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Two Novel Protein Kinase Genes, *OsMSRPK1* and *OsMSURPK2*, Are Regulated by Diverse Environmental Stresses in Rice

Mi-Ok Lee[1], Pil-Gyu Choi[1], Jung-A Kim[1], Young-Ho Jung[1], Seung-Hee Jung[1], So Hee Kim[1], Jin-Won Kim[2], Sang-Kyu Lee[3], Jong-Seong Jeon[3], Rakwal Randeep[4], Ganesh Kumar Agrawal[5], and Nam-Soo Jwa[1]*

[1]Department of Molecular Biology, College of Natural Science, Sejong University, Seoul 143-747, Korea
[2]Department of Environmental Horticulture, University of Seoul, Seoul 130-743, Korea
[3]Graduate School of Biotechnology and Plant Metabolism Research Center, Kyung Hee University, Yongin 449-701, Korea
[4]Human Stress Signal Research Center, National Institute of Advanced Industrial Science and Technology, Ibaraki 305-8566, Japan
[5]Research Laboratory for Agricultural Biotechnology and Biochemistry, GPO Box 8207, Kathmandu, Nepal

Two novel rice (*Oryza sativa* L.) protein kinase (PK) genes have been isolated. *OsMSRPK1* and *OsMSURPK2*, which most likely exist as single-copy genes in the rice genome, encode 693 and 503 amino acids polypeptide, respectively, and have the serine/threonine kinase domain of cyclin dependent protein kinase (*OsMSRPK1*), or the serine/threonine kinase domain and NAF domain (*OsMSURPK2*). Steady-state mRNA analyses of these PKs, with constitutive expression in the leaves of two-week-old seedlings, revealed that *OsMSRPK1* is up-regulated upon exposure to environmental stresses, whereas *OsMSURPK2* is down-regulated by these same stresses. Furthermore, the two PKs are developmentally regulated in both young and mature rice plants, including in the panicles. These results strongly suggest that the genes have roles in both plant development and in their defense/stress-signaling pathways.

Keywords: defense/stress signaling pathway, protein kinase, rice

Plants are constantly exposed to a variety of stresses, both biotic (e.g., pathogen infection and insect herbivory) and abiotic (e.g., high or low temperature, drought, and salinity) (Xiong and Yang, 2003). To survive these challenges, plants have elaborate mechanisms to perceive external signals and to manifest adaptive responses with proper physiological and morphological changes (Agrawal et al., 2002, 2003). At the molecular level, the perception of extracellular stimuli and the subsequent activation of defense responses require a complex interplay of signaling cascades (Yang et al., 1997).

All organisms use a network of signal transduction pathways to cope with their environment, to control their metabolism, and to realize their developmental programs. In plants, the processes of protein phosphorylation/dephosphorylation play essential roles in this adaptation (Umezawa et al., 2004). Several protein kinases (PKs) have been described as signal transduction factors related to stress responses in plants. For example, plant MAPKs (mitogen-activated PKs) are activated by abiotic stress (Munnik et al., 1999; Hoyos and Zhang, 2000; Ichimura et al., 2000; Mikolajczyk et al., 2000; Wen et al., 2002). Likewise, some *SnRK2* are activated by hyperosmotic stress in tobacco cells (Mikolajczyk et al., 2000) or soybean (Farras et al., 2001).

Little is known about how various stresses affect the activity of CDK (cyclin-dependent kinase). The impairment of root growth by osmotic conditions is associated with reduced cell division (Sacks et al., 1997; Samarajeewa et al., 1999). This implies that the meristem plays an important role in that response. Nevertheless, the mechanisms by which biotic and abiotic stress conditions affect cell cycle regulation are not well understood. In the leaves of wheat (*Triticum aestivum*), water stress causes a shortening of the meristem and a prolonged cell cycle when CDK activity is reduced by inhibitory phosphorylation (Schuppler et al., 1998). When leaves of maize (*Zea mays*) are grown under mild water-deficit conditions, their rate of cell division is also diminished. Likewise, $p34^{cdc2a}$ kinase activity is decreased by approximately 45% in all leaf zones, although the total amount of $p34^{cdc2a}$ is not affected (Granier et al., 2000). Finally, in *Arabidopsis*, severe salt stress can transiently reduce the expression of *CycA2;1* and *CycB1;1* (Burssens et al., 2000).

The cytosolic free-calcium (Ca^{2+}) concentration can be altered by many extracellular stimuli, including hormones or environmental signals, e.g., abscisic acid, gravity, light, salinity, drought, cold, oxidative stress, anoxia, and mechanical perturbation (Poovaiah and Reddy, 1993; Bush, 1995; Trewavas and Malho, 1998; Knight, 2000). Calcium is the ubiquitous second messenger involved in many of these processes (Gilroy et al., 1993). In plants, intracellular Ca^{2+} levels are modulated in response to various signals, such as abiotic stresses, light, pathogens, and hormones (Knight, 2000).

*Corresponding author; fax +82-2-3408-3661
e-mail nsjwa@sejong.ac.kr

In the case of plant Ca^{2+}-dependent protein kinases (CDPKs), which harbor a Ca^{2+}-binding domain as well as a catalytic Ser/Thr kinase domain, Ca^{2+} signals can be sensed and transmitted by a single protein (Sheen, 1996; Kamachi et al., 2004). Functional analyses of different plant CDPKs have provided evidence for the crucial roles these proteins play in divergent processes, e.g., hormonal and stress signaling, as well as in response to pathogens (Sheen, 1996). Osmotic and other abiotic and biotic stresses also cause increases in the cytosolic Ca^{2+} concentration (Knight, 2000). CDPKs can be induced by water deficit (Urao et al., 1994), and over-expression of *OsCDPK7* results in greater tolerance to cold and osmotic stress in rice (Saijo et al., 2000). Furthermore, the expression of a constitutively active form of *AtCDPK1* can activate an osmotic stress–related promoter (Sheen, 1996).

