# Arabidopsis Calcium-Dependent Protein Kinase AtCPK32 Interacts with ABF4, a Transcriptional Regulator of Abscisic Acid-Responsive Gene Expression, and Modulates Its Activity[1]

**Hyung-in Choi, Hee-Jin Park, Ji Hye Park, Sunmi Kim, Min-Young Im, Hyo-Hyun Seo, Yong-Woo Kim, Inhwan Hwang, and Soo Young Kim***

Kumho Life and Environmental Science Laboratory, Gwangju 500–712, South Korea (H.C., H.-J.P., J.H.P., S.K., M.-Y.I., H.-H.S., S.Y.K.); and Department of Life Science, Pohang University of Science and Technology, Pohang, Kyungbuk 790–784, South Korea (Y.-W.K., I.H.)

The phytohormone abscisic acid (ABA) regulates stress-responsive gene expression during vegetative growth. The ABA regulation of many genes is mediated by a subfamily of basic leucine zipper class transcription factors referred to as ABFs (i.e. ABF1–ABF4), whose transcriptional activity is induced by ABA. Here we show that a calcium-dependent protein kinase is involved in the ABA-dependent activation process. We carried out yeast two-hybrid screens to identify regulatory components of ABF4 function and isolated AtCPK32 as an ABF4-interacting protein. AtCPK32 has autophosphorylation activity and can phosphorylate ABF4 in vitro. Mutational analysis indicated that serine-110 of ABF4, which is highly conserved among ABF family members, may be phosphorylated by AtCPK32. The serine-110 residue is essential for ABF4-AtCPK32 interaction, and transient expression assay revealed that it is also required for the normal transcriptional function of ABF4. The expression patterns and subcellular localization of AtCPK32 are similar to those of ABF4. Furthermore, its overexpression affects both ABA sensitivity and the expression of a number of ABF4-regulated genes. Together, our data demonstrate that AtCPK32 is an ABA signaling component that regulates the ABA-responsive gene expression via ABF4.

The phytohormone abscisic acid (ABA) controls several aspects of plant growth and development, which include seed germination, seedling growth, abiotic stress response, and seed maturation (Finkelstein et al., 2002; Xiong et al., 2002). Many of the ABA-mediated physiological processes are accompanied by changes in gene expression patterns. Numerous ABA-regulated genes have been identified, and genome-wide analysis of gene expression revealed that more than 1,300 genes are responsive to ABA in Arabidopsis (*Arabidopsis thaliana*) seedlings (Hoth et al., 2002; Kreps et al., 2002; Takahashi et al., 2004).

cis-Regulatory elements responsible for the ABA regulation of gene expression have been determined by extensive promoter analyses (Busk and Pages, 1998; Rock, 2000). Many of the elements share a conserved motif, ACGTGGC, which is generally known as ABRE (ABA-responsive element). A large number of basic Leu zipper (bZIP; Landschulz et al., 1988) class transcription factors have been isolated based on their in vitro interaction with the ABRE. Among the ABRE-binding bZIP factors, the ABF/AREB subfamily members (Choi et al., 2000; Uno et al., 2000) have been demonstrated to have regulatory function in ABA and/or stress responses during vegetative growth. The factors can activate an ABRE-containing promoter in an ABA-dependent manner in protoplasts (Uno et al., 2000). Their overexpression in Arabidopsis promotes ABA sensitivity and abiotic stress tolerance (Kang et al., 2002; J.-B. Kim et al., 2004; S. Kim et al., 2004). The ABF/AREB subfamily members are highly homologous to ABI5, an embryo-abundant, genetically identified ABA signaling component, whose mutations result in ABA-insensitive germination and seedling establishment (Finkelstein and Lynch, 2000; Lopez-Molina and Chua, 2000; Lopez-Molina et al., 2001). Other ABF/AREB/ABI5 homologs have also been reported that include sunflower (*Helianthus annuus*) DPBFs (DPBF1–DPBF3; Kim et al., 1997; Kim and Thomas, 1998), the first reported members of the bZIP family, Arabidopsis AtDPBFs (AtDPBF1–AtDPBF4; Bensmihen et al., 2002; Kim et al., 2002), rice (*Oryza sativa*) TRAB1 (Hobo et al., 1999), wheat (*Triticum aestivum*) TaABF (Johnson et al., 2002), and barley (*Hordeum vulgare*) HvABI5 (Casaretto and Ho, 2003).

[1] This work was supported by the Agricultural Plant Stress Research Center (grant no. R11–2001–092–02001–0) of Chonnam National University funded by the Korea Science and Engineering Foundation, and the Crop Functional Genomics Center (grant no. CG2223 to S.Y.K.) of the 21C Frontier Program funded by the Ministry of Science and Technology of Korea. This paper is Kumho Life and Environmental Science Laboratory publication number 74.

* Corresponding author; e-mail sooykim@kkpc.com; fax 82–62–972–5085.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Soo Young Kim (sooykim@kkpc.com).

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.105.069757.

The ABF subfamily members (ABF1–ABF4) and their homologs have nearly identical bZIP domains in their C-terminal regions. Additionally, three highly conserved regions are found in their N-terminal halves. The N-terminal conserved regions, which will be referred to as C1 to C3, invariably possess one to two Ser or Thr residues that may be potential sustrate sites for various protein kinases. Several lines of evidence indicate that functions of ABFs and their homologs are modulated by the phosphorylation of the conserved amino acids. Transient expression assay using protoplasts showed that the transcriptional activities of ABF2/AREB1 and ABF4/AREB2 are induced by exogenous ABA treatment (Uno et al., 2000). The ABA-activated transcription is inhibited by protein kinase inhibitors. An ABA-dependent kinase activity that phosphorylates the conserved regions has been identified in cultured cells. The conserved Ser or Thr residues of ABI5 are phosphorylated in vivo, although their phosphorylation does not affect ABI5 activity (Lopez-Molina et al., 2002). Similarly, one of the Ser residues within the conserved regions of TRAB1, which is essential for ABA-induced transcription, is phosphorylated in response to ABA (Kagaya et al., 2002). However, the kinases responsible for the phosphorylation events have not been identified yet.

On the other hand, the conserved domains of TaABF, a seed-specific wheat ABI5 homolog, interact with PKABA1, which is an SnRK2 kinase involved in the ABA suppression of GA-induced gene expression (Johnson et al., 2002). Other kinases, especially those belonging to the calcium-dependent protein kinase (CDPK)-SnRK superfamily, have also been demonstrated to mediate ABA/stress signaling in Arabidopsis and other species (Cheng et al., 2002; Finkelstein et al., 2002; Luan et al., 2002; Hrabak et al., 2003; Gong et al., 2004). OST1/SRK2E, an Arabidopsis ortholog of *Vicia fava* AAPK, which is an ABA-activated, $Ca^{2+}$-independent protein kinase, regulates stomatal closure under water deficit conditions (Li et al., 2000; Mustilli et al., 2002; Yoshida et al., 2002). Arabidopsis SnRK2 kinases, SOS2, CIPK3, and their homologs, mediate ABA and various abiotic stress responses in a $Ca^{2+}$-dependent manner (Kim et al., 2003; Gong et al., 2004). In particular, several CDPKs have been shown to mediate abiotic or biotic stress responses. Constitutively active forms of two Arabidopsis CDPKs, AtCPK10 (ATCDPK1) and AtCPK30 (ATCDPK1a), activate an ABA/stress-inducible promoter in maize (*Zea mays*) leaf protoplasts (Sheen, 1996). In rice, overexpression of OsCDPK7 results in cold, salt, and drought tolerance (Saijo et al., 2000). On the other hand, a tobacco (*Nicotiana tabacum*) CDPK, NtCDPK2, is involved in hypoosmotic stress response in addition to the defense response against pathogen infection (Romeis et al., 2001). The substrates of these CDPKs, however, have not been reported.

Here we show that ABF4 and probably other ABFs may be the substrates of AtCPK32. We carried out yeast two-hybrid screens to identify regulator(s) of ABF4 function and found that AtCPK32 interacts with the C2-C3 conserved region of ABF4. AtCPK32 has autophosphorylation activity and phosphorylates ABF4 in vitro. Mutational analysis suggested that AtCPK32 phosphorylates one of the conserved Ser residues, which is required for the normal function of ABF4. Moreover, AtCPK32 overexpression resulted in ABA hypersensitivity and altered expression of ABF4-regulated genes. The expression patterns and subcellular localization of AtCPK32 are similar to those of ABF4. Taken together, our data indicate that AtCPK32 is an ABA/stress signaling component that positively modulates ABF4 function.

## RESULTS

### Isolation of an ABF4-Interacting Protein

We conducted two-hybrid screens (Chien et al., 1991; Gyuris et al., 1993) to identify proteins that interact with the conserved regions of ABF4. Bait constructs were prepared using two different fragments containing C1 or C2-C3 region, respectively (Fig. 1A). The constructs were individually introduced into a reporter yeast strain, and the bait-containing yeast was transformed with a cDNA expression library, which was prepared using RNA from ABA/salt-treated Arabidopsis seedlings (Choi et al., 2000). Approximately five million transformants of the C1 region reporter yeast were screened, but no positive clones were isolated. However, two positive clones that interacted with the C2-C3 region but not with the nuclear lamin (Fig. 1B) were identified from the screen of six million transformants.

