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RESEARCH PAPER

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

Liang Chen[1,2], Feng Ren[1], Li Zhou[1], Qing-Qing Wang[1], Hui Zhong[1] and Xue-Bao Li[1,*]

[1] Hubei Key Laboratory of Genetic Regulation and Integrative Biology, College of Life Sciences, Central China Normal University, Wuhan 430079, China
[2] Key Laboratory of Plant Germplasm Enhancement and Specialty Agriculture, and Wuhan Botanical Garden, Chinese Academy of Sciences, Wuhan 430074, China

* To whom correspondence should be addressed. E-mail: xbli@mail.ccnu.edu.cn

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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 Ca^{2+}-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). Ca^{2+} sensors can be classified into sensor responders and sensor relays (Sanders *et al*., 2002). Upon binding of Ca^{2+}, 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 Ca^{2+}. 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

identified, respectively (Kolukisaoglu *et al*., 2004; Batistic and Kudla, 2004).

CIPK family proteins have an N-terminal kinase catalytic domain, related to sucrose non-fermenting kinase (SNF1) and AMP-activated protein kinase (AMPK), via a junction domain, fused to a highly variable C-terminal regulatory domain. The N-terminal kinase domain contains a putative activation loop located between the conserved DFG and APE sequences (Batistic and Kudla, 2004). Mutational analysis revealed that the constitutively active forms of CIPK activity might be generated through substitution of one of three conserved residues (serine, threonine, and tyrosine) to aspartate within the activation loop (Guo *et al*., 2001; Gong *et al*., 2002*a*). C-terminal regulatory domains of all CIPKs possess a highly conserved FISL motif, also named a NAF domain, which consists of a unique 24 amino-acid residues, required and sufficient for interacting with CBL proteins (Albrecht *et al*., 2001). It was revealed that the intramolecular interaction between NAF domain and regulatory domain exerts an inhibitory effect towards the kinase activity. Both the binding of the FISL motif with CBLs and deletion of the NAF domain can relieve auto-inhibition (Guo *et al*., 2001; Gong *et al*., 2002*b*). Superactive kinase SOS2 is generated when the threonine to aspartate mutation is combined with the NAF domain deletion (Guo *et al*., 2001; Gong *et al*., 2002*b*). However, little is known about how CIPKs are activated *in vivo*. It was assumed that activation of AtCIPKs take place *in vivo* through phosphorylation of the activation loop and binding of CBL proteins working simultaneously (Gong *et al*., 2004; Batistic and Kudla, 2004). Previous study indicated that a motif, PPI (protein phosphatase interaction), adjacent to the FISL motif is necessary for interaction with ABI (Abscisic acid-Insensitive) protein phosphatases (Ohta *et al*., 2003). It is proposed that CIPK kinases and a 2C-type protein phosphatase control the phosphorylation status of each other and/or downstream target proteins (Rodriguez, 1998; Ohta *et al*., 2003; Lee *et al*., 2007).

CBL/CIPK signal components are involved in many kinds of signalling pathways. SOS is one of the CBL/CIPK signalling pathways conferring plant salt tolerance (Zhu *et al*., 1998). Under salt stress, the calcium sensor SOS3 (CBL4) activates the kinase SOS2 (CIPK24) that positively regulates SOS1, a plasma membrane sodium/proton antiporter (Shi *et al*., 2000; Qiu *et al*., 2002). Moreover, it was demonstrated that CBL10 can also interact with CIPK24 to form the CBL10/CIPK24 complex that regulates the downstream protein SOS1. The CBL10/CIPK24/SOS1 pathway mainly regulates Na^+-homeostasis in shoots and leaves, revealing that CBL4 and CBL10 are required for salt tolerance (Quan *et al*., 2007). Previous study showed that *CIPK3* transcripts were induced by cold, salt, drought, and abscisic acid (ABA), and the *cipk3* loss-of-function mutant is hypersensitive to ABA. Disruption of *CIPK3* altered the expression pattern of a number of stress gene markers in response to ABA, cold and high salt, but did not affect transcription of low temperature-inducible transcription factors (e.g. *CBF3*), suggesting *CIPK*3 act as a cross-talk component between cold and ABA signalling (Kim *et al*., 2003). Furthermore, CBL9 and CIPK3 physically and functionally interact with each other and form a specific complex that functions in ABA response in seed germination (Pandey *et al*., 2004, 2008). Over-expression of CIPK20/PKS18 (T169D) rendered the transgenic plants hypersensitive to ABA, whereas RNAi plants showed insensitivity to ABA (Gong *et al*., 2002*b*). 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 *Arabidopsi*s 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 (Biolistic 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 deteceted, 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 analysed 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 μM ABA for 6 h, 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 pGBKT7 (bait vector) and pGADT7 (prey vector), respectively. Afterwards, the pGBKT7-BnCIPK6 construct was introduced singly into the yeast strain Y187 using the high-efficiency lithium acetate transformation procedure (Gietz *et al*., 1992), and each pGADT7-AtCBL construct was transferred into the yeast strain AH109. The mating reactions and further interaction assays between the two haploid strains containing pGBKT7-BnCIPK6 and pGADT7-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 instruction (BD Biosciences Clontech). For screening target proteins of BnCIPK6 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 pGBKT7-BnCIPK6 and pGADT7-AtCBL constructs were listed in Supplementary Table S1 at *JXB* online.

