \textit{BnaC4.BOR1;1c} was identified experimentally (Sun et al. 2012). We, therefore, generated transgenic \textit{Arabidopsis} carrying the \textit{ProBOR1;1c} (5′-UTR): GUS and 5′-UTR-deleted \textit{ProBOR1;1c} (Δ5′-UTR): GUS to evaluate their effects on the expression abundance (Fig. 1b). The relative expression level of \textit{GUS} in plants grown on 0.25 μM B or 250 μM B was determined. High B treatment accumulated lower \textit{GUS} mRNA abundance relative to low B treatment in plants carrying \textit{ProBOR1;1c} (5′-UTR): GUS construct (Fig. 1c). Construct with deletion of 5′-UTR was almost entirely insensitive to B regulation (Fig. 1c). These results demonstrate that 5′-UTR of \textit{BnaC4.BOR1;1c} is required for the B-dependent mRNA abundance regulation.

5′ terminal region of 5′-UTR contributes to the B-dependent regulation of \textit{BnaC4.BOR1;1c}

The 5′-UTR sequence of \textit{BnaC4.BOR1;1c} consists of 351 nt upstream of its main open reading frame (ORF), in which two mini uORFs (Fig. 2a) and three more uORFs existed (Fig. S1). Considering the important role of mini uORF and its upstream sequence in B-dependent mRNA regulation (Tanaka et al. 2011, 2016), the constructs of \textit{Pro35S} (5′-UTR): GUS, \textit{Pro35S} (5′-UTRΔ1−29): GUS and \textit{Pro35S} (5′-UTRΔ1−97): GUS were generated to investigate the B response in \textit{Arabidopsis} plants (Fig. 2b). The 29 nt region and 97 nt region in the 5′ terminal region includes the first AUG-stop and second AUG-stop, respectively (Fig. 2a and Fig. S1). Either 1–29 nt deletion or 1–97 nt deletion from the 5′ terminal region in the 5′-UTR completely abolished the high B effect on the mRNA (Fig. 2c), suggesting that the 29 nt sequence in the 5′ terminal region of 5′-UTR is required for the high B response. We further validated this conclusion that deletion of 92–97 nt (corresponding to second AUG-stop, Fig. 2a) in the 5′-UTR maintained a rapid high B response (Fig. 2c). Consistent with this, both 1–29 nt deletion and 1–97 nt deletion significantly reduced the high B inhibition of \textit{GUS} activity relative to low B condition, and the relative \textit{GUS} activity of deletion of the 92–97 nt in the 5′-UTR remained lower level at high B condition (Fig. S2). Interestingly, compared with low B condition, the \textit{GUS} activities of \textit{Pro35S} (5′-UTRΔ1−29): GUS and \textit{Pro35S} (5′-UTRΔ1−97): GUS were partially reduced at high B condition (Fig. S2), though their mRNA levels were comparable at both low B and high B conditions (Fig. 2c). Taken together, these results suggest that the 5′ terminal 1–29 nt region in the 5′-UTR contributes to the B-dependent regulation of \textit{BnaC4.BOR1;1c}.

Localization of \textit{BnaC4.BOR1;1c} in \textit{B. napus}

Undoubtedly, the total reduced B content in the shoots of \textit{BnaC4.BOR1;1c} RNAi plants can be ascribed to the down translocation of B in the roots of \textit{BnaC4.BOR1;1c} (Zhang et al. 2017). Although \textit{BnaC4.BOR1;1c} promoter activity has been observed in the root stele, node, and base of the floral organ in \textit{proBnaC4.BOR1;1c}: \textit{GUS} transgenic \textit{Arabidopsis} (Zhang et al. 2017), the details of \textit{BnaC4.BOR1;1c} localization in these tissues of \textit{B. napus} is not clarified. To this end, we first performed the in-situ PCR (Athman et al. 2014) of \textit{BnaC4.BOR1;1c} in the root, hypocotyl, and node of \textit{B. napus}. For the negative control, no \textit{BnaC4.BOR1;1c} reverse primer was used in the reverse transcription PCR. Compared with negative control (Fig. 3a), a strong signal was detected in the cells surrounding the stele of root (Fig. 3b). In the hypocotyl, the signal mainly existed in the vascular tissues (Fig. 3d, e), while no detectable signal was showed in the negative control (Fig. 3c). Then the localization of \textit{BnaC4.BOR1;1c} mRNA in the nodes was investigated. The vascular bundles showed clear signals (Fig. 3f), including the xylem, cambium (Fig. 3h), and indeed no specific signal was detected in these cells of negative control (Fig. 3g).

