Variable Interactions between Sucrose Non-fermented 1-Related Protein Kinases and Regulatory Proteins in Higher Plants

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Received May 21, 2003; Accepted August 26, 2003

WPK4 is a sucrose non-fermented 1 (SNF1)-related wheat protein kinase, and was previously reported to interact with 14-3-3 proteins. We identified four Arabidopsis thaliana WPK4-like genes, and designated them AtWLI through AtWL4. Yeast two-hybrid analysis, however, indicated that none of the AtWLs interacted with any of A. thaliana 14-3-3 (At14-3-3) proteins, although WPK4 itself interacted with six of them. Structurally, AtWLs were classified into a subfamily of AtCIPK, which generally interacts with calcineurin B-like proteins (CBL). This was also the case for AtWL1 and AtWL2, showing an efficient interaction with AtCBL2. In contrast, WPK4 interacted with none of the CBLs. In addition, to ascertain the possible interaction in vivo, expression of those genes was examined with a promoter-GUS assay. These results suggested that the interacting partner of SNF1-related protein kinases varies among plant species, and that, in the case of A. thaliana, it was CBLs, some of which were predicted to broadly regulate multiple CIPKs.

Key words: Arabidopsis thaliana; calcineurin B-like protein; protein kinase

Protein phosphorylation plays a crucial role in signal transduction of various stimuli, including biotic and abiotic stresses, and numerous responsive protein kinases have been identified within single organisms. For example, 860 genes encoding putative Ser/Thr protein kinases have been found in the Arabidopsis thaliana genome.9 While the regulation mechanisms are largely unknown, a group of regulatory proteins have been suggested to associate with them, acting on their kinase activity depending on various second messengers.

We previously identified a wheat protein kinase, WPK4, resembling the yeast protein kinase, SNF1 (sucrose non fermented 1), an enzyme that contributes to glucose repression in yeast.2 Further sequence comparison among plant protein kinases indicated that WPK4 could be classified into the SnRK3 (SNF1 related protein kinase 3) family of proteins featuring a kinase domain in the N-terminal region, and a putative regulatory domain in the C-terminal region.3 Similarity with SNF1 was only seen in the kinase domain, and not in the putative regulatory domain. Searching for proteins that interact with WPK4 by yeast two-hybrid screening, we identified the 14-3-3 proteins, TaWINI and TaWIN2.4 The 14-3-3 proteins generally interact with and regulate proteins involved in signal transduction pathways in a phosphorylation dependent manner.5 Since TaWIN1 binds to nitrate reductase phosphorlylated by WPK4, and since WPK4 transcripts are up-regulated by cytokinins and nutrient deprivation, an involvement in nitrogen metabolism was considered.

Over the past few years, several Ser/Thr protein kinases in A. thaliana have been shown to interact with calcium-binding proteins, corresponding to yeast calcineurin B.6-9 Accordingly, they were designated as AtCBL (Arabidopsis thaliana calcineurin B like) and the former as CIPK (CBL interacting protein kinase). The CIPKs could be classified into the SnRK3 family on the basis of their primary structure. However, since relationships in terms of physiological functions between authentic SNF1 and SnRK3 have not been clarified, we consider that CIPK is more suitable name rather than SnRK3 and has therefore been used in this study. In A. thaliana, 25 and 10 genes have been identified encoding CIPK (AtCIPK) and CBL (AtCBL) proteins, respectively.10 Although interactions between AtCIPKs and AtCBLs have mostly been defined by yeast two-hybrid assays, their physiological function...
has yet to be fully clarified. Notable exceptions are SOS2 (AtCIPK24) and SOS3 (AtCBL4), products of mutated genes in the sos (salt overly sensitive) mutants, sos2 and sos3, that show hypersensitivity to salt stress.\[1\] Genetic analyses strongly suggested that SOS2 and SOS3 interact with each other in vivo.\[2\] In addition, a Na+/H+ antiporter, SOS1,\[3\] was shown to be activated by SOS2 and SOS3 in yeast.\[4\] These studies indicated that the kinase activity of SOS2 is increased by SOS3 in a calcium-dependent manner. Similar regulation mechanisms could be postulated for other CIPK and CBL proteins. Although the nomenclature is somewhat confusing, some researchers using PKS (SOS2-like protein kinase) for CIPK, and ScaBP (SOS3-like calcium binding protein) for CBL, we use CIPK and CBL in this study.

We initially attempted to clarify whether A. thaliana contains CIPK interacting with A. thaliana 14-3-3 proteins as in the case of WPK4. We identified four AtCIPKs similar with WPK4, but interaction assays using a yeast two-hybrid system revealed none of them to interact with 14-3-3 proteins. Instead, we found that they efficiently interacted with AtCBLs, and subsequently therefore did experiments focusing on their mode of action.

