The Saltol QTL-localized transcription factor OsGATA8 plays an important role in stress tolerance and seed development in Arabidopsis and rice

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Abstract

GATA represents a highly conserved family of transcription factors reported in organisms ranging from fungi to angiosperms. A member of this family, OsGATA8, localized within the Saltol QTL in rice, has been reported to be induced by salinity, drought, and ABA. However, its precise role in stress tolerance has not yet been elucidated. Using genetic, molecular, and physiological analyses, in this study we show that OsGATA8 increases seed size and tolerance to abiotic stresses in both Arabidopsis and rice. Transgenic lines of rice were generated with 3-fold overexpression of OsGATA8 compared to the wild-type together with knockdown lines with 2-fold lower expression. The overexpressing lines showed higher biomass accumulation and higher photosynthetic efficiency in seedlings compared to the wild-type and knockdown lines under both normal and salinity-stress conditions. OsGATA8 appeared to be an integrator of diverse cellular processes, including K+/Na+ content, photosynthetic efficiency, relative water content, \( Fv/Fm \) ratio, and the stability to sub-cellular organelles. It also contributed to maintaining yield under stress, which was ~46% higher in overexpression plants compared with the wild-type. OsGATA8 produced these effects by regulating the expression of critical genes involved in stress tolerance, scavenging of reactive oxygen species, and chlorophyll biosynthesis.

Keywords: Drought, GATA, Oryza sativa, RNAi, salinity, Saltol QTL, transcription factor, overexpression, tolerance, yield.

Introduction

As sessile organisms, the growth and development of plants is greatly influenced by environmental factors such as salinity, drought, and heat (Vij and Tyagi, 2007; Pareek et al., 2010). Salinity is a major factor affecting plant productivity, as it limits the uptake of water by the roots and is associated with the accumulation of toxic ions, and thus it influences the overall cellular physiology of the plant (Munns and Tester, 2008). To cope with salinity, plants have evolved a diverse set of adaptive strategies, which include accumulation of osmoprotectants, activation of membrane-bound antiporters, and triggering of numerous stress-responsive pathways, which are regulated by the orchestrated action of specific transcription factors (Chinnusamy et al., 2005; Nakashima et al., 2007; Joshi et al., 2016; Nutan et al., 2017). Thus, a large subset of stress-related genes are coordinately and differentially expressed, depending on the dose and duration of the stress imposed (Seki et al., 2002; Zhu, 2002; Kumari et al., 2009a; Das et al., 2015; Singh et al., 2015). The fine regulatory control required to achieve such precise expression patterns is provided by transcription factors (TFs). TFs influence almost every aspect of plant life,
such as growth, development, and responses to abiotic and biotic stresses (Lakra et al., 2013; Joshi et al., 2016).

GATA TFs are a major class of transcriptional regulators that are widely distributed in eukaryotes. They are characterized by the presence of conserved, type-IV zinc-finger motifs (C-X_{2−5}C-X_{17−20}C-X_{2−C}) followed by a highly basic region. The first evidence of their existence in plants came with the identification of GATA motifs in the regulatory regions of genes responsive to light and circadian rhythms (Argüello-Astorga and Herrera-Estrella, 1998), including many of those involved in, or related to, photosynthesis, such as the Rubisco small subunit and the chlorophyll a/b binding protein (Koch, 1996). In the genome of rice (Oryza sativa), a total of 28 loci encoding GATA transcription factors have been annotated (Reyes et al., 2004; Gupta et al., 2017). Studies on single GATA-domain-containing proteins have revealed their role in diverse phenomena in plants such as regulation of processes by light, leaf greening, seed germination, and flowering (Richter et al., 2013a, b; Klermund et al., 2016). In Arabidopsis, the paralogous and functionally redundant GATA transcription factors GATA, NITRATE-INDUCIBLE, CARBON METABOLISM INVOLVED (GNC) and GNC-LIKE/CYTOKININ-RESPONSIVE GATA FACTOR1 (GNL/CGA1) have been shown to be involved in the control of flowering time, leaf greening, and cold tolerance (Richter et al., 2013a, 2013b). Functional studies on GNC/GNL mutants and overexpressing plants of Arabidopsis have indicated a role of these proteins downstream of signaling by hormones such as auxin, gibberellin (GA), and cytokinin (CK), and in this way they contribute to processes such as leaf greening, cold tolerance, determination of seed size, reduction-suppression of germination, flowering time, and senescence (Richter et al., 2010, 2013a, 2013b; Behringer and Schwechheimer, 2015). However, the role of these TFs in other abiotic stresses such as salinity and drought have not yet been reported.

