Overexpression of the mitogen-activated protein kinase gene *OsMAPK33* enhances sensitivity to salt stress in rice (*Oryza sativa* L.)

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Mitogen-activated protein kinases (MAPK) signalling cascades are activated by extracellular stimuli such as environmental stresses and pathogens in higher eukaryotic plants. To know more about MAPK signalling in plants, a MAPK cDNA clone, *OsMAPK33*, was isolated from rice. The gene is mainly induced by drought stress. In phylogenetic analysis, *OsMAPK33* (Os02g0148100) showed approximately 47–93% identity at the amino acid level with other plant MAPKs. It was found to exhibit organ-specific expression with relatively higher expression in leaves as compared with roots or stems, and to exist as a single copy in the rice genome. To investigate the biological functions of *OsMAPK33* in rice MAPK signalling, transgenic rice plants that either overexpressed or suppressed *OsMAPK33* were made. Under dehydration conditions, the suppressed lines showed lower osmotic potential compared with that of wild-type plants, suggesting a role of *OsMAPK33* in osmotic homeostasis. Nonetheless, the suppressed lines did not display any significant difference in drought tolerance compared with their wild-type plants. With increased salinity, there was still no difference in salt tolerance between *OsMAPK33*-suppressed lines and their wild-type plants. However, the overexpressing lines showed greater reduction in biomass accumulation and higher sodium uptake into cells, resulting in a lower K^+/Na^+ ratio inside the cell than that in the wild-type plants and *OsMAPK33*-suppressed lines. These results suggest that *OsMAPK33* could play a negative role in salt tolerance through unfavourable ion homeostasis. Gene expression profiling of *OsMAPK33* transgenic lines through rice DNA chip analysis showed that *OsMAPK33* altered expression of genes involved in ion transport. Further characterization of downstream components will elucidate various biological functions of this novel rice MAPK.

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1. Introduction

Plants have evolved protective mechanisms to cope with various unfavourable environmental conditions such as cold, drought, salinity and nutrient deficits. Sensing and relay of extracellular stimuli to initiate adaptive responses are the key processes leading to stress tolerance in plant (Chinnusamy *et al*. 2004). Despite adaptive responses, abiotic stresses represent

Keywords. Abiotic stress tolerance; MAPK; microarray; rice; transgenic plants

Abbreviations used: ABA, abscisic acid; IPTG, isopropyl-β-D-thiogalactose; MAPK, mitogen-activated protein kinases

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the primary cause of loss of crop productivity worldwide, reducing average yields for most major crops by more than 50% (Boyer 1982; Bray *et al*. 2000)

Mitogen-activated protein kinases (MAPK) signalling cascades are known to play diverse roles in the response of organisms to external stresses as well as in growth and development in higher eukaryotic cells (Lewis *et al*. 1998; Kyriakis and Avruch 2001; Zwerger and Hirt 2001; Nakagami *et al*. 2005). MAPK signalling cascades consist of the following three sequential protein kinase modules, which are highly conserved in all eukaryotes: MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK. MAPK is activated by the dual-specificity serine/threonine tyrosine kinase MAPKK, which is in turn activated by the serine/threonine kinase MAPKKK (Seger and Krebs 1995; Robinson and Cobb 1997; Widmann *et al*. 1999). Efficient signal transduction can occur through direct association among the components of the cascade or by component binding to cellular scaffold proteins (Whitmarsh and Davis 1998; Zhang and Klessig 2001).

MAPK is the final component of the MAPK cascades and is shared among multiple cellular responses. Plants are equipped with greater numbers of genes encoding MAPKs than other eukaryotes. For example, yeasts have 6 genes encoding MAPK proteins, mammals have 13, *Arabidopsis* plants have 20 and rice plants have 15 (Hunter and Plowman 1997; Hirt 2000; Meskiene and Hirt 2000; Ichimura *et al*. 2002; Hamel *et al*. 2006). Plant MAPKs can be divided into four groups designated as A–D. Based on the sequence alignment of the conserved amino acid motif TxY, which is phosphorylated by MAPKKs, MAPKs are classified into the subtypes TEY and TDY (Ichimura *et al*. 2002). The TEY subtype can be classified into three groups, namely, groups A, B and C, while the TDY subtype forms group D (Mizoguchi *et al*. 2000).

A variety of MAPKs have been identified in several plant species such as *Arabidopsis*, tobacco, rice and alfalfa. Members of several MAPK subtypes have been identified in plant stress responses (Mizoguchi *et al*. 2000; Agrawal *et al*. 2003; Nakagami *et al*. 2005). However, their biological functions are not fully understood so far owing to complicated roles of MAPK signalling cascades. In comparsion with dicots, the knowledge on the functions of MAPK cascade in monocots is very limited. Rice (*Oryza sativa*) has been known as a well-established model for monocot plants over the last decade, especially after the completion of the rice genome project. Examination of the rice genome sequence database revealed 15 putative MAPK genes, indicating that the monocot MAPK gene family may be smaller than the dicot family (Hamel *et al*. 2006). The first-discovered rice MAPK, named BWMK1, was isolated from an *Indica*-type cultivar 'IR36' from plants treated for infection by the blast fungus *Magnaporthe grisea* or upon mechanical wounding (He *et al*. 1999). The report provided the first evidence for the existence of a MAPK cascade component in rice plants and indicated its possible involvement in plant defense mechanisms. Although several novel MAPKs have been identified so far, a complete MAPK cascade consisting of sensor and downstream elements remains to be elucidated, with the exception of MAPK signalling of pathogen-associated molecular patterns (PAMPs), phytoalexin biosynthesis and stomatal development in *Arabidopsis* (Asai *et al*. 2002; Wang *et al*. 2007; Ren *et al*. 2008).

We previously reported that *OsMAPK44*, a rice gene encoding a MAPK, inversely modulated salinity and oxidative stress tolerance (Jeong *et al*. 2006). In this study, a rice MAPK gene induced by drought stress was also identified. This cDNA clone, named *OsMAPK33* (Os02g0148100), had already been isolated by other groups (GenBank accession number AF216317 and AF241166), but its functions have not been identified yet. Therefore, this study aimed to analyse the *OsMAPK33* gene expression profile in detail under conditions of various abiotic stresses and investigated its biological functions in transgenic rice plants with overexpressed and suppressed *OsMAPK33*. In this study, the downstream components of rice MAPK33 signalling in response to abiotic stresses have been investigated through profiling of up- or down-regulated genes using microarrays.