Construction of BnCIPK6p:GUS *chimeric genes and* Arabidopsis *transformation*

A *Sal*I site and *Bam*HI site were introduced at the 5'-end and the 3'-end of the *BnCIPK6* 5'-upstream region, respectively. The *Sal*I/*Bam*HI fragments (0.8 kb and 1.2 kb, respectively) were then subcloned into the pBI101 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 on MS medium containing 50 mg l^{-1} kanamycin. Homozygous lines of the T_3 and T_4 generations were used for phenotypic analysis of transgenic plants. Corresponding primer sequences were listed in Supplementary Table S1 at *JXB* online.

Histochemical assay of GUS activity

Histochemical assays for GUS activity in transgenic *Arabidopsis* plants were conducted according to the protocol described byLi *et al*. (2002).

To test the induction of GUS expression by salt, osmotic stresses, and ABA treatments, transgenic seedlings were transferred to MS liquid medium containing 150 mM NaCl, 300 mM mannitol, or 100 μM ABA (abscisic acid), for 6 h. GUS staining patterns were confirmed by observing at least five different transgenic lines. Moreover, GUS activity in the transgenic seedlings with expressing *BnCIPK6p:GUS* was quantitatively measured by the fluorometrical method using 4-MUG (Sigma-Aldrich) as substrate (Jefferson *et al*., 1987).

Over-expression of BnCIPK6, BnCIPK6M, *and* BnCBL1 *genes in* Arabidopsis *and phenotypic analysis of transgenic plants*

To introduce the Thr182-to-Asp (T182D) mutation for constitutively activated *BnCIPK6*, the primer-based site-directed mutation *BnCIPK6M* (5'-acgggcttctccacGACacttgtggaactcc-3', 5'-ggagttccacaagtGTCgtggagaagcccgt-3') was generated. The coding sequences of *BnCIPK6* and *BnCIPK6M* were cloned into PBI121 vector. Similarly, the coding sequence of *BnCBL1* 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 3.5 h, the recombinant proteins were purified from the bacterial lysates by NEB 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 [$r^{32}P$]ATP, 25 mM TRIS-HCl pH 8.0, 0.5 mM DTT, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$) 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 Kodark 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 further, 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).

