Differential Expression of Mitogen Activated Protein Kinase (MAPK) and Stress-Related Genes in Rice Overexpressing MPK3 and MPK6 under Abiotic Stress

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Abstract
Plants being sessile have ability to adapt themselves in constantly changing and challenging environment by perceiving external/ internal cues and transducing them down to the nucleus for appropriate cellular reorganization. Activation of mitogen-activated protein kinases (MAPKs) is a common reaction of plant cells towards such cues in development-related signal transduction pathways. The downstream events after the activation of MAPKs are largely unknown in plants. In the present study, we examined the effect of arsenic, salt and drought stress on growth parameters and transcript profiling of MAPKs, silicon transporters and stress responsive genes in transgenic rice overexpressing OsMPK3 and OsMPK6 under the control of a dexamethasone (DEX)-inducible promoter, and possible role MAPKs in stress tolerance. Our results revealed that OsMPK3 and OsMPK6 overexpressing lines under arsenic stress and drought stress enhanced the transcript level of stress responsive genes, encoding enzymes superoxide dismutase, ascorbate peroxidase, glutamine synthetase and aldehyde oxidase. Upralulation in transcripts profiling of three silicon transporter genes OsLsi1, OsLsi2 and OsLsi6 showed improved stress tolerance ability of OsMPK3 and OsMPK6 overexpressing lines by effective absorption of silicon. The fundamental knowledge about the function of MPK3 and MPK6, important member of MAPKs family may also lead to an engineered plant tolerant to various abiotic and biotic stresses.

Keywords: Arsenite stress, Dexamethasone inducible promoter, Drought stress, Expression analysis, MAP kinase, Salinity stress.

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Introduction
Mitogen-activated protein kinases (MAPKs) are cell-signalling enzymes that regulate an extraordinarily diverse range of biological processes in eukaryotic organisms (Avruch, 2007; He et al., 2020). MAPK pathways in plants are very well developed while at the same time they are very complex to unveil all the cross talks. The simple reason behind these complexities is that the plants are sessile in nature and they have no choice to escape from these environmental cues. The MAPK signaling pathways get induced in order to overcome these threats of biotic and abiotic stresses (Sinha et al., 2011; Jalmi and Sinha, 2015). MAPK cascade transduces developmental and environmental signals into intracellular responses and plays a central role as the controller of gene expression, cellular and physiological responses (Samajová et al., 2013; Rezatabar et al., 2019; Sharma et al., 2020). MAPK cascade is stimulated by activation of various Mitogen Activated Protein Kinase Kinase Kinase (MAPKKKs), which phosphorylates and activates the downstream Mitogen Activated Protein Kinase Kinase Kinase (MAPKKKs) which in turn activate MAPKs upon phosphorylation (Sinha et al., 2011). Previous study revealed that SUB1A protein directly increases the expression of MPK3, whereas MPK3 phosphorylates SUB1A protein (a positive feedback loop) in acclimation of rice seedlings to the adverse effects of submergence (Singh and Sinha, 2016). MAPK cascades are also known to respond to the resulting oxidative burst and may regulate ROS accumulation. Both MPK3 and MPK6 signaling modules within Group A are implicated in ROS signalling (Jalmi and Sinha, 2015).

Abiotic stresses such as drought, salt stress, and arsenic toxicity negatively impact growth, development, yield and seed quality of crop and other plants. These stresses in plants affect the physiology of the plants, such as modulation of signalling pathways, gene expression profiles, translocation and maintaining various cellular and metabolic processes (Pandey et al., 2015; Jalmi et al., 2018; Verma et al., 2019). Therefore, several gene products are involved in the plant tolerance mechanisms. For example, modifications of the plant cell wall to block the metal transport as well as expression of osmotically responsive genes and increase activities of antioxidant enzymes are important defense responses against abiotic stress (Chao et al., 2019). Increased ROS levels under abiotic stresses could play a delicate balance between signaling and destruction in plants.
Differential Regulation of Rice Mitogen Activated Protein Kinase by Abiotic Stress

(Devireddy et al., 2020). However, this balance can be disrupted by various environmental stresses such as temperature, water availability and toxicants, leading to ROS accumulation. Therefore, removing it is necessary for organisms. Up-regulation of antioxidant defence systems is a general response to ROS scavenging (Halliwell, 1999).