In parallel, we performed immunohistochemistry staining of the roots and nodes of \textit{B. napus} using an antibody that was generated against an artificial peptide of the \textit{BnaC4.BOR1;1c} region (SSTPLNNRSLSSPR). The plants ‘QY10’ and RNAi plants grown on 0.25 μM B were sampled for the positive and negative control, respectively. The strong green signal was imaged on the PM of cells surrounding stele in ‘QY’ roots (Fig. 4a, b) compared to the RNAi plants (Fig. 4c, d). In line with the polar localization of \textit{AtBOR1} on the PM of root cells toward the stele-side (Takano et al. 2010), the polar localization toward the stele-side was observed in \textit{B. napus} (Fig. 4a, b). In the nodes of \textit{B. napus}, the signal...
Fig. 1 5′-UTR mediates high B response of BnaC4.BOR1;1c. a Relative expression levels of BnaC4.BOR1;1c gene in response to low (0.25 μM B), normal (25 μM B), and high B (250 μM B) treatments. Roots of 15-day-old seedlings grown in hydroponic solution with different B supply were used. Values represent means ± SD (n = 3 independent lines). Different letters indicate significantly different values (one-way ANOVA, Duncan’s test). b Schematic representations of ProBnaC4.BOR1;1c (5′-UTR):GUS and ProBnaC4.BOR1;1c (Δ5′-UTR):GUS constructs. c Relative expression levels of these two constructs in the transgenic Arabidopsis lines responded to B treatments (0.25 μM B and 250 μM B). 10-day-old Arabidopsis seedlings grown on 100 μM B medium were transferred to 0.25 μM B or 250 μM B medium for 2 d growth. Experiments were performed using 3 independent homozygous lines. Values represent means ± SD (n = 3). *p < 0.05, Student’s t test
was mainly detected in the xylem and cambium of vascular bundles (Fig. 4e-h). Polar localization on the cambium cell toward to phloem was observed. Furthermore, 250 μM B is sufficient to abolish BnaC4.BOR1;1c protein accumulation in roots and nodes (Fig. 4i-l). Because the artificial peptide of BnaC4.BOR1;1c was partially conserved among BnaBOR1s (Fig. S3), the results presumably represent the localization of multiple

**Fig. 2** 5′ terminal region of 5′-UTR is required for high B response of BnaC4.BOR1;1c. a Sequences of two mini uORFs (AUGUA) in 5′-UTR. b, c Truncation analyses of 5′-UTR for high B regulation of BnaC4.BOR1;1c. Truncated sequences with deletion of 29 nt, 97 nt, and 92–97 nt in 5′-UTR were fused to 35S promoter to express GUS protein in transgenic Arabidopsis, respectively (b), and their relative expression levels in response to B treatments were investigated (c). 10-day-old Arabidopsis seedlings grown on 100 μM B medium were transferred to 0.25 μM B or 250 μM B medium for 2 d growth. Values represent means ± SD (n = 3). * p < 0.05, Student’s t test

**Fig. 3** In situ PCR analysis of BnaC4.BOR1;1c in B. napus. In situ PCR analysis was performed in roots (a, b), hypocotyl (c-e), and node (f-h) of B. napus. e and h are the enlarged image of d and f, respectively. Negative control in roots (a), hypocotyl (c), and node (g) was prepared due to the reverse transcription PCR without primer supply. 20-day-old B. napus seedlings grown on 25 μM B medium were transferred to 0.25 μM B medium for 2 d growth. Fresh plant tissues were cut and fixed in FAA solution for subsequent in situ PCR analysis
BnaBOR1 proteins in *B. napus*. To clarify the localization of BnaC4.BOR1;1c, transgenic Arabidopsis expressing BnaC4.BOR1;1c-GFP was generated. Polar localization of BnaC4.BOR1;1c-GFP toward stele-side on the PM of both epidermis and endodermis was observed in Arabidopsis (Fig. 4m-o). Taken together, these results demonstrate that BnaC4.BOR1;1c is polarly localized on the PM of cells in vascular tissues.

