Expression of OsMATE1 and OsMATE2 alters development, stress responses and pathogen susceptibility in Arabidopsis

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Multidrug and Toxic compound Extrusion proteins (MATE) are a family of secondary active transporters which utilize electrochemical gradient of membrane maintained by ATPases for their transport activity1. Members of this transporter family have ubiquitous occurrence in all the organisms including human and plants. The first member of this family (NorM) was characterized from Vibrio parahaemolyticus, which effluxes norfloxacin and ciprofloxacin outside of the cells in an energy dependent way2. Most of the earlier studies suggest that MATE proteins act as an efflux pump that export majority of drugs and xenobiotic compounds outside the cell, and largely contribute to the drug resistance in bacteria. Recent reports indicate that MATE proteins function in antiport manner and drive the substrate transport in exchange with H+/Na+ depending on the organism. Studies suggest that H+ coupling is operational in plants, which is substituted by Na+ in bacteria. Though structure and mechanism of action of MATEs have not been studied in detail, recent investigations revealed conformation and transport behaviour of MATE proteins3,4. Conversely to designated name “multi-drug”, numerous studies revealed that MATE proteins have stringent substrate specificity and facilitate the movement of specific compounds3. Presence of homologous putative MATEs has been identified from several organisms through comparative genomics. A genome wide scanning and analysis revealed that 58 MATE paralogues are present in Arabidopsis genome6. In general, MATE proteins from various source organisms have a conserved domain and share at least 40% amino acid sequence homology3.

In higher plants, studies demonstrated that MATE proteins are mainly involved in the transport and trafficking of xenobiotic and small organic molecules7. The foremost characterized Arabidopsis MATE protein, AtDTX1, was demonstrated to export norfloxacin due to its ability to restore drug tolerance in the norfloxacin sensitive bacterial mutant (kan3 mutant)8. Recently, evidences for role of MATEs in the plants are rapidly accumulating. Depending on the established functions of this gene family in plants, MATEs have been grouped into three major classes. The first group of the MATE has been demonstrated to be associated with disease resistance in Arabidopsis. Several Arabidopsis mutants have been identified and examined for understanding the molecular mechanism of pathogen resistance. Among them, enhanced disease susceptibility mutant (eds5) of Arabidopsis displayed a reduced basal resistance during pathogen interaction. The fine genetic mapping of the eds5 locus identified that EDSS, a member of the MATE transporter family, was localised within this locus, and substantially contributed to disease tolerance9. The relation of EDSS with disease tolerance has been explored very recently and demonstrated that EDSS export salicylic acid (SA) outside chloroplast, where SA synthesis takes place10,11. Similarly, ADS1 (activated disease susceptibility1), a negative regulator for the disease resistance, was another
MATE identified in *Arabidopsis* showing involvement during pathogen infection. A second group of MATE proteins is known for exporting small organic molecules such as citrate outside the cell which acts as a ligand molecule to bind aluminium (Al) in the rhizosphere. Unlike to other metals, Al toxicity is much pronounced in acidic soil and several genetic studies were performed to map the locus responsible for Al tolerance in plants. A locus for Al tolerance, *Altsb*, was identified that encode a MATE via positional cloning in sorghum. This *SbMATE* has been shown to efflux the citrate from cell and forming non-toxic complexes with Al in soil solution. Similar to sorghum, MATE proteins from *Hordeum*, *Arabidopsis*, *Triticum* and maize have been shown to participate in Al tolerance. Tandem duplication of MATE paralogues in the maize genome have been demonstrated to offer another level of transcriptional regulation to provide adaptation of maize to Al toxicity.

Another, third group of MATE transporters are involved in trafficking of secondary metabolites in vacuoles. Usually, vacuoles are the major repository site of most of the conjugated form of flavonoids comprising mainly flavonols, anthocyanins and flavone glycrose. The transport and storage of these flavonoids into vacuole are mediated by the different class of transporters on tonoplast. A member of MATE gene family (*TT12*) was elucidated for sequestration of proanthocyanidins in vacuoles of seed that leads to pigmentation of seed coat. The kinetic study of vesicles isolated from *TT12* expressing yeast revealed that it can specifically transport glycosidic form like epicatechin 3′-O-glucoside and cyanidin 3-O glucoside. Consistent to the *TT12*, MATEs from *Medicago* and *Vitis* have been characterized for mobilization of flavonoids in cell organelles. Similar to the role in vacuolar flavonoids sequestration, MATE proteins are also recognised for alkaloids trafficking into vacuoles in tobacco.