Previously, it has been indicated that cold, heavy metals, salts and UV rays activate MAPKs in rice, Arabidopsis and tomato plants (Wankhede et al., 2016; Muhammad et al., 2019; Verma et al., 2019; Tang and Tang, 2020). The present study aims to characterize the role of transgenic rice overexpressing MPK3 and MPK6, two of the most important members of MPK family in rice plants during growth, development and stress tolerance. In the present study role of rice MAPKs in arsenic stress (arsenite, AsIII), salt stress (sodium chloride, NaCl) and drought stress (polyethylene glycol, PEG) have been studied by growth parameters and transcripts profiling of different MAPKs, transporters and ROS inducible genes. We hypothesized that OsMPK3 and OsMPK6, overexpressing lines of rice variety helped the plant to recover from drought, AsIII toxicity and salt toxicity through modulation in stress related gene expression and silicon transporters, which can alter the transportation of nutrient and elements. To uncover the role of MPK3 and MPK6 in stress tolerance, overexpressing lines of rice variety was evaluated.

Materials and Methods

Plant Material and Growth Conditions
For all the experiments, Oryza sativa L. (japonica cultivar group var. Taipei 309), overexpressing MPK3 (MPK3OE) and MPK6 (MPK6OE) under the control of DEX inducible promoter were used in the present study (Singh et al., 2019). Seeds were surface sterilized in 70% ethanol for 3 min and was subsequently washed thoroughly with distilled water. Seeds were soaked in distilled water for 24 h and then spread in petri plate containing moist cotton bed and kept in dark at 28±2°C for another 48 h. After germination, seedlings were transferred to light (a 16 h photoperiod) with day/night temperature of 25±2°C for 7 days in a controlled environmental growth chamber with 70% relative humidity and watered with 5% Hoagland nutrient solution. Nutrient solutions were changed twice per week. For transcript profiling and phenotypic changes, 7-day-old seedlings were divided into eight groups (4 seedlings per group), group I–VIII. Seven groups were treated with (DEX) (1µM), AsIII (NaAsO₂: 150 µM), AsIII+DEX, NaCl (150 mM), NaCl+DEX, PEG (10%), PEG+DEX. For mock treatment, plants were treated with DMSO (dimethyl sulfoxide) at final concentration of 0.1%. Dexamethasone treatment was given two day before to induce the OsMPK3 and OsMPK6 gene expression in the transgenic lines. The complete seedlings were harvested after 72 h time points for further analysis. Growth parameters were analyzed in terms of preliminary studies such as plant height (cm) and fresh weight (g) after 72 hour after treatment (HAT). Experiments were repeated twice using three replicates of each treatment.

RNA Extraction and Gene Expression Analysis
Stress dependent gene expression profile of (1) MAPK gene family i.e., MPK3 and MPK6 (Group A), (2) transporter genes i.e., low silicon rice 1 (Lsi1), low silicon rice 2 (Lsi2) and low silicon rice 6 (Lsi6); (3) stress inducible genes i.e. superoxide dismutase (SOD), ascorbate peroxidase (APX), glutamine synthetase (GS), aldehyde oxidase (AO), Drought inducible-19 (Drought-19) and salt inducible gene (SALT) was undertaken by quantitative real-time PCR. Total RNA was isolated from mock-treated and stress-treated seedlings samples by using the TRIZOL method (TI Reagent, Sigma) according to the manufacturer’s protocol. RNA was quantified with Nanodrop ND-1000 spectrophotometer (Thermo Scientific) and RNA quality was ascertained by resolving on agarose gel electrophoresis. Total 2 µg of DNase-treated RNA was used to synthesize cDNA using Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, United States) following the manufacturer’s instructions. Equal amounts of cDNA were used for all the genes. The gene-specific real time primers were designed using the PRIMER EXPRESS software (Table 1).