2. Materials and methods

2.1 *Plant materials and stress treatment*

The plant materials (*Oryza sativa* L.) used in this study were rice cvs. 'Dongjin', 'Vandana' and 'IR64'. Dongjin was used as a control plant in all experiments, while Vandana and IR64, two *Indica*-type cultivars provided by the International Rice Research Institute (Los Baños, Philippines), were used to compare *OsMAPK33* expression in drought-tolerant and drought-sensitive rice cultivars, respectively. Unless indicated otherwise, abiotic stress treatment was performed on 2-week-old seedling rice plants at four- or five-leaf stages. Drought stress was induced by two different methods: plant dehydration on Whatman 3MM paper at room temperature or mannitol-induced dehydration through supplementation of 200 mM mannitol into Yoshida nutrient solution (Yoshida *et al*. 1976). Salt stress was imposed to plants by placing the 2-week-old seedlings in Yoshida nutrient solution containing 200 mM NaCl. Abscisic acid (ABA) and H_2O_2 treatments were prepared by spraying a 100 μM ABA solution and 1 mM H_2O_2 onto leaves, respectively. After imposing stresses, leaf and root samples were rapidly frozen in liquid nitrogen and stored at −80°C for further analysis. The whole plants were exposed to the major pathogens causing rice disease (rice blast, *Magna-*

porthe grisea, and bacterial leaf blight, *Xanthomonas oryzae* pv. *oryzae*) by spraying. Briefly, for bacterial inoculation, *X. oryzae* (pv. *oryzae* 10331 and 10385) was incubated in a peptone sucrose medium at 28°C. The inoculums were collected by scraping with 0.02% Triton X-100, and adjusted to 5×10^8 CFU/ml. For fungal inoculation, conidia of *M. grisea* were collected with 0.02% Tween20 from 10-day-old colonies on oatmeal agar (50 g oatmeal per 1000 ml), and the conidial concentration was adjusted to 2×10^5 conidia/ml prior to inoculation. Fungal inoculation onto 14-day-old seedlings was conducted by spraying by means of an air compressor. The fully expanded leaves of those seedling plants were used to isolate RNA.

2.2 *Recombinant protein production and kinase assays*

Escherichia coli strain M15 (pREP4) (Qiagen, USA) was used for expression of His-fused *OsMAPK33* protein. The open reading frame of *OsMAPK33* was amplified by PCR with the following primers: forward, 5′-CGGGGTACCATGGCGAT CATGGTG-3′, and reverse, 5′-CCCCAAGCTTGAAGAT CATCGGGC-3′. The *Kpn*I/*Hin*dIII restriction enzyme sites underlined were introduced into the primers for cloning. The PCR products were cloned in the *E. coli* overexpression vector pQE30 (Qiagen, USA). The reading frame of the cloned coding region was confirmed by DNA sequencing using an automated sequencer (Genetic analyser ABI 3100, PE Applied Biosystems, USA). The transformed *E. coli* cells were grown at 37°C to mid-log phase and induced with 1 mM isopropyl-β-D-thiogalactose (IPTG). Exponentially growing cells induced for 4 h were harvested and re-suspended in protein extraction lysis buffer (Qiagen, USA). Soluble proteins were purified using isopropyl-β-D-thiogalactose (IPTG) affinity column.

Aliquots of His-OsMAPK33 fusion protein were incubated in the kinase assay buffer (10 μCi [γ -^{32}P] ATP, 20 mM Tris–HCl [pH 7.5] and 10 mM $MnCl_2$) at 30°C for 30 min. To assay substrate phosphorylation, 1 μg of generic substrate (myelin basic protein) was added to the kinase assay buffer. The reaction was stopped by the addition of 5× SDS sample buffer, boiled immediately for 5 min and analysed by SDS-PAGE. Gels of SDS-PAGE were stained with Coomassie brilliant blue R-250, dried and analysed with a phosphorimage analyser (Personal Molecular Imager FX system, BioRad, USA).

2.3 *Southern and Northern blot analysis*

Genomic DNA was isolated from rice using a genomic DNA prep kit according to the manufacturer's instructions (SolGent, Korea). A total of 15 μg of genomic DNA was digested with the restriction enzymes *Bam*HI, *Eco*RV and *Hin*dIII. Digested DNA fragments were separated on a 0.9% agarose gel and then transferred onto a nylon membrane (Amersham Pharmacia, USA) and UV cross-linked. Hybridization was performed in Church buffer (0.5 M $NaHPO_4$ [pH 7.2], 7% [w/v] SDS, 1 mM EDTA and 1% crystalline BSA) using a full-length *OsMAPK33* cDNA probe at 65°C overnight. The membrane was washed in 2× SSC, 1× SSC/0.1% SDS and then in 0.5× SSC/0.1% SDS at 65°C for 20 min each.

Total RNA was extracted from 2-week-old seedlings treated with various target stresses for various time periods using Trizol® Reagent according to the manufacturer's instructions (Invitrogen, USA). RNAs (20 μg per lane) were separated by 1.2% formaldehyde agarose gel electrophoresis and then transferred to a nylon membrane (Amersham Pharmacia, USA) and then UV cross-linked. Hybridization was performed in Church buffer using a cDNA probe (780 bp) produced by digestion of *OsMAPK33* with *Eco*RI and *Sac*I. The membranes were sequentially washed with 2× SSC and 1× SSC for 20 min each, and rewashed with 0.5× SSC/0.1% SDS for 10 min at 65°C. The washed membrane was exposed to an image plate and analysed with a phosphor image analyser (Personal Molecular Imager FX system, BioRad, USA).

2.4 *Vector construction and plant transformation*

Plant expression vectors were constructed using the Gateway™ Cloning system (Invitrogen, USA) to generate transgenic rice plants with both *OsMAPK33* overexpression and suppression. *OsMAPK33* gene-specific primers were fused with attB1 and attB2 for lambda recombination (BP and LR reaction). The primer sequences, which were used to amplify the *OsMAPK33* gene fragment for vector constructions, were as follows: for overexpression, forward (5′-GGGGACAAGTTTGTACAAAAAAGCAGGC TTCATGGCGATCATGGTGGATCCT-3′) and reverse (5′-GGGGACCACTTTGTACAAGAAAGCTGGGT CTCATCGGGCACTCATTGCTGC-3′); for suppression, forward (5′-GGGGACAAGTTTGTACAAAAAAG3′) and reverse (5′-GGGGACCACTTTGTACAAGAAAGCTGGG TACTGAATTCACTTTAATAGC-3′).