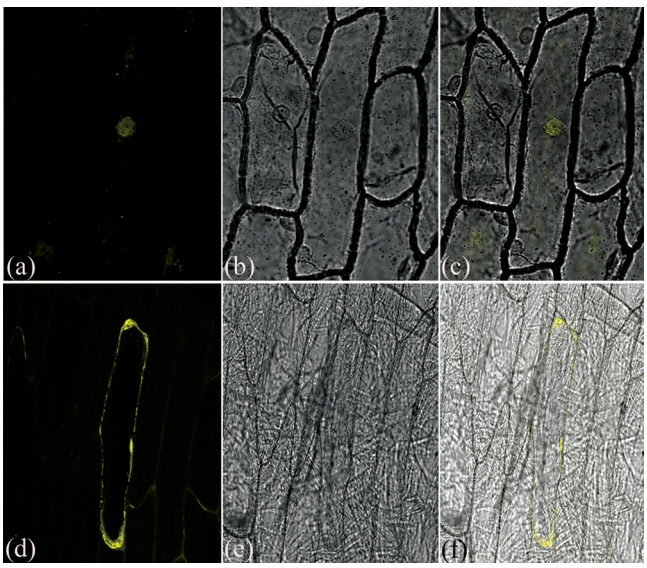

Fig. 1. BiFC assays of BnCIPK6 interaction with BnCBL1 *in vivo*. (a–c) BiFC visualization of bZIP63 dimerization *in vivo*; (d–f) BiFC visualization of BnCIPK6 interaction with BnCBL1 *in vivo*. (a, d) YFP fluorescent images; (b, e) bright field images of images (a) and (d); (c, f) fluorescent images merged with their bright-field images.

