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

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Supplementary Data

Supplementary data

Supplementary Table 1. Primer sequences used in this study

Name	Squence	Function
BnCIPK6 up	5’-GGGACTAGTATGGTCGGAGCAAAACCTATAG-3'	BiFC
BnCIPK6 dn	5’-GGGCTCGAGAGCAGGTGTAGTAGAAGTCCAG-3’	BiFC
BnCBL1 up	5’- CTTGGATCCATGGGCTGCTTCCACTCCAAA -3’	BiFC
BnCBL1 dn	5’- CTTGGATCCTTTGACAATCTCATCCACCTCC-3’	BiFC
BnCIPK6 p1	5’-GGAGGCGGCGAAGGTTGGGAATAAGTTTGA-3’	RT-PCR
BnCIPK6 p2	5’-GGGCTCGAGTCAAGCAGGTGTAGTAGAAGTC-3’	RT-PCR
BnCBL1 p1	5’- ATCACTACTTAAGATCATGAC -3’	RT-PCR
BnCBL1 p2	5’- TATGATGTAAGAGAAACAATT -3’	RT-PCR
BnACT2 p1	5’- GTGTTGTTGGTAGGCCAAGACAT-3’	RT-PCR
BnACT2 p2	5’- CTTGATGTCTCTTACAATTTCCC-3’	RT-PCR
AtACT2 p1	5’- GAAATCACAGCACTTGCACC -3’	RT-PCR
AtACT2 p2	5’- AAGCCTTTGATCTTGAGAGC -3’	RT-PCR
RD29A p1	5’- TGAAAGGAGGAGGAGGAATGGTTGG -3’	RT-PCR
RD29A p2	5’- ACAAAACACACATAAACATCCAAAGT -3’	RT-PCR
RD29B p1	5’- CCAGATAGCGGAGGGGAAAGGACAT -3’	RT-PCR
RD29B p2	5’- AAGTTCACAAACAGAGGCATCATAC -3’	RT-PCR
KIN1 p1	5’- ACCAACAAGAATGCCTTCCAAGC -3’	RT-PCR
KIN1 p2	5’- TCCCAACAGTTAATTAGAAAAGG -3’	RT-PCR
ABF3 p1	5’- CTTTGTTGATGGTGTGAGTGAGC -3’	RT-PCR
ABF3 p2	5’- GTGTTTCCACTATTACCATTGCTG -3’	RT-PCR
ABF4 p1	5’- AACAACTTAGGAGGTGGTGGTCAT -3’	RT-PCR
ABF4 p2	5’- TGTAGCAGCTGGCGCAGAAGTCAT -3’	RT-PCR
BnCIPK6 F	5’- CTTGAATTCATGGTCGGAGCAAAACCTATAG -3’	pGBKT7 construction
BnCIPK6 R	5’- GGGCTGCAGTCAAGCAGGTGTAGTAGAAGTC -3’	pGBKT7 construction
AtCBL1 F	5’-CGAATTCATGGGCTGCTTCCACTCAAAGGCAG-3’	pGADT7 construction
AtCBL1 R	5’-CGGATCCTCATGTGGCAATCTCATCGACCTCC-3’	pGADT7 construction
AtCBL2 F	5’- CGAATTCATGTCGCAGTGCGTTGAC -3’	pGADT7 construction
AtCBL2 R	5’- CGAATTCATGTCGCAGTGCATAGACGGTTTC -3’	pGADT7 construction
AtCBL3 F	5’- CGAATTCATGTCGCAGTGCATAGACGGTTTC -3’	pGADT7 construction
AtCBL3 R	5’- CGGATCCTCAGGTATCTTCCACCTGCGAGTG -3’	pGADT7 construction
AtCBL4 F	5’- CCATATGATGGGCTGCTCTGTATCGAAGAAG -3’	pGADT7