Rice shows a lot of genetic diversity, including in the response to salinity; for example, a traditional landrace, Pokkali, has been found to be tolerant, while the commercial variety IR64 has been shown to be sensitive (El-Shabrawi et al., 2010). Morphological and physiological analyses of seedlings under salinity stress have demonstrated that the basis of the higher tolerance in Pokkali is higher chlorophyll and proline contents, increased shoot growth, and reduced membrane injury compared with IR64 (Kumari et al., 2010). Extensive transcriptome and proteome analyses in these genotypes has revealed differential expression of various categories of stress-related genes and proteins that contribute to the higher tolerance in Pokkali (Karan et al., 2009; Kumari et al., 2009a; Gupta et al., 2013; Nutan et al., 2017; Lakra et al., 2018, 2019). The Saltol QTL on the short arm of chromosome 1 in rice was identified from a recombinant inbred line population developed between Pokkali and IR29, and it has been shown to account for 62–80% of phenotypic variation under salinity stress (Bonilla et al., 2002; Thomson et al., 2010). The importance of this QTL in the salinity response in tolerant genotypes is indicated by the identification within Saltol of SKCI, which encodes a Na+ selective transporter (Ren et al., 2005), and by the increased abundance of transcripts of other genes involved in alleviation of salinity (Kumari et al., 2009a). Consequently, Saltol and its constituent genes have been the focus of research targeting the improvement of salinity tolerance in high-yielding rice genotypes. Functional validation of the Saltol QTL has been achieved using traditional marker assisted breeding programs for varietal improvement (Singh et al., 2018), as well as through transgenic technology by ectopically expressing salinity-induced genes localized in Saltol such as OsHBP1b and OsIF in tobacco as well as in rice (Soda et al., 2018; Das et al., 2019). Rice genomes modified in this way exhibit improved tolerance towards multiple abiotic stresses, thus reflecting the importance of the constituent genes that make up the QTL. Expression analysis of genes related to signaling (Soda et al., 2013) and to transcription factors (Nutan et al., 2017) that show higher constitutive transcript abundance in the tolerant genotype (Pokkali) and induced expression in the sensitive genotype (IR64) has led to the identification of a novel transcription factor, OsGATA8 and its characterization as playing a role in the response to salinity stress (Gupta et al., 2017). Out of the 28 gene loci (encoding 35 putative GATA transcription factors) that we have found in the rice genome, only GATA8 is localized within the Saltol QTL (Gupta et al., 2017), indicating its possible role in salinity tolerance. Additional support for this hypothesis comes from the fact that the GATA transcription factors GLN3 and GAT1 have been shown to be involved in salt tolerance in yeast (Saccharomyces cerevisiae; Crespo et al., 2001). Based on the localization of OsGATA8 within the Saltol QTL, its differential regulation in rice genotypes with contrasting salinity tolerance, and the involvement of its orthologs in salt tolerance in yeast, we hypothesize that OsGATA8 may be a master regulator of stress responses in rice that determines survival and yield under stress. Although the GATA family of TFs has been a subject of interest for many years, their roles in abiotic stress tolerance have only recently started to become apparent as tools have become available for producing mutants and overexpression lines in plants. In the present study, we examined the function of OsGATA8 in providing tolerance to salinity and drought stress by using transgenic plants of rice and Arabidopsis in which the gene was either overexpressed or knocked down by RNAi. Our results demonstrate that OsGATA8 plays an important role in both species in processes associated with chlorophyll biosynthesis, ion homeostasis, scavenging of reactive oxygen species, grain size, stress tolerance, and yield.

Materials and methods

Plant growth, stress treatments, and transcript abundance analysis for rice

Seeds of rice (Oryza sativa cv IR64 and Pokkali) were washed with de-ionized water and soaked in dark for two days after which seeds were allowed to germinate in a hydroponic system filled with half-strength Yoshida medium (Yoshida et al., 1971). The system was kept inside a growth chamber maintained at 28±1 °C, 70% humidity, under a 12/12 h light/dark cycle with illumination of 500–600 μmol m−2 s−1. For the salinity stress treatment, 7-d-old seedlings were treated with half-strength Yoshida medium supplemented with 200 mM NaCl; controls were maintained without adding NaCl. Seedlings were harvested after 10, 20,
OsGATA8 promotes abiotic stress tolerance in Arabidopsis and rice

Subcellular localization of OsGATA8–GFP fusion protein

The coding sequence of OsGATA8 was amplified using the primer pair OsGATA8-SL (Supplementary Table S1 at JXB online), which gave the desired amplicon without the stop codon and the required restriction site at both ends for cloning in the expression vector. The PCR product was sub-cloned into the pCAMBIA1304 vector to generate pCAMBIA-OsGATA8-GFP containing an OsGATA8-GFP fusion construct under the control of the cauliflower mosaic virus (CaMV) 35S promoter. The construct was used for transformation of epidermal cells of onion (Allium cepa) by biolistic bombardment, as previously described (Sharan et al., 2017). The transformed cells were incubated in Murashige and Skoog (MS) medium at 28 °C for 36–48 h and then observed using a FluoView FV1000 laser-scanning confocal microscope (Olympus).

Transactivation assays

In order to identify the activation domain in the transcription factor, the pGBD–C1 vector containing a GAL4 DNA-binding domain (James et al., 1996) was used. Yeast one-hybrid assays were performed using the AH109 strain, which was transformed with the pGBD-C1-OsGATA8 construct. Yeast transformants were selected on single drop-out medium (SD/-Trp) for 3–5 d at 30 °C. Further, they were transferred onto double drop-out medium (SD/-Trp/-His + 5 mM 3-amino-1,2,4-triazole (3-AT)) to check the activation of His reporter gene, and the transformed clones were subsequently streaked onto triple drop-out medium (SD/-Trp/-His/-Ade) supplemented with 5 mM 3-AT. Yeast cells carrying the empty vector (pGBD-C1, containing only the GAL4 DNA-binding domain) were used as the negative control. For the positive control, OsRR26, a response regulator protein from rice that is known to exhibit auto-activation (Sharan et al., 2017), was cloned into the pGBD-C1 vector.

Arabidopsis transformation, stress treatments, and phenotyping at the seedling and reproductive stages

