Transcription factor BnaA9.WRKY47 contributes to the adaptation of *Brassica napus* to low boron stress by up-regulating the boric acid channel gene *BnaA3.NIP5;1*

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**Summary**

Boron (B) deficiency is one of the major causes of growth inhibition and yield reduction in *Brassica napus* (*B. napus*). However, the molecular mechanisms of low B adaptation in *B. napus* are largely unknown. Here, fifty-one BnaWRKY transcription factors were identified as responsive to B deficiency in *B. napus*, in which BnaAn.WRKY26, BnaA9.WRKY47, BnaA1.WRKYS3 and BnaCn.WRKYS7 were tested in yeast one-hybrid assays and showed strong binding activity with conserved sequences containing a W box in the promoters of the B transport-related genes *BnaNIPS*;1 and *BnaBOR1*s. Green fluorescent protein fused to the target protein demonstrated the nuclear localization of BnaA9.WRKY47. CRISPR/Cas9-mediated knockout lines of BnaA9.WRKY47 in *B. napus* had increased sensitivity to low B and lower contents of B than wild-type plants. In contrast, overexpression of BnaA9.WRKY47 enhanced the adaptation to low B with higher B contents in tissues than in wild-type plants. Consistent with the phenotypic response and B accumulation in these transgenic lines, the transcription activity of BnaA3.NIP5;1, a B efficiency candidate gene, was decreased in the knockout lines but was significantly increased in the overexpressing lines under low B conditions. Electrophoretic mobility shift assays, transient expression experiments in tobacco and in situ hybridizations showed that BnaA9.WRKY47 directly activated BnaA3.NIP5;1 expression through binding to the specific cis-element. Taken together, our findings support BnaWRKYs as new participants in response to low B, and BnaA9.WRKY47 contributes to the adaptation of *B. napus* to B deficiency through up-regulating BnaA3.NIP5;1 expression to facilitate efficient B uptake.

**Introduction**

As sessile organisms, plants have to quickly transduce extracellular stimuli/stresses to endogenous signals through signal transduction cascades, finally leading to physiological adaptation. During this process, numerous transcription factors serve as the switches of regulatory cascades, such as well-characterized members of MYB (myeloblastosis-related), bZIP (basic leucine zipper) and WRKY families (Spitz and Furlong, 2012). WRKY, one of the largest and most important transcription factor families, has been widely identified in various plant species and modulates many plant processes (Rushton et al., 2010). The WRKY proteins contain one or two domains composed of the relatively conserved amino acid sequence WRKYGQK together with a novel zinc-finger-like motif (Ulker and Somssich, 2004). With the ability to bind specifically to T/CCTGACC/T (W box) cis-regulatory elements present in promoters of target genes, the WRKY domains activate or repress the transcription of the target genes (Eulgem et al., 2000). WRKY-mediated gene expression is involved in various stress responses, such as pathogen defence, cold resistance, salt tolerance and nutritional stresses, and in developmental and metabolic processes (Phukan et al., 2016). Accumulating evidence has highlighted the importance of WRKY genes in responding to nutrient conditions. During phosphate (Pi) starvation, AtWRKY45 activated *PHO1* (Phosphate transporter) expression to take up Pi (Wang et al., 2014). In addition, AtWRKY6 and AtWRKY42 (two suppressors of *PHO1*) were degraded to release *PHO1* (Phosphate1, Pi transporter) which enhances plant Pi transfer from root to shoot (Chen et al., 2009; Su et al., 2015). Down-regulation of AtWRKY75 and OsWRKY74 increased the sensitivity of plants to Pi starvation (Dai et al., 2016; Devaiah et al., 2007). During Fe deficiency, AtWRKY46 promoted Fe translocation from root to shoot by directly up-regulating the expression of Fe transporter gene *VITL1* (Yan et al., 2016).

