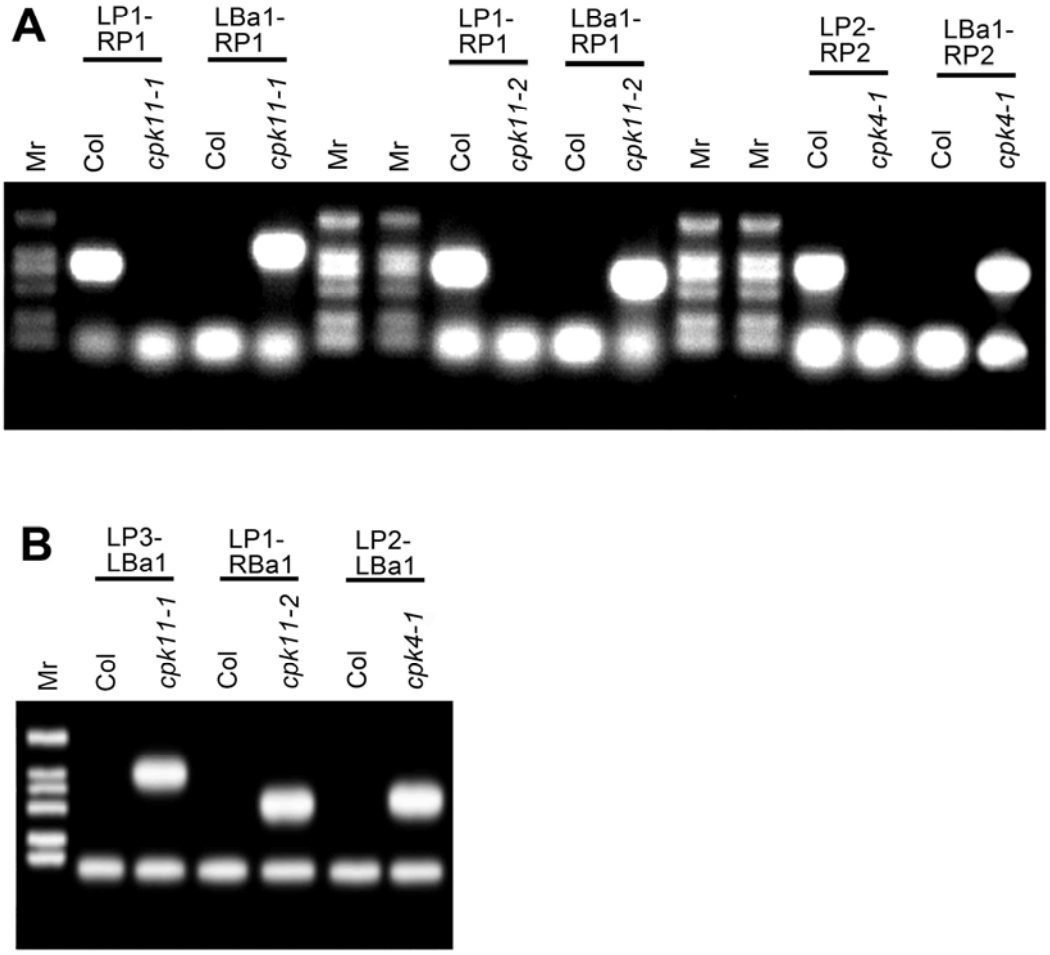
**Supplemental Data.** Zhu et al. 2007. Two Calcium-Dependent Protein Kinases, CPK4 and CPK11, Regulate Abscisic Acid Signal Transduction in *Arabidopsis*.