Sequencing of the positive clones revealed that they encoded a protein with a kinase domain and four EF hands, i.e. a CDPK (Cheng et al., 2002; Hrabak et al., 2003). Both clones were missing the initiation codon, and database search revealed that they were missing the N-terminal 18 and 38 amino acid residues, respectively. Subsequently, full-length clone was isolated by PCR using the sequence information available from the partial sequence and the Arabidopsis database. The full-length clone encodes a protein of 538 amino acid residues with a calculated molecular mass of 60.9 kD (Fig. 1C). The corresponding gene is located on chromosome 3 (At3g57530) and consists of eight exons. We initially designated the protein as CIA (CDPK interacting with ABFs); however, according to the suggested nomenclature (Hrabak et al., 1996), it will be referred to as AtCPK32 hereafter.

### AtCPK32 Interacts with ABF4 in Vitro

To test whether AtCPK32 interacts with ABF4 in vitro, we carried out glutathione *S*-transferase (GST) pulldown assay. Full-length recombinant ABF4 was prepared as a fusion to GST and allowed to bind the in vitro-translated AtCPK32 labeled with $^{35}$S-Met. The bound protein was then separated by SDS-PAGE. Autoradiography of the gel (Fig. 1D) showed that

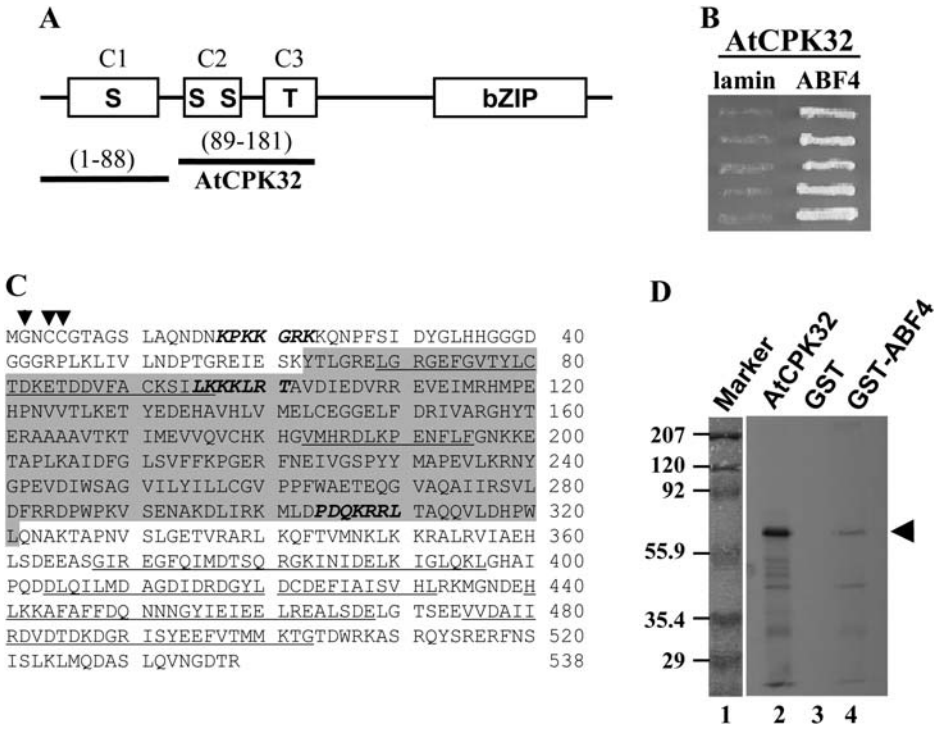


**Figure 1.** Isolation of AtCPK32 by two-hybrid screening. A, Schematic diagram of ABF4. The three conserved regions (C1, C2, and C3) and the bZIP region are indicated by rectangles. The N-terminal-conserved regions contain Ser (S) or Thr (T) residue that may be phosphorylated by CDPK. The two regions used for two-hybrid screenings are indicated by the thick lines with amino acid numbers in parentheses. AtCPK32 was isolated with the C2-C3 region bait construct. B, Specificity of interaction. Interaction of AtCPK32 with an unrelated protein, nuclear lamin (lamin), or with the C2-C3 region of ABF4 was investigated by two-hybrid assay. Reporter yeast with a *LEU2* reporter gene was transformed with each bait construct and one of the positive clones (clone 5). Transformants were then patched on a selection medium lacking Leu and grown for 4 d. C, Deduced amino acid sequence of AtCPK32. The kinase domain is highlighted, and the ATP-binding region, the active site, and the EF hands are underlined. Putative nuclear localization signals are indicated by bold-faced italics, and the myristoylation and the palmitoylation sites by arrowheads. D, In vitro interaction of ABF4 and AtCPK32. GST pulldown assay was carried out using in vitro-translated, $^{35}$S-Met-labeled AtCPK32 (lane 2) and purified recombinant proteins, GST (lane 3) and GST-ABF4 fusion protein (lane 4). The arrowhead indicates the position of AtCPK32. Marker (lane 1), Protein size marker. The numbers indicate size in kD.

AtCPK32 was retained by the GST-ABF4 fusion protein (lane 4) but not by GST alone (lane 3). Thus, our in vitro interaction assay indicated that AtCPK32 interacts with ABF4 in vitro.

### The Kinase Domain of AtCPK32 Is Necessary for the Interaction with the Conserved Region

The domain(s) of AtCPK32 involved in the interaction with the C2-C3 region of ABF4 was dissected by two-hybrid assay. Various deletion constructs of AtCPK32 were prepared (Fig. 2), and then their interaction with the C2-C3 region of ABF4 was assessed. Deletion of the N-terminal 57 amino acids of AtCPK32 (Construct II) reduced the $\beta$-galactosidase reporter activity to 20% of that observed with the full-length AtCPK32 (Construct I). The result indicates that the N-terminal sequence preceding the kinase domain is necessary for the normal interaction with the conserved region of ABF4. Further deletion of the N-terminal region including the kinase domain (Construct III) reduced the reporter activity to background level. Thus, the kinase domain was essential for the interaction, and the C-terminal region containing the EF hands was not able to interact with ABF4. Deletion of the EF hands (Construct IV) lowered the reporter activity by approximately 70%, but the remaining N-terminal half containing the kinase domain still could interact with the C2-C3 region. The kinase domain alone (Construct V) exhibited little reporter activity. In summary, our results indicated that full-length AtCPK32 is necessary for the normal interaction with the C2-C3 region of ABF4. Although necessary, the kinase domain was not sufficient for the interaction, and both the N-terminal variable region and the C-terminal EF hand region were required.

### Expression Patterns of AtCPK32 Are Similar to Those of ABF4

Expression of AtCPK32 under various abiotic stress conditions was determined by RNA gel-blot analysis. As shown in Figure 3A, AtCPK32 message level was not affected by low temperature, ABA, or mannitol.

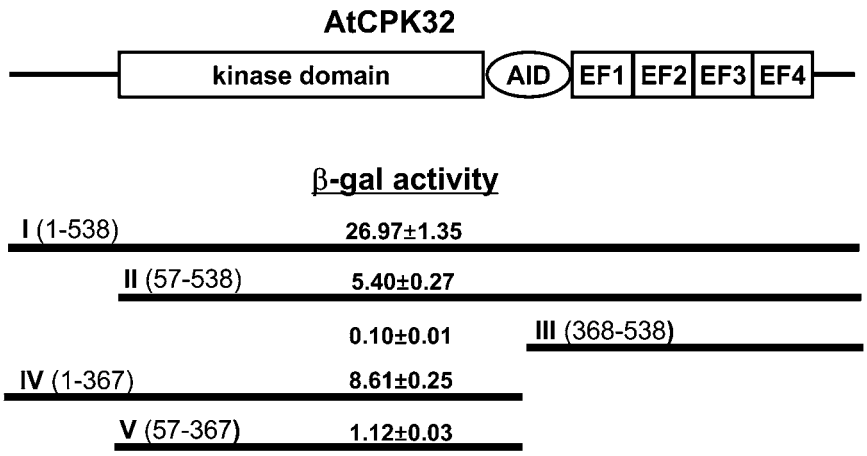


**Figure 2.** Dissection of AtCPK32 interaction domains. Top, schematic diagram of AtCPK32. AID, Autoinhibitory domain; EF, EF hand. Bottom, The region necessary for the interaction with the C2-C3 region of ABF4 was determined by two-hybrid assay using various deletion constructs of AtCPK32 (designated as I–V). The numbers in the parentheses denote amino acid position. The $\beta$-galactosidase reporter activities, expressed in Miller units, represent the mean ± SD of five independent determinations. The reporter activity of the empty vector alone is 0.08 ± 0.01.

However, it increased under high-salt condition, indicating the salt inducibility of its expression. To further investigate the expression patterns of AtCPK32, we generated transgenic Arabidopsis plants harboring an *AtCPK32* promoter-$\beta$-glucuronidase (*GUS*) reporter construct and determined the promoter activity by histochemical GUS staining. GUS activity was detected in embryos and most of the vegetative tissues (Fig. 3B, a–c). Especially, emerging radicles and roots exhibited strong GUS activity. Among the floral organs, anthers and stigma were stained strongly, and the abscission zone also exhibited strong GUS activity (Fig. 3B, d and e). The expression patterns of AtCPK32 are similar to those of ABF4 and other ABFs, which also are induced by high salinity and expressed in most of the vegetative tissues, especially in roots, stigma, anthers, and the abscission zone (Choi et al., 2000; Kang et al., 2002).

