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RGG1 transgenic rice plants and their physiological characteristics in relation to salinity stress

Durga Madhab Swain[1], Ranjan Kumar Sahoo[1] and Narendra Tuteja[1,2]*

[1]International Centre for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi-110067, India

[2]Amity University, Noida - 201313, Uttar Pradesh, India

**Corresponding author e-mail: narendra@icgeb.res.in, ntuteja@amity.edu*

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ABSTRACT

The heterotrimeric G-protein complex, comprising of G α , G β , and G λ subunits. It is an evolutionarily conserved-signaling molecular machine which transmits signals from transmembrane receptors to downstream target proteins. Now-a-days their functions in plant stress-signalling have been reported. Here we report the physiological function of rice G-protein λ subunit (RGG1) rice (Oryza sativa L. *cv. IR64) plants under salinity stress in T_3 generation. The overexpression of CaMV35S promoter driven RGG1 in transgenic rice confers high salinity (200 mM NaCl) stress tolerance. Agronomic parameters were studied and found to be higher in the transgenic plants with respect to wild type (WT) plants.*

Key words: *Antioxidative enzymes, G-protein gamma interacting partners, oxidative stress; hormones, RGG1, salinity stress tolerance, transgenic rice*

Gene Bank Accession Number of RGG1: GU111573.1

Locus: GU111573

Salinity is a widespread soil problem limiting crop productivity worldwide, especially in the tropical and irrigated fields where salinization has caused deterioration of agricultural lands (Mahajan and Tuteja 2005; Munns and Tester 2008). Several studies have demonstrated that the introduction of foreign genes into crop plants provides resistance against biotic as well as abiotic stresses (Xiong *et al*. 2006; Mazzucotelli *et al*. 2008; Chen *et al*. 2013). It was studied earlier that some RNA/DNA helicases also play an important role in the abiotic stress resistance (Liu *et al*. 2002; Tuteja and Tuteja 2006; Vashisht and Tuteja 2006; Kant *et al*. 2007; Li *et al*. 2008). Recently, the over-expression of a mitochondrial helicase OsSUV3 has been reported to impart salinity stress tolerance in rice plants without yield loss (Tuteja *et al*. 2013).

Rice (*Oryza sativa*) is an important cereal crop that provides a staple diet for almost half of the world's population and is the major food crop cultivated in Asia. The quality and yield of rice is greatly affected by environmental stresses such as salinity, drought, heat and cold. Abiotic stresses decrease both the growth and productivity of crops by reducing photosynthesis, decreasing seedling fresh weight, germination percentage and biomass and increasing the generation of reactive oxygen species (ROS) (Hadiarto and Tran, 2011). The heterotrimeric G-proteins are composed of G α (39-52 kDa), G β (34-36 kDa) and G λ (7-10 kDa) subunits (Gilman 1987; Tuteja and Sopory 2008). G-proteins transduce the signals from the outside environment to inside possibly through regulators (Colaneri *et al*. 2014). Subunits of G-protein have been reported in several plants such as arabidopsis, lotus, lupin, pea, rice, soybean, spinach, tobacco, tomato and wild oat (Jones and Assmann 2004; Assmann 2002; Mishra *et al*. 2007; Yadav *et al*. 2012). Plant G-proteins have been reported to regulate the ion channels, cell proliferation and developmental events and are involved

in plant responses to stress, light, hormones, innate immunity, and in controlling shoot meristem size (Jones 2002; Jones and Assmann 2004; Perfus-Barceoch *et al*. 2004; Chen *et al*. 2006; Bommert *et al*. 2013; Cheng *et al*. 2015; Maruta *et al*. 2015). Pea G-proteins have been shown to be regulated under stress (Mishra *et al*. 2007; Bhardwaj *et al*. 2011). In recent studies, it was found that G-protein alpha null mutation confers prolificacy potential in maize (Urano *et al*. 2015), and type B heterotrimeric G-protein gamma-subunit regulates auxin and ABA signaling in tomato (Subramaniam *et al*. 2016). Furthermore, the interactome of arabidopsis G-protein reveals that G-proteins are multifunctional and play significant role in the development and combat against environmental stresses (Klopffleisch *et al*. 2011).