The OsGATA8 gene cloned in the plant expression vector (pCAMBIA1304–OsGATA8) was transformed into Agrobacterium (GV3101) and transformation of Arabidopsis was performed using the floral dip method (Clough and Bent, 1998). For abiotic stress-tolerance assays on plates, wild-type and 35S–OsGATA8 seeds (T3) were germinated on MS medium. At 5 d after germination, seedlings from each line were carefully transferred to new MS media supplemented with different concentrations of NaCl, mannitol, ABA, or PEG. After 10 d of growth on the treatment media, root length, number of lateral roots, and the fresh weight of the seedlings were measured. For drought tolerance, 35S–OsGATA8 and wild-type (WT) seeds were germinated on MS medium, and 5-d-old seedlings were transferred to pots (100×100×8 cm) containing a mixture of vermiculite and humus (1:1). The plants were maintained in a growth chamber for 5 weeks (22 °C, 70% humidity, 12/12 h light/dark cycle (120–150 μmol m−2 s−1) before stress treatments were applied. For the drought treatment, water was withheld from plants for 20 d and they were then rewatered for next 3 d. For the salinity stress treatment, water was withheld for 5 d and the plants were then well irrigated for 20 d with 150 mM of NaCl solution, which was poured into a tray in which the pots were standing. Measurement of plant survival was based on the total number of seedlings surviving at the end of the stress period (for salinity) or after the 3-d recovery period (for drought). In each case, control plants were maintained in a well-watered state. For each subsequent experiment, three biological replicates were used. The following measurements were taken at the end of the 20-d treatment periods for salinity and drought stress. Chlorophyll content was determined in the cauline leaves following the method described by Lakra et al. (2013). In situ accumulation of O2− and H2O2 were assayed in the fourth fully expanded leaf from the base of the plant, using histochemical staining by nitroblue tetrazolium (NBT) and 3,3′-diaminobenzidine (DAB), respectively (Lakra et al., 2015; Das et al., 2019). The stained leaves were bleached in 95% ethanol, and after complete removal of chlorophyll, they were imaged and densiometric analysis was carried out using the ImageJ software (Schneider et al., 2012). At the end of the experiment, the length and width of seeds were measured using images taken under a microscope (Nikon SMZ1500) and using the Nikon NIS-Elements software.

Rice transformation, stress treatments, and phenotyping at the seedling and reproductive stages

The gene cloned in the plant expression vector pCAMBIA1304–OsGATA8 and RNAi vector pFGC1008–OsGATA8 were transformed into Agrobacterium (LBA4404). Transformation and regeneration were performed in the IR64 rice callus according to Sahoo et al. (2011). After 4 d of salinity treatment at 200 mM NaCl in the hydropnons system, seedlings were harvested for physiological analyses. Control seedlings without NaCl treatment were also harvested. The parameters measured were fresh weight, shoot length, number of lateral roots per seedling, root length, number of leaves per seedling, relative water content, electrolyte leakage, proline content, and K+/Na+ in shoots. The ultrastructure of chloroplast was examined in leaves after 2 d of salinity stress using an electron microscope (JEOL) in the Advanced Instrument Research Facility, Jawaharlal Nehru University according to the protocol described by Das et al. (2019).

To evaluate the tolerance of plants at the reproductive stage, T2 seeds of the WT, overexpressing (OE), and knockdown (KD) lines were sown in soil in pots (15 cm diameter). Five pots each of WT, OE7, OE24, KD41, and KD81 were used and salinity stress at 200 mM NaCl was imposed after 60 d of growth. Photosynthetic parameters (Fv/Fm, net photosynthetic rate, stomatal conductance, and transpiration rate) were recorded after 15 d of treatment in the second leaf from top of the plant using a LI-COR 6400-40 IRGA with default settings. The relative water content was measured for the same leaf. For this purpose, the fresh weight (WF) of the leaf was determined immediately after harvesting from the plant and then its hydrated weight (WR) was measured after floating the leaf in deionized water at 4 °C overnight. The leaves were then dried at 80 °C for 24 h and the dry weight (WD) was obtained. RWC (%) was calculated as [(WF−WD)/(WR−WD)]×100. The Na+ and K+ contents were measured in the fifth leaf from the top of the plant using a flame photometer (Corning EEL, UK), as described previously (Kumar et al., 2009). After 45 d of salinity treatment, the saline solution was removed and the plants were then rewatered for 5 d to allow the completion of their life cycle, and morphological and yield-related parameters were scored as described previously (Tripathi et al., 2016).
Expression analysis of target genes in transgenic rice plants

For gene expression analysis, shoot tissues were collected from 10–40-day-old seedlings of the WT, OE, and KD lines that had been subjected to either control conditions or salinity treatment with 200 mM NaCl for 24 h. Total RNA was extracted from three replicates per treatment, with each replicate formed of 2–3 seedlings, cDNA was prepared, and transcript analysis was carried out using the protocol described by Suda et al. (2013). Gene expression data was normalized using the 2^ΔΔCt method (Livak and Schmittgen, 2001) taking actin as the internal control. We focused on genes involved in ROS scavenging, stress-responsive genes encoding transcription factors, and three genes encoding PROTOCHLOROPHYLLIDE OXIDOREDUCTASE (POR A, B, C), which is involved in a critical step of chlorophyll biosynthesis, namely the conversion of protochlorophyllide a to chlorophyllide a (Thomas, 1997). The primers used are listed in Supplementary Table S1.

Statistical analysis

The data were analysed using one-way ANOVA followed by Tukey’s post hoc test in SigmaPlot 12.0 (Systat Software Inc.).

Results

OsGATA8 is localized in the nucleus in rice

OsGATA8 is a member of the conserved GATA family of TFs in rice and has a single GATA domain (Reyes et al., 2004). To investigate the function of OsGATA8, we isolated OsGATA8 (LOC_Os01g24070) from the commercial rice cultivar IR64. The full-length coding sequence (CDS) of OsGATA8 consisted of 396 bp and its corresponding protein sequence was predicted to have 131 amino acids (NCBI accession: KU377763) (Fig. 1A). In silico analysis of the sequence of OsGATA8 showed the presence of a GATA_Zn_Finger_1 domain of 26 amino acids (aa), from 21 to 46 aa, which is the characteristic feature of GATA proteins. Use of cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) confirmed the presence of a nuclear localization signal (NLS) of 11 aa (47 to 57 aa) adjacent to the GATA domain. The deduced amino acid sequence of OsGATA8 showed a maximum identity (86%) with BdGATA23-like from Brachypodium distachyon, followed by 74% identity with ObGATA23-like from wild rice (Oryza brachyantha), 72% identity with SiGATA23-like from Setaria italica, and 60% identity with ZmGATA22 from Zea mays. AtGATA23 of Arabidopsis showed a minimum identity of 38.33%. A phylogenetic tree based on the full-length sequence of these related GATA domain-containing proteins indicated that OsGATA8, ObGATA23-like, and BdGATA23-like were closely related (Fig. 1B), while the other proteins formed a separate clade of different origin.