ABF4 is a bZIP class transcription factor, which is likely to be nuclear localized, and putative nuclear localization signals are present in AtCPK32 (Fig. 1D). Hence, we asked whether AtCPK32 is localized in the nucleus. Arabidopsis plants transformed with an *AtCPK32*-green fluorescent protein (*GFP*) fusion construct were generated. Subcellular localization of AtCPK32 was then determined by the localization of GFP. Figure 3C (middle section) shows that, in contrast to GFP alone (top section), which was detected throughout the cell, AtCPK32-GFP was detected in the nucleus. AtCPK32 was detected also in the periphery of the cells. We then determined the subcellular localization of ABF4 by a transient expression assay, in which an *ABF4-GUS* fusion construct was introduced into onion epidermal cells by particle bombardment. Figure 3C (bottom section) shows that ABF4 was localized in the nucleus. Together, our results indicated that both ABF4 and AtCPK32 are localized in the nucleus. Unlike AtCPK32, however, ABF4 was found mainly in the nucleus.

### Kinase Activity of AtCPK32

An in vitro kinase assay was performed to investigate the enzymatic activity of AtCPK32. First, we tested the autophosphorylation activity of AtCPK32. Full-length, recombinant AtCPK32 (Fig. 4A, lane 2) was prepared, and its autophosphorylation activity was investigated by monitoring the incorporation of [$\gamma$-$^{32}$P]ATP. Figure 4A (lane 4) shows that the radioactivity was incorporated into AtCPK32 in the assay, indicating that autophosphorylation occurred.

Next, we investigated whether AtCPK32 phosphorylates ABF4. Full-length, recombinant ABF4 (Fig. 4A, lane3) was prepared and used as a substrate in the kinase assay. As shown in Figure 4A (lane 5), $^{32}$P was incorporated into ABF4, demonstrating that it was phosphorylated by AtCPK32.

### Ser-110 of ABF4 May Be Phosphorylated by AtCPK32

There are two Ser residues within the C2-C3 region of ABF4 and other ABF family members (Figs. 1A and 4B) that may be the substrate sites of CDPK (i.e. $\phi$-X-R/K-X-X-**S**, where $\phi$ is a hydrophobic residue and X is any residue; Cheng et al., 2002). To investigate whether these residues are phosphorylated by AtCPK32, we introduced mutations by replacing the Ser residues with Ala (Fig. 4B). The effects of mutations on phosphorylation were then assessed by an in vitro kinase assay using purified GST fusion proteins (Fig. 4C, lanes 2–4). As shown in Figure 4C (lane 5), $^{32}$P was incorporated into the wild-type, C2-C3 domain-containing N-terminal fragment (KSD, amino acids 1–181), indicating that the fragment was phosphorylated by AtCPK32. $^{32}$P incorporation decreased substantially when Ser-110 was substituted by Ala (lane 6, mKSDI). In the same experiment, the change of Ser-118 into Ala (mKSDII) did not affect phosphorylation significantly (lane 7). Thus, our mutational analysis strongly suggested that Ser-110 is phosphorylated by AtCPK32.

### Ser-110 Is Essential for ABF4-AtCPK32 Interaction

The above results indicated that Ser-110 is likely to be an AtCPK32 phosphorylation site and that it may be necessary for normal ABF4 function. We therefore asked whether the residue is also required for ABF4-AtCPK32 interaction. Ser-110 of the C2-C3

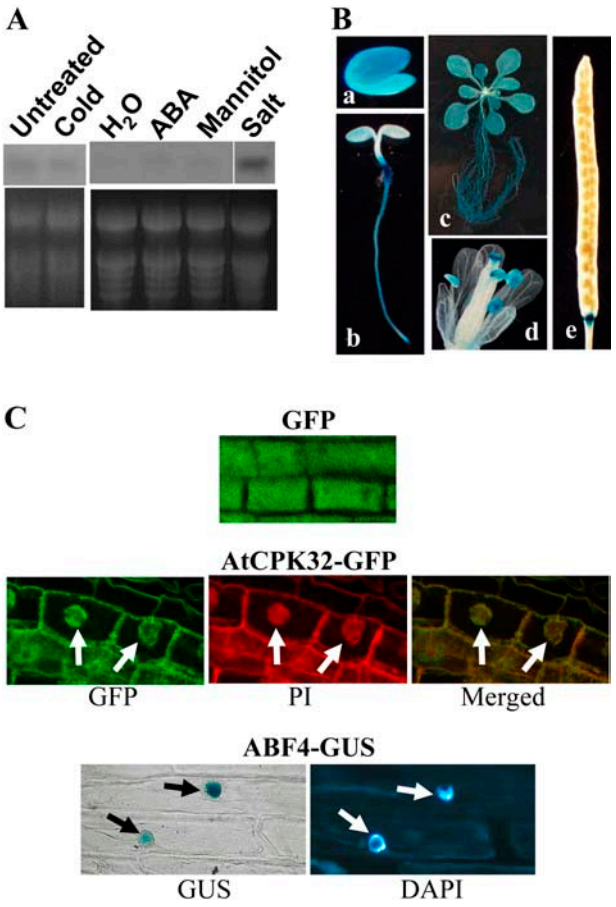


**Figure 3.** Expression patterns and subcellular localization of AtCPK32. A, RNA gel-blot analysis of AtCPK32. Three-week-old seedlings (L*er*) were treated with 100 $\mu$M ABA for 4 h, 60 mM mannitol for 24 h, cold (4°C) for 24 h, and 250 mM NaCl (Salt) for 4 h before RNA isolation. Bottom section shows the ethidium bromide-stained gel as a loading control. B, AtCPK32 promoter activity determined by of histochemical GUS staining. Plants transformed with a 2-kb promoter-*GUS* reporter construct were stained with X-gluc for 24 h. a, Mature embryo. b, Two-day-old seedling. c, Two-week-old-seedling. d, Flower. e, Immature silique. C, Subcellular localization. Top and middle sections, Confocal images of root cells of plants transformed with a *35S-GFP* (GFP) or a *35S-AtCPK32-GFP* (AtCPK32-GFP) construct. PI, PI-stained cells. Bottom section, Light microscopy images of onion cells transiently expressing ABF4-GUS fusion protein. Cells were stained with X-gluc (GUS) or 4′,6-diamidino-2-phenylindole (DAPI). Nuclei are indicated by arrows.

region was mutated into Ala as described above, and its effect on ABF4 interaction with AtCPK32 was determined by two-hybrid assay in which the C2-C3 region was used as bait. As shown in Figure 4D, the Ser to Ala mutation of the residue abolished the interaction of the conserved region with AtCPK32. By contrast, the same Ser to Ala mutation of Ser-118 did not affect the interaction. Thus, Ser-110 was essential for ABF4-AtCPK32 interaction.

### Ser-110 Is Necessary for Normal Transactivation Function of ABF4

To examine the role of Ser-110 in the transcriptional activation function of ABF4, we carried out transient expression assays employing Arabidopsis protoplasts. A GUS reporter construct containing three copies of ABRE in the promoter region was prepared (Fig. 5A). Protoplasts transfected with the reporter construct alone exhibited little GUS activity (Fig. 5B). Addition of exogenous ABA enhanced the reporter activity. The degree of enhancement, however, was very low. Co-transfection of the reporter with an effector construct, in which full-length ABF4 was expressed under the control of cauliflower mosaic virus (CaMV) 35S promoter, resulted in slight increase (approximately 7 times the background activity) of the reporter activity, indicating that ABF4 has low degree of transcriptional activity in the absence of ABA. With the addition of ABA to the assay mixture, the reporter activity increased to approximately 17 times the activity without ABA. The degree of transactivation, however, was reduced to less than half (43%), when the Ser-110 was changed to Ala (mABF4-I). Thus, Ser-110 was required for the normal ABA-activated transcriptional function of ABF4. Substitution of Ser-118 to Ala also lowered the reporter activity to a similar degree, indicating that it was also necessary for normal ABF4 function.

### Overexpression of AtCPK32 Affects ABA Sensitivity and the Expression of ABA-Regulated Genes

To investigate whether AtCPK32 affects ABA-regulated gene expression in vivo, we generated transgenic Arabidopsis plants overexpressing AtCPK32 under the control of CaMV 35S promoter. The expression levels of ABA-regulated genes were then determined by reverse transcription (RT)-PCR. The result (Fig. 6B) showed that transcript levels of several ABA-responsive genes, such as *rd29A*, *rab18*, and *rd29B*, were elevated in the AtCPK32-overexpression lines. Slight increase in the *ICK1* level was also observed. Our previous study showed that these genes, which are involved in protective stress response or in cell cycle regulation, are up-regulated by ABF4 overexpression (Kang et al., 2002). Thus, AtCPK32 overexpression resulted in the enhanced expression of ABF4-regulated genes.

At the whole-plant level, AtCPK32 overexpression enhanced ABA and salt sensitivities during germination. As shown in Figure 6C, ABA dose response analysis revealed that ABA inhibition of germination was enhanced at high concentrations of ABA. Similarly, germination of AtCPK32 overexpression lines was hypersensitive to salt inhibition at 150 mM NaCl (Fig. 6D). In both instances, the degree of hypersensitivity was low and it was not observed at low concentrations of ABA or salt.