Apart from these functions *FRD3*, a MATE citrate efflux transporter, was shown to involved in Fe transport towards stelar region and hence assisted in distribution of Fe throughout aerial parts in *Arabidopsis*. Curiously, one member of MATE gene family (*Md1*) was shown to regulate the glucose assimilation, and which is thought to be necessary for nutrient utilization, sporation and pathogenicity in fungi. In another report, role of a MATE-like transporter (*ZRZ*) has been demonstrated in organ initiation as overexpression of this MATE led to diverse morphological changes in *Arabidopsis*. Therefore, it seems that MATEs govern the diverse physiological functions in the plants which need to be elucidated. Likewise to *Arabidopsis*, genome-wide analysis of MATEs encoding loci in rice revealed the presence of large members of this gene family however, function of only a few members has been elucidated as yet. Our earlier study suggested that expression of some of the genes encoding MATEs is up-regulated in rice seedlings exposed to arsenic (As) stress. Of these loci, we have investigated the role of two gene, OsMATE1 (Os03g08900) and OsMATE2 (Os05g48040) using heterologous system, *Arabidopsis*, in this study. Our observations suggest that these MATEs play crucial role in plant growth, development and in stress responses.

**Results**

*In silico* identification and analysis of rice MATEs. In order to identify MATE genes present in the rice genome, BLASTP analysis at Rice Genome Annotation Project using *Arabidopsis* MATEs as query sequences was performed. A total of 53 members of the rice MATE family were identified (Supplementary Table S2). To study the evolutionary relationship between identified rice MATE proteins, a maximum likelihood phylogenetic tree was constructed using all the MATE proteins from *Arabidopsis* as well as from other plants with well established functions. The analysis suggests that rice MATE proteins clustered in different groups of *Arabidopsis* and other known MATEs from different plants.

As very limited information about the function of rice MATE proteins is available, we have selected two arsenic responsive members, OsMATE1 (Os03g08900) and OsMATE2 (Os05g48040), for functional studies. Though both of MATEs show same responses with As, whereas grouped in two distinct clades (Supplementary Fig. S1). These rice MATEs, OsMATE1 and OsMATE2 encode polypeptide comprising the 489 and 500 amino acid residues respectively and shares 36% identities and 53% similarity with each other within 90% query cover. Furthermore, trans-membrane helix prediction through TMHMM indicated the presence of 12 trans-membrane helices in both MATEs (Supplementary Fig. S2). This preliminary analysis suggests that OsMATE1 and OsMATE2 were membrane bound proteins and possibly engage in transport activity across membrane.

Expression of OsMATE1 and OsMATE2 alter growth in *Arabidopsis*. In order to investigate the function of OsMATE1 and OsMATE2, full-length cDNAs were expressed in *Arabidopsis* under control of a CaMV35S promoter. The presence and expression of transgene was confirmed through genomic and semiquantitative RT-PCR (Supplementary Fig. S3). Depending upon relative expression and phenotypic appearance, three independent lines expressing OsMATE1 and OsMATE2 were selected for further study. Up to two weeks of germination in soil, growth pattern of the all transgenic lines was similar to WT plants. However, after two weeks of growth, transgenic lines expressing OsMATE1 or OsMATE2 displayed a distinguishable growth pattern in comparison to WT plants, but very similar to each other. The visible changes at the stage of three week old plants among all transgenic lines were quite prevalent (Supplementary Fig. S3, Fig. 1) that consist petiole length, leaf size and rosette leaf arrangement in vegetative parts.

The pattern of rosette leaves of the mature plants was different in comparison to WT plants without any change in the number of leaves (Fig. 1a). Petiole length and rosette leaf size of the transgenic lines were significantly increased compared to the WT (Fig. 1b). Collectively, the expression of OsMATE1 and OsMATE2 resulted variation in vegetative tissues, including the leaf size and altered pattern of rosette development. To authenticate these inferences, growth of two T-DNA insertional mutants of *Arabidopsis* MATEs (SALK_045655C and SALK_124549C) having close sequence similarity with OsMATE1 and OsMATE2 were analysed. Interestingly, a cognate growth pattern of salk lines with WT plants was observed (Supplementary Fig. S4a). Altogether, these observations suggest that OsMATE1 and OsMATE2 play important role in the growth and development of plants.

