Members of the Plant CRK-superfamily are capable of trans-/auto-phosphorylation of tyrosine residues.

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*Running title: Plant CRKs Function as PKs for Tyr Phosphorylation

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Background: Protein kinases that catalyze Tyr-phosphorylation in plants in vivo are largely unknown.

Results: CRKs that auto/trans-phosphorylate Tyr residues and six substrates of these were identified. CRK knockout mutants show reduced Tyr-phosphorylation of beta-tubulin proteins.

Conclusions: CRKs can phosphorylate Tyr residues of beta-tubulin and certain transcription factors.

Significance: CRKs might be responsible for much of the protein Tyr-phosphorylation in vivo.

ABSTRACT

Protein phosphorylation on Tyr residues is a key post-translational modification in mammals. In plants, recent studies have identified Tyr-specific protein phosphatase and Tyr-phosphorylated proteins in Arabidopsis by phosphoproteomic screenings, implying that plants have a Tyr phosphorylation signal pathway. However, little is known about the protein kinases (PKs) involved in Tyr phosphorylation in plants. Here, we demonstrate that Arabidopsis calcium-dependent protein kinase (CDPK/CPK)-related PKs (CRKs) have high Tyr autophosphorylation activity and that they can phosphorylate Tyr residue(s) on substrate proteins in Arabidopsis. In order to identify PKs for Tyr phosphorylation, we examined the autophosphorylation activity of 759 PKs using an Arabidopsis protein array based on a wheat cell-free system. In total, we identified 38 PKs with Tyr autophosphorylation activity. The CRK family was a major protein family identified. A cell-free substrate screening revealed that these CRKs phosphorylate beta-tubulin (TBB) 2, TBB7 and certain transcription factors (TFs) such as ethylene response factor 13 (ERF13). All five CRKs tested showed Tyr auto/trans-phosphorylation activity and especially two CRKs, CRK2 and CRK3, showed a high ERF13 Tyr phosphorylation activity. A cell-based transient expression assay revealed that Tyr16/207 sites in ERF13 were phosphorylated by CRK3 and that Tyr phosphorylation of endogenous TBBS occurs in CRK2 overexpressing cells. Furthermore, crk2 and crk3 mutants showed a decrease in the Tyr phosphorylation level of TBBS. These results suggest that CRKs have Tyr kinase activity, and these might be one of the major PKs responsible for protein Tyr-phosphorylation in Arabidopsis plants.

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Protein phosphorylation of serine (Ser), threonine (Thr), and tyrosine (Tyr) residues is a key post-translational modification required for signal transduction in eukaryotes. In animals, protein Tyr kinases (PTKs) play a central role in many signaling pathways, including hormone response, differentiation, development, and cancer formation (1). In contrast, it is unclear whether Tyr phosphorylation signaling cascades exist in plants, because no PTK homologous genes have been reported in Arabidopsis and rice genomes (2,3).

Recently, Tyr-specific protein phosphatase (PTP1) (4) and more than a thousand Tyr-phosphorylated proteins have been identified by proteomics analysis (5,6) in plants. A previous report suggests that not only PTP1 but also dual-specific (pSer/pThr and pTyr) phosphatases involve in abiotic stress and hormone signaling are present in plants (7). On the other hand, a phosphoproteomic approach revealed that approximately 4% of phosphopeptides are Tyr-phosphorylated peptides in plants and the proportion of Tyr phosphorylation is equivalent to that found in human cells (5). In addition, meta-analysis of phosphoproteomic data revealed that Tyr-phosphorylated peptides are overrepresented in mitochondrial and characterized two Tyr phosphorylation motifs (6).

Other studies have demonstrated that PTK and PTP inhibitor application to plants alter abscisic acid (8), gibberellin (9), cold stress (10), and sugar responses (11), as well as cytoskeleton organization (12) and cell division (13). These findings strongly suggest that plants possess Tyr phosphorylation signaling pathways. According to recent studies, Tyr residues (as well Ser and Thr) of several dual-specific (Ser/Thr/Tyr) type or Ser/Thr specific type PKs, e.g., receptor-like/receptor-like cytoplasmic PKs (RLK/RLCKs), CDPKs, glycogen synthase kinase 3 (GSK3)/Shaggy-like PKs and MAPK, have been observed to autophosphorylate (14–16). These PKs probably participate in the Tyr phosphorylation signaling in plants. However, the molecular mechanisms of signaling pathways that are controlled by Tyr-phosphorylation remain to be clarified because the substrate proteins for Tyr phosphorylation have not been identified.

In order to understand the Tyr phosphorylation signaling, it is important to identify PKs and their substrate protein for Tyr phosphorylation. Although PKs are one of the largest gene families, representing ~4% (more than 1,000) of all the genes in Arabidopsis and rice, the biochemical characteristics of most plant PKs are unclear. In our previous study, we demonstrated the Ser/Thr autophosphorylation activity using a high-throughput profiling method combining the Arabidopsis 759 PKs array that was produced using a wheat cell-free system, and a luminescent method ‘AlphaScreen’ (17). Here, by modifying this approach, we have identified and characterized 38 Tyr autophosphorylation PKs. Among them, we focused on angiosperm-specific CRK2, CRK3, and CRK8, and screened for the substrate protein for Tyr phosphorylation using a TFs protein array (18) and a pull-down assay using cellular extracts. We identified 6 substrate proteins in total. Cell-based transient expression assay and analysis of crk2 and crk3 mutants revealed that CRK2 and CRK3 were able to phosphorylate Tyr residue(s) of substrate proteins such as TBBs or ERF13 in cells or plants. In addition, the five proteins tested from the CRK family showed Tyr auto/trans-phosphorylation activity in vitro. These findings suggest that CRK proteins possibly function as PKs for Tyr phosphorylation in plants.

**EXPERIMENTAL PROCEDURES**

**General** The following procedures were previously described (19–22): wheat cell-free protein production, split-primer PCR for construction of the DNA templates, parallel syntheses of mRNAs and their translated proteins, protein biotinylation, purification of synthesized proteins, hydrolysis of radioisotope-labeled protein, and quantification of proteins synthesized using densitometer scans of Coomassie brilliant blue (CBB) stained proteins or of radiolabeled proteins.

**Analysis of protein kinase autophosphorylation using a Luminescent Method** In vitro autophosphorylation assays were carried out as previously described with slight modifications (17). Ser/Thr or Tyr autophosphorylation was detected by anti-phospho Ser/Thr (Upstate Biotechnology, Lake Placid, NY, USA) or anti-phospho Tyr antibody (4G10) (Millipore), respectively. All data represent the average of two independent experiments and the background for each experiment was controlled using the relevant
non-biotinylated PK. For in vitro dephosphorylation assays, autophosphorylated biotinylated-PKs were incubated with crude FLAG-PTP1 at 26 °C for 60 min. Tyr autophosphorylation was detected by anti-phospho Tyr antibody (4G10). All data represent the average of three independent experiments, and dephosphorylation efficiency was defined as the ratio of PTP1-treated kinases to untreated ones.