Boron (B) is essential for plant growth, but it often limits crop production worldwide owing to low available B concentration in soils (Shorrocks, 1997). B plays important roles in cell wall formation, membrane integrity, pollen tube growth, sugar transport, nitrogen fixation, plant metabolism and B deficiency leads to nutritional disorders, which inhibit plant apical growth and flower development, and eventually result in decreased plant production (Marschner, 2012). In *Arabidopsis*, B homeostasis is regulated by a synergistic network of several B transporters involved in B uptake, transportation and distribution. Under low B conditions, the transport pathways of B from the *Arabidopsis* root surface to the shoot includes at least three events: (1) *NIP5;1*, a major boric acid channel, efficiently facilitates B uptake from soil into the root (Takano et al., 2006; Wang et al., 2017); (2) *BOR1*, a B efflux transporter, is responsible for xylem loading of B (Noguchi et al., 1997; Takano et al., 2002); and (3) *NIP6;1* and *NIP7;1*, homologues of *NIP5;1*, are required for proper distribution of boric acid in developing shoot tissues and anthers (Routray et al., 2018;...
Tanaka et al., 2008). Recently, more B transport-related genes, such as
tassel-less1 (co-orthologue of AtNIP5;1) (Matthes et al., 2018) and
r rotten ear (co-orthologue of AtBOR1) (Chatterjee et al., 2014)
in maize, OsNIP5;1 (a homologue of AtNIP5;1) (Hanaoka et al.,
2014; Shao et al., 2018) and OsBOR1 (a homologue of AtBOR1)
(Nakagawa et al., 2007) in rice, VvBOR1 in grapevine
(Pérez-Castro et al., 2012), CmBOR1 in citrus (Cañon et al., 2013)
and TaBOR1s in wheat (Leaungthikanchana et al., 2013), have
been reported. These studies demonstrated that both boric acid
channel NIPs (nodulin 26-like intrinsic proteins) and B transporter
BORs are critical for plants to cope with B stress. The B-dependent
regulatory mechanism of AtNIP5;1 was controlled by the 5’
untranslated region (UTR) (Tanaka et al., 2011). To date, transaction
factors that regulate B transport-related genes have not been
reported in plants. The only reported transaction factor involved
in B stress responses was WRKY6 in Arabidopsis (Kasajima et al.,
2010). Low B induced the promoter activity of WRKY6 around the
root tip, and functional loss of WRKY6 decreased root elongation
under both B deficiency and B excess (Kasajima et al., 2010).
Brassica napus (B. napus) (A_{nn}A_{nn},C_{nn}C_{nn}, 2n = 4x = 38) is ex-
tremely sensitive to B deficiency (Marschner, 2012) with typical defect
of ‘flowering without seed setting’ (Xu et al., 2002). In our previous
studies, BnaA3.NIP5;1, a homologue of AtNIP5;1, was identified as
a B efficiency candidate gene through quantitative trait locus fine
mapping (Hua et al., 2016a). Zhang et al. (2017) found that
BnaC4.BOR1;1c, a homologue of AtBOR1, is preferentially
expressed in roots, shoot nodes and flower organs and is required
for florescence development and fertility under low B conditions
in B. napus. A number of BnaNIPs and BnaBORs, including
Bnaa3.NIP5;1 and BnaC4.BOR1;1c, had differential expression in
response to varying B availabilities (Hua et al., 2017). Nevertheless,
little is known about the molecular regulatory mechanisms
underlying low B adaptation of B. napus. A comprehensive mRNA
transcriptome of B. napus suffering from B fluctuation was
analysed to identify genome-scale B-responsive genes, and mul-
tiple transcription factor families, including the BnaWRKY family,
were found to be regulated by various B supplies (Hua et al., 2017).
In this study, we carried out a systematic investigation of the
involvement of BnaWRKYs in the low B adaptation of B. napus.
Fifty-one BnaWRKYs responding to B deficiency were identified,
and BnaAn.WRKY26, BnaA9.WRKY47, BnaA1.WRKYS3 and
BnaCn.WRKYS7 tested had the binding activity with the conserved
W boxes present in the promoters of B transporter genes
(BnaNIP5;1s and BnaBOR1s). Furthermore, BnaA9.WRKY47 was
verified to be involved in low B tolerance by specifically activating
BnaA3.NIP5;1 expression through genetic and biochemical
approaches. Our study identifies BnaWRKY transcription factors
as novel regulators of B deficiency adaptation in B. napus.

Results
BnaWRKY family genes respond to B deficiency in B. napus

Insufficient B supply impaired growth of B. napus, accompanied
by curved young leaves and stunted roots (Figure 1a-c). Genome-
wide transcriptomic analysis in B. napus (Westar 10) has revealed
numerous low B-responsive genes, including transcription factors,
signal transducers and structural molecules (Hua et al., 2017).
Among these responsive genes, 51 WRKY transcription factor
family genes showed more than twofold differential expression
under B deficiency (0.25 μM B) relative to the normal B condition
(25 μM B) (Figure 1d and Table S1). Of these, 16 BnaWRKYs were
up-regulated and 5 genes were down-regulated in roots, while
32 BnaWRKYs were up-regulated and 3 genes were down-
regulated in shoots (Figure 1e). Notably, 4 BnaWRKYs were
induced and 1 BnaWRKY was reduced in both roots and shoots
by low B. qRT-PCR analyses of random 10 B-responsive WRKY
genes (Figure S1) confirmed the differential expression in
response to low B stress and showed a high correlation with the
expression from RNA-Seq (R² = 0.9415) (Figure 1f).

BnaWRKYs bind to the conserved cis-element present in
the promoters regions of B transport-related genes

A conserved W box (TGTGACCT/T) in the promoter region
target genes was identified as the typical binding site of WRKY
(Eulgem et al., 2000). The boric acid channel NIP5;1 for B up-
take and transporter AtBOR1 for B xylem loading are pivotal for
the efficient transcellular transport of B under B limited conditions
(Miwa and Fujiwara, 2010). Interestingly, the differential expres-
sion of the homologues of NIP5;1s and BOR1s in B. napus under
B deficiency appeared to be correlated with the W-box sequences
present in their promoter regions. Among six BnaNIP5;1s,
BnaA2.NIP5;1, BnaA3.NIP5;1 (Hua et al., 2016a), BnaC2.NIP5;1
and BnaC3.NIP5;1 were obviously up-regulated in roots under B
deficiency (Figure 2a). It was interesting that these four
BnaNIP5;1s and AtNIP5;1 contain a 16-bp conserved sequence
(W̅C-N in Figure 2a) with a W box (TTGACCT) in their promoters,
whereas BnaA7.NIP5;1 and BnaC6.NIP5;1, whose expression
was less affected by low B, do not have the conserved W̅C-N
(Figure 2a). Similarly, a 17-bp conserved sequence (W̅C-B)
with a W box (TTGACTC) was found in AtBOR1 and four BnaBOR1s,
including BnaA3.BOR1;3a, BnaA4.BOR1;1a, BnaA5.BOR1;2a and
BnaC4.BOR1;2c, which were induced by low B with the exception
of BnaA3.BOR1;3a (Figure 2b). Thus, we presumed that
BnaWRKYs participate in low B stress by regulating the expression
of boric acid channel and B transporters.