In the current study, we have developed transgenic rice plants IR64 (*Oryza sativa* L., cv. IR64) by over-expressing RGG1 gene. We observed that the over-expression of RGG1 leads to the enhancement of salinity stress tolerance by coping with stress-induced oxidative damage.

MATERIALS AND METHODS

Polymerase chain reaction and Western-blot analysis

Integration of the RGG1 gene was checked by PCR using 35sCamv forward primer (5'-AGAAGACGTTCCAACCACGTCTT-3') and RGG1 gene specific reverse primer (5'-TCACAAAAACCAGCATTTGCATCTG-3'). The crude plant extract from WT and over expressing lines was prepared using the method described (Hurkman and Tanaka 1986). Equal amount of crude proteins were denatured and separated using SDS PAGE, electroblotted onto polyvinylidene fluoride (PVDF) membrane and then probed with mouse polyclonal antibodies (1:1,000 dilution) raised against full length RGG1 and the crude extract from WT plant was taken as negative control. Western blot analysis using anti-RGG1 (1:5000) primary and alkaline phosphatise conjugated anti-mice (Sigma) secondary antibodies (1:1000 dilution) was performed to check the production of the protein by the transgenic lines. The blot was developed as per manufacturer's protocol (Sigma). The alkaline phosphatase conjugated secondary anti-mouse IgG antibody (Sigma-Aldrich http://www.sigmaaldrich.com) was used at 1:10000 dilution.

Isolation of RNA and quantitative real-time PCR

25-days-old rice (*Oryza sativa* cv. IR64) seedlings samples were harvested. Leaf samples of the wild type (WT) plants as well as T_3 transgenic lines (L1-L5) were used for RNA isolation and qRT-PCR was performed as described earlier (Tuteja *et al*. 2013). For qRT-PCR, the RGG1 gene specific primers (forward 5'-GCGCTTTCTCGAGGAACTTGAAG-3' and reverse 5'-CTTGCCAGTCTTGGGACAGATGGTTTG-3') were used. The expression was normalized to α-tubulin (forward 5'-GGTGGAGGTGATGATGCTTT-3' and reverse 5'-ACCACGGGCAAAGTTGTTAG-3') and calculated using the $2^{-\Delta\Delta Ct}$ method from three independent experiments (Livak and Schmittgen 2001).

Measurement of photosynthetic activities, agronomic attributes and endogenous ion content of T_3 transgenic plants

45-days-old seedlings of transgenic and WT plants were allowed to grow in 200 mM NaCl in a tank till maturity. The different photosynthetic parameters like stomatal conductance (gs), net photosynthetic rate (PN), and intercellular CO_2 concentration (Ci) were recorded in fully expanded leaves using an infrared gas analyser (IRGA; LI-COR, http://www.licor.com) on a sunny day between 10:00-11:30 am. The different agronomic characteristics were measured at 0 and 200 mM NaCl treatment in T_3 transgenic and WT plants using the method described (Tuteja *et al*. 2014). The endogenous ions (phosphorous, potassium and sodium) content were measured as described earlier (Tuteja *et al*. 2013).

Biochemical assays of RGG1 transgenic plants in T_3 generation

Biochemical analysis like lipid peroxidation, catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and proline were carried out by using 25-days-old WT and transgenic rice seedlings exposed for 24h to salt stress. The electrolytic leakage was measured as previously described (Garg *et al*. 2012).

Statistical analysis

The experiment was arranged in a randomized block design. For various growth parameters of the WT and RGG1 T_3 transgenic plants, values are presented asmeans of three replicates (each plant was considered

a replicate). Here the 'mean of three replicates' represents the 'mean of three independent plants. Data were analysed statistically and standard errors were calculated. Least significant differences (LSDs) between the mean values (n = 3) of control (WT) and RGG1 overexpressing transgenic rice lines (L1-L5) were calculated by one-way analysis of variance (ANOVA) using SPSS 10.0 (SPSS, Inc., now IBM, http://www-01.ibm.com/software/analytics/spss). A comparison between the means was performed using Duncan's multiple range tests. The WT and transgenic lines at $P < 0.05$, $P < 0.01$ and $P < 0.001$ were considered statistically significant.