**Fig. 4** BnaC4.BOR1;1c localizes on the vascular cell layers of tissues. Immunohistological staining of BnaC4.BOR1;1c using specific anti-BnaC4.BOR1;1c antibody in roots and nodes. Roots (a, b) and nodes (e, f) of wild-type *B. napus* and roots of RNAi plant (c, d) grown in 0.25 μM B were used. b close-up view of a and g, h close-up view of e were shown. 250 μM B treated wild-type roots (i, j) and nodes (k, l) were used to inhibit BnaBOR1s expression in *B. napus*. Xy, xylem; CZ, cambium; Ph, Phloem. Localization analysis of Arabidopsis expressing 35S: BnaC4.BOR1;1c-GFP in epidermis (m) and endodermis (o). FM4–64 (magenta) was used to stain the plasma membrane. The GFP signal intensity on the plasma membrane toward the soil-side (outer) and the stele-side (inner) was quantified by line plot (n). Ep, epidermis; En, endodermis

BnaBOR1 proteins in *B. napus*. To clarify the localization of BnaC4.BOR1;1c, transgenic Arabidopsis expressing BnaC4.BOR1;1c-GFP was generated. Polar localization of BnaC4.BOR1;1c-GFP toward stele-side on the PM of both epidermis and endodermis was observed in Arabidopsis (Fig. 4m-o). Taken together, these results demonstrate that BnaC4.BOR1;1c is polarly localized on the PM of cells in vascular tissues.

BnaC4.BOR1;1c transports B in yeast and in *B. napus*

Despite the symptoms of B deficiency were observed in BnaC4.BOR1;1c RNAi *B. napus* (Zhang et al. 2017), the transport activity of BnaC4.BOR1;1c is not validated in the plant. Previously, the B transport activity of BnaC4.BOR1;1c in yeast was reported (Diehn et al. 2019), while yeast expressing BnaC4.BOR1;1c did not...
show stronger growth than that of yeast expressing empty vector on the high B medium (Diehn et al. 2019). To clarify the B transport activity of BnaC4.BOR1;1c, yeast Sbor1 mutant was employed to express target genes. AtBOR1 was used as a positive control because its B transport activity had been demonstrated (Takano et al. 2002). In agreement with the result (Diehn et al. 2019), the yeasts with induction of pYES2, AtBOR1, and BnaC4.BOR1;1c did not show distinct growth inhibition on the medium without B, while the best growth was observed for the AtBOR1-yeast, then followed by BnaC4.BOR1;1c-yeast, and the pYES2-yeast at the 20 mM B medium (Fig. S4). The similar growth between pYES2-yeast and BnaC4.BOR1;1c yeast cannot strongly demonstrate that BnaC4.BOR1;1c can export B from yeast cells. Thus, we further compared the intracellular B concentrations among these yeasts. Yeasts were incubated in 500 μM B for 60 min in galactose medium, the pYES2-yeast had about 1.1 mmol B/kg DW, while both AtBOR1-yeast and BnaC4.BOR1;1c-yeast had lower B concentrations (~0.34 mmol B/kg DW and ~0.52 mmol B/kg DW) (Fig. 5a). This result demonstrated that BnaC4.BOR1;1c has B transport activity. The B transport activity of BnaC4.BOR1;1c was further investigated in B. napus using the RNAi lines for B’s transient uptake assay (Zhang et al. 2017). Plants grown hydroponically with 100 μM 11B were transferred to 0.25 μM 11B for 24 h, then 5 μM 10B for 24 h, and the 10B concentrations in roots and shoots were measured by inductively coupled plasma-mass spectrometry (ICP-MS). The amount of 10B transported into shoots per unit dry weight of roots within 24 h was defined as the BnaC4.BOR1;1c function in roots. The RNAi lines showed lower 10B accumulation in shoots than wild-type ‘QY10’ (Fig. 5b), indicating that BnaC4.BOR1;1c can facilitate B translocation from roots into shoots.