Here, we report the molecular characterization of *OsMSRPK1* and *OsMSURPK2*, members of the plant CDK- and CDPK-families, respectively. We investigated their activation by various environmental stresses to determine their specific functioning in stress signaling.

MATERIALS AND METHODS

cDNA Clones

OsMSRPK1 (Genbank accession number AK111593) and *OsMSURPK2* (Genbank accession number AK103032) cDNAs were obtained from the Rice Genome Resource Center (RGRC, Tsukuba, Japan).

Stress Treatments

Rice (*Oryza sativa* L. japonica-type cv. Nipponbare) plants were grown under white fluorescent light (wavelength 390 to 500 nm, 150 mol m^{-2} s^{-1}, 12-h photoperiod) at 25°C and 70% relative humidity. All treatments utilized the middle portions (2 cm long) of fully expanded leaves from two-week-old seedlings, as described previously (Agrawal et al., 2002). Leaf segments floated on Milli Q (MQ) water in covered Petri dishes served as the wounding-by-cut control. Other segments were treated with 100 μM JA (jasmonic acid), 100 μM SA (salicylic acid), 100 μM Cd (cadmium chloride), 10 mM CHX (cycloheximide), 0.1% CT (chitosan), 1 M OA (okadaic acid), 10 mM H_2O_2, or 150 mM NaCl. For the drought treatment, the segments were placed in an empty, open Petri dish. Ultraviolet light (UV-C, 254 nm) was irradiated from a distance of 15 cm using a Hitachi (Japan) germicidal lamp. Treatments were conducted under continuous light (Rakwal et al., 1999; Agrawal et al., 2002). The leaves or leaf segments were then sampled at various intervals and immediately frozen at -80°C. For treatment with ozone (O_3), the potted seedlings were transferred to a fumigation chamber (controlled at 25°C with a RH of 70%, mean wind velocity of 0.22 m s^{-1}, and constant light at 350 mol m^{-2} s^{-1}). While some were exposed continuously to 0.2 ppm O_3, the control plants were treated with filtered, pollutant-free air (Kubo et al., 1995; Agrawal et al., 2002). The ozone was prepared and its concentrations monitored with an ML9810 O_3 analyzer (Lear Siegler Measurement Controls, USA). For our circadian/rhythmic study, two-week-old seedlings were held for an additional 3 d in the growth chamber (12-h photoperiod). In the case of the O_3 and circadian experiments, the 3rd and 4th leaves were removed at various time intervals and immediately stored at -80°C.

Dot Blot Hybridization

Putative rice MAPK cDNAs were obtained from the RGRC. The cDNAs (2 μg each) were denatured with DNA denaturation solution (0.5 N NaOH and 1.5 M NaCl) and heated at 65°C for 5 min. Denatured DNAs were then mixed with 2X SSC and transferred onto a nylon membrane (Hybond-N^+; Amersham Pharmacia Biotech, UK), using a 96-well dot-blot manifold blotter. The membrane was hybridized with α^{32}P- labeled total cDNA of *OsEDR1* over-expressing transgenic rice. This cDNA was synthesized from total RNA isolated from those transgenic leaves, using TRIzol reagent (Invitrogen, USA). Hybridized membranes were washed under strict stringency and exposed to X-ray film.

Southern Blot Hybridization

Rice genomic DNA was extracted from the leaves of two-week-old seedlings (cv. Nipponbare). Genomic DNA (5 μg) was digested with *Hin*dIII, *Eco*RI, *Sac*I, *Xba*I, and *Eco*RV, then separated by electrophoresis on a 0.8% agarose gel. For DNA denaturation, the agarose gel containing the genomic DNA was washed with a denaturation solution and a neutralizing solution. The genomic DNA was blotted onto nylon membranes (Hybond-N^+; Amersham Pharmacia Biotech), which were then hybridized with α^{32}P-labeled *OsMSRPK1* or *OsMSURPK2* cDNA probes. These membranes were washed under strict stringency and exposed to X-ray film.

RT-PCR

Total RNA from rice tissues was isolated with a QIAGEN RNeasy Plant Mini Kit (Qiagen, USA). The total RNA samples were DNase-treated with RNase-free DNase (Stratagene, USA) prior to RT-PCR. First-strand cDNA was synthesized in a 50 μL reaction mixture with a StartaScriptTM RT-PCR Kit (Stratagene), according to the manufacturer's protocol, using 10 μg total RNA isolated from the leaves. The 50 μL reaction mixture (in 1 × buffer, as recommended by the manu-

facturer of the polymerase) contained 1.0 μL of the first-strand cDNA, 200 mM dNTPs, 10 pmols of each primer set [(OsMSRPK1-F: 5′-TAATTTGGGACAGGAA-TGGTTC-3′; OsMSRPK1-R: 5′-ACAAACACATTTTCCTC-CTCGT-3′; 301 bp), and (OsMSURPK2-F: 5′-TGAG-CACCTGAACTCACAATCT-3′; OsMSURPK2-R: 5′-GAG-GAATTAGAAACAAGCAGCAA-3′; 284 bp)], and 0.5 U of *Taq* polymerase (TaKaRa Ex *Taq* Hot Start Version; TaKaRa Shuzo, Japan). The thermal-cycling parameters included: initial denaturation at 97°C for 5 min; then a cycling regime of 25/30 cycles of 95°C for 45 s, 55°C for 45 s, and 72°C for 1 min; followed by a final extension for 10 min at 72°C (TaKaRa PCR Thermal Cycler Device, Model TP600; Takara Bio, Japan). After the PCR was completed, the total reaction mixture was mixed with 2.0 μL of 10X loading buffer and vortexed; then, 10 μL was loaded into the wells of a 1.5% agarose gel (Agarose ME; Iwai Chemicals, Japan). Electrophoresis was performed for ca. 30 min at 100 V in 1X TAE buffer, using a Mupid-ex electrophoresis system (ADVANCE, Japan). The gels were then stained (20 μL of 50 mg mL^{-1} ethidium bromide in 100 mL 1X TAE buffer) for ca. 10 min, and the stained bands were visualized using a UV-transilluminator (ATTO, Japan). Band intensities were analyzed by the NIH image program (National Institutes of Health, USA) on a Macintosh computer.

