The *Brassica napus* Calcineurin B-Like 1/CBL-interacting protein kinase 6 (CBL1/CIPK6) component is involved in the plant response to abiotic stress and ABA signalling

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Abstract

A CBL-interacting protein kinase (CIPK) gene, *BnCIPK6*, was isolated in *Brassica napus*. Through yeast two-hybrid screening, 27 interaction partners (including *BnCBL1*) of *BnCIPK6* were identified in *Brassica napus*. Interaction of *BnCIPK6* and *BnCBL1* was further confirmed by BiFC (bimolecular fluorescence complementation) in plant cells. Expressions of *BnCIPK6* and *BnCBL1* were significantly up-regulated by salt and osmotic stresses, phosphorous starvation, and abscisic acid (ABA). Furthermore, *BnCIPK6* promoter activity was intensively induced in cotyledons and roots under NaCl, mannitol, and ABA treatments. Transgenic *Arabidopsis* plants with over-expressing *BnCIPK6*, its activated form *BnCIPK6M*, and *BnCBL1* enhanced high salinity and low phosphate tolerance, suggesting that the functional interaction of *BnCBL1* and *BnCIPK6* may be important for the high salinity and phosphorous deficiency signalling pathways. In addition, activation of *BnCIPK6* confers *Arabidopsis* plants hypersensitive to ABA. On the other hand, over-expression of *BnCIPK6* in *Arabidopsis cipk6* mutant completely rescued the low-phosphate-sensitive and ABA-insensitive phenotypes of this mutant, further suggesting that *BnCIPK6* is involved in the plant response to high-salinity, phosphorous deficiency, and ABA signalling.

Key words: Abiotic stress tolerance, abscisic acid (ABA), *Brassica napus*, *BnCBL1*, *BnCIPK6*, interaction, regulation of gene expression.

Introduction

As an essential second messenger, calcium regulates diverse cellular processes in plants. Several Ca²⁺-sensor protein families, including calmodulin (CaM), the superfamily of calcium-dependent protein kinases (CDPK), and calcineurin B-like (CBL) proteins, have been characterized and implicated in a variety of physiological functions in plants (Albrecht *et al.*, 2001; *Kim et al.*, 2003; Pandey *et al.*, 2004). Ca²⁺ sensors can be classified into sensor responders and sensor relays (*Sanders et al.*, 2002). Upon binding of Ca²⁺, sensor responders change their conformation and modulate their own enzymatic activity or function through intramolecular interactions. By contrast, sensor relays must interact with their target proteins (such as protein kinases) to regulate their activity after binding Ca²⁺. CDPKs act as sensor responders (*Sanders et al.*, 2002; *Kim et al.*, 2003), while CaM and CBL proteins are sensor relays (*Luan et al.*, 2002; *Sanders et al.*, 2002). However, unlike CaMs targeting a large variety of target proteins, CBLs specifically interact with a family of protein kinases referred to as CBL-interacting protein kinases (CIPKs) or SnRK3s (*Luan et al.*, 2002). 10 CBLs and 25 CIPKs in *Arabidopsis* and 10 CBLs and 30 CIPKs in rice were
SOS1 pathway mainly regulates Na+-homeostasis in shoots and interact with CIPK24 to form the CBL10/CIPK24 complex that functions in ABA response in seed germination (Batistic and Kudla, 2004). Moreover, it was demonstrated that CBL10 can also participate in the plant response to salt and low phosphate stresses. The data also revealed that BnCIPK6M (T182D) rendered the transgenic plants hypersensitive to ABA, whereas RNAi plants showed insensitivity to ABA (Gong et al., 2002b). CIPK23, activated by the binding of CBL1 and CBL9, directly phosphorylates the K⁺ transporter AKT1, and enhance K⁺ uptake under low-K⁺ conditions (Li et al., 2006; Xu et al., 2006). A later study revealed that CIPK23 also phosphorylates the nitrate transporter CHL1 to maintain a low-level primary response to low nitrate concentration (Ho et al., 2009).

*Brassica napus*, as an important oilseed plant, often encounters abiotic stresses, such as high salinity, drought, cold, and nutrient deficiency (such as P and K limitation), resulting in plant growth retardation and reduced agricultural productivity. It is both of biological and agricultural importance to understand the molecular mechanism of *Brassica napus* in response to abiotic stresses. Although *Arabidopsis* CBLs/CIPKs participating in various calcium-signalling pathways are well characterized, little is known about the CBL/CIPK signalling pathway in *Brassica napus*. In this study, a gene in *Brassica napus*, BnCIPK6, was isolated. Through yeast two-hybrid screening, 27 novel interaction partners (including BnCBL1) of BnCIPK6 were identified in *Brassica napus*. Interaction of BnCIPK6 and BnCBL1 was further confirmed by BiFC in plant cells. Over-expression of both BnCIPK6 and BnCBL1 in *Arabidopsis* enhance the plant’s tolerance to salt and low phosphate stresses, suggesting that BnCBL1 and BnCIPK6 may functionally interact with each other to be involved in the plant response to salt and low phosphate stresses. The data also revealed that BnCIPK6M (T182D) transgenic plants were hypersensitive to abscisic acid (ABA). To our knowledge, this is the first report that *Brassica napus* CBL/CIPK is functionally involved in the response to abiotic stress and ABA signalling. More importantly, no CBLs/CIPKs have been identified as signalling components involved in phosphorus starvation signalling so far.