BnaC4.BOR1;1c contributes to preferential B acquisition of developing tissues under B deficiency

To investigate whether BnaC4.BOR1;1c contributes to B acquisition in developing tissues in shoots such as flower and young leaves; labeling experiments were performed using the isotope B. The top inflorescence of wild-type ‘QY10’ and BnaC4.BOR1;1c RNAi lines grown in 11B-enriched solution (100 μM) were incubated in 5 μM 10B-enriched solution for two days. ICP-MS measurement of 10B concentration showed that wild-type buds had higher B concentrations than RNAi buds (Fig. 6a). This result indicates that BnaC4.BOR1;1c contributes to B acquisition of buds. B is usually immobile in most plant species (Brown and Shelp 1997). We tested the B mobility in B. napus and the potential role of BnaC4.BOR1;1c in this process using RNAi lines. Seedlings grown in 11B-enriched solution (100 μM) were transferred to 0 μM B for two days. B concentration was reduced in the new leaves and old leaves (Fig. 6b). No obvious reduction was observed in other tissues. The distribution ratio of B in new leaves was increased.

![Figure 5](image_url)
BnaC4.BOR1;1c preferentially transports B into developing tissues under B deficiency. a Comparison of transient B uptake in ‘QY10’ and BnaC4.BOR1;1c RNAi lines. Inflorescences cut from plants grown in a pot with 100 μM 11B irrigation were cultured 2 d in a hydroponic box with 5 μM 10B. b, c Comparison of B centration and B distribution ratio in ‘QY10’ and BnaC4.BOR1;1c RNAi lines. 22-day-old seedlings (4 true leaves stage) grown on 100 μM 11B-enriched hydroponic solution was treated with B deprivation for 2 d. Tissues for B concentration measurement were sampled before B deprivation (+ B) and after B deprivation (0 B). Significant differences between + B and 0 B were statistically analyzed. Values represent means ± SD (n = 3). *: p < 0.05, Student’s t test. d Chimera plants generation. Scions of 10-day-old (cotyledon age) ‘QY10’ and RNAi lines were inserted into ‘QY10’ rootstock to produce graftWT, graftRNAi1, and graftRNAi4. The graft junction was supported by a silicon tube, and one week high humidity is required to maintain a high survival ratio. 22-day-old chimera plants (4 true leaves) were used for the trace experiment. The leaves order was indicated in (d). e, f Comparison of transient B uptake and B distribution ratio in chimera plants. Chimera plants grown in 100 μM 11B-enriched solution were transferred to 0.1 μM 10B-enriched medium for 1 day, followed by 2 d exposure in 1 μM 10B-enriched solution. Tissues for B concentration measurement were sampled before and after 10B treatment. Values represent means ± SD (n = 3). Different letters indicate significantly different values (one-way ANOVA, Duncan’s test). R: root; H: hypocotyl; C: cotyledon; P: petiole; OL: old leaves; NL: new leaves; ST: stem.
after B starvation, while it was decreased in old leaves (Fig. 6e). No big changes were detected for other tissues. Although a small amount of B was transported into new leaves from other tissues, the distribution pattern among wild-type plants and RNAi lines was similar, suggesting that BnaC4.BOR1;1c did not contribute to this process. To clarify whether BnaC4.BOR1;1c is involved in preferential B translocation into new leaves, we performed the transient B uptake assay of BnaC4.BOR1;1c in roots, chimera seedlings. To avoid the B transport activity of BnaC4.BOR1;1c in the shoots play an essential role in their substrate availability mediated by 5’-UTR of BnaC4.BOR1;1c for the B distribution into leaves in rice (Shao et al. 2018). (Tanaka et al. 2008) and OsNIP3;1 for the B distribution into leaves in rice (Shao et al. 2018).

BnaC4.BOR1;1c is regulated in response to B availability mediated by 5’-UTR

B homeostasis is essential for the optimal growth of the plant through fine-tuning B transporter expression. In China, a large agricultural soil area of usually possesses low B concentrations, which cannot satisfy the growing demand of B. napus (Xu et al. 2001). BnaC4.BOR1;1c was up-regulated by low B in the root, shoot, and flower of B. napus ‘QY10’ (Sun et al. 2012), and its expression level increased gradually within 24 h of low B treatment (Chen et al. 2018). Low B inducible expression of BnaC4.BOR1;1c probably contributes to the B efficiency in ‘QY10’. However, the low sensitivity of BnaC4.BOR1;1c to low B stress was reported in a European winter-type B. napus cv. Darmor-PBY018 (Diehn et al. 2019). Therefore, different B. napus cv. might have distinct responses of BnaC4.BOR1;1c.