Since OsGATA8 is a known TF, we wanted to examine its subcellular localization. For this purpose, OsGATA8 was fused ‘in-frame’ with the GFP reporter gene under the control of the CaMV 35S promoter and GFP was visualized in onion epidermal cells. The results clearly confirmed the presence of the protein in the nucleus (Fig. 1C). To confirm the role of the NLS in targeting to the nucleus, we cloned OsGATA8 in two halves, one encoding the NLS sequence together with the N-terminal part of the protein (OsGATA8-N) and the other having the rest of the sequence without NLS (OsGATA8-C). The localization of the OsGATA8-N fragment in the nucleus is shown in Fig. 1D; in the absence of the NLS sequence the protein was only present in the cytoplasm (Fig. 1E). These results confirmed OsGATA8 to be localized in the nucleus.

To determine whether OsGATA8 alone could act as a transcriptional activator, yeast one-hybrid assays were performed. The full-length OsGATA8 cDNA was fused to the DNA-binding domain of GAL4 (pGBD-C1-OsGATA8) and transformed into yeast strain AH109 (Supplementary Fig. S1A). The results showed that the yeast cells carrying only the empty vector could not grow on the selective media while the ones carrying either OsRR26 or the OsGATA8::GAL4 could activate the downstream reporter genes and could grow on SD−Trp−His−Ade media supplemented with 5 mM 3-AT (Supplementary Fig. S1B, C). It was therefore concluded that OsGATA8 has autoactivation activity and is capable of initiating transcription in yeast when fused with GAL4BD.

Transcript abundance of OsGATA8 is influenced by salinity and development stage in rice

We have previously reported that OsGATA8 is a salinity-inducible gene and is differentially regulated in seedlings of rice genotypes with contrasting tolerances to salinity (Nutan et al., 2017). High expression of OsGATA8 has also been observed during early (germination, seedling, and tillering) and late stages (milking and dough) of growth, as indicated by available microarray data (https://www.genevestigator.com/gv/) (Supplementary Fig. S2A). We conducted our own observations through RNA blotting analysis. Constitutive expression of OsGATA8 in response to salinity was detected at the seedling stage with a biphasic pattern of expression, which showed an initial peak of expression after 30 min of stress and a second peak at ∼24 h of stress (Supplementary Fig. S2B). Analysis at the tillering stage using leaves of 40-d-old plants again showed a high abundance of OsGATA8 transcripts at 30 min and 24 h of stress (Supplementary Fig. S2C).

To assess the tissue specificity of expression of OsGATA8, we performed blotting analysis using RNA isolated from various tissues of mature plants (Supplementary Fig. S2D). Negligible amounts of transcript were found in the inflorescence under control conditions but there was slight induction after 48 h of salinity stress and this continued to 72 h. In the leaves, high constitutive expression was seen, and inducibility by salinity stress was observed for short durations but showed a decline by the final time-point after 72 h of stress. Lower leaves and the lower culm also showed high abundance of OsGATA8 transcripts under control condition. The roots showed a completely different pattern, with negligible constitutive expression but strong salinity-induced expression by 24 h, which was maintained through to 72 h. The role of OsGATA8 in salinity stress in rice is therefore tissue-specific and varies according to the stage of plant development.
OsGATA8 promotes abiotic stress tolerance in Arabidopsis and rice

OsGATA8 contributes to tolerance to multiple abiotic stresses in Arabidopsis at the seedling stage

To determine the function of salinity-inducible OsGATA8, the corresponding full-length cDNA was isolated from the rice cultivar IR64, cloned into the pCAMBIA1304 vector (Supplementary Fig. S3A), and transformed into Arabidopsis. The integration of the gene and its copy number were confirmed by PCR (Supplementary Fig. S3B) and Southern blotting (Fig. S3C), respectively. In the Southern analysis, no band corresponding to the rice OsGATA8 CDS probe was detected in WT Arabidopsis while the putative transgenic lines showed...
the signal, thus indicating the unique identity of the probe corresponding to OsGATA8. Three representative single-copy transgenic lines (L1, L3, and L4; Fig. S3D) were selected and multiplied to obtain T3 homozygous lines (35S-OsGATA8).

To examine the role of OsGATA8 in tolerance to various abiotic stresses, detailed phenotyping analyses were performed. The 35S-OsGATA8 Arabidopsis and WT plants were grown for 5 d under control conditions and were then transferred to various stress conditions. Under control conditions (MS medium alone), the WT and the overexpression lines showed negligible differences in their root length, number of lateral roots, fresh weight of seedlings, and leaf greening (Fig. 2A). In contrast, significant differences were observed in response to treatment with PEG, mannitol, NaCl, and ABA. Root length and the number of lateral roots of the transgenic seedlings were found to be increased relative to the WT after 10 d of growth on medium containing PEG as an osmoticum (Fig. 2A–D). To determine further whether OsGATA8 was involved in general osmotic stress, 5-d-old seedlings grown on control medium were transferred to medium with 200 mM or 300 mM mannitol. After 10 d of treatment, it was found that the growth of WT seedlings was clearly inhibited compared to the 35S-OsGATA8 plants (Fig. 2A, E–G).