To verify this possibility, yeast one-hybrid (Y1H) assays
were performed. Based on the DEGs (Figure 1) and co-expression
network analysis (Figure S2), BnaAn.WRKY26, BnaA9.WRKY47,
BnaA1.WRKYS3 and BnaCn.WRKYS7 were selected for prey
construct using the pGADT7-Rec2 vector. Four tandem copies
of conserved W-box sequences (4 x W̅C-N and 4 x W̅C-B)
were cloned into the pHIS2 reporter vector and used as baits for
the binding assays (Figure 2c). All the yeasts could grow well on
selective dropout medium (SD-Leu-Trp-His) without 3-AT (3-
amino-1, 2,4-triazole). When 50 mM 3-AT was added, the
positive control (pGADT7-53 and pHIS2-53) grew normally, while
the negative control (pGADT7-53 and pHIS2 empty vector) could
cannot survive. It was evident that all the 4 x W̅C-B-WRKY or
4 x W̅C-N-WRKY co-transformants were able to grow on the 3-
AT-containing selective dropout medium (Figure 2d,e), indicating
the potential for interaction between BnaWRKYs and BnaNIP5;1s,
or BnaWRKYs and BnaBOR1s.

BnaA9.WRKY47 is a nuclear-localized transcription factor

To elucidate the molecular mechanism of BnaWRKYs functioning
in low B adaptation of B. napus, BnaA9.WRKY47 was selected to
be further analysed owing to its significant up-regulation by B
deficiency in both roots and leaves and relatively higher mRNA
expression abundance than other BnaWRKY DEGs (Figures 1d,
3a, 3b and S1). By transient expression of 35S:BnaA9.WRKY47:
GFP (green fluorescent protein) in Arabidopsis protoplasts, the
GFP signal was exclusively co-localized with the CFP (cyan

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Figure 1 Low B stress induces differential expression of BnaWRKY family genes. (a) The phenotype of B. napus ‘Westar 10’ seedlings grown under B sufficiency (25 μM B) and B deficiency (0.25 μM B) conditions for 10 days. Bar = 5 cm. (b–c) Close-up view of leaves indicated by red squares in (a). Bars = 1 cm. (d) Heat map of 51 BnaWRKY genes with differential expression in response to low B stress. (e) Venn diagram of the 51 BnaWRKY DEGs in roots and shoots. The upward and downward arrows indicate up-regulated and down-regulated genes, respectively. The number of DEGs in the Venn graph represents the BnaWRKY quantity. (f) Correlation of expression ratios between qRT-PCR and RNA-Seq for 10 BnaWRKY genes. [Colour figure can be viewed at wileyonlinelibrary.com]
Figure 2. BnaWRKYs interact with BnaNIP5;1s and BnaBOR1s in yeast. (a-b) Multiple sequence alignment of promoter and gene expression profiling of BnaNIP5;1s (a) and BnaBOR1s (b). Conserved sequences in the promoters of BnaNIP5;1s and BnaBOR1s are indicated by 'WC-N' and 'WC-B', respectively. W-box sequences are highlighted in red. The significantly differentially expressed BnaNIP5;1s and BnaBOR1s genes are labelled by asterisks in the heatmaps. (c-e) Yeast one-hybrid assays of binding activity of BnaWRKYs with conserved cis-elements of the promoters of BnaNIP5;1s (d) and BnaBOR1s (e). Yeast cells were cotransformed with pGADT7-BnaWRKYs and pHIS2-cis (4×WC-N or 4×WC-B), then grown on selective dropout medium (SD/-Trp-Leu-His) containing 0 or 50 mM 3-AT. 'pHIS2/S5/pHIS2 plus pGADT7/S3' were the positive and the negative controls, respectively. [Colour figure can be viewed at wileyonlinelibrary.com]
fluorescent protein)-labelled Ghd7 nuclear signal (Figure 3c) demonstrating that BnaA9.WRKY47 was localized in the nucleus.

**BnaA9.WRKY47 positively regulates low B adaptation of *B. napus***

To investigate whether BnaA9.WRKY47 plays an important role in low B adaptation of *B. napus*, homozygous CRISPR/Cas9 mediated mutants possessing three editing types in the genome region of the BnaA9.WRKY47 gene (CR#19 carried a 2-bp deletion and a 1-bp insertion; CR#24 carried a 1-bp deletion and a 1-bp insertion; and CR#88 carried a 2-bp deletion, a 1-bp insertion and a 16-bp mutation) (Figure 4a) and three independent overexpressing lines (OE#177, OE#184 and OE#385) with 10- to 30-fold elevated expression levels compared with wide-type 'Westar 10' (Figure 4b) were established. No distinguishable differences in phenotype were observed in all lines when grown in the 25 µM B condition (Figure 4c). In 0.25 µM B, all three gene-edited mutants displayed stronger growth inhibition in both leaves and roots compared with wild-type plants; conversely, overexpressing lines showed dramatic tolerance to low B compared with the wild-type plants (Figure 4c). The phenotypic differences among all genetic lines were further supported by the dry weights of both shoots and roots (Figure 4d,e). The B contents of the gene-edited mutants and overexpressing lines were significantly lower and higher, respectively, than in wild-type plants in both shoots and roots under the 0.25 µM B treatment (Figure 4f,g). However, the phenotype, dry weight and the B contents were indistinguishable among wild-type plants, mutants and overexpressing lines under sufficient B supply (25 µM) (Figure 4). Juvenile leaves and root tips are the most sensitive tissues in response to low B stress. In 25 µM B, all lines developed fully expanded new leaves, but 0.25 µM B treatment led to deformed and curly juvenile leaves; the degree of curliness of juvenile leaves was further aggravated in the gene-edited lines and was largely alleviated in the overexpressing lines (Figure 5a), which was reflected by new leaf areas (Figure 5b). Consistent with the phenotypic characterization, all lines had comparable B concentrations in juvenile leaves at 25 µM B, but at 0.25 µM B, the gene-edited lines had lower B concentrations and the overexpressing lines had higher B