Altered floral morphology in transgenic lines expressing OsMATE1 and OsMATE2. Aside to morphological changes, variations in flowering time and flower size were observed in all the transgenic lines expressing OsMATE1 and OsMATE2 in comparison to WT plants (Fig. 2). An early flowering was observed in transgenic lines under normal growth conditions. Flowering time in transgenic lines was significantly reduced (up to four days) relative to WT plants (Fig. 2) but rosette leaf numbers at bolting stage were identical in transgenic lines and WT plants. Flowering time of salk mutants and WT plants were found similar and no significant difference was observed during initiation of bolting (Supplementary Fig. S4b). In addition to the alteration in flowering time, significantly reduced size of mature flower (length and width) was observed in transgenic lines compared to WT (Fig. 3). Though silique size of each transgenic line was comparable to WT, the number of siliques per plant were significantly higher (up to 50%) in comparison to WT (Fig. 4b). Transgenic lines also produced smaller seeds as compared to WT, although the viability of seeds was not compromised (Fig. 4b). Similar to these differences of reproductive organs, seed number produced per silique in all the transgenic lines was significantly higher corresponding to WT plants (Fig. 4c). Taken together, these
results suggest that expression of OsMATE1 or OsMATE2 in Arabidopsis develops many phenotypic changes which include reduced time of transition from juvenile to flowering stage, morphology of flowers, silique number, seed size and silique number produced per plants. However, such a macroscopic changes were not observed in salk lines. Genome wide expression analysis in the transgenic lines. To address reason for phenotypic changes in transgenic plants, modulation of transcriptome was analyzed using Affymetrix ATH1 (22K) array of three week old WT and transgenic line expressing OsMATE1. At this stage, clear phenotypic changes between WT and transgenic lines were visible. After normalization of cel files through d-Chip, a significantly modulated expression (1.6 fold, p values 0.05) of 39 genes in transgenic lines compared to WT plants were observed. Of these, expression of 14 and 25 genes were up- and down-regulated, respectively in the transgenic line (Table 1 and 2). The majority of genes up-regulated were related to DNA binding proteins and transcription factors controlling the diverse metabolic and physiological processes in plants. Out of most notable modulated expression, pentatricopeptide (PPR) repeat-containing protein up-regulated nearly 3 fold (Table 1). Characterized PPR proteins in Arabidopsis are known to be localized in mitochondria and participate in the post transcriptional modification of RNA. The impaired or loss of function of PPR lead to reduced NADH dehydrogenase activity and consequently a compromise defence response in Arabidopsis. Enhanced expression of RD20 (Responsive to Desiccation 20) and CAX7 (calcium exchanger 7) was also seen, which are involved in salinity stress. A significant up-regulation of NAC6 and NAC32 was observed in transgenic plants. NAC domain containing proteins are highly studied transcription factor family in plants and are known to regulate growth and development of plants and drought, desiccation, salinity and ABA response. Interestingly, expression of a Jumonji transcription factor (jmjC domain-containing protein) was enhanced in transgenic lines. In Arabidopsis, jmjC domain-containing protein (Jmj30) is known as the key determinant of circadian rhythm and regulates the flowering time in a cooperative manner with central oscillator, circadian clock associated1 and late elongated hypocotyl. Expression of all of the up-regulated genes was further validated through qRT-PCR (Fig. 5) that followed the same pattern of expression as observed in microarray analysis. Overall, these observations revealed modulated expression of certain regulators involved in contending the abiotic stress, and one specific regulator of circadian function (Jmj30) in transgenic plants. Our analysis also indicated that a group of defence related genes were down-regulated in transgenic plants in comparison to WT (Table 2). Quantitative RT-PCR analyses of these genes were in agreement with microarray results (Fig. 5). These genes comprised the pathogenesis related proteins (PR-1, PR-5), β-1,3-glucanase, chitinase, plant natriuretic peptide A, aspartyl protease family protein, crinkly related protein and ankyrin binding protein. A remarked decrease in transcript level of three cysteine rich receptor like protein kinases (CRK) and two receptors like protein were observed among 28 down-regulated genes. PR proteins are, in
general, recruited downstream in salicylic acid signalling pathway and extend the cascading of signals. Expression of β-1,3-glucanase, a class of PR proteins, and chitinase are induced during fungal infection that integrate a sort of tolerance while invasion of fungus in the plants. We conceived that the significantly reduced expression of stress related genes may lead to compromised defence response in transgenic plants.

**Arsenic response of transgenic lines.** Our earlier study suggested that OsMATE1 and OsMATE2 expression is enhanced during As exposure in rice. To examine the response of As on growth of OsMATEs expressing transgenic lines, seeds of transgenic lines, WT and pBI121 (empty vector transformed control) were grown for ten days on half strength MS containing agar plates with and without addition of As. All the genotypes grew similarly in absence of As(V). However, growth and root length of transgenic lines were significantly decreased in comparison to WT and pBI121 plants under As(V) rich environment (Supplementary Fig. S5). As participation of MATE proteins in combating the aluminium (Al) stress is well documented, the effects of Al on growth of transgenic lines were also evaluated. However, root length of all the genotypes grew similarly in absence of Al in media and no change has been observed compared to WT and pBI121 plants (Supplementary Fig. S6). The root length data suggests that As(V) specifically sensitizes the growth of transgenic lines.

**Enhanced disease susceptibility in transgenic lines.** Expression analyses suggested that a group of defence related genes were down-regulated in transgenic plants compared to WT (Table 2). We envisioned that significantly reduced expression of stress related genes may lead to compromised defence response in transgenic plants. To further study the expression of MATEs under biotic stresses, we have investigated the expression pattern of OsMATE1 and OsMATE2 in Nipponbare and IAC165 cultivar of rice during infection of M. grisea isolate (FR13) and S. hermonthica using publically available microarray data. Expression dataset exhibited that OsMATE1 was enhanced at post infection of M. grisea whereas its expression was repressed or unaffected during S. hermonthica interaction. By contrast, OsMATE2 induced specifically by the exposure of S. hermonthica (Fig. 6a). The expression analysis revealed that OsMATE1 and OsMATE2 were differentially expressed during M. grisea and S. hermonthica infections in rice and suggest that these genes might have a role in biotic stresses.