### AtCPK32 Interacts with Other ABF Family Members

As mentioned above, the C2 and the C3 regions are highly conserved among ABF family members. Hence, we asked whether AtCPK32 would interact with the corresponding regions of other ABF family members.

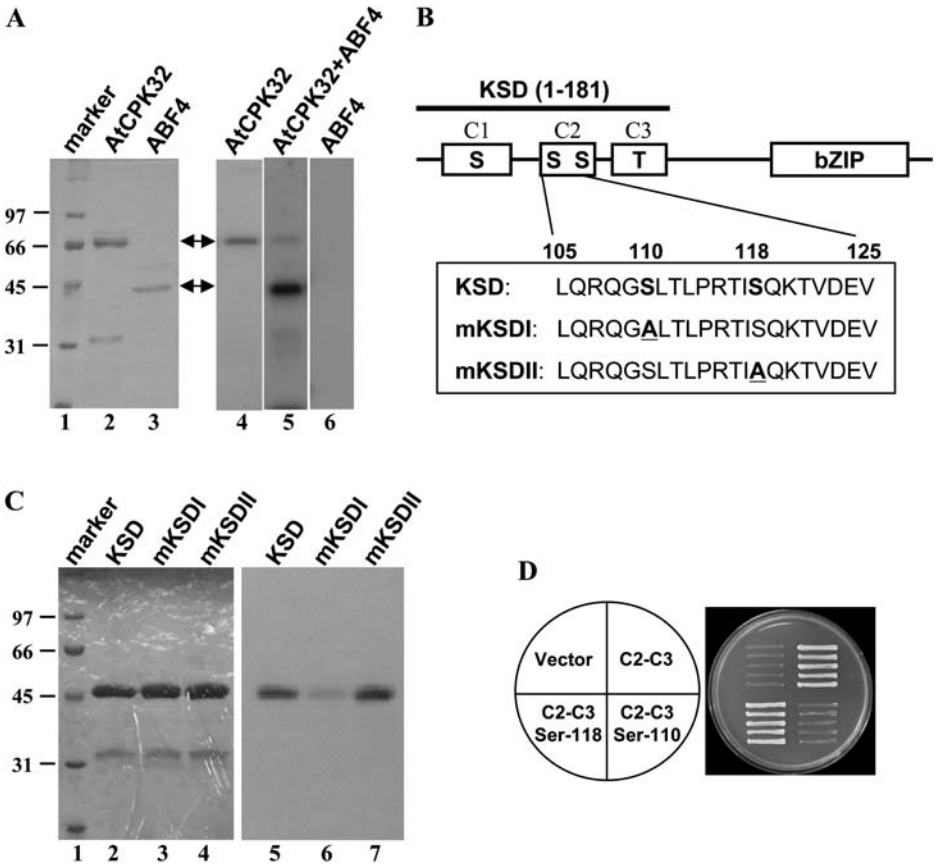


**Figure 4.** Phosphorylation of ABF4 by AtCPK32. A, Kinase activity of AtCPK32. Purified, full-length recombinant proteins were used in in vitro kinase assay. Left (lanes 1–3), Coomassie brilliant blue-stained gel of recombinant proteins. Right (lanes 4–6), Autoradiography showing the incorporation of $^{32}P$ into the recombinant proteins. Arrows indicate the position of bands, and the numbers on the left denote marker size. B, Site-directed mutagenesis of ABF4. ABF4 and the conserved N-terminal domain (KSD) used in the mutagenesis analysis are shown schematically. Partial amino acid sequence of the C2 region is shown in the bottom. Ser-110 and Ser-118 are indicated in bold, and Ala residues are underlined. The numbers indicate amino acid position. C, Phosphorylation of the wild-type (KSD) and the mutant (mKSDI, mKSDII) conserved domains by AtCPK32 was investigated by kinase assay. Left (lanes 1–4), Coomassie brilliant blue-stained gel of the conserved domain-GST fusion proteins. Right (lanes 5–7), Autoradiography. The numbers on the left denote marker size. D, The effects of Ser-110 and Ser-118 mutations on ABF4-AtCPK32 interaction. Interaction between ABF4 and AtCPK32 was determined by two-hybrid assay using various bait constructs (left section) containing the wild-type or the mutated C2-C3 regions. The assay was carried out as in Figure 1B.

Bait constructs containing the C2-C3 region of ABF1, ABF2, or ABF3 were prepared, and their interaction with AtCPK32 was assessed by two-hybrid assay. Figure 7 (top section) shows that all three bait constructs interacted with AtCPK32, demonstrating that AtCPK32 interacts with other ABF family members in yeast.

AtCPK32 belongs to subgroup III of Arabidopsis CDPKs (see ''Discussion''). It has been demonstrated by Sheen (1996) that two subgroup III CDPKs, AtCPK10 and AtCPK30, which also are salt inducible (Urao et al., 1994), activate an ABA/stress-inducible promoter. We therefore carried out two-hybrid assays to address if they would interact with ABF4. The assays, in which AtCPK10 and AtCPK30 were employed as prey (Fig. 7, bottom section), showed that both kinases interacted with the C2-C3 region of ABF4. In contrast, AtCPK28, which belongs to subgroup IV (Cheng et al., 2002), did not interact with ABF4. Thus, our results indicated that ABF4 interacts with two other subgroup III CDPKs, AtCPK10 and AtCPK30, but not with the subgroup IV CDPK, AtCPK28, in yeast.

## DISCUSSION

### AtCPK32 Interacts with ABF4

We demonstrated here that an Arabidopsis CDPK, AtCPK32, interacts with ABF4. CDPKs are found in plants but not in animals and possess a common structural feature: an N-terminal kinase domain fused to a C-terminal calmodulin-like domain, which generally contains four EF hands for $Ca^{2+}$ binding (Cheng et al., 2002; Hrabak et al., 2003). The N-terminal sequences preceding the kinase domain are highly

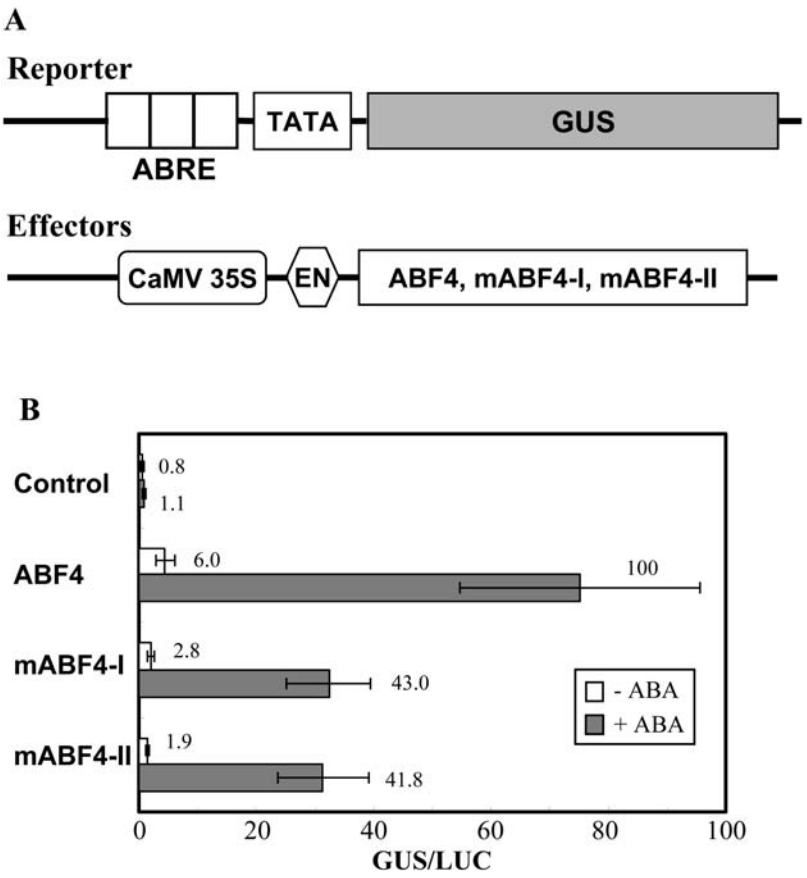


**Figure 5.** Transient expression assay. A, Schematic diagram of constructs. The GUS reporter construct contains three copies of ABRE in front of the CaMV 35S minimal promoter (−46/+1). Effector constructs, driven by CaMV 35S promoter, contain the translation enhancer (EN) sequence of *rbcS* fused to the full-length wild-type (ABF4) or mutant ABF4 (mABF4-I, mABF4-II). Ser-110 and Ser-118 are changed into Ala in mABF4-I and mABF4-II, respectively. B, Transient expression assay. Arabidopsis leaf protoplasts were transfected with the reporter and the effector constructs, and GUS activity was measured with and without ABA treatment. Each data point represents the mean of three independent transfection experiments, and the SES are shown by the small bars. Transfection efficiency was normalized by cotransfection of a CaMV 35S promoter-luciferase construct. The numbers above each bar indicate the percent GUS activities relative to that observed with the wild-type ABF4 in the presence of ABA.

variable compared with other regions. Arabidopsis contains 34 CDPKs, which can be divided into four subgroups (I–IV) based on their sequence homology. AtCPK32 belongs to subgroup III, together with AtCPK10, AtCPK30, and five additional CDPKs.