In this study, we demonstrate that the 5’ terminal 29 nt in the 5’-UTR of BnaC4.BOR1;1c of ‘QY10’ mediates the B response (Figs. 1 and 2). In Arabidopsis, the transcription level of AtNIP5;1 was down-regulated by high B (Takano et al. 2006), and the 5’-UTR of AtNIP5;1 is required for B-dependent mRNA degradation (Tanaka et al. 2011). Furthermore, the ‘AUGUAA’ cis-element in the 5’-UTR and its upstream conserved sequence ‘UAUA’ were found to induce ribosome stalling and mRNA degradation under high B conditions (Tanaka et al. 2016). Interestingly, the 29 nt in the 5’-UTR of BnaC4.BOR1;1c includes a mini uORF (AUGUAA) and, in particular, a sequence consensus ‘CAUA’ at the same position (Fig. S1) corresponding to the ‘UAUA’ in 5’-UTR of AtNIP5;1. These results suggest that a common mechanism of B-dependent mRNA regulation might exist in both AtNIP5;1 and BnaC4.BOR1;1c in ‘QY10’. However, the high B response of AtNIP5;1 is stronger (Takano et al. 2006) than that of BnaC4.BOR1;1c, probably due to the sequence inconsistency between them. ‘ZS11’ is a conventional commercial cultivar with high B efficiency in China (Hua et al. 2016). We compared their 5’-UTR sequences of BnaC4.BOR1;1c (Fig. S5) and found significant differences existed in the region between uORF1 and uORF2. Most importantly, mRNA degradation associated upstream conserved sequence before uORF (‘UAUA’ in AtNIP5;1 and ‘CAUA’ in ‘QY10’ BnaC4.BOR1;1c) was ‘ACA’ in ‘ZS11’ (Fig. S5). This result implies that BnaC4.BOR1;1c might have different B responses among B. napus lines.

On the other hand, AtBOR1 showed two B-dependent regulatory mechanisms, in which high B rapidly triggers BOR1 protein endocytosis from PM for degradation in the vacuole (Takano et al. 2005, 2010; Kasai et al. 2011), and continuous toxic B supply induces translational suppression in the 5’-UTR (Aibara et al. 2018). The high B-dependent translational suppression in the 5’-UTR of AtBOR1 was mediated by the multiple uORFs (Aibara et al. 2018). B-dependent regulation of AtNIP5;1 together AtBOR1 cooperatively maintain B homeostasis. We found a total of 5 uORFs in the 5’-UTR of BnaC4.BOR1;1c (Fig. S1). Despite the deletion of 29 nt or 97 nt abolished the high B effect on
mRNA abundance, the protein level was reduced partially under high B treatment (Fig. S2). One possibility is that high B inhibits protein translation efficiency through 5’-UTR of BnaC4.BOR1;1c. The sequences uORF3, uORF4 and uORF5 in 5’-UTR of BnaC4.BOR1;1c between ‘QY10’ and ‘ZS11’ possess high conservation (Fig. S5), suggesting B-dependent protein translation regulation of BnaC4.BOR1;1 gene is generalized for B. napus. Together, these results revealed a combined regulatory action of mRNA abundance and translation efficiency mediated by the 5’-UTR of ‘QY10’ BnaC4.BOR1;1c in response to B availability.

BnaC4.BOR1;1c is responsible for B loading in roots and preferential distribution of B to developing tissues in shoots

It is conceivable that BnaC4.BOR1;1c may have common characteristics as AtBOR1 because of the high similarity of protein sequences such as polar localization of BnaC4.BOR1;1c on the PM of root cells toward the stele-side (Fig. 4). This polar localization presumably contributes to efficient B loading into the xylem in roots.

In Arabidopsis, polar localization of AtBOR1 localizes on the PM of epidermis and endodermis toward the stele-side in roots was proposed to efficiently load B into xylem for translocation (Takano et al. 2008). The B transport activity assays of BnaC4.BOR1;1c in yeast and B. napus directly support that BnaC4.BOR1;1c is responsible for B loading in the root (Fig. 5).