### Materials and methods

#### Plant growth conditions

Seeds of *Brassica napus* (cv. Zhongyou 821) were surface-sterilized and germinated on half-strength Murashige and Skoog (MS) medium (pH 5.8) containing 0.8% agar under a 16/8 h light/dark cycle at 25 °C for 7 d. Roots, cotyledons, and hypocotyls were collected from sterile seedlings for RNA isolation. Other tissues, such as stems, leaves, and flowers, were derived from *B. napus* plants grown in the field.

In stress experiments, 1-week-old seedlings of *B. napus* were transferred to MS medium containing 150 mM NaCl, 200 mM mannitol, 100 µM ABA (abscisic acid), or low phosphate (10 µM phosphate) for certain durations, respectively.

#### BiFC analyses of interaction between BnCIPK6 and BnCBL1

pUC-SPYNE-BnCBL1, pUC-SPYNE-BnCIPK6, pUC-SPYCE-BnCBL1, and pUC-SPYCE-BnCIPK6 vectors were constructed using gene-specific primers, respectively (see Supplementary Table S1 at JXB online). The constructs were then introduced into onion epidermal cells by DNA particle bombardment according to the manufacturer’s instructions (Biologic PDS-1000/He Particle Delivery System, Bio-Rad, USA), respectively. Fluorescence microscopy was performed on a SP5 Meta confocal laser microscope (Leica, Germany). YFP fluorescence in transformed cells was detected, using bZIP63 dimerization as the positive control and pUC-SPYNE-BnCBL1+pUC-SPYCE and pUC-SPYNE+pUC-SPYCE-BnCIPK6 as the negative controls.
Quantitative RT-PCR and Northern blot analyses

The expression of the *BnCIPK6* and *BnCBL1* genes in *B. napus* tissues was assayed by quantitative reverse transcriptase (RT)-PCR as described earlier (Li et al., 2005), and using the *BnACT2* gene as a quantitative control. To assay the expression of stress- and ABA-responsive genes, RT-PCR analysis was performed with the RNA samples isolated from 2-week-old seedlings treated with or without 100 mM ABA for 6h, using the *ACTIN2* gene as an internal control. All the quantitative RT-PCR analyses were repeated three times along with three independent repetitions of the biological experiments, and means of three biological experiments were calculated for estimating gene expression levels. Primer sequences for real-time PCR are listed in Supplementary Table S1 at JXB online.

RNA Northern-blot hybridization was performed as described previously by Li et al. (2002).

Yeast two-hybrid analysis

The coding sequences of *BnCIPK6* and 10 *AtCBL* genes were cloned into the yeast two-hybrid vectors pGBK7T (bait vector) and pGAD7T (prey vector), respectively. Afterwards, the pGBK7T-BnCIPK6 construct was introduced singly into the yeast strain Y187 using the high-efficiency lithium acetate transformation procedure (Gietz et al., 1992), and each pGAD7T-*AtCBL* construct was transferred into the yeast strain AH109. The mating reactions and further interaction assays between the two haploid strains containing pGBK7T-BnCIPK6 and pGAD7T-*AtCBL* constructs were performed by the method described previously by Zhang et al. (2010).

A yeast two-hybrid library of *B. napus* cDNAs from mRNAs of different tissues (roots, hypocotyls, cotyledons, stems, leaves, and flowers) was constructed, using the Clontech BD Matchmaker Library Construction and Screening Kits according to the manufacturer’s instructions (BD Biosciences Clontech). For screening target proteins of *CIPK6* from the *B. napus* cDNA library, yeast two-hybrid analysis was performed using the BnCIPK6 proteins as a bait to screen the two-hybrid library of *B. napus* cDNAs constructed on the prey vector, by the method described previously by Zhang et al. (2010). Primer sequences for pGBK7T-BnCIPK6 and pGAD7T-*AtCBL* constructs were listed in Supplementary Table S1 at JXB online.

Construction of BnCIPK6p:GUS chimeric genes and Arabidopsis transformation

A SalI site and BamHI site were introduced at the 5’-end and the 3’-end of the *BnCIPK6* 5’-upstream region, respectively. The SalI/BamHI fragments (0.8 kb and 1.2 kb, respectively) were then subcloned into the pBI121 vector, replacing the CaMV 35S promoter to generate chimeric *BnCIPK6p:GUS* constructs. The constructs were introduced into Arabidopsis by the floral dip method. Transformed seeds were selected in 2-week-old seedlings by the promoter sequence of *BnCIPK6* was also cloned into the PBI121 vector (see Supplementary Table S1 at JXB online). The constructs were then transferred into Arabidopsis by the floral dip method. Seeds of wild-type and independent lines of *BnCIPK6*, *BnCIPK6M*, and *BnCBL1* transgenic plants were germinated on MS medium with different concentrations of ABA. Seeds were considered germinated when radicles completely penetrated the seed coat; germination and seedling growth were scored at the indicated times.