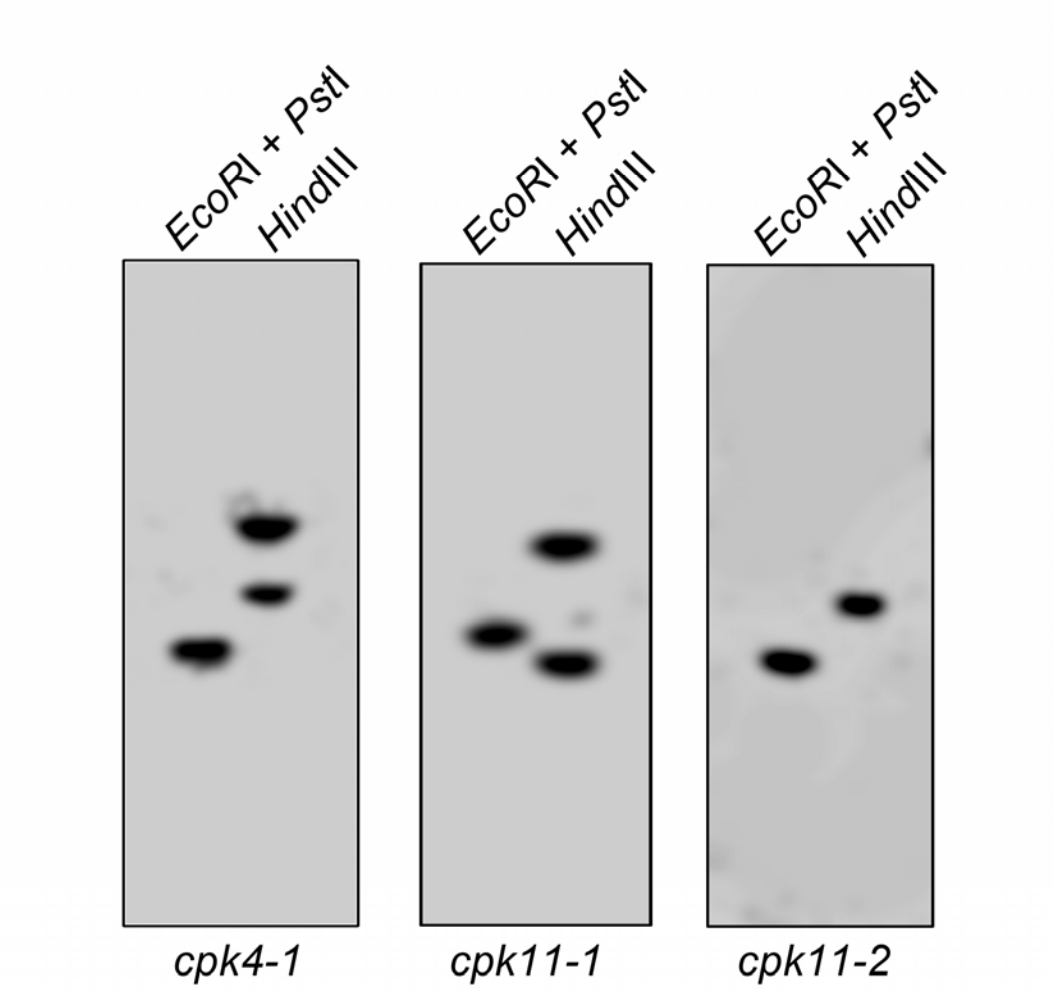

**Supplemental Figure 1. Identification of T-DNA Insertion for *cpk4-1*, *cpk11-1* and *cpk11-2* Mutations in the Arabidopsis Genome by PCR Analysis.**

The left (LBa1) and right (RBa1) border primers for T-DNA insertion, the left (LP1, LP3) and right (RP1) genomic primers for *AtCPK11* gene, and the left (LP2) and right (RP2) genomic primers for *AtCPK4* gene, are presented in Supplemental Table 1.

**(A)** The genomic sequences spanning the potential inserted T-DNA region between LP1 and RP1 (*AtCPK11*) or between LP2 and RP2 (*AtCPK4*) for Col are intact, whereas disrupted in *cpk11-1*, *cpk11-2* or *cpk4-1* mutants. However, the sequences of the T-DNA insertion between LBa1 and RP1 in both *cpk11-1* and *cpk11-2* mutants or between LBa1 and RP2 in *cpk4-1* mutant are detected, but not in the wild-type Col.