![Figure 3](image-url)  
**Figure 3** BnaA9.WRKY47 is up-regulated in roots and leaves under B limitation and is a nuclear-localized transcription factor. (a) qRT-PCR analysis of BnaA9.WRKY47 expression in various tissues of 'Westar 10' at the flowering stage supplied with 9 kg borax/ha. BnaEF1α and BnaTubulin mRNAs were used as internal controls. Data are shown as the means ± SD (n = 3). (b) qRT-PCR analysis of BnaA9.WRKY47 expression in 'Westar 10' under B sufficiency and B deficiency. Seven-day-old 'Westar 10' seedlings were transferred to nutrient solution containing 25 µM or 0.25 µM boric acid. BnaEF1α and BnaTubulin mRNAs were used as internal controls. Data are shown as the means ± SD (n = 3). Asterisks indicate significant differences compared with B sufficiency (t-test, ** P < 0.001) (c) Subcellular localization of the BnaA9.WRKY47-GFP fusion protein in Arabidopsis protoplasts. Bars = 20 µm. [Colour figure can be viewed at wileyonlinelibrary.com]

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concentrations than wild type (Figure 5c). B deficiency also impaired root morphology and caused tip necrosis (Figure S3). Malondialdehyde (MDA) is a product of lipid peroxidation (Bejaoui et al., 2016), and the degree of tissue damage by low B can be reflected by MDA concentration to a certain extent (Hua et al., 2016b). Under the 25 µM B condition, all tested plants showed no obvious differences in root MDA concentration. However, under the 0.25 µM B condition, the overexpressing lines had the lowest root MDA concentrations followed by the wild-type ‘Westar 10’, and the mutants had highest (Figure 5d); and the lower the MDA concentration was, the higher the B concentration that was detected in roots (Figure 5e).

BnaA9.WRKY47 positively regulates BnaA3.NIP5;1 transcription

We thus investigated the transcription levels of BnaNIP5;1s and BnaBOR1s in BnaA9.WRKY47 null mutants and overexpressing lines to test whether BnaA9.WRKY47-mediated low B adaptation is dependent on B transport-related genes. The expressions of BnaA2.NIP5;1 and BnaC2.NIP5;1 were decreased in the new leaves of BnaA9.WRKY47 CR#24 mutants but increased in BnaA9.WRKY47 overexpressing line OE#184 with low B; however, no obvious differences were observed in the roots of various lines (Figure 6a,b). There was no significant difference in the expression of BnaC3.NIP5;1 in BnaA9.WRKY47 transgenic lines.
BnaA9.WRKY47 affects the growth of new leaves and roots under low B treatment. (a) Photographs of the new leaves of ‘Westar 10’ and transgenic lines grown under 25 μM B and 0.25 μM B conditions for 15 days. Bars = 2 cm. (b) Statistical analysis of new leaf size of plants grown hydroponically under 25 μM B and 0.25 μM B conditions for 15 days. Mean values ± SD are shown (n = 5). Asterisks indicate significant differences compared with wild-type plants (t-test, **P < 0.01, ***P < 0.001). (c) New leaf B concentration measurement of the all lines grown hydroponically under 25 μM B and 0.25 μM B conditions for 15 days. Mean values ± SD are shown (n = 4). Asterisks indicate significant differences compared with wild-type plants (t-test, *P < 0.05, **P < 0.01). (d) Statistical analysis of MDA concentrations in roots. Root samples were detached from various lines grown under 25 μM B and 0.25 μM B conditions for 15 days. Mean values ± SD are shown, with three biological replicates and two plants per replicate. Asterisks indicate significant differences compared with wild-type plants (t-test, *P < 0.05). (e) Root B concentration measurements of the all lines grown hydroponically under 25 μM B and 0.25 μM B conditions for 15 days. Mean values ± SD are shown (n = 4). Asterisks indicate significant differences compared with wild-type plants (t-test, *P < 0.05). [Colour figure can be viewed at wileyonlinelibrary.com]
The yeast growth of the wild type (WS) and all the transformants was indistinguishable at 50 µM 3-AT, while wild-type, mW2, mW12, mW13, mW23 and mW123 were visibly inhibited at 100 µM 3-AT. When 3-AT concentration was increased to 150 µM, the inhibition effect was more serious in mW2, mW12, mW23 and mW123 compared with W5 (Figure 5b). These results suggest that the BnaA9.WRKY47 protein could bind with the promoter region of BnaA3.NIP5;1 in yeast, and at least the box element TGACG to −643 could effectively affect the binding activity of BnaA9.WRKY47 to BnaA3.NIP5;1.

An electrophoretic mobility shift assay (EMSA) was performed to confirm whether the BnaA9.WRKY47 protein could bind directly to the promoter of BnaA3.NIP5;1 in vitro. The conserved WC-N sequence in BnaNIP5;1s and the specific W5 sequence in the BnaA3.NIP5;1 promoter were synthesized as probes and incubated with the BnaA9.WRKY47 protein generated in Escherichia coli. It was evident that BnaA9.WRKY47 could not bind with the conserved WC-N (Figure 6e) but obviously interacted with the specific W5 probe, and excess W5 unlabelled probes (twofold and fivefold) could competitively inhibit the combination (Figure 6f). Moreover, the binding of W5 with BnaA9.WRKY47 was completely inhibited by fivefold unlabelled −669 to −641 sequence (referred to W669 to −641), but the shift binding rates were less affected by the addition of unlabelled WC-N (Figure 6f). The mutation either in C-658 or in C-589 did not weaken the binding of probes with BnaA9.WRKY47, while mutation in C-643 greatly impaired the affinity of probe to BnaA9.WRKY47 in EMSA (Figure S6b). Together with the Y1H assay, these results indicated that the W-box element TGACG to −643 presumably played an important role in the binding of BnaA9.WRKY47 with BnaA3.NIP5;1.