RESULTS AND DISCUSSION

Polymerase chain reaction, Western-blot analysis and transcript profile analysis of T_3 RGG1 transgenic rice plants

The T_3 transgenic rice plants were developed using the T-DNA construct of the RGG1 gene (Figure 1a). Phenotypically the transgenic rice plants were significantly taller (L1-L5) than the WT (Figure 1b). The integration of the transgene (RGG1) was confirmed by PCR using 35S forward and the gene specific reverse primers. The expected size band of 430 bp was observed (Figure 1c). The western blot results show that the protein is expressed to almost similar levels in all the transgenic lines L1-L5 (Figure 1d).The RGG1 transcript level was up-regulated significantly by 10- to 12-folds in comparison with the WT plants (Figure 1e).

Agronomic performance of RGG1 T_3 transgenic plants under stress

Under 200mM NaCl stress condition the RGG1 transgenic plants showed better performance in several growth parameters, such as plant height, root length, root dry weight and leaf area, as compared to the WT plants (Table 1). Several yield characters, such as days required for flowering, number of tillers per plant, panicles per plant, filled grain per panicle, chaffy grain per panicle, 100 grain weight at 200 mM NaCl were recorded and found to be almost similar to the WT plants grown in water (0 mM NaCl). However, the WT plants did not survive till flowering stage under 200 mM NaCl stress (Table 2).

Table 1. Phenotypic attributes of WT, VC and T_3 generation of *RGG1* overexpressing transgenic lines (Line 1, Line 2, Line 3, Line 4 and Line 5) of rice (*Oryza sativa* L. cv. IR64) under 0 and 200 mM NaCl

Yield attributes	Control WT plants		200 mMNaCl grown T1 RGG1 transgenic plants									
			Line 1		Line2		Line 3		Line 4		Line 5	
	0	200	0	200	0	200	0	200	0	200	0	200
Plant height(cm)	62±3.2^{a}	31.6±1.4^{b}	72±3.2^{a}	71±3.7^{a}	81±3.1^{a}	66±3.2^{a}	76±3.1^{a}	73±3.1^{a}	74±3.2^{a}	68±3.2^{a}	75±3.1^{a}	69±3.3^{a}
Root length (cm)	28±0.8^{ab}	12.8±0.02^{b}	28±1.0^{a}	25±1.0^{ab}	27±1.5^{a}	19±1.5^{ab}	29±1.3^{a}	26±1.0^{a}	28±1.0^{a}	22±1.1^{ab}	29±1.4^{a}	19±1.4^{ab}
Root dry weight (g)	2.6±0.13^{b}	1.2±0.01^{c}	2.8±0.14^{a}	2.1±0.14^{a}	3.2±0.12^{a}	2.6±0.17^{a}	2.9±0.2^{a}	2.7±0.15^{a}	2.9±0.16^{a}	2.5±0.13^{a}	3.6±0.13^{a}	2.6±0.16^{a}
Leaf area (cm^2/plant)	85±2.3^{ab}	41.72±2.0^{c}	96±1.0^{a}	78±1.7^{ab}	96±1.0^{a}	76±1.4^{ab}	93±1.6^{a}	81±1.6^{ab}	95±1.0^{a}	84±1.8^{ab}	98±1.0^{a}	88±1.6^{ab}
Net photosynthetic rate (PN, µ mol CO_2 m-2s^{-1})	9.17±0.8^{b}	5.04±0.21^{c}	10.35±0.6^{a}	7.27±0.8^{a}	10.55±0.1^{a}	9.38±0.3^{a}	10.07±0.4^{a}	8.17±0.6^{a}	10.38±0.6^{a}	8.29±0.8^{a}	10.54±0.2^{a}	7.37±0.2^{a}
Stomatal conductance (gs, m mol m^{-2}s^{-1})	227±11.4^{a}	109.3±5.3^{b}	242±10.9^{a}	209±11.6^{a}	255±10.9^{a}	212±10.7^{a}	248±10.2^{a}	219±10.3^{a}	244±10.8^{a}	205±11.5^{a}	256±10.7^{a}	211±10.8^{a}
Intracellular CO_2 (Ci, µ mol mol^{-1})	227±11.3^{a}	101.1±4.4^{b}	225±10.2^{a}	208±10.2^{a}	227±10.5^{a}	202±10.2^{a}	224±11.5^{a}	209±10.2^{a}	228±10.2^{a}	204±10.3^{a}	232±10.5^{a}	206±10.1^{a}
Phosphorus (%)	0.221±	0.125±	0.226±	0.235±	0.224±	0.247±	0.223±	0.263±	0.225±	0.236±	0.226±	0.247±
	0.014^{b}	0.002^{c}	0.010^{a}	0.010^{a}	0.011^{a}	0.011^{a}	0.011^{a}	0.011^{a}	0.010^{a}	0.011^{a}	0.012^{a}	0.011^{a}
Potassium (%)	0.148±	0.094±	0.146±	0.129±	0.146±	0.139±	0.147±	0.148±	0.142±	0.139±	0.147±	0.136±
	0.002^{b}	0.002^{c}	0.002^{a}	0.002^{a}	0.002^{a}	0.002^{a}	0.001^{a}	0.005^{a}	0.002^{a}	0.002^{a}	0.002^{a}	0.003^{a}
Sodium (%)	0.005±	0.066±	0.004±	0.048±	0.005±	0.041±	0.005±	0.047	0.006±	0.041±	0.004±	0.048±
	0.001^{a}	0.001^{a}	0.001^{a}	0.001^{a}	0.001^{a}	0.001^{a}	0.001^{a}	±0.001^{a}	0.001^{a}	0.002^{a}	0.001^{a}	0.001^{a}

