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ORIGINAL ARTICLE

Function of heterotrimeric G-protein γ subunit RGG1 in providing salinity stress tolerance in rice by elevating detoxification of ROS

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Abstract

Main conclusion **The present study provides evidence of a unique function of *RGG1* in providing salinity stress tolerance in transgenic rice without affecting yield. It also provides a good example for signal transduction from the external environment to inside for enhanced agricultural production that withstands the extreme climatic conditions and ensures food security.**

The role of heterotrimeric G-proteins functioning as signalling molecules has not been studied as extensively in plants as in animals. Recently, their importance in plant stress signalling has been emerging. In this study, the function of rice G-protein γ subunit (*RGG1*) in the promotion of salinity tolerance in rice (*Oryza sativa* L. cv. IR64) was investigated. The overexpression of *RGG1* driven by the CaMV35S promoter in transgenic rice conferred high salinity tolerance even in the presence of 200 mM NaCl. Transcript levels of antioxidative genes, i.e., *CAT, APX,* and *GR*, and their enzyme activities increased in salinity-stressed transgenic rice plants suggesting a better antioxidant system to cope the oxidative-damages caused by salinity stress. The *RGG1*-induced signalling events that conferred tolerance to salinity was mediated by increased gene expression of the enzymes that scavenged reactive oxygen species. In salinity-stressed *RGG1* transgenic lines, the transcript levels of *RGG2*, *RGB, RGA, DEP1,* and *GS3* also increased in addition to *RGG1*. These observations suggest that most likely the stoichiometry of the G-protein complex was not disturbed under stress. Agronomic parameters, endogenous sugar content (glucose and fructose) and hormones (GA3, zeatin and IAA) were also higher in the transgenic plants compared with the wild-type plants. A BiFC assay confirmed the interaction of RGG1 with different stress-responsive proteins which play active roles in signalling and prevention of aggregation of proteins under stress-induced perturbation. The present study will help in understanding the G-protein-mediated stress tolerance in plants.

Keywords Antioxidative enzymes · G-protein gamma interacting partners · Hormones · Oxidative stress · Rice G-protein gamma subunit 1 · Salinity stress tolerance · Transgenic rice

Abbreviations

AGG1 (2, 3)	Arabidopsis G-protein gamma subunit 1 (2, 3)
APX	Ascorbate peroxidase
CAT	Catalase
DEP1	Dense and erect panicle
GR	Glutathiol reductase

Gene Bank Accession Number of *RGG1*: GU111573.1; Locus: GU111573.

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GS3	Grain size 3
OsGGC2	G-protein gamma subunit type C number 2 of rice
RGA	Rice G-protein alpha subunit
RGB	Rice G-protein beta subunit
RGG1 (2)	Rice G-protein gamma subunit 1 (2)
ROS	Reactive oxygen species
WT	Wild type
VC	Vector control

Introduction

Plant growth and development is severely affected by abiotic stresses which ultimately results in heavy economic losses and food crisis. Salinity is a major abiotic stress that negatively affects the productivity of plants (Mahajan et al. 2006; Tuteja et al. 2013). Rice is the staple food for billions of Asians, which is severely affected by salinity stress (Singh et al. 2012). High salt percentage in soil enhances the production of reactive oxygen species (ROS) (Tuteja et al. 2013). Various genetic engineering advances have been employed to boost salt tolerance in a range of plants including rice (Sanan-Mishra et al. 2005; Misra et al. 2007; Gill et al. 2013; Tuteja et al. 2013).

G-protein (guanine nucleotide-binding proteins) signalling pathway is an evolutionarily conserved extracellular signal transduction which comprises the Gα subunit of 39–52 kDa, the Gβ subunit of 34–36 kDa and the Gγ subunit of 7–10 kDa (Gilman 1987; Tuteja and Sopory 2008). The pathway transduces signals from the outside environment to inside possibly through regulators (Tuteja 2009). G-proteins are engaged in a diverse range of vital biological pathways including extracellular signal transduction, hormone regulation, light perception, ion channel regulation, cell proliferation, multiple developmental processes, defence responses, cell wall modification and its regulation, and innate immunity (Jones 2002; Jones and Assmann 2004; Perfus-Barbeoch et al. 2004; Trusov et al. 2007, 2009; Zhang et al. 2008; Dupre et al. 2009; Klopffleisch et al. 2011; Trusov and Botella 2012; Urano et al. 2013; Cheng et al. 2015; Maruta et al. 2015). Subunits of G-protein have been reported in humans, *Arabidopsis*, wild oat, pea, rice, lupin soybean, tobacco, tomato spinach and lotus (Merlet et al. 1999; Assmann 2002; Jones and Assmann 2004; Misra et al. 2007; Yadav et al. 2012). Under stress, the G-proteins in peas have been shown to be regulated (Misra et al. 2007; Bhardwaj et al. 2011). Furthermore, *Arabidopsis* G-proteins are multifunctional units, interacting and playing significant roles in the development and environmental stress combat (Klopffleisch et al. 2011).