**Phosphoamino acid analysis** - The phosphoamino acid analysis was performed according to a previous method (23). Biotinylated PKs were purified using Streptavidin MagneSphere® Paramagnetic Particles (Promega), and incubated at 30 °C for 30 min in a total volume of 50 µL consisting of 50 mM Tris-HCl (pH 7.6), 37 kBq of [γ-32P] ATP, 100 mM potassium acetate, 10 mM MgCl2, and 1 mM DTT. Then, biotinylated PKs were hydrolyzed using 6 N HCl at 110 °C for 4 h. After drying the product, each amino acid was separated by thin-layer chromatography (TLC) using ethanol:ammonium hydroxide:water at a ratio of 105:42:6 (v/v) and each 32P-labeled-amino acid was detected by autoradiography.

In vitro phosphorylation and dephosphorylation assays - For in vitro kinase assays, biotinylated proteins and FLAG-tagged proteins were purified using Streptavidin MagneSphere® Paramagnetic Particles and anti-FLAG M2 agarose (Sigma) respectively. Then, in vitro kinase assays were carried out in a total volume of 50 µL consisting of 50 mM Tris-HCl (pH 7.6), 100 mM potassium acetate, 10 mM MgCl2, 1 mM DTT, and 100 µM ATP or 37 kBq of [γ-32P] ATP at 26 °C for 30–60 min. Dephosphorylation by AtPTP1 was carried out in a total volume of 50 µL consisting of 50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 2 mM DTT, 0.01% Brij 35, and 50 ng purified FLAG-AtPTP1 at 26 °C for 30–60 min. For immunoblotting, anti-phospho Tyr antibody (4G10) was used to detect phospho-tyrosine. Biotinylated proteins were detected with Alexa Fluor 647 Streptavidin conjugate (Invitrogen). The chemiluminescent signal, fluorescent signals, and filmless autoradiography were detected using an ImageQuant LAS-4000 mini biomolecular imager (GE Healthcare), a Typhoon 9400 imager (GE Healthcare), respectively.

In-Gel Protein Digestion - CBB-stained protein bands were excised from the SDS-PAGE gels and further destained using 50% (v/v) acetonitrile in 100 mM ammonium bicarbonate (pH 8.9). Destained gel pieces in 30 µL of 100 mM ammonium bicarbonate (pH 8.9) were reduced by adding 10 µL of 40 mM dithiothreitol for 2 h at 37 °C and alkylated by adding 10 µL of 250 mM acrylamide for 30 min at room temperature. In-gel digestion of each protein was performed with 0.1 µg of sequencing-grade modified trypsin or chymotrypsin (Promega, Madison, WI, USA) at 37 °C for 12 h. After repeated extractions of tryptic digests from the gel with 50% (v/v) acetonitrile/5% (v/v) trifluoroacetic acid, the solution containing the extracted peptides was concentrated using a vacuum centrifuge. The peptide sample was reconstituted with 0.1% (v/v) trifluoroacetic acid for mass spectrometry (MS) analysis.

Liquid chromatography-mass spectrometry analysis - Tandem MS (MS/MS) analysis was performed using the LTQ XL linear ion trap mass spectrometer (Thermo Fisher Scientific) coupled with DiNa nano LC system (KYA Technologies). Peptide separations were performed at a constant flow rate of 300 nL/min with a fused silica capillary column packed with C18 resin (75 µm × 15 cm). Mobile phases used for separation were 0.1% formic acid (A) and 80% acetonitrile with 0.1% formic acid (B). A gradient (2–50% mobile phase B) was applied for 25 min, followed by a 10-min wash at 100% mobile phase B, and a equilibration for 15 min with 2% mobile phase B. For the identification of autophosphorylation sites, MS/MS spectra were processed using the Proteome Discoverer software version 1.1 (Thermo Fisher Scientific). Peptide identification was performed using the SEQUEST search algorithm with the following parameters: two missed cleavages allowed; precursor mass tolerance, 2 Da; fragment mass tolerance, 0.8 Da; static modification, propionamide (cysteine); dynamic modifications, phosphorylation (serine, threonine, and tyrosine), methionine oxidation, and pyroglutamic acid. For the identification of substrate proteins, the acquired MS/MS spectra
were searched against the IPI Arabidopsis database (ipi.ARATH v.3.85) using the SEQUEST software and we required at least one high-quality peptide for positive identification (peptide probability score ≥ 10).

**Construction of transient expression plasmids for cultured cells**- Full-length cDNAs of CRK2, CRK3, and ERF13 were cloned into pDONR221 vectors via Gateway reactions. After the clone sequence was confirmed, C-terminal HA-tagged form and kinase-dead (KD) form mutants, CRK2 (Lys 176 to Arg) and CRK3 (Lys 175 to Arg), were generated using a PrimeSTAR Mutagenesis Basal kit (Takara Bio) according to the manufacturer's instructions. Fragments containing the gene coding sequence and the HA-tag were subcloned into p35SΩ-GW-NOST vectors (which we generated from the 35SΩ-sGFP vector (24), unpublished) using LR clonase reaction (Invitrogen). We produced expression vectors for YFP or GFP fusion proteins by LR clonase recombination of CRK2 or CRK3 and p35SΩ-GW-YFP-NOST vectors, and ERF13 and p35SΩ-GW-GFP-NOST vectors.

**Tyr phosphorylation analysis of substrate proteins and subcellular localization analysis by transient expression**- Isolation of Arabidopsis suspension-cultured cell protoplasts and polyethylene glycol-mediated DNA transfection were performed as previously described (25). For Tyr phosphorylation analysis of ERF13-GFP, 100 μg plasmid DNA of ERF13-GFP and 40 μg plasmid DNA of CRK2-HA or CRK3-HA were transfected into 2 × 10⁶ protoplasts. After overnight incubation in the dark, crude extracts were obtained in cell lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% TritonX-100, phosphatase inhibitor cocktail, and Protease Inhibitor Cocktail]. Immunoprecipitation of endogenous TBBs was performed with 50 μL Protein G Sepharose and 5 μL mouse polyclonal anti-TBB antibody (TUB2.1) (Sigma), and aliquots were analyzed by Phos-Tag SDS-PAGE (100 μM Phos-Tag and 100 μM MnCl₂) and immunoblot analysis using mouse polyclonal anti-TBB antibody or anti-phospho Tyr antibody (4G10). Expression of CRK2-HA and CRK3-HA was detected by anti-HA-HRP antibody (3F10) (Roche Applied Science). For subcellular localization analysis, 10 µg plasmid DNA was transfected to 100 µL of 2 × 10⁴ protoplasts. YFP and GFP fluorescence was observed with a confocal laser-scanning microscope LSM5 PASCAL (Zeiss).