Similar to many other CDPKs, AtCPK32 possesses Gly residue at the second position, which may be myristoylated, and the fourth and the fifth positions are occupied by Cys residues, which may be palmitoylated (Fig. 1C). Protein myristoylation is known to promote protein-membrane or protein-protein interactions (Martin and Busconi, 2000; Farazi et al., 2001). The irreversible acylation is usually followed by reversible palmitoylation, which further stabilizes or regulates the interactions. In addition to the acylation sites, AtCPK32 has three nuclear localization signals, one in the N-terminal region and two more in the kinase domain (Fig. 1C). Thus, AtCPK32 has two subcellular targeting signals: membrane association and nuclear localization.

Overall, the expression patterns of AtCPK32 (Fig. 3) are similar to those of ABF4 (Choi et al., 2000; Kang et al., 2002), supporting the physiological relevance of the ABF4-AtCPK32 interaction. Furthermore, we observed the localization of both ABF4 and AtCPK32 in the nucleus. Differences in their expression patterns and intracellular localization were also observed. The transcript level of ABF4 is ABA inducible (Choi et al., 2000), but the AtCPK32 transcript level was not. ABF4 was localized mainly in the nucleus (Fig. 3C). On the other hand, AtCPK32 was found not only in the nucleus but also in the cell periphery. We do not know the physiological significance of the localization pattern of AtCPK32. However, it is not surprising because AtCPK32 has both membrane anchor and nuclear localization signals.

### AtCPK32 May Phosphorylate Ser-110 of ABF4, Which Is Essential for Normal ABF4 Activity

The C2 conserved domain possesses two consecutive sequence motifs that may serve as CDPK phosphorylation sites (Fig. 4B). The first phosphorylation site (Ser-110) is conserved in all eight ABF/AREB/ABI5 subfamily members, whereas the second one (Ser-118) is missing in ABI5 and another embryo-specific member, AtDPBF2 (Bensmihen et al., 2002). Our data in Figure 4 suggest that AtCPK32 may phosphorylate Ser-110 of ABF4. The residue not only fits into the minimal consensus phosphorylation site of CDPK, but also its mutation to Ala causes decrease in phosphorylation. In addition, the same mutation abolishes the ABF4-AtCPK32 interaction.

Our transient assay (Fig. 5) showed that Ser-110 is necessary for the normal ABF4 activity. The result implies that AtCPK32-dependent phosphorylation of

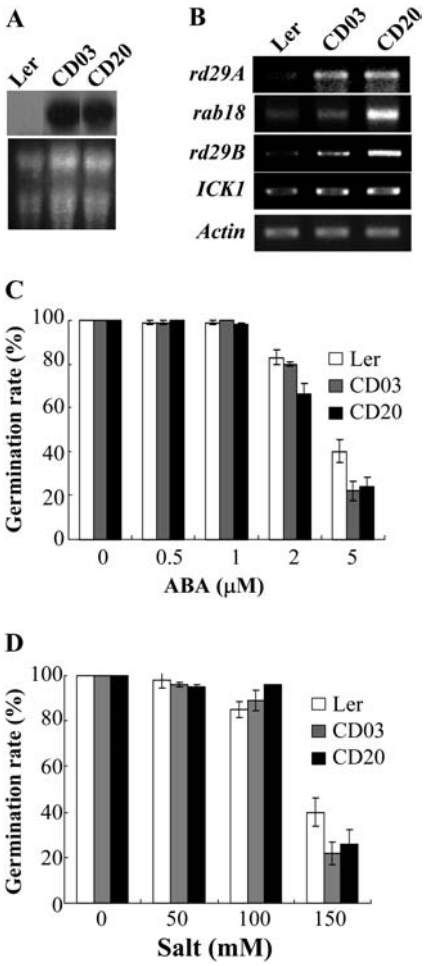


**Figure 6.** AtCPK32 overexpression phenotypes. A, The expression levels of AtCPK32 in L*er* and *35S-AtCPK32* transgenic lines CD03 and CD20 were determined by RNA gel-blot analysis. The bottom section shows the ethidium bromide-stained gel. B, Expression levels of ABA-regulated genes in L*er* and transgenic lines CD03 and CD20 were determined by RT-PCR. C and D, ABA and salt sensitivities, respectively, during seed germination. Seeds were plated on medium containing ABA or salt after 4 d of cold treatment at 4°C, and germination (full emergence of radicle) was scored after 4 d. Experiments were done in triplicates ($n = 36$ each), and the small bars represent SES.

Ser-110 may be an integral part of the ABA activation process of ABF4 transcriptional function. In principle, the reduced reporter activity observed in the transient assay may result from reduced binding to ABRE or reduced transcriptional activity. However, we did not observe significant difference in the ABRE-binding activities of phosphorylated and nonphosphorylated ABF4 (data not shown). Thus, the reduced reporter activity probably reflects the reduced transcriptional activity of ABF4.

### AtCPK32 Overexpression Affects the Expression of ABF4-Regulated Genes and ABA/Salt Sensitivity

The changes in the expression levels of several ABF4-regulated genes resulting from AtCPK32 overexpression provide more direct evidence that it is involved in the modulation of ABF4 activity. Transcript levels of several ABF4-inducible genes (Kang et al., 2002) were elevated by AtCPK32 overexpression (Fig. 6B). The result suggests that AtCPK32 is a positive regulator of ABF4 function. We did not observe major phenotypic changes with AtCPK32-overexpressing plants except minor growth retardation (data not shown). However, similar to ABF4 overexpression lines, the AtCPK32 transgenic lines displayed hypersensitivity to ABA and salt during germination (Fig. 6), although it was observed only at high concentrations of ABA and salt.

### Other Kinases May Be Involved in the Modulation of ABF4 Activity

Several observations are worthy to be mentioned regarding the ABF4-AtCPK32 interaction and the ABF4 transcriptional activity. First, ABF4 interacts also with AtCPK10 and AtCPK30, and AtCPK32 interacts with other ABFs in yeast (Fig. 7). Further studies are required to validate the interactions, and their physiological significance needs to be demonstrated. Nonetheless, the data suggest that there might be cross talk among the ABF family members and CDPKs. We do not know the degree or specificity of the putative cross talk in detail yet. However, the data in Figure 7 suggest that perhaps only subgroup III CDPKs may interact with ABFs. Under physiological conditions, the interactions will be limited further by the differences in their temporal and/or spatial

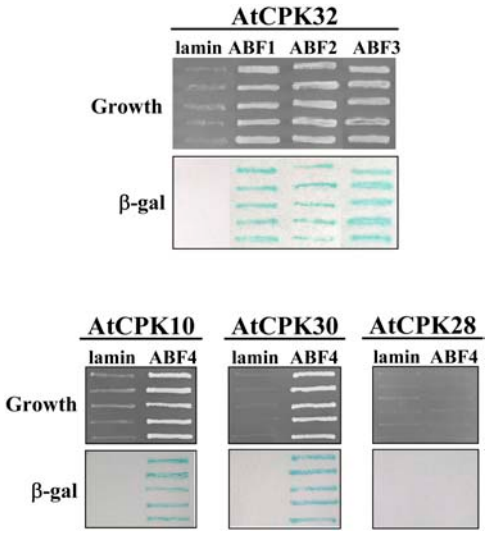


**Figure 7.** Interaction between ABF family members and CDPKs. Interaction between AtCPK32 and ABF family members (ABF1, ABF2, or ABF3) or between ABF4 and CDPKs (AtCPK10, AtCPK30, and AtCPK28) was determined by two-hybrid assays. The assays were carried out as in Figure 1B, using the C2-C3 region of ABF1 (amino acids 1–160), ABF2 (amino acids 65–162), ABF3 (amino acids 103–215), or ABF4 (amino acids 89–181) and nuclear lamin (lamin) as bait. $\beta$-gal, $\beta$-Galactosidase assay. The reporter activities for ABF4-AtCPK10, ABF4-AtCPK30, and ABF4-CPK28 interactions are $39.65 \pm 2.42$, $34.64 \pm 1.89$, and $0.08 \pm 0.00$, respectively, in Miller units.

expression patterns. Whatever the specificity is, our data raises an interesting possibility that part of the underlying mechanism of AtCPK10 and AtCPK30 action in ABA/stress signaling (Sheen, 1996) may be the modulation of ABA-regulated gene expression via ABFs.

Second, Ser-118 is also necessary for ABA activation of ABF4 function (Fig. 5). Although it may not be phosphorylated by AtCPK32 (Fig. 4), its mutation nonetheless affected the ABA-induced transcriptional activity negatively. We do not know at present whether Ser-118 of ABF4 is phosphorylated in response to ABA. However, it can be speculated that Ser-118 might be a substrate of other kinases. Uno et al. (2000) demonstrated that a 42-kD protein kinase phosphorylates the conserved blocks, and an SnRK2 kinase of similar size has been shown to be essential for dehydration tolerance (Mustilli et al., 2002; Yoshida et al., 2002; Umezawa et al., 2004). Meanwhile, the conserved domains of TaABF are phosphorylated by the SnRK2 kinase, PKABA1 (Johnson et al., 2002), further suggesting the possible involvement of other kinases.