While multiple subunits of G-proteins have been reported in animals, the *Arabidopsis* genome has been reported to have only one gene for the Gα (*GPA1*) subunit and Gβ (*AGB*) subunit and three genes for Gγ (*AGG1, AGG2* and *AGG3*) subunits (Perfus-Barbeoch et al. 2004; Thung et al. 2012; Trusov et al. 2012). Rice genome contains only one canonical Gα (*RGA*), one Gβ (*RGB*) and five Gγ subunits; in addition to *RGG1* and *RGG2* three other *RGG3* subunits namely *GRAIN SIZE 3* (*GS3*), *DENSE AND ERECT PANICLE1* (*DEP1*) and G-protein gamma subunit type C number 2 of rice (*OsGGC2)* have been identified (Trusov et al. 2012). There are three different types of gamma subunits in plants: type "A" (i.e. the animal looking classic one), type "B" (lacking the isoprenylation motif (Subramaniam et al. 2016) and type "C" (with the long tail) (Botella 2012). Gγ provides functional selectivity to the heterotrimer and is essential for its proper targeting to the plasmalemma (Trusov et al. 2007; Choudhury et al. 2011; Thung et al. 2012). Studies have shown that AGB1 (*Arabidopsis thaliana* GB1) is necessary for survival during salt stress (Colaneri et al. 2014; Yu and Assmann 2015) and is also involved in ER stress tolerance (Cho et al. 2015). In *A. thaliana*, *AGG1* is involved in auxin signalling with brassinosteroid (Domagalska et al. 2007) and overexpression of *AGG3* in *Camelina sativa* resulting yield increase and heavy metal stress tolerance (Alvarez et al. 2015). Recently, it was reported that *Arabidopsis* Gα (*GPA1*) also functions in mediating ethylene-induced stomatal closure via H_2O_2 production (Ge et al. 2015). Three extra-large G-proteins XLG1, XLG2 and XLG3 in *Arabidopsis* are found to be acting as signal molecules for tuning plant G protein responses (Chakravorty et al. 2015).

Our previous work has reported that the expression of two isoforms of Gγ, i.e. *RGG1* and *RGG2,* was increased 10- to 20-fold by NaCl stress (Yadav et al. 2012). In the present study, *RGG1* overexpressing transgenic rice plants (*Oryza sativa* L., cv. IR64) were developed which shows enhanced tolerance to salinity stress with reduced oxidative damage. We have also identified some interacting partners of RGG1 protein using yeast two-hybrid screening assay. These partners probably play role in signalling, cell cycle control and prevention of aggregation of proteins under stress-induced perturbations.

Materials and methods

Isolation of RNA and quantitative real-time PCR

25-day-old rice seedlings (*O. sativa* cv. IR64; seeds obtained from ICAR-National Rice Research Institute, Cuttack, India) were stressed by keeping in 200 mM NaCl, 200 mM KCl and 10 μM ABA, and heat stress was given at 45 °C temperature separately under controlled parameters. Leaf samples were harvested at various time intervals (1, 2, 6, 12 and 24 h) and used for RNA isolation and qRT-

PCR as described earlier (Tuteja et al. 2013). For qRT-PCR, the *RGG1* gene-specific primers (forward 5′-GCGC TTTCTCGAGGAACTTGAAG-3′ and reverse 5′-CTTG CCAGTCTTGGGACAGATGGTTTG–3′) were used. The expression was normalised using α-tubulin gene-specific primers (forward 5′-GGTGGAGGTGATGATGCTTT-3′ and reverse 5′–ACCACGGGCAAAGTTGTTAG–3′) and fold change was calculated using $2^{-\Delta\Delta C_t}$ method from three independent experiments (Livak and Schmittgen 2001). The *RGG1* transgenic plants were used to check the transcript levels of different subunits such as *RGG2 RGB* and *RGA*. The qRT-PCR experiment was performed using gene-specific primers such as (forward 5′-CAAGAAGC TCGAGCAAGAGG-3′ and reverse 5′-CGGACCTTCAA ACCATCTGT-3′) for *RGG2* (forward 5′-GTCCTGTG GGATGTTACTACTG-3′ and reverse 5′-GCATCACATG AACCCGAAAC-3′) for *RGB* and (forward 5′-CTGG GAAACAGGAGGTTGAA-3′ and reverse 5′-GGTCT AGGGTCGTAGTTCTGTA-3′) for *RGA*.