Leaf greening, root growth, and fresh weight of the WT were strongly inhibited at the seedling stage when plants were treated with 125 mM NaCl (Fig. 2A, H–J). At 180 mM NaCl, the growth of the WT was severely inhibited and chlorophyll bleaching of seedlings was observed, whereas OsGATA8 transgenic seedlings were not quite as severely affected. To examine the responsiveness to ABA, 5-d-old seedlings were treated with either 2 μM or 14 μM of ABA. At 2 μM ABA, the transgenic plants had longer roots, a greater number of lateral roots, and higher fresh weight than the WT. At 14 μM ABA, severe retardation of growth was observed in both the WT and the transgenic lines. However, 35S-OsGATA8 seedlings had a greater number of lateral roots and higher fresh weight than the WT (Fig. 2A, K–M). Taken together, the transgenic plants ectopically expressing OsGATA8 exhibited better growth

Fig. 2. OsGATA8 promotes tolerance to multiple abiotic stresses in Arabidopsis seedlings. (A) Effects on seedling growth of drought (PEG), salinity (NaCl), osmotic stress (mannitol), and ABA. WT, wild-type; L1, L3, and L4 are 35S-OsGATA8 overexpressing transgenic lines; MS, Murashige and Skoog medium only. (B–M) Root length, number of lateral roots per seedling, and fresh weight per seedling under (B–D) drought, (E–G) osmotic stress, (H–J) salinity stress, and (K–M) ABA. Data are means (±SE) of three biological replicates. Different letters indicate significant differences between means as determined using one-way ANOVA followed by Tukey’s post hoc test (P<0.05).
OsGATA8 promotes abiotic stress tolerance in Arabidopsis and rice

Leaf greening and ROS homeostasis is promoted by OsGATA8 under salinity and drought stress in Arabidopsis

35S–OsGATA8 and WT Arabidopsis plants were grown in soil for 5 weeks and then watered with 150 mM NaCl. After 20 d of treatment, signs of salinity stress were very clear on WT plants but the 35S–OsGATA8 plants were much less affected (Fig. 3A). 35S–OsGATA8 plants were found to have ~60% higher chlorophyll content than the WT under stress (Fig. 3B). The survival of the transgenic plants on saline soil was much higher (70–80%) than that of the WT (~30%) (Fig. 3C).

To assess performance under drought stress, another set of 35S–OsGATA8 and WT plants were grown in soil for 5 weeks and then watered for 20 d. The plants were then rewatered for 3 d (Fig. 3D). Before rewatering, wilting in the WT was more prominent than in the transgenic plants. Under stress conditions, the transgenic plants had 2-fold higher chlorophyll content than the WT (Fig. 3E). After rewatering, ~80% of the transgenic plants recovered, whereas only ~20% of WT plants recovered (Fig. 3F).

After the 20-d periods of salinity and dehydration stress, leaves of WT and transgenic plants were excised and stained with DAB (Fig. 3G, H) or NBT (Fig. 3I, J). Under control conditions, the patterns of DAB and NBT staining between the WT and transgenic lines were very similar. However, under salinity and drought stress, leaves of WT plants showed denser
staining than the transgenic lines (Fig. 3G–J). The staining patterns under salinity were similar to those under dehydration, indicating that the transgenic lines accumulated less H$_2$O$_2$ and O$_3^{-}$ than the WT under both these stresses. The seed length of the transgenic plants was found to be significantly greater than that of the WT (~494 μm versus ~445 μm, respectively), but no significant differences were observed in seed width (Supplementary Fig. S4B). The thousand-seed weight of the transgenic lines was significantly higher than that of the WT (~19.8 mg versus ~17.5 mg, respectively).

Under normal growth conditions, OsGATA8 is determinant of biomass and leaf greening in rice in both seedlings and the mature plant

Agrobacterium containing pCAMBIA1304–OsGATA8 was used to transform rice IR64 to produce overexpressing (OE) plants. Stable integration and copy number of the OsGATA8 transgene was confirmed by PCR and Southern blot analysis, respectively (Supplementary Fig. S5). Similarly, Agrobacterium containing pFGC1008–OsGATA8 was used to produce RNAi-based knockdown (KD) plants (Supplementary Fig. S6). qRT-PCR analysis demonstrated that the KD lines (KD41 and KD81) showed 1.8-fold down-regulation of OsGATA8 transcripts whilst the OE lines (OE7 and OE24) showed 3-fold up-regulation (Supplementary Fig. S6D). We used T2 homozygous lines of these transgenic genotypes.

Under control growth conditions, 20-d-old seedlings of the OE lines showed vigorous growth with more and longer roots compared to the KD lines and the WT (Supplementary Fig. S7). The fresh weight of the WT and OE seedlings were significantly higher than the KD lines (Supplementary Fig. S7C), and similar results were found for other growth parameters (Supplementary Fig. S7D–G). Following transfer to a greenhouse, mature plants showed similar patterns, with growth of the OE lines being greater that the WT and KD lines (Supplementary Fig. S7H). Chlorophyll content in mature plants was also significantly higher in OE lines as compared to the WT and KD lines (Supplementary Fig. S7I).

OsGATA8 is essential for salinity tolerance in rice seedlings

After 4 d of salinity stress, the OsGATA8-OE lines retained more chlorophyll than the WT and KD lines (Fig. 4A). In contrast to the fully expanded leaves seen in the OE lines, leaf rolling was evident in the WT and KD lines after just a day of stress. After 4 d of salinity, OE lines were able to survive, but the WT and KD lines showed severe stress-induced senescence. After 4 d of stress, the fresh weight of WT seedlings was reduced by ~57% compared to the controls whereas the OE seedlings showed only ~41% reduction (Fig. 4B). The KD seedlings showed reduction in fresh weight by over 66%. The relative tolerance of the OE lines and sensitivity of the KD lines compared to the WT was reflected in the results from other parameters (Fig. 4C–H). For example, the OE lines showed no significant reductions in relative water content under salinity stress compared with control conditions whereas the WT showed a reduction of 17% and the KD lines showed reductions of 43% (Fig. 4C). Similarly, electrolyte leakage in the OE lines under salinity stress was lower as compared to the WT and KD lines (Fig. 4D). However, the proline content and the K$^+$/Na$^+$ ratio in the OE lines were significantly higher under salinity stress than in the WT and KD lines (Fig. 4E, H).