**(B)** The sequences of the T-DNA insertion between LP3 and LBa1 in *cpk11-1*, and between LP1 and RBa1 in *cpk11-2*, as well as between LP2-LBa1 in *cpk4-1* were also detected.

These results show the occurrence of the T-DNA insertion in the *AtCPK4* gene in the *cpk4-1* mutant and in the *AtCPK11* gene in the *cpk11-1* and *cpk11-2* mutants, and indicate that one single copy T-DNA is present in the *cpk11-2* mutant, but tandem T-DNAs were inserted in an inverted manner into the genome for the *cpk4-1* and *cpk11-1* mutants. Mr, molecular markers.

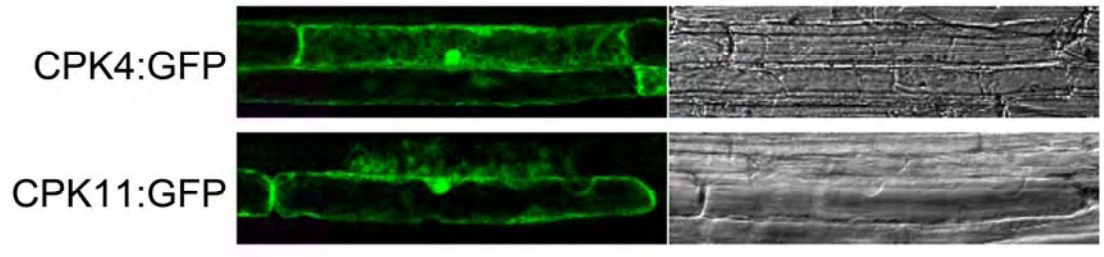



**Supplemental Figure 2. DNA-Blot Analysis for the T-DNA Insertion in *cpk4-1*, *cpk11-1* and *cpk11-2* Mutants.**

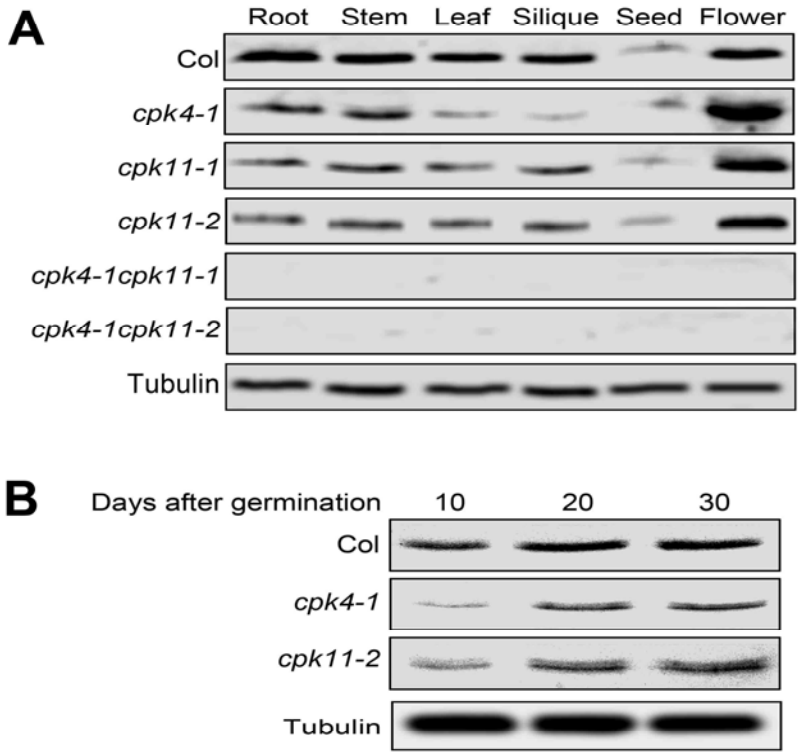
A 10-μg portion of *Arabidopsis* genomic DNA isolated from the *cpk4-1*, *cpk11-1* and *cpk11-2* mutants was digested with *EcorR*I plus *Pst*I and *Hind*III, respectively, electrophoresed in a 0.8% agarose gel, and transferred onto a nylon membrane. The membranes were hybridized with the $^{32}$P-labelled specific probe for the T-DNA (see the Supplemental METHODS). The results indicate that one single copy of the T-DNA was inserted into the genome for the *cpk11-2* mutant, and tandem T-DNA of two copies was inserted into the genome for the *cpk4-1* and *cpk11-1* mutants (for tandem T-DNA insertion, see also the results of sequencing of the T-DNA flanking sequences in Supplemental Table 1).