Cloning of the gene *RGG1* and its *Agrobacterium*-mediated transformation in IR64 rice

The coding region of *RGG1* gene (282 bp) from rice was PCR-amplified using forward (5′-GGTACCATGCAGGCC GGAGGAGGAGGGGA-3′) and reverse primers (5′-GAA TTCTCACAAAAACCAGCATTTGCATCTG-3′) and the amplified fragment was sequenced (GenBank accession number: GU111573.1). The CDS *RGG1* (282 bp) was cloned in plant transformation vector pCAMBIA1302 to generate the plasmids pCAMBIA1302-*RGG1*. The IR64 rice was transformed with this construct using the *Agrobacterium tumefaciens* (LBA4404) transformation method (Sahoo and Tuteja 2012).

Segregation analysis of the T_1 transgenic lines

The T_1 seeds of transgenic IR64 plants were allowed to germinate on MS medium containing hygromycin (50 mg l^{-1}). Segregation analysis was carried out by the progenies that were resistant to hygromycin.

Polymerase chain reaction (PCR), Southern- and western-blot analysis

The incorporation of the gene *RGG1* in the transgenic plants was checked by PCR and Southern-blot analysis using the method described previously (Sahoo and Tuteja 2012). The crude plant extract from WT and the overexpressing lines was done using the process described earlier (Hurkman and Tanaka 1986). Equal amount of crude protein was denatured and separated using SDS-PAGE and electroblotted onto polyvinylidene fluoride (PVDF) membrane and then probed with mouse polyclonal antibodies (1:1000 dilution) raised against full length RGG1 protein. Crude extract from WT plant was used as negative control. Western-blot analysis using anti-RGG1 (1:1000 dilution) primary and alkaline phosphatase-conjugated anti-mouse IgG (Sigma) secondary antibodies (1:10000 dilution) was performed to check the production of the protein by the transgenic lines. The blot was developed as per manufacturer's protocol (Sigma).

Germination and leaf disc assay

The leaf disc assay and chlorophyll content were measured by employing the process described (Sanan-Mishra et al. 2005). The germination of seeds was checked for salinity tolerance.

Analysis of transgenic, WT and vector control (VC) plants

45-day-old seedlings of transgenic, WT and VC plants were allowed to grow in 200 mM NaCl kept in a tank. The changes in phenotypic characters were recorded at an interval of 45 days.

Measurement of photosynthetic activities, agronomic attributes, and Na^+ and K^+ content

The different photosynthetic parameters were measured according to previously depicted method (Tuteja et al. 2015). The endogenous ions' (phosphorous, potassium and sodium) contents were measured as described (Tuteja et al. 2013). The T_2 *RGG1* transgenic plants were analysed as described above.

Isolation of RNA and real-time PCR of antioxidant genes

25-day-old rice (*O. sativa* cv. IR64) plants were kept in H_2O and 200 mM NaCl for 24 h. Leaf samples of WT, vector control (VC) and transgenic lines were collected for RNA isolation and qRT-PCR was achieved as described earlier (Tuteja et al. 2013). Three major antioxidant genes such as *CATa*, *APX1* and *GR2* were chosen for this qRT-PCR experiment and in all these cases gene-specific primers were used. The expression was normalised to α–tubulin (forward 5′-GGTGGAGGTGATGATGCTTT-3′ and reverse 5′-ACCACGGGCAAAGTTGTTAG-3′) and calculated using the $2^{-\Delta\Delta C_t}$ method from three sovereign experiments (Livak and Schmittgen 2001). List of primers used in this experiment has been provided in (Supplementary Table S1).

Biochemical assays of *RGG1* transgenic plants

The biochemical analyses, like lipid peroxidation, catalase a (CATa), ascorbate peroxidase one (APX1), glutathione reductase two (GR2), proline and hydrogen peroxide, were carried out using 25-day-old WT and transgenic rice seedlings exposed for 24 h to salt stress. The electrolytic leakage and relative water content (RWC) was measured as previously described (Garg et al. 2012).