Developing tissue in the shoot has low or no transpiration capability; thus the mineral elements demand is proposed to be facilitated preferentially by various transporters. The connection sites such as node and base of floral organ linking developing tissues and stem are important hubs for the nutrient’s distribution (Yamaji and Ma 2014). In Arabidopsis, AtBOR1 contributes to the preferential translocation of B to young leaves, while it is unclear that whether AtBOR1 functions at such hubs (Takano et al. 2001) because no evidence of AtBOR1 in nodes was documented. The xylem-to-phloem transfer of B is mediated by the AtNIP6;1 in Arabidopsis (Tanaka et al. 2008) and by the OsNIP3;1 in rice (Shao et al. 2018) under low B conditions since their specific localization in the vascular cells. Most recently, rice OsBOR1 was found to be polarly localized in bundle sheath cells of nodes and xylem parenchyma cells of the leaf sheath, and it plays a vital role in distributing B to new leaves and panicles (Shao et al. 2021). In this study, we found that BnaC4.BOR1;1c is localized in the vascular xylem and cambium cells region in the nodes of developing tissues (Fig. 3), distinct from the AtBOR1 localization in the shoot (Yoshinari et al. 2016), implying the different roles between AtBOR1 and BnaC4.BOR1;1c. The biomass of B. napus is far more than the biomass of Arabidopsis; thus the efficient B acquisition is necessary to sustain optimal growth. There is little doubt that the vascular localization of BnaC4.BOR1;1c might contribute to B re-distribution or distribution in shoot tissues. B is usually immobile in most plant species (Brown and Shelp 1997). Based on our experiments, BnaC4.BOR1;1c did not contribute to the B re-distribution in shoots (Fig. 6b, c), although a small proportion B was redistributed into young leaves (Fig. 6c) when plants were transferred from adequate B condition to B starvation condition, probably due to free B in plants. The tracer analyses of B uptake in B. napus buds (Fig. 6a) and in grafted plants (Fig. 6d-f) demonstrated that BnaC4.BOR1;1c is important for the preferential distribution of B in developing tissues. These results suggest that BOR1 protein plays a similar role in B. napus and rice. Furthermore, we observed the polar localization of BnaBOR1s, including BnaC4.BOR1;1c on the PM of cambium cells toward phloem (Fig. 4e-h). A reasonable answer to this is that polar localization of BnaBOR1s in cambium cells is beneficial to B translocation from the xylem into the phloem. Taken together, the present data reveal that BnaC4.BOR1;1c is responsible for B loading in root and preferential distribution of B to developing tissues.

Materials and methods

Plant materials and growth conditions

B-efficient B. napus ‘QY10’ (winter-type B. napus in China), the RNAi lines of BnaC4.BOR1;1c (Zhang et al. 2017), transgenic Arabidopsis (Col-0 background) were used in this study. For the pot growth of B. napus, seeds were sown in the soil, which includes 0.85 g NH4NO3 kg−1, 383.3 mg KH2PO4·2H2O kg−1, 250 mg MgSO4·7H2O kg−1, 751.1 mg KCl kg−1. Pots were irrigated with 1500 ml ultrapure water supplemented with micronutrients (3.5 mg 11B, 12.60 mg MnCl2·4H2O, 1.54 mg ZnSO4·7H2O, 0.56 mg CuSO4·5H2O, 0.168 mg Na2MoO4·2H2O, 1.75 mmol

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Fe-EDTA) to maintain proper humidity. For the fluorescence tracer B experiment, the inflorescences with stem segments about 15 cm in length were cut and cultured two days in a hydroponic box with 5 μM 10B. For the hydroponic growth of B. napus, seeds were surface-sterilized. They germinated one week in ultrapure water and then were transferred into a quarter-strength solution treatment and full-strength solution for 5 days growth followed by 5 days half-strength solution treatment and full-strength solution culture. Modified Hoagland’s solution was used in hydroponic growth (Li et al. 2019), in which 100 μM sucrose. Transgenic Arabidopsis lines were established (Clough and Bent 1998) with 1% gellan gum and 1% sucrose. Transgenic Arabidopsis lines were established by the Agrobacterium-mediated in planta method (Fujiwara et al. 1992) with 1% gellan gum and 1% sucrose. Transgenic Arabidopsis lines were established by the Agrobacterium-mediated in planta method (Clough and Bent 1998). To generate chimera B. napus plants, a scion cut at 1–2 cm under cotyledon was inserted into a rootstock at the position 1 cm above the cotyledon. A silicon tube was used to support vertical growth. The grafted plants were placed in a transparent box with very high artificial humidity. After one-week, grafted plants were successfully established.