To ascertain the role of these MATEs in biotic stress, basal resistance of the transgenic and WT plants challenged with Pseudomonas syringae (Pst DC3000), a natural host of Arabidopsis causing speck disease was measured. After 3 dpi, an explicit enhanced susceptibility was observed in transgenic lines in comparison to WT in the form of the chlorotic lesion with a substantially higher bacterial titre (Fig. 6b, c). Our observations suggest that OsMATE1 and OsMATE2 may be negatively regulating disease tolerance apart from governing the growth and development. Furthermore, we have carried out infection assay after sprayed inoculation of bacterial culture on whole plants and quantified the bacterial titre at 7 dpi, which reconfirmed the susceptibility and significantly higher titre in all the transgenic lines expressing OsMATE1 and OsMATE2 (Supplementary Fig. S7).
Sub-cellular localization of OsMATE1 and OsMATE2. In order to investigate the sub-cellular localization of OsMATE1 and OsMATE2, complete coding region of OsMATE1 and OsMATE2 were cloned in transient expression vector 326 sGFP and transformed in protoplast isolated from mesophyll cells of Arabidopsis leaves. The examination of GFP fluorescence, after overnight incubation in dark, under confocal microscope explicitly suggests that OsMATE1 and OsMATE2 underlie on the plasma membrane, whereas a diffused green fluorescence was seen throughout the cell transformed with empty vector (Fig. 8). Altogether, these observations indicated that OsMATE1 and OsMATE2 reside on the plasma membrane.

Altered flavonoid content in transgenic lines. The relatedness of OsMATE1 and OsMATE2 with some of characterized flavonoid transporters in phylogenetic tree (Supplementary Fig. S1) suggest the possible role of these proteins in flavonoid transport. The two fold higher expression of GST (Glutathione-S transferase) in OsMATE1 expressing lines as observed in expression analyses also strengthen this hypothesis. The physical conjugation of glutathione to flavonoid catalysed by GST is a crucial metabolic step for flavonoids trafficking. Therefore, content of major flavonols (kaempferol, quercetin and rutin) have been quantified in leaves of WT and transgenic lines. Flavonol profiling revealed the perturbation of kaempferol content in transgenic lines in comparison to WT plants. As speculated, kaempferol content was enhanced in transgenic leaves, which was undetectable in WT leaves (Table S6), and suggests that a proper flavonol channelling might be affected in transgenic plants.

Discussion

By using reverse genetics approach and a combination of molecular and biochemical tools, functional characterization of two members of the MATE family from rice, OsMATE1 and OsMATE2 has been carried out in this study. The scarcity of knowledge about the function of MATE proteins in rice, except the known citrate efflux activity of the FRDs, encouraged us to ascribe the detailed role of OsMATEs out of total 53 proteins in rice genome. The constitutive expression of two As-responsive MATE encoding genes, OsMATE1 and OsMATE2, led to the diverse changes in growth and morphology of Arabidopsis. The major alterations were seen in leaf morphology, pattern of rosette leaves arrangement and flower development. We further demonstrate that transgenic lines were sensitive to As(V) exposure as well as exhibit a compromised basal resistance. Repressed immunity of transgenic lines was correlated with lowered expression of genes encoding pathogenesis related proteins.

Astonishingly, transgenic plants harbouring OsMATE1 and OsMATE2 independently displayed distinct common alterations that encompass leaf size, rosette leaf arrangement, flower, silique and seed morphology (Fig. 1–4). Nonetheless, these phenotypic variations were lacking in salk lines possibly due to presence of large members of this gene family with overlapping functions in Arabidopsis. For studying the mechanisms and responsible genes, we studied the genome wide expression analyses of transgenic and WT leaves. The analysis suggested that enhanced expression of a set of genes in transgenic plants may play important role in such phenotypic changes (Table 1 and 2). Two loci encoding the protein that encompasses leaf size, rosette leaf arrangement, flower, silique and seed morphology (Fig. 1–4). Nonetheless, these phenotypic variations were lacking in salk lines possibly due to presence of large members of this gene family with overlapping functions in Arabidopsis. For studying the mechanisms and responsible genes, we studied the genome wide expression analyses of transgenic and WT leaves. The analysis suggested that enhanced expression of a set of genes in transgenic plants may play important role in such phenotypic changes (Table 1 and 2). Two loci encoding the protein that contains NAC domain representing NAC transcription factor family were unregulated. NAC proteins constitute one of the largest families of transcription factors in plants and NAC domain containing transcription factors is considered as key regulator of stress perception and co-ordinating developmental programmes in plants. Recently phytochrome-b interacting proteins, vascular plant one zinc finger1 (VOZ1) and VOZ2, were identified to regulate the flowering in Arabidopsis. VOZ1 and VOZ2, a transcriptional activator, belong to the NAC family and voz1voz2 double mutant showed a late flowering phenotype. In this study, we also reported an early flowering in transgenic lines and changes in silique number, flower and seed size corresponding to WT plants (Fig. 2–4). These changes might be due to increased transcript level of NAC transcription factors. Early
flowering of transgenic plants can be due to enhanced expression of JumonjiC (JmjC) containing protein. Basically, Jmj30 is a type of histone demethylase, which epigenetically regulated the circadian clock of *Arabidopsis* by affecting histone remodelling. In *Arabidopsis*, circadian clock regulates various processes such as gene expression, photoperiodic flowering, and leaf movement. Thus, higher expression of Jmj30 might have a role in association with other factors as a determinant of early flowering in transgenic plants.

