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Expression analysis of a MATE-type transporter gene of *Arabidopsis* and its orthologues in rice and chickpea under salt stress

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Abstract

Salinity is one of the major abiotic stresses that adversely affect the crop growth and productivity. Salt responsive genes belonging to MATE efflux proteins reportedly play a significant role imparting salt tolerance to plants. In the present study, AT5G52050, a putative salt responsive gene from *Arabidopsis thaliana* encoding MATE efflux family protein which functions as an antiporter and its orthologues in rice (LOC_Os02g45380) and chickpea (LOC101489496) have been identified. The expression pattern of these genes was validated by quantitative reverse transcription polymerase chain reaction. *In silico* analysis of the upstream promoter regions of these genes revealed the presence of several conserved sequence motifs related to salt response either in single or in multiple copies. A phylogenetic analysis to understand the evolutionary relationship of MATE family genes in Arabidopsis, rice and chickpea revealed conservation of MATE family genes between dicots and monocots. The genes identified in this study may serve as promising candidates for further elucidation of the salt tolerance mechanism in plants.

Key words: Salinity, gene expression, arabidopsis, MATE-family genes

Introduction

Plants are exposed to different stresses, both biotic and abiotic, during their growth and development. Abiotic stresses, especially cold, high salinity and drought are the primary causes of crop loss worldwide. Soil salinity is one of the major limiting constraints to world agriculture affecting an estimated 45 million hectares of irrigated land, and is predicted to intensify owing to global climate changes (Munns and Tester 2008). Plants have evolved a range of adaptive mechanisms to cope up with various stresses. Stress signals are perceived and transduced through signaling components resulting in the activation of a number of stress inducible genes leading to stress response and tolerance (Shinozaki et al. 2003).

The adverse effects of salinity on plants include osmotic as well as ionic stress, nutrient imbalance, and the production of reactive oxygen species (Ashraf and Fooland 2007). Under high saline conditions, rates of plant growth, photosynthesis and seed germination are significantly reduced. The process of flowering and fruiting are adversely affected and overall plant productivity decreases (Sairam and Tyagi 2004). Research on model plants and other species has identified HKT1 (High Affinity K+ Transporter1), SOS1 (Salt Overly Sensitive1) and NHX (Na+/H+ exchanger) as major determinants of salt tolerance, with HKT1 and SOS1 controlling transport across the plasma membrane and NHX controlling net movement across the tonoplast membrane. Furthermore, plant hormones such as abscisic acid (ABA), ethylene, salicylic acid, and jasmonic acid play a significant role (Ma et al. 2006).

Identification and characterization of genes that respond to various abiotic stresses is required for a better understanding of the molecular mechanisms underlying stress tolerance in crop plants (Seki et al. 2006). Hence, there have been studies on studying gene expression profiling in many plant species, under different stresses. Multigenic nature of salt tolerance is well documented (Chinnusamy et al. 2005) and analysis of gene expression under salt stress may help to identify candidate genes associated with stress

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tolerance mechanism. Many genes involved in perception and signal transduction of salt stress have been identified through various approaches, particularly through transcriptomics studies. Salt responsive transciptome studies have identified numerous potential salt-responsive candidate genes in Arabidopsis (Seki et al. 2006; Jiang and Deyholos 2006), rice (Kawasaki et al. 2001; Rabbani et al. 2003; Chao et al. 2005; Walia et al. 2005, 2007) and chickpea (Molina et al. 2011; Mantri et al. 2007). Sequence similarities and expression patterns of genes identified in model plant species would enable us to identify orthologous genes with similar functions in crop plants of our interest (Maa et al. 2001).

MATE (Multidrug and toxic compound extrusion) gene family members are present in all the organisms including plants. However, in plants this family is remarkably large signifying its importance. Arabidopsis genome encodes 58 members of the MATE family and 57 in *V. vinifera*, 58 in Populus *trichocarpa*, and 38 in *Zea mays* and *Brachypodium distachyon* (Chen et al. 2015). MATEs govern diverse physiological functions in the plants such as plant growth, development and stress responses, which need to be elucidated in detail (Tiwari et al. 2014). In a recent study it is reported that an *Arabidopsis* MATE family member, DTX50, functions as an ABA efflux transporter and plays a role in ABA-mediated growth inhibition and drought stress responses (Zhang et al. 2014).