Phylogenetic Tree Construction

All sequencing data were analyzed by the CLUSTAL-W program (http://www.ebi.ac.uk/clustalw/). Homologies of the amino acid sequences were compared against sequences in the GenBank and EMBL DNA databases. The phylogenetic tree was then constructed using the CLUSTAL-W program (http://www.ebi.ac.uk/clustalw/).

Localization and Visualization Analysis

The GFP fusion protein was constructed with a full-length *OsMSRPK1* clone, using the GFP N-terminus in a CaMV35S promoter. For transient localization assays, the *OsMSRPK1::GFP* and *OsMSURPK2::GFP* construct DNAs were purified as plasmids with a Qiagen plasmid miniprep kit (Qiagen, USA). Plasmid DNAs were mixed with tungsten particles and particles coated with the *OsMSRPK1*::GFP and *OsMSURPK2*::GFP construct. The plasmids were then transformed into onion cells by particle bombardment (Bilang and Bogorad, 1996). Leaf samples were placed in Petri dishes containing MS media, and the epidermal cells that expressed GFP images via epifluorescence were identified using an Olympus microscope with GFP-optimized ND filter sets (Olympus, Japan). Digital images were collected with an Olympus IX70 fluorescence microscope, an I.CAM-SCOPE digital camera (Sometech, Korea), and Micro-Fire software (USA). The images were further processed with Adobe Photoshop 7.0 software.

RESULTS

Identification of the *OsMSRPK1* and *OsMSURPK2* Genes

OsEDR1 was the first novel member of the MAPKKKs family cloned from japonica-type rice cv. Nipponbare. This gene is rapidly and transiently regulated by diverse environmental factors (Kim et al., 2003). We previously generated *OsEDR1* over-expressing transgenic rice plants and observed *OsEDR1*-induced hypersensitive reaction (HR)-like cell death (Jwa et al., unpublished data). Here, *OsPR1a,* a rice pathogen resistance gene, was up-regulated in our *OsEDR1* over-expressing transgenics (Fig. 1A.). To help determine the genes

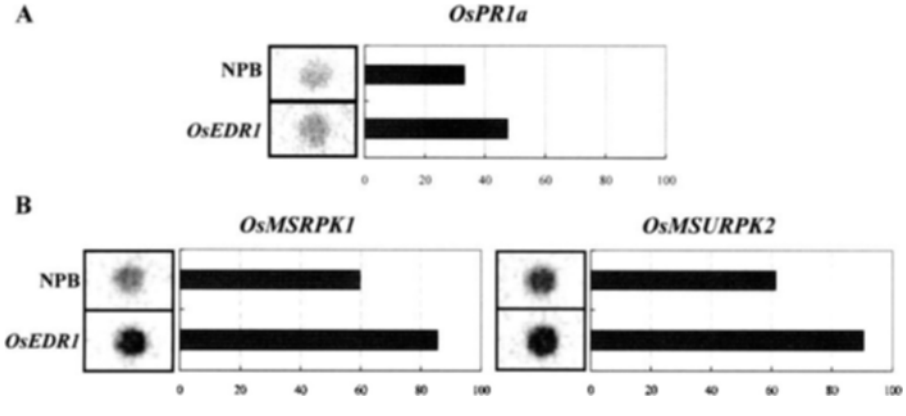

Figure 1. Identification of putative *OsMSRPK1* and *OsMSURPK2*. Dot-blot analysis was performed using putative protein kinases and total cDNA probes from wild-type rice Nipponbare (NPB) and *OsEDR1*-overexpressing transgenic plants. **A**, *OsPR1a* gene; **B**, up-regulated genes, in *OsEDR1*-overexpressing lines.