**Supplemental Figure 3. Alignment of Deduced Amino Acid Sequences of AtCPK4 and AtCPK11.**

Identical amino acid residues are indicated by white letters on a black background. Gaps, indicated by points (.), were introduced to maximize alignment. The two CPKs share high sequence identity (94%). The C-terminal fragment of AtCPK4 from amino acid 386 to 501 (indicated by red line) was used to produce anti-AtCPK4$^{C}$ serum, and the C-terminal fragment of AtCPK11 from amino acid 387 to 495 (indicated by green line) was used to produce anti-AtCPK11$^{C}$ serum.




**Supplemental Figure 4. Subcellular Localization of AtCPK4 and AtCPK11.**
Expression of AtCPK4:GFP (top panel) and AtCPK11:GFP (bottom panel) fusion proteins in the root cells of *Arabidopsis* transgenic plants. The fusion proteins of both CDPKs are present in cytoplasm and nucleus, shown by the AtCPK4:GFP and AtCPK11:GFP fluorescence images (left panels) under laser-scanning confocal microscope. The right panels show the corresponding bright field. For generation of the transgenic *AtCPK4:GFP*- and *AtCPK11:GFP*-expressing lines, see Supplemental METHODS.

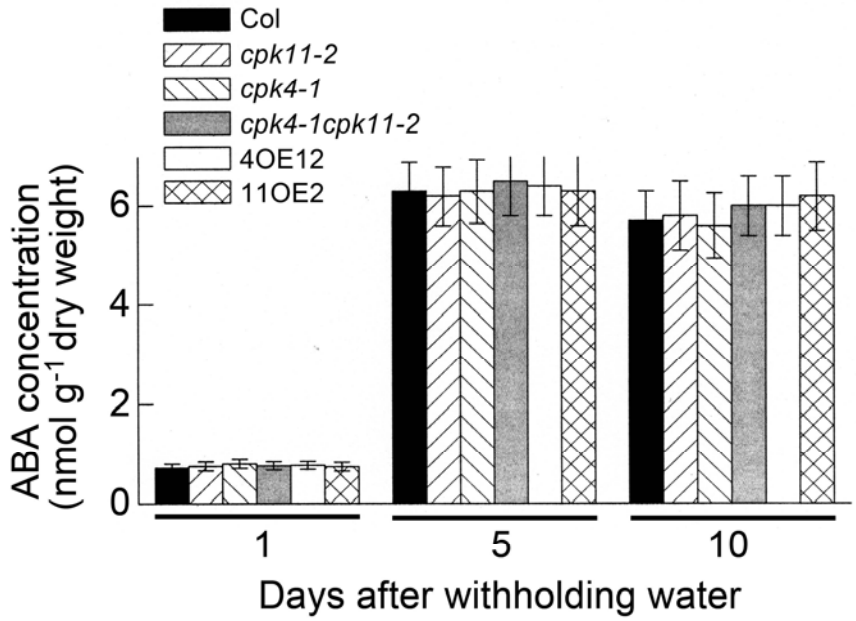



**Supplemental Figure 5. Expression of AtCPK4 and AtCPK11 in different tissues and during different growth periods.**

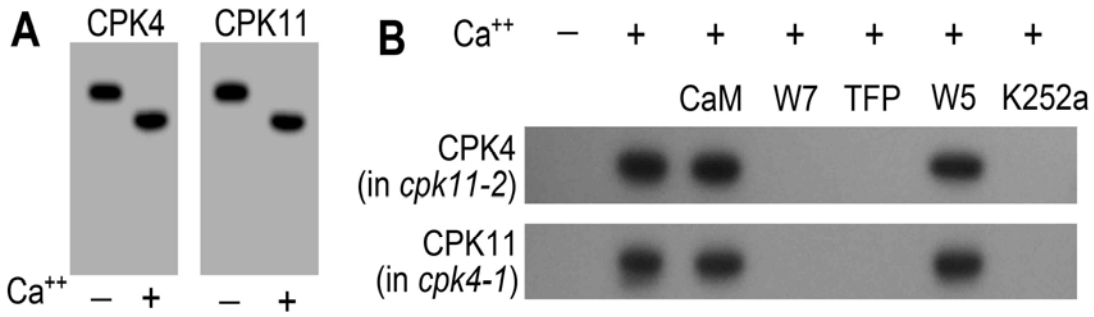
**(A)** Immunoblotting analysis with anti-AtCPK11$^{C}$ serum in the total proteins extracted from different tissues in wild-type Col and homozygous mutants *cpk4-1*, *cpk11-1* and *cpk11-2* and double mutants *cpk4-1cpk11-1* and *cpk4-1cpk11-2*. Tubulin was taken as a loading control.

