RESEARCH ARTICLE

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 andstress responsivegenes 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. Upregulation 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.

Kewwords: 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). MAPKs 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 (Šamajová *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 (MAPKKs) 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

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(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 ($NaAsO_2$, 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 (TRI 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).

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 $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). PCR efficiency was calculated by using the default software ViiA 7 platform (Applied Biosystems).

Table 1: List of primers used in qRT-PCR expression analysis

S.N.	*Primer Name*	*Primer sequence*
1	OsMPK3_F	GCTCCAACCAAGAACTGTC
2	OsMPK3_R	AGTCGCAGATCTTGAGG
3	OsMPK6_F	AGGTCACCGCCAAGTACAAG
4	OsMPK6_R	AGCAGCTTGATCTCCCTGAG
5	OsLsi1_F	CGGTGGATGTGATCGGAACCA
6	OsLsi1_R	CGTCGAACTTGTTGCTCGCCA
7	OsLsi2_F	GAGTTCGACAACGTCTAATCGC
8	OsLsi2_R	AGTACACGGTACATGTATACACG
9	OsLsi6_F	AGGGAGCAGCAGCAAGA
10	OsLsi6_R	AGGGAGTAGAGGGCGAAGGT
11	Os*SOD_F*	CGAGGATGACATTGTGAACGA
12	Os*SOD_R*	TCATCACCATTTGCTTCAGCAT
13	Os*APX_F*	GCTCGAGCCCATCAAGGA
14	OsAPX_*R*	CCACAACTCCGGCAAGCT
15	Os*GS_F*	CGAGGCGAAGGGAAAAGG
16	OsGS_R	TCACGACGTACGGGTCCAT
17	Os*AO_F*	CAGGTTACTGGTGAAGCGGAATA
18	OsAO_R	ACCAGAGCAGCATGCAAGGT
19	Os*Drought19_F*	AACACTACTGCCACAAACATTTCTG
20	*Os Drought19_R*	GTTCCTCTGAATCCAATGTTGAGA
21	Os*SALT_F*	CCTACGGCATCCTGGTCAAG
22	Os SALT_R	GCCCACGCATTTGATCTAACA

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 MPK6/MPK3 involve 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*intheir 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 treated 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) underarsenic, 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 Lsi2is 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

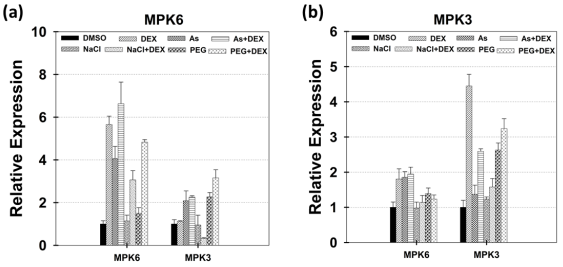

Fig. 1: Expression pattern of rice MPKs (a) *MPK6* and (b) *MPK3* in7-d-old DEX-inducible MPK6 and MPK3 *Oryzasativa*seedlings treated withAsIII, NaCl and PEG stressed and its combination with DEX. Relative expression values are $\log_2$ (fold change) compared to matched DMSO controls.Values are presented as the mean and the errors bars indicate standard deviation of three biological replicates (n=3, ±S.E.). Arsenite(AsIII); sodium chloride (NaCl); polyethylene glycol (PEG); dexamethasone (DEX)

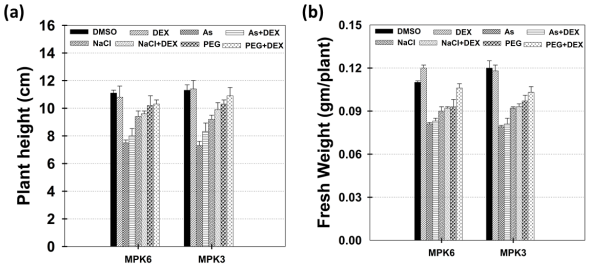

Fig. 2: Plant height and fresh weight in 7-d-old DEX-inducible MPK6 and MPK3 *Oryzasativa*seedlings treated with AsIII, NaCl and PEG stressed and its combination with DEX. Values are presented as the mean and the errors bars indicate standard deviation of three biological replicates (n=3, ±S.E.). Arsenite(AsIII); sodium chloride (NaCl); polyethylene glycol (PEG); dexamethasone (DEX)