We report here the *in silico* identification of a set of genes being differentially regulated under salt stress in *Arabidopsis*, identification and salt responsive expression analysis of its putative orthologues in rice and chickpea. A phylogenetic analysis was carried out to study the evolutionary relationship among MATE gene family members of *Arabidopsis*, rice and chickpea.

Materials and methods

In silico analysis

The Arabidopsis genes used in this study, for expression analysis, were identified from the publically available TAIR 9 database (The Arabidopsis Information Resource, http://www.Arabidopsis.org/) as well as based on previous studies (Seki et al. 2006; Kreps et al. 2002; Dinneny et al. 2008). Identified list of 44 genes of Arabidopsis that were annotated to be involved in response to salt stress. tBLASTx searches at National Center for Biotechnology Information (NCBI) with default parameters were used for the identification of orthologues in rice and chickpea using nucleotide sequence of Arabidopsis gene as query. Genevestigator (Hruz et al. 2008) was used for expression analyses. The filters used were high at a fold change of 2 and a p-value of <0.001. Potential cis-regulatory elements in the promoter sequence of six salt upregulated genes in Arabidopsis were analysed using PlantCARE (Lescot et al. 2002). To identifiy MATE family genes, different databases PROSITE, Pfam, InterPro and ProteomeScout were searched for MATE domain signature sequences of various organisms and a local database was created as a first step. Linux shell pipes command “sort signature.fasta | uniq-u” was used followed by manual curation to remove duplicates. With protein sequences of all MATE family genes of three plant species Arabidopsis, chickpea and rice a BLAST search was done against the local data base created using BLASTp module with default parameters except e value set at 1e-8. Furthermore to increase accuracy, candidate MATE family genes were subjected to manual curation. Multiple sequence alignments of the full-length protein sequences of all MATE family genes from Arabidopsis, chickpea and rice amounting to a total of 162 sequences were performed using MAFFT V.7 (Katoh and Standley 2013). Model Generator (Keane et al. 2006) was used to determine the substitution model and rate heterogeneity that best fit the MATE protein data. The phylogeny of the aligned sequences was generated based on a Bioneighbor-joining method using PhyML 3.0 software (Guinden et al. 2010). Bootstrap analyses for phylogenetic tree were performed using 100 pseudoreplicates. Homology between the MATE family members from the three crops was visualized by using circos (Krzywinski et al. 2009).

Plant materials, growth conditions and stress treatments

Arabidopsis thaliana wild-type (ecotype Columbia-0) seeds were surface sterilized, placed in Petri dishes containing Murashige and Skoog (MS) agar medium, and kept for two days at 4^{o}C. Two week old seedlings were exposed to salt stress (150 mM NaCl) and samples were collected at 6 and 24 h post-treatment (hpt), flash frozen in liquid nitrogen and stored at -80^{o}C until RNA extraction. Unstressed plants were used as control. Rice (*Oryza sativa* L. ssp. *indica*) seeds of salt tolerant variety FL478 were obtained from Division of Genetics, Indian Agricultural Research Institute, New Delhi, India. Seeds were kept on wet germination paper in Petri plates under controlled

environmental conditions at National Phytotron Facility. Four day old germinated seedlings were shifted to polystyrene foam floats with holes stitched by nylon wire mesh and made them to float on trays filled with Yoshida nutrient solution (Yoshida et al. 1976). Two week old seedlings were exposed to salt stress by supplementing nutrient solution with 150 mM NaCl and leaf samples were collected at 6 and 24 h post-treatment (hpt), and flash frozen in liquid nitrogen and stored at –80^{o}C until RNA extraction. Two week old seedlings of chickpea desi variety ICC4958 grown on solrite pots were shifted to a tray containing Hoagland's solution in which NaCl solution was added to the final concentration of 150 mM and sampled at 6 and 24 hour post- treatment (hpt).Three biological replicates of each tissue sample were harvested. The primers used in the present study were designed using Primer3 software and details are given in Table 3.