To further investigate whether BnaA9.WRKY47 could activate BnaA3.NIP5;1 expression, a transient expression assay was performed in tobacco leaves. The proBnaA3.NIP5;1:GUS vector was co-expressed with or without 3SS:BnaA9.WRKY47 in tobacco (Nicotiana benthamiana) leaves. The GUS activity and GUS staining results showed that proBnaA3.NIP5;1:GUS alone was barely expressed in tobacco leaves but the expression was elevated by about sevenfold when it was co-injected with 3SS:BnaA9.WRKY47 (Figure 6g), indicating that BnaA9.WRKY47 significantly activated the expression of BnaA3.NIP5;1.

We further carried out RNA in situ hybridization to detect BnaA3.NIP5;1 expression in BnaA9.WRKY47 transgenic lines and wild type. As a boric acid channel, NIP5;1 mainly functions in root tips for efficient B uptake. In longitudinal sections of root tips, the hybridization signal of BnaA3.NIP5;1 was barely expressed in tobacco leaves but the expression was elevated by about sevenfold when it was co-injected with 3SS:BnaA9.WRKY47 (Figure 6i). Consistent with the in situ hybridization results in root tips, the qRT-PCR analyses indicate that there were no significant differences in BnaA3.NIP5;1 expression in the roots among transgenic lines. The increase of the hybridization signal in the roots of the BnaA9.WRKY47 transgenic plants on solid medium containing 100 µM B or 0.3 µM B for 5 days. The root tips were hybridized with BnaA3.NIP5;1 specific probe labelled with DIG. Bars = 100 µm. (i) qRT-PCR analysis of BnaA3.NIP5;1 expression in the roots of the BnaA9.WRKY47 transgenic plants grown on solid medium containing 100 µM B or 0.3 µM B for 5 days. Gene expression was normalized by BnaEF1α and BnaTubulin mRNAs with three biological replicates. Asterisks indicate significant differences based on a t-test (** P < 0.01, *** P < 0.001). (j) Immunoblotting analysis of BnaA3.NIP5;1 expression in the new leaves and roots of the wild-type ‘Westar 10’, BnaA9.WRKY47 mutants and overexpressing plants. Gene expression was normalized by BnaEF1α and BnaTubulin mRNAs with three biological replicates. Asterisks indicate significant differences based on a t-test (** P < 0.01, *** P < 0.001). (k) Western blots analysis of BnaA3.NIP5;1 expression in the new leaves and roots of the wild-type ‘Westar 10’, BnaA9.WRKY47 mutants and overexpressing plants. Gene expression was normalized by BnaEF1α and BnaTubulin mRNAs with three biological replicates. Asterisks indicate significant differences based on a t-test (** P < 0.01, *** P < 0.001). (l) Characterization of the BnaA3.NIP5;1 promoter sequence. The TGAC core sequence and conserved sequence W5 of BnaA3.NIP5;1s were marked by a red and yellow rectangle, respectively. The specific sequence W6 of BnaA3.NIP5;1 was shown. (m) The interaction of BnaA9.WRKY47 protein with BnaA3.NIP5;1 specific promoter region (W5) in yeast. (n) Electrophoretic mobility shift assay to analyse the interaction of BnaA9.WRKY47 and the conserved W5 in BnaA3.NIP5;1s (e) or the specific W6 (f) in the BnaA3.NIP5;1 promoter. The 5’ Cy5 labelled probe was incubated with BnaA9.WRKY47-His protein at 25°C for 20 min. Competition for the labelled sequences was tested by adding different concentrations of unlabelled probes. The binding shift is indicated with a red arrow. (g) Transient expression assay of BnaA3.NIP5;1 activated by BnaA9.WRKY47 in tobacco leaves. GUS activities were detected after 3SS:BnaA9.WRKY47 and proBnaA3.NIP5;1:GUS were injected into tobacco leaves together or separately for 2 days. Values represent mean values ± SD of three biological replicates, and two independent experiments verified these results. Asterisks indicate significant differences based on a t-test (** P < 0.01). (h) In situ hybridization analysis of BnaA3.NIP5;1 expression in the roots of the BnaA9.WRKY47 transgenic plants. Longitudinal sections of the root tips from wild-type, CR#19 and OE#184 seedlings grown on solid medium containing 100 µM B or 0.3 µM B for 5 days. The root tips were hybridized with BnaA3.NIP5;1 specific probe labelled with DIG. Bars = 100 µm. (i) qRT-PCR analysis of BnaA3.NIP5;1 expression in the roots of the BnaA9.WRKY47 transgenic lines and ‘Westar 10’ (t-test, * P < 0.05). [Colour figure can be viewed at wileyonlinelibrary.com]