**(B)** Immunoblotting analysis with anti-AtCPK11$^{C}$ serum in the total proteins extracted from leaves during different growth periods in wild-type Col and homozygous mutants *cpk4-1* and *cpk11-2*. Tubulin was taken as a loading control.

Because the anti-AtCPK4$^{C}$ or anti-AtCPK11$^{C}$ serum is able to recognize both AtCPK11 and AtCPK4 (see METHODS), the immuno-signal detected by either of the antisera in wild-type Col is AtCPK4 plus AtCPK11; and in the knockout mutant *cpk4-1* presents AtCPK11, and in the *cpk11-1* and *cpk11-2,* AtCPK4.




**Supplemental Figure 6. ABA concentrations in the different mutants.**
Three-week-old plants of the mutants *cpk4-1*, *cpk11-2* and *cpk4-1cpk11-2*, *AtCPK4*- and *AtCPK11*-overexpressors (4OE12 and 11OE2, respectively) and wild-type Col were subjected to drought treatment (withholding water for 1 d, 5 d and 10 d, respectively), and the rosette leaves from these plants were used to assay ABA concentrations by ELISA method as described previously (Chen et al., 2006).




**Supplemental Figure 7. Enzymatic characterization of CPK4 and CPK11.**

**(A)** $Ca^{2+}$-dependent electrophoretic mobility shift of CPK4 (left panel) and CPK11 (right panel) in the assay of in-gel autophosphorylation activity. The CPK4 protein was obtained by immunoprecipitation in the total proteins prepared from the three-week-old seedling of the *cpk11-2* mutant with the anti-$CPK4^C$ serum, and the CPK11 protein from the total proteins of the *cpk4-1* mutant with the anti-$CPK11^C$ serum. $Ca^{2+}$ or EGTA was added to the immunoprecipitated proteins dissolved in SDS-PAGE sample buffer. After SDS-PAGE, the in-gel phosphorylation assay was done in the presence of $Ca^{2+}$. – and + indicate the absence of $Ca^{2+}$ (in the presence of EGTA) and presence of $Ca^{2+}$ in the SDS-PAGE buffer, respectively.