Third, the role of Ser-110 of ABF4 differs from that of the corresponding residue of rice TRAB1. In rice suspension-cultured cells, Ser-102 of TRAB1, which is equivalent to Ser-118 of ABF4, is phosphorylated and required for ABA-induced transcription, whereas Ser-94 (i.e. Ser-110 of ABF4) is not (Kagaya et al., 2002). This suggests that the phosphorylation patterns and/or specific roles of the conserved residues of ABF4 may be different from those of TRAB1. It is noteworthy in this regard that the phosphorylation of the corresponding amino acids of ABI5 does not affect its in vivo function (Lopez-Molina et al., 2002) and that ABF1 and ABF3 exhibit high transcriptional activity even without ABA activation (Choi et al., 2000). Thus, more than one kinase appears to be involved in the phosphorylation of ABF4, and the functions of the conserved kinase substrate residues of ABF-related factors may differ from one another.

To summarize, we showed that AtCPK32 interacts with ABF4. The physiological significance of the AtCPK32-ABF4 interaction was supported by their similar expression patterns and similar intracellular localization. AtCPK32 overexpression affected the expression of several ABF4-regulated genes and promoted ABA and salt sensitivities, demonstrating its involvement in ABA/stress response. AtCPK32 appears to modulate the ABF4 activity by phosphorylating one of the Ser residues within the C2 conserved region. Additionally, our data suggest that other kinases may be involved in the modulation of ABF4 activity.

## MATERIALS AND METHODS

### Manipulation of DNA and RNA

Standard methods (Sambrook and Russell, 2001) were employed to manipulate DNA and RNA. DNA sequencing was carried out on ABI 310 Genetic Analyzer. RNA was isolated according to Chomczynski and Mackey (1995) and purified further by LiCl precipitation followed by ethanol precipitation. RNA gel-blot analysis was performed as described (Choi et al., 2000), using 25 $\mu$g of total RNA. Hybridization was at 65°C for 24 h in Rapid-hyb buffer (Amersham Pharmacia Biotech) using the coding region of AtCPK32 (amino acids 278–389 for cold-treated RNA sample and 361–454 for other samples) as a probe. Washing of the filter was done sequentially: twice in 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate) for 10 min at room temperature, once in 0.2× SSC for 10 min at room temperature, and once in 0.2× SSC for 10 min at 65°C. For RT-PCR, 0.5 $\mu$g of total RNA was processed according to the supplier's instruction using the Access RT-PCR system (Promega). That RNA preparations were free of contaminating DNA was confirmed by using the actin primer set spanning an intron (Arabidopsis [*Arabidopsis thaliana*] actin-1 gene, accession no. M20016), and, whenever possible, primer sets spanning introns were used as described previously (Kang et al., 2002). The number of PCR cycles was 20 to 30, within the linear range of PCR amplification for each gene, and all the RT-PCR results were confirmed by several independent reactions.

### Yeast Two-Hybrid Screen, Specificity Test, and Two-Hybrid Assay

Standard techniques were used for the manipulation of yeast (Guthrie and Fink, 1991). Transformation, rescue of yeast plasmid DNA, and screenings were carried out as described previously (Choi et al., 2000). MATCHMAKER LexA two-hybrid system (Clontech) was employed in two-hybrid screens. To prepare bait constructs, the coding regions corresponding to the amino acids 1 to 88 and 89 to 181 were prepared by PCR, using the primer sets 5′-ATGGGAACTCACATCAATTTC-3′ and 5′-gagagctcgagAGCAGCTGGCGCAGAAGTC-3′, and 5′-ACAGCGGTAGCGCAACCTGG-3′ and 5′-gagagctcgagACCCATCTG TTGAACACAGTTATC-3′, respectively. The PCR products were digested with *Xho*I and ligated with pGilda (Clontech), which was prepared by *Bam*HI digestion, Klenow fill-in reaction, and *Xho*I digestion.

The constructs were introduced into EGY48 (*MATα*, *his3*, *trp1*, *URA3*::*LexA*$_{op(x8)}$-*LacZ*, *LexA*$_{op(x6)}$-*LEU2*) by transformation, and transformants were kept on SC-His-Ura medium. For the screening, the reporter yeast was transformed with library plasmid DNA, which was described previously (Choi et al., 2000). Transformed mixture was plated on Gal/Raf/CM-His-Leu-Trp-Ura medium, and after 6 d colonies were assayed for the $\beta$-galactosidase activity by filter lift assay. Positive colonies were patched on the same selection medium. For the insert analysis, DNA was isolated from the yeast clones, the cDNA inserts were amplified by PCR, and the PCR products were grouped according to restriction patterns. Plasmid DNA representing each group was rescued by *Escherichia coli* transformation, sequenced, and reintroduced into the reporter yeast to test specificity.

To prepare the conserved region bait constructs of ABF1 (amino acids 1–160), ABF2 (amino acids 65–162), and ABF3 (amino acids 103–215) used in the interaction study (Fig. 1B), corresponding fragments were prepared by PCR (primer sequences are available upon request). *Xho*I site was attached to the reverse primers to facilitate cloning, and, after the enzyme digestion, the PCR fragments were cloned into pGilda, which was prepared as described above. To test whether the positive clones interact with the conserved blocks, each construct was transformed into EGY48, resulting yeast transformants were transformed with clone 5 plasmid DNA, and the transformants were patched on Gal/Raf/CM-His-Leu-Trp-Ura medium. The yeast was then allowed to grow for 4 d before taking photographs.

Full-length AtCPK32 containing 5′ and 3′ untranslated regions was isolated by PCR, using the library DNA as a template and primers 5′-TGGAATTTCGTCCTCAATCTCTCAAG-3′ and 5′-cgcggattcGAATGGTTATAGAAAATTACAGATTGATC-3′, and, after *Bam*HI cut, cloned into the *Eco*RV-*Bam*HI sites of pBluescript KS (Stratagene).

Various fragments used in two-hybrid assays (Figs. 1, 2, and 7), including the full-length AtCPK10, AtCPK30, and AtCPK28, were prepared by PCR (primer sequences are available upon request). For the convenience of cloning, *Bam*HI and *Not*I linker sequences were attached to the forward and reverse primers, respectively, and the PCR fragments were cloned into the corresponding sites of pYESTrp2 (Invitrogen). The constructs were introduced into the reporter yeast containing the conserved region bait construct (amino acids 89–181) used for the two-hybrid screening. $\beta$-Galactosidase assay was carried out as described (Choi et al., 2000), using *O*-nitrophenyl-$\beta$-D-galactopyranoside as a substrate. For each construct, five independent transformants were assayed.

## In Vitro Binding Assay

The entire coding region of AtCPK32 was excised from pBluescript by *Sal*I and *Not*I digestion. The fragment was then cloned into the same enzyme sites of pGEM-T Easy (Promega). In vitro translation was performed using the TNT in vitro translation kit (Promega) according to the supplier's instruction in the presence of $^{35}$S-Met.

*GST-ABF4* fusion construct was prepared by cloning the coding region of ABF4 into the *Sma*I-*Not*I sites of pGEX-6P-2 (Amersham Pharmacia Biotech). To prepare recombinant protein, BL21 *E. coli* cells were transformed with the GST-ABF4 fusion construct, and the transformed cells were grown overnight. The culture was diluted 100-fold and grown until $A_{600}$ reached 0.6. To induce the expression of recombinant protein, 0.5 mM isopropyl-$\beta$-D-thiogalactopyranoside was added and grown further for 3 h. After the induction, cells were pelleted down by centrifugation, resuspended in phosphate-buffered saline (0.14 M NaCl, 2.7 mM KCl, 10.1 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$, pH 7.3), and sonicated. The lysate was purified according to the supplier's instruction after clearing cell debris by centrifugation.

For binding assay, GST-ABF4 fusion proteins (2 $\mu$g) were incubated with the Glutathione-Sepharose 4B resin for 30 min at 4°C in 100 $\mu$L of a buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 10% glycerol, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride). In vitro-translated AtCPK32 (20 $\mu$L) was then added, and binding was allowed for 2 h with constant rotation. At the end of binding, resins were washed with 1 mL of binding buffer three times to remove unbound proteins and resuspended in SDS-PAGE sample buffer. Proteins were visualized by autoradiography after fractionation on 15% SDS-polyacrylamide gel.

## Kinase Assay

To prepare recombinant AtCPK32, its coding region was prepared by PCR, using primers 5′-cgcggatccGTCATGGGTAATTGTTGCGGAAC-3′ and 5′-tttttcctttgcggccgc TCTTGTATCACCATTGACCTGC-3′. To facilitate cloning, *Bam*HI and *Not*I linker sequences were attached to the primers, and, after digestion with the enzymes, the amplified fragment was cloned into the corresponding sites of pET-23a(+) (Novagen) in frame with the His tag. The construct was introduced into BL21 cells by transformation. Fresh culture of transformed cells (OD600 = 0.8) were induced to express the protein by the addition of 0.5 mM isopropyl-$\beta$-D-thiogalactopyranoside and, after 3 h at 30°C, cells were collected by centrifugation and sonicated. The cell lysate was applied to the nickel-nitrilotriacetic acid agarose column (Qiagen) and processed according to the manufacturer's instruction.