Expression analysis using qRT-PCR

Total RNA was isolated using Trizol (Invitrogen) reagent. The integrity and quality of RNA was assessed through agarose gel (1%w/v) and Nano drop ND-1000 spectrophotometer, respectively. 10 µg of RNA was used for the DNase I treatment and 1 µg DNase treated RNA was used for the first strand cDNA synthesis using superscript III Reverse transcriptase (Invitrogen LifeTechnologies, USA)) according to the manufacturer's protocol. The qRT-PCR analysis was conducted using gene specific primers (Table 3) in a reaction mix of KAPA SYBR FAST qRT-PCR Master mix (Kapa Biosystems, Inc. USA). Primers specific to *Actin8* (AT1G49240) gene of *Arabidopsis*, *GAPDH* gene of chickpea and *25S rRNA* (AK119809) gene of rice were used as internal control. Aliquots of 2µl undiluted cDNA were used as template for each sample. Reaction mix of standard reaction volume 20 µl was prepared according to manufacturer's instruction (Kapa Biosystems, Inc. USA) and reaction was performed using Light Cycler 480 II PCR system (Roche). For each time interval, three biological replicates were used. The data was normalized by the value of *Arabidopsis Actin8* gene, *GAPDH* gene of chickpea and rice *25S rRNA* gene using the $\Delta\Delta$CT method (Livak and Schmittgen 2001) and the corresponding fold change in the expression level at each time interval was calculated compared with that of the unstressed sample and the standard error of the mean was calculated.

Results and discussion

Expression analysis through semi-quantitative RT-PCR and quantitative real time – PCR

The TAIR9 database was searched and a total of 44 putative salt inducible genes were identified in *Arabidopsis*. These genes were distributed across all the chromosomes (Table 1) and belonged to different functional categories. We found that 14 belong to groups related with metabolism, 6 belong to transcription factor activity, 12 belong to unknown protein, 3 belong to transporter activity, and one belongs to unclassified proteins. Initially semi quantitative RT-PCR was performed for the genes chosen to study the salt responsive expression levels in *Arabidopsis* at 6 and 24 hour post treatment (hpt.). Among the differentially expressed genes, six genes that showed significant up regulation after salt stress in the semi-quantitative RT-PCR study (Fig. 1) were further validated using quantitative real time PCR (Fig. 2). The six up regulated genes selected were AT1G64610, AT2G18190, AT2G38240, AT4G24380, AT4G37710 and AT5G52050 and belong to different

Table 1. Chromosome wise distribution of 44 putative salt responsive genes identified from *Arabidopsis thaliana*

AT1G73390	AT2G18190	AT3G56880	AT4G15490	AT5G65990
AT1G73480	AT2G38240	AT3G62730	AT4G17030	AT5G28510
AT 1G 51140	AT2G38830	AT3G62260	AT4G36740	AT5G66780
AT 1G 50740		AT3G57540	AT4G37790	AT5G66070
AT1G64610		AT3G57120	AT4G33905	AT5G65300
		AT3G55980	AT4G33550	AT5G65470
		AT3G53160	AT4G30470	AT5G63130
		AT3G51750	AT4G29780	AT5G62490
		AT3G23250	AT4G28460	AT5G59220
			AT4G24380	AT5G52760
			AT4G37710	AT5G17350
				AT5G52050
				AT5G37540
				AT5G51190
				AT5G40880
				AT5G43650

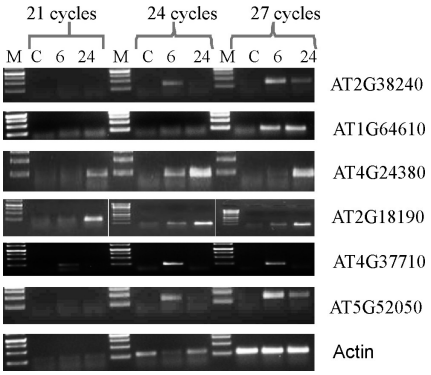

Fig. 1. Semi-quantitative RT-PCR of six putative salt-responsive genes from Arabidopsis at 21, 24, and 27 numbers of thermal cycles of control and plants harvested 6 and 24h salt stress

