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Genome-wide transcriptome modulation in rice transgenic lines expressing engineered mitogen activated protein kinase kinase 6

Kundan Kumar† and Alok Krishna Sinha*

National Institute of Plant Genome Research; Aruna Asaf Ali Road; New Delhi, India

†Current address: Department of Biological Sciences; Birla Institute of Technology & Science Pilani; K. K. Birla Goa Campus; Goa, India

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Mitogen activated protein kinase kinase (MAPKK) is the central module of MAPK cascade and also point of signal integration and divergence. To investigate the regulatory role of *OsMKK6*, the regulon of genes controlled by *OsMKK6* was constructed by microarray analysis between constitutively activated overexpressing transgenic lines and the wild type rice. Regulated genes were identified in overexpressed constitutively activated *OsMKK6* and they were further subdivided on the basis of functional categories, viz. transcription, metabolism, signaling, defense and unknown function. These findings suggest the possible physiological role of *OsMKK6* in modulating gene expression and signaling pathways during different stresses.

Plants have developed sophisticated signaling machineries to protect them and to adapt their cellular metabolism in response to various forms of environmental biotic and abiotic stresses. The mitogen activated protein kinase (MAPK) transduction cascades connect the perception of these external environmental stimuli to physiological and cellular responses and have important mechanisms for stress adaptation by control of gene expression. The well conserved MAPK pathway consist of a protein kinase module that phosphorylate in an ordered cascade advanced from a MAPK kinase kinase (MAPK3K, MAPKKK, MKKK, MEKK) to a MAPK kinase (MAP2K, MAPKK, MKK, MEK), and then to a MAP kinase (MAPK).[1,2] MAPK are activated by phosphorylation on their threonine and tyrosine (TXY) residues located in the activation loop (T-loop) by MAP2K. MAP2K are dual specificity kinases and gets activated by phosphorylating two amino acids in the S/T-X_{3-5}-S/T motif of the activation loop.[3]

Arabidopsis genome analysis revealed the presence of 20 members of MAPKs, 10 MAP2Ks and around 80 MAP3Ks.[1,4,5] The 10 MAP2K genes of *Arabidopsis* are classified into four groups (A-D) based on their protein sequence alignments. Rice (*Oryza sativa* L.), a monocot cereal research model, is one of the most important food crops worldwide.[6,7] The completion of sequencing project revealed the presence of 15 MAPKs, 8 MAP2Ks, and 75 MAP3Ks in rice genome.[5,8] Out of eight MAP2Ks reported from rice, a partial functional characterization of only OsMKK6 has been performed so far. OsMKK6 (earlier named as OsMEK1) specifically and physically interacted with OsMPK3 (earlier named as OsMAP1) in moderate low temperature stress signaling as shown by yeast two hybrid assay.[9] Transcript studies have revealed that *OsMKK4* and *OsMKK6* are strongly regulated by salt and cold stress.[10] OsMKK4-OsMPK3 module was shown to be involved in arsenite stress signal transduction.[11] A MAP2K (*OsMEK1*), identified as *OsWNK1* (With No Lysine kinase) in rice and its probable role in circadian rhythm and abiotic stress has been reported.[12] Dominant mutants of constitutively active form of protein kinases are shown to be useful for determining various protein functions.[13,14] The functional role of *OsMKK6* in salinity stress using transgenic approach was showed in our report recently.[15] A constitutively active form of *OsMKK6* was generated by substituting putative phosphorylation sites from S and T to E residues (S221E and T227E by site directed mutagenesis). Transgenic rice overexpressing constitutive active $OsMKK6^{EE}$ exhibited higher expression of phytoalexin biosynthesis pathway genes upon UV stress and infection with *Magnaporthe oryzae* suggesting their role in biotic stress.[16] In the present study, global gene expression analysis was performed for the overexpressed constitutive active *OsMKK6* lines.

To define the total regulon of genes controlled by *OsMKK6*, DNA microarray analysis was performed in three constitutively active $OsMKK6^{EE}$ overexpressing lines and three wild type Pusa Basmati (PB1) plants. Two week old overexpressed lines and wild types rice shoots were subjected for total RNA extraction using the Qiagen RNeasy Mini kit (Qiagen, USA) according