		construction
AtCBL4 R	5’-CGAATTCTTAGGAAGATACGTTTTGCAATTCC-3’	pGADT7 construction
AtCBL5 F	5’-CGAATTCATGGGATGTGTTTGCAGCAAGC -3’	pGADT7 construction
AtCBL5 R	5’-CGGATCCTTACCGGAGAAAGGTTGGGAAAATC-3’	pGADT7 construction
AtCBL6 F	5’- CGAATTCATGATGATGCAATGTTTAGATGG -3’	pGADT7 construction
AtCBL6 R	5’- CGGATCCTCATCCATCCAGCTCACTAG -3’	pGADT7 construction
AtCBL7 F	5’- CGAATTCATGGATTCAACAAGAAATTCAGC -3’	pGADT7 construction
AtCBL7 R	5’- CGGATCCTCAGGTATCTTCCACTTGCG -3’	pGADT7 construction
AtCBL8 F	5’- CCATATGATGTTGGCATTCGTGAAATGC -3’	pGADT7 construction
AtCBL8 R	5’- CGAATTCCTAGTCTTCAACTTCAGAGTCGAG -3’	pGADT7 construction
AtCBL9 F	5’- CGAATTCATGGGTTGTTTCCATTCCACG -3’	pGADT7 construction
AtCBL9 R	5’- CGGATCCTCACGTCGCAATCTCGTCC -3’	pGADT7 construction
AtCBL10 F	5’- CCCATGGATGACAACTGGCCGACCAAAT-3’	pGADT7 construction
AtCBL10 R	5’- CGGATCCTCAGTCTTCAACCTCAGTGTT-3’	pGADT7 construction
BnCIPK6 CP1	5’- GGAGTTCCACAAGTGTCGTGGAGAAGCCCGT-3’	Genome walking
BnCIPK6 CP2	5’- CGCCACGCTTTTACCCGTTGTTACGTTACG -3’	Genome walking
BnCIPK6 CP3	5’- CACTTTAGCGAATGTCCCGTGGCCTAGAAG-3’	Genome walking
BnCIPK6 Promoter up	5’-GGGGTCGACTTTTATCTTCTGTCTTCGTGTA-3’	Isolate promoter
BnCIPK6 Promoter dn	5’-GGGGGATCCTGTTTTTAACCAAAAAAAAGGG-3’	Isolate promoter
BnCIPK6/BnCIPK6 (M) S	5’-CTTCCCGGGATGGTCGGAGCAAAACCTATAG-3’	OE vector construction
BnCIPK6/BnCIPK6 (M) AS	5’-GGGCTCGAGTCAAGCAGGTGTAGTAGAAGTC-3’	OE vector construction
BnCBL1 S	5’- CTTGGATCCATGGGCTGCTTCCACTCCAAA -3’	OE vector construction
BnCBL1 AS	5’- GGGGAGCTCTCATTTGACAATCTCATCCAC -3’	OE vector construction

Supplementary Table 2. Identified cDNA clones from the BnCIPK6 yeast two-hybrid screen

Clone no.	Homologous genes	Species	E-value	Homology
1	DNAJ heat shock protein	*Arabidopsis thaliana*	2e-77	Id.84%; Po.96%
2	CBL3	*Arabidopsis thaliana*	2e-100	Id.98%; Po.99%
3	fms interacting protein	*Ricinus communis*	6e-46	Id.60%; Po.77%
4	putative alpha/beta hydrolase	*Arabidopsis thaliana*	5e-62	Id.92%; Po.96%
5	polyubiquitin UBQ14	*Cucumis sativus*	3e-26	Id.100%; Po.100%
6	metal ion binding	*Brassica rapa*	1e-33	Id.97%; Po.98%
7	TCP-1chaperonin-like protein	*Arabidopsis thaliana*	3e-88	Id.98%; Po.100%
8	AtITPK4(inositol 1,3,4-trisphosphate 5/6-kinase 4)	*Arabidopsis thaliana*	4e-46	Id.88%; Po.94%
9	F-box family ptotein	*Arabidopsis lyrata*	3e-51	Id.59%; Po.67%
10	MYB3	*Arabidopsis thaliana*	3e-19	Id.66%; Po.69%
11	APG8 activating enzyme	*Arabidopsis thaliana*	1e-48	Id.95%; Po.99%
12	ATP binding / metal ion binding	*Arabidopsis thaliana*	6e-78	Id.95%; Po.97%
13	Putative xylan endohydrolase	*Arabidopsis thaliana*	1e-24	Id.88%; Po.96%
14	unknown protein	*Arabidopsis thaliana*	1e-36	Id.79%; Po.86%
15	hypothetical protein	*Arabidopsis lyrata*	9e-32	Id. 97%; Po.98%
16	chaperone protein dnaJ-related	*Arabidopsis thaliana*	2e-60	Id. 81%; Po.88%
17	plastid-lipid associated protein PAP2	*Brassica rapa*	1e-77	Id.100%; Po.100%
18	phospholipase A2-alpha	*Arabidopsis lyrata*	1e-46	Id.89%; Po.93%
19	acyl-CoA thioesterase	*Ricinus communis*	4e-43	Id.76%; Po.87%
20	Soybean gene regulated by cold-2	*Arabidopsis thaliana*	1e-73	Id.91%; Po.94%
21	putative phosphoglucomutase	*Arabidopsis thaliana*	3e-46	Id.95%; Po.98%
22	SNF1 kinase homolog 10	*Arabidopsis thaliana*	2e-64	Id.87%; Po.92%
23	phosphoribulokinase	*Arabidopsis thaliana*	7e-96	Id.97%; Po.99%
24	cytokinin dehydrogenase	*Arabidopsis thaliana*	4e-70	Id.91%; Po.95%
25	ubiquitin-protein ligase	*Arabidopsis thaliana*	3e-74	Id.94%; Po.97%
26	C2 domain-containing protein	*Noccaea caerulescens*	4e-13	Id.84%; Po.93%
27	calcineurin B-like protein 1	*Brassica oleracea*	8e-117	Id.99%; Po.100%