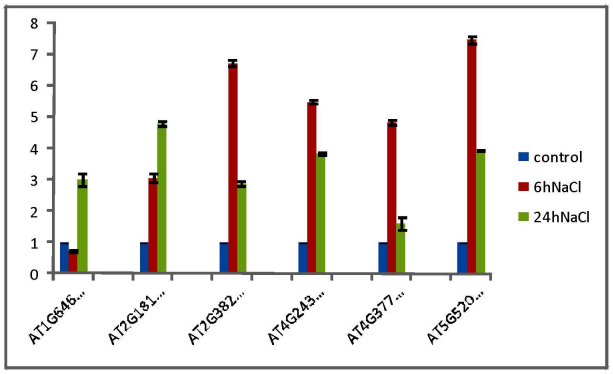

Fig. 2. Quantitative RT-PCR analysis of six candidate genes of Arabidopsis in response to salt stress of 150 mM NaCl at 6 and 24h time interval. Actin gene was used for normalization. The expression level of genes in control sample was used as calibrator to calculate fold change

functional categories (Table 2). Among the six genes, AT5G52050 showed ~7.4 fold up regulation at 6hpt., while AT4G37710, AT2G18190, AT2G38240, AT1G64610 and AT4G24380 showed 4.8, 3.0, 6.7, 0.7 and 5.4 fold up regulation, respectively at 6hpt (Fig. 2).

The gene that showed maximum up-regulation (AT 5G52050) under salinity stress is a member of MATE family. There are several studies pointing towards a role for the members of MATE family in abiotic stress response (Tiwari et al. 2014; Zhang et al. 2014). Our study shows that this gene expression is upregulated to an extent of 7.4 fold at 6hpt in response to NaCl stress and the expression reduces by 3.9 fold at 24hpt. AT2G38240 belongs to 2-

Table 2. The six genes up-regulated genes under salt stress

AGI number	Annotated gene function[a]
AT1G64610	Transducin/WD40 repeat-like superfamily protein
AT2G18190	ATPase, AAA-type, P-loop containing nucleoside triphosphate hydrolases superfamily protein
AT2G38240	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein
AT4G24380	Serine hydrolase
AT4G37710	VQ motif-containing protein
AT5G52050	MATE efflux family protein, anti-porter activity

a) Annotation was obtained from the Gene Ontology of The Arabidopsis Information Resources

oxoglutarate (2OG) and Fe (II)-dependent oxygenase superfamily and also shown high induction upon salt treatment. Oxidoreductases are a superfamily of proteins that play significant role in different aspects of plant growth, development and responses to biotic as well as abiotic stresses (Reddy et al. 2007; Jacquot et al. 2009). AT4G37710 is also a putative salt responsive gene whose molecular function is unknown. AT2G18190 an ATPase (AAA-type) domain containing protein that also showed salt stress responsive upregulation in present investigation. AT4G24380 is predicted to be involved in jasmonic acid biosynthetic process. In our study six genes which showed induction after salt stress were all annotated with

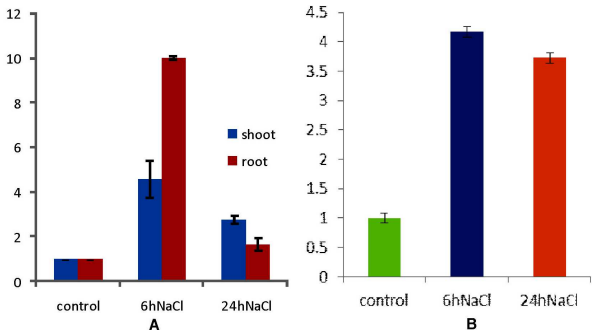

Fig. 3. Quantitative RT-PCR analysis of (A) chickpea LOC101489496 and (B) rice LOC_Os02g45380 in response to salt stress of 150mM NaCl at 6 and 24h time interval. GADPH gene was used for normalization. The expression level of genes in control sample was used as calibrator to calculate fold change

Table 3. List of genes and corresponding primers used for expression analysis by qRT-PCR