Discussion

BnaWRKYs act as new players in response of B. napus to B deficiency

Plants can sense and cope with stressful environments depending on various transcription factor-mediated stress adaptation (Chinnusamy et al., 2004). The WRKY transcription factor family is one of the most important regulator families responding to nutrition disorders in plants. For example, an increasing number of studies have identified multiple WRKYs that modulate Pi homeostasis in plants (Devaiah et al., 2007; Chen et al., 2009; Wang et al., 2014; Dai et al., 2016; Su et al., 2015). To date, the only WRKY described as a regulator of B stress is AtWRKY6 (Kasajima et al., 2010). Our understanding of WRKY functioning in low B remains limited. In the present study, fifty-one of 287 BnaWRKYs (He et al., 2016) were detected as B-responsive DEGs (Figure 1 and Table S1). Among them, 44 DEGs (86%) and 7 DEGs (14%) exhibited elevated and decreased mRNA abundance with low B treatment, respectively. Up-regulated DEGs were predominant in low-B condition, but significantly lower and higher expressions of BnaA3.NIP5;1 under low B treatment were detected in BnaA9.WRKY47 mutants and overexpressing lines compared with wild-type, respectively (Figure 6i).
BOR4) have abundant W boxes in their 2000 bp promoter sequences (Table S2), implying that BnaWRKY transcription factors might interact with B transporter genes. The binding of BnaWRKYs to the conserved W boxes of B-responsive BnaNIP5;1s and BnaBOR1s in Y1H assays (Figure 2d,e) suggests that BnaWRKYs might participate in B stress adaptation by modulating BnaNIP5;1s and/or BnaBOR1s. Furthermore, the BnaA9.WRY47 mutants displayed severe B deficiency phenotypes under B deficiency and the BnaA9.WRY47 overexpressing lines showed higher tolerance of low B than wild-type ‘Westar 10’ (Figure 4 and 5), indicating that BnaWRKYs act as new players in the response of B. napus to B deficiency, although further investigation is required to support this conclusion.

**BnaA9.WRY47-BnaA3.NIP5;1 cascade modulates low B adaptation of B. napus**

The B deficiency phenotype in new leaves and roots of BnaA9.WRY47 mutants under low B conditions disappeared in the BnaA9.WRY47 overexpressing lines (Figure 4c), suggesting enhanced BnaA9.WRY47 activity facilitates B accumulation in B. napus. As expected, the B concentrations were higher in these BnaA9.WRY47 overexpressing lines than in wild-type ‘Westar 10’ and BnaA9.WRY47 null mutants (Figure 4f,g). Interestingly, a boric acid channel gene BnaA3.NIP5;1 was significantly up-regulated and down-regulated in the BnaA9.WRY47 overexpressing lines and its null mutants, respectively (Figure 6a,b), but the expression of other BnaNIP5;1s and BnaBOR1s was less affected in BnaA9.WRY47 transgenic lines (Figure 6a,b and S4). Although BnaA9.WRY47 showed binding activity with conserved W-box cis-elements in the promoters of several BnaNIP5;1s and BnaBOR1s (Figure 2d,e), it is reasonable that BnaA9.WRY47 specifically activates BnaA3.NIP5;1 expression by 'TGAC'-containing sequence repeats rather than the conserved W box in the EMSA assays (Figure 6e,f and Figure S6); adjacent sequences of W boxes also partly determine binding site preference leading to specificity for their target promoters (Ciolkowski et al., 2008). BnaA3.NIP5;1 was identified in our previous work as a key candidate gene for the determinant of B efficiency in different low B-sensitive B. napus cultivars (Hua et al., 2016a). The B efficient cultivar ‘Qingyou 10’ and the near-isogenic lines containing the BnaA3.NIP5;1 gene of ‘Qingyou 10’ with ‘Westar 10’ background have higher expression levels of BnaA3.NIP5;1 and stronger low B tolerance than B-inefficient ‘Westar 10’ (Hua et al., 2016a). In Arabidopsis, the upstream open reading frame (uORF), AUG-stop in the 5’ untranslated region of the AtNIP5;1 promoter induces B-dependent mRNA degradation (Tanaka et al., 2011, 2016). However, the BnaA9.WRY47 activation of BnaA3.NIP5;1 expression might be an uORF-independent mechanism because the BnaA9.WRY47 target cis-elements are not in the 5’ UTR of BnaA3.NIP5;1 (Figure 6c) and no uORFs were changed in both ‘QY10’ and ‘Westar 10’ (Hua et al., 2016a). The activation of BnaA3.NIP5;1 by BnaA9.WRY47 in Nicotiana tabacum cells and in situ hybridization (Figure 6g,h), and the nuclear localization of BnaA9.WRY47 (Figure 3c) suggested that BnaA9.WRY47 truly activates BnaA3.NIP5;1 expression in the nuclei of B. napus. Taken together, the BnaA9.WRY47 transcription factor and boric acid channel gene BnaA3.NIP5;1 form a cascade that modulates low B adaptation in B. napus.

In conclusion, we propose a working model to illustrate the involvement of BnaWRKYs in the response to low B stress (Figure 7). When B. napus suffers from low B stress, the dynamic web of 51 BnaWRKYE DEGs regulate transcriptional reprogramming associated with adaptation to low B, and BnaWRKYs act as activators or repressors in low B adaptation by regulating unknown target genes (N). [Colour figure can be viewed at wileyonlinelibrary.com]
repressors and activators. Among these BnaWRKYs, the upregulated BnaA9.WRKY47, in particular, activates the expression of the boric acid channel gene BnaA3.NIP5;1 and improves the adaptation to low B of B. napus. More BnaWRKYs may be critical factors in B nutrition through the same or different pathways as BnaA9.WRKY47, which needs to be studied further.