The PCR products were introduced into the donor vector pDONR201 (Invitrogen, USA) and then integrated into the plant binary vectors pB7WG2D and pB7GWIWG2 (II) as described by Karimi *et al.* (2002) for overexpression and suppression, respectively. Recombination reactions were performed on the basis of the manufacturer's instructions (Invitrogen, USA). The recombinant plasmids were introduced into *Agrobacterium tumefaciens* LBA4404 for rice plant transformation. Rice plant transformation was carried

out using actively growing calli derived from mature embryos of rice (*Oryza sativa* cv. 'Dongjin') as described by Hiei *et al.* (1994) and Jeong *et al.* (2006). The regenerated plants were transferred into pots containing soil. After recovering from transplanting, the recombinant plants were screened by herbicide 'basta' application (0.3% v/v) to confirm the gene insertion. The transgenic plants showing resistance to herbicide were self-fertilized and harvested in a greenhouse.

2.5 *PCR amplification*

PCR amplification was performed using Taq polymerase (Invitrogen, USA) as follows: 94°C for 2 min, and 25–30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 72°C for 10 min. For RT-PCR, first-strand cDNAs were synthesized from 5 μg of total RNAs isolated from transformed plants using the SuperScript III first-strand synthesis system on the basis of the manufacturer's instructions (Invitrogen, USA). Following reverse transcription, each of the gene transcripts was amplified for 25 cycles using the same PCR condition and gene-specific primers. The gene-specific primers were as follows: OsMAPK33, 5′-CTCGCGGTGTCTCGGGTAGG-3′ and 5′-ATTGGCAGTGATCATTGGAAAGAC-3′; OsIFCC018678, 5′-ATATGGGTTGGGGCGCGGTG-3′ and 5′-TCAATC AATTGGGTGCTTGTC-3′; OsIFSB004392, 5′-GGATGCAA CAGTTAAGAACA-3′ and 5′-CCCCCATCACCCAAGCG GCGA-3′.

2.6 *Drought and salt tolerance analysis*

Water loss and osmotic potentials were measured in order to assess the drought tolerance of *OsMAPK33* transgenic lines. Upon uprooting from a water culture, the plant roots were blotted using a blotting paper to remove any surface water on the roots. The rate of water loss was determined as previously described (Kwon *et al.* 2009). Briefly, intact whole plants were continuously weighed on a chemical balance in a controlled environment chamber (25°C and RH 60%) every minute from 0 to 300 min after dehydration stress was started. Changes in plant fresh weight within unit time were logged on a spreadsheet using a communication software and interface (RS-232). Changes at 1 min intervals indicated water loss due to dehydration. For the measurement of osmotic potentials, sampled plants were wrapped in a layer of parafilm followed by foil in order to prevent evapotranspiration from the surface of the sampled plants. The wrapped samples were frozen by plunging them into liquid nitrogen, and then the plants were thawed at ambient room temperature for 20 min. The thawed samples were unwrapped and put into a centrifuge tube to extract cell sap. Cell sap was extracted at 10000*g* relative centrifugal force for 20 min at 4°C. The osmotic potential of the cell sap was determined using a vapour pressure osmometer (Vapro 5520, Wescor, USA).

The salt tolerance of the *OsMAPK33* transgenic lines was determined as follows. First, young transgenic plant tillers were grown in a Yoshida's nutrient solution for 2 weeks until vigorous adventitious roots appeared. Then, salt stress was imposed with the supplement of 40 mM NaCl into the nutrient solution for 10 days. The area of the upper parts of the plants was determined before and after treatment with NaCl using a portable area meter (LI-300A. LI-COR, USA). The cell sap of leaves was extracted to measure the concentration of cations. Sodium, potassium and calcium ion concentrations were determined using an inductively coupled plasma optical emission spectrometer (Integra XL, GBC scientific, USA) after diluting the cell sap samples 100-fold in 0.7% HNO_3.

2.7 *Microarray analysis*

Microarray experiments were performed using the Rice 60K Oligomeric DNA Chip (GG Biotech., Korea). Probes were prepared from total RNA of the upper parts of *OsMAPK33* transgenic rice plants or from wild-type control plants with or without drought stress treatment (200 mM mannitol for 18 h). Microarray analysis was performed with RNA purified from independently harvested plant tissues. Hybridization, washing and scanning were carried out according to the manufacturer's instructions. Briefly, mRNA was purified from 100 μg of total RNA using a Qiagen Oligotex column. cDNA synthesis and post-labelling were performed according to the directions provided in the Genisphere Expression Array Detection Kit (Array 50 Version 2, Genisphere, USA). Individual slides were scanned using a GenePix 4000B (Axon Instruments, USA). Data were primarily handled using GenePix 5.0 software and then analysed using Acuity 3.1 software (Axon Instruments, USA) for normalization and hierarchical clustering. Functional classification of genes was performed using the COG database (*http://www.ncbi.nlm.nih.gov/COG*).

3. Results

3.1 *Isolation and characterization of the OsMAPK33 cDNA*

To study the relationship between MAP kinases and abiotic stress signal transduction, candidate MAPKs were identified from the Rice Genome Research Program EST database (NIAS, Japan). Several putative MAPK genes were obtained and one uncharacterized gene was selected for

further analysis. The former ID of the selected gene was S20051 with GenBank accession number AU055782. Thereafter, it was submitted to the database with accession numbers AK119650 after completion of the rice full-length cDNA project (Consortium TRF-Lc 2003). Sequence analysis revealed that the cDNA, renamed as *OsMAPK33* (Os02g0148100), had a high degree of similarity to the Ser/Thr protein kinase genes (MAPKs) of other plant species. *OsMAPK33* contained 11 conserved subdomains and the phosphorylation activation motif (TEY) found in Ser/Thr protein kinases (data not shown). *OsMAPK33* was found to be the most closely related to OsMAP2 (92.7%) as previously reported (Wen *et al*. 2002). It also showed 44.1–92.7% identity at the amino acid level with previously reported plant MAPKs. The *OsMAPK33* cDNA was 1549 bp long and encoded a polypeptide of 370 amino acids with a predicted molecular mass of 42.5 kDa. This cDNA had the same deduced amino acid sequence as OsMAP3 (AF216317) (Wen *et al*. 2002) and OsMAPK2 (AF241166). The *Os*MAPK33 cDNA was recently annotated as 'OsMPK14' by The Institute for Genomic Research (TIGR) rice MAPK community. Phylogenetic analysis indicated that *OsMAPK33* belongs to C group (C2) of MAPK proteins, along with OsMAPK4, Osmsrmk3 and OsMAP2 (figure 1).

To confirm its kinase activity *in vitro*, the coding region of *OsMAPK33* cDNA was expressed in *E. coli.* However, the recombinant protein did not show kinase activity possibly due to protein instability or abnormal folding.