Measurement of soluble sugar and endogenous hormones (GA3, zeatin and IAA)

Leaves from T_2 transgenic and WT plants, grown for 24 h on 200 mM NaCl, were used for estimation of glucose and sucrose following the method of Karkacier et al. (2003). The T_2 *RGG1* rice (L1–L5) along with wild-type (WT) plants were grown under 200 mM NaCl. After 24 h, the plant samples were collected for endogenous hormone assays (Chen et al. 1996).

Yeast two-hybrid and bi-molecular fluorescence complementation (BiFC) assays

The interacting partners of RGG1 were isolated using the technique described earlier (Gill et al. 2013). Open reading frames (ORFs) were amplified using the primer pair (Supplementary Table S2) and were initially cloned in pJET1.2 and then finally subcloned into pSY728 and pSY738 vectors. The vectors pSY728 and pSY738 were used to express proteins of interest fused at their C-termini to fragments of YFP, using gene-specific primers and apt restriction enzymes (Supplementary Table S2). This gives rise to translational fusion between the coding sequences and BiFC probes (Knip et al. 2013). The constructs were co-bombarded into onion epidermal pieces on agar plates containing Murashige and Skoog salts (Sigma-Aldrich) using a biolistic PDS-1000/He system (Bio-Rad). After incubation for 24–30 h at 30 °C, the expression of YFP fluorescence was observed by confocal-laser microscopy. Following excitation with a 476.5–493.5 nm band-path filter, along with a triple band filter set (Zeiss Iberia, Madrid, Spain; filter set 40) the YFP appears as bright green (Cell observer SD, Apotome 2, LSM 710, Zeiss) (Bracha-Drori et al. 2004; Gu et al. 2014).

Quantitative real-time PCR (qRT-PCR) of *RGA, RGB, RGG1* and *RGG2*

25-day-old rice (*O. sativa* cv. IR64) plants were kept in H_2O and 200 mM NaCl for 24 h. Leaf samples of WT, VC and transgenic lines were collected for the isolation of RNA and qRT-PCR as described earlier (Tuteja et al. 2013). The expression was normalised to α-tubulin (forward 5′-GGTGGAGGTGATGATGCTTT-3′ and reverse 5′-ACCACGGGCAAAGTTGTTAG-3′) and calculated using the $2^{-\Delta\Delta C_t}$ method from three repeats (Livak and Schmittgen 2001). List of primers used in this experiment has been provided in (Supplementary Table S3).

Statistical analysis

The data were analysed statistically as mentioned earlier (Tuteja et al. 2013).

Results

Relative expression of *RGG1* gene in IR64 rice wild-type (WT) plants under different abiotic stresses

RGG1 transcript level was up-regulated up to threefold during 1–24 h of heat stress (45 °C) treatment (Fig. 1a). Similarly, ABA treatment increased *RGG1* expression by up to fourfold (Fig. 1b). The relative expression of *RGG1* in WT plants was less when treated with KCl (Fig. 1c). The transcript abundance of *RGG1* was also up-regulated up to 14-fold during 1–24 h of salt (200 mM NaCl) treatment (Fig. 1d). It seems to be a strong response towards NaCl exposure. The expression of *RGG1* was only threefold after treatment with an equal concentration, i.e. 200 mM of KCl suggesting that the high level of expression in response to NaCl was due to the stress created by sodium ions (Fig. 1d; 24 h). These results demonstrate that *RGG1* is highly up-regulated due to salt (NaCl) treatment in rice.

Polymerase chain reaction, Southern- and Western-blot analysis of *RGG1* transgenic rice plants

The T–DNA construct of the *RGG1* gene was used for developing T_1 transgenic rice plants (Fig. 2a). The original T–DNA map of pCAMBIA-1302 binary vector has been provided in (Supplementary Fig. S3). Phenotypically, the transgenic rice plants were significantly taller (L1–L5) than the WT (Fig. 2b). 35S forward and the gene-specific reverse primers were used in PCR to confirm the successful integration of the transgene (*RGG1*). The expected size band of 480 bp was observed (Fig. 2c). Western-blot analysis revealed that the protein was expressed to almost similar levels in all the transgenic lines from L1 to L5 (Fig. 2d). Southern-blot analysis confirmed the integration and the copy number of the transgene. The results show that a single copy transcript is present in the transgenic lines (L1-L5) driven by the constitutive 35S promoter and in the WT (native gene) (Fig. 2e). The incorporation of the *RGG1* gene was established by PCR in T_2 plants and the band of expected size (480 bp) was observed (Fig. 2f).