**(B)** Inhibition of the histone-phosphorylating activity of the CPK4 and CPK11 by CaM antagonists or kinase inhibitors. The CPK4 protein from *cpk11-2* mutant (panel above) and CPK11 from *cpk4-1* mutant (panel below) were prepared as described above in **(A)** by immunoprecipitation. CaM (form bovine brain, Sigma) was used at 5 μM; TFP, W7 and W5 at 250 μM, and K252a at 10 μM. These reagents were added, respectively, to the phosphorylation reaction medium (buffer B as described in METHODS) for a preincubation and a subsequent reaction incubation for $^{32}P$-labeling to the kinase substrate histone. – and + indicate the absence and presence of $Ca^{2+}$ in the reaction buffer, respectively. The gels phosphorylated in the different reaction media were grouped to detect the phosphorylated histone bands by autoradiography.

**Supplemental Table 1**. **Analysis of T-DNA Insertion into Arabidopsis Genome for Identification of the *cpk4* and *cpk11* Knockout Mutants.**
The start codon (**ATG**) is indicated by red-bold letters.

**A: Identification of the T-DNA insertion site and the possible sequence deletion due to the T-DNA insertion in the *cpk11-1* mutant**

**The primers used for identification of the *cpk11-1* mutation:**

Left border primer (LBa1): 5'-GGTTCACGTAGTGGGCCATC-3'

Right genomic primer 1 (RP1): 5'-AAACCAATTAGGCGATGAACC-3'

Left genomic primer 3 (LP3): 5'-TGGGATGAAAACACACAAGCGG-3'

**The deleted genomic sequence (blue-bold letters, nt -120 to -87, 34 bp deleted) due to the insertion of a tandem-two-copy T-DNA into this site in a inverted fashion in the *cpk11-1* mutant:**

TAAAA**TGATATAAAGAGAGAGTCAAAAAAATTGGAGAAG**AGGGAAGGAGCAACAAAGAAAAAG
TCTGTTTATCATCTTCTTCTTCTTCAAATCGAGATCGAAGAAGAACCAACAAAAAACCAAAA**ATG**

The presence of the PCR products obtained with both the primer pair LBa1-RP1 and LP3-LBa1, together with the fact that the PCR products could not been found (data not shown) when PCR was performed with the primer pair LP3-RBa1 (see below for the RBa1 sequence), indicate that tandem T-DNAs were inserted into the genome for the *cpk11-1* mutant in an inverted fashion at the same locus, and the T-DNA insertion generates a 34-bp deletion from –120 to –87 bp 5'-upstream of the CPK11 translation start codon. Southern blot analysis further indicates that a tandem T-DNA of two copies was inserted at the locus (see Supplemental Figure 2).

**B: Identification of the T-DNA insertion site and the possible sequence deletion due to the T-DNA insertion in the *cpk11-2* mutant**

**The primers used for identification of the *cpk11-2* mutation:**

Left border primer (LBa1) and right genomic primer 1 (RP1) are the same as those mentioned above for *cpk11-1* mutant identification.

Left genomic primer 1 (LP1): 5'-GAGAGAGTCAAAAAAATTGGAGAA-3'

Right border primer (RBa1): 5'-GTTTCTGACGTATGTGCTTAGC-3'

**The deleted genomic sequence (blue-bold letters, nt 320 to 358, 39 bp deleted) due to the insertion of a single-copy T-DNA into this site in the *cpk11-2* mutant:**

**ATG**GAGACGAAGCCAAACCCTAGACGTCCTTCAAACACAGTTCTACCATATCAAACACCACGATT
AAGAGATCATTACCTTCTGGGAAAAAAGCTAGGCCAAGGCCAATTTGGAACAACCTATCTCTGCA
CAGAGAAATCAACCTCCGCTAATTACGCCTGCAAATCGATCCCGAAGCGAAAGCTCGTGTGTCG
CGAGGATTACGAAGATGTATGGCGTGAGATTCAGATCATGCATCATCTCTCTGAGCATCCAAATG
TTGTTAGGATCAAAGGGACTTATGAAGATTCGGTGTTTGTTCATATTGTTATGGAGGTTT**GTGAAG
GTGGTGAGCTTTTTGATCGGATTGTTTCTAAAG**GTCAT

The results indicate that a single copy of T-DNA was inserted into the genome for the *cpk11-2* mutant, and the T-DNA insertion generates a 39-bp deletion from 320 to 358 bp downstream of the CPK11 translation start codon.