Supplementary Table 3. Statistical analysis of relative green leaves and relative fresh weight of *BnCIPK6* and *BnCIPK6M* transgenic *Arabidopsis* under a range of NaCl concentrations.

NaCl Concentration	Relative green leaves (%)					Relative fresh weight (mg)				
	WT	**BnCIPK6(M) L8**	**BnCIPK6(M) L9**	**BnCIPK6 L3**	**BnCIPK6 L6**	**WT**	**BnCIPK6(M) L8**	**BnCIPK6(M) L9**	**BnCIPK6 L3**	**BnCIPK6 L6**
75 mM	95±2	95±3	96±2	96±3	95±4	29.1±1.6	31.4±2.4	34.3±2.8*	32.1±2.6	30.1±2.1
150mM	65±4	81±3**	85±4**	82±3**	80±3**	20.1±1.2	24.7±1.4*	29.0±2.1**	25.2±1.5**	22.7±1.2*
170mM	25±3	62±5**	75±8**	70±7**	35±6**	17.6±1.5	21.4±1.7**	28.5±3.1**	22.4±1.3**	20.5±1.3*
200mM	18±3	48±4**	60±6**	53±6**	28±3**	12.5±2.4	17.1±1.5**	21.1±2.6**	18.4±1.9 **	16.1±1.0*
250mM	4±1	6±2	8±4	5±4	4±1	-	-	-	-	-

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.

Supplementary Table 4. Statistical analysis of relative green leaves and relative fresh weight of *BnCBL1* transgenic *Arabidopsis* under a range of NaCl concentrations.

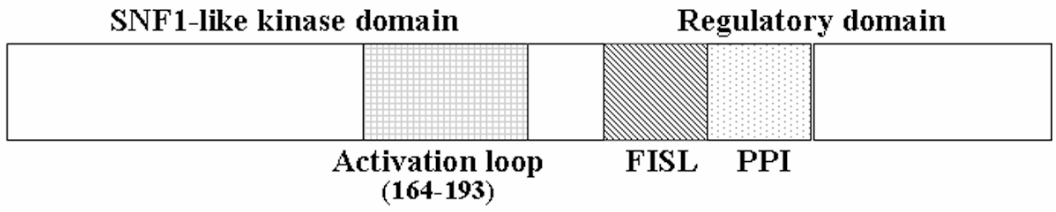
NaCl	Relative green leaves (%)			Relative fresh weight (mg)		
Concentration	**WT**	**BnCBL1 L10**	**BnCBL1 L11**	**WT**	**BnCBL1 L10**	**BnCBL1 L11**
75 mM	95±2	95±3	96±2	29.1±1.6	33.2±1.4*	30.5±1.9
150mM	65±4	82±2**	79±4**	20.1±1.2	28.3±2.6**	23.8±1.7*
170mM	25±3	63±5**	41±5**	17.6±1.5	26.5±2.0**	21.2±1.3**
200mM	18±3	42 ±4**	29 ±3**	12.5±2.4	18.5±2.5**	16.7±1.5 **
250mM	4±1	5±2	4±1	-		

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.