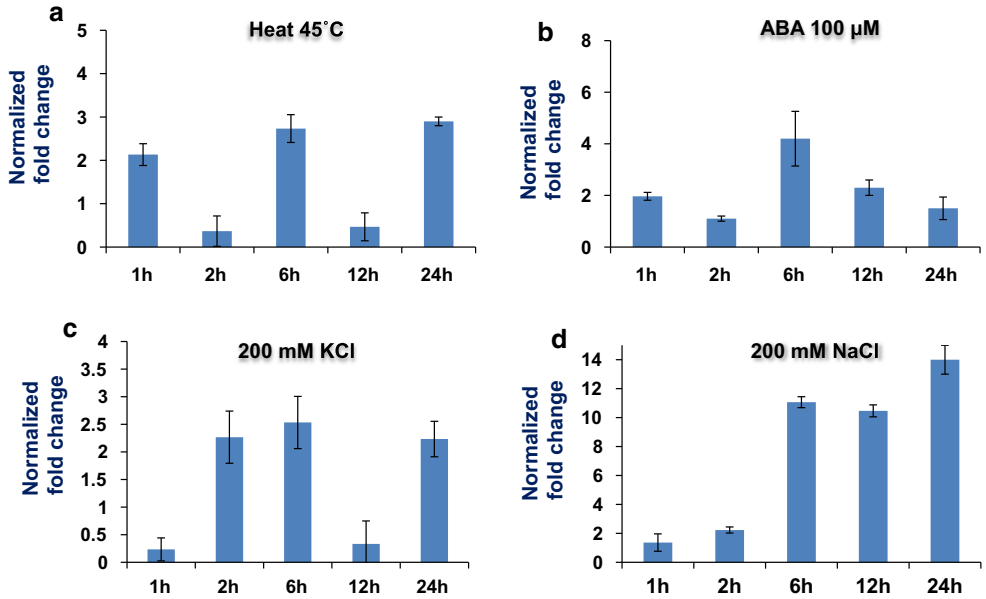

Fig. 1 Relative expression of *RGG1* gene in wild-type (WT) IR64 rice in different stress condition: **a** heat stress at 45 °C. **b** 100 µM ABA. **c** 200 mM KCl. **d** 200 mM NaCl. *Error bars* indicate standard errors (SEs) calculated from three independent experiments

Response of T_1 transgenic plants under salinity stress condition

The effect of saline stress during germination of seeds was studied by growing WT and T_1 transgenic seeds on MS plates supplemented with 200 mM NaCl (Murashige and Skoog 1962). The seeds of transgenic lines were able to germinate and continue growth even in the presence of 200 mM NaCl saline stress (Fig. 3a). Leaf disc assays demonstrated that the reduction of the amount of chlorophyll under the influence of salt stress was lower after 72 h in the *RGG1* T_1 transgenic lines compared to WT plants (Fig. 3b). As a control, the 0 h NaCl treatment showed no sign of bleaching (data not shown). The data of the chlorophyll content supported the findings of the leaf disc assay results under both, 100 and 200 mM, NaCl saline stress (Fig. 3c). Senescence changes in the leaves of WT plants after 3 days of salt (200 mM NaCl) treatment were observed (Fig. 3d); however, after 45 days of treatment with salt, the *RGG1* plants (L1–L3) matured and produced viable seeds (Fig. 3e), while the WT plants expired. The transgenic lines L4 and L5 demonstrated results similar to the L1- L3 plants (Supplementary Fig. S1).

Segregation ratio and agronomic performance of *RGG1* T_1 transgenic plants under stress

The survival rates of seedlings of T_1 transgenic (after 3 days of treatment in 200 mM NaCl saline stress) were contrasted with the WT seedlings (without stress) and VC (empty vector control). The difference was not significant in this case (Table 1). The results showed that the segregation ratio of seeds was ~3:1 in the presence of hygromycin (Table 1). Under the influence of saline stress, the *RGG1* IR64 plants evidenced superior growth and development when compared with the WT and VC (Table 2). Other parameters of growth of the *RGG1* IR64 plants were found to be better than WT and VC plants (Table 3).

Photosynthetic characteristics and endogenous ion contents of *RGG1* T_1 transgenic plants under stress

The photosynthetic characteristics of transgenic as well as WT and VC plants were measured in the matured stage (harvesting time) after one week of induction of salt (200 mM NaCl) stress. The photosynthetic rate declined by 33% in WT and 35% in VC as compared with *RGG1* transgenic lines. The stomatal conductance, net photosynthetic rate and intracellular CO_2 were also higher in transgenic lines in comparison with the WT and VC plants (Table 2). In the presence of NaCl (200 mM), the WT and VC plants accumulated excess sodium whereas the transgenic lines had reduced amounts of sodium in their leaves. Salt-treated T_1 transgenic lines showed higher accumulation of phosphorus and potassium (Table 2).