3.2 *Copy number and organ-specific gene expression*

The copy number of *OsMAPK33* in the rice genome was estimated using Southern blot analysis. Genomic DNA was digested with various restriction endonucleases, fractionated by electrophoresis and transferred to a membrane. The digested DNA was hybridized with ^{32}P-labelled probe generated from full-length *OsMAPK33* cDNA. Under low stringency conditions, a single hybridization signal was detected in *Bam*HI- and *Eco*RV-digested genomic DNA, and two signals were detected in *Hin*dIII-digested genomic DNA (figure 2A). Based on information derived from the rice genome database (GenBank), we found that *OsMAPK33* was located on chromosome 2 and the encoding genomic DNA was composed of three exons and two introns spanning a 3.3 kb genomic region. These results indicate that *OsMAPK33* exists as a single copy in the rice genome.

Gene expression was examined in different tissues to determine whether *OsMAPK33* exhibited spatial regulation within rice plants. Northern blot analysis indicated organ specificity with higher expression in leaves than in roots and stems (figure 2B).

3.3 *OsMAPK33 expression profiling in response to various stresses*

To characterize the expression profile of *OsMAPK33*, mRNA transcript levels were analysed under various stress conditions. Total RNA was isolated from *Oryza sativa* (cv. Dongjin) subjected to the drought stress by dehydration or mannitol treatment, and salt stress. Under dehydration, *OsMAPK33* mRNA levels quickly increased from 15 min until 2 h after stress treatment initiation and then gradually decreased to basal levels after 3 h (figure 3A). Gene expression was also induced to a maximum level at 1 h after starting the treatment of 200 mM mannitol (figure 3B). In contrast, salt stress reduced the *OsMAPK33* transcript level until 8 h after NaCl treatment, indicating its negative regulation in the presence of salt stress (figure 3C). The time course of *OsMAPK33* expression was also analysed in different organs of plants subjected to drought stress. *OsMAPK33* transcript levels remained essentially constant and high in roots and leaves after treatment with 200 mM mannitol (data not shown). *OsMAPK33* mRNA gradually accumulated in the stem over time. To check for cross-talk of *OsMAPK33* with other extracellular signalling pathways, 2-week-old rice seedlings were treated with the phytohormone abscisic acid (ABA) and a potent reactive oxygen species (H_2O_2). Northern blot analysis showed that *OsMAPK33* mRNA was slightly induced from 30 min to 24 h after the start of the treatment with signalling molecules and then almost disappeared at 48 h, indicating that there was a very weak interaction between *OsMAPK33* signalling cascade and the signalling molecules (supplementary figure 1).

The *OsMAPK33* gene was induced under biotic stress conditions such as by treatment with fungal (*M. grisea*) and bacterial (*X. oryzae*) pathogens. The amount of mRNA transcripts varied depending on the kingdom of pathogen, with more induction when exposed to the fungus. However, pathogen property that is compatible or incompatible did not affect *OsMAPK33* gene expression pattern (supplementary figure 2).

3.4 *Functional analysis of OsMAPK33 transgenic lines in response to abiotic stresses*

To investigate the biological function of *OsMAPK33*, transgenic rice plants were produced in which *OsMAPK33* expression was either enhanced or suppressed under the control of a constitutive CaMV35S promoter (figure 4A). Overexpression and suppression of *OsMAPK33* was confirmed in independent transgenic lines by RT-PCR (figure 4B). The growth and size of transgenic plants did

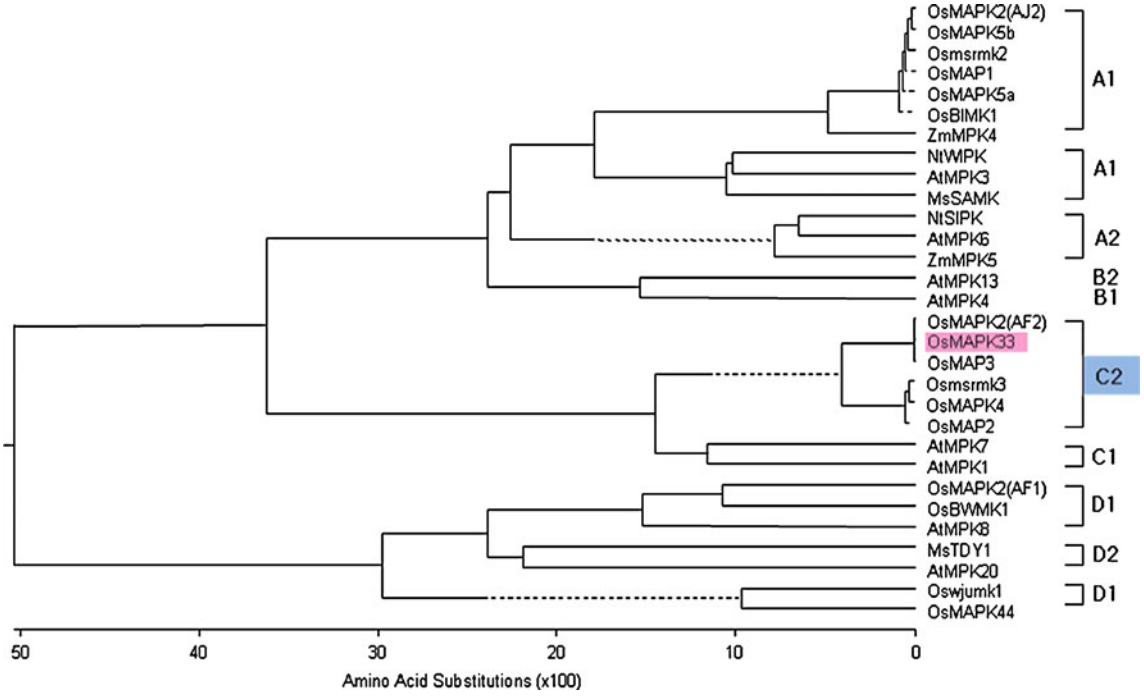

Figure 1. Phylogenetic relationship of *OsMAPK33* with other plant's MAPKs. The phylogenetic tree includes MAPKs from rice (*Oryza sativa,* Os), maize (*Zea mays,* Zm), tobacco (*Nicotiana tabacum,* Nt), alfalfa (*Medicago sativa,* Ms) and *Arabidopsis* (*Arabidopsis thaliana,* At). The phylogenetic tree was constructed by the Clustal W method using the DNASTAR program ('Lasergene 7.2.1'). The amino acid sequences of OsMAPK2 (AJ2), OsMAPK2 (AF2) and OsMAPK2 (AF1), represent AJ250311, AF241166 and AF 194416, respectively. Plant MAPK homologs containing the dual activation phosphorylation motif (TEY or TDY) are grouped into four groups (A–D). The TEY subtype was classified into three groups (A, B and C); the TDY subtype formed group D.