**Identification of Tyr-phosphorylated proteins from extracts of Arabidopsis cultured cells**- For substrate screening by co-immunoprecipitation, biotinylated CRK3 was attached to Streptavidin MagneSphere® Paramagnetic Particles (Promega). After washing, CRK3 was incubated with Arabidopsis suspension-cultured cells lysate in immunoprecipitation buffer (25 mM Tris-HCl, 150 mM NaCl, 0.5% TritonX-100, Phosphatase inhibitor cocktail, and Protease Inhibitor Cocktail) at 26 °C for 60 min. Following the above reaction, the beads were washed 4 times with immunoprecipitation buffer and, then, boiled in the sample buffer. The precipitated interacting proteins were analyzed by immunoblotting with anti-phospho-Tyr antibody, CBB stain, and MS analysis.

**Identification of Tyr-phosphorylated proteins using protein library of Arabidopsis TFs**- For substrate screening using AlphaScreen, 188 TFs were selected from the Arabidopsis TF library previously described (18). In vitro protein-protein interaction (PPI) assays were carried out in a total volume of 15 μL consisting of 100 mM Tris-HCl (pH8.0), 0.1% Tween20, 1 mg/ml BSA, 1 μL biotinylated TFs, and FLAG-PKS at 25 °C for 1 h in a 384-well Optiplate (PerkinElmer). In accordance with the AlphaScreen IgG (ProteinA) detection kit (PerkinElmer) instruction manual, 10 μL of detection mixture containing 100 mM
Tris-HCl (pH 8.0), 0.1% Tween20, 1 mg/mL BSA, 5 μg/mL anti-FLAG M2 antibody (Sigma), 0.1 μL streptavidin-coated donor beads, and 0.1 μL Protein A-coated acceptor beads was added to each well of the 384-well Optiplate, followed by incubation at 25 °C for 1 h. Luminescence was analyzed using the AlphaScreen detection program. All data represent the average of two independent experiments and the background was controlled using a dihydrofolate reductase (DHFR) from E. coli.

**Mutational analysis**- Mutagenesis was carried out using a PrimeSTAR Mutagenesis Basal kit (Takara Bio) according to the manufacturer's instructions. The mutated genes were sequenced using an ABI PRISM 310 DNA sequencer (Applied-Biosystems).

**T-DNA-tagged line analysis**- T-DNA insertion mutant lines for crk2 and crk3 were obtained from the Arabidopsis Biological Resource Center (http://www.biosci.ohio-state.edu/pemb/Facilities/abrc/abrchome.htm). The crk2 (Salk_090938C) and crk3 (Salk_128719C) mutants are in the Columbia-0 (Col-0) background. Wild-type Col-0 and mutants seeds were surface sterilized, rinsed with sterile water, and stratified at 4 °C for at least 2 days. Then, seeds were germinated and grown on 1/2 Murashige and Skoog agar plates (half-strength Murashige and Skoog salts, 1% sucrose, 0.8% agar, and pH 5.7) in a growth cabinet at 22 °C under a 16 h light/8 h dark photoperiod. For Real-Time RT-PCR analysis, 5-day-old seedlings were homogenized, and total RNA was extracted using RNeasy Plant mini kit (Qiagen). The cDNA was synthesized from 500 ng of total RNA with a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) according to the manufacturer's instructions. The Taqman-based real-time RT-PCR analysis was carried out in a real-time LightCycler 480 PCR system (Roche Applied Science) using LightCycler 480 Probes Master (Roche Applied Science) and Universal ProbeLibrary (Roche Applied Science), and using ACTIN4 (At5g59370) (forward primer 5’-TACGGCTTTGGCTCAAGTA-3’; reverse primer 5’-CTGCCCTCGCAATCCACAT-3’; probe No. 55) as the control gene. We used the following primer pairs and Universal ProbeLibrary selected by Assay Design Center program (https://qpcr.probefinder.com/organism.jsp): CRK1 (At2g41140) (forward primer 5’-CGTGACCTTAAACCGAGAAC-3’; reverse primer 5’-GTTGCACATAGTCAGAAGACCCA-3’; probe No. 82), CRK2 (At3g19100) (forward primer 5’-TATGATGCGTCGAGGACCA-3’; reverse primer 5’-AGAGTATTCTCCCTCGTCT-3’; probe No. 10), CRK3 (At2g46700) (forward primer 5’-ATGTCAGGACCCAATAACG-3’; reverse primer 5’-GGCTATTACCCAACCTGCTG-3’; probe No. 22), CRK4 (At5g24430) (forward primer 5’-TGAGGATGCTGATAATGTT-3’; reverse primer 5’-CAGGGTGATCGCCACCTCT-3’; probe No. 39), CRK5 (At3g50530) (forward primer 5’-TTCCCTCATTTCTGATGCTTATGA-3’; reverse primer 5’-GTGTAATCTGCCACCTCTTGA-3’; probe No. 63), CRK6 (At3g49370) (forward primer 5’-GATTCCGATAATGTTTGGTG-3’; reverse primer 5’-GACCACCTTCGCAAAATA-3’; probe No. 39), CRK7 (At3g56760) (forward primer 5’-TGCTCTGCTAAGGGAAAGA-3’; reverse primer 5’-TGCAATGCGATGGCTATCT-3’; probe No. 5), CRK8 (At1g49580) (forward primer 5’-CCCAGAGGTTCGCTTCTTG-3’; reverse primer 5’-AGCTGCAACAAAACCTCTTAAAA-3’; probe No. 6).

For Tyr phosphorylation analysis of endogenous TBBs, 14-days-old seedlings were homogenized in the cell lysis buffer and cleared by centrifugation at 12,000 g for 10 min. Immunoprecipitation, Phos-Tag SDS-PAGE, and immunoblot analysis were carried out as described above.

**RESULTS**

**Identification of Tyr autophosphorylation protein kinases using a wheat cell-free protein array**- Autophosphorylation of PKs is an important aspect of regulatory systems such as activation or inactivation by autophosphorylation-dependent conformational change (26). Many biochemical analyses demonstrate that almost all PKs catalyze intra/inter-molecular autophosphorylation
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reactions (27). Actually, in our previous report, many Arabidopsis PKs showed the Ser/Thr autophosphorylation activity (17). These findings prompt us to propose that Tyr-autophosphorylation activity might be a useful clue to identify plant PK(s) for Tyr phosphorylation. In order to search for Arabidopsis PKs for Tyr phosphorylation, we therefore profiled Tyr autophosphorylation activity using a wheat cell-free based luminescent method with anti-phospho Tyr (pTyr) antibody in this study (Fig. 1A). Since our previous data indicated that more than half of the PKs that showed 4 times stronger signal than background one had the high Ser/Thr autophosphorylation activity (17). Thus, we concluded that these PKs are more likely to have Tyr autophosphorylation activity. Based on this threshold, 89 out of 759 PKs were considered candidates for Tyr autophosphorylation activity by autophosphorylation profiling (supplemental Table S1). Furthermore, to further enrich PKs having the high Tyr autophosphorylation activity, profiling data of the Tyr autophosphorylation activity was compared with our previous Ser/Thr autophosphorylation data (Fig. 1B). Finally, we selected 38 Tyr autophosphorylation PK candidates that showed 4-fold stronger luminescence with anti-pTyr antibody than with anti-pSer/pThr antibody (pTyr/pSer-pThr) (Table 1). However, we cannot be concluded that these PKs have high Tyr autophosphorylation activity based on the antibody detection alone as the signal value is correlated to the number of phosphorylated molecules and phosphorylation site. Furthermore, the effects of the three-dimensional structure of the PK on the accessibility and specificity of the antibodies cannot be discounted. Unfortunately, the affinity and specificity of currently available anti-pSer/pThr antibodies are lower than those of anti-pTyr antibodies. Thus, currently available anti-pSer/pThr antibodies are almost certainly unable to solve these problems because of their low specificity. Nevertheless, at present, an approach to compare the luminescent signal would be probably effective as an indicator for high-throughput autophosphorylation profiling of PKs.