Plasmid construction

To construct ProBOR1;1c (5’-UTR): GUS and ProBOR1;1c (Δ5’-UTR): GUS, promoter sequences of BnaC4.BOR1;1c with or without 5’-UTR were amplified from ‘QY10’ DNA by PCR reaction with specific primers (Table S1), then fused with ScaI and SmaI digested PBI121 fragment using In-Fusion Cloning kits (Clontech). To construct Pro35S 5’UTR: GUS, Pro35S 5’UTRΔ1–29: GUS, and Pro35S 5’UTRΔ1–97: GUS, truncated 5’ UTR sequences were amplified by PCR reaction with specific primers (Table S1) and were fused with XbaI digested PBI121 using In-Fusion Cloning kits (Clontech). To generate Pro35S 5’UTRΔ1–29: GUS, Pro35S 5’UTR: GUS was used as a template by PCR reaction using specific primers, and the PCR product was self-fused.

Gene expression analysis

To investigate GUS expression, 10-d-old Arabidopsis seedlings grown on 100 μM B were transferred to 0.25 μM B or 250 μM B medium for 2 d growth. Plants were harvested for sampling. RNA was extracted using TRIZOL Reagent (Invitrogen, CA, USA). Reverse transcription was carried out using M-MLVReverse Transcriptase (Promega) according to the manufacturer’s protocol. RT fluorescence quantitative PCR was performed using the SYBR Green Real-Time PCR Master Mix Kit (Toyobo, Japan) and the CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Actin and Tubulin were used as internal controls with primers F: 5’-ACAGTGTCTGAGTCGGTCGTT-3’, R: 5’-TGCCATCTCATATACGACC-3’ (ACTIN); F: 5’-CAGCAATACAGTGCTTTGACGTG-3’, R: 5’-TCTGTGTAACAAAGGAAAGCC-3’ (TUBULIN). Each gene’s relative expression level was calculated and normalized based on these two internal controls using the ΔΔCt method (Livak and Schmittgen 2001).

β-Glucuronidase (GUS) histochemical staining and quantification of GUS activity

β-Glucuronidase (GUS) histochemical staining of GUS reporter lines was performed using the GUS Histochemical Kit (bioshap, Cat. BL622A). The sample photos were taken using an Olympus SZX16 stereomicroscope. For the GUS activity assay, GUS line’s total protein was extracted using the GUS extraction buffer (50 mM sodium phosphate buffer (pH 7.0), 10 mM β-mercaptoethanol, 1 mM Na2-EDTA·2H2O, and 0.1% Triton X-100). After centrifugation at 12,000 rpm, the total protein concentration was determined by using the Bradford assay (Bradford 1976). 50 μg of total protein was mixed with 450 μL MUG buffer incubated at 37 °C. 40 μL interaction solution was obtained at 5 min, 15, 25, or 35 min, respectively, and mixed with 160 μL of 0.2 M Na2CO3 to stop the interaction. GUS fluorescence (excitation: 365 nm; emission: 455 nm) was measured using a microplate reader (TECAN Infinite M200). The standard curve was established by mixing 40 μL 4-MU (4-methylumbelliferone) and 160 μL 0.2 M Na2CO3, in which the 4-MU was set as 1/26 mM, 1/27 mM, until to1/2 12 mM).