not differ from non-transformed wild-type plants. Physiological changes in *OsMAPK33* transgenic rice lines in response to drought and salt stress were analysed. The *OsMAPK33*-suppressed lines did not show any significant difference with wild-type plants (cv. Dongjin) both phenotypically and physiologically such as in terms of water loss rate and osmotic potentials after exposure to 200 mM mannitol. Interestingly, *OsMAPK33*-suppressed lines had lower osmotic potentials than wild-type plants with or without dehydration (table 1). Under salt stress, *OsMAPK33*-suppressed plants did not exhibit any difference with wild-type plants known to be moderately salt tolerant. Unlike the drought stress response, there was no difference in osmotic potentials between *OsMAPK33*-suppressed lines and wild-type plants (table 1). However, *OsMAPK33*-overexpressing lines showed a significant difference in salt tolerance compared with wild-type plants and *OsMAPK33*-suppressed lines (figure 5). The overexpressing lines showed more damage in growth and higher sodium uptake into the cell, resulting in a lower K^+/Na^+ ratio inside cells than wild type or *OsMAPK33*-suppressed lines in the presence of salt stress. These results suggest that *OsMAPK33* could play a negative role in salt tolerance through unfavourable ion homeostasis.

OsMAPK33-suppressed lines did not show a difference in drought tolerance compared with wild-type plants even though *OsMAPK33* gene expression was induced in rice plants by drought stress. To validate involvement of *OsMAPK33* in the rice plant's drought tolerance mechanism, the *OsMAPK33* gene expression pattern was investigated between the drought-tolerant (cv. Vandana) and drought-sensitive (cv. IR64) genotype. The transcript level of *OsMAPK33* was not positively correlated with drought tolerance. Instead, *OsMAPK33* was strongly induced in the drought-sensitive cultivar but not in the drought-tolerant cultivar (supplementary figure 3). This result indicates that *OsMAPK33* does not play a role in drought tolerance mechanism, especially in cv. Vandana genotype. Pathogen resistance was assessed in *OsMAPK33* transgenic lines because the results showed that pathogen treatment slightly induced *OsMAPK33* expression. Expression profiling of some PR genes using RT-PCR did not show any change in their transcripts levels (data not shown). The disease assay also revealed no significant difference in resistance to *X. oryzae* among *OsMAPK33*-suppressed, *OsMAPK33*-overexpressing and wild-type rice plants (figure 6).

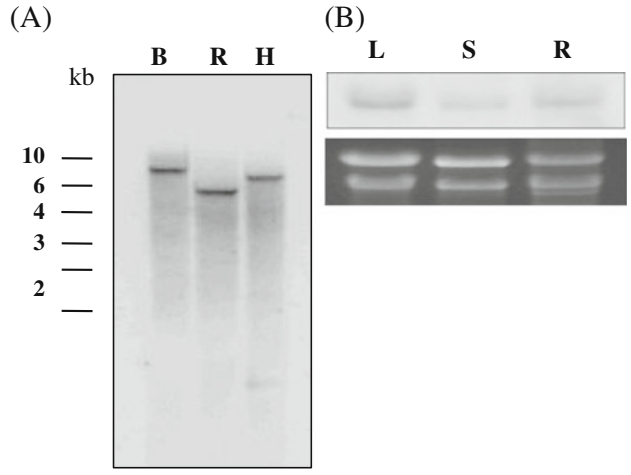

Figure 2. Genomic organization in the rice genome (**A**) and organ-specific expression of *OsMAPK33* under normal growth conditions (**B**). (**A**) Genomic DNA (15 μg per lane) from leaves was digested with the restriction enzymes *Bam*HI (B), *Eco*RV (R) and *Hin*dIII (H), and separated by electrophoresis on a 0.7% (w/v) agarose gel. After blotting onto nylon membranes, membranes were hybridized using a [α-^{32}P] dCTP-labelled *OsMAPK33* cDNA probe. The DNA size in kb is indicated at the left. (**B**) Total RNA was prepared from the leaves (L), stems (S) and roots (R) of 2-week-old rice plants. RNA (20 μg per lane) was separated by 1.2% formaldehyde agarose gel electrophoresis, and then transferred to nylon membranes. Northern blots were probed with ^{32}P-labelled *OsMAPK33* cDNA.

3.5 *Effect of OsMAPK33 suppression to the expression of signalling and metabolism-related genes*

Microarray experiments were performed on an *OsMAPK33*-suppressed line to study the expression of downstream genes of the *OsMAPK33*-regulated signal pathway. A total of 114 and 82 unique genes were identified in independent replicate experiments, displaying more than a 1.5-fold decrease in their expression in the *OsMAPK33*-suppressed lines. The functional categories of the genes were determined based on Clusters of Orthologous Groups of proteins (COGs) annotation summarized in table 2. The largest category of genes that showed significant spots was annotated as 'cellular processes and signalling'. Many of the genes involved in signal transduction and metabolism, such as ion transporters and protein kinases, displayed a significant fold change at the transcript level. The microarray analysis revealed that *OsMAPK33* affected the transcript level of genes involved in ion homeostasis, such as genes encoding efflux pumps and the K^+/H^+ antiporter. Expression profiles from the microarray experiment were confirmed by RT-PCR using several gene-specific primers (figure 7). A decrease in the transcript level of some genes in *OsMAPK33*-suppressed plants was observed under normal and stressed conditions, indicating that expression of these genes is down-regulated by *OsMAPK33*.

4. Discussion

Although several complete MAPK signalling cascades have been established for yeast and *Arabidopsis*, a detailed plant MAPK signalling pathways remains to be elucidated. Therefore, this study aimed to identify additional plant MAPK functions using reverse genetics and to investigate downstream elements of MAPK signalling pathways in rice plants under abiotic stresses. This study may help expand the knowledge of plant MAPK-induced signalling pathways in response to abiotic stresses through the physiological characterization of rice plants with overexpressed or suppressed *OsMAPK33*, identification and expression profiling of downstream genes using microarrays and analysis of cross-talking between abiotic and biotic stress pathways.