In order to confirm Tyr autophosphorylation, we performed phosphoamino acid analysis and dephosphorylation assays. By TLC, 22 of the 25 tested PKs showed phosphorylation of the Tyr residue (Fig. 1C). A phospho-Tyr residue was difficult to detect in the other 3 PKs (PK_B11, C05, and C06 in Fig. 1C). This might be due to the differences in PK kinetic properties. Another explanation for this might be that they are not able to autophosphorylate further because the autophosphorylation levels of these PKs had been saturated during the in vitro translation. However, the Tyr autophosphorylation signal of all 38 PKs as observed to decrease in by incubation with AtPTP1 (Fig.1D). These results suggest that these 38 PKs have Tyr autophosphorylation activity (Table 1). Next, we randomly selected PK_A09, B01, B08, B12, C02, and D01, and tested whether these PKs have re-autophosphorylation activity. Expectedly, all tested PKs showed activity after dephosphorylation by treatment with lambda protein phosphatase (Fig. 1E). In addition, we randomly selected PK_A01, A11, B01, B07, B10, and D01, and generated KD proteins by mutating conserved Lys residues in the ATP binding sites to Arg. All the mutated PKs failed to react with anti-pTyr antibody (Fig. 1F). Taken together, these results suggest that Tyr phosphorylation in PKs is due to the kinase activity itself. According to the PlantsP classification (http://plantsp.genomics.purdue.edu/), these 38 PKs are mainly classified into five groups: GSK3/Shaggy-like PK (8 clones), MAPK (6 clones), RLK/RLCK (8 clones), casein kinase I/casein kinase I-like (CK1/CKL) (4 clones), and CRK (3 clones). Because the Tyr autophosphorylation activity of some of the PKs classified within the GSK3/Shaggy-like PK, MAPK, RLK/RLCK, and CK1/CKL families has been identified in previous studies (14,28), suggesting that this approach is able to find PKs having the Tyr autophosphorylation activity.

On the other hand, the CRK family, which was classified as part of the Ser/Thr-type CDPK-sucrose non-fermenting-1-related PK superfamily (29), has not been previously characterized. In the profiling data, three CRKs showed Tyr autophosphorylation activity. This finding supports the suggestion that CRKs probably has Tyr autophosphorylation activity and we focused on the analysis of the biochemical properties of CRKs. In order to confirm the Tyr autophosphorylation sites of CRK3, we predicted phosphorylation sites using a web page based
program (PhosPhAt; http://phosphat. uni-hohenheim.de/index.html, NetPhos 2.0; http://www. cbs. dtu. dk/services/NetPhos/).

Following this, the predicted Tyr559 residue sites were changed to Phe and the Tyr559-Phe (Y559F) mutant was used for in vitro kinase assays. Immunoblot analysis showed that autophosphorylation was decreased in CRK3-Y559F (Fig. 1G). Similarly to immunoblotting, luminescent analysis showed that the Tyr autophosphorylation signal of CRK3-Y559F decreased by 80% in comparison with that of the wild type (Fig. 1H). The CRK3 autophosphorylation site was also analyzed by MS analysis and phospho-Tyr559 was detected (supplemental Table S2).

Substrate screening of CRK3 by pull-down assay based on a wheat cell-free system- In order to better understand the biochemical properties of CRKs, we selected CRK3 and explored the substrate proteins which Tyr residue(s) are phosphorylated by CRK3. Protein extracts from Arabidopsis cultured cells were used for pull-down assays with recombinant biotin-labeled CRK3 and anti-pTyr antibody was used to detect Tyr phosphorylation. Some Tyr-phosphorylated proteins interacting with CRK3 were detected (Fig. 2A) and, subsequently, CBB-stained bands of ~50 kDa molecular weight showing Tyr phosphorylation were analyzed using MS. These proteins were identified TBB2, TBB7, and TBB9 (supplemental Table S3).

In order to confirm whether the TBBs are substrate proteins of CRK3 for Tyr phosphorylation, these three TBB proteins were used for in vitro kinase assays. We also tested whether CRK2 and CRK8 could phosphorylate these three TBB proteins because CRK2 and CRK8 also showed Tyr autophosphorylation activity. Our results show that the three CRK proteins tested can phosphorylate TBB2 and TBB7 at the Tyr residue but not TBB9 (Fig. 2B). Comparison of amino acid sequences showed that TBB2 and TBB7 have an extra C-terminal region including Tyr residue(s) (Fig. 2C), whereas TBB9 is missing this region. By mutation of the Tyr residue to Phe (Y443F or Y449F), Tyr phosphorylation of TBB7 was decreased (Fig. 2D), suggesting that the two Tyr residues are major phosphorylation sites for CRK2, CRK3, and CRK8. Interestingly, Tyr phosphorylation of beta-tubulins in Arabidopsis and tobacco plants has already been reported (12,30,31). Our finding suggests that the three CRKs are the responsible PKs for Tyr phosphorylation of beta-tubulins.

Substrate screening of CRK2 and CRK3 by PPI assay using TFs array- We found only two TBB substrate proteins by pull-down assays using cell extracts. Thus, we used a protein array of Arabidopsis TFs previously described (18) to explore more substrate proteins phosphorylated at the Tyr residue(s) by CRK2 and CRK3. From the 647 TFs in the protein array, we selected 188 TFs characterized in previously published papers (supplemental Table S4). In order to identify substrate proteins for Tyr phosphorylation, we adopted a two-step screening procedure: 1) PPI between CRK2 or CRK3, and TFs using a luminescent method (Fig. 3A and supplemental Table S4), and 2) in vitro Tyr-phosphorylation assay using the top 20 proteins that showed the strongest PPI assay signals. After this double screening, four TFs, ethylene response factor 13 (ERF13) (At2g44840), WRKY DNA-binding protein 14 (WRKY14) (At1g30650), ERF subfamily B-4 of ERF/AP2 transcription factor 2.6 (RAP2.6) (At1g43160), and cryptochrome-interacting basic-helix-loop-helix 5 (CIB5) (At1g26260), were found to be Tyr phosphorylation substrates of CRK2 or CRK3 (Fig. 3B and Table 2). Since ERF13 and WRKY14 were phosphorylated by both CRK2 and CRK3, they were used for further analysis. Tyr-phosphorylation of the two substrates was not detected when KD forms of CRK2 and CRK3 were used, indicating that ERF13 and WRKY14 were phosphorylated by CRK2 and CRK3 in an activity-dependent manner (Fig. 3C). In summary, we identified a total of six substrate proteins Tyr-phosphorylated in vitro by CRK2, CRK3, and CRK8 by using pull-down and PPI assays (Table 2).