Gene	Forward primer (5'–3')	Reverse primer (5'–3')
AT1G64610	TCTCAATTACCCAGCAAGAAGCCG	TTCAGGCCACGTTGTTGATCCT
AT2G18190	TGCGAGCAAAGATGATGACGGA	TTCGCCATGGCAGCAATCAAAC
AT2G38240	AGTAACTGCAAACCGACCTGCT	TCAAACCTCAAGAGAGAGCAGCAA
AT4G24380	AGCTTGGTCCTTTCGATGGTCT	TGGCCTTTGGGATGATGGACAA
AT4G37710	TCAAGAAACCACCACAAGGGCA	TCTGCTTTGCTGCATCATCCGA
AT5G52050	ACGGCTTTCGCGTTTACTGTCT	TTAGCCCTTTCCGCCTCAAGTT
Os02g0676400	CGAGACCTACATTCTTGCTTCT	ATAAACCTTC CGCTCACCG
LOC101489496	TGGAACTCGCAGCAGGTTCATT	ACGGATTGAGCGCGAAGGTAAA
AT1G49240 (*Actin 8*)	ATGAAGATTAAGGTCGTGGCA	GACATCTCTCCAAACGCTGT
AK119809 (*25S-rRNA*)	AAGGCCGAAGAGGAGAAAGG	CGTCCCTTAGGATCGGCTTAC
AK064960 *(GAPDH)*	TGATGACCACCGTCCATTCCATCA	AGACATCAACAGTTGGGACACGGA

Table 4. *cis*-elements found in the promoters of AT5G52050, LOC10148949 and Os02g0676400

Cis-element	Sequence	AT5G52050	LOC101489496	Os02g0676400	Function of element
ABRE	TACGTG	+	–	+	Abscisic acid responsiveness
ERE	ATTTCAAA	+	+	–	Ethylene-responsive element
HSE	AAAAAATTTC	+	+	+	Heat stress responsiveness
MBS	TAACTG	+	+	–	MYB binding site involved in drought-inducibility
TC-rich repeats	ATTTTCTTCA	+	+	+	Defense and stress responsiveness
LTR	CCGAAA	–	+	–	Low-temperature responsiveness