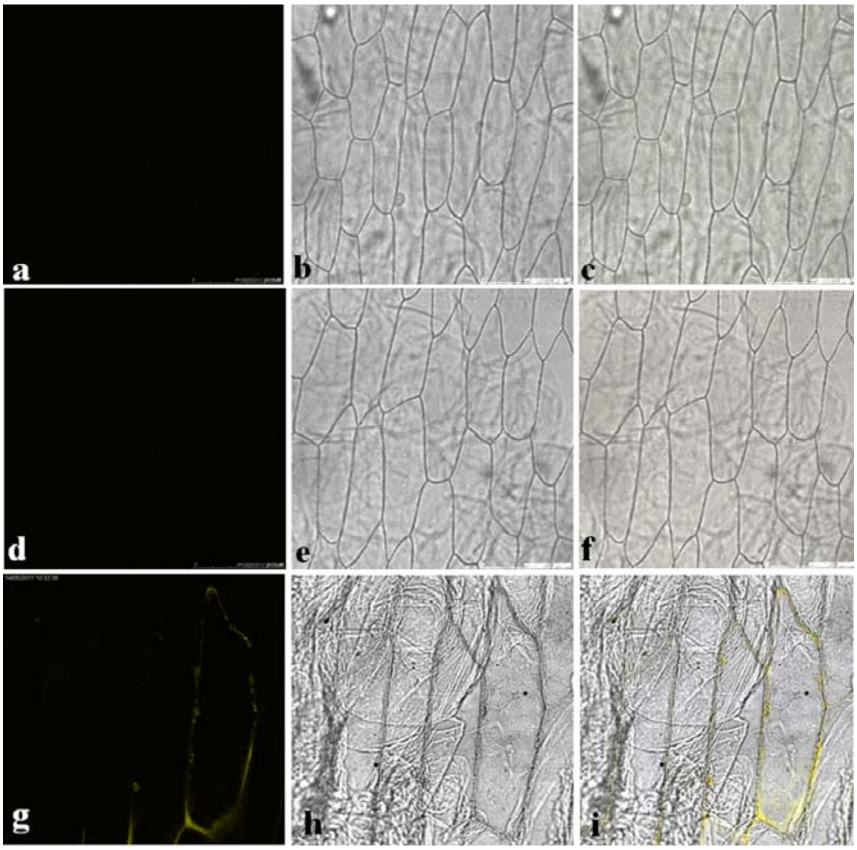
Supplementary Table 5. Statistical analysis of the primary root length (cm) of *BnCIPK6* and *BnCIPK6M* transgenic *Arabidopsis* under a range of ABA concentrations.

Genotype	0 µM ABA	0.25 µM ABA	0.5 µM ABA	0.75µM ABA	1 µM ABA
WT	2.03±0.2	1.73±0.2	1.30±0.14	1.21±0.15	1.10±0.1
BnCIPK6(M)L8	1.95±0.2	1.35±0.1*	0.90±0.1**	0.85±0.11**	0.80±0.09**
BnCIPK6(M)L9	1.98±0.18	1.30±0.15*	0.84±0.08**	0.74±0.08**	0.69±0.08**
BnCIPK6L3	1.98±0.18	1.43±0.1	1.09±0.1*	0.99±0.05*	0.95±0.12*
BnCIPK6L6	2.0±0.2	1.51±0.2	1.15±0.11*	1.06±0.10*	1.02±0.1*

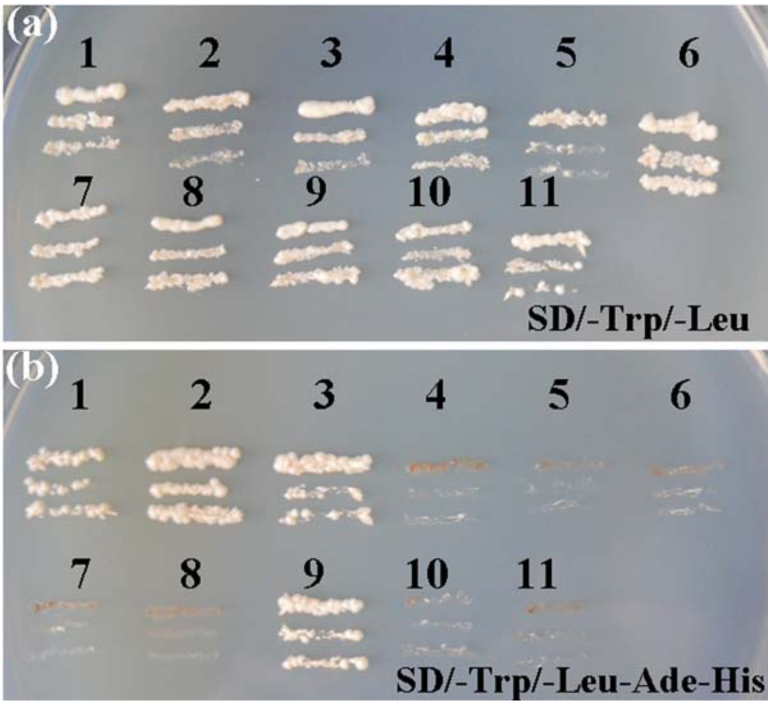
Independent t-tests for equality of means demonstrated that there was significant difference (* P value < 0.05) or very significant difference (** P value < 0.01) between wild type and transgenic plants.