Full-length recombinant ABF4 was prepared by excising ABF4 from GST-ABF4 described above. BL21 cell lysate (50 mL) of 1 L culture was loaded onto the Glutathione Sepharose 4B column (approximately 0.4 mL) and washed with phosphate-buffered saline and 1 × Precision cleavage buffer (50 mM Tris-HCl, pH 7.0, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol). Excision was carried out by incubating the resin for 6 h at 4°C in 1 mL of the buffer containing 80 units of Precision protease (Amersham Pharmacia Biotech), and the cleaved protein was eluted.

The construct (KSD) containing the N-terminal portion of ABF4 was prepared by cloning the amplified fragment containing the amino acids 1 to 181 into the *Bam*HI and *Xho*I sites of pGEX-6P-2. mKSDI was prepared by single-step ligation of two amplified fragments, I-1 and I-2, with pGEX-6P-2, which were digested with *Bam*HI and *Xho*I. Fragment I-1 was prepared by PCR using primers 5′-cgcggatccATGGGAACTCACATCAATTTCA-3′ and 5′-ACCTGCCTCTGGAGATTCC-3′, and fragment I-2 was amplified using primers 5′-GCGTTGACGTTGCCTAGAAC-3′ and 5′-gagagctcgagACCCATCTGTTGAACACAGTTATC-3′. Before ligation, the fragments were digested with *Bam*HI and *Xho*I, respectively. Similarly, mKSDII was prepared by single-step ligation of pGEX-6P-2 with two amplified fragments, which were prepared using primer sets 5′-cgcggatccATGGGAACTCACATCAATTTCA-3′ and 5′-AATTGTTCTAGGCAACGTCAACG-3′, and 5′-GCTCAGAAGACTGTTGATGAGGTGTG-3′ and 5′-gagagctcgagACCCATCTGTTGAACACAG TTATC-3′, respectively.

Kinase assays were carried out at 25°C for 5 min by incubating 0.15 $\mu$g of the purified ABF4 with 0.1 $\mu$g of AtCPK32 or 5 $\mu$g of KSD domain proteins with 0.13 $\mu$g of AtCPK32 in 25 $\mu$L of buffer (25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 $\mu$M ATP) containing 2 $\mu$Ci of [$\gamma$-$^{32}$P]ATP. For autophosphorylation, 1 $\mu$g of AtCPK32 was incubated without substrate for 20 min at the same condition.

## Transient Expression Assay

Effector constructs were prepared in pBI221 (Jefferson et al., 1987) in two steps. The coding regions of wild-type and mutant ABF4 were prepared by PCR, cloned into the intermediate vector pBluescript KS (Stratagene), and then cloned into pBI221 from which the GUS coding region was removed. To enhance translation efficiency, the 5′ untranslated sequence of ribulose-1,5-bisphosphate carboxylase small subunit gene (aaagagtaaagaagaaca) was attached in front of the initiation codon. Wild-type ABF4 was amplified using primers 5′-cgcggatccaaagagtaaagaagaaca ATGGGAACTCACATCAATTTC-AAC-3′ (UTRF) and 5′-ccgaattcTCACCATGGTCCGGTTAATGT-3′ (RE), digested with *Eco*RI and *Bam*HI, and cloned into the same sites of pBluescript KS. mABF4-I and mABF4-II fragments were prepared by ligating two PCR fragments, respectively. First, using the mKSDI and mKSDII constructs (above) as templates, N-terminal portions were amplified using the primer set UTRE and 5′-ACCCATCTG TTGAACACAGTTATC-3′. The C-terminal fragment was amplified using the wild-type ABF4 as a template and primers 5′-CAGGTCAACGGAAACAATAACAAT-3′ and RE. The N-terminal and C-terminal fragments were then digested with *Bam*HI and *Eco*RI, respectively, and ligated with pBluescript KS. The wild-type, mABF4-I, and mABF4-II fragments in the intermediate vector were excised out by sequential treatments with *Eco*RI, Klenow fragment, and *Bam*HI. The fragments were then ligated with GUS-less pBI221 prepared by *Sac*I digestion, removal of 3′ overhang, and *Bam*HI digestion.

To prepare reporter construct, the +46/−1 region of the CaMV 35S promoter region was amplified using primers 5′-ggcccaagcttCAAGACCC-TTCCTCTATATAAGGAAGT-3′ and 5′-CCAGGGATCCTCTAGAGTCCC-3′ and, after digestion with *Hin*dIII and *Bam*HI, ligated with promoterless pBI221 digested with the same restriction enzymes. Resulting pBI221 was digested with *Hin*dIII, treated with Klenow fragment to fill the 5′ overhang, and ligated with ABRE. ABRE, which contains three copies of the Em1a element (GGACACGTGGCG), was prepared by annealing primers (Choi et al., 2000).

Reporter and effector constructs (15 $\mu$g each) were introduced into 300 $\mu$L of protoplast suspension ($5 \times 10^6$/mL) by polyethylene glycol-mediated transformation (Jin et al., 2001). Transformed protoplasts were treated with 60 $\mu$M ABA or with water for 5 h, harvested by centrifugation, and resuspended in 150 $\mu$L of extraction buffer (50 mM $NaPO_4$, pH 7.0, 10 mM EDTA, 0.1 mM EDTA, 0.1% Triton X-100, 10 mM $\beta$-mercaptoethanol). The protoplast suspension was sonicated, cleared of cell debris by centrifugation, and the supernatant was used to determine GUS activity by the standard procedure (Jefferson et al., 1987). For each construct, 50 $\mu$L of the supernatant was added to 350 $\mu$L of 1 mM 4-methylumbelliferyl glucuronide, and 100-mL aliquots were assayed in duplicates. To stop reactions, 600 $\mu$L of 0.2 M $Na_2CO_3$ was added, and 200 $\mu$L of the mixture was used to determine fluorescence. CaMV 35S promoter-luciferase construct was cotransfected in each experiment to normalize transfection efficiency.

## Plant Growth, Transformation, and Germination Assay

Plants (Arabidopsis ecotype Landsberg *erecta* [L*er*] and Columbia [Col-0]) were grown at 22°C under long-day condition (16-h-light/8-h-dark cycles) aseptically on Murashige and Skoog medium (Murashige and Skoog, 1962) or on soil (1:1:1 mixture of vermiculite, perlite, and peat moss). ABA, salt, and cold treatments of seedlings were conducted as described (Choi et al., 2000). For in vitro growth, seeds were treated with 70% ethanol and 30% household bleach for 5 min each and then with sterile water five times before plating. The Murashige and Skoog medium was supplemented with 1% Suc. For germination assay, seeds collected at the same time were used. Transformation of Arabidopsis was according to Bechtold and Pelletier (1998).

## Histochemical GUS Staining

A 2-kb promoter fragment, prepared by PCR using primers 5′-CAA-AACCGATGAAACTTGAGAGATTG-3′ and 5-CATTCCATCTGCTGAGAC-TGATACG-3′, was first cloned into TOPO cloning vector (Invitrogen) and then into pBI101.2 (Jefferson et al., 1987) using the *Hin*dIII and *Xba*I sites. The vector was used to transform Arabidopsis (Col-0), and T2 or T3 generation plants were used for GUS staining, which was carried out as described (Kang et al., 2002). Several independent lines were examined, and typical results are shown in Figure 3.

## Subcellular Localization

The coding region of AtCPK32 prepared by PCR was cloned into pCAMBIA1302 (Cambia) using the *Bgl*II and *Spe*I sites. The construct was

used to transform Arabidopsis (Col-0), and T1 plants were used to determine GFP localization. Roots of 10-d-old seedlings were examined for the green (GFP expression) and red (propidium iodide [PI]) fluorescence, employing a confocal microscope (Leica, TCS-NT). Nuclei were stained with PI staining. For the localization of ABF4, the coding region of ABF4 was cloned in frame in front of the GUS coding region of pBI221. The *ABF4-GUS* fusion construct was introduced into onion epidermal cells by particle bombardment using PDS 1000 (Bio-Rad). GUS activity was determined by 5-bromo-4-chloro-3-indolyl-β-GlcUA (X-gluc) staining after 24 at 23°C. Nuclei were stained with 4′,6-diamidino-2-phenylindole and visualized with a fluorescence microscope (Olympus BX51).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number NM_115613.

Received August 9, 2005; revised September 30, 2005; accepted October 9, 2005; published November 18, 2005.