Seeds of wild-type and independent lines of *BnCIPK6*, *BnCIPK6M*, and *BnCBL1* transgenic plants were germinated on MS medium. Seedlings were then transferred to MS medium containing different salt concentrations (0, 75, 150, 170, 200, and 250 mM) in the vertical position. The status of seedling growth was recorded a few days after the transfer. The chlorophyll content in leaves was determined. In brief, chlorophylls in leaves were extracted with 80% acetone, and the extract was incubated at 4 °C for 2 h in darkness. Chlorophyll content was assayed by measuring absorbance at 645, 652, and 663 nm with a spectrophotometer. Proline content in both control and transgenic plants was determined using the protocol as described by Gong et al. (2012).

Seeds of wild-type and transgenic lines germinated and grew on MS medium with or without ABA for 2 weeks, and the primary root length was measured. For seedling growth under low phosphate (LP) treatments, 6-day-old seedlings were transferred and vertically cultured at 50 µM low phosphate (LP) medium for a few days, then the status of seedling growth was recorded, including lateral root growth, and fresh and dry weight assays.

**In vitro phosphorylation assay of recombinant MBP fusion proteins**

The coding regions of *BnCIPK6* and *BnCIPK6M* (T182D) were cloned into the pMAL vector. Afterwards, the vectors including recombinant and empty vectors were separately transformed into Escherichia coli strain BL21. After induction for 3.5h, the recombinant proteins were purified from the bacterial lysates by NEE according to the manufacturer’s instructions. *In vitro* protein phosphorylation assays were performed in the reaction mixture comprising purified recombinant MBP proteins incubated in kinase buffer (10 µCi [γ-32P]ATP, 25 mM TRIS-HCl pH 8.0, 0.5 mM DTT, 10 mM MgCl2, 0.1 mM CaCl2) for 30 min at 30 °C. Reactions were terminated by adding 5× LSB buffer and were boiled for 5 min at 95 °C. To detect autophosphorylation, the reaction mixture was separated by 12% (w/v) SDS-PAGE, and then the gel was dried and exposed to a Kodak X-ray film.

**Results**

**Characterization of the BnCIPK6 gene**

To investigate the genes involved in response to abiotic stress, 154 salt- and drought-induced genes were identified (Chen et al., 2010), including *BnCIPK6* (accession number in GenBank: JF751063) in the *Brassica napus* cDNA library. *BnCIPK6* encodes a CBL-interacting protein kinase (CIPK). Subsequently, the complete DNA sequence of the *BnCIPK6* gene in the *B. napus* genome was isolated, including its 5’-flanking sequence. Compared with its cDNA sequence, it was found that the *BnCIPK6* gene contains no intron in its open reading frame. In addition, *BnCIPK6* protein contains an N-terminal SNF1-like...
kinase catalytic domain and a C-Terminal regulatory domain with a CBL-interacting NAF/FISL module (see Supplementary Fig. S1 at JXB online).

Identification of BnCIPK6-interacting proteins
To identify interaction partners of BnCIPK6 in Brassica napus, the yeast two-hybrid library of Brassica napus cDNAs was screened using BnCIPK6 as bait. 27 unique proteins were identified as positive clones (see Supplementary Table S2 at JXB online), and all positive clones were checked for the presence of a cDNA–AD fusion and further confirmed in the one-to-one interaction analysis. The BnCIPK6-interacting proteins identified were related to various aspects of plant development, metabolism, and signal transduction. BLASTP analysis revealed that an isolated calcineurin B-like (CBL) protein (No. 27) by yeast two-hybrid screening shares high homology to AtCBL1, and consequently designated as BnCBL1 (accession number in GenBank: JF751064). To confirm the interaction between BnCIPK6 and BnCBL1, the BIFC method was employed. The results demonstrated that BnCIPK6 interacted with BnCBL1 in vivo (Fig. 1; see Supplementary Fig. S2 at JXB online). In addition, the experimental results indicated that BnCIPK6 protein showed no self-activation of transcription, and also interacted strongly and specifically with AtCBL1, AtCBL2, AtCBL3, and AtCBL9, suggesting that CBL–CIPK binding specificity can cross the species barrier (see Supplementary Fig. S3 at JXB online).