Each value represents mean of three replicates ± SE. Means were compared using ANOVA. Data followed by the same letters in a row are not significantly different at $P > 0.05$

Table 2 Comparison of various yield parameters of WT and T_3 generation of *RGG1* over expressing transgenic lines (Line 1, Line 2, Line 3, Line 4 and Line 5) of rice (*Oryza sativa* L. cv. IR64) under 0 or 200 mM NaCl, respectively.

Yield attributes	Control WT plants		200 mM NaCl grown T_3 *RGG1* transgenic plants									
			Line 1		Line2		Line 3		Line 4		Line 5	
	0	200	0	200	0	200	0	200	0	200	0	200
Time required for flowering (days)	91±2.3^{a}	ND*	93±3.6^{a}	87±2.2^{a}	92±3.0^{a}	90±2.7^{a}	93±2.6^{a}	90±2.7^{a}	94±3.6^{a}	90±2.1^{a}	93±3.1^{a}	89±2.4^{a}
No. of tillers/plant	22±1.0^{c}	ND	23±0.12^{ab}	22±1.2^{ab}	22±0.15^{ab}	21±1.2^{ab}	22±0.12^{a}	19±1.0^{a}	21±0.12^{ab}	20±1.3^{ab}	23±0.14^{ab}	21±1.2^{ab}
No. of panicle/plant	18±0.5^{c}	ND	22±0.11^{ab}	21±1.1^{ab}	26±0.13^{ab}	22±1.1^{ab}	25±0.15^{a}	21±1.2^{a}	27±0.11^{ab}	20±1.0^{ab}	24±0.13^{ab}	21±1.0^{ab}
No. of filled grain/panicle	84±3.1^{b}	ND	96±3.2^{a}	83±3.2^{a}	93±3.23^{a}	85±4.1^{a}	95±3.27^{a}	86±3.2^{a}	96±3.3^{a}	81±3.1^{a}	95±3.23^{a}	88±4.0^{a}
No. of chaffy grains/panicle	13±0.21^{a}	ND	07±0.12^{b}	05±0.11^{b}	05±0.06^{b}	04±0.22^{b}	07±0.21^{b}	05±0.11^{b}	08±0.12^{b}	06±0.11^{b}	05±0.05^{b}	04±0.21^{b}
Straw dry weight (g)	56±1.3^{b}	ND	55±3.05^{a}	47±2.3^{a}	68±2.1^{a}	60±1.6^{a}	63±1.8^{b}	55±1.3^{a}	59±3.04^{a}	48±2.1^{a}	62±2.2^{a}	61±1.4^{a}
100 grain weight	2.78±0.1^{a}	ND	2.68±0.12^{b}	2.61±0.11^{a}	2.68±0.12^{b}	2.66±0.10^{a}	2.64±0.14^{b}	2.61±0.10^{a}	2.68±0.12^{b}	2.61±0.11^{a}	2.68±0.12^{b}	2.63±0.11^{a}
Seed weight per plant	37.64±1.2^{a}	ND	52.99±1.4^{b}	45.49±0.3^{b}	62.07±0.5^{a}	47.48±1.1^{b}	63.36±1.2^{b}	47.13±1.1^{b}	61.99±1.2^{b}	42.28±1.1^{b}	61.19±1.2^{b}	48.6±1.3^{b}