**Materials and Methods**

*Plant materials and growth conditions. Abaidopsis thaliana* L. Heynh (ecotype Columbia) was grown on soil in a plastic tray at 21°C under long-day conditions (16-hr-light/8-hr-dark cycle) with a photon flux rate of 70 μmol m⁻² s⁻¹. For the analysis of light response, plants were incubated in the dark for 24 hr and then transferred to lighted conditions. After 24 hr, the shoots were harvested, frozen in liquid nitrogen, and stored at −80°C until use.

*Yeast two-hybrid assays. MATCHMAKER GAL4 Two-Hybrid System3 (Clontech) and HybriZAP-2.1 (Stratagene) were used for yeast two-hybrid assays. Vectors pGBK7 and pBD-GAL4 Cam carry the DNA binding domain, while pAD-GAL4-2.1 contains the activation domain. The coding region of AtSR1, amplified by PCR with primers containing restriction sites, was cloned in frame at the SalI site of pGBK7. The coding regions of AtSR2, AtWLI, AtW12, and AtW13, amplified by PCR with primers containing SalI and EcoRI restriction sites, were cloned into pGBK7. The coding region of AtWL4, amplified by PCR with primers containing restriction sites, was cloned in frame at SalI and PstI sites of pGBK7. The coding regions of At14-3-3psilon, At14-3-3kappa, At14-3-3lamba, At14-3-3nu, At14-3-3upsilon, At14-3-3phi, At14-3-3chi, At14-3-3psi, and At14-3-3omega, amplified by PCR with primers containing BamHI and XhoI restriction sites, were cloned into pAD-GAL4-2.1. The coding region of At14-3-3mu, amplified by PCR with primers containing restriction sites, was cloned in frame at BglII and XhoI sites of pAD-GAL4-2.1. The coding regions of AtCBL1, AtCBL2, AtCBL3, AtCBL4, AtCBL6, and AtCBL9, amplified by PCR with primers containing SalI and EcoRI restriction sites, were cloned into pAD-GAL4-2.1. The sequence of each construct was confirmed by DNA sequencing. Yeast strains Y109 or AH109 were transformed with these plasmids by the polyethylene glycol/lithium acetate method and transformants were selected on synthetic complete (SC) medium lacking Trp and Leu. Interaction assays were done on plates containing 0, 20, 40, or 60 mm 3-aminotriazole (3-AT) in SC medium without Trp, Leu, or His. β-Galactosidase activity was assayed in a 5-bromo-4-chloro-3-indolyl β-d-galactoside filterlifting assay. For quantitative assays, o-nitrophenyl β-d-galactopyranoside was used as a substrate and β-galactosidase activity (U) was calculated as $U = 1000 \times (\text{OD}_{420})/([\text{Time (min)}] \times \text{Vol (ml)} \times \text{OD}_{600})$.

**RNA-blot analysis.** Total RNA was extracted by the aurintricarboxylic acid method.\[5\] Five micrograms of total RNA per lane were separated on a 1% (w/v) agarose gel containing 2% (v/v) formaldehyde, capillary blotted onto a nylon membrane (Hybond, Amersham Pharmacia) with 20 × SSC and fixed with ultraviolet light radiation. A specific hybridization probe for AtCBL2 was prepared from a fragment containing the AtCBL2 3′-noncoding region (585–989 region of the full-length cDNA). Hybridization probes for AtSR1, AtSR2, AtWLI, AtW12, AtW13, and AtWL4 were prepared from the coding region of each gene. It was checked that these fragments do not cross-hybridize with each other (data not shown). The probes were labeled with [α-32P]dCTP using BcaBEST DNA labeling kit (Takara, Japan) and hybridization was done carried out at 42°C for 16 hr in a hybridization buffer containing 1 mm EDTA (pH 8.0), 50 mm Tris-HCl (pH 7.5), 3 × SSC, 1 × Denhardt’s solution, 0.5% (w/v) SDS, 10% (w/v) dextran sulfate, and 50% (v/v) formamide. After hybridization, membranes were rinsed once with 2 × SSC containing 0.5% (w/v) SDS, washed three times with 0.1 × SSC containing 0.1% (w/v) SDS at 65°C for 30 min, and used to expose X-ray films.