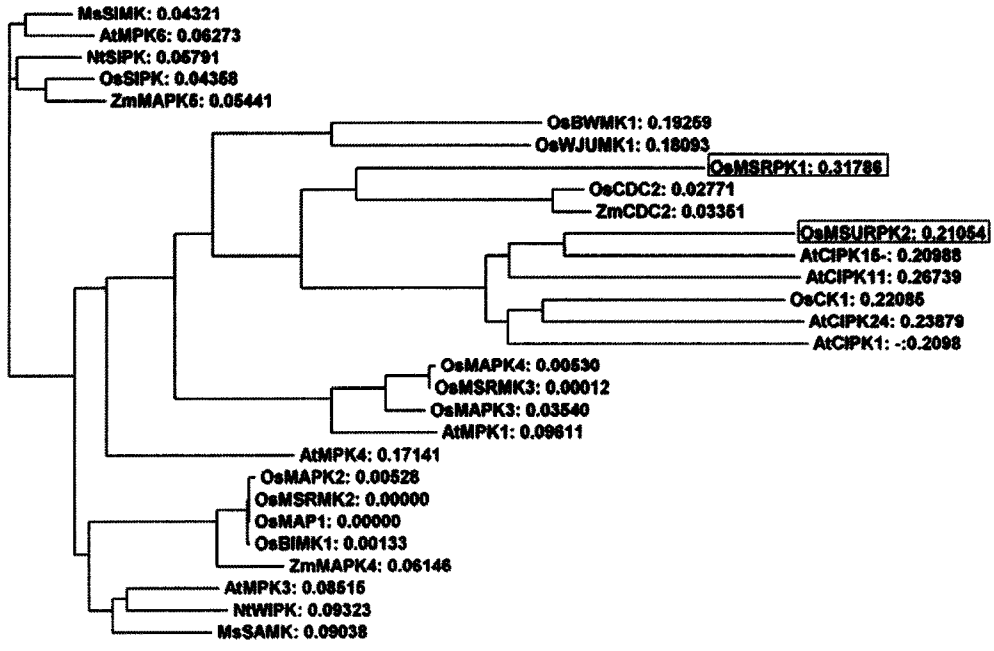

Figure 2. Phylogenetic tree of two novel protein kinase (OsMSRPK1 and OsMSURPK2) proteins from rice and other species. Neighbor-joining method was used to generate dendrogram of protein kinase proteins. Bootstrap values expressed as percentage (over 1000 replicates) are shown at corresponding nodes. Sources of genes are indicated in parentheses: MsSIMK-(Q07176/Alfalfa); NtSIPK-(U94192/Tobacco); OsSIPK-(AJ535841/Rice); ZmMAPK5-(AB016802/Maize); AtMPK6-(D21842/Arabidopsis); OsBWMK1-(AF177392/Rice); OsWJUMK1-(AJ512643/Rice); OsMSRPK1-(AK111593/Rice); OsMSURPK2-(AK103032/Rice); OsMAPK4-(AJ251330/Rice); OsMSRMK3-(AJ512642/Rice); OsMAPK3-(AF194416/Rice); AtMPK1-(D14713/Arabidopsis); AtMPK4-(D21840/Arabidopsis); OsMAPK2-(AJ250311/Rice); OsMSRMK2-(AJ486975/Rice); OsMAP1-(AF216315/Rice); OsBIMK1-(AF332873/Rice); ZmMAPK4-(AB016801/Maize); AtMPK3-(D21839/Arabidopsis); MsSAMK-(X82270/Alfalfa); NtWIPK-(D61377/Tobacco); OsCDC2 (P29618/Rice); ZmCDC2 (P23111/Maize); AtCIPK15-(P92937/Arabidopsis); AtCIPK11-(O22932/Arabidopsis); OsCK1-(Q6X4A2/Rice); AtCIPK24-(Q9LDI3/Arabidopsis); AtCIPK1-(Q8RWC9/Arabidopsis).

involved in a plant-defense/stress signal transduction pathway, we performed reverse northern hybridization with 72 signal transduction-related clones. Using total cDNA as the probe synthesized after mRNA extractions from both wild-type rice (Nipponbare) and the transgenic plants, we identified and selected two novel PKs (Fig. 1B). Based on their expression profiles (described below), these genes were labeled as *O. sativa* multiple stress-responsive/unresponsive PKs, and were named *OsMSRPK1* and *OsMSURPK2*. *OsMSRPK1* cDNA is 3116 bp long, contains an open reading frame (ORF) of 2079 nucleotides, and encodes a 693-amino acid polypeptide. *OsMSURPK2* cDNA is 2338 bp long, contains an ORF of 1509 nucleotides, and encodes a polypeptide of 503 amino acids. Amino acid sequence alignment of *OsMSRPK1* revealed the distinct conserved serine/threonine kinase domain of CDKs, while *OsMSURPK2* showed two conserved domains, i.e., the serine/threonine kinase domain and an NAF domain. Although both could be grouped as MAPK-like genes, our domain analysis of these individual genes demonstrated their classification into separate PK families -- *OsMSRPK1*, with the *CDC2* family; *OsMSURPK2*, with the *CIPK* family (Fig. 2).

Using DNA gel blotting, we determined that *OsMSRPK1* and *OsMSURPK2* are single-copy genes in the rice genome (Fig. 3).

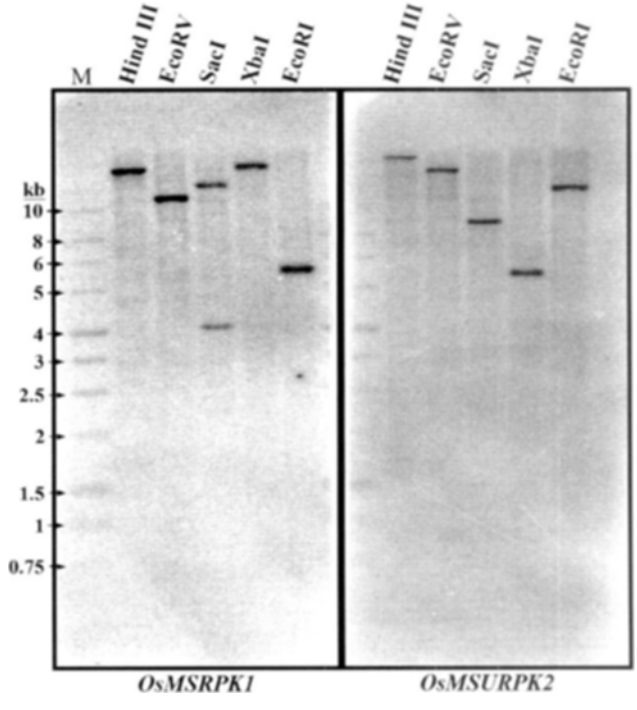

Figure 3. Southern blot analysis of *OsMSRPK1* and *OsMSURPK2*, using wild-type rice genomic DNA (10 μg), and *OsMSRPK1*-specific probes (left) and *OsMSURPK2*-specific probes (right). Genomic DNA was digested with *Hind*III, *Eco*RI, *Sac*I, *Xba*I, and *Eco*RV.