*Correspondence to: Alok Krishna Sinha; Email: alok@nipgr.ac.in
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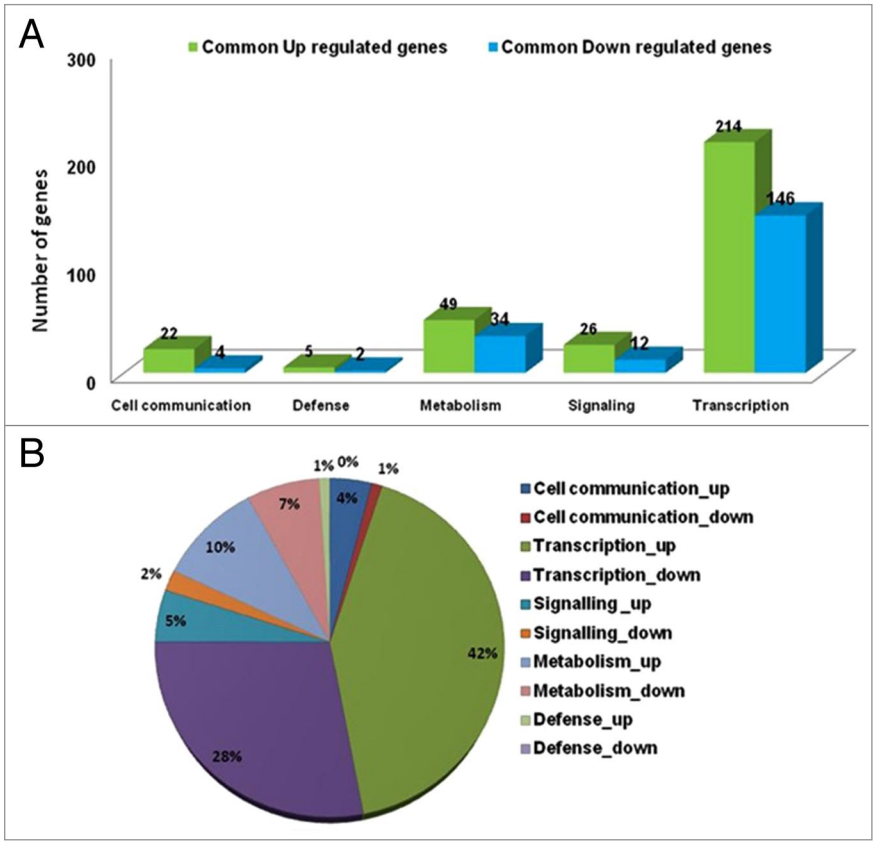

Figure 1. Functional classification of upregulated and downregulated genes in the *OsMKK6*^{EE} overexpressed transgenic plant. (**A**) Bar diagram representing number of genes upregulated and dow-regulated in relation to function in overexpressed lines compared with wild types. (**B**) Pie-diagram showing functional classification of common upregulated and downregulated genes in the overexpressed transgenic plant.

to manufacture's protocols. Quality and integrity of RNA was checked by measuring $A_{260/280}$ and RNA integrity number by Agilent BioAnalyzer (Agilent Technologies, USA). Samples were labelled using the Agilent low input RNA amplification kit and QC was performed using ND-1000 spectrophotometer measurements (NanoDrop Technologies, USA), and profile of amplified RNA was checked using Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Three independent transgenic lines of *OsMKK6*^{EE} and three independent wild type rice shoots were used for the experiment. The transcriptome analysis was performed using the Agilent's rice custom 44k array AMADID (Agilent Technologies, USA). Microarray experiments including the data analysis were performed according to manufacturer's instruction (Agilent technologies, USA). Feature extraction and image analysis software (v7.3; Agilent Technologies, USA) was used to locate and delineate every spot in the array and to integrate each spots intensity, filtering and normalization by the Lowess method (P value cut off as 0.01). The statistical significance of gene expression was tested using a one way analysis of variance test combined with Benjamini and Hochberg false discovery rate multiple correction algorithm (Genespring 7.3) with a corrected $P < 0.05$ set as cutoff. The normalization was done using Genespring GX using the recommended per chip and per gene data. Changes in signal intensity between transgenic and wild type experiments exceeding 3-fold or higher difference in repeated experiments were considered significant. The genes with a signal ratio of *OsMKK6*^{EE}/WT ≥ 2 (upregulated) or ≤ 0.5 (downregulated) were considered differentially expressed. Genes were considered differentially expressed for $P \leq 0.05$ in the *t* test.

Gene expression ratios were calculated and expression levels of transgenic and wild type were compared. A final set of 316 common genes in all transgenic lines showed upregulation more than 2-folds, while 198 common genes in all transgenic lines showed downregulation and these genes were further subdivided into five functional categories (**Fig. 1A**). 214 genes constituting 42% of upregulated genes and 146 genes constituting 28% of downregulated genes were involved in transcription. 10% of upregulated genes and 7% of downregulated genes were involved in metabolism. 5% of upregulated and 2% of downregulated genes were involved in signaling, 4% of upregulated and 1% of downregulated genes were involved in cell communication and 1% of upregulated genes were involved in defense responses (**Fig. 1A, 1B**). Nevertheless, about one third of upregulated and downregulated genes were annotated with unknown function. Some of the common upregulated and downregulated genes with their putative function are listed (**Table S1**) and plotted as heat map in supporting information (**Fig. S1**).