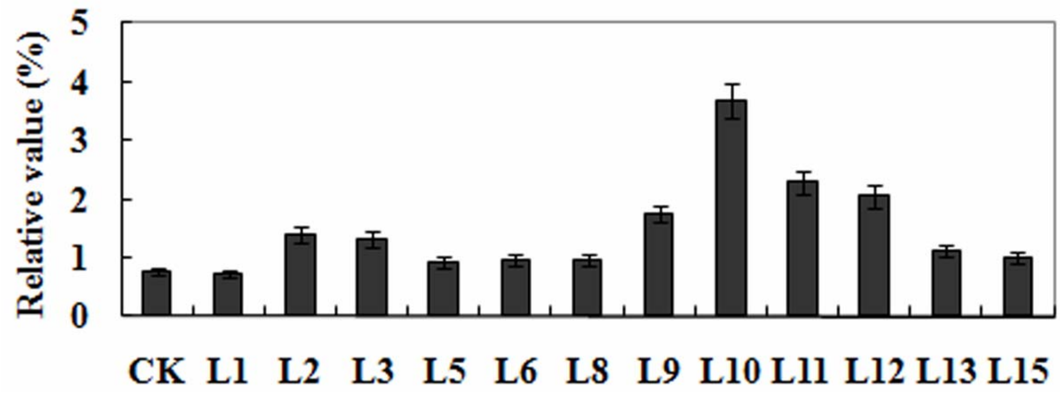
Supplementary Fig 1. A schematic diagram of the domain structure of BnCIPK6. The overall structure of BnCIPK6 comprises an N-terminal kinase domain and a regulatory C-terminal domain that are separated by a junction domain. Within the kinase domain, phosphorylation of amino acids in the activation loop (indicated as a box, 164-193aa) results in kinase activation. The regulatory C-terminal domain contains two conserved interaction domains, the FISL domain (also called NAF domain), which is responsible for the CBL–CIPK interaction, and the adjacent protein–phosphatase interaction (PPI) domain mediating interaction with 2C-type protein phosphatase (PP2C)-type phosphatases.



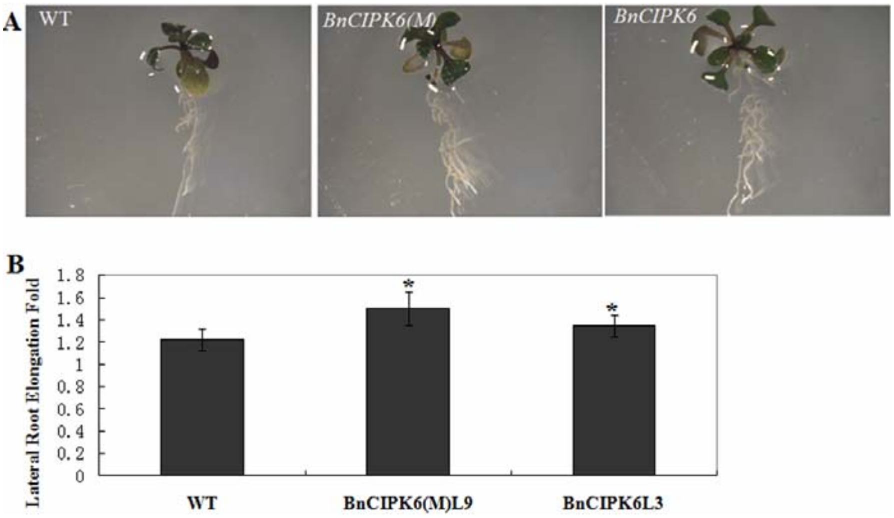
Supplementary Fig 2. BiFC assays of BnCIPK6 interaction with BnCBL1 in onion cells. (a) to (c) pUC-SPYNE and pUC-SPYCE-BnCIPK6; (d) to (f) pUC-SPYNE-BnCBL1+pUC-SPYCE; (g) to (i) pUC-SPYNE-BnCIPK6+pUC-SPYCE-BnCBL1. (a, d, g) YFP fluorescent Images; (b, e, h) Bright field Images of images a, d and g; (c, f, i) Fluorescent Images merged with their bright-field Images.