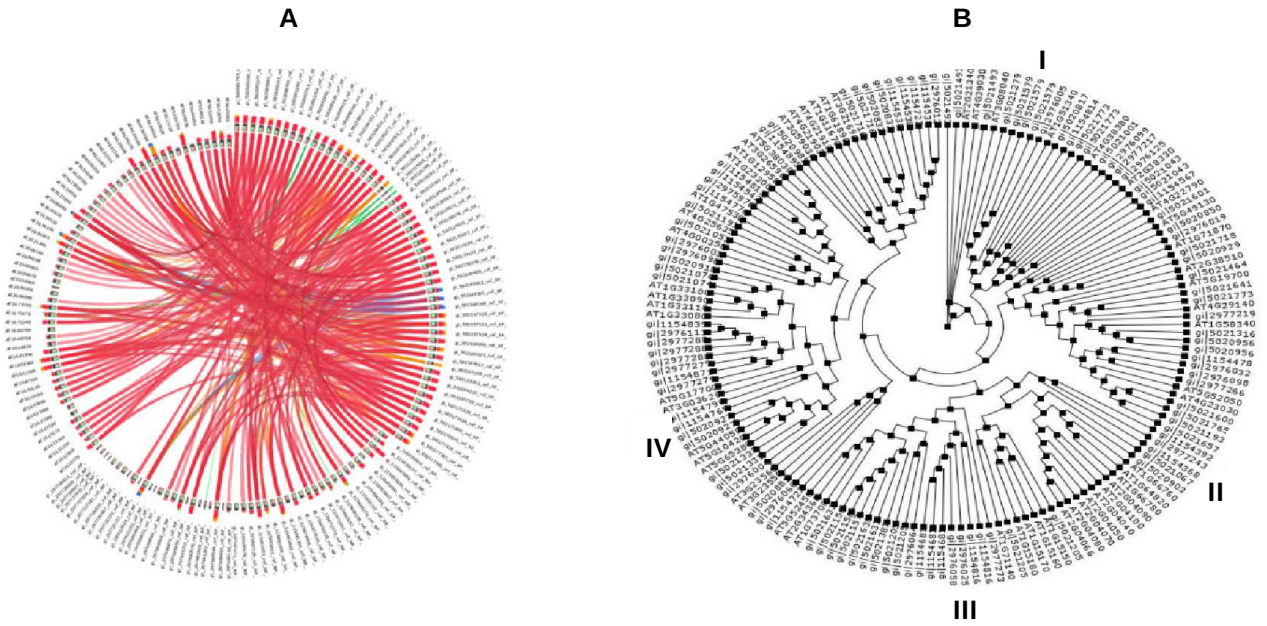

Fig. 4. Circos diagram of chickpea MATE family protein sequence pairs between Arabidopsis and rice. Red color depicts >75% homology; blue color < 25%; green color 25-50% and orange 50-75%. Phylogenic analyses of MATE proteins in chickpea, rice and Arabidosis based on the MATE protein sequences. The phylogenetic tree was constructed by PHYML

putative functions related with salt stress tolerance and each might serve as potential candidate gene in salinity stress response.

Expression profiling using Genevestigator

The expression profiles of Arabidopsis genes under different perturbations, tissues, and developmental stages are available for public access on the Genevestigator database. In order to find the response of the genes identified in this study to an array of stresses, Genevestigator was used. The resulting heat map (Fig. 5A) created using the “Perturbations” option in Genevestigator shows that the gene AT5G52050 is expressed at higher levels relative to the other genes. Furthermore, the gene shows upregulation in other abiotic stress conditions such as drought and hypoxia. Pattern of gene expression suggest its role in different stresses. The expression of the six genes in the various tissues and developmental stages are shown in Fig. 5B.