Transcripts of BnCIPK6 and BnCBL1 genes were induced by abiotic stresses and abscisic acid (ABA)
To investigate whether expressions of BnCIPK6 and BnCBL1 genes are regulated by abiotic stresses and plant hormones, one-week-old seedlings of B. napus were subjected to different abiotic stresses and exogenous plant hormone treatments. As shown in Fig. 2a, the expression level of BnCIPK6 was significantly up-regulated by high-salinity, osmotic stress, low phosphate and ABA in roots. BnCIPK6 mRNAs were largely accumulated in roots at 3 h after NaCl treatment, and reached its highest level at 48 h. By contrast, BnCIPK6 transcripts reached its highest level at 12 h and then declined at 24 h under mannitol and ABA treatments. It was also found that the BnCIPK6 gene is strongly induced by low phosphate stress, but its expression was decreased when plants were transferred from Pi starvation to normal Pi condition (1.25 mM). Similarly, the expression level of the BnCBL1 gene in roots was remarkably enhanced by NaCl, mannitol, and ABA, and reached its peak values at 1–3 h after the treatments. In addition, our experimental data revealed that BnCBL1 was induced by low phosphate stress in leaves, but not in roots of B. napus (Fig. 2b). Moreover, the expression patterns of BnCIPK6 and BnCBL1 genes in different tissues of B. napus were also analysed by quantitative RT-PCR. The results revealed that transcripts of BnCIPK6 were largely accumulated in flowers, at moderate or low levels in other tissues, whereas BnCBL1 expression was at relatively high levels in stems of B. napus (Fig. 2c, 2d).

BnCIPK6 promoter is salt/osmotic stress- and ABA-inducible
To investigate whether the BnCIPK6 promoter is induced by abiotic stresses and hormone treatments, a 1.2 kb BnCIPK6 5’-flanking region before the translational initiation codon (ATG) was cloned upstream of the GUS reporter gene in the pBI101 vector, giving rise to the BnCIPK6p:GUS chimeric gene. The construct was introduced into Arabidopsis. A total of 30 transgenic Arabidopsis plants (lines) were obtained. Histochemical assay revealed that GUS staining was detected at relatively high levels in hypocotyls and cotyledons in 12-d-old seedlings, but weak or no GUS signals in other tissues (Fig. 3a). GUS activities were significantly increased in cotyledons and hypocotyls of the transgenic seedlings (12-d-old) for 6 h after 150 mM NaCl, 300 mM mannitol, and 100 µM ABA treatments, compared with those of mock treatments (Fig. 3b–d). It should be noted that GUS activities were detected in the maturation zone of roots after these treatments, but not in those of control transgenic seedlings. Quantitative analysis further confirmed that GUS activity was higher in the salinity, osmotic, and ABA-treated transgenic plants than those of the mock treatment (Fig. 3e). These results suggested that the BnCIPK6 promoter is salt/osmotic stress-/ABA-inducible.

Assay of BnCIPK6 kinase activity in vitro
To characterize the kinase activity of BnCIPK6 biochemically, MBP-BnCIPK6 and MBP-BnCIPK6M (T182D, i.e. Thr182 was substituted by Asp in BnCIPK6, hereafter referred to as BnCIPK6M) proteins, as well as empty MBP protein from Escherichia coli, were expressed. As shown in Fig. 4a and 4b, BnCIPK6M mutant protein exhibited 2.3-fold higher autophosphorylation activity than that of BnCIPK6 protein, suggesting
that the 182nd threonine residue in BnCIPK6 may be a critical target site for the protein activation by upstream kinase(s).

**Over-expression of BnCIPK6 and BnCBL1 in Arabidopsis enhances plant tolerance to salt stress**

The coding sequences of BnCIPK6 and BnCIPK6M fused to the CaMV 35S promoter were introduced into Arabidopsis, and over 30 homozygous transgenic lines (T2 and T3 generations) were obtained. Expression levels of BnCIPK6 and BnCIPK6M in the transgenic plants were examined by RT-PCR analysis (Fig. 4c).

Two transgenic lines (L8 and L9) with higher BnCIPK6M expression and two lines (L3 and L6) with higher BnCIPK6 expression were selected for analysing their phenotypes under various treatment conditions. When 1-week-old seedlings grew on MS medium under normal conditions, there was no difference between wild type and transgenic plants. When 1-week-old seedlings were transferred and vertically cultured on MS medium containing a range of NaCl concentrations, both BnCIPK6 and BnCIPK6M transgenic lines displayed better salt tolerance than that of wild-type plants (Fig. 4d; see Supplementary Table S3 at JXB online). Statistical analysis indicated that there were...
that over-expression of BnCIPK6, BnCIPK6M, and BnCBL1 significantly enhanced plant tolerance to salt stress.