Transcript levels of different subunits of G protein in *RGG1* over expressing T_2 transgenic plants under 200 mM NaCl stress

Under control conditions (unstressed; 0 mM NaCl), the level of *RGG1* transcript in T_2 transgenic plants (L1–L5)

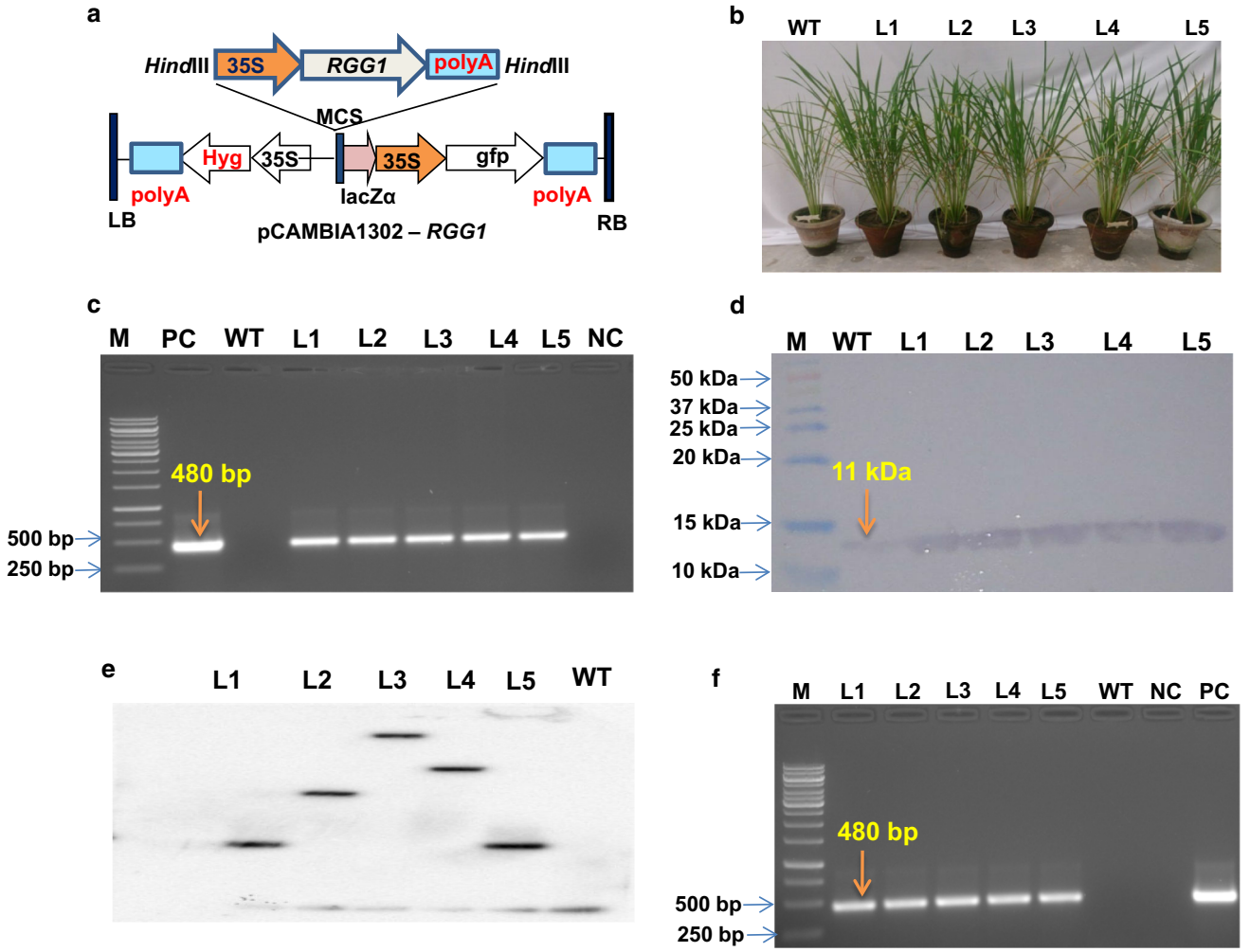

Fig. 2 Analysis of *RGG1* over expressing transgenic T_1 IR64 rice plants. **a** The *OsRGG1* gene cloned in pCAMBIA1302 vector at *Hin*dIII site. The directionality of the insert depicted was confirmed by sequencing. **b** Transgenic plants (L1–L5) along with WT. **c** PCR analysis of the *RGG1* over expressing transgenic (T_1) lines along with wild type (WT), positive control (PC) and negative control (NC) shows the amplification of the 480 bp fragment. **d** Western-blot analysis showing the production of RGG1 protein (~11 kDa). **e** Southern-blot analysis showing the integration and copy number of the *RGG1* gene. **f** PCR analysis of the *RGG1* over expressing transgenic (T_2) lines along with WT, positive control (PC) and negative control (NC) showing the amplification of a 480 bp fragment

increased considerably. The qRT-PCR showed 12- to 14-fold increases in the transcript level of *RGG1* in contrast to WT and VC plants under normal conditions (Supplementary Fig. S2c). However, in similar unstressed conditions, transcript levels of other subunits (*RGA, RGB*, *RGG2, DEP1* and *GS3*) were