Figure 4 | Alteration in silique and seed number in transgenic plants. (A) All the transgenic lines more silique per plant than WT. Photograph of 6 week grown plants (2 lines of each transgenic) was taken. Data of at 3 transgenic lines presented as mean and ± SE (n = 8–12). (B) Seed size of transgenic and WT plants (representative of 1 line of each transgenic). (C) Seeds number per silique was high in transgenic lines. Values were collected from 10 silique per plant and 8–12 plant for each lines and WT. Data are presented as mean, ± SE (n = 8–12). Significance was determined by One-way ANOVA (Newman-Keuls used as post hoc test). ***Significantly different from WT (P < 0.0001).

| Locus ID | Annotation | Fold change |
|----------|------------|-------------|
| At5g59310| LIPID TRANSFER PROTEIN 4 | 6.87 |
| At5g39610| NAC domain containing protein 6 | 3.02 |
| At3g20810| JUMONJI (jmjC) domain-containing protein | 2.79 |
| At1g07590| PENTATRICOPEPTIDE (ppr) repeat-containing protein | 2.75 |
| At2g19970| Pathogenesis related protein, putative | 2.52 |
| At5g17860| CAAX (calcium exchanger 7) | 2.24 |
| At2g33380| RD20 (response to dessication20) | 2.19 |
| At1g30260| Unknown protein | 2.18 |
| At2G29490| Glutathione S-transferase TAU 1 | 2.03 |
| At1g77450| NAC domain containing protein 32 | 2.02 |
| At5g42900| Unknown protein | 2.02 |
| At3g28290| UFPO496 protein | 2 |
| At2g36120| Glycine-rich protein | 1.99 |
| At4g14540| UDP-glycosyltransferase | 1.97 |
| At1g77620| Nitrate Reductase | 1.95 |
| At5g61820| Unknown protein | 1.95 |
| At1g72260| Toxin receptor binding (thionin2.1) | 1.83 |
| At5g23240| DNAJ heat shock N-terminal domain-containing protein | 1.68 |
We further checked the co-expressed genes of Jmj30 by searching at ATTED-II to get a more clue at molecular level. Our analysis suggests that COL9 (At3g07650) and ELF4 (At2g40080) show a high correlation co-efficient along with Jmj30. COL9 belongs to CONSTANS gene family and is a negative regulator of photoperiodism and flowering via suppressing the expression of Flowering locus T (FT) and Suppressor of Overexpression of CO1 (SOC1)45. A subtle increase in expression of COL9 in transgenic lines supports the assumption (Supplementary Fig. S8). In addition, induced expression of the pentatricopeptide repeat containing (PPR) and other redox related genes suggest an imbalance of proper redox state of the cells.

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Table 2 | List of significantly down-regulated genes in OsMATE1 expressing Arabidopsis line

| Locus ID | Annotation | Fold change |
|----------|------------|-------------|
| At2g14610 | Pathogenesis related protein 1 (PR1) | −57.81 |
| At3g57260 | BETA-1,3-GLUCANASE 2 (BG1) | −7.76 |
| At1g75040 | Pathogenesis-related protein 5 (PR5) | −7.5 |
| At3g25010 | Receptor Like Protein 41 (RLK41) | −6.57 |
| At4g23150 | Cysteine-rich RLK (CRK7) | −6.36 |
| At2g43570 | Chitinase | −5.9 |
| At3g22600 | Lipid transfer protein (LTP) | −5.41 |
| At1g14870 | Plant Cd resistance protein | −4.87 |
| At3g18250 | Membrane lipoprotein | −4.62 |
| At2g18660 | Plant natriuretic peptide A (PNP-A) | −4.34 |
| At5g10760 | Aspartyl protease family protein | −4.21 |
| At1g76960 | Unknown protein | −3.6 |
| At3g28540 | P-loop containing NTP hydrolases superfamily protein | −3.43 |
| At5g42830 | HXXD-type acyl-transferase family protein | −3.33 |
| At5g47850 | CRINKLY4 RELATED 4(CCR4) | −3 |
| At2g37430 | Zinc finger family protein (ZAT11) | −2.99 |
| At2g32680 | Receptor Like Protein 23 (RLP23) | −2.93 |
| At1g74710 | Isocitrate synthase 1 (ICS1) | −2.86 |
| At1g30900 | VACUOLAR SORTING RECEPTOR 6 (VSR6) | −2.77 |
| At5g54610 | ANK (ankyrin) protein binding | −2.62 |
| At3g48650 | Unknown protein | −2.46 |
| At4g23140 | CYSTEINE-RICH RLK 6 (CRK6) | −2.42 |
| At1g35230 | ARABINOGALACTAN-PROTEIN 5 (AGP5) | −2.35 |
| At3g45860 | Cysteine-rich receptor-like protein kinase 4 | −2.02 |
| At5g24530 | Flavanone 3-hydroxylase-like protein | −1.99 |
| At1g35210 | Unknown protein | −1.97 |
| At3g27690 | Photosystem II light harvesting complex gene (LHCB2.3) | −1.9 |
| At2g32030 | GCN5-related N-acetyltransferase (GNAT) family protein | −1.81 |