**C: Identification of the T-DNA insertion site and the possible sequence deletion due to the T-DNA insertion in the *cpk4-1* mutant**

**The primers used for identification of the *cpk4-1* mutation:**

Left border primer (LBa1): 5'-GGTTCACGTAGTGGGCCATC-3'

Right genomic primer 2 (RP2): 5'-GCTTAGCATCATCACTGGGAC-3'

Left genomic primer 2 (LP2): 5’-AATCCGACTTACTTTGGTTAGAA-3’

**The deleted genomic sequence (blue-bold letters, nt -67 to -57, 11 bp deleted) due to the insertion of a tandem-two-copy T-DNA into this site in a inverted fashion in the *cpk4-1* mutant:**

AACTTC**GTATCATCTTC**CTCCTCCTCCTTTGATAAACACCAAAAAAAGGCAGAGACTTTCGAAATC AAGAACA**ATG**

The presence of the PCR products obtained with both the primer pair LBa1-RP2 and LP2-LBa1, together with the fact that the PCR products could not been generated (data not shown) when PCR was performed with the primer pair LP2-RBa1, indicate that tandem T-DNAs were inserted into the genome for the *cpk4-1* mutant in an inverted fashion at the same locus, and the T-DNA insertion generates an 11-bp deletion from –67 to –57 bp 5’-upstream of the CPK4 translation start codon. Southern blot analysis further indicates that a tandem T-DNA of two copies was inserted at the locus (see Supplemental Figure 2).

## SUPPLEMENTAL METHODS

### DNA Gel Blot Analysis

Genomic DNA was extracted from 4-week-old *cpk4-1* or *cpk11-1* or *cpk11-2* plants using the method of Doyle and Doyle (1990). Ten micrograms of DNA was digested to completion with *EcoR*I plus *Pst*I, and *Hind*III restriction enzymes, electrophoresed through 0.8% agarose, and blotted onto nylon membranes (Hybond-$N^+$, Amersham Pharmacia Biotech). The specific probe was produced as follows: the 597-bp specific sequence of T-DNA was amplified using the genomic DNA of *cpk4-1* by forward primer 5'-TCAGAAGAACTCGTCAAGAAGG -3', and reverse primer 5'-CTATCGTGGCTGGCCACGACG-3'; and then the PCR product was gel purified and radiolabeled with $^{32}$P by a random primer labeling kit (Takara). DNA gel blot hybridization was performed at 65℃ for 24 h using hybridization solution (200 mM sodium phosphate buffer, pH 7.2, 1 mM EDTA, pH 8.0, 50% formamide, 10% BSA, and 7% SDS) with $^{32}$P-labeled specific probes. Then the membranes were washed at 65℃ in 5 × SSC and 0.5% SDS, 1 × SSC and 0.5% SDS, and 0.1 × SSC and 0.5% SDS for 30 min sequentially. The copy of T-DNA insertion was detected by autoradiography after exposition of the membranes to Kodak X-Omat AR film for one week at -70℃.