Over-expression of BnCIPK6 and BnCBL1 in Arabidopsis enhances plant tolerance to low phosphate stress

Six-day-old BnCBL1, BnCIPK6, and BnCIPK6M transgenic seedlings were transferred and vertically cultured at 50 µM low phosphate medium for 9 d. It was found that BnCIPK6 and BnCIPK6M transgenic seedlings grew better than wild-type plants under low phosphate conditions, whereas there was no significant difference between the transgenic plants and the wild type under phosphate-sufficient conditions (Fig. 6a, 6b). In addition, BnCIPK6 and BnCIPK6M transgenic seedlings have more and longer lateral roots than those of the wild type (Fig. 6c; see Supplementary Fig. S5 at JXB online). The fresh weight of BnCIPK6 and BnCIPK6M transgenic plants was also larger than that of the wild type. Likewise, BnCBL1 transgenic seedlings displayed higher low-phosphate tolerance than that of the wild type at the concentrations tested (Fig. 6d, 6e, 6f; see Supplementary Fig. S6 at JXB online). Collectively, these results suggested that BnCBL1 and BnCIPK6 may be involved in plant response and tolerance to low-phosphate.

Activation of BnCIPK6 confers Arabidopsis plants hypersensitive to abscisic acid (ABA)

To determine whether BnCIPK6 protein is involved in the ABA signalling pathway, the transgenic lines were tested under exogenous ABA treatment. When germinated and grown on MS medium, BnCIPK6 and BnCIPK6M transgenic plants grew almost the same as the wild type (Fig. 7a, 7c). When cultured on MS medium with 0.25–1 µM ABA for several days, however, both seed germination and primary root growth of BnCIPK6M and BnCIPK6 transgenic plants were severely inhibited, compared with wild-type plants (Fig. 7b; see Supplementary Table S5 at JXB online). When seedlings were grown on MS medium containing 0.5 µM ABA, the root length of the BnCIPK6M transgenic lines was shorter than that of the wild type (Fig. 7d). Measurement and statistical analysis indicated that the root length of two BnCIPK6M transgenic lines was significantly less than 70% of the wild type, whereas the root length of the BnCIPK6 lines was over 80% of the wild type. Likewise, BnCIPK6M transgenic plants was also larger than BnCIPK6 and BnCIPK6M transgenic seedlings have more and longer lateral roots than those of the wild type (Fig. 6c; see Supplementary Fig. S5 at JXB online). Collectively, these results suggested that BnCIPK6M transgenic plants were more ABA-sensitive than those of BnCIPK6 transgenic plants, suggesting that activation of BnCIPK6 may be important for its participation in ABA signalling transduction.

To investigate further the mechanism of the constitutively activated kinase, BnCIPK6M, involved in the ABA signalling pathway, it was examined whether expression of ABA-responsive genes (such as RD29A, RD29B, KIN1, ABF3, and ABF4) in BnCIPK6M transgenic lines, served as markers for monitoring ABA and stress response pathways. As shown in Fig. 8, expression levels of ABF3, ABF4, and RD29A in the transgenic plants were much higher than those in the wild

significant differences in chlorophyll content, and proline content between BnCIPK6/BnCIPK6M transgenic lines and control plants under salt stress (Fig. 4e, 4f).

Similarly, in order to determine whether BnCBL1 is also involved in salt signalling, the BnCBL1 gene was introduced into Arabidopsis plants. Over 20 homozygous transgenic lines (T2 and T3 generations) were obtained, of which the lines with high BnCBL1 expression were selected for more detailed analysis (Fig. 5a; see Supplementary Fig. S4 at JXB online). The results also revealed that BnCBL1 transgenic seedlings showed increased NaCl-tolerance, compared with the wild type (Fig. 5; see Supplementary Table S4 at JXB online). These data indicated

Fig. 3. Analysis of BnCIPK6 promoter activity in Arabidopsis plants under NaCl, mannitol, and abscisic acid (ABA) treatments. 12-d-old transgenic Arabidopsis seedlings containing the BnCIPK6 promoter fused to the GUS reporter gene were treated without (control) or with 150 mM NaCl, 300 mM mannitol, and 100 µM ABA for 6 h. (a) A control seedling; (b) a seedling with NaCl treatment; (c) a seedling with mannitol treatment; (d) a seedling with ABA treatment. (e) Measurement and quantitative analysis of GUS activity in BnCIPK6p::GUS transgenic Arabidopsis plants under NaCl, mannitol or ABA treatments. Mean values and standard errors (bar) were shown from three independent experiments. Independent t tests for equality of means demonstrated that there was very significant difference between CK and NaCl-, mannitol-, or ABA-treated transgenic plants (**P value < 0.01).
type under ABA treatment although there was no significant difference in expression levels of those genes between the transgenic plants and the wild type in the absence of ABA. However, there was only slight or no significant decrease in mRNA levels of *RD29B* and *KIN* between wild-type and transgenic plants with or without ABA treatment. These results further suggest that the constitutively activated BnCIPK6, which displayed higher kinase activity, may be involved in the ABA signalling pathway, acting upstream of these marker genes. On the other hand, it was found that BnCB1 over-expression transgenic plants did not show detectable phenotypic change under ABA treatments (data not shown) although BnCB1 can interact with BnCIPK6, suggesting that other CBLs that interacted with BnCIPK6 protein may be involved in the response to ABA.