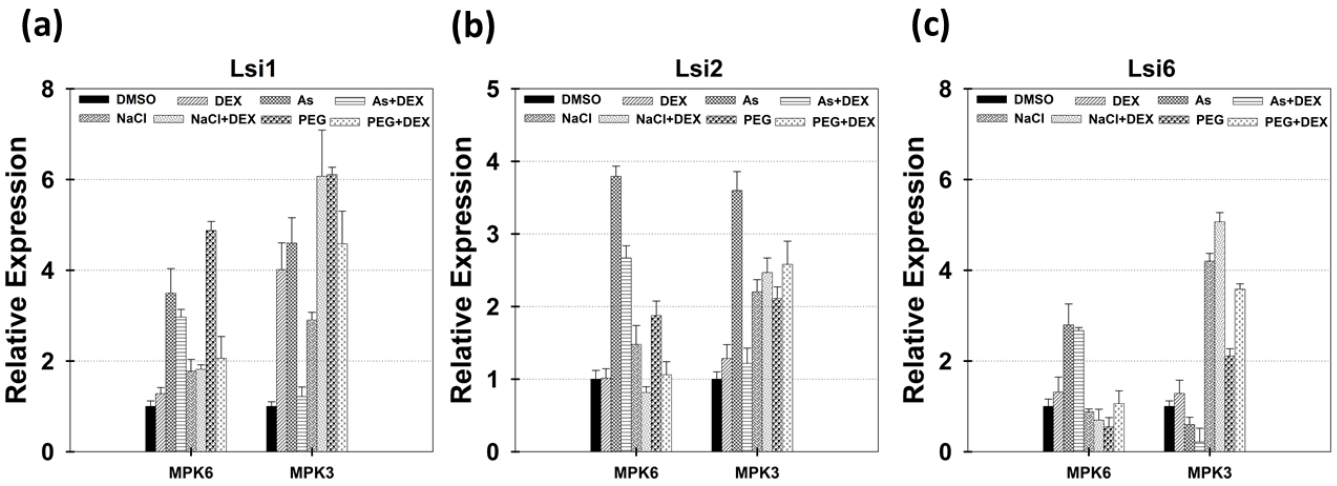

Fig. 3: Expression pattern of low silicon rice transporters (a) *Lsi1*, (b) *Lsi2* and (c) *Lsi6* in 7-d-old DEX-inducibleMPK6 and MPK3 *Oryzasativa*seedlings treated with AsIII, NaCl and PEG stressed and its combination with DEX. Relative expression values are $\log_2$ (fold change) compared to matched DMSO controls.Values are presented as the mean and the errors bars indicate standard deviation of three biological replicates (n=3, ±S.E.). Arsenite (AsIII); sodium chloride (NaCl); polyethylene glycol (PEG); dexamethasone (DEX); low silicon rice 1 (Lsi1); low silicon rice 2 (Lsi2); low silicon rice 6 (Lsi6)