ND-No data, * control plants did not survived till harvesting under 200 mM NaCl. Each value represents mean of three replicates ± SE. Means were compared using ANOVA. Data followed by the same letters in a row are not significantly different at $P > 0.05$ as determined by least significant difference (LSD) test. a, b, c indicate significant differences at $P > 0.05$ level as determined by Duncan's multiple range test (DMRT).

Photosynthetic characteristics and endogenous ion contents of RGG1 T_3 transgenic plants under stress

The photosynthetic characteristics of transgenic as well as WT plants were measured after one week of induction of salt (200 mM NaCl) stress. The photosynthetic rate declined by 35% in WT as compared with RGG1 transgenic lines. The stomatal conductance, net photosynthetic rate and intracellular CO_2 were also higher in transgenic lines as compared to the WT plants (Table 1). In the presence of NaCl (200 mM), the WT plants accumulated excess sodium whereas the transgenic lines had reduced amounts of sodium in their leaves. Salt-treated T_3 transgenic lines showed higher accumulation of phosphorus and potassium (Table 1).

Analysis of antioxidant enzymes activities and response of ion leakage, proline content malondialdehyde (MDA) in T3 RGG1 transgenic plants

The overexpression of RGG1 resulted in increased enzymatic activities of CAT, APX and GR due to salt treatment (200 mM NaCl) in transgenic plants (Fig. 2a-c). The changes induced by the presence of salt in the accumulation of MDA, ion leakage and proline antioxidant machineries in T_3 transgenic lines (L1-L5) were compared with rice seedlings of WT plants. The levels of MDA, ion leakage were significantly reduced while proline content were increased in RGG1 transgenic lines as compared to the WT under salt (200 mMNaCl) stress (Fig. 2d-f). The increased detoxification of ROS led to reduced membrane lipid peroxidation *i.e.*, MDA production and membrane damage as indicated by electrolyte leakage.

G-proteins are ubiquitous in nature, and are known to be involved in diverse cellular and metabolic processes, including their new emerging role in plant abiotic stress tolerance (Misra 2007). Salinity is a multigenic trait which controls the whole plant machinery and rice productivity is severely affected due to this stress. Although G-gamma subunits were initially regarded as a passive partner in the G beta-gamma dimer whose only function was to anchor the dimer to the plasma membrane, they have now emerged as an important member of the heterotrimer, providing multiple physiological functions in plants (Jones and