**BnCIPK6 functionally complemented the defects of the atcikp6 mutant**

The homozygous lines of the *Arabidopsis cikp6* mutant (SM_3_39539) obtained from ABRC were identified by PCR using gene-specific and T-DNA-specific primers (3′ dSpm). To confirm that *cikp6* is a transcript-null mutant, RT-PCR analysis was performed. As shown in Supplementary Fig. S7 at *JXB* online, no CIPK6 transcripts were detected in three lines of cikp6ko seedlings. Phenotypic analysis revealed that cikp6ko seedlings were more sensitive to salt and low phosphate stress, and confers the plant ABA-insensitive phenotype (our unpublished data).

To investigate whether BnCIPK6 performs a similar function as AtCIPK6, functional complementation analysis was performed.
**Fig. 5.** Over-expression of *BnCBL1* in *Arabidopsis* enhances plant tolerance to salt stress. (a) Northern blotting analysis of *BnCBL1* expression in transgenic *Arabidopsis*. (b) One-week-old seedlings were transferred and grown for 5 d on MS medium as controls. (c) One-week-old seedlings were transferred and grown for 5 d on MS medium supplemented with 170 mM NaCl. (d) One-week-old seedlings were transferred and grown for 2 weeks on MS medium supplemented with 170 mM NaCl. (e) Statistical analysis of chlorophyll content of leaves. The seedlings were grown on MS medium containing 170 mM and 200 mM NaCl for 5 d. (f) Measurement of proline content. The seedlings of wild-type and transgenic lines were treated with 170 mM and 200 mM NaCl for 24 h. Mean values and standard errors (bar) were shown from three independent experiments (n > 50 seedlings per each line). Independent t-tests for equality of means demonstrated that there was very significant difference between wild-type and transgenic plants (**P value < 0.01). WT, wild type; L10, L11, and L12, *BnCBL1* transgenic lines 10, 11, and 12. (This figure is available in colour at JXB online.)

**Fig. 6.** Over-expression of *BnCIPK6*, *BnCIPK6M*, and *BnCBL1* in *Arabidopsis* enhances plant tolerance to phosphorous starvation. (a–c) Transgenic *Arabidopsis* plants over-expressing *BnCIPK6M* and *BnCIPK6* were transferred and grown for 9 d on MS medium and low phosphate medium. (a) Six-day-old seedlings were transferred and grown on MS medium. (b) Six-day-old seedlings were transferred and grown on MS medium with 50 µM phosphate (low phosphate, LP). (c) Statistical analysis of the lateral root number. (d–f) Transgenic *Arabidopsis* plants over-expressing *BnCBL1* were transferred and grown for 9 d on MS medium and low-phosphate medium. (d) One-week-old seedlings were transferred and grown on MS medium as controls. (e) One-week-old seedlings were transferred and grown on low-phosphate medium (50 µM Pi). (f) Statistical analysis of plant fresh weight. Mean values and standard errors (bar) were shown from three independent experiments (n > 50 seedlings per each line). Independent t-tests for equality of means demonstrated that there was significant difference (*P value < 0.05) or very significant difference (**P value < 0.01) between wild-type and transgenic plants. (This figure is available in colour at JXB online.)

*BnCIPK6*, under the CaMV 35S promoter, was expressed in *Arabidopsis cipk6* knockout mutants (*cipk6/35S::BnCIPK6*). RT-PCR analysis showed that strong *BnCIPK6* expression was only detected in the complemented transgenic lines. Under normal growth conditions, *cipk6/BnCIPK6* transgenic plants did not show noticeable phenotypes compared with wild-type and
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cipk6 mutant plants. By contrast, the cipk6/BnCIPK6 transgenic lines completely rescued the low-phosphate-sensitive and ABA-insensitive phenotypes of the cipk6 mutants under low phosphate stress and ABA treatment (see Supplementary Fig. S8 at JXB online), suggesting that both BnCIPK6 and AtCIPK6 proteins may perform similar function in plants.

Discussion

Previous studies revealed that the constitutively activated forms of CIPK activity might be generated through the substitution of one of the three conserved residues (serine, threonine, and tyrosine) to aspartate within the activation loop (Guo et al., 2001; Gong et al., 2002b). Similarly, our results showed that substitution of Thr182 by Asp resulted in a higher autophosphorylation activity of BnCIPK6. It remains unclear so far whether these three residues are also the targets of phosphorylation in regulating the activity of CIPKs in vivo. However, the mechanism of phosphorylation-dependent activation of CIPKs suggests that