not induced in T_2 transgenic plants (L1-L5) (Supplementary Fig. S2 a, b, d–f). In the 200 mM NaCl stress condition, the relative level of *RGG1* transcript increased 10- to 14-fold increase in WT and VC plants, but in all transgenics, the relative fold change was around 19- to 20-fold (Fig. S2c). Similar results were found for *RGG2*. The WT, VC, as well as all the transgenic lines showed 10- to 13.5-fold increase in their relative transcript level (Fig. S2d). However, under 200 mM NaCl stress condition, there was no significant increase in the transcript level of *RGA* subunit in WT, VC and transgenic lines (Supplementary Fig. S2a). In case of *RGB*, the WT and VC did not show any significant increase in transcript level but all transgenic lines (L1–L5) showed a 12- to 14-fold increase in their relative transcript levels (Supplementary Fig. S2b). The relative expression of *DEP1* and *GS3* in WT and VC plants in 200 mM NaCl stress did not significantly change but all transgenic lines (L1–L5) showed 11- to 14.9-fold changes (Supplementary Fig. S2 e, f).

Gene expression of antioxidative enzymes

Manifestation of different stresses is mediated by ROS. Plants protect themselves from ROS by upregulating the gene expression of different antioxidant enzymes. To ascertain if ROS detoxifying enzymes are modulated by salt stress in WT, VC and transgenic plants, their gene