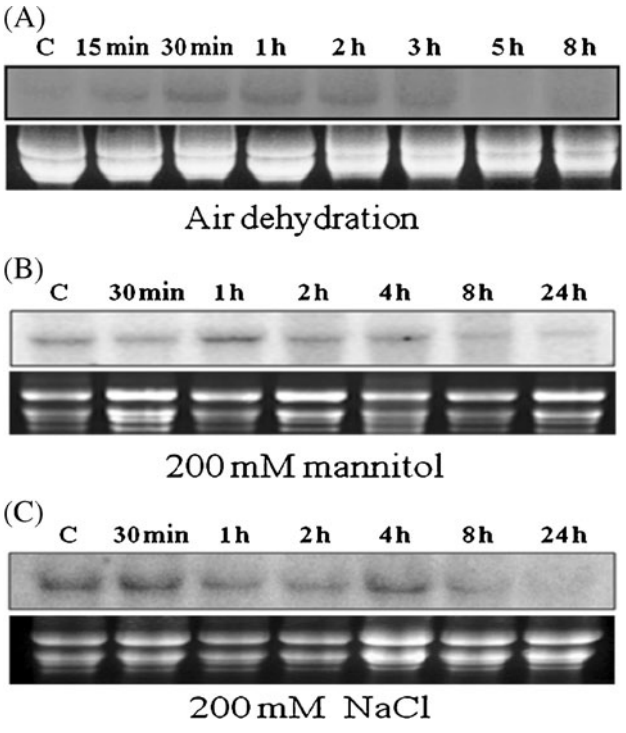

Figure 3. Gene expression profiling of *OsMAPK33* under abiotic stress conditions. Total RNA was prepared from upper parts of 2-week-old wild-type rice plants (cv. Dongjin) treated with air dehydration (**A**), 200 mM mannitol (**B**) and 200 mM NaCl (**C**) for various time periods. A total of 20 μg RNA was separated by 1.2% formaldehyde agarose gel electrophoresis, and then transferred to nylon membranes. Northern blots were probed with ^{32}P-labelled OsMAPK33 cDNA. 'C' refers to the non-treated control.

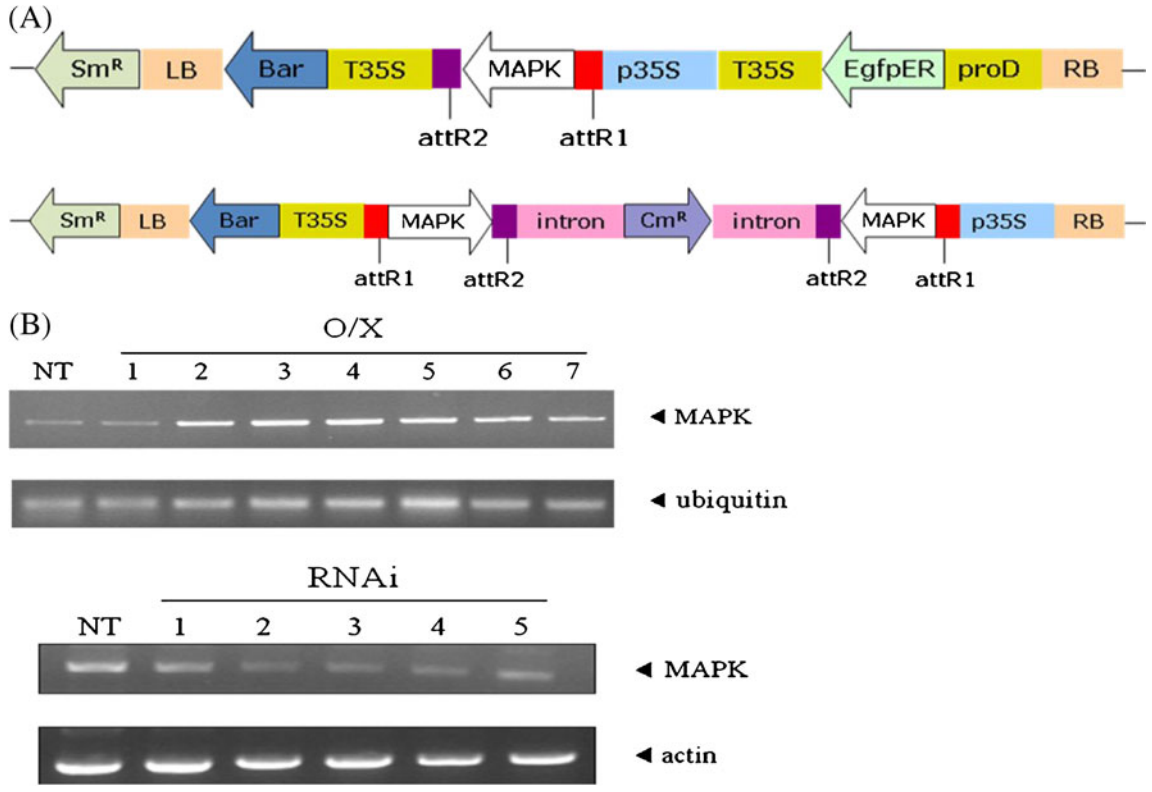

Figure 4. Vector construction (**A**) and confirmation of *OsMAPK33* overexpression and suppression in transgenic rice plants (**B**). (**A**) Gateway binary vector pB7WG2D and pB7GWIWG2(II) were used for overexpression (*top*) and suppression (*bottom*) of *OsMAPK33*, respectively. (**B**) mRNA transcript levels of *OsMAPK33* in transgenic lines were analysed by RT-PCR. Total RNA was prepared from upper parts of 2-week-old transgenic rice plants. Following reverse transcription, *OsMAPK33* gene transcripts were PCR-amplified for 25 cycles using gene-specific primers. The ubiquitin, actin mRNA were used as a control for ubiquitous, constitutive expression. NT, O/X and RNAi indicate the non-transformed wild-type plants (cv. Dongjin), *OsMAPK33* overexpressing lines and *OsMAPK33* suppressed lines, respectively

Table 1. Osmotic potential of the expressed cell sap from OsMAPK33-RNAi transgenic lines and wild-type plants cv. Dongjin

Dehydration stress			Salinity stress		
Time (min)	Wild type	M3 RNAi	NaCl (mM)	Wild type	M3 RNAi
0	−8.28	−9.87	0	−8.25	−9.87
30	−9.34	−9.67	70	−17.98	−17.95
100	−9.9	−10.65			
300	−12.78	−15.45			
	df	Pr > F		df	Pr > F
Genotype (A)	1	0.0197		1	0.5234
Treatment (B)	3	<0.0001		1	<0.0001
A × B	3	0.4154		1	0.5081

Plants were exposed to air dehydration stress until 300 min (left) and salinity stress of 70 mM NaCl for 7 days (right). The value of osmotic potential is presented as bar unit.

df, the degrees of freedom; F, the F statistic associated with the given source; Pr, the *P*-value associated with the F statistic of a given source.