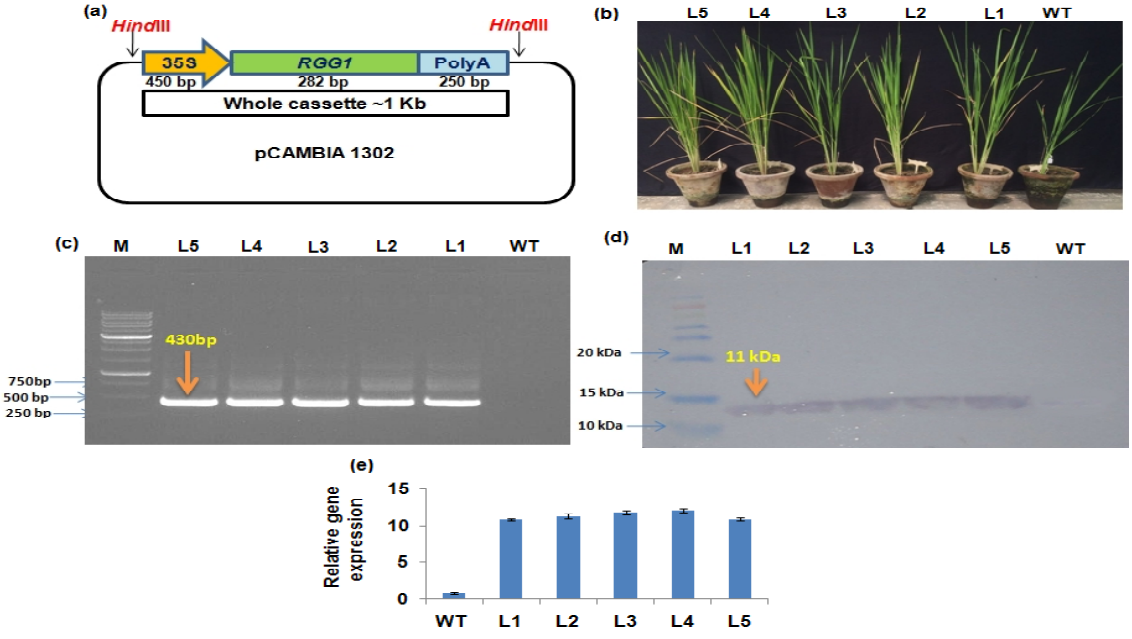

Fig. 1 Analysis of RGG1 over expressing transgenic T_3 IR64 rice plants. (a) The OsRGG1 gene cloned in pCAMBIA1302 vector at HindIII site. (b) Transgenic plants (L1- L5) along with WT. (c) PCR analysis of the RGG1 over expressing transgenic (T_3) lines along with wild type (WT), positive control (PC) and negative control (NC) shows the amplification of the 430 bp fragment. (d) Western blot analysis showing the production of RGG1 protein (~11kDa). (e) Real time PCR analysis of the RGG1 over expressing transgenic (T_3) lines (L1- L5) along with WT