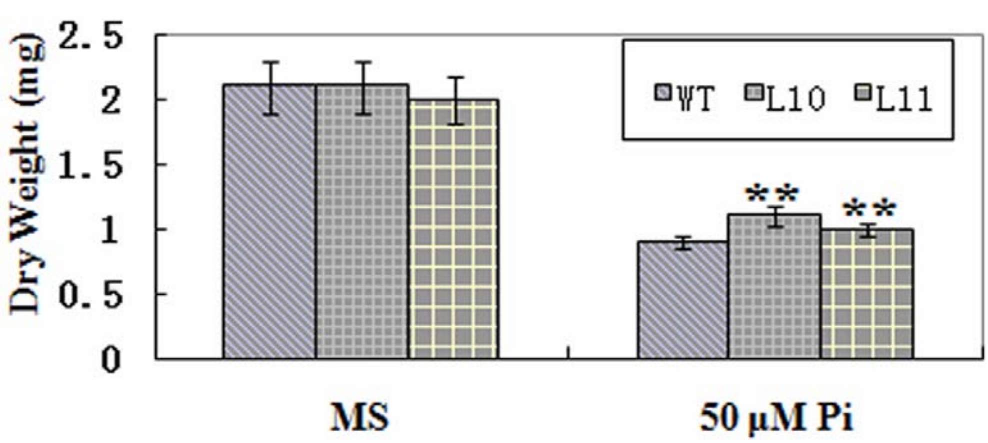
Supplementary Fig 3. Yeast two-hybrid analysis for interactions between BnCIPK6 and Ten AtCBL proteins. BnCIPK6 can interact with AtCBL1, AtCBL2, AtCBL3 and AtCBL9. (**a**) Transformants were assayed for growth on QDO nutritional selection medium (SD/-Trp/-Leu-Ade-His). (**b**) Transformants were assayed for growth on selection medium (SD/-Trp/-Leu). 1-10, AtCBL1-AtCBL10; 11 AD (empty).



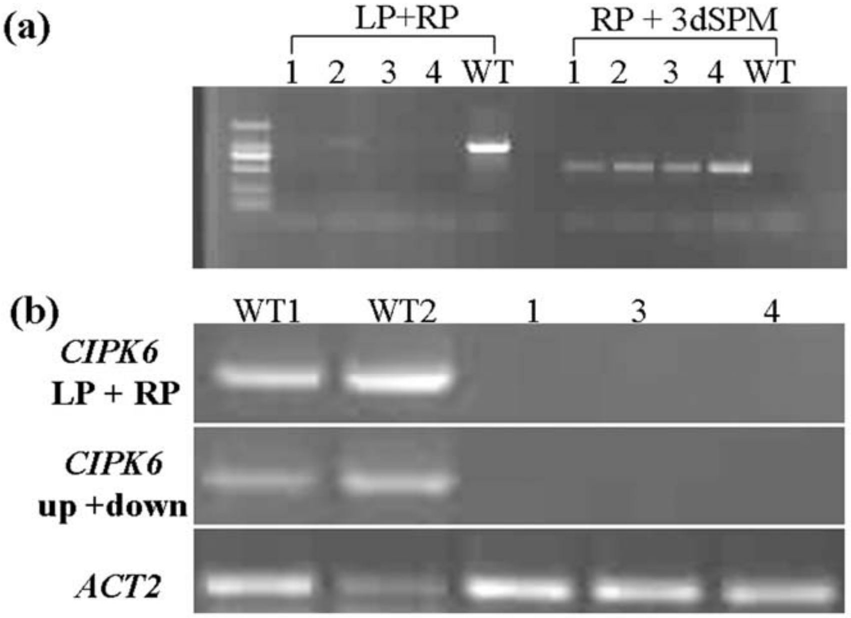
Supplementary Fig 4. Quantitative RT-PCR analysis of *BnCBL1* expression in the transgenic Arabidopsis. CK, wild type; L1 – L15, *BnCBL1* transgenic lines.