**Experimental procedures**

**Plant materials and growth conditions**

The B. napus cultivar ‘Westar 10’ stored in this laboratory was used to perform the analyses of the phenotype and mRNA expression profiling. Plump seeds with similar size were surface-sterilized for 15 min using 1% NaClO and washed with ultrapure water (18.25 MΩ cm). Then, these seeds were sown on a piece of gauze moistened with ultrapure water. After germination for 7 days, uniformly sized seedlings were transferred to Hoagland solution (Hoagland and Arnon, 1950) (HS) with B deficient (0.25 µM B) or B sufficient (25 µM B) treatments for 10–15 days. The nutrient solution was refreshed every three days with gradually increasing nutrient strength, including 1/4 HS for 3 days, 1/2 HS for 3 days and complete HS for 4–9 days. The plants were cultivated in an illuminated growth room under 16-h-light/8-h-dark photoperiod (with a photon flux density of 300–320 µmol m−2 s−1 and a relative humidity of 60%). Leaves and roots were harvested with three to five biological replicates for transcriptome sequencing, qRT-PCR and B concentration detection. The genome sequences and amino acid sequences of B. napus were obtained from the Arabidopsis Information Resource (TAIR 10) (http://www.arabidopsis.org/) and Brassica Database (BRAD) (http://brassicadb.org/brad/index.php) to identify the homologues in the allotetraploid B. napus genome. FDR (false discovery rate) ≤ 0.05 and the fold-change ≥ 2 were used as the threshold to screen the differentially expression genes (Audic and Claverie, 1997). Heat maps of DEGs were delineated by Multiexperiment Viewer, and a phylogenetic tree of DEGs was constructed by MEGA 6.0.

**Identification of differential expression genes (DEGs)**

Transcriptome sequencing was performed as described (Hua et al., 2017). A basic local alignment search tool (BLASTn) program was performed between Arabidopsis Information Resource (TAIR 10) (http://www.arabidopsis.org/) and Brassica Database (BRAD) (http://brassicadb.org/brad/index.php) to identify the homologues in the allotetraploid B. napus genome. FDR (false discovery rate) ≤ 0.05 and the fold-change ≥ 2 were used as the threshold to screen the differentially expression genes (Audic and Claverie, 1997). Heat maps of DEGs were delineated by Multiexperiment Viewer, and a phylogenetic tree of DEGs was constructed by MEGA 6.0.

**Sequence analysis**

The genome sequences and amino acid sequences of B. napus ‘Westar 10’ were obtained from the Brassica Database (Chalhoub et al., 2014; Cheng et al., 2011), and the sequences of Arabidopsis were downloaded from TAIR 10 database (Reiser et al., 2017). The 2000 bp upstream sequences from the start codons of BnaWRKY genes and B-related genes were analysed to determine the cis-regulatory elements (W box).

**Quantification of gene expression**

Total RNA was extracted using the Easyprep Super Total RNA Extraction Kit (Promega, Madison, WI). The first-strand cDNA was reverse transcribed by ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). The qRT-PCR analysis was carried out using the SYBR Green Real-Time PCR Master Mix Kit (TOYOBO) and QuantStudio™ 6 Flex System (Applied Biosystems, Foster City, CA). The primer efficiency was checked by Bio-Rad CFX Manager software (Bio-Rad, Hercules, CA) within the range of 90 to 110%. The transcript level of each mRNA was normalized with the level of BnaEF1α and BnaTubulin mRNAs using the delta Ct method (Livak and Schmittgen, 2001), and their geometric means were shown as relative expression data (Vandenompele et al., 2002). Gene-specific primers are listed in Table S4.

**Vector construction and transformation in B. napus**

To develop BnaA9.WRKY47 overexpressing plants, the full-length coding sequence of BnaA9.WRKY47 amplified from ‘Westar 10’ was inserted into the pFGCS941 vector at the AscI and PacI restriction sites driven by the CaMV35S promoter. To create CRISPR/Cas9-mediated BnaA9.WRKY47 targeted mutants, two target sequences sgRNA1 (AACCTGTAGAAGGTACCGGG) and sgRNA2 (ATCTTGACCATGTCGAGG) were selected based on CRISPR-P (http://crispr.hzau.edu.cn/CRISPR). The vectors (pKSE401 and pCPC-DT1172) were used according to the method described by Xing et al. (2014). The plasmid constructs were then transferred into Agrobacterium tumefaciens GV3101 and were subsequently used to transform B. napus ‘Westar 10’ as described previously (De Block et al., 1989). For overexpressing plants, three independent homozygous lines (35S#177, 184, 385) were selected from 11 lines based on phenotypic investigation under B deficiency, and qRT-PCR results verified the elevated expression of BnaA9.WRKY47. For CRISPR lines, the genomic region containing the two target sites was amplified and sequenced by BnaA9.WRKY47-specific primers (Table S4), a total of 19 independent mutant lines were obtained and three BnaA9.WRKY47 homozygous mutants in the absence of Cas9 transgene (CR#19, 24, 88) were selected for further study. The off-target detection was verified by sequencing the three most likely off-target sites in these lines (Table S3), and no sequence variations were detected in these regions.

**Yeast one-hybrid assays**

The yeast one-hybrid assays were performed using the Matchmaker One-Hybrid System following the manufacturer’s instructions (Clontech, Mountain View, CA). The WRKY coding region was amplified from ‘Westar 10’ and cloned into the pGADT7-Rec2 prey vector by the In-Fusion HD Cloning kit (Takara Bio) to create a translational fusion between the GAL4 activation domain and the transcription factor. The bait sequence, four tandem copies of conserved W boxes or single copy of specific sequence from the target genes were synthesized (Tsingke Bio) and cloned into the pHS2 reporter vector by EcoRI and SacI. The prey vector and reporter vector were cotransformed into the yeast strain Y187, and cotransformation of plasmids pGADT7-S3 and pHS2-S3 were used as positive controls, while pGADT7-S3 and pHS2 empty vectors were negative controls. Cells were grown in SD-Trp-Leu liquid media to an OD600 of 0.1 and then diluted with 10- and 100-fold sterile water. For each dilution, 7 µL was spotted on SD-Trp-Leu-His media plates supplemented with 0 and 50 µM 3-AT (3-aminocino, 2,4-triazole, a competitive inhibitor of the yeast HIS3 protein, used to suppress background growth) to test the strength of the interaction. The plates were then incubated for 3–4 days at 30 °C.