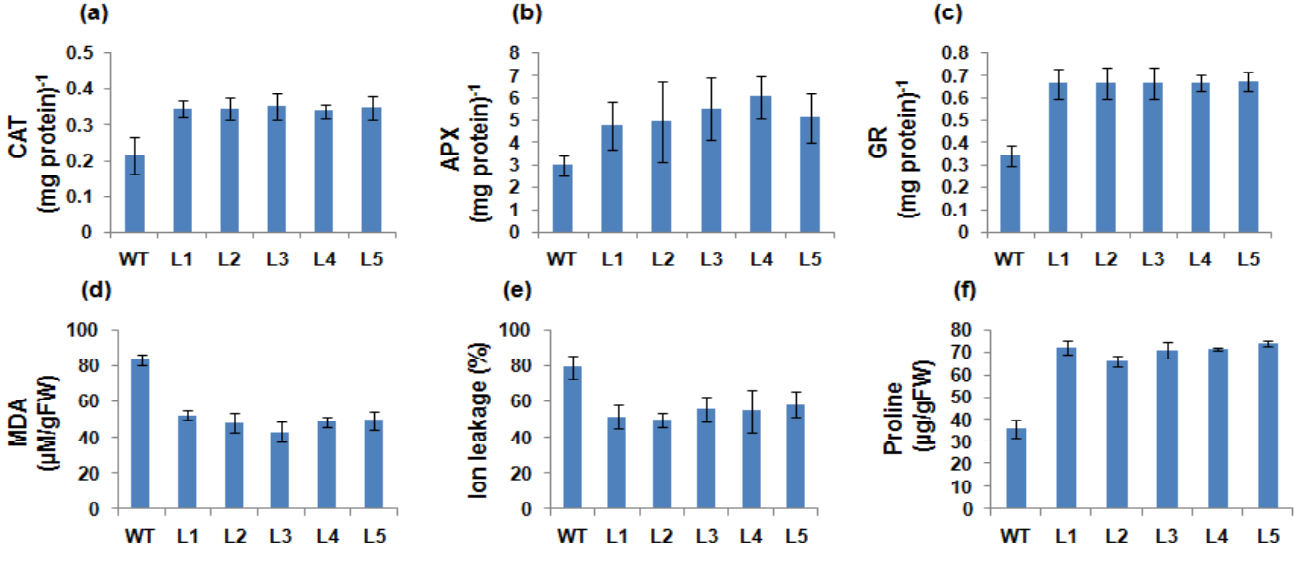

Fig. 2. Biochemical analysis of RGG1 over expressing T_3 transgenic lines (L1- L5) along with WT. (a) Catalase (CAT) activity, one unit of enzyme activity defined as 1 µ mol H_2O_2 oxidized min^{-1}. (b) Ascorbate peroxidase (APX) activity, one unit of enzyme activity defined as 1 µ mol of ascorbate oxidized min^{-1}. (c) Glutathione reductase (GR) activity, one unit of enzyme activity is defined as 1 µ mol of GS-TNB formed min^{-1} due to reduction of DTNB (d) Lipid peroxidation expressed in terms of malondialdehyde (MDA) content. (e) Percent electrolytic leakage. (f) Level of proline accumulation.

Assmann 2004; Perfus-Barbeoch *et al*. 2004; Trusov *et al*. 2007; Zhang *et al*. 2008; Trusov *et al*. 2009; Dupre *et al*. 2009; Klopffleisch *et al*. 2011; Trusov and Botella 2012; Urano *et al*. 2013; Cheng *et*

al. 2015; Maruta *et al*. 2015). The present study was conducted in order to study the function of RGG1 in providing salinity stress tolerance in rice (*Oryza sativa* L. cv. IR64).

The RGG1 overexpressing transgenic lines retained more chlorophyll than WT under salinity stress, which is in agreement with the earlier reports (Sanan-Mishra *et al*. 2005; Moradi and Ismail 2007; Dang *et al*. 2013; Singh *et al*. 2012; Sahoo *et al*. 2012). The photosynthetic activities like net photosynthesis rate (Pn), stomatal conductance (gs), and intercellular CO_2 concentration (Ci) were decreased by salinity stress but a lesser reduction was observed in RGG1transgenic lines as compared to WT plants. The better control over photosynthesis apparatus under salinity stress may be due to retention of chlorophyll content in these transgenic lines. It has been reported earlier also that tolerance in PDH45 and SUV3 overexpressing rice plants in stress results due to maintenance of the photosynthetic apparatus (Gill and Tuteja 2010; Tuteja *et al*. 2013). Under salinity stress plant produces more ROS, which can cause serious damage to plasma membrane, chloroplasts and mitochondria by peroxidation and de-esterification of membrane lipids and damage to nucleic acids and proteins (Gill and Tuteja 2010).

The antioxidant enzymes such as APX, GPX and GR showed significantly higher activity under salinity stress in T_3 transgenic lines as compared to WT plants, which help in scavenging the ROS production during the stress. To protect the plants from the injurious effects of H_2O_2, plants produce more APX through the AsA-GSH cycle, where APX uses ascorbate as hydrogen donor and GR catalyses the NADPH dependent reduction of GSSG (oxidised form) to GSH (reduced form) and maintains high ratio of GSH/GSSG (Gill and Tuteja 2010).

Higher concentration of potassium and lower concentration of sodium were found in leaves of RGG1 overexpressing transgenic lines than WT plants under salinity stress. It indicates that the overexpression of RGG1 may restrict the entry of sodium ions in the leaves of transgenic lines thereby contributing towards protection of photosynthetic machinery from salinity stress.

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