*Chimeric gene constructs and generation of transgenic Arabidopsis.* Chimeric gene constructs for GUS expression analyses were generated by PCR amplification of the 5′ regulatory regions using the primers (AtSR1-5′, 5′-AGAAGCTTCCATGAAA-CACGCATCAGAA-3′; AtSR1-3′, 5′-CCCTTAG-ACATTTAAAACAGAATCTGCT-3′; AtSR2-5′, 5′-AAGCTTTCAGAATAATGTGTTTTT-TAAC-3′; AtSR2-3′, 5′-GGATCCGCGCCGCGC-
GGAAGGAGAAAATGG-3'; AtWL1-5', 5'-AAGCTTGATATCAATGATGACACC-3'; AtWL1-3', 5'-GAATCCCATGATCGTGTAGAGCATAAGG-3'; AtWL2-5', 5'-AAACCTTCCTCTTCGTATCTTCTCTTCC-3'; AtWL2-3', 5'-GGATCCATGAATTTGATTTGCCTTGGA-3'; AtWL3-5', 5'-AAAGCTTAAAGAGAAATACATTCCAGCC-3'; AtWL3-3', 5'-GGATCCATTTGATTTGCCTTGGA-3'; AtWL4-5', 5'-AAAGCTTACCCCTCCCTCTACCAACGG-3'; AtWL4-3', 5'-GGATCCATTTGATTTGCCTTGGA-3';

Table 1. A Comparison List of Equivalent Genes Described in This Study

<table>
<thead>
<tr>
<th>This study</th>
<th>Albrecht et al. (2001)</th>
<th>Guo et al. (2001)</th>
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<tbody>
<tr>
<td>AtWL1</td>
<td>CIPK18</td>
<td>PKS22</td>
</tr>
<tr>
<td>AtWL2</td>
<td>CIPK13</td>
<td>PKS10</td>
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<td>AtSR2</td>
<td>CIPK7</td>
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Histological β-glucuronidase assays. Transgenic A. thaliana was grown on A1M plate (1% agarose in A1M) or rockwool at 21°C under long-day conditions (16-h-light/8-h-dark cycle) at a photon flux rate of 70 μmol m⁻² s⁻¹. Plants grown on rockwool were also supplied with A1M. Histological detection of β-glucuronidase activity was done by incubating plants in 50 mM NaPO₄ with 0.5% Triton X-100, 0.5 mM X-Gluc, 0.5 mM K₄Fe[CN]₆, 0.5 mM K₃Fe[CN]₆, and 20% methanol at 37°C for 12 h. The stained plants and tissues were fixed in 5% formaldehyde, rinsed in deionized water, and stored in 70% ethanol until they were photographed.

Immunoblotting analysis. Soluble proteins were made by homogenizing A. thaliana shoots in an extraction buffer (50 mM Tris-HCl, 100 mM NaCl, 0.05% Tween20, 1 mM EDTA, 1 mM PMSF, 5 μg/ml leupeptin), followed by centrifugation. The supernatant fractions were put through to SDS polyacrylamide gel electrophoresis, and the proteins were electroblotted onto a polyvinylidene difluoride membrane (Millipore). Immunoanalysis was done with a rabbit antibody raised against a polypeptide corresponding to AtCBL2 with a horse-radish peroxidase (HRP) linked anti-rabbit antibody as the second antibody. HRP activity on the membrane was detected with an ECL detection kit (Amersham Pharmacia).

Antisera against AtCBL2 were prepared as follows: The coding region of AtCBL2 attached to a thrombin digestion site at the 5' region was amplified by PCR, and inserted into a pDONR vector (Invitrogen) by recombinational cloning, then transferred into a pDEST15 vector (Invitrogen). Escherichia coli BL21 (DE3) was used as the host strain for production of GST fusion proteins. The recombinant proteins were affinity-purified from bacterial lysates with glutathion-Sepharose beads (Amershams Pharmacia). After digestion with thrombin and exclusion of GST, the AtCBL2 polypeptides were injected into a rabbit. Antisera were immunosorbed by GST-expressing bacterial lysates before use.

Results

Identification of Arabidopsis genes encoding proteins similar to WPK4

A homology search with the BLAST program detected four A. thaliana genes encoding proteins very similar to WPK4, these being designated as AtWL (Arabidopsis thaliana WPL4 like) 1 to 4. cDNA clones of the AtWL genes were isolated by RT-PCR with primers designed with information from the A. thaliana genome data base. After our isolation, they were also recorded under different names (AtCIPK and PKS) by two other groups. In order to avoid confusion, a comparison list of equivalent genes is shown in Table 1. AtWLs demonstrate high similarity with WPK4 throughout the entire protein, two serine residues in WPK4 important for interaction with 14-3-3 being conserved in all cases (Fig. 1A). One distinct difference in primary structure was the absence in AtWLs of the praline-rich region present in WPK4. Among 25 AtCIPKs examined, AtWLs showed greatest similarity to WPK4 (Fig. 1B).