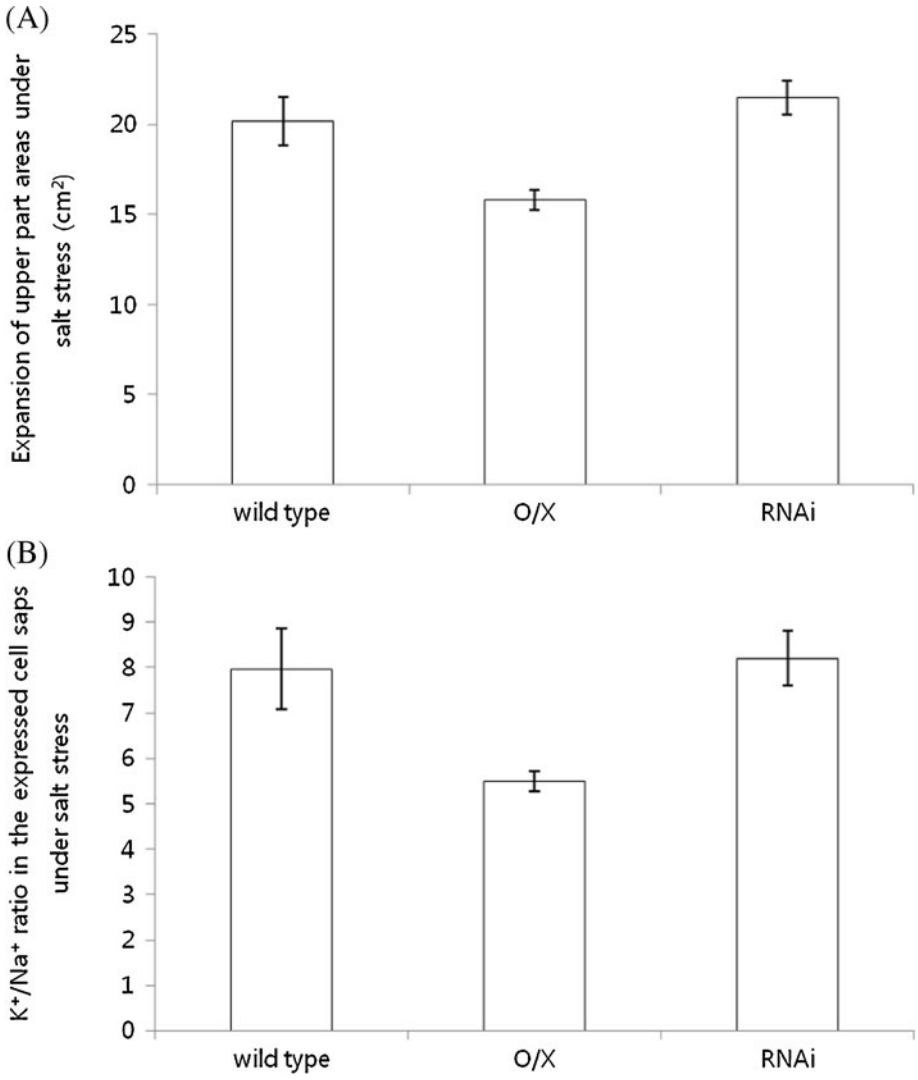

Figure 5. Salt tolerance of transgenic rice plants with *OsMAPK33* overexpression or suppression. (**A**) Expansion of upper-part areas of transgenic plants under salt stress conditions for the indicated time period (10 days). (**B**) K^+/Na^+ ratio in the expressed cell saps from the two different transgenic lines under salt stress conditions. Salt stress was induced by imposition of 40 mM NaCl for 10 days. Wild type refers to the parental cv. Dongjin. O/X and RNAi indicate overexpression and suppression, respectively. Data represent mean ± SE (n=13).

The combined results suggest that *OsMAPK33* could play as a transmitter of extracellular stimuli to downstream genes and has a negative role in salt tolerance.

This study also found that *OsMAPK33* is constitutively expressed in leaves, which is consistent with other rice MAPKs, such as *OsMAPK2* (Huang *et al*. 2002), *OsMAPK4* (Fu *et al*. 2002) and *OsBWMK1* (Cheong *et al*. 2003). Therefore, it could be suggested that the basal level expression of this MAPK may have functional significance under both normal and stressed conditions in rice plants. Several pieces of evidence indicate that MAPKs are involved in plant developmental processes (Wilson *et al*. 1997; Huang *et al*. 2002; Wang *et al*. 2007). Organ-specific expression of *OsMAPK33* supports its involvement in the growth and development of rice plants in addition to its role in defense/stress pathways.

Time-course gene expression profiling showed that *OsMAPK33* transcript levels in leaves changed rapidly in response to drought stress. This finding is of special interest because the leaves were not in direct contact with the 200 mM mannitol solution. The observed rapid changes indicate the existence of long-distance messengers that move from the roots to the shoots in rice. Potential long-distance signals that could perform this function include nutrients (Forde 2002) and hormones such as abscisic acid (ABA) (Schroeder *et al*. 2001), calcium-mediated action

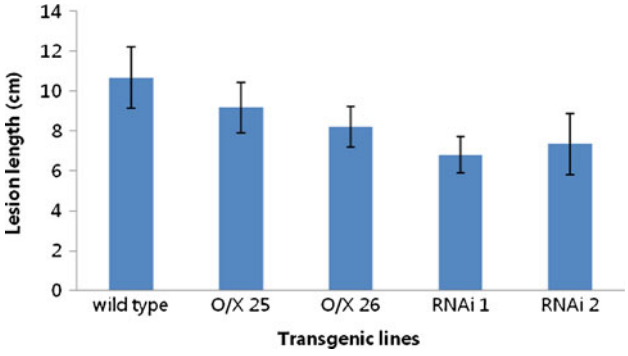

Figure 6. Disease assays of *OsMAPK33* transgenic lines following exposure to *Xanthomonas oryzae* pv. *oryzae* (K1 race, KACC 10331). Ten individual plants (T_1) from each transgenic line were inoculated with *X. oryzae* by the clipping method. Lesion lengths were measured at 14 days post inoculation. Data represent mean ± SD.

potentials (Knight 2000) or a disturbed water potential gradient from roots to leaves (Nonami *et al*. 1997). This long-distance signalling by hormones may be mediated by reactive oxygen species (Lake *et al*. 2002).

Treatment with signalling molecules (ABA and H_2O_2) showed that *OsMAPK33* mRNA levels were rarely induced, suggesting that the *OsMAPK33* signalling pathway may be independent from the ABA- and H_2O_2-mediated signalling cascade. However, pathogen treatment slightly increased the *OsMAPK33* transcript level. This finding indicates the steady nature of the expression of *OsMAPK33* gene as stated in a previous report as one of the inherent properties of MAPKs (Ligterink and Hirt 2001). Considering that only a small number of MAPKs are involved in responses to various stresses, this finding is very consistent with the findings of other studies that MAPKs are one of the points of convergence for defense signalling networks (Zhang and Klessig 2001; Ichimura *et al*. 2002).