There are few recent report emerges that correlate a synchrony between oxidative burst (ROS signalling) and circadian clock of Arabidopsis46. Our expression analyses revealed that decreased expression of a group of defence related genes in OsMATE1 expressing Arabidopsis lines compared to WT (Table 2). These genes can be grouped in two broad categories, some of genes contribute to restrict the invasion of pathogen as their expression was highly induced, mainly comprising the PR proteins, β-1,3-Glucanase and Receptor like proteins (Table 2). Proteins encoded by these genes play critical role in pathogen recognition and signal perception followed by activation of

Figure 5 | Modulation of gene expression in transgenic line expressing OsMATE1. Relative gene expression between WT and transgenic plant was calculated using qRT-PCR (tubulin was taken as endogenous control). Data provided represent fold expression Log2 (transgenic/WT). Specific-primer set of individual genes was designed from intron spanning region.
Significance was determined by One-way ANOVA (Newman-Keuls used as post hoc test). and standard errors calculated from three biological replicate (each leaf from different plants). The experiment was carried out more than three times. Significance was determined by One-way ANOVA (Newman-Keuls used as post hoc test). *** indicates significant difference (P < 0.0001) as compared to WT.

defence responses. Similarly, cysteine-rich receptor like kinase and leucine rich repeat (LRR) protein kinases were significantly down-regulated (Fig. 5). A NB-LRR is the major class of plant receptor, which intercepts effectors signal from much of pathogens and control the transcriptional mobilization of defence pathways in Arabidopsis. Reduction in the expression of various genes responsible for signal perception to many downstream processes reflect the reduced basal disease resistance in transgenic plants and that was manifested in PstDC3000 infection assay as well (Fig. 6). An enhanced susceptibility was observed in transgenic lines expressing OsMATE1 and OsMATE2 compared to WT plants either on the detached leaf (Fig. 6b, c) or after seven dpi in planta sprays of bacterial inoculums (Supplementary Fig. S7). Similar to our observations, another study revealed that an activation tagging mutant of ads1-D (MATE protein) is highly susceptible to PstDC3000 and knockdown of ADS1 results a higher basal resistance in Arabidopsis. A systematic proteomic analysis of root exudates of Arabidopsis revealed that chitinase, β-1,3-Glucanase and other defence proteins are secreted in higher amount during flowering and shown to link between flowering and defence processes. The involvement of MATE proteins in disease resistance is unexceptionally accepted, and formerly identified during genetic screen of Arabidopsis mutants however underpinning molecular mechanism is explored very recently. It has been revealed that EDS5 regulate salicylic acid biosynthesis by exporting from chloroplast. Flavonoids are often regarded as prominent signalling compound and interplay during biotic stresses however underlying actual molecular events of flavonoids signalling is not very much known. It has been reported that through managing accumulation of some flavonols plants can better adapt under plenty of pathogens and insect attacks. In our study, flavonoid profiling of transgenic and WT leaves suggested altered flavonol content in transgenic plants in comparison to WT plants. We propose that perturbation of kaempferol, through OsMATEs expression in Arabidopsis, could be the central and foremost event that persuades morphological alterations. Although, specific substrates transported by these transporters have not resolved yet, but the results presented here indicated that it may be a flavonol, and which need to explore in future by molecular transport experimentation.

A prominent (AsV) sensitivity was observed in transgenic lines expressing MATEs as compared to WT plants (Supplementary Fig. S5). The sensitivity of transgenic plants could be explained on the basis of inevitable contribution of flavonoids in maintaining redox by retaining ROS scavenging activity. Disturbance of this process via perturbation of intracellular flavonoids either at different steps of their biosynthesis or transport could lead to the manifestation of As(V) sensitive phenotype. Plasma-membrane localized MATE transporters are devoted for citrate efflux while others are recognised for flavonoid and other secondary metabolites sequestration in vacuoles. The previously identified role of MATE transporters engage in flavonoids sequestration are found to mostly restricted on vacuolar membrane rather than plasma-membrane localization of OsMATE1 and OsMATE2, which is elucidated in this study (Fig. 8). Thus, more and more information generated about functional significance of members of this gene family in plants, would elaborate our insight about transport of flavonoids and other compounds ranging from intracellular to long distance transport and their impact on various physiological processes in future.