Interaction of protein kinases with 14-3-3 and CBL proteins

Interactions between four AtWLs, ten A. thaliana 14-3-3s (At14-3-3s) and six A. thaliana CBLs (AtCBLs) of which cDNA clones had been isolated in a previous study, were analyzed in a yeast two-hybrid system. The combination of WPK4 and TaWIN1 was used as a positive control. Yeast cells harboring constructs for a fusion protein featuring the Gal4 DNA binding domain and a protein kinase (AtWLs or WPK4) and one with a Gal4 activation domain and a regulatory protein (At14-3-3s or AtCBLs) were assayed with two different reporters, His3 and β-galactosidase. For the His3 assay, yeast
Fig. 1. Amino Acid Sequences and Phylogenetic Tree of CIPK Proteins.

(A) Amino acid sequence alignment of AtWL proteins and WPK4. Highlighted are the amino acid residues conserved in more than three proteins included in the figure. The solid line under the sequence represents the proline-rich region of WPK4. The NAF domain (FISL motif) is double underlined. Conserved serine residues for interaction with 14-3-3 proteins are indicated by asterisks.

Phylogenetic tree of CIPK proteins. The dendrogram indicates relative evolutionary distances among the CIPK proteins and was generated using the UPGMA method. The bar indicates the genetic distance for 0.1 amino acid substitution/site.

Two reporter-assays essentially gave the same result, as summarized in Fig. 2. Combination of any AtWL and At14-3-3 did not indicate interaction, whereas AtWL1 and AtWL2 showed interactions with AtCBL2, 3, and 9, and AtCBL2 and 3, respec-
Expression profiles of AtWLs and AtCBL2 monitored by the GUS reporter gene

In order to examine tissue-specific localization of AtWL1, AtWL2, and AtCBL2, approximately 2-kb sections of the 5'-untranslated regions of these genes were fused with the β-glucuronidase (GUS) reporter gene and introduced into *A. thaliana*. The same experiments were also done with AtSR1 and AtSR2. GUS activity derived from the AtWL1 promoter was observed in the region between roots and hypocotyls (Fig. 5A), and AtWL2 in the upper ground parts and root tips (Fig. 5B). Subsequent analysis of GUS activity indicated that AtCBL2 was expressed in most cells of a seedling (Fig. 5C). AtSR1 was found predominantly in cotyledons (Fig. 5D) and AtSR2 mainly in the vascular tissue (Fig. 5E). It should be noted that the GUS activity for AtCBL2 and AtSR1 showed a similar pattern in flowers, with staining of...
all tissues except the petals and weak expression in pistils (Fig. 5H and I). However, the others showed different staining patterns, AtSR2 in filaments, bases of stigma, and flower bases (Fig. 5J), AtWL1 in stigmas, anthers, and weakly in pistils (Fig. 5F), and AtWL2 in anthers (Fig. 5G).

Expression profiles of AtSR1 and AtCBL2 in response to light
We previously reported that transcripts for AtSR1 and AtCBL2 increased in response to light in detached leaves.9 In this study, we confirmed this light response by RNA blot analysis using intact plants. As shown in Fig. 6, transcripts for both AtCBL2 and AtSR1 accumulated in response to light, as observed in detached leaves.9

However, the transcript levels of AtSR2, AtWL1, and AtWL2 did not change upon illumination (data not shown). A light response of AtCBL2 was also observed at the protein level. Figure 7 shows that the level of AtCBL2 proteins decreased in the dark and increased in the light. These results indicated that AtCBL2 was apparently regulated by light at both transcript and protein levels.

Discussion
One main objective of this study was to clarify whether A. thaliana contains WPK4-homologs that interact with 14-3-3 proteins. As the initial step, we identified four A. thaliana genes, AtWL1 through AtWL4, encoding proteins very similar to WPK4, except for the absence of a praline-rich region. Since the primary structure of the regulatory domain, through which TaWINs bind to WPK4, was found to be highly conserved in AtWLs, we speculated that
interaction with 14-3-3 proteins would occur. However, this was not the case under our experimental conditions (Fig. 2). On the other hand, WPK4 efficiently interacted with six At14-3-3s, including nu, upsilon, phi, chi, and omega forms indicating a conservation of structures necessary for interaction with WPK4. One possible explanation for the findings is that the proline rich region, absent in AtWLs, is critical for binding to 14-3-3 proteins. It is also present in WPK4 homologs of rice, maize, and sorghum, but not in those of A. thaliana. Although a question remains as to whether results from the yeast two-hybrid assay truly reflect in vivo interactions, it is tempting to speculate that the interaction partners of WPK4 family proteins are distinct among plant families.