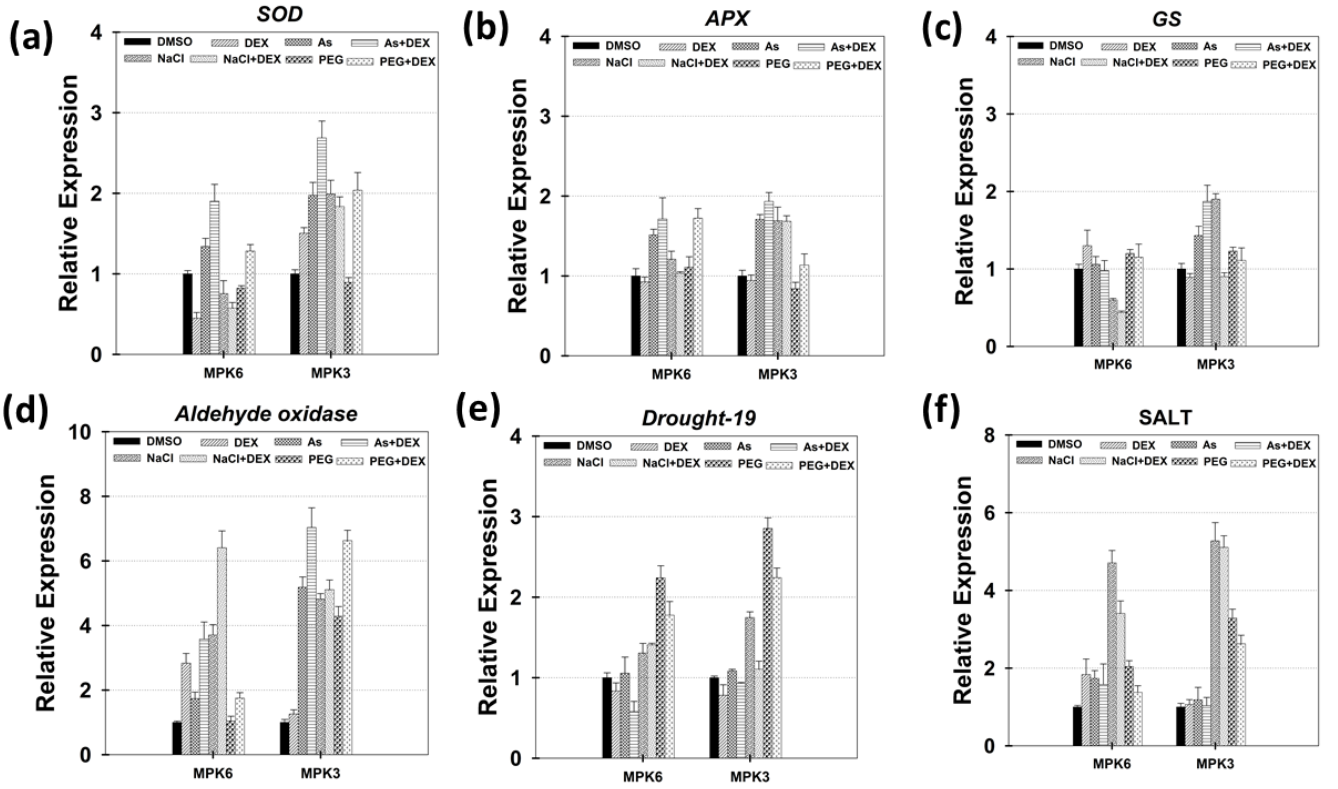

Fig. 4: Expression pattern of rice stress-responsive genes (a) *SOD*, (b) *APX*, (c) *GS*, (d) *aldehyde oxidase*, (e) *Drought-19* and (f) *SALT* in 7-d-old DEX-inducible MPK6 and MPK3 *Oryzasativa*seedlings treated with As(III), NaCl and PEG stressed and its combination with DEX. Relative expression values are $\log_2$ (fold change) compared to matched DMSO controls.Values are presented as the mean and the errors bars indicate standard deviation of three biological replicates (n=3, ±S.E.). Arsenite(AsIII); sodium chloride (NaCl); polyethylene glycol (PEG); dexamethasone (DEX); superoxide dismutase (SOD); ascorbate peroxidase (APX); glutamine synthetase(GS); aldehyde oxidase (AO), Drought inducible-19 (Drought-19); salt inducible gene (SALT)

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 the 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 stress 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 AsIII 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.

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