## LITERATURE CITED

**Bechtold N, Pelletier G** (1998) In planta Agrobacterium-mediated transformation of adult Arabidopsis thaliana plants by vacuum infiltration. Methods Mol Biol **82:** 259–266

**Bensmihen S, Rippa S, Lambert G, Jublot D, Pautot V, Granier F, Giraudat J, Parcy F** (2002) The homologous ABI5 and EEL transcription factors function antagonistically to fine-tune gene expression during late embryogenesis. Plant Cell **14:** 1391–1403

**Busk PK, Pages M** (1998) Regulation of abscisic acid-induced transcription. Plant Mol Biol **37:** 425–435

**Casaretto J, Ho T-HD** (2003) The transcription factors HvABI5 and HvVP1 are required for the abscisic acid induction of gene expression in barley aleurone cells. Plant Cell **15:** 271–284

**Cheng SH, Willmann MR, Chen HC, Sheen J** (2002) Calcium signaling through protein kinases: the Arabidopsis calcium-dependent protein kinase gene family. Plant Physiol **129:** 469–485

**Chien CT, Bartel PL, Sternglanz R, Fields S** (1991) The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc Natl Acad Sci USA **88:** 9578–9582

**Choi H, Hong J, Ha J, Kang J, Kim SY** (2000) ABFs, a family of ABA-responsive element binding factors. J Biol Chem **21:** 1723–1730

**Chomczynski P, Mackey K** (1995) Modification of the TRI reagent procedure for isolation of RNA from polysaccharide- and proteoglycan-rich sources. BioTechniques **19:** 942–945

**Farazi TA, Waksman G, Gordon JL** (2001) The biology and enzymology of protein N-myristoylation. J Biol Chem **276:** 39501–39504

**Finkelstein RR, Gampala SS, Rock CD** (2002) Abscisic acid signaling in seeds and seedlings. Plant Cell (Suppl) **14:** S15–S45

**Finkelstein RR, Lynch TJ** (2000) The Arabidopsis abscisic acid response gene ABI5 encodes a basic leucine zipper transcription factor. Plant Cell **12:** 599–609

**Gong D, Guo Y, Schumaker KS, Zhu JK** (2004) The SOS3 family of calcium sensors and SOS2 family of protein kinases in Arabidopsis. Plant Physiol **134:** 919–926

**Guthrie C, Fink GR, editors** (1991) Guide to Yeast Genetics and Molecular Biology. Methods in Enzymology, Vol 194. Academic Press, New York

**Gyuris J, Golemis E, Chertkov H, Brent R** (1993) Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. Cell **75:** 791–803

**Hobo T, Kowyama Y, Hattori T** (1999) A bZIP factor, TRAB1, interacts with VP1 and mediates abscisic acid-induced transcription. Proc Natl Acad Sci USA **96:** 15348–15353

**Hoth S, Morgante M, Sanchez JP, Hanafey MK, Tingey SV, Chua N-H** (2002) Genome-wide gene expression profiling in Arabidopsis thaliana reveals new targets of abscisic acid and largely impaired gene regulation in the abi1-1 mutant. J Cell Sci **115:** 4891–4900

**Hrabak EM, Chan CW, Gribskov M, Harper JF, Choi JH, Halford N, Kudla J, Luan S, Nimmo HG, Sussman MR, et al** (2003) The Arabidopsis CDPK-SnRK superfamily of protein kinases. Plant Physiol **132:** 666–680

**Hrabak EM, Dickmann LJ, Satterlee JS, Sussman MR** (1996) Characterization of eight new members of the calmodulin-like domain protein kinase gene family from Arabidopsis thaliana. Plant Mol Biol **31:** 405–412

**Jefferson RA, Kavanagh TA, Bevan MW** (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J **20:** 3901–3907

**Jin JB, Kim YA, Kim SJ, Lee SH, Kim DH, Cheong G-W, Hwang I** (2001) A new dynamin-like protein, ADL6, is involved in trafficking from the trans-Golgi network to the central vacuole in Arabidopsis. Plant Cell **13:** 1511–1526

**Johnson RR, Wagner RL, Verhey SD, Walker-Simmons MK** (2002) The abscisic acid-responsive kinase PKABA1 interacts with a seed-specific abscisic acid response element-binding factor, TaABF, and phosphorylates TaABF peptide sequences. Plant Physiol **130:** 837–846

**Kagaya Y, Hobo T, Murata M, Ban A, Hattori T** (2002) Abscisic acid-induced transcription is mediated by phosphorylation of an abscisic acid response element binding factor, TRAB1. Plant Cell **14:** 3177–3189

**Kang J, Choi H, Im M, Kim SY** (2002) Arabidopsis basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. Plant Cell **14:** 343–357

**Kim J-B, Kang J-Y, Kim SY** (2004) Over-expression of a transcription factor regulating ABA-responsive gene expression confers multiple stress tolerance. Plant Biotechnol J **2:** 459–466

**Kim K-N, Cheong YH, Grant JJ, Pandey GK, Luan S** (2003) CIPK3, a calcium sensor-associated protein kinase that regulates abscisic acid and cold signal transduction in Arabidopsis. Plant Cell **15:** 411–423

**Kim S, Kang J-Y, Cho D-I, Park JH, Kim SY** (2004) ABF2, an ABRE-binding bZIP factor, is an essential component of glucose signaling and its overexpression affects multiple stress tolerance. Plant J **40:** 75–87

**Kim SY, Chung HJ, Thomas TL** (1997) Isolation of a novel class of bZIP transcription factors that interact with ABA-responsive and embryo-specification elements in the Dc3 promoter using a modified yeast one-hybrid system. Plant J **11:** 1237–1251

**Kim SY, Ma J, Perret P, Li Z, Thomas TL** (2002) Arabidopsis ABI5 subfamily members have distinct DNA-binding and transcriptional activities. Plant Physiol **130:** 688–697

**Kim SY, Thomas TL** (1998) A family of novel basic leucine zipper proteins bind to seed-specification elements in the carrot Dc3 gene promoter. J Plant Physiol **152:** 607–613

**Kreps JA, Wu Y, Chang HS, Zhu T, Wang X, Harper JF** (2002) Transcriptome changes for Arabidopsis in response to salt, osmotic, and cold stress. Plant Physiol **130:** 2129–2141

**Landschulz WH, Johnson PF, McKnight SL** (1988) The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. Science **240:** 1759–1764

**Li J, Wang XQ, Watson MB, Assmann SM** (2000) Regulation of abscisic acid-induced stomatal closure and anion channels by guard cell AAPK kinase. Science **287:** 300–303

**Lopez-Molina L, Chua N-H** (2000) A null mutation in a bZIP factor confers ABA-insensitivity in Arabidopsis thaliana. Plant Cell Physiol **41:** 541–547

**Lopez-Molina L, Mongrand S, Chua N-H** (2001) A postgermination developmental arrest checkpoint is mediated by abscisic acid and requires the ABI5 transcription factor in Arabidopsis. Proc Natl Acad Sci USA **98:** 4782–4787

**Lopez-Molina L, Mongrand S, McLachlin DT, Chait BT, Chua N-H** (2002) ABI5 acts downstream of ABI3 to execute an ABA-dependent growth arrest during germination. Plant J **32:** 317–328

**Luan S, Kudla J, Rodriguez-Concepcion M, Yalovsky S, Gruissem W** (2002) Calmodulins and calcineurin B-like proteins: calcium sensors for specific signal response coupling in plants. Plant Cell (Suppl) **14:** S389–S400

**Martin ML, Busconi L** (2000) Membrane localization of a rice calcium-dependent protein kinase (CDPK) is mediated by myristoylation and palmitoylation. Plant J **24:** 429–435

**Murashige T, Skoog F** (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol Plant **15:** 473–497

**Mustilli AC, Merlot S, Vavasseur A, Fenzi F, Giraudat J** (2002) Arabidopsis OST1 protein kinase mediates the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. Plant Cell **14:** 3089–3099

**Rock C** (2000) Pathways to abscisic acid-regulated gene expression. New Phytol **148:** 357–396

**Romeis T, Ludwig AA, Martin R, Jones JD** (2001) Calcium-dependent

protein kinases play an essential role in a plant defence response. EMBO J **20:** 5556–5567

**Saijo Y, Hata S, Kyozuka J, Shimamoto K, Izui K** (2000) Over-expression of a single $Ca^{2+}$-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. Plant J **23:** 319–327

**Sambrook J, Russell DW** (2001) Molecular Cloning: A Laboratory Manual, Ed 3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

**Sheen J** (1996) $Ca^{2+}$-dependent protein kinase and stress signal transduction in plants. Science **274:** 1900–1902

**Takahashi S, Seki M, Ishida J, Satou M, Sakurai T, Narusaka M, Kamiya A, Nakajima M, Enju A, Akiyama K, et al** (2004) Monitoring the expression profiles of genes induced by hyperosmotic, high salinity, and oxidative stress and abscisic acid treatment in Arabidopsis cell culture using a full-length cDNA microarray. Plant Mol Biol **56:** 29–55

**Umezawa T, Yoshida R, Maruyama K, Yamaguchi-Shinozaki K, Shinozaki K** (2004) SRK2C, a SNF1-related protein kinase 2, improves drought tolerance by controlling stress-responsive gene expression in Arabidopsis thaliana. Proc Natl Acad Sci USA **101:** 17306–17311

**Uno Y, Furihata T, Abe H, Yoshida R, Shinozaki K, Yamaguchi-Shinozaki K** (2000) Arabidopsis basic leucine zipper transcription factors involved in an abscisic acid-dependent signal transduction pathway under drought and high-salinity. Proc Natl Acad Sci USA **97:** 11632–11637

**Urao T, Katagiri T, Mizoguchi T, Yamaguchi-Shinozaki K, Hayashida N, Shinozaki K** (1994) Two genes that encode Ca(2+)-dependent protein kinases are induced by drought and high-salt stresses in Arabidopsis thaliana. Mol Gen Genet **244:** 331–340

**Xiong L, Schumaker KS, Zhu J-K** (2002) Cell signaling during cold, drought, and salt stress. Plant Cell (Suppl) **14:** S165–S183

**Yoshida R, Hobo T, Ichimura K, Mizoguchi T, Takahashi F, Alonso J, Ecker JR, Shinozaki K** (2002) ABA-activated SnRK2 protein kinase is required for dehydration stress signaling in Arabidopsis. Plant Cell Physiol **43:** 1473–1483