OsGATA8 is an essential integrator of photosynthetic efficiency, and seed size and yield under salinity stress in rice

To obtain a simple assessment of salinity tolerance in mature rice plants, leaf-strap senescence assays were performed (Tripathi et al., 2016) in which 2-cm strips were incubated either in water or in 200 mM NaCl solution. After 5 d of treatment, higher chlorophyll bleaching and leaf rolling were observed in the OsGATA8-KD lines as compared to the WT and OE lines (Supplementary Fig. S8A). Chlorophyll bleaching was greater in the WT than in the OE lines but was less than in the KD lines (Supplementary Fig. S8B). This is an important indicator for salinity tolerance, and hence demonstrated a positive role of OsGATA8 TF in salinity stress tolerance. After, 2 d of salinity stress, a higher degree of damage to the thylakoid membranes in the chloroplasts of the WT seedlings was observed compared to the OE lines. This damage was even more severe in the chloroplasts of the KD plants (Supplementary Fig. S8C).

We also performed the yield analysis of transgenic rice under salinity stress as previously described (Tripathi et al., 2016). Plants were cultivated in soil in pots and salinity stress (200 mM NaCl) was imposed after 60 d of growth. After 15 d of treatment the OE plants showed mild morphological effects of salinity toxicity (Fig. 5A). Under salinity stress, the shoot K$^+$/Na$^+$ ratio, RWC, $F_v/F_m$, net photosynthesis, stomatal conductance, and transpiration rate were all significantly higher in the OE lines and lower in the KD lines as compared to the WT (Supplementary Fig. S9). After 45 d of treatment, leaves of the WT and KD lines were yellowish and showed senescence while those of the OE lines still appeared light-green, indicating their higher tolerance to salinity stress (Fig. 5A). The plants were then recovered by normal watering until they had completed their life cycle. After 5 d of recovery, the OE lines had revived and turned green but the WT and KD lines failed to do so. Under control conditions, the OE lines produced larger grains than the WT, whilst the KD lines produced smaller grains (Fig. 5B). The grain length of the OE lines was significantly greater (~20%) than that of the WT, while the KD lines produced grains that were significantly shorter (~16%) than the WT (Fig. 5C). No significant differences were observed in the width of the grains (Fig. 5D). Under control conditions, the OE lines had a higher number of panicles per plant (~10%), more filled grains per panicle (~13%), higher 1000-grain weight, and a higher total yield (~10%) as compared to the WT (Fig. 5E, Table 1). Under salinity stress, reductions in the yield parameters were observed in all the genotypes, but the total yield penalty in the OE lines was less (~29%) compared to the WT (~53%) and the KD lines (~87%) (Fig. 5F).
To investigate the possible molecular functions of OsGATA8 in rice, we examined the expression dynamics of different categories of genes in 10-d-old plants (WT, OE and KD lines) that were treated with 200 mM NaCl for 24 h. We focused on genes involved in ROS scavenging, stress-responsive genes encoding transcription factors, and three genes encoding PROTOCHLOROPHYLLIDE OXIDOREDUCTASE (POR A, B, C). Genes encoding ROS-scavenging enzymes such as SOD and CAT showed 2–2.5-fold higher constitutive expression in the OE lines as compared to the WT under control conditions (Fig. 6A). Under salinity stress, a significant increase in expression of SOD was observed in both the WT and OE lines. The response of APX was different, with a 4-fold salinity-induced increase in expression being observed in the OE lines only.

We also studied the expression of key transcription factors that have been reported to be induced under environmental stresses. Under control conditions, OsDREB1A and OsNAC6 showed 3-fold and 1.7-fold higher expression, respectively, in the OE lines compared to the WT (Fig. 6B); under salinity stress, the differences in expression increased to ~6-fold and ~2.5-fold for OsDREB1A and OsNAC6, respectively.
OsDREB1A was induced by salinity in both the WT and the OE lines. In comparison to control conditions, expression of OsbZIP23 was significantly up-regulated under salinity stress in all the genotypes; however, no differences in expression between the genotypes under salinity stress were observed, suggesting that OsbZIP23 may not be involved in the differences in salinity tolerance in these plants.

Transcript abundance for POR A and POR B was ~3.5 and ~4.2-fold higher, respectively, in the OE lines as compared to the WT under control conditions (Fig. 6C). Transcripts of POR C were also relatively higher in the OE lines but to a lesser extent, and the levels in general were much lower than those of POR A and POR B. Under salinity stress, the transcript levels for all these genes were decreased relative to the controls, but they were always higher in the OE lines.

The expression of all the genes in the different categories was greatly affected in the KD lines, with lower expression than in the WT under control conditions and generally no induction under salinity stress (the one exception being OsbZIP23). Indeed, the POR genes were down-regulated under stress. Overall, the results indicated the possible roles of OsGATA8 in the regulation of genes encoding ROS-scavenging enzymes, transcription factors, and chlorophyll-biosynthesis enzymes involved in stress alleviation.