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Primer Name</th>
<th>Primer sequence</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>OsMPK3_F</td>
<td>GCTCCAACCAAGAACTGTC</td>
</tr>
<tr>
<td>2</td>
<td>OsMPK3_R</td>
<td>AGTCCGAGATCTTGGAG</td>
</tr>
<tr>
<td>3</td>
<td>OsMPK6_F</td>
<td>AGTGCAAGCGCAAGAACAG</td>
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<td>4</td>
<td>OsMPK6_R</td>
<td>AGCACTCTAGTACTCAG</td>
</tr>
<tr>
<td>5</td>
<td>OsLsi1_F</td>
<td>CGTGGAGATGTAGCTGGAACCA</td>
</tr>
<tr>
<td>6</td>
<td>OsLsi1_R</td>
<td>CCGTCAAGCTTCTGCG</td>
</tr>
<tr>
<td>7</td>
<td>OsLsi2_F</td>
<td>GAGGCTGAGCACTGTAACCG</td>
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<td>OsLsi2_R</td>
<td>AAGGAAGGAGCAGCAAG</td>
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<td>9</td>
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<td>AGAAGGAGGAGG</td>
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<td>10</td>
<td>OsLsi6_R</td>
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<tr>
<td>22</td>
<td>Os SALT_R</td>
<td>GCCAGCAGGAGGAG</td>
</tr>
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qRT-PCR analyses were performed in 384 well plate by using the ViiA 7 platform (Applied Biosystems, Germany) following standard procedures (Pandey and Gupta 2018). Each 10 µl reaction contained a mixture of 2 µl diluted cDNA, 5 µl SYBR Green 1 PCR master mix (Roche Applied Science, Germany), 0.45 µl of each forward and reverse primer (20 picomoles) and 3.1 µl of RNase free water. The reaction mixture was incubated at 50°C for 2 min, 95°C for 10 min, and for 40 cycles of 15 sec at 95°C and 1 min at 60°C. The transcript abundance normalized to the internal control ubiquitin gene was analyzed using the 2−ΔΔCt method (Livak and Schmittgen, 2001). PCR efficiency was calculated by using the default software ViiA 7 platform (Applied Biosystems).
RESULTS AND DISCUSSION

Arsenic, salt and drought stress activates MPKs in OsMPK3 and OsMPK6 overexpressing rice seedling

For the study, we selected DEX - inducible OsMPK3-23 and OsMPK6-11 rice lines, based on preliminary assessments (data not shown). Selected lines showed maximum transcript accumulation of MAPKs among different overexpression lines. No morphological difference was noted between the transgenic lines and WT (data not shown). The relative expression of OsMPK6 and OsMPK3 genes in response to 72 (HAT) of 150 µM AsIII, 150 mM NaCl and 10% PEG in combination with 48 h pre-DEX induced seedlings is shown in Fig. 1. Expression of OsMPK6 in OsMPK6OE line and OsMPK3 in OsMPK3OE line was induced by DEX treatments. Expression of OsMPK6 and OsMPK3 was significantly upregulated in DEX induced AsIII, NaCl and PEG stressed OsMPK6OE and OsMPK3OE lines respectively. It has been well studied that upon exposure to stresses transcript levels of a multitude of genes are altered. Numerous studies have shown that MAPKs specially MKP6/MKP3 involves in regulation of various developmental condition and stress response pathway, suggesting that they are tightly involved in plant response to various adverse environment conditions (González Besteiro et al., 2011; Sinha et al., 2011; Singh and Sinha 2016; Singh et al., 2019; Banerjee et al., 2020; Sharma et al., 2020). However, not much significant changes were observed in the expression of OsMPK6 in OsMPK3OE line and OsMPK3 in OsMPK6OE line.

Differential growth response to arsenic, salt and drought stress in overexpressing OsMPK3 and OsMPK6 lines

Expression of OsMPK3 and OsMPK6 in their respective overexpression lines are tightly regulated under stress condition by DEX-inducible promoter. Thus, these transgenic lines would serve as a model system defining various stress tolerance-related phenotypic traits. Growth parameters such as seedlings fresh weight and length were measured to determine the stress tolerance effect of overexpressing OsMPK3OE and OsMPK6OE lines. In this study, DEX induced stress overexpressed lines showed increase in seedlings height and fresh weight (Fig. 2) as compared to stress alone. Taken together, we speculate that the phenotypes conferred by OsMPK3OE and OsMPK6OE lines might be due to MAPKs-mediated increases in the expression of MAPKs at the seedling stage.

Overall, phenotypic characteristics showed tolerance mechanism of inducible overexpressed lines against arsenic, salt and drought stress which could be attributed to their role to regulate the plant growth and development.

Altered gene expression of low silicon rice transporters (OsLsi1, OsLsi2 and OsLsi6) under arsenic, salt and drought stress in overexpressing OsMPK3 and OsMPK6 seedlings