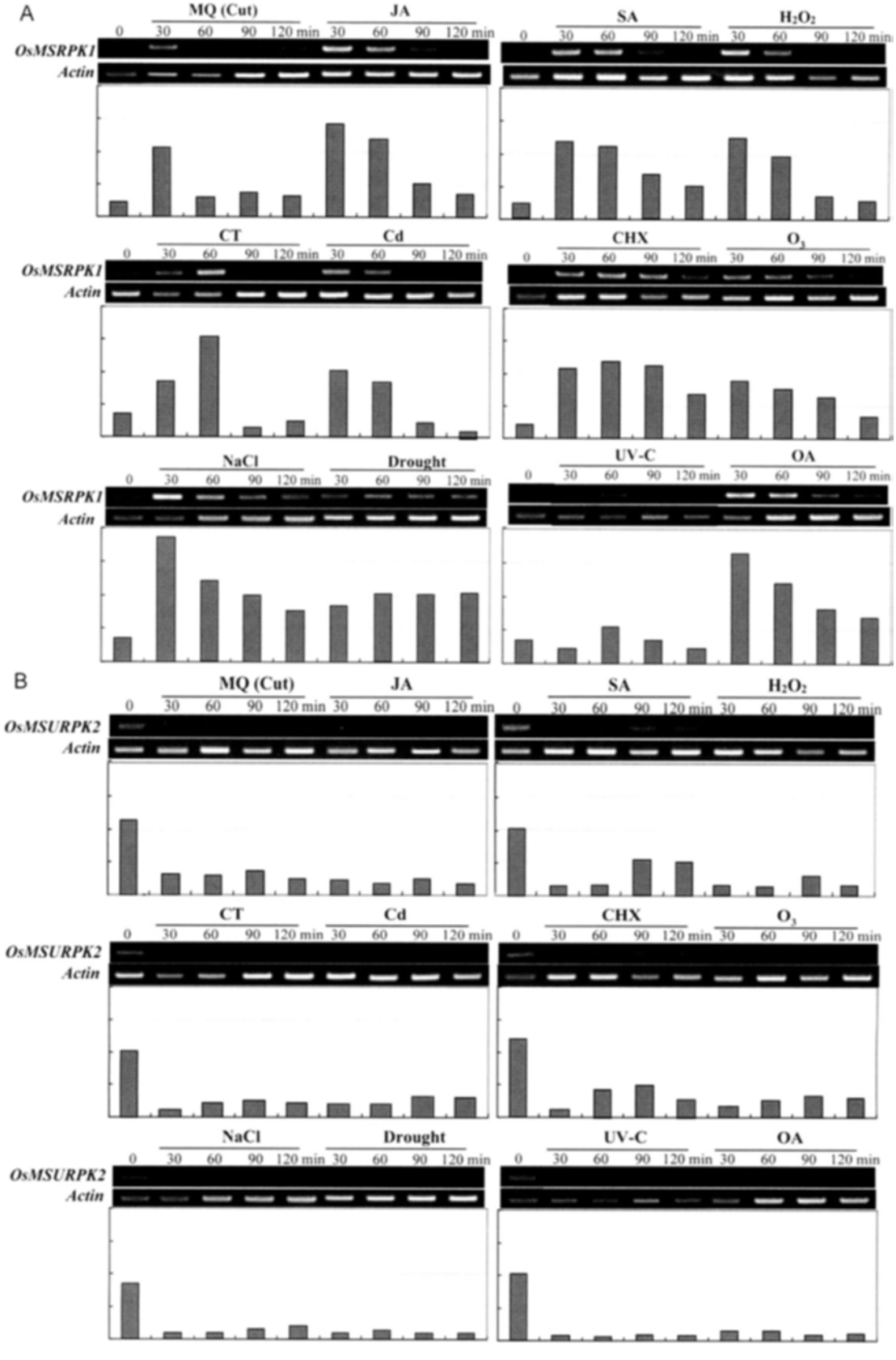

Figure 4. Time-course analysis of *OsMSRPK1* (**A**) and *OsMSURPK2* (**B**) gene expressions against diverse environmental factors. Leaf segments were treated with 100 μM each of JA, SA and Cd, 0.1% CT, 1 M OA, 10 mM H_2O_2, or 150 mM NaCl. Leaf segments placed in open Petri dish without water were used for drought treatment. Ultraviolet light (UV-C, 254 nm) was irradiated from a distance of 15 cm by Hitachi germicidal lamp. Treatments were done under continuous light. Leaves or leaf segments were sampled at times indicated, and total RNA was extracted for cDNA synthesis followed by RT-PCR. Band intensities were analyzed via NIH image program and graphed to represent ratio with band intensity of rice actin.