Table 2. Characterization of mRNAs with expression levels that decreased greater than 1.5-fold in OsMAPK33-suppressed transgenic rice treated with 200 mM mannitol

COG	COG ID	Fold		Gene
		1st	2nd	
[R]	Serine/threonine protein kinase	−17.30	−8.66	*OsIFCC024781*
[R]	SNF2 family DNA-dependent ATPase	−8.76	−4.11	*OsIFCC023224*
[R]	FOG: Transposon-encoded proteins with TYA/reverse transcriptase/integrase domains in various combinations	−7.67	−7.20	*OsIFSB001264*
[T]	Apoptotic ATPase	−7.37	−6.78	*OsJRFA103659*
[R]	Uncharacterized membrane protein/predicted efflux pump	−6.20	−6.23	*OsJRFA107345*
[J]	Mitochondrial/chloroplast ribosomal protein S16	−3.93	−2.40	*OsJRUA000010*
[R]	C2 Ca2+−binding motif-containing protein	−2.96	−2.56	*OsIRUA003968*
[R]	**FOG: TPR repeat***	−2.77	−2.38	*OsIFSB004392*
[OE]	Serine carboxypeptidases (lysosomal cathepsin A)	−2.50	−1.53	*OsJRFA103461*
[G]	Predicted sugar kinase	−2.12	−1.70	*OsJRFA103343*
[Q]	Dehydrogenases with different specificities (related to short-chain alcohol dehydrogenases)	−2.08	−1.63	*OsJRFA064532*
[S]	Uncharacterized conserved protein	−1.84	−1.69	*OsIFCC005756*
[T]	Acetylcholine receptor	−1.83	−1.63	*OsJRUA000531*
[T]	**Predicted G-protein-coupled receptor**	−1.73	−1.57	*OsIFCC018678*
[KL]	Mitochondrial/chloroplast DNA-directed RNA polymerase RPO41/provides primers for DNA replication-initiation	−1.68	−3.17	*OsJRFA069977*
[Y]	Nucleolar GTPase/ATPase p130	−1.64	−1.66	*OsIFCC003144*
[C]	NADH dehydrogenase subunit 1	−1.58	−1.64	*OsJRFA064531*
[V]	Arylacetamide deacetylase	−1.57	−1.54	*OsJRFA071569*
[S]	Uncharacterized conserved protein	−1.54	−1.86	*OsIFCC023719*
[DZ]	Microtubule-associated protein essential for anaphase spindle elongation	−1.54	−1.56	*OsJRFA105451*
[P]	Predicted K+/H+−antiporter	−1.54	−1.55	*OsIFCC041426*
[J]	Mitochondrial/chloroplast ribosomal protein S12	−1.53	−1.52	*OsIRUA000217*

Fold values represent the results of independent replicate experiments.

*Gene expression of COG IDs in bold was confirmed by RT-PCR.

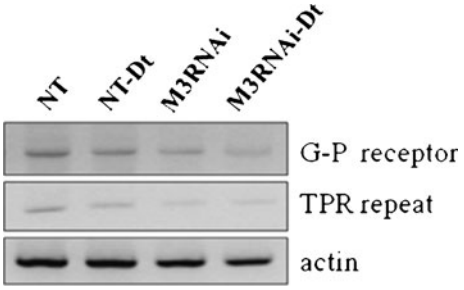

Figure 7. RT-PCR expression analysis of *OsMAPK33* downstream candidate genes selected from microarray experiments. The mRNA transcripts that showed more than 1.5-fold decrease in *OsMAPK33* suppressed lines were selected to confirm their expression. Total RNA was prepared from upper parts of 2-week-old transgenic rice plants. Following reverse transcription, each of the gene transcripts was PCR-amplified for 25 cycles using gene-specific primers. G-P receptor and TPR repeat indicate G-protein-coupled receptor (OsIFCC018678) and FOG: TPR repeat (OsIFSB004392), respectively. Actin was used as a control for ubiquitous constitutive gene expression. NT refers to non-transformed wild-type plant, cv. Dongjin.

Condition-dependent inactivation of the MAPK pathways is as important as its activation. The transient nature of MAPK activation by various stresses has been established to be the result of *de novo* synthesis of a negative regulator, possibly a MAPK phosphatase (Zhang and Klessig 2001). Previous studies on the induction of several defense/stress-related genes showed that cytoplasmic *de novo*-synthesized positive regulators were needed for up-regulation in stressed leaves (Agrawal *et al*. 2001, 2002). It also indicates that both negative and positive regulators are synthesized *de novo* under stress, which maintains a balance in the regulators controlling the defense/stress response. To determine whether *de novo*-synthesized proteins are involved in the regulation of *OsMAPK33* expression, further biochemical studies are required.

Although dehydration increased *OsMAPK33* mRNA transcript levels, gene expression was hardly induced in the drought-resistant genotype but strongly induced in the drought-susceptible genotype. In addition, *OsMAPK33*-suppressed lines did not show a significant difference in drought tolerance compared with wild-type plants (cv. 'Dongjin'). These results were not expected, because it was thought that *OsMAPK33* might play a role in drought tolerance through regulation of drought-tolerance-related genes based on induction under drought stress as described in figure 3. The results suggest that this gene plays a role in transmitting extracellular stimuli to downstream genes, resulting in change of osmotic balance but not affecting drought tolerance of whole plants, indicating the existence of other MAPK cascades for drought tolerance mechanisms in rice plants.

It was reported that some MAPK cascades could have a negative role in cell development (Lampard *et al*. 2008) and external stresses responses (Frye *et al*. 2001; Xiong and Yang 2003). The results of this study also showed that the overexpression of *OsMAPK33* in rice plants enhanced the sensitivity of the plants to salt stress. This suggests that the *OsMAPK33* gene could negatively regulate salt tolerance. The gene expression profile also showed a reduction in *OsMAPK33* transcript levels until 8 h after the initiation of NaCl treatment, indicating that the *OsMAPK33* gene is down-regulated. However, there is a need for additional experiments under high salt conditions for more convincing results. In contrast with previous studies that have shown cross-talking among biotic and abiotic stresses, there was no remarkable enhancement or reduction of disease resistance in *OsMAPK33* transgenic rice plants.

Microarray experiments using *OsMAPK33* transgenic rice plants revealed that the transcription of many genes categorized as being involved in signal transduction and metabolism, including ion transporters, protein kinases and transcription- and translation-related genes was altered under drought stress. These results allude to the complexity of MAPK signalling pathways activated in response to extracellular stimuli. Functional analysis of the inserted gene showed that overexpression of *OsMAPK33* enhanced sensitivity to salt stress through unfavourable ion homeostasis. Reduced expression of ion transporter genes, such as efflux pumps and the K^+/H^+ antiporter, supports an idea that *OsMAPK33* can contribute to ion homeostasis. Further study is needed to evaluate the downstream components of the negative regulation pathways using molecular approaches such as yeast two hybrid screening and proteomic profiling.

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