In conclusion, our study suggests that OsMATE1 and OsMATE2 are functionally redundant to each other based on observations obtained in transgenic lines as both MATEs direct the common phenotypic changes through expression in Arabidopsis as well as...
length MATEs was constructed by the neighbour-joining method using Phylip52 and http://www.ebi.ac.uk/Tools/clustalw2/index.html. The phylogenetic tree using full-
multiple amino acid sequences were aligned with the help of ClustalW alignment tool of rice,
of each experiment were imported and analysed using d-Chip under default setting54. (GSE10373) were used in expression analysis. The Affymetrix CEL files
http://rice.plantbiology.msu.edu) by using keyword, domain name and BLAST the database on the BLAST server (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi and
SCIENTIFIC
methods.
Plant material and growth condition. Arabidopsis thaliana (L.) accession Col-0 and
salk mutants (SALK_045655C and SALK_124549C) were used in the study. Arabidopsis plants were grown in growth chamber (Canviron, USA) under controlled
conditions 16 h light-8 h dark photoperiod cycle, 22 °C temperature, 150–180 μmol m−2 s−1 light intensity and 60% relative humidity. Seeds were sown in solirite and
stratified for 48 h in the dark at 4 °C before shifting in the light. Plants were watered with nutrient media (5 mM KNO3, 2 mM MgSO4, 2 mM Ca (NO3)2, 2 mM K
H2PO4, 50 μM Fe(II), EDTA, 70 μM H3BO3, 14 μM MnCl2, 1 μM ZnSO4, 0.5 μM
CuSO4, 0.2 μM Na2MoO4, 10 μM NaCl, 0.01 μM CoCl2) and reverse osmosis (RO) water at alternate interval in every week until plants complete their lifecycle.

Database search and Bioinformatics analysis. MATE sequences were searched from the database on the BLAST server (http://www.ncbi.nlm.nih.gov/blast/BLAST.cgi and http://rice.plantbiology.msu.edu) by using keyword, domain name and BLAST against characterized Arabidopsis MATEs. We retrieved full length MATE sequences of rice, Arabidopsis, and fully characterized MATE proteins from other organisms. Multiple amino acid sequences were aligned with the help of ClustalW alignment tool (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The phylogenetic tree using full-
length MATEs was constructed by the neighbour-joining method using Phylip52 and displayed using PhyloDraw V0.8214. Expression study of OsMATE1 and OsMATE2 during biotic stress in rice has been carried out using publicly available microarray CEL files in the GEO database. GEO database for virulent infection by Magnaporthe grisea (GSE7258) and expression data for interaction with the plant parasite Striga hermonthica (GSE10373) were used in expression analysis. The Affymetrix CEL files of each experiment were imported and analysed using d-Chip under default setting54.

Cloning, construct preparation and transgenic plant development. Full-length cDNAs of OsMATE1 and OsMATE2 were amplified from the single stranded cDNA library prepared from rice root though PCR by using nested primers (Table S1) and cloned in pU17Z subcloning vector (Fermentas, USA). Another set of primers was designed with restriction sites of Xba1 and SacI enzymes in forward and reverse primers, respectively. The coding region of both MATEs was cloned in plant expression vector pBI121 between Xba1 and Sac1 restriction sites. These constructs were transformed in Agrobacterium tumefaciens strain GV3101 by the freeze thaw method. Arabidopsis transgenic plants were generated through floral dip method55. Transformants were screened on half MS agar plates containing kanamycin (50 μg per ml media). Based on the relative expression of transgene through semi quantitative RT-PCR, three lines for each of OsMATE1 and OsMATE2 among the 15 screened independent lines were selected for further analysis. All the studies were performed in T4 homozygous lines. For promoter analysis, upstream sequences from the translation initiation codon of OsMATE1 (1540 bp) and OsMATE2 (1434 bp) were retrieved and amplified using High Fidelity enzyme mix (Fermentas, USA) from genomic DNA and cloned in pCAMBIA1303 plant binary vector between HindIII and Ncol restriction sites. Transgenic plants were generated and screened on hygromycin (10 μg per ml). Similarly, for preparing the construct employed in sub-cellular localization, coding region of OsMATE1 and OsMATE2 were cloned in 326 sGFP transient expression vector between Xba1 and BamHI restriction sites to encode the fusion proteins55. All the clones were sequenced to ensure the in frame fusion and error free preparation of constructs.

Microarray and gene expression analysis. Microarray analysis was performed using RNA isolated from rosette leaves of nearly three weeks old Col-0 and OsMATE1 expressing plants. Whole genome Arabidopsis ATH1 array (Affymetrix, USA) was utilized in experiment and each step was followed as per manufacturer’s instruction. Total RNA was isolated from rosette leaves by using Spectrum Plant Total RNA Kit (Sigma, USA), followed by treatment with RNase-free DNase (Fermentas, USA) to remove the genomic DNA. Briefly, 250 ng total RNA of each sample was taken as starting material and strictly followed the Affymetrix protocol during RNA amplification, hybridization, washing-staining and scanning of the arrays. Normalization of cell files and relative fold change were measured using d-Chip54. Quantitative Real time PCR (qRT-PCR) was carried out to validate microarray data. Total RNA (2 μg) was used in first strand cDNA library preparation (RevertAid H minus first strand cDNA synthesis Kit Fermentas, USA). One μl template was taken