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

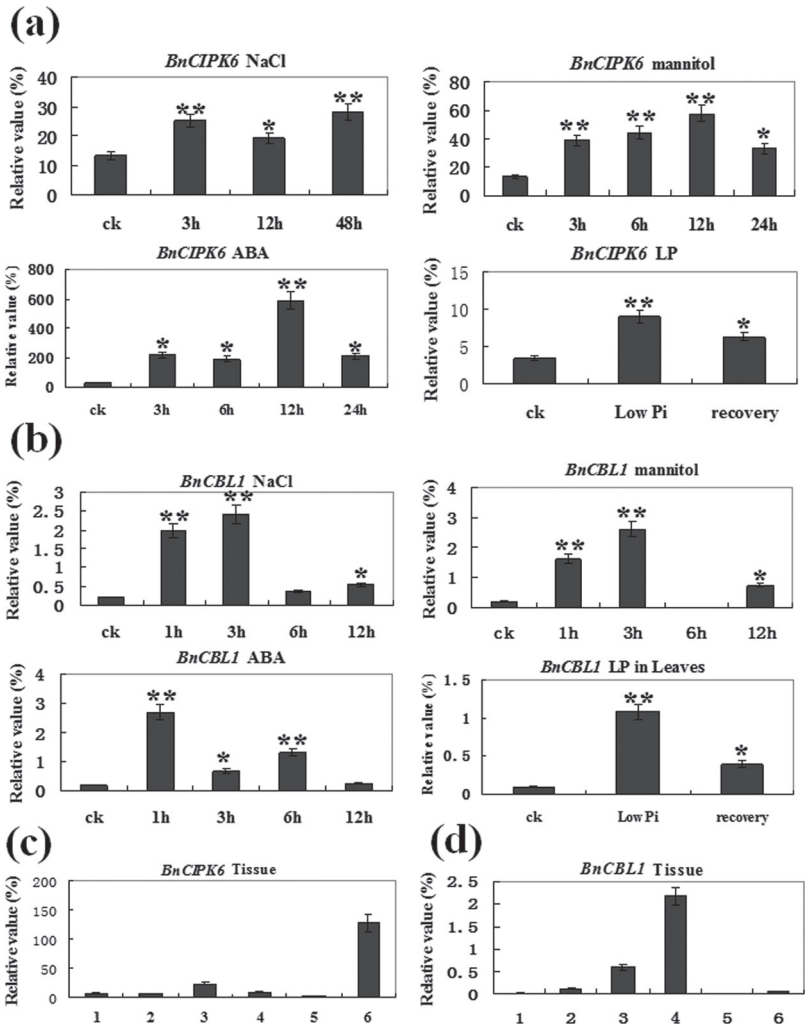

Fig. 2. Quantitative RT-PCR analysis of *BnCIPK6* and *BnCBL1* in *Brassica napus*. (a) Expression of the *BnCIPK6* gene in roots of *B. napus* under NaCl, mannitol, ABA, and low phosphate stress treatments. Total RNA was isolated from roots of 7-d-old seedlings treated with 150 mM NaCl for 3, 12, and 48 h, 200 mM mannitol for 3, 6, 12, and 24 h, 100 μM ABA for 3, 6, 12, and 24 h, and low phosphate (10 μM phosphate for 72 h, then recovered for 72 h in MS medium with 1.25 mM phosphate), respectively. (b) Expression of the *BnCBL1* gene in roots of *B. napus* under salt stress, osmotic stress, and ABA treatment, and in leaves of *B. napus* under low phosphate stress. Total RNA was isolated from roots of 7-d-old seedlings treated with 150 mM NaCl, 200 mM mannitol, 100 μM ABA for 1,3, 6, and 12 h and low phosphate (10 μM phosphate for 72 h, then recovered for 72 h in MS medium with normal phosphate content), respectively. (c) *BnCIPK6* and (d) *BnCBL1* expression in *B. napus* tissues. Total RNAs were isolated from roots (1), hypocotyls (2), cotyledons (3), stems (4), leaves (5), and flowers (6) of *B. napus*, respectively. Relative value of *BnCIPK6* and *BnCBL1* expression was shown as a percentage of *BnACT2* expression activity; ck, untreated roots (control). Mean values and standard errors (bar) were shown from three independent experiments. 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 untreated (control) and treated roots or leaves.

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 (T_2 and T_3 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 analysng 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

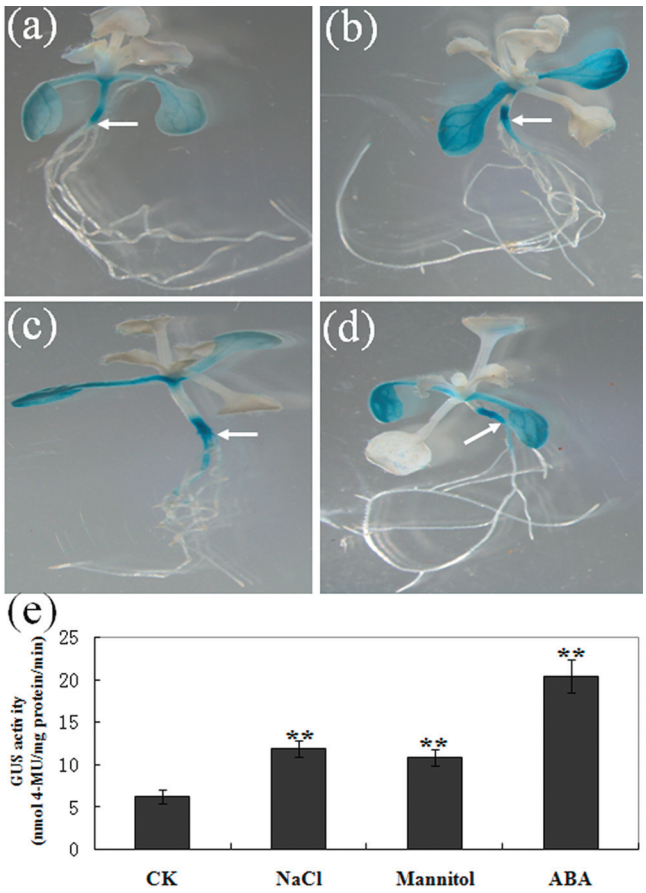

Fig. 3. Analysis of *BnCIPK6* promoter activity in *Arabidopsis* plants under NaCl, mannitol, and abscisic acid (ABA) treatments. 12-d-old transgenic *Arabidopsis* seedlings containing the *BnCIPK6* promoter fused to the *GUS* reporter gene were treated without (control) or with 150 mM NaCl, 300 mM mannitol, 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).

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 (T_2 and T_3 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 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 on MS medium with 0.5 µM ABA (Fig. 7e). These results demonstrated that both germination and post-germination growth of *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