CRK family has Tyr phosphorylation activity- Next, we analyzed the biochemical properties of CRK2 and CRK3 by using ERF13 as a substrate. First, we analyzed whether CRK2 and CRK3 phosphorylate not only Tyr but also Ser/Thr residues. AtPTP1 is known to be a Tyr-specific
protein phosphatase (4,23). A dephosphorylation assay revealed that both CRK2-dependent and CRK3-dependent phosphorylations of ERF13 dramatically decreased upon treatment with AtPTP1 (Fig. 3D). Furthermore, AtPTP1 significantly dephosphorylated even radioisotopically CRK-phosphorylated ERF13 proteins (Fig. 3E). In another approach, Tyr residue(s) were mutated to Phe (Fig. 3F). A significant decrease in Tyr phosphorylation was observed in Tyr16-Phe (Y16F) and Tyr207-Phe (Y207F) mutants (Fig. 3F), while the Tyr16/207-Phe (Y16/207F) double mutation completely lost Tyr phosphorylation (Fig. 3G). These mutation analyses revealed that Tyr16 and Tyr207 residues are the main phosphorylation sites in ERF13.

The Arabidopsis genome contains eight CRK genes (Fig. 3H). To test the activity of other CRKs, we synthesized five CRKs and analyzed their Tyr autophosphorylation and trans-phosphorylation activity. All CRKs exhibited Tyr autophosphorylation activity, and particularly CRK2, CRK3, and CRK4 showed high activity (Fig. 3I). In addition, five CRKs could phosphorylate ERF13 (Fig. 3I), even though the degree of Tyr ERF13 phosphorylation differed. CRK2 and CRK3 showed the highest kinase activity, using ERF13 protein as a substrate, when compared with the other CRKs. Furthermore, we searched for the CRK3 orthologous gene in soybean and found Glyma07g05750 (74% similarity to AtCRK3), which we named GmCRK3 (Fig. 3H). The cDNA was cloned from soybean seedlings and GmCRK3 protein was synthesized using the cell-free system. In vitro kinase assay revealed that GmCRK3 also phosphorylated Arabidopsis ERF13 (Fig. 3J), indicating that GmCRK3 has PTK activity.

Overexpression of CRK2 and CRK3 could phosphorylate exogenous ERF13 and endogenous beta-tubulin in cultured cells- In order to confirm the Tyr phosphorylation activity of CRK2 and CRK3 in cells, we used cultured Arabidopsis cells. Firstly, we investigated the cellular localization of these proteins. Transient expression analyses revealed that CRK3-YFP and ERF13-GFP are partially localized in the nucleus of cultured cells. CRK2-YFP is likely to localize to the plasma membrane as well as to the nucleus to a lesser degree (Fig. 4A). Similar results were observed in onion epidermal cells transiently expressing CRK3-GFP (32). Next, we expressed CRK2 or CRK3 together with ERF13 in cells. Immunoprecipitation with anti-GFP followed by immunoblot analysis with antibody to phosphorylated Tyr showed that CRK3WT phosphorylated ERF13-GFP whereas CRK2WT did not (Fig. 4B), and ERF13Y16/207F-GFP was not phosphorylated by CRK3WT. Since expression of KD forms of CRK2 and CRK3 was very low in cells compared with wild-type forms, we failed to analyze KD forms (Fig. 4B). Taken together, these results suggest that CRK3, instead of CRK2, phosphorylates the Tyr16 and Tyr207 sites of ERF13 in cells. In addition, we tried to investigate the Tyr phosphorylation of endogenous TBBs. Since Tyr phosphorylation of TBBs was very weak, we used an immunoprecipitation method with anti-TBB antibody to increase the detection sensitivity. A phospho-protein mobility shift assay with Phos-tag acrylamide (33) was used because the immunoprecipitated TBBs could not be detected on normal SDS-PAGE gel incubated with heavy chain anti-TBB antibody. Due to the covalent modification of the phosphorylated TBB by the Phos-tag reagent, a band of low mobility was detected (Phospho TBB in Fig. 4C). Following this, Tyr phosphorylation of the TBBs was detected by anti-Tyr antibody 4G10 [shown as TBB (pY) in Fig. 4C]. A low level of Tyr phosphorylation of endogenous TBBs was observed in control cells (Mock lane), indicating that the Tyr residue of endogenous TBBs is slightly phosphorylated as shown by a previous report (31). Overexpression of CRK2WT induced Tyr phosphorylation of endogenous TBBs (CRK2WT lane), whereas that of CRK3WT did not (CRK3WT lane). These results suggest that CRK2, but not CRK3, phosphorylates endogenous TBB in cultured cells.

Tyr phosphorylation of TBBs was decreased in CRK2- and CRK3-deficient mutants- In order to further clarify Tyr phosphorylation of endogenous CRK2 or CRK3 in plants, the Tyr phosphorylation level of TBBs in CRK2- and CRK3-deficient mutants (crk2 and crk3 respectively) was analyzed. CRK2 gene expression dramatically decreased in the crk2 mutant (Fig. 5A). CRK3 gene expression level in the crk3 mutant was 50% lower than that
Plant CRKs Function as PKs for Tyr Phosphorylation in the Col-0 wild-type. Protein levels of the endogenous TBBs were almost the same as in wild-type plants (right panel in Fig. 5B). Using the phospho-protein mobility shift assay, we observed that Tyr phosphorylation levels of endogenous TBBs were lower in crk2 and crk3 mutants than in wild-type plants (left panel in Fig. 5B). This result suggests that in Arabidopsis plants, Tyr phosphorylation of TBBs is mainly catalyzed by CRK2 and CRK3. Taken together, these data strongly suggest that Arabidopsis CRKs function as PKs for Tyr phosphorylation in plants.

DISCUSSION

In this study, we profiled the autophosphorylation activity of 759 PKs by combining a wheat cell-free system and a high-throughput AlphaScreen method, and identified 38 PKs with Tyr autophosphorylation activity. The 38 PKs were mainly classified into five groups: GSK3/Shaggy, MAPK, RLK/RLCK, CK1/CKL, and CRK. Like in mammals, several plant MAPKs and GSK3/shaggy-like kinases have already been reported to show Tyr autophosphorylation activity (14,34,35). Indeed, Tyr phosphorylation sites of most of these PKs have been identified already by MS analysis (6,36).