Differential Regulation of *OsMSRPK1* and *OsMSURPK2* in Response to Diverse Environmental Cues

Because their up-regulation was determined through reverse northern hybridization, their involvement in defense signaling was screened with global signaling molecules, JA, SA, H_2O_2, the fungal elicitor CT, drought, high salt (NaCl), heavy-metal cadmium, UV-C irradiation, and the protein phosphatase inhibitor OA. The RT-PCR control used a specific rice actin gene primer set. Transcriptional expression was detected after 30 min, as manifested by rapid kinase responses to external and internal stimuli (Fig. 4). The transcriptional behavior of our two PKs, *OsMSRPK1* and *OsMSURPK2*, was examined in a time-course of 30 to 120 min for detailed expression analyses following diverse stress treatments. Both wounding and JA enhanced *OsMSRPK1* expression at 30 min; afterward, transcript levels rapidly decreased in response to the former, while that decline was slower for the latter treatment over the next 90 min (Fig. 4A). SA and H_2O_2 promoted enhanced transcript accumulations at 30 min, followed by a gradual decrease to the basal level at 120 min (Fig. 4A). In contrast, exposure to NaCl or drought increased transcript levels at 30 min, which then remained almost unchanged for another 90 min. UV-C caused slight induction at 60 min. CHX, an inhibitor of eukaryotic protein synthesis, also enhanced the transcript level over time, suggesting that a *de novo*-synthesized negative regulator might be involved in *OsMSRPK1* regulation (Fig. 4A). Finally, in response to OA treatment, *OsMSRPK1* transcript was abundantly accumulated at 30 min, followed by a slow decrease. Although *OsMSRPK1* acted as a positive regulator for the majority of the stresses tested here, *OsMSURPK2* seemed to negatively regulate the defense/stress response activators in rice (Fig. 4B).

Light Regulation of *OsMSRPK1* and *OsMSURPK2*

Transcript levels were generally enhanced either under darkness (*OsMSRPK1*) or in response to light exposure (*OsMSRUPK2*) (Fig. 5). Overall, *OsMSRPK1* expression was increased in the first 24 h of darkness, followed by a gradual decrease. In contrast, *OsMSURPK2* accumulation was rapid in the first 12 h of the dark period, followed by a dramatic decline under 72 h of continuous darkness.

Developmental Regulation of *OsMSRPK1* and *OsMSURPK2*

The developmental regulation patterns of *OsMSRPK1* and *OsMSURPK2* were examined in the first leaf (FL) and flag leaf (FgL), as well as in the panicles at different

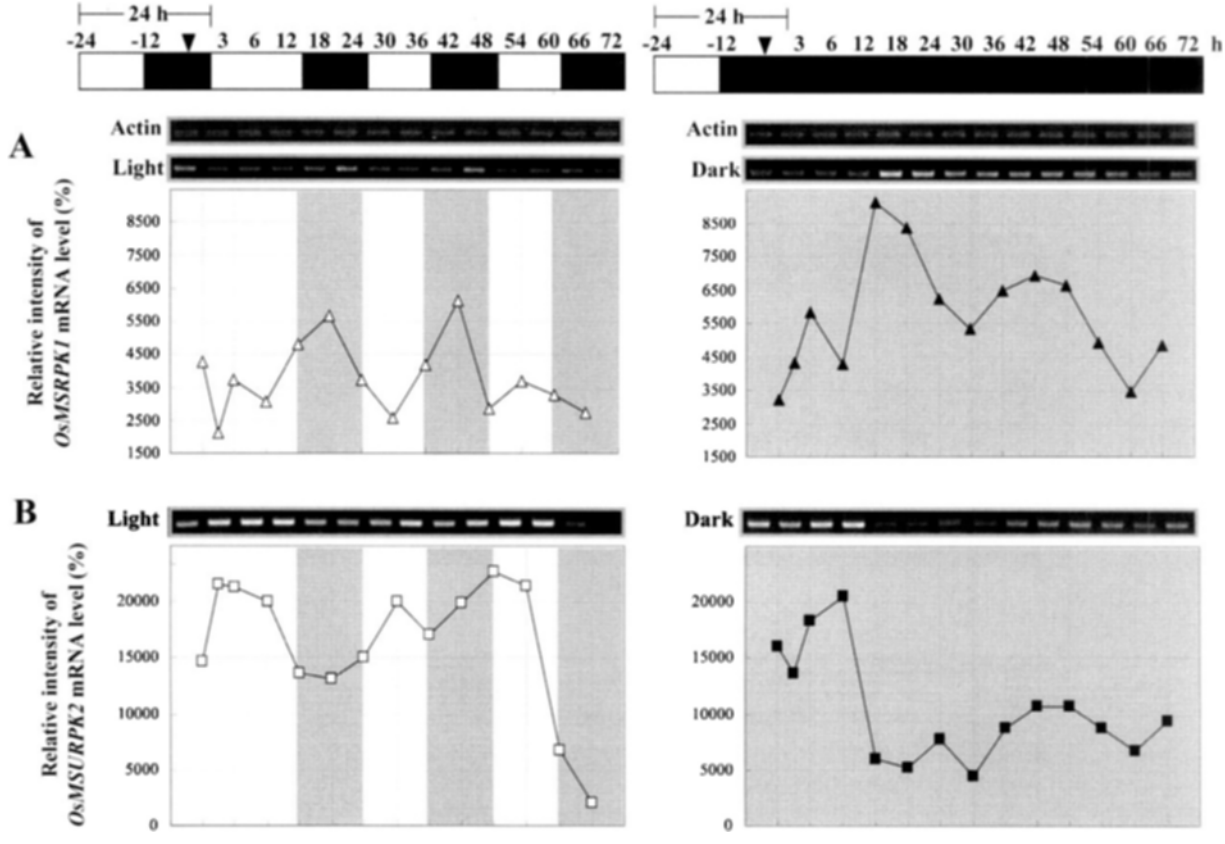

Figure 5. Regulation of *OsMSRPK1* (**A**) and *OsMSURPK2* (**B**) by photoperiod. White and black bars represent light and dark treatment, respectively. Band intensities were analyzed as in Figure 4.