To get an idea as to how MAPKs regulates stress tolerance, transcript analysis of transporter genes Lsi1, Lsi2 and Lsi6 was performed in DEX induced and uninduced overexpression lines. Low silicon rice (Lsi) transporters belong to the family of aquaporin channel proteins, and constitutively express in roots and shoots of rice plants. Low silicon rice 1 (Lsi1) and low silicon rice 2 (Lsi2) are influx and efflux transporters, respectively, transports silicon from root to shoot via xylem sap. Lsi1 are localised in distal and Lsi2 in proximal side of both exodermis and endodermis cells of plasma membrane (Ma et al., 2006; Khan and Gupta, 2018). Silicon taken up by roots through Lsi1 and Lsi2 is translocated to the aboveground parts of the plant. A homolog of Lsi1, Lsi6 is involved in xylem unloading, and is polarly localized in xylem parenchyma cells in the leaf sheath and leaf blades (Yamaji et al., 2012). Under stress condition expression of transporter genes (Lsi1, Lsi2 and Lsi6) were upregulated in both DEX induced overexpression lines as compared to uninduced. Expression of OsLsi1 and OsLsi2 were upregulated in DEX inducible OsMPK3OE lines under stress condition as compared to uninduced stress condition (Fig. 3). However, expression of OsLsi1 and OsLsi2 were downregulated in DEX inducible OsMPK6OE line under AsIII stress condition as compared to uninduced AsIII stress. Other studies have also shown that increase in expression of Lsi is associated with abiotic stress tolerance (Fang et al., 2017, 2019; Khan and Gupta, 2018; Li et al., 2020). Stress induced defence response such as antioxidant capacity is also enhanced in the Lsi1OE rice line and reduced in the Lsi1-RNAi line (Fang et al., 2019).

These observations demonstrate the function of MPK3 specially upon AsIII stress, but results also indicate the
The importance of MPK6 in salt and drought stress-induced defence response. Interestingly, it has been reported that toxic metal shares the silicon (a nutrient) transport pathway to enter the plant body via OsLsi1 and OsLsi2, and their expressions are directly proportional to silicon accumulation (Khan and Gupta, 2018). Expression of OsLsi6 showed differential pattern with
higher expression in DEX inducible OsMPK3OE line under NaCl stress as compared to uninduced NaCl stress (Fig. 3c).

**Profiles of stress-responsive plant genes define molecular signatures characteristic for stress tolerance in overexpressing OsMPK3 and OsMPK6 seedlings**

We hypothesised that differential gene expression is responsible for tolerance of rice cultivar towards abiotic stress. In order to verify this hypothesis, we examined the expression of MPKs and its directly regulated general defence genes at 72 HAT in MPK3OE and MPK6OE lines, and marked increase in the transcript of genes were observed in these overexpressing lines (Fig. 4).

Plants respond to environmental changes by expressing numerous genes, some of which are considered to play a major role in defence pathways. Genes were selected based on their association with proteins putatively involved in general defence responses and scavenging of ROS. Therefore, we quantified the expression of SOD, APX, GS, AO, DROUGHT-19 and SALT genes in two OsMPK3 and OsMPK6 overexpressing lines. Gene expression profiles were monitored at 72 HAT by qRT-PCR to assign genes as being differentially expressed in induced and uninduced lines (Fig. 4). All stress responsive genes, except DROUGHT-19 and SALT in both OE lines and GS in MPK6OE line, were upregulated in DEX-induced stress condition as compared to DEX uninduced stress condition. By contrast, expression of DROUGHT-19 and SALT was downregulated in DEX-induced overexpressing seedlings under stress condition as compared to DEX-uninduced stressed condition. Up-regulated transcripts in induced overexpressing lines under stress condition as compared to uninduced stressed condition, signify tolerance mechanism in overexpressed lines, and might explain the success in accumulating less toxic compounds. It has been reported that the genes involved in ROS scavenging were down-regulated in susceptible cultivars under abiotic stress conditions which is well in agreement with the current findings (You et al., 2019). Under AsII and drought conditions, the DEX-induced transgenic plants accumulated lower levels of reactive oxygen species compared with uninduced, proved by higher expression levels of three antioxidant enzymes SOD, APX and GS. In addition, expression of one stress-responsive genes coding for aldehyde oxidase was induced to higher levels in the transgenic lines with salt stress. Induction of genes involved in ROS scavenging has been previously reported in stress tolerant genotypes (Shinozaki and Yamaguchi-Shinozaki, 2007; Pandey and Gupta, 2018). MPK3 and MPK6 is a signalling molecule and functions in positive modulation of abiotic stress tolerance. In conclusion, the present study gives an account of rice MAPKs in arsenic, salt and drought stress which is a growing concern for researchers in context of increasing environmental fluctuations and climate change at earth’s surface.

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CP and AKS conceptualized the idea. The experimental work was carried out by CP and GB contributed to the analyses of the data. CP compiled the data and prepared the first draft of manuscript. AKS revised and finalized the manuscript for publication.

**References**