In mammals, CK1 is a well-conserved Ser/Thr-specific PK (37). In our study, four CK1/CLKs showed Tyr autophosphorylation activity and Arabidopsis CKL9 has previously been reported to have Tyr autophosphorylation activity (14,34,35). Indeed, Tyr phosphorylation sites of most of these PKs have been identified already by MS analysis (6,36). In mammals, CK1 is a well-conserved Ser/Thr-specific PK (37). In our study, four CK1/CLKs showed Tyr autophosphorylation activity and Arabidopsis CKL9 has previously been reported to have Tyr autophosphorylation activity (14,34,35). Indeed, Tyr phosphorylation sites of most of these PKs have been identified already by MS analysis (6,36). In mammals, CK1 is a well-conserved Ser/Thr-specific PK (37). In our study, four CK1/CLKs showed Tyr autophosphorylation activity and Arabidopsis CKL9 has previously been reported to have Tyr autophosphorylation activity (14,34,35). Indeed, Tyr phosphorylation sites of most of these PKs have been identified already by MS analysis (6,36).

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TBBs decreased in crk2 and crk3 mutants. Our results strongly suggest that CRKs are the responsible kinases for the Tyr phosphorylation of TBBs. Pharmacological approaches have shown that PTK and PTP inhibitors can alter the stability and orientation of the microtubules (MTs) (12,13). In mammals, membrane-associated TBBs were phosphorylated by membrane-associated pp60c-src in nerve growth cones membranes (53). In addition, pp60c-src-dependent Tyr phosphorylation of TBB inhibited the polymerization of MTs (54). However, no significant difference in growth was observed between crk2 or crk3, and wild type Col-0 plants under normal conditions. This might be because other CRKs might play complementary roles. The role of Tyr phosphorylation of TBBs by CRKs remains unclear. The AtGenExpress global stress expression data set revealed that expression of CRK2 increased under osmotic stress (55). Thus, CRK2-dependent Tyr phosphorylation might be involved in MT depolymerization or MT orientation maintenance under osmotic stress (56).

In some cases, phosphorylation of TFs can provide a key link between cell signaling and the control of gene expression. By PPI and in vitro kinase assays, we identified four Tyr-phosphorylated TFs that are substrate proteins of CRK2 and CRK3. Our approach, which combines a wheat cell-free system and a high-throughput AlphaScreen method, is able to analyze the direct interactions between the PK and the substrate protein with a high sensitivity level, as shown by the previous reports (57, 58). We hypothesized that this approach might be applicable to identify substrate proteins or kinases involved in phosphorylation. By dephosphorylation analysis and mutational analysis of the identified substrate proteins, we showed that CRK2 and CRK3 could phosphorylate ERF13 Tyr residues (Tyr16 and Tyr207). In contrast, CRKs-dependent phosphorylation of Ser or Thr residues was hardly observed. These results suggest that CRKs catalyze mainly the trans-phosphorylation of ERF13 Tyr residues. In order to uncover whether CRK2 and CRK3 can preferentially trans-phosphorylate the Tyr residue of a substrate protein, other substrate proteins would need to be found because it is not possible to clarify the essential biochemical properties of PKs by using pseudo substrates such as myelin basic protein or peptide. We found only two substrates, ERF13 and TBB7, which Tyr residues were phosphorylated. Thus, we could not confirm whether CRK2 and CRK3 preferentially phosphorylate Ser/Thr or Tyr residues because our data from these two substrate proteins is very limited.

Using a cell-based transient assay, we showed that CRK3 could phosphorylate the Tyr residues (Tyr16 and Tyr207) of ERF13-GFP in cells. However, CRK2-dependent Tyr phosphorylation was below the detection limit in this study. Using subcellular localization analysis, we observed that CRK2 was mainly localized to the plasma membrane and slightly to the nucleus. However, cytoplasmic localization might also be possible because we did not compare its localization with a plasma membrane-localized marker protein. Among the CRK family members, six CRKs (1, 2, 3, 5, 7, and 8) were predicted to have a myristoylation/palmitoylation motif in the N-terminus (29) and a predicted nuclear localization signal motif was found in all CRKs (59). In fact, tomato CRK1 and CPK/CRK-related tobacco CPK5 that have a myristoylation/palmitoylation motif in the N-terminus (29) and a predicted nuclear localization signal motif in the N-terminal are localized to the plasma membrane, while motif mutants of these are localized to the nucleus and cytoplasm (60,61). These findings suggest that CRKs have the potential for nuclear and membrane localization and are able to phosphorylate nuclear and/or membrane proteins.

Previous studies have shown that overexpression of ERF13 causes growth retardation, and increases abscisic acid and glucose sensitivities in Arabidopsis (62). In addition, expression of CRK3 is induced by abscisic acid treatment (53). These findings suggest that Tyr phosphorylation of ERF13 by CRK3 might be involved in the regulation of abscisic acid signaling. However, unfortunately, direct downstream target genes of ERF13 remain unknown. Both CRK3 and CRK2 could phosphorylate WRKY14, and CRK3 phosphorylates CIB5 in vitro. Thus, it is possible that both CRK3 and CRK2 play a role in the phosphorylation of these TFs in plants. On the other hand, previous studies have also suggested that some CRKs are involved in the signal transduction of different extracellular stimuli such as salt, low/high-temperature stress, wounding, and phytohormones in Arabidopsis, tobacco,
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tomato, and pea (61,63–67). Phenome analysis based on genetic techniques might provide significant information regarding the role of CRK-dependent Tyr phosphorylation in plants.

Although many previous studies have strongly suggested the existence of plant PTKs, evidence linking a particular PK to Tyr phosphorylation of substrates in vivo has been missing. In this study, we demonstrate that Arabidopsis has an angiosperm-specific CRK family involved in Tyr phosphorylation. Furthermore, we have identified six substrate proteins for CRKs. Our results provide the first direct evidence that plant PKs are able to phosphorylate substrate proteins on Tyr residues, and that typical TFs are the target of CRKs as observed in mammals. Furthermore, our data supports the identification of CRKs as the PKs that phosphorylate tubulin in vivo. In future studies, the functions of CRKs will be investigated in order to further clarify the role of Tyr phosphorylation in plants.

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FOOTNOTES

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The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (68) with the data set identifier PXD002158.

The abbreviations used are: PK, protein kinase; CDPK/CPK, calcium-dependent protein kinase; CRK, CDPK/CPK-related protein kinase; TBB, beta-tubulin; ERF13, ethylene response factor 13; PTK, protein tyrosine kinase; PTP1, tyrosine-specific protein phosphatase 1; MS, mass spectrometry; TLC, thin-layer chromatography; DHFR, dihydrofolate reductase; TF, transcription factor; PPI, protein-protein interaction; PD, pull-down; KD, kinase dead; MT, microtubule; IP, immunoprecipitation; IB, immunoblot.