Immunohistological staining of BnaC4.BOR1;1c

To perform in vivo immunohistological staining of BnaC4.BOR1;1c in B. napus, an antibody against BnaC4.BOR1;1c was obtained by purifying the rabbit
antiserum, which was prepared through immunizing rabbits with an artificial peptide (SSTPLNRRSSPR). The immunohistological staining method (Ma et al. 2007) was used with modification. Briefly, *B. napus* seedlings (20-d-old), precultured in 25 μM B, were transferred to 0.25 μM B or 250 μM B for 2 d growth. The roots, basal node, and nodes (petiole junction) from ‘QY10’ and RNAi plants were cut as ~0.5–1 cm thickness and fixed in the solution at 4 °C (4% paraformaldehyde, 60 mM sucrose, and 50 mM Cacodylic acid). The samples were centrifuged at 7000 rpm for 2 min. After exposed to room temperature for 2 h, samples were washed several times using 1x PBS buffer, then embedded by 5% agar (Nakalai, Tesque, Kyoto, Japan) for solid at 4 °C. 100 μm specimens were prepared using a semi-automatic slicer and stored in 1xPBS buffer supplemented with 0.1% (w/v) pectolyase+0.3%(w/v) Triton X-100 for 2 h. After washed with 1xPBS, the specimens were blocked in 5% (w/v) BSA/PBS for 10 min, followed incubated with primary antibody (anti-BnaC4.BOR1;1c, rabbit, 1:1000 dilution) for overnight. After washed with 1xPBS buffer, the specimens were incubated in secondary antibody (Alexa fluor 488-conjugated Goat anti-rabbit IgG (H+L) AS053, 1:2000 dilution) for 2 h without light. The specimens were then washed 5 times in 1x PBS and mounted with an antifade polyvinylpyrrolidone mounting medium (Beyotime, Shanghai, China). Fluorescence imaging was examined in the Leica SP8 system (Leica, Mannheim, Germany). GFP was excited by 488 nm, and the signal was collected from 505 to 545 nm wavelength.

In situ PCR

The in situ PCR method (Athman et al. 2014) was used with modification. Briefly, fresh plant tissues were fixed overnight in FAA solution (63% ethanol, 5% acetic acid, and 2% formalin) without vacuum infiltration step. After three times washes by buffer (63% ethanol and 5% acetic acid) and one-time wash by 1xPBS buffer, the samples were embedded in 5% agar (Nakalai, Tesque, Kyoto, Japan) for solid at 4 °C. 75 μm specimens were prepared using a semi-automatic slicer and stored in DEPC water containing 100 U RNaseOUT (Takara Bio, Japan), 2.5% DNase I (Promega, China) followed by cold DEPC water, the reverse transcription (Promega, China) was performed in the RT mix with BnaC4.BOR1;1c specific primer (4 μl 5 X buffer, 2 μl 10 mM dNTPs, 1 μl 0.1 M DTT, 10 μl H2O and 1 μl reverse primer at 65 °C for 5 min. Then 1 μl M-MLV Reverse Transcriptase and 1 μl RNaseOUT were added into the tube on the ice, followed by 1 h incubation at 50 °C and 5 min at 85 °C. The negative control was prepared as above without a specific primer. Specimens were transferred into tube containing PCR mix [5 μl 10 x Taq buffer (Aidlab bio.), 1 μl 10 mM mixed dNTPs, 0.2 μl digoxigenin-11-dUTP (Roche; 4 μM), 2.5 μl 10 mM forward primer, 2.5 μl 10 mM reverse primer, 1 μl Taq polymerase (Aidlab bio), 38.3 μl H2O]. After twice washes by 1 x PBS, specimens were blocked 30 min in 1 x block solution at room temperature (10 mg BSA in 10 ml 1 x PBS). 1.5 U AP-conjugated anti-DIG Fab fragments (Roche) were added for 1 h incubation at room temperature. The specimens were washed twice for 15 min using buffer (0.1 M Tris-Cl; 0.15 M NaCl, pH 9.5) and then incubated in 50 μl BM purple (Roche) for 2 h. Twice washes by washing buffer to remove the blue solution, the specimens were mounted on the microscope slide for imaging.

Measurement of B concentration

All samples were dried at 65 °C in an oven for 3–4 d and then ground into fine powders using carnelian mortar. 1 M HCl was used to digest dry powders on the shaker for 2 h at 250 rpm. The solution was filtered, and B was measured by inductively coupled plasma mass spectrometer (ELAN DRC-e; Perkin Elmer, USA).

Statistical analysis of data

Data were analyzed using Student’s *t* test and Duncan’s test. Significance was defined when *P* value <0.05.

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Author contributions F.X. and S.W designed the research; S.W., L.L., D.Z., Y.H and Z.Z performed the experiments and analyzed the data; S.W. wrote the manuscript; all authors read and approved it.

Declarations

Conflict of interest The authors declare no competing financial interests.

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