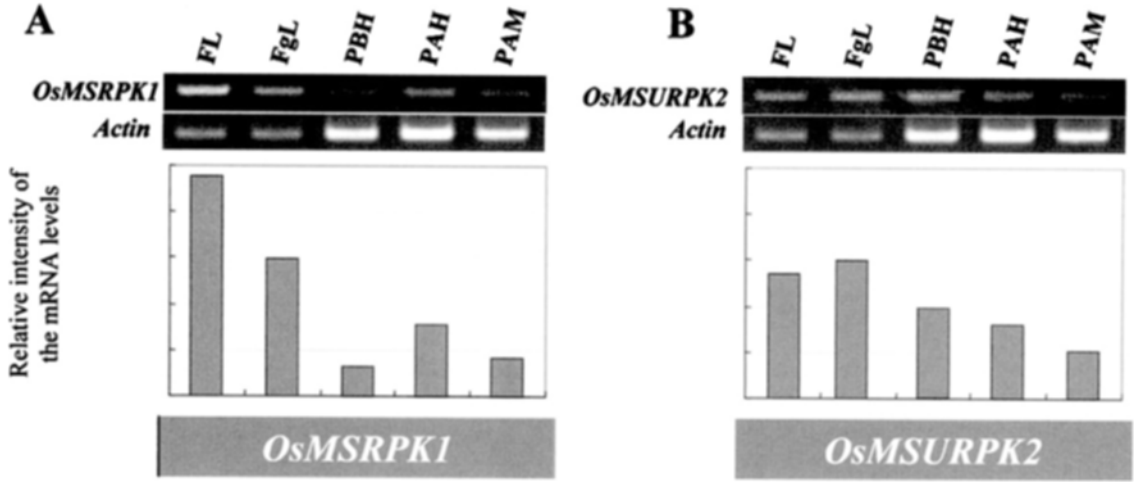

Figure 6. Developmental-stage and tissue-specific expression patterns of *OsMSRPK1* (**A**) and *OsMSURPK2* (**B**) in rice organs. First leaf FL), flag (FgL), panicle at heading (PAH), panicle before heading (PBH), and panicle after maturity (PAM). Band intensities were analyzed as in Figure 4.

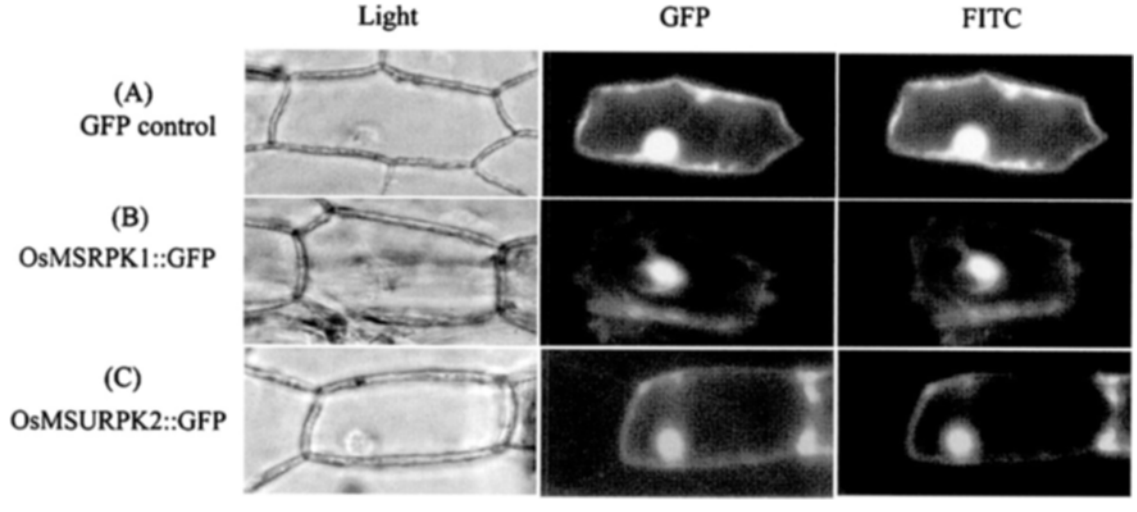

Figure 7. Localization of OsMSRPK1::GFP (**B**) and OsMSURPK2::GFP (**C**) fusion proteins in onion cells. (**A**) Unfused GFP protein. Onion peels were bombarded with DNA constructs indicated, and incubated at 28°C for 12 h. Epidermal cells were imaged using Light, GFP, and FITC channels of fluorescence microscope. Both OsMSRPK1 and OsMSURPK2 localized to nucleus and cytoplasm.

stages of maturity (Fig. 6). The transcript level of *OsMSRPK1* was much higher in the FL than in any other organ, as well as being particularly higher in the panicle at heading (PAH) than in panicles before heading (PBH) and after maturity (PAM). *OsMSURPK2* was expressed at relatively equal levels in all tested organs, although its transcripts in the panicles were lower at PAH and PAM. These results indicate clear developmental regulation by *OsMSRPK1* and *OsMSURPK2* in rice.

Nuclear Targeting of *OsMSRPK1* and *OsMSURPK2*

To facilitate the identification of functional *OsMSRPK1* and *OsMSURPK2*, we used a transient assay involving the biolistic bombardment of various *OsMSRPK1* and *OsMSURPK2*-GFP fusion constructs into *Allium cepa* (onion) epidermal cells. The green fluorescent protein (GFP)–coding sequence was in-frame-fused to the 3' end of either *OsMSRPK1* or *OsMSURPK2*, and distribution of the fusion protein was assessed by a transient transfection system. Onion epidermal cells were bombarded with gold particles coated with plasmid DNA containing the gene fusion, then subjected to microscopic analysis. Unfused GFP protein (Fig. 7A) was found in both the nucleus and the cytoplasm. Although OsMSRPK1 and OsMSURPK2 were localized in both nucleus and cytoplasm (Fig. 7), CDKs generally are localized and functioned in the former while the CIPKs are found in the latter. It is possible that subcellular localization of these proteins is regulated by a stress signal.