FIGURES LEGENDS

FIGURE 1. Screening of Tyr autophosphorylation PKs using a wheat cell-free based protein array. A, Autophosphorylation analysis of 759 biotinylated PKs using a luminescent system with anti-pSer/pThr or anti-pTyr antibodies. All data represent the average of two independent experiments and the background for each experiment was controlled using the relevant non-biotinylated PK. B, Relative luminescent signals between anti-pSer/pThr (horizontal axis) and anti-pTyr antibody (4G10) (vertical axis). Black circles represent PKs that are more reactive with anti-pTyr antibody than with anti-pSer/pThr antibody. C, Phosphoamino acid analysis. Phospho-Tyr (pTyr), phospho-Ser (pSer), and phospho-Thr (pThr) in PKs were confirmed by one-dimensional TLC with (γ-32P) ATP. The ninhydrin panel shows the location of standard pTyr, pSer, and pThr (upper). The autoradiography panel shows 32P-labeled pTyr, pSer, and pThr (middle: short exposure and bottom long exposure) D, The dephosphorylation assay of 38 PKs by AtPTP1 using a luminescent system with anti-pTyr antibody. All data represent the average of three independent experiments and dephosphorylation efficiency was defined as the ratio between PTP1-treated kinases and untreated ones. E, Tyr autophosphorylation of PKs was confirmed by immunoblotting with anti-pTyr antibody (4G10) with or without ATP. F, In vitro autophosphorylation assay using WT or KD mutants. Biotinylated PKs were detected using Fluor 647 Strepavidin. G, In vitro autophosphorylation assay of autophosphorylation site mutants. Wild-type biotinylated CRK3 and CRK3 Y559F mutants were used. H, Autophosphorylation analysis of CRK3 by a luminescent system. The data presented are the average of three independent experiments. Black and grey bars represent wild-type CRK3 and autophosphorylation site mutants, respectively.

FIGURE 2. CRK2, CRK3, and CRK8 could phosphorylate C-terminal Tyr residues of TBB2 and TBB7. A, Affinity-purified Tyr phosphorylation proteins interacting with biotinylated CRK3 (biotin-CRK3) from Arabidopsis cell lysates. B, In vitro kinase assay of biotinylated CRK2, CRK3, and CRK8 with biotinylated TBB2, TBB7, and TBB9. C, Alignment of Tyr phosphorylation sites sequences of Arabidopsis TBBs. Multiple alignment of the C-terminal of TBBs was performed using the ClustalW programme (DNA Data Bank of Japan). The phosphorylation sites identified by mutant analysis are highlighted in grey. Identical amino acids among AtTBB1 (At1g57580), AtTBB2 (At5g2690), AtTBB4 (At5g44340), AtTBB5 (At1g20010), AtTBB6 (At5g12250), AtTBB7 (At2g29550), AtTBB8...
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(At5g23860), and AtTBB9 (At4g20890) are marked with asterisks. D, In vitro kinase assay of biotinylated CRK2/3/8 with biotinylated TBB7 mutants (Y443F and Y449F). Tyr phosphorylated residue was detected by anti-pTyr antibody (4G10) and the amount of protein loaded was determined by CBB stain or Fluor 647 Streptavidin (A, B, and D).

FIGURE 3. Substrate identification of CRKs, and biochemical characterization of CRK2 and CRK3. A, Heat map of the PPI relative AlphaScreen signals between 188 biotinylated TFs and FLAG-CRK2, FLAG-CRK3, or FLAG-cyclin-dependent kinase F:1 (CDKF:1). All data represent the average of two independent experiments and the background was controlled using DHFR from E. coli. B, In vitro Tyr phosphorylation assay using the top 20 proteins of interacting clones. After the incubation of FLAG-CRK2 or FLAG-CRK3, and biotinylated TF, Tyr phosphorylation was detected by immunoblot analysis with anti-pTyr antibody (4G10) and the amount of protein loaded was determined by CBB stain or Fluor 647 Streptavidin (A, B, and D).

FIGURE 4. Functional analysis of CRK2 and CRK3 Tyr phosphorylation in cells. A, Localizations of CRK2-YFP, CRK3-YFP, and ERF13-GFP in Arabidopsis protoplasts. DIC, differential interference contrast microscopy; YFP, YFP fluorescence; DAPI, 4',6-diamidino-2-phenylindole. B, Tyr phosphorylation analysis of ERF13-GFP by protoplast transient expression assays. ERF13-GFP or ERF13Y16/207F-GFP was immunoprecipitated with anti-GFP antibody from protoplast lysates. Tyr-phosphorylation was detected by anti-pTyr antibody (4G10). Expressions of WT or KD form of CRK2-HA and CRK3-HA were detected by anti-HA antibody. C, Tyr phosphorylation analysis of endogenous TBBs in CRK2 or CRK3 expressing protoplasts. Endogenous TBBs were immunoprecipitated with anti-TBB antibody (IP: aTBB) from protoplast lysates, and analyzed using 100 µM Phos-tag acrylamide gel and immunoblotting with 4G10 (IB: 4G10) (left panel) or anti-TBB antibodies (IB: aTBB) (middle panel). TBB proteins from 5% of the protoplast lysates were immunopurified and detected by anti-TBB antibody (Input, cell lysate) (left panel).

FIGURE 5. Tyr phosphorylation of endogenous TBBs was decreased in crk2 and crk3 mutants. A, Analysis of CRK family gene expression in crk2 and crk3 by real-time RT-PCR. Data are expressed as relative values normalized by the value of wild-type Col-0 plants. All data represent averages (n = 3, ± S.D.). B, Tyr phosphorylation analysis of endogenous TBBs in Col-0, crk2, or crk3 mutants. Endogenous TBBs were immunoprecipitated from protein lysates using anti-TBB antibodies (IP: aTBB), and analyzed
using 100 µM Phos-tag acrylamide gel and immunoblotting (Plant lysate) (left and middle panel). Tyr-phosphorylated proteins or TBBs were detected by anti-pTyr (IB: 4G10) (left) or anti-TBB antibodies (IB: αTBB) (middle and right panel). Five percent of the input lysates were analyzed by anti-TBB antibody (Input, Plant lysate). Tyr-phosphorylated recombinant TBB7 was analyzed by a similar method (in vitro). IgGHC: IgG heavy chains.
### TABLE 1. List of Tyr autophosphorylation protein kinases