Figure 7 | Promoter analyses of OsMATE1 and OsMATE2. (A) cis-regulatory elements of ProOsMATE1 and ProOsMATE2 was analysed through PLACE and venn diagram shows the number of common and unique motifs present in promoters. (B) Promoter activity in WT and Arabidopsis lines expressing ProOsMATE1:uidA and ProOsMATE2:uidA. Representatives of GUS staining performed in seedling (10 day old), flowers and siliques of mature plant. White scale bars present in each panel in (B) are equivalent to 100 μm length.

their similar promoter activities in Arabidopsis. There is a necessity of thorough study in the future to obtain more insight about nature of substrates transported by these proteins to establish a direct link between growth-development and disease resistance in plants.
denaturation at 94°C. Master mix (Fermentas, USA) were used. A reaction set up, with an initial template and a primer set specific for OsMATE1 and OsMATE2 (Table S1) and 2× master mix (Fermentas, USA) were used. A reaction set up, with an initial denaturation at 94°C for 2 min, 28 cycles at 94°C for 15 s, 55°C for 15 s, and 72°C for 30 s, followed by a final 5 min extension at 72°C, condition were executed in 20 µl reaction volume. PCR products were separated in 1.2% agarose gel in 0.5× TBE running buffer. For taking equal amount of cDNA tubulin was taken as an endogenous control.

**Metal exposure and root growth assay.** Seeds of all the genotypes were surface sterilized with sodium hypochlorite and plated on half strength MS agar plates with and without supplementation of As(V) and Al57. Plates were kept in dark for 48 h at 4°C (stratification) and photographed after the growth of ten days in standard growth conditions. Root lengths of each genotype and WT plants were manually measured and taken from at least three separate plates.

**Analyses of transgenic lines.** Homozygous transgenic lines expressing OsMATE1 and OsMATE2 were grown for complete life cycle and all observations regarding different phenotypic characters were collected at indicated growth stages and monitored at least 2–3 successive generations. For histological GUS activity, ProOsMATE1:uidA and ProOsMATE2:uidA transgenic lines were grown on half strength MS agar plates for 10 days on or solirite. Seedlings and different tissues from mature plants were rinsed in ice cold PBS buffer 3–4 times and after three washing samples were incubated 16 h at 37°C in GUS staining buffer containing 100 mM NaPO4, 1.0 mM ferrocyanide, 1.0 mM ferricyanide, 0.05% Triton X-100, pH 7.2, and 2 mM 5-bromo-4-chloro-3-indolyl-b-D-glucuronide (initially dissolved in N,N-Dimethyl formamide)58. After successive washing from the gradient of ethanol (70%–90%), images were captured under light microscope (Carl Zeiss, USA).

**Bacterial infection assay.** *Pseudomonas syringae* pv. tomato strain (PstDC3000) was allowed to grow overnight in king’s B media containing 15 µg rifampicin per ml media at 28°C with 220 rpm shaking. Cells were collected by 5000 rpm centrifugation and washed with sterile 0.8% gelatin. Bacterial OD was adjusted to 5 × 10^{9} cfu/ml in 0.8% gelatin and sprayed on whole plants equally. Infected plants were kept in growth chamber under 16 h light- 8 h dark cycle at 22°C for 7 days and then bacterial titres were measured.

**Sub-cellular localization.** For sub-cellular localization, complete coding sequences of OsMATE1 and OsMATE2 were fused upstream, in frame with, to sGFP59 under control of CaMV 35S promoter in 326 gSFP vector56. These constructs were transiently expressed in protoplasts isolated from mesophyll cells of four weeks old *Arabidopsis* leaves and 15 µg of purified DNA was used in polyethylene glycol mediated transformation60. After overnight incubation transient expression of GFP fusion protein was examined. Images were captured under a confocal microscope (LSM 510, Zeiss, USA).

**Flavonoids estimation.** For flavonoids quantification, individual flavonoids in the rosette leaves were determined by methods of Misra et al. (2010). Briefly, plant material (500 mg) was grinded finely in liquid N2 and extracted in 5 ml methanol:water (80:20) with constant agitation for 2 h and supernatant was collected after centrifugation. Pellet was re-extracted in 5 ml methanol; water for overnight at room temperature with constant agitation and supernatant was filtered. Both the filtrates were pooled, concentrated in rotavapour and again dissolved in 1.0 ml of 80% methanol. Separation for qualitative and quantitative analysis of flavonoids was performed by HPLC-PDA with a Shimadzu (Kyoto, Japan) LC-10 system comprising an LC-10 AT dual pump system, an SPD-M20A PDA detector (254 nm) and rhodine injection valve furnished with a 20 µl sample loop.

**Statistical analysis.** Each experiment was carried out under a completely randomized design with at least three replications. Phenotypic observations were measured upto 2–3 generations for consistency. Data are expressed as mean ± SEM and subjected to one-way ANOVA followed by post hoc Newman–Keuls for multiple comparison test of significance using GraphPad Prism 3.02.

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