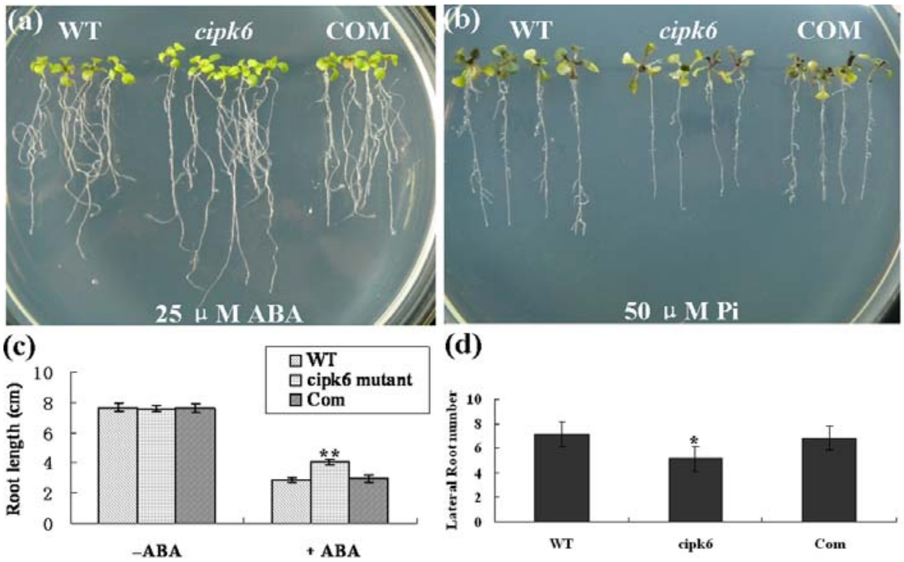
Supplementary Fig 5. Assay of lateral roots elongation of *BnCIPK6* and *BnCIPK6M* transgenic plants growing on low phosphate medium. (A) Comparison of the later roots between *BnCIPK6/6M* transgenic plants and wild type. (B) Statistical analysis of lateral root elongation. WT, wild type; BnCIPK6L3, *BnCIPK6* transgenic line 3; BnCIP6(m)L9, *BnCIPK(M)* transgenic line 9. Independent *t*-tests for equality of means demonstrated that there was significant difference (* P value < 0.05) between wild type and *BnCIPK6/6M* transgenic lines.



Supplementary Fig 6. Statistical analysis of plant dry weight of *BnCBL1* transgenic plants under phosphorous starvation. WT, wild type; L10 and L11, transgenic line 10 and 11. Independent *t*-tests for equality of means demonstrated that there was very significant difference (** P value < 0.01) between wild type and *BnCBL1* transgenic lines.



Supplementary Fig 7. Identification of *cipk6* loss-of-function mutants. (**a**) The homozygous insertion mutant *cipk6* was isolated by PCR-based screening using *CIPK6*-specific primers and atransposon specific primer (3' dSpm). (**b**), RT-PCR analysis of *CIPK6* expression in wild-type plants and homozygous mutants line1, 3 and 4. *Actin2* served as control. LP and RP, left and right genomic primers for the *CIPK6* gene, respectively; up and down, *AtCIPK6* full-length cDNA primers; WT, wild type; 1-4, *cipk6* line1-4.



Supplementary Fig 8. Characterization of *Arabidopsis cipk6* knockout mutant expressing *BnCIPK6*. (a) Six-old-day seedlings were transferred and grew on MS medium with 25 µM ABA (**b**) Six-old-day seedlings were transferred and grew on MS medium with 50 µM phosphate (low phosphate, LP). (**c**)Statistical analysis of the root length of wild type, the *cipk6* mutant and the *cipk6/BnCIPK6* complemented transgenic lines (COM) grown on MS medium without (-ABA) or with 25 µM ABA for two weeks after transferring. (**d**) Statistical analysis of the lateral root number of wild type, the *cipk6* mutant and the *cipk6/BnCIPK6* complemented transgenic lines (COM) grown on low phosphate medium (50µM Pi). 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, the *cipk6* mutant and the *cipk6/BnCIPK6* complemented transgenic lines (COM).