| Tyr autophosphorylation PK No. | AGI code  | Gene name          | Relative value<sup>a</sup> | PPA<sup>b</sup> | PlantP Family<sup>c</sup> |
|-------------------------------|-----------|--------------------|-----------------------------|-----------------|-----------------------------|
|                              |           |                    | pSer_pThr pTyr pTyr/ pSer_pThr |                 |                             |
| A01                           | At5g15080 |                    | 3.4 31.8 9.4                | +               | Family 1.2.2 - Receptor Like Cytoplasmic Kinase VII |
| A02                           | At4g34440 |                    | 0.8 17.6 21.8              | NT              | Family 1.6.2 - Plant External Response Like Kinase |
| A03                           | At1g55200 |                    | 0.8 5.6 6.7                | NT              | Family 1.6.2 - Plant External Response Like Kinase |
| A04                           | At3g24550 |                    | 0.7 4.8 6.6                | +               | Family 1.6.2 - Plant External Response Like Kinase |
| A05                           | At5g56790 |                    | 5.0 24.1 4.8               | NT              | Family 1.6.2 - Plant External Response Like Kinase |
| A06                           | At1g70520 |                    | 1.1 14.7 13.9              | +               | Family 1.7.2 - DUF26 Kinase |
| A07                           | At2g26330 |                    | 1.8 8.7 4.8                | +               | Family 4.1.2.4 - Leucine Rich Repeat Kinase XI & XII |
| A08                           | At1g48480 |                    | 19.9 120.1 6.0             | +               | Family 4.1.3 - Leucine Rich Repeat Kinase III |
| A09                           | At4g28880 |                    | 0.4 9.0 25.1               | +               | Family 3.1.1 - Casein Kinase I |
| A10                           | At4g28540 |                    | 1.1 8.1 7.7                | NT              | Family 3.1.1 - Casein Kinase I |
| A11                           | At4g28880 |                    | 2.2 16.4 7.6               | NT              | Family 3.1.1 - Casein Kinase I |
| A12                           | At5g43320 |                    | 2.2 9.2 4.1                | +               | Family 3.1.1 - Casein Kinase I |
| B01                           | At1g73500 |                    | 0.4 7.6 19.9               | +               | Family 4.1.3 - MAP2K |
| B02                           | At3g61960 |                    | 0.9 4.2 4.6                | NT              | Family 4.1.7 - APG1 Like Kinase |
| B03                           | At1g49580 |                    | 0.6 4.4 7.0                | +               | Family 4.2.1 - Calcium Dependent PK |
| B04                           | At2g46700 |                    | 1.8 9.4 5.3                | +               | Family 4.2.1 - Calcium Dependent PK |
| B05                           | At3g19100 |                    | 0.9 4.3 4.8                | NT              | Family 4.2.1 - Calcium Dependent PK |
| B06                           | At3g10540 |                    | 1.0 4.7 4.8                | +               | Family 4.2.6 - IRE/NPH/PI dependent/S6 Kinase |
| B07                           | At2g30040 |                    | 2.4 17.6 7.3               | NT              | Family 4.4.1 - Unknown Function Kinase |
| B08                           | At3g5640  |                    | 0.2 5.8 27.4               | NT              | Family 4.5.1 - MAPK Family |
| B09                           | At4g01370 |                    | 0.3 4.1 14.2               | NT              | Family 4.5.1 - MAPK Family |
| B10                           | At2g43790 |                    | 0.6 4.4 8.0                | NT              | Family 4.5.1 - MAPK Family |
| B11                           | At4g19110 |                    | 2.9 18.6 6.4               | ND              | Family 4.5.1 - MAPK Family |
| B12                           | At1g59580 |                    | 0.8 4.6 5.9                | +               | Family 4.5.1 - MAPK Family |
| C01                           | At2g01450 |                    | 1.1 5.9 5.5                | +               | Family 4.5.1 - MAPK Family |
| C02                           | At5g39420 |                    | 0.3 5.9 19.8               | NT              | Family 4.5.2 - CDC2 Like Kinase |
| C03                           | At1g03740 |                    | 0.9 5.3 5.9                | +               | Family 4.5.2 - CDC2 Like Kinase |
| C04                           | At4g18710 |                    | 0.3 4.7 15.7               | +               | Family 4.5.4 - GSK3/Shaggy Like PK |
| C05                           | At3g61160 |                    | 0.4 6.4 14.1               | ND              | Family 4.5.4 - GSK3/Shaggy Like PK |
| C06                           | At4g00720 |                    | 0.9 12.4 13.2              | ND              | Family 4.5.4 - GSK3/Shaggy Like PK |
| C07                           | At1g57870 |                    | 1.4 16.3 12.0              | +               | Family 4.5.4 - GSK3/Shaggy Like PK |
| C08                           | At1g96390 |                    | 1.1 11.4 10.4              | NT              | Family 4.5.4 - GSK3/Shaggy Like PK |
| C09                           | At2g30980 |                    | 1.8 14.7 8.2               | +               | Family 4.5.4 - GSK3/Shaggy Like PK |
| C10                           | At5g26750 |                    | 0.7 5.3 7.6                | +               | Family 4.5.4 - GSK3/Shaggy Like PK |
| C11                           | At3g05840 |                    | 0.8 6.3 7.5                | +               | Family 4.5.4 - GSK3/Shaggy Like PK |
| C12                           | At2g40120 |                    | 1.8 7.4 4.1                | +               | Family 4.5.8 - Unknown Function Kinase |
| D01                           | At4g28980 |                    | 1.8 16.3 8.8               | +               | Family 4.5.8 - Unknown Function Kinase |
| D02                           | At5g58350 |                    | 1.1 6.5 6.1                | +               | Family 4.5.8 - Unknown Function Kinase |

<sup>a</sup> pSer_pThr and pTyr are the relative values of the non-biotinylated PKs. All data represent the average of two independent experiments and the background was controlled for each experiment using the relevant non-biotinylated PK.

<sup>b</sup> Results of phosphoamino acid analyses (PPA). +, pTyr residue was detected; ND, not detected; NT, not tested.

<sup>c</sup> The classification is based on the PlantP database (http://plantsp.sdsc.edu/). -, not annotated.
TABLE 2. Tyr phosphorylated proteins as substrates for CRKs

| AGI code   | Name   | Tyr phosphorylation site | CRKs                              | Method |
|------------|--------|--------------------------|-----------------------------------|--------|
| At5g62690  | TBB2   |                          | CRK2, CRK3, CRK8                  | PD     |
| At2g29550  | TBB7   | Tyr443, Tyr449           | CRK2, CRK3, CRK8                  | PD     |
| At2g44840  | ERF13  | Tyr16, Tyr207            | CRK1, CRK2, CRK3, CRK4, CRK8, GmCRK3* | PPI    |
| At1g43160  | RAP2.6 |                          | CRK2                              | PPI    |
| At1g26260  | CIB5   |                          | CRK3                              | PPI    |
| At1g30650  | WRKY14 |                          | CRK3                              | PPI    |

*Glycine max (soybean)
PD: pull-down.
PPI: protein-protein interaction analysis by AlphaScreen.
Plant CRKs Function as PKs for Tyr Phosphorylation

Fig. 1
Plant CRKs Function as PKs for Tyr Phosphorylation

Fig. 2
Plant CRKs Function as PKs for Tyr Phosphorylation

Fig. 3
Plant CRKs Function as PKs for Tyr Phosphorylation

Fig. 4
Plant CRKs Function as PKs for Tyr Phosphorylation

Fig. 5
Members of the Plant CRK-superfamily are Capable of trans-/auto-Phosphorylation of Tyrosine Residues
Keiichirou Nemoto, Nobuaki Takemori, Motoaki Seki, Kazuo Shinozaki and Tatsuya Sawasaki

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