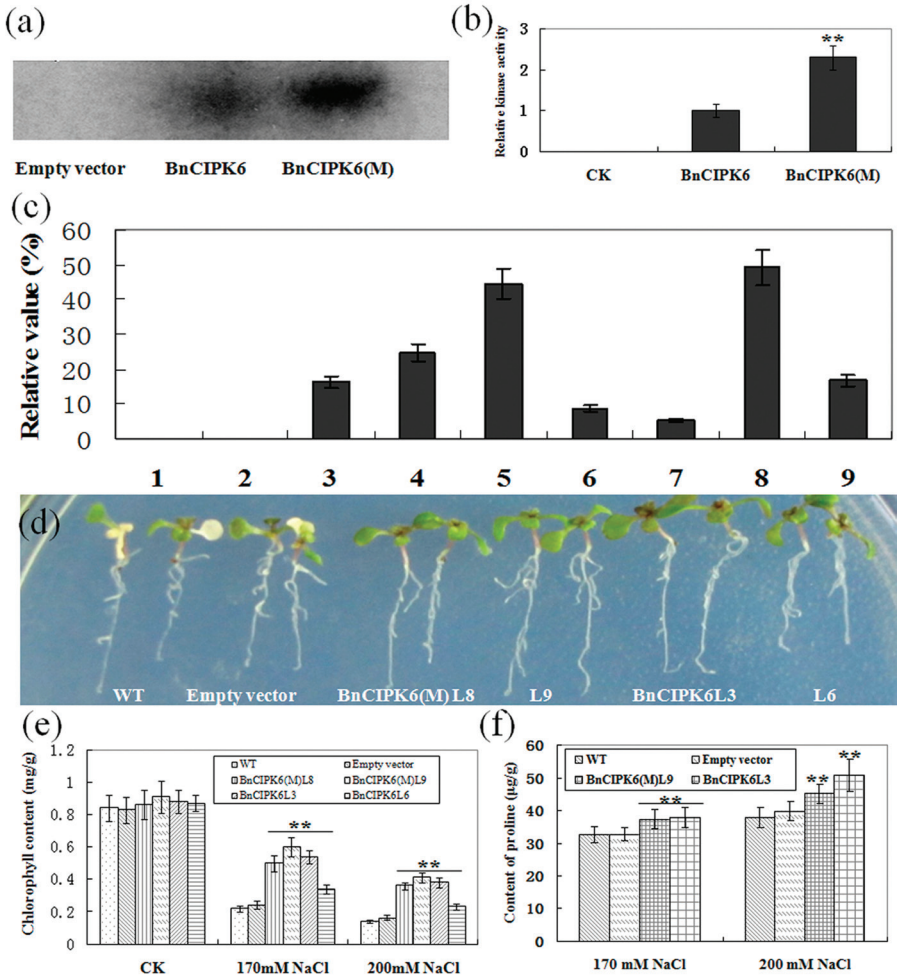

Fig. 4. Over-expression of *BnCIPK6* and *BnCIPK6M* in *Arabidopsis* enhances plant tolerance to salt stress. (a) *In vitro* phosphorylation assay of recombinant MBP-BnCIPK6 and MBP-BnCIPK6M fusion proteins. (b) Quantitative analysis of autophosphorylation activities of BnCIPK6 and BnCIPK6M. (c) Quantitative RT-PCR analysis of *BnCIPK6* and *BnCIPK6M* expression in transgenic *Arabidopsis*. (1) WT; (2) PMD vector; (3–6) *BnCIPK6M* transgenic lines L5, L8, L9, and L10; (7–9) *BnCIPK6* transgenic lines L1, L3, and L6. (d) One-week-old seedlings were transferred and grew for 5 d on MS medium supplemented with 170 mM NaCl. (e) Statistical analysis of chlorophyll content of leaves of seedlings grown on MS medium containing 170 mM and 200 mM NaCl for 7 d. (f) Measurement of proline content in seedlings of wild-type and transgenic lines 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). (This figure is available in colour at *JXB* online.)

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 *BnCBL1* over-expression transgenic plants did not show detectable phenotypic change under ABA treatments (data not shown) although BnCBL1 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 atcipk6 mutant

The homozygous lines of the *Arabidopsis cipk6* mutant (SM_3_39539) obtained from ABRC were identified by PCR using gene-specific and T-DNA-specific primers (3' dSpm). To confirm that *cipk6* 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 *cipk6ko* seedlings. Phenotypic analysis revealed that *cipk6ko* 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.