Validation of microarray data was performed by real-time quantitative RT–PCR (qRT–PCR) analysis of 2 week old rice shoots using StepOne real-time PCR systems (Applied

Biosystems, USA). The specific primers used in this qRT-PCR study are listed in supporting information (**Table S2**). Pathogenesis related-1 protein gene (*PR1*), Lipoxygenase 9 (*Lip9*), WRKY transcription factor 50 (*WRKY50*), *MAPK1*, Salt responsive gene (*SalT*), abscisic acid responsive gene (*ABA*), cytosolic ascorbate peroxidase (*APX*) and dehydration responsive element binding protein 1 (*DREB1*) were upregulated in overexpressed plants compared with wild type plants (**Fig. 2**). *SalT* is involved in salt stress[17] and *Lip9* has role in low temperature signal transduction pathway.[18] *ABA*, *APX*, and the transcription factor *OsDREB1* are known to be upregulated in abiotic stresses.[19,20] *PR1* is involved in pathogen response and Ribulose biphosphate carboxylase (*RbCs*) transcript remain unaltered in overexpressed lines compared with wild type control. In microarray analysis, *WRKY 50*, *Lip9*, *MAPK1*, *SalT*, *DREB1*, and *ABA* showed upregulation more than 3-folds. *RbCs* showed no changes in transgenic plant compared with wild type. The transcript abundance from qRT-PCR and microarray of 10 selected genes were positively correlated, which suggests that transcripts quantified by qRT-PCR showed similar regulation pattern as in DNA chip assay.

A final set of 316 genes showed upregulation and 198 genes showed downregulation in the constitutively active *OsMKK6^{EE}* transgenic plants as compared with the wild types. Comprehensive expression analysis by gene chip microarray revealed that a substantial proportion of transcription factors were expressed differentially in *OsMKK6^{EE}* transgenics. In agreement with the previous transcriptome analysis toward plant stress, multiple types of transcription factors constitute the largest group of upregulated genes.[21,22]As 1% of upregulated genes were also involved in defense, it suggest putative involvement of *OsMKK6* in plant pathogen defense along with abiotic stress tolerance.[16] *OsMAPK1* also showed enhanced expression level in microarray analysis as well as in qRT-PCR analysis which has already been reported as OsMKK6 interacting partner.[10] The genes quantified by qRT-PCR analysis showed similar expression pattern as in DNA chip analysis. *OsMKK6^{EE}* plants showed enhanced expression of *PR1*, *Lip9*, *APX*, *DREB1*, *SalT*, and *WRKY50* suggesting that these plants might be more tolerant to abiotic and biotic stresses. The constitutive expression of the OsMKK6 gene suggests that a pathways involving *OsMKK6* might regulate the activity of downstream MAPKs and the transcription factors, which may ultimately regulate various abiotic and biotic stress responsive genes.

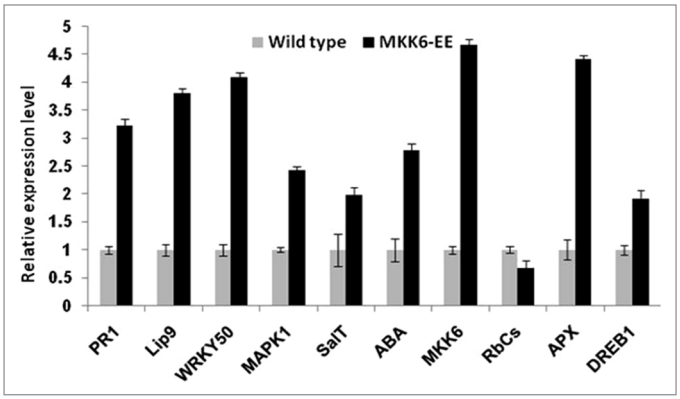

Figure 2. Real-time PCR quantification of relative expression levels of important genes regulated under different abiotic stress. Black bar denotes control wild type plant and gray bar denotes *OsMKK6^{EE}* over-expressed transgenic plants. Error bars indicate standard error of three independent biological samples.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplementary Material

Supplementary material may be found here:
http://www.landesbioscience.com/journals/psb/article/28502/

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