Accordingly, we searched for proteins interacting with AtWLs. A clue was available from the fact that protein kinases similar to WPK4 in A. thaliana were reported to interact with CBL proteins and designated as AtCIPK. This led us to examine whether AtWLs interacted with AtCBL proteins. The results clearly showed interaction of AtWLs (AtWL1 and AtWL2) with AtCBLs, being consistent with the presence of amino acids postulated to be sufficient for interaction with AtCBL (FISL motif or NAF domain) in AtWLs (Fig. 1A). Although interaction between AtWL4 and AtCBL3 was also reported by Guo et al., the interaction could not be detected in our system. The reason for this difference is currently not clear. However, we could not detect any interaction between WPK4, in which these amino acids were also conserved, and AtCBLs tested in this study. This suggested that the interaction might not be determined by such a domain alone. Kim et al. also reported that a domain located between sub-domains 9 and 11 of the CIPK5 kinase domain inhibits the interaction with AtCBLs. Thus we considered that AtWLs are likely to interact with AtCBLs, and that, although WPK4 and AtWLs share high homology in their primary structure, they might interact with different regulatory proteins, probably due to a small change in their primary structure. The possibility that kinase domain of WPK4 inhibits interaction of the C terminal domain and AtCBLs, also can not be ruled out, suggesting interaction of WPK4 with CBLs in vivo under specific conditions.

Base on this conclusion, we further investigated the mode of interaction between AtWLs and AtCBL2. Since we previously reported that AtCBL2 also specifically interacted with AtSR1, related AtCIPKs were also examined. Among six of these proteins, prominent activation of the reporter gene was produced with AtSR1 (Fig. 4). Since the specificity determined by a yeast two-hybrid reflected interaction observed in vivo for SOS2 and SOS3, the result suggested possible interaction of AtSR1 and AtCBL2 in vivo. This does not rule out the possibility of in vivo interaction between AtCBL2 and other AtCIPK proteins.

It is generally considered that, if two proteins interact in vivo, they are supposed to localize to the same cell. Consequently, expression profiles of AtCBL2 and four AtCIPKs that interacted with AtCBL2 were done. Previously we reported that transcripts of AtCBL2, AtSR1, and AtSR2 were detected in most organs of intact plants. In order to postulate more accurate localization of AtCBL2 and the four positively interacting AtCIPKs, their expression profile was examined. Histochemical analysis of GUS activity indicated that expression of AtCBL2 was relatively uniform in seedlings, while those of the four AtCIPKs were rather tissue-specific (Fig. 5). The idea that AtCBL2 might interact with any of the four AtCIPKs in different cells is supported by the presence of 25 CIPKs and 10 CBLs in the A. thaliana genome, suggesting that one AtCBL may interact with multiple AtCIPKs. To our knowledge, our present work is the first report simultaneously analyzing cell-specific expression of both genes of which interaction was ascertained.

Since SOS3 was shown to regulate SOS2 in a calcium-dependent manner, it is conceivable that AtCBL2 possibly regulated these AtCIPKs in a similar way. However, in contrast to the SOS system, stimuli located upstream of AtCBL2 and AtCIPKs are currently not known. A clue may be available from expression profiles. AtSR1 is the only AtCIPK for which transcripts increased upon light illumination within our analysis (Fig. 6). Among AtCBLs, both AtCBL2 and AtCBL1 were shown to respond to light, although AtCBL1 did not interact with AtSR1. Such a simultaneous response of AtSR1 and AtCBL2 to light suggests that they might function in some physiological phenomenon that occurs after light exposure. Since AtCBL2 protein in leaves did not completely disappear after incubation in the dark for 24 h, AtCBL2 may be directly involved in transduction of light signals and increase upon exposure to light. Since, however, their transcripts are observed in roots, the function of AtCBL2 and AtSR1 is not likely limited to transduction of light signals.

Expression of AtCBL1 regulated by various stresses suggested its involvement in transduction of multiple signals. Similarly, it can be postulated that AtCBL2 and other AtCBLs could be involved in transduction of various signals interacting with more than one CIPK. Further characterization of each CBL and CIPK will be needed to understand the physiological functions of this family.

Acknowledgments

This work was supported by grants for Research for the Future Program from the Japan Society for
the Promotion of Science (JSPS-RFTF00L01604). The authors thank Drs. Y. Yamaguchi (Nara Institute of Science and Technology) and M. Moore (Internal, Nagoya) for valuable discussion and critical reading of the manuscript, respectively.

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