Discussion

Although the roles of GATA proteins in plant development and carbon/nitrogen metabolism are well established, their involvement in responses associated with tolerance towards abiotic stresses have not yet been determined (Gupta et al., 2017). In the present study, we aimed to characterize one of the members of the GATA family of proteins in rice, namely OsGATA8. OsGATA8 has a single GATA domain with a strongly predicted
OsGATA8 promotes abiotic stress tolerance in Arabidopsis and rice

Table 1. Yield-related parameters for rice wild-type (WT) plants, OsGATA8-overexpression (OE) lines, and OsGATA8-knockdown (KD) lines under control conditions or subjected to salinity stress

|                   | Plant height (cm) | Number of panicles/plant | Number of grains/panicle | Number of filled grains/panicle | Total yield* (g) | 1000 grain weight (g) |
|-------------------|-------------------|--------------------------|--------------------------|---------------------------------|------------------|-----------------------|
| **Control**       |                   |                          |                          |                                 |                  |                       |
| WT                | 79.2±0.92         | 18±2.1                   | 115±1.9                  | 80±3.0                          | 1630±12.2        | 31±0.78               |
| OE7               | 80.2±0.57         | 20±1.8                   | 120±2.1                  | 95±3.5                          | 1801±10.2        | 34±0.55               |
| OE24              | 79.1±0.65         | 20±1.5                   | 119±2.3                  | 93±4.1                          | 1795±15.5        | 34±1.8                |
| KD41              | 70.2±0.41         | 12±0.9                   | 86±1.8                   | 44±1.6                          | 600±10.5         | 27±0.86               |
| KD81              | 67.3±0.61         | 11±1.1                   | 83±1.6                   | 42±1.5                          | 590±8.2          | 27±0.80               |
| **Salinity stress** |                  |                          |                          |                                 |                  |                       |
| WT                | 65±1.6            | 16±0.82                  | 80±2.4                   | 48±1.7                          | 780±11.8         | 28±0.55               |
| OE7               | 65.7±1.2          | 18±0.79                  | 111±3.2                  | 64±2.4                          | 1160±12.4        | 32±1.1                |
| OE24              | 66.9±1.3          | 17±0.66                  | 109±4.2                  | 67±2.1                          | 1150±6.3         | 32±0.92               |
| KD41              | 56.8±0.81         | 10±0.45                  | 40±1.6                   | 21±0.88                         | 213±4.2          | 18±0.74               |
| KD81              | 53.7±0.9          | 10±0.5                   | 35±1.2                   | 20±0.5                          | 205±4.5          | 18±0.62               |

Plants were grown in soil in pots for 60 d, then subjected to 200 mM NaCl for 45 d, after which they were well-watered until they had completed their life cycle. Control plants were well-watered throughout.

* Total yield = total number of filled grains per plant.

NLS sequence adjacent to it (GATA_Zn_Finger_1). OsGATA8 showed significant homology with various orthologous proteins from diverse plant species, indicating its evolutionary conservation within plants (Fig. 1). The multiple sequence alignment showed good homology (86%) with orthologous members of the family from wild rice. OsGATA8 also showed 38% homology with Arabidopsis AtGATA23, a member of the well-studied B-GATA class of transcription factors (TFs) that play an important role in plant development (Behringer et al., 2014; Behringer and Schwechheimer, 2015). Previous studies in rice and Arabidopsis have classified OsGATA8 as a GATA-like zinc finger domain-containing B-GATA class protein (Reyes et al., 2004; Gupta et al., 2017).

Examination of its subcellular localization clearly showed OsGATA8 to be present in the nucleus, as targeted by the NLS (Fig. 1). Yeast one-hybrid assays further confirmed the probable role of OsGATA8 in the transcription process (Supplementary Fig. S1). The binding of GATA proteins on specific cis-acting elements on DNA and hence their functions as TFs has been clearly demonstrated (Ko and Engel, 1993; Lowry and Atchley, 2000; Crespo et al., 2001; Doubeikovskaia et al., 2001; Richter et al., 2013b). Taken together, these findings clearly establish OsGATA8 to be a functional TF.

Plants modulate their responses to changes in their physical and biological environment through complex gene networks that operate in a cell- and tissue-specific manner (Pareek et al., 2010). The regulation of gene expression in response to environmental stresses has been well studied (Walia et al., 2007; Kumari et al., 2009b). Our previous analysis of OsGATA8 showed that it has differential expression in shoots of seedlings of contrasting rice cultivars under salinity stress (Nutan et al., 2017). We have also reported induced transcript abundance of OsGATA8 together with various other members of the GATA family in rice seedlings in response to salinity, ABA, and drought (Gupta et al., 2017). In our current study, we found that expression of OsGATA8 has consistently been reported in rice leaves at both the tillering and milking stages and expression is further induced in response to salinity stress (Supplementary Fig. S2). Consistent with our results that showed reduced expression in the inflorescence, culm, and roots at the milking stage under normal (no stress) conditions, Nishi et al. (2000) found that a ZIM (zinc-finger protein expressed in inflorescence meristem) gene in Arabidopsis encoding a GATA-type zinc-finger protein with a C-X2-C-X20-C-X2-C motif also showed differential patterns of expression at the reproductive stage. Further, ZIM expression was observed in shoot apices and in the roots at the vegetative stage (Shikata et al., 2004). In our study, the lower culm and lower leaves of mature rice plants showed high expression of OsGATA8 under non-stress conditions (as compared to the upper parts of the plant), which may indicate a role for these tissues in acting as sinks for toxic substances during development, the accumulation of which may indicate a role for these tissues in acting as sinks for toxic substances during development, the accumulation of which thus induces the expression (Supplementary Fig. S2D). Different environmental stress signaling cascades in plants form a complex network with convergent and divergent signaling circuits (Zhu, 2002; Shinozaki et al., 2003; Wani et al., 2018). The complex pattern of expression of OsGATA8 in these tissues is a result of this complexity of regulatory circuits.

In Arabidopsis, GATA family proteins have been shown to be involved in chlorophyll biosynthesis (Jeong et al., 2003; Bi et al., 2005). We examined the effect of ectopic expression of OsGATA8 in Arabidopsis under the control of the 35S promoter. Expression of OsGATA8 in Arabidopsis resulted in a phenotype with higher chlorophyll content and reduced water loss under salinity and drought stress, indicating its role in abiotic stress tolerance (Figs 2, 3). Our results were also consistent with a previous report that GATA TFs promote increased seed size (Supplementary Fig. S4; Behringer and Schwechheimer, 2015). Thus, our study gives a clear indication of the conserved functions of members of this TF family across different genera.