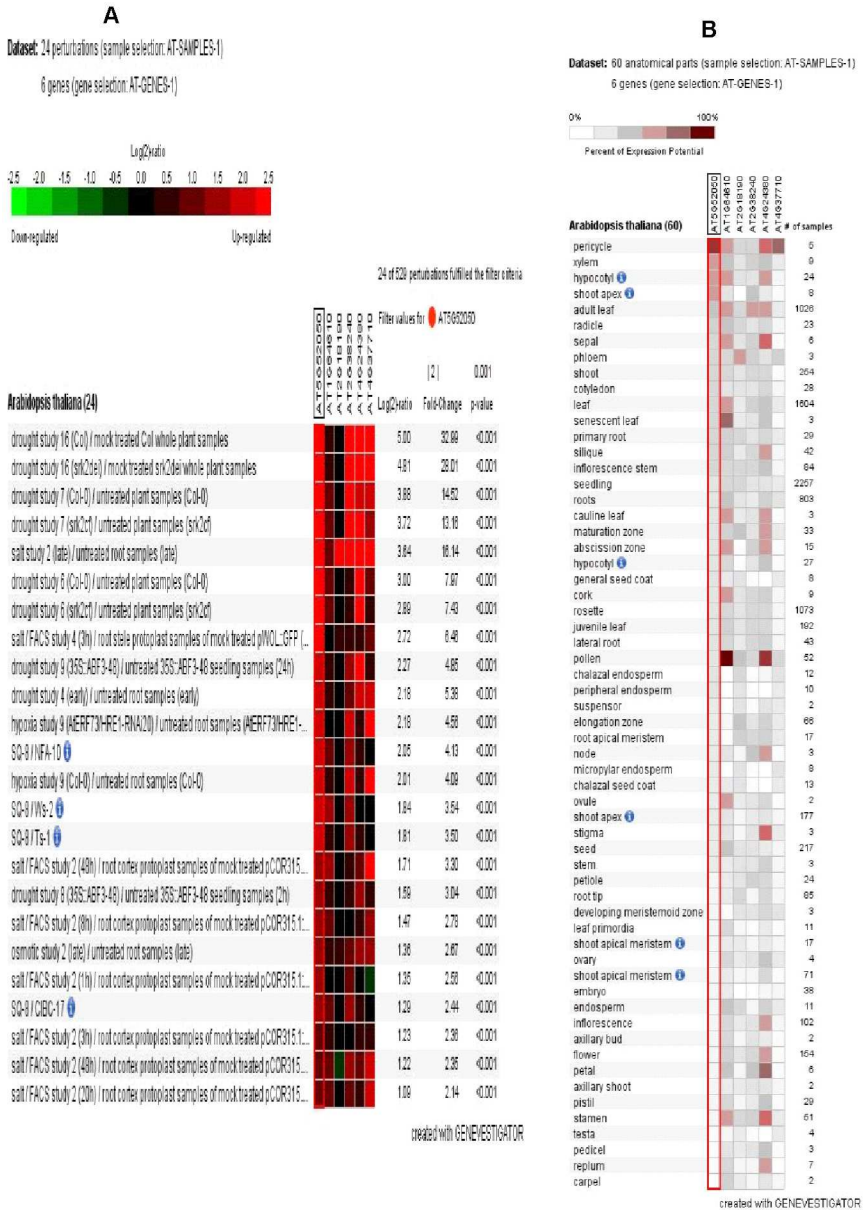

Fig. 5. Expression analysis of Arabidopsis genes (A) under different stresses and (B) in different tissues. The expression profile is presented as a heat map, generated by Genevestigator. Selection criteria used were: expression at 2-fold or more and p-value < 0.001 b)

Identification of orthologues of AT5G52050 and expression analysis

MATE efflux family gene (AT5G52050) with a putative function of antiporter activity that shows significant upregulation under salt stress in our study was taken for further analysis. Using nucleotide sequence of AT5G52050 as query, a tBLASTx search was done to find out orthologues in chickpea as well as rice. Real time quantitative RT-PCR analysis was carried out for the chickpea (LOC101489496) and rice (LOC_Os02g 45380) orthologues after exposure to salt (150 mM NaCl) stress to study the gene expression pattern (Fig. 3). It was shown that the genes showed similar upregulation in chickpea and rice after 6hr of salt stress. A 10 fold increase expression of was observed in root tissues of chickpea while in the shoot tissues expression was 4.5 fold. In case of rice there was 4.1 fold expression after 6 hr of salt stress. These results suggest a probable role for these genes in salt tolerance mechanism. In rice, MATE family of transporters have been shown to reduce the ROS production in mitochondria (Finatto et al. 2015) and thus protect the plants exposed to stress conditions.

Promoter analysis

Putative stress-responsive *cis*-elements in the promoter regions of the genes (AT5G52050, LOC_Os02g45380, LOC101489496) were also identified. It is found that four major types of stress-related *cis*-elements including MYB binding site involved in drought-inducibility (MBS), low temperature-responsive element (LTR), heat shock element (HSE), and defense and stress-responsive element (TC-rich repeats) are present (Table 4). The presence of these elements in the upstream region of these genes indicates their possible role in stress response. In addition, some elements possibly participate in stress hormone mediated responses.

Phylogeny

To evaluate the evolutionary relationship, the MATE proteins from chickpea and other two model plants, Arabidopsis and rice, were used. No comprehensive study including phylogenetic analysis of MATE gene family members of *Arabidopsis*, rice and chickpea has been reported. After searching the databases and further validation by PROSITE, Pfam, InterPro and ProteomeScout domain analysis, a total of 161 MATE genes were identified which include 57, 55 and 49 each from chickpea, Arabidopsis and rice, respectively and a phylogenetic tree was constructed (Fig. 4). In general, MATE proteins from various source organisms have a conserved domain and share at least 40% amino acid sequence homology (He et al. 2010). In our study, all MATE proteins were grouped into 4 clades, designated as clade I to IV (Fig. 4). Among four, clade I is outlier having single cluster consisting of two genes from Arabidopsis and two from chickpea. Other three clades had both AtMATE and OsMATE genes, and it demonstrates that the evolution of MATE genes was conserved between dicots and monocots. Clade IV constitutes the largest one in the MATE phylogeny containing 63 genes and further divided into subgroup A and B consisting 18 AtMATE, 20 OsMATE and rest is from chickpea. The present study identified a few candidate genes with different functions that appear to be involved in salt tolerance and further characterization of these genes as well as their promoters may contribute to a better understanding of the salt stress response in both rice and chickpea. Furthermore, this study provides information on the role and evolution of MATE family genes associated with stress tolerance in plants.

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