DISCUSSION

A New Rice *CDK*-like Gene, *OsMSRPK1*, is Regulated by Diverse Environmental Stresses in Rice

Cell cycle regulators presumably control both cell cycle duration and the number of dividing cells, and also serve as important regulators of adaptations to environmental conditions. In *Arabidopsis*, several classes of *CDK*s have been identified, of which the A-type and B-types have been best characterized (Vandepoele et al., 2002). The A-type CDKA1 is constitutively expressed during the cell cycle. Activity of its protein product is maximal at the G1-to-S and G2-to-M transitions, implying an involvement in the transition through both checkpoints. The plant-specific B-type *CDKB1-1* is transcribed in the S and G2 phases; activity of the corresponding protein peaks only at the G2-to-M transition (Porceddu et al., 2001; Sorrell et al., 2001). Expression of both genes is linked to dividing cells, whereas *CDKA1* is also found in cells that do not divide but are competent to do so (Mironov et al., 1999). In plants, the adaptation to stress involves two phases: first, a rapid, transient inhibition of the cell cycle that results in fewer cells remaining in the meristem. Then, when the meristem reaches the appropriate size for the given conditions, the cell cycle duration returns to its "default" (West et al., 2004). Therefore, to determine the possible effectors for a stress-induced block of the cell cycle, we studied the transcription of *OsMSRPK1* under various conditions.

Interestingly, the rice *OsMSRPK1* gene was activated by all our stress treatments. Moreover, its induction following wounding had not previously been reported, such that we are now the first to propose the existence and importance of a wound-responsive *CDK* in plants. Both JA and SA are other known key players in the plant defense/stress pathway (Reymond and Farmer, 1998). This regulation of *OsMSRPK1* implies JA- and SA-dependent pathway in rice, and a role for both in rice plant defense/stress responses. Similarly, pathogen signaling pathways involve Ca^{2+} fluctuations (Rudd and Franklin-Tong, 2001). Here, we can hypothesize that *OsMSRPK1* in rice is an important threshold parameter in such a defense response. Although this may represent a general mechanism in other species, to our knowledge, such a phenomenon has not been otherwise reported.

A large number of plant kinase genes are induced by abiotic stresse that include drought, salinity, and low temperature (Jonak et al., 1996; Berberich et al., 1999; Munnik et al., 1999; Mikolajczyk et al., 2000; Agrawal et al., 2002; Umezawa et al., 2004). It is intriguing to find that enhanced stress tolerance is associated with increased OsMSRPK1 activity. Therefore, based on our results, we conclude that *OsMSRPK1* likely encodes a positive regulator of, at least, drought, salt, and cold tolerances.

Heavy metals, a class of notorious environmental pollutants, also activate defense/stress responses in rice (Hajduch et al., 2001). Our results here also suggest the existence and induced expression of *OsMSRPK1* in rice that senses and relays the signal(s) downstream of heavy metal perception. Interestingly, *OsMSRPK1* expression was also induced by UV stress. Nevertheless, we do not know how these stresses might link *OsMSRPK1* to the defense/stress response in rice.

PKs and phosphatases play important signaling roles when plants are exposed to salinity or drought stresses (Wurgler-Murphy and Saito, 1997; Gustin et al., 1998). Our experiments showed that, in response to OA (a protein phosphatase inhibitor), *OsMSRPK1* transcript was strongly accumulated at 30 min, followed by a slow decrease over time. This indicates that the phosphorylation/dephosphorylation events of a kinase-signaling cascade may be involved in the activation of *OsMSRPK1* expression. Therefore, we can hypothesize that this gene plays a role when stresses are sufficient for the inducement of a multiple-stress gene. Furthermore, our study results provide an opportunity for continuing research into how *OsMSRPK1* serves as a threshold factor in multiple signaling pathways.

OsMSURPK2 May Act as a Negative Regulator under Stress Conditions

CDPK (mostly from *Arabidopsis*) can be activated or transcriptionally induced upon exposure to abiotic stresses, such as ABA, drought, and NaCl (Kim et al., 2003). Interestingly, *OsMSURPK2* was transcriptionally repressed by nearly all of our treatment conditions. This almost total inhibition of *OsMSURPK2* expression suggests that this gene is part of a negatively regulated cascade that is also involved in rice plant defense/stress responses.

We have shown that *OsMSRPK1* and *OsMSURPK2* are expressed constitutively in rice seedling leaves, but are differentially regulated by a wide variety of environmental stress factors. Basal-level expression of *OsMSRPK1* and *OsMSURPK2* may have functional significance under normal and defense/stress condition(s) in that crop. Interactions among different signaling pathways appear to be very common and important in regulating defense responses against stress (Xiong and Yang, 2003). We have demonstrated that *OsMSRPK1* is potently and rapidly activated by stresses, whereas *OsMSURPK2* is negatively regulated by the same treatments. Considering the large number of *PK* genes, it is not surprising that plants have evolved such integrated signaling and transduction systems to delicately coordinate various physiological activities. Because of these interactions, however, researchers must examine both their positive and negative effects on different agronomic traits. By further understanding PKs and

carefully modifying their components, it should be possible to generate improved crop varieties that combine desirable traits, e.g., enhanced tolerances to biotic and abiotic stresses. The cloning of *OsMSRPK1* and *OsMSURPK2*, as well as detailed expression analyses at the transcript level, will reveal their involvement in regulating multiple stress pathways. This is an important step towards elucidating the early and immensely important signaling regulatory pathways in rice.

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