The probe sequence was:
TCAGAAGAACTCGTCAAGAAGGCGATAGAAGGCGATGCGCTGCGAATCGGGAGCG
GCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTCGCCGCCAAGCTCTTCAGC
AATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCACACCCAGCCGGC
CACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGCAAGCAGG
CATCGCCATGGGTCACGACGAGATCATCGCCGTCGGGCATGCGCGCCTTGAGCCTG
GCGAACAGTTCGGCTGGCGCGAGCCCCTGATGCTCTTCGTCCAGATCATCCTGATC
GACAAGACCGGCTTCCATCCGAGTACGTGCTCGCTCGATGCGATGTTTCGCTTGGT
GGTCGAATGGGCAGGTAGCCGGATCAAGCGTATGCAGCCGCCGCATTGCATCAGCC
ATGATGGATACTTTCTCGGCAGGAGCAAGGTGAGATGACAGGAGATCCTGCCCCGG
CACTTCGCCCAATAGCAGCCAGTCCCTTCCCGCTTCAGTGACAACGTCGAGCACAG
CTGCGCAAGGAACGCCCGTCGTGGCCAGCCACGATAG

### Subcellular Localization of AtCPK4 and AtCPK11

For subcellular localization of AtCPK4 and AtCPK11, the full-length ORF of *AtCPK4* was PCR-amplified by using forward primer 5'-CCGCTCGAGATGGAGAAACCAAACCCTAG -3' and reverse primer 5'- CGGGATCCCGCTTTGGTGAATCATCAGATTTAG-3', and the full-length ORF of *AtCPK11* was PCR-amplified by using forward primer 5'-CCGCTCGAGATGGAGACGAAGCCAAACCCTAG-3' and reverse primer 5'-CGGGATCCCGGTCATCAGATTTTTCACCATC-3'. The PCR products were then fused to the upstream of the enhanced GFP (Cormack et al., 1996) at the *Xho*I (5'- end) / *Bam*H I (3'-end) sites in the CaMV 35S-EGFP-Ocs 3'- vector (p-EZS-NL vector, Dr. Ehrhardt, http://deepgreen.stanford.edu), respectively. The full-length *AtCPK4* cDNA with GFP sequence at C-terminal was then amplified by PCR using p-EZS-NL-AtCPK4-EGFP vector as the template using the forward primer

5’-GCTCTAGAATGGAGAAACCAAACCCTAG-3’ and reverse primer 5’-TCCCCCGGGTTACTTGTACAGCTCGTCCATGC-3’. The full-length *AtCPK11* cDNA with GFP sequence at C-terminal was amplified by PCR using p-EZS-NL-AtCPK11-EGFP vector as the template using the forward primer 5’-GCTCTAGAATGGAGACGAAGCCAAACCCTAG-3’ and reverse primer 5’-TCCCCCGGGTTACTTGTACAGCTCGTCCATGC-3’. The resulting PCR product was digested with *Xba* I and *Sma* I, subcloned into pCAMBIA-1300-221 vector under the control of CaMV 35S promoter. Finally, each vector was sequenced to confirm that the fusion was in-frame and without PCR-induced mistakes. These constructions were then transformed into *Agrobacterium* strain GV3101 and introduced into plants of wild-type Columbia by the floral dip method as previously described (Clough and Bent, 1998), respectively. The homozygous T3 seeds of the transgenic plants were used for assays of subcellular localization using a confocal laser scanning microscope (Bio-Rad MRC 1024) (see Supplemental Figure 4).

**ABA Measurement**

Rosette leaves were excised from 3-week-old mutant and wild-type plants grown under drought treatment (withholding water for 1 d, 5 d and 10 d, respectively). ABA contents in tissues were measured by ELISA method as described previously (Chen et al., 2006).

**REFERENCES**

**Chen, Y., Ji, F., Xie, H., Liang, J. and Zhang, J.** (2006). The regulator of G-protein signaling proteins involved in sugar and abscisic acid signaling in Arabidopsis seed germination. Plant Physiol **140,** 302-310.

**Clough, S.J. and Bent, A.F.** (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. **16,** 735–743.

**Cormack, B. P., Valdivia, R. H., and Falkow, S.** (1996). FACS-optimized mutants of the green fluorescent protein (GFP). Gene **173,** 33-38.

**Doyle, J. J. and Doyle, J.L.** (1990). Isolation of plant DNA from fresh tissue. Focus **12,** 13–15.