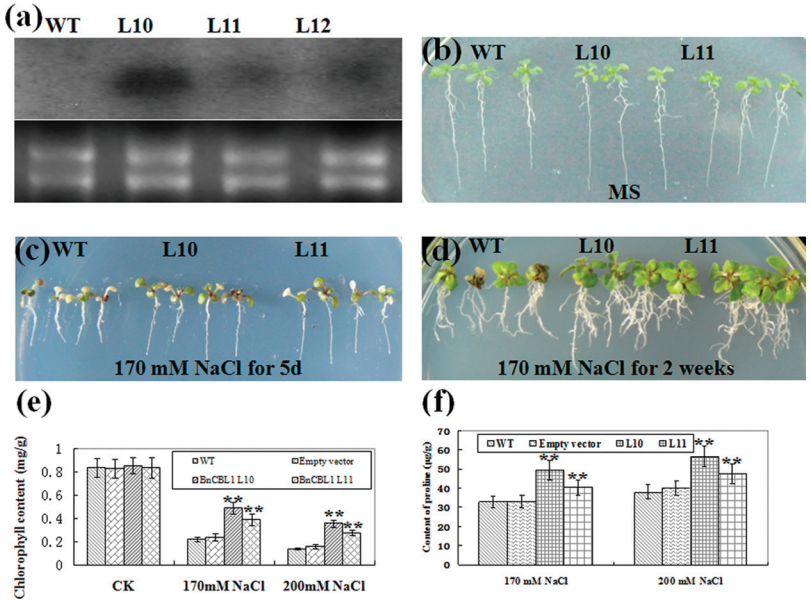

Fig. 5. Over-expression of *BnCBL1* in *Arabidopsis* enhances plant tolerance to salt stress. (a) Northern blotting analysis of *BnCBL1* expression in transgenic *Arabidopsis*. (b) One-week-old seedlings were transferred and grown for 5 d on MS medium as controls. (c) One-week-old seedlings were transferred 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.)

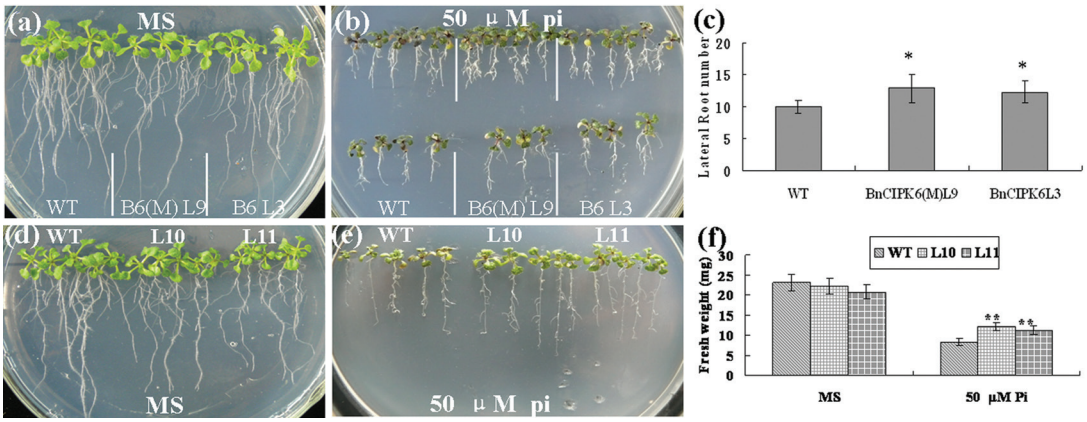

Fig. 6. Over-expression of *BnCIPK6*, *BnCIPK6M*, and *BnCBL1* in *Arabidopsis* enhances plant tolerance to phosphorous starvation. (a–c) Transgenic *Arabidopsis* plants over-expressing *BnCIPK6M* and *BnCIPK6* were transferred and grown for 9 d on MS medium and low phosphate medium. (a) Six-day-old 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* 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