Fig. 7. Over-expression of BnCIPK6M in Arabidopsis results in plants that are hypersensitive to abscisic acid (ABA). (a) Seeds of wild-type and transgenic lines germinated and were grown on MS medium without ABA for 2 weeks. (b) Seeds of wild-type and transgenic lines germinated and were grown on MS medium with 0.5 μM ABA for 2 weeks. (c) Root length of wild-type and transgenic seedlings grown on MS medium for 2 weeks. (d) Root length of wild-type and transgenic seedlings grown on MS medium containing 0.5 μM ABA for 2 weeks. (e) Statistical analysis of the root length of transgenic Arabidopsis plants over-expressing BnCIPK6 and BnCIPK6M grown on MS medium without (CK) or with 0.5 μM ABA for 2 weeks. Mean values and standard errors (bar) were shown from three independent experiments (n > 50 seedlings per each line). Independent t tests for equality of means demonstrated that there was significant difference (*P value < 0.05) or very significant difference (**P value < 0.01) between wild-type and transgenic plants. WT, wild type; B6(M)L8 and L9, BnCIPK6M transgenic line 8 and 9; B6L3 and L6, BnCIPK6 transgenic line 3 and 6. (This figure is available in colour at JXB online.)

Fig. 8. Quantitative RT-PCR analysis of expression of stress- and ABA-responsive genes in transgenic Arabidopsis plants over-expressing BnCIPK6M. Total RNA was isolated from 2-week-old seedlings without (-ABA) and with ABA treatment (+ABA) for 6h, respectively. Transcript levels of RD29A, RD29B, KIN1, ABF3, and ABF4 were determined by quantitative RT-PCR, using ACTIN2 as a quantification control. Independent t tests for equality of means demonstrated that there was (very) significant difference between wild-type and transgenic plants (*P value < 0.05; **P value < 0.01).
CIPKs may be activated by other CIPK-phosphorylating kinases, such as CDPKs, MAPKs or other protein kinases, and are involved in the signalling cross-talk with other signalling pathways such as CDPKs and MAPKs (Kolukisaoglu et al., 2004). In our study, it was found that three protein kinases, including ITPK4, SNF1 kinase homologue 10, and phosphoribulokinase, were the interactors of BnCIPK6. These data may lay the foundation of explaining the activation mechanism of CIPKs in vivo in the future.

Yeast two-hybrid analysis revealed that BnCIPK6 is able to interact with Arabidopsis CBL1, CBL2, CBL3, and CBL9, indicating that the structure of CIPKs is conserved and CBL-CIPK binding specificity can cross the species barrier. To identify more interaction partners of BnCIPK6 in Brassica napus, BnCIPK6 protein was used as bait to screen the Brassica napus cDNA two-hybrid library, and 27 unique proteins were identified as positive clones, including two calcineurin B-like proteins, BnCBL1 and BnCBL3. A previous study indicated that four Arabidopsis CBL proteins, including CBL1, CBL4, CBL5, and CBL9, contain a myristoylation site at their N-terminals that plays an important role for protein-membrane attachment (Batistic and Kudla, 2004). Studies on protein localization displayed membrane targeting of AtCBL1 and AtCBL9 (Albrecht et al., 2003; Cheong et al., 2004). AtCIPK1:GFP fusion proteins were observed at the plasma membrane, and to some extent also in the cytosol and nucleus. AtCIPK1 was recruited to the plasma membrane by interaction with AtCBL1 and AtCBL9, which localize to the plasma membrane (D’Angelo et al., 2006). A similar observation was also made in a study of AtCIPK23 subcellular localization (Xu et al., 2006; Cheong et al., 2007). In our study, BnCIPK6 was mainly localized at the plasma membrane and nucleus, whereas its interaction partner BnCBL1 was localized to the plasma membrane. These results suggest that the BnCBL1/BnCIPK6 complex may function in vivo by interacting with some membrane-localized proteins as their targets.

Progress has been made in understanding the salt-stress signalling pathway of Arabidopsis in recent years. The Arabidopsis SOS pathway includes three components, SOS1, SOS2, and SOS3, which collectively contribute to salt stress (Shi et al., 2002). It was reported that SOS pathway also exists in rice and has a high degree of functional similarity to its Arabidopsis counterpart (Martínez-Atienza et al., 2007). Similarly, our data demonstrated that over-expression of BnCIPK6 and BnCIPK6M significantly enhanced plant tolerance to salt stress. Recently, a study indicated that CaCIPK6 is up-regulated by abiotic stresses (such as salinity and dehydration) and hormones (such as ABA and IAA). Over-expression of a constitutively activated mutant of CaCIPK6 promoted salt tolerance in transgenic tobacco, whereas the Arabidopsis cipk6 knockdown mutant was more sensitive to salt stress (Tripathi et al., 2009). These results together suggest that CIPK6 plays positive roles in conferring plant salt-tolerance. In this study, two calcineurin B-like proteins, BnCBL1 and BnCBL3, were identified as BnCIPK6 interacting proteins. Further study revealed that BnCBL1 was also involved in the plant response to salt stress. BnCBL1 transgenic seedlings displayed more salt-tolerance than that of the wild type. The data presented here implied that BnCBL1 and BnCIPK6 may function in the same salt signalling pathway.