In agreement with the response seen in transgenic Arabidopsis and rice with overexpression (OE) of OsGATA8 also exhibited increased seedling weight (Fig. 4), higher chlorophyll content and increased photosynthetic efficiency
(Supplementary Figs S8, S9) compared to the WT or knockdown (KD) plants under salinity stress. Using Arabidopsis plants overexpressing GNC and GNL (B-GATA members) together with their single- and double-mutants, Richter et al. (2010, 2013b) confirmed a role of GATA TFs in increased accumulation of chlorophyll in the hypocotyl region, and also found a positive effect on leaf numbers. Similar observations have also been reported in transgenic rice overexpressing \textit{HIGHER YIELD RICE} (HYR) (Ambavaram et al., 2014). In comparison to the WT, rice seedlings overexpressing OsGATA8 showed an improved phenotype under salinity stress in terms of higher biomass, and increase in RWC, electrolyte leakage, proline content, and K$^+$/Na$^+$ ratio (Fig. 4), all indicating the higher tolerance of OE plants to salinity stress. Overexpression of rice \textit{OsGATA8} positively regulates the expression of downstream genes that have roles in leaf greening, ROS-scavenging, and stress tolerance. Wild-type (WT) plants, OsGATA8-overexpressing lines (OE7, OE24), and OsGATA8-knockdown lines (KD41, KD81) were grown for 10 d and then treated with 200 mM NaCl for 24 h. Total RNA was then extracted, cDNA was synthesized, and qRT-PCR was carried out. Gene expression was normalized using \textit{actin} as the internal control and the value for the WT under control conditions was set as 1. (A) ROS-scavenging genes, SOD, CAT, and APX; (B) stress-responsive genes, OsDREB1A, OsbZIP23, and OsNAC6, and (C) and chlorophyll biosynthesis genes, POR A, POR B, and POR C. Data are means (±SE) of three biological replicates. Different letters indicate significant differences between means as determined using one-way ANOVA followed by Tukey’s post hoc test ($P<0.05$). SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; OsDREB1A, rice dehydration responsive element binding factor 1A; OsbZIP23, rice basic leucine zipper 23; OsNAC6, rice NAM, ATAF, and CUC (NAC) 6; POR A–C, protochlorophyllide oxidoreductase A–C.
plants overexpressing GNC and GNL (Richter et al., 2010, 2013a, 2013b; Hudson et al., 2013; Ranftl et al., 2016). The B-GATA members GNC and GNL acting in coordination with the MADS box TF SOC1 have been reported to be involved in processes associated with greening and cold tolerance (Richter et al., 2013a). The expression of POR C in the OE lines was different to that of POR A and POR B under both control and salinity stress conditions (Fig. 6). This may be due to the fact that these chlorophyll biosynthesis genes are regulated by two different hormones and together with various light-signaling components (Liu et al., 2017). Cytokinin controls the expression of POR A and POR B through EIN3/EIL1, a master TF (Chao et al., 1997; Guo and Ecker, 2004), while POR C is expressed under the regulation of the DELLA protein in the GA signaling pathway (Cheminant et al., 2011). We found higher transcript abundance in the OE plants compared to the WT and KD plants for the POR genes, TF genes, and ROS-scavenging genes under both control and salinity stress conditions (Fig. 6). Higher expression of POR genes has also been found in Arabidopsis plants overexpressing GNC and GNL (Richter et al., 2010). Higher accumulation of various stress-related and photosynthesis genes have also been reported in transgenic rice under drought stress (Oh et al., 2009; Ambavaram et al., 2014). Based on these observations and the available literature, we propose a model to describe the functioning of OsGATA8 in promoting tolerance to stresses (Fig. 7). Expression of OsGATA8 is probably induced by various stresses such as salinity and drought through an ABA-mediated pathway. It also appears to be induced by various other hormones such as GA, auxin, and CK (Behringer and Schwechheimer, 2015). Our data clearly show that OsGATA8 regulates the expression of various downstream genes involved in leaf greening, ion homeostasis, chlorophyll biosynthesis, stress tolerance, and ROS-scavenging enzymes. Taken together, OsGATA8 appears to be a master regulator of stress response in plants interacting directly or indirectly with diverse cellular mechanisms that contribute to stress tolerance.

Since salinity and drought stress are considered to be the two major threats to agriculture in the near future, the results of our present study have the potential to help improve crop productivity in affected areas. OsGATA8 appears to be a highly suitable candidate gene for studies targeting the improvement of tolerance to multiple stresses in crops plants, which is a topic of great importance in the context of climate change. Field-level evaluation of new plant types is required to assess their suitability for environments where combinations of stresses will determine their performance and yield. Testing of more ‘multi-stress responsive genes’ through functional genomics is urgently needed to ensure global food security in the years to come.

**Supplementary data**

Supplementary data are available at JXB online.

Fig. S1. Yeast one-hybrid assay for OsGATA8.

Fig. S2. OsGATA8 expression is salinity-responsive and tissue-specific.

Fig. S3. Molecular confirmation of transgenic Arabidopsis ectopically expressing OsGATA8.

Fig. S4. OsGATA8 positively influences the seed size in transgenic Arabidopsis.

Fig. S5. Molecular confirmation of rice lines overexpressing OsGATA8.

Fig. S6. Molecular confirmation of rice lines with knockdown of OsGATA8.

Fig. S7. OsGATA8 increases biomass, greening, and net photosynthesis in rice under control conditions.

Fig. S8. OsGATA8 promotes leaf greening and maintains chloroplast structure under salinity stress in rice.
Fig. S9. OsGATA8 increases leaf greening and photosynthetic rate under salinity stress in mature rice plants.

Table S1. List of primers used in this study.

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