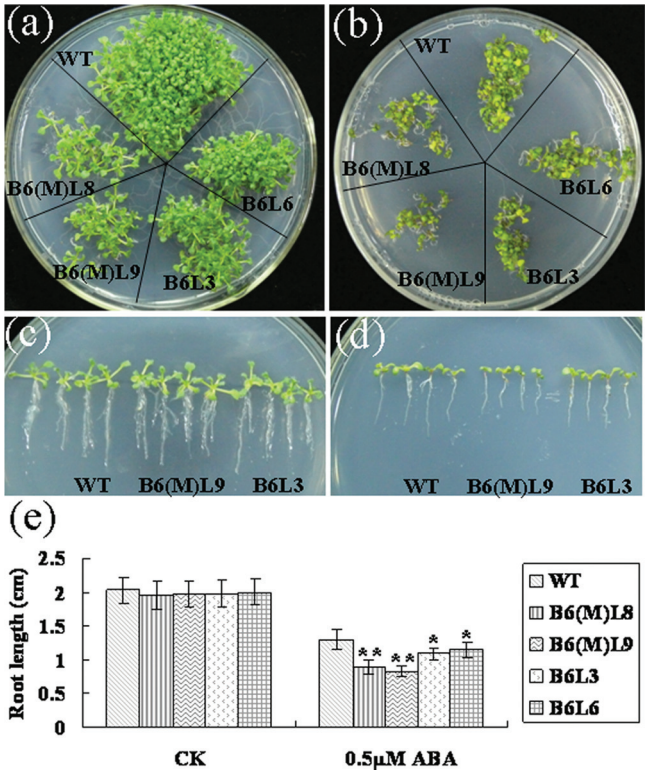

Fig. 7. Over-expression of *BnCIPK6M* in *Arabidopsis* results in plants that are hypersensitive to abscisic acid (ABA). (a) Seeds of wild-type and transgenic lines germinated and were grown on MS medium without ABA for 2 weeks. (b) Seeds of wild-type and transgenic lines germinated and were grown on 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.)

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*., 2002*b*). Similarly, our results showed that substitution of Thr_{182} 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

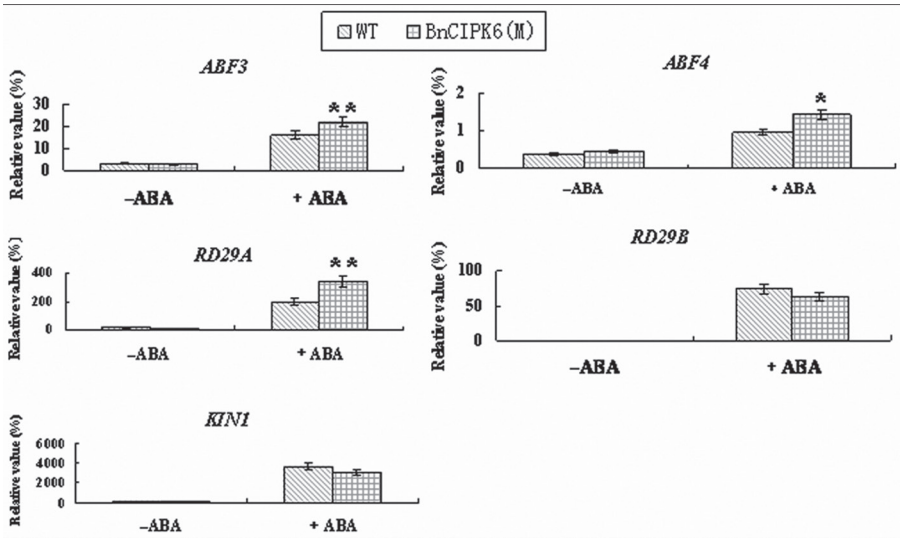

Fig. 8. Quantitative RT-PCR analysis of expression of stress- and ABA-responsive genes in transgenic *Arabidopsis* plants over-expressing *BnCIPK6M*. Total RNA was isolated from 2-week-old seedlings without (–ABA) and with ABA treatment (+ABA) for 6 h, respectively. Transcript levels of *RD29A*, *RD29B*, *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-terminus 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*., 2003; Pandey *et al*., 2004). In contrast to CBLs, CIPKs do not have any recognizable localization signal or the myristoylation site (Kolukisaoglu *et al*., 2004). Different localization of CIPKs may be dependent on their specific interaction partners, which could determine and regulate its localization (Batistic and Kudla, 2004). A previous localization analysis showed that 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*., 2000; Qiu *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 hypersensitive, 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*c*). 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 *ABF3*, *ABF4*, and *RD29A* genes were much increased in the transgenic plants compared with the wild type after ABA treatment. Previous studies revealed that over-expression of *ABF3* or *ABF4* 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 ($H_2PO_4^-$) 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 CIPK6 is involved in the response to phosphorous starvation. *BnCIPK6* expression was strongly induced by low-phosphate stress in both roots and leaves of *B. 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 roots 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*.

Acknowledgements

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