Reverse genetics analyses have uncovered crucial functions of CBLs and CIPKs in the plant response to ABA. Previous studies reported that cipk3 and cbl9 loss-of-function mutants are hypersensitive to ABA (Kim et al., 2003; Pandey et al., 2004, 2008). Furthermore, the pks3 (cipk15) mutant shows ABA hypersensitivity, revealing that PKS3/CIPK15 is a negative regulator of ABA signalling (Guo et al., 2002). Over-expression of CIPK20/PKS18 (T169D) rendered the transgenic plants hypersensitive to ABA, whereas RNAi plants showed insensitivity to ABA (Gong et al., 2002). Similarly, it was shown that both germination and post-germination growth of BnCIPK6M over-expression transgenic Arabidopsis were hypersensitive to ABA, whereas silencing of its homologous gene AtCIPK6 confers plant ABA-insensitive growth phenotypes. Furthermore, expression levels of AFB3, AFB4, and RD29A were much increased in the transgenic plants compared with the wild type after ABA treatment. Previous studies revealed that over-expression of AFB3 or AFB4 in Arabidopsis resulted in ABA hypersensitivity and other common ABA-associated phenotypes (Kang et al., 2002). The RD29A (responsive to desiccation) gene has been shown to be responsive to ABA, drought, cold, and salinity (Yamaguchi-Shinozaki and Shinozaki, 1994). Both CPK4 and CPK11 kinases phosphorylated two ABA-responsive transcription factors, ABF1 and ABF4, in vitro, suggesting that the two kinases may regulate ABA signalling through these transcription factors (Zhu et al., 2007). Based on our results, as well as those of published data, it is therefore speculated that BnCIPK6M kinase, a constitutively activated form of BnCIPK6, may regulate these transcription factors in the ABA signalling pathway.

Phosphate (H₂PO₄⁻) is an essential nutrient required for various basic biological functions in the plant life cycle (Raghothama, 1999), and is the major form that is most readily taken up and transported in plant cells (Tu et al., 1990). It was known that the phosphate concentration in soil, typically 10 µM or less, results in phosphorous starvation for plant growth and survival, which is one of the major limiting factors for crop production in cultivated soils (Chen et al., 2009). It is shown here that BnCIPK6 is involved in the response to phosphorous starvation. BnCIPK6 expression was strongly induced by low-phosphate stress in both roots and leaves of Brassica napus. The transgenic seedlings over-expressing BnCIPK6 and BnCIPK6M were obviously growing better than the wild type under low-phosphate conditions. BnCIPK6 and BnCIPK6M transgenic seedlings had more and longer lateral roots than that of wild-type plants under low-phosphate conditions. It should be mentioned that significant lateral root differences were not observed between the wild type and cipk6 mutants in normal growth medium. This is inconsistent with a previous report that the lateral roots of cipk6 mutants are thinner and shorter than wild-type plants (Tripathi et al., 2009). Furthermore, over-expression of BnCBL1 in Arabidopsis enhances plant tolerance to low-phosphate stress. These results
suggest that BnCBL1-BnCIPK6 may functionally interact with each other to be involved in response to low-phosphate stress. Further identification of CIPK6 substrates will be crucial toward a better understanding of its role in phosphorous starvation signalling.

**Supplementary data**

Supplementary data can be found at JXB online.

- **Supplementary Table S1.** Primer sequences used in this study.
- **Supplementary Table S2.** cDNA clones identified from the BnCIPK6 yeast two-hybrid screen.
- **Supplementary Table S3.** Statistical analysis of relative green leaves and relative fresh weight of BnCIPK6 and BnCIPK6M transgenic Arabidopsis under a range of NaCl concentrations.
- **Supplementary Table S4.** Statistical analysis of relative green leaves and relative fresh weight of BnCBL1 transgenic Arabidopsis under a range of NaCl concentrations.
- **Supplementary Table S5.** Statistical analysis of the primary root length of BnCIPK6 and BnCIPK6M transgenic Arabidopsis under a range of ABA concentrations.
- **Supplementary Fig. S1.** A schematic diagram of the domain structure of BnCIPK6.
- **Supplementary Fig. S2.** BiFC assays of BnCIPK6 interaction with BnCBL1 in onion cells.
- **Supplementary Fig. S3.** Yeast two-hybrid analysis for interactions between BnCIPK6 and ten AtCBL proteins.
- **Supplementary Fig. S4.** Quantitative RT-PCR analysis of BnCBL1 expression in transgenic Arabidopsis.
- **Supplementary Fig. S5.** Assay of lateral roots elongation of BnCIPK6 and BnCIPK6M transgenic plants growing on low phosphate medium.
- **Supplementary Fig. S6.** Statistical analysis of plant dry weight of BnCBL1 transgenic plants under phosphorous starvation.
- **Supplementary Fig. S7.** Identification of cipk6 loss-of-function mutants.
- **Supplementary Fig. S8.** Characterization of Arabidopsis cipk6 knockout mutant expressing BnCIPK6.

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**References**