**Subcellular localization**

For subcellular localization, the full-length coding sequence of BnaA9.WRKY47 amplified from ‘Westar 10’ was cloned into the pM999-33 vector driven by the CaMV35S promoter (Tang et al., 2016) using the In-Fusion HD Cloning kit (Takara Bio). The fused construct 35S:BnaA9.WRKY47:GFP was cotransformed with 35S:Ghd7:CFP, a nuclear marker (Tang et al., 2016), into Arabidopsis.
protoplasts. After transformation for 12 to 16 h, a confocal laser-scanning microscope (Leica SP8) was used for capturing the fluorescence signal with the setting of excitation/detection wavelengths: GFP (488 nm/505–540 nm) and CFP (448 nm/460–490 nm).

Electrophoretic mobility shift assay

The BnaA9:WRKY47 full-length coding sequences were inserted into the inducible expression vector pET-15d (with 6×His tag) using the In-Fusion HD Cloning kit (Takara Bio) and expressed in the BL21 strain. Recombinant protein expression was induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 14 h at 16°C and further purified following the Sangon instructions (Ni-NTA Sefinose™ Resin, C600033). Two complementary oligonucleotide strands were labelled with 5′ Cy5 (Shanghai Sangon, China). The double-stranded oligonucleotides were generated by mixing with an equal amount of the complementary single-stranded oligonucleotides for 2 min at 95°C followed by cooling to 25°C. The labelled probes (1 μL of 10 μmL−1 mother probes) were incubated with the purified recombinant proteins (1–2 μg) in binding buffer (Beyotime, GS005) with or without unlabelled competitor DNA at 25°C for 20 min. Finally, the DNA–protein complexes mixed with loading buffer (Beyotime, GS006) were electrophoresed on 6% nondenaturing polyacrylamide gels in the dark for 1 h at 4°C. The fluorescence signal was captured by FLA-9000 (Fuji).

B concentration and MDA measurement

For B concentration measurement, the dried samples were ground into fine powders using a carnelian mortar, and B was lixiviated from 0.01 to 0.06 g powder in 1.0M HCl (4–6 mL) by shaking (200 rpm) for 2 h. The B concentrations were measured by Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES, Varian, Inc.). Malondialdehyde (MDA) is a product of lipid peroxidation (Bejaoui et al., 2016), and the degree of tissue damage by low B can be reflected by MDA concentration to a certain extent (Bejaoui, Salas, Nouairi, Smaoui, Abdelly, Martínez-Force, E. and Yousif, N.B. (2016) Changes in chloroplast lipid contents and chloroplast ultrastructure in Sulla carnosa and Sulla coronaria leaves under salt stress. J. Plant Physiol. 198, 32–38).

In situ hybridization

For in situ hybridization, seeds of BnaA9:WRKY47 transgenic plants and wild-type were plated on MGRL solid medium (according to Fujiiwara et al., 1992) containing 100 μm B or 0.3 μm B, and were vertically grown in an incubator with a 16-h-light/8-h-dark cycle for 5 days. The 5-cm root tips were fixed with 50% FAA (5% formalin, 5% acetic acid, 45% ethanol and 45% water). To generate the BnaA3:NIP5;1-specific antisense probe, a 47-bp mRNA sequence fragment of BnaA3:NIP5;1 was synthesized and labelled with DIG (digoxigenin): 5′-DIG-AACC-GAAUCAUUAAAAGCAGAACGCACACAUAUGGAUGC-GUAAC-3′ (Roehen Bio). The hybridization and immunological detection were performed as previously described (De Block and De Brouwer, 1993).

Statistical analysis

For statistical analyses, SPSS (Statistical Package for the Social Sciences) was used. Data are presented as means ± SD with at least three independent replicates. Significant differences were determined by t-test (***P < 0.001, **P < 0.01, *P < 0.05). Sequences of primers and probes used in this study are listed in Table S4.

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Conflict of interest

The authors declare no competing financial interests.

Author contributions

F.X. designed the research; Y.F., R.C., M.H., Y.H. and X.Y. performed the experiments and analysed the data; Y.F., F.X. and S.W. wrote the manuscript; L.S. reviewed the manuscript; all authors read and approved it.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Validation of the random 10 BnaWRKY DEGs by qRT-PCR assays.

Figure S2 Co-expression network of BnaWRKYs in B. napus.

Figure S3 Microscopy of root tips. The plants were grown under 25 μM B and 0.25 μM B conditions for 15 days.

Figure S4 Transcript levels of BnaBOR1s in BnaA9.WRKY47 transgenic lines.

Figure S5 BnaA9.WRKY47 binds to BnaA3.NIP5;1 promoter by specific TGAC sequence in yeast.

Figure S6 BnaA9.WRKY47 binds to BnaA3.NIP5;1 promoter by specific TGAC sequence in EMSA assays.

Table S1 The 51 BnaWRKY DEGs mRNA transcriptome data.

Table S2 The numbers of predicted WRKY-binding W boxes (T/CTGACC/T) in B stress-responsive genes.

Table S3 Examination of the sequences on putative off-target sites in BnaA9.WRKY47 CRISPR/Cas9 mutants.

Table S4 Primer and probe sequences used in this study.