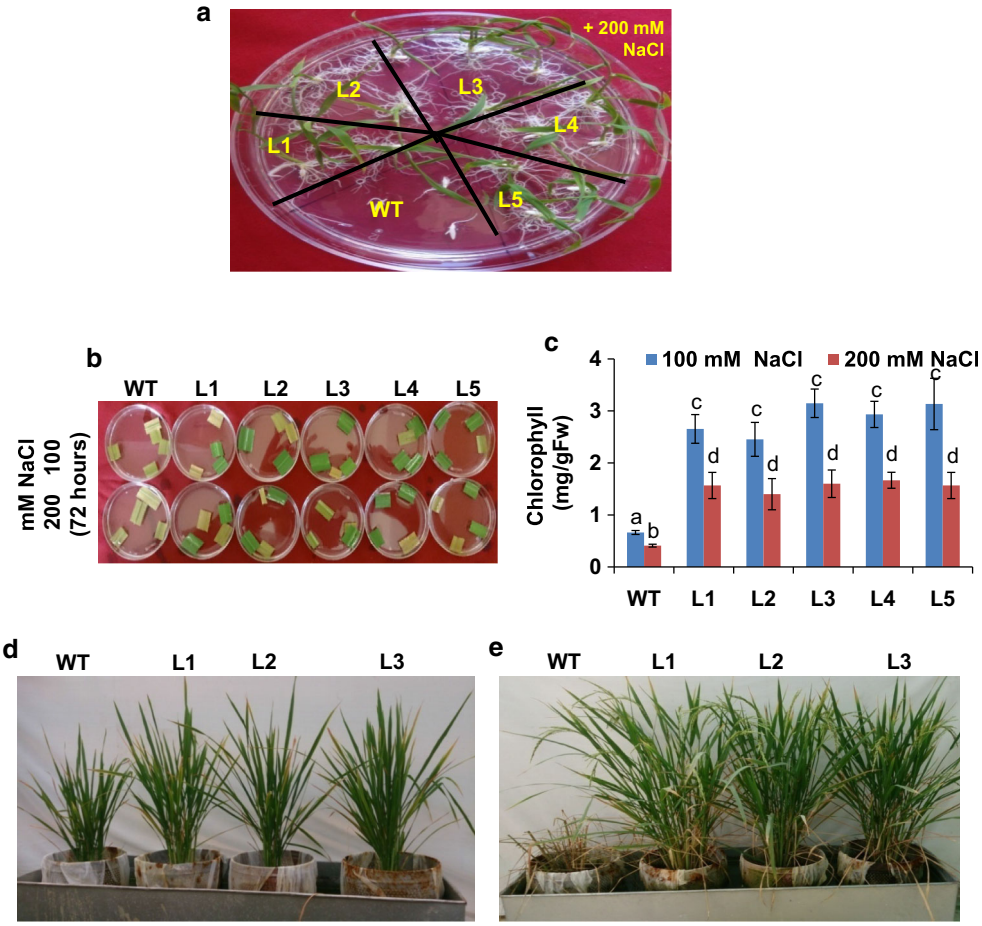

Fig. 3 Analysis of *RGG1* over expressing transgenic T_2 IR64 rice plants. **a** Germination test of T_2 seeds on solid MS medium with 200 mM NaCl. **b** Leaf disc senescence assay under 100 and 200 mM NaCl. **c** Chlorophyll content (mg/g FW) in T_2 *RGG1* transgenic lines after salt stress. **d** Salt tolerance response of T_2 *RGG1* transgenic plants (L1, L2 and L3) and WT in 0 day of 200 mM NaCl stress. **e** Salt tolerance of same set of mature plants after 45 days of NaCl stress

Table 1 Comparison of segregation ratio (Hyg^r:Hyg^s) and plant seedlings survival (%) of the WT (control), VC (empty vector control) and T_1 generation of *RGG1* overexpressing transgenic plants (line 1, line 2, line 3, line 4 and line 5) (*O. sativa* L. cv. IR64) grown in the presence of 0 and 200 mM NaCl, respectively

Attributes	Water grown control plants		200 mM NaCl grown *RGG1* transgenic plants				
	WT	VC	Line 1	Line 2	Line 3	Line 4	Line 5
Segregation ratio (Hyg^r:Hyg^s[n][a])	0	0	2.6:1[183]	2.5:1[136]	3.1:1[182]	2.8:1[176]	3.1:1[179]
Plant seedlings survival (%)	93 ± 3.1[a]	90 ± 3.1[a]	96 ± 2.6[a]	94 ± 3.3[a]	93 ± 3.2[a]	95 ± 2.3[a]	94 ± 2.5[a]

Each value represents mean of three replicates ± SE. The letters "a" indicates significant differences at $P > 0.05$ level as determined by Duncan's multiple range test (DMRT)

expression was monitored in control and stressful environment.

Catalase (CATa) As compared to control conditions, the gene expression of *CAT*, responsible for destruction of H_2O_2, increased by twofold in both WT and VC and around sixfold in different T_2 transgenic lines in saline environment (Fig. 4a).

Ascorbate peroxidase (APX1) In response to salinity stress, the relative gene expression of *APX* increased by twofold in WT and VC and fivefold in various T_2 transgenic lines (Fig. 4b).

Glutathione reductase (GR2) The relative expression of *GR* with respect to control conditions increased in salt-stressed samples by 1.9-fold in both WT and VC and 5.5-fold in various T_2 transgenic lines (Fig. 4c).

Analysis of antioxidant enzymes activity and response of malondialdehyde (MDA), H_2O_2 and ion leakage in T_2 *RGG1* transgenic plants

In agreement with gene expression results, the overexpression of *RGG1* resulted in increased enzymatic

Table 2 Phenotypic attributes of WT, VC and T_1 generation of *RGG1* overexpressing transgenic lines (line 1, line 2, line 3, line 4 and line 5) of rice (*O. sativa* L. cv. IR64) under 0 or 200 mM NaCl

Yield attributes	Control WT plants		VC plants		200 mMNaCl grown T_1 *RGG1* transgenic plants			
					Line 1		Line 2	
	0	200	0	200	0	200	0	200
Plant height (cm)	64 ± 3.2[a]	34.6 ± 1.4[b]	65 ± 3.2[a]	35.6 ± 1.4[b]	76 ± 3.2[a]	70 ± 3.7[a]	78 ± 3.1[a]	69 ± 3.2[a]
Root length (cm)	27 ± 0.8[a,b]	12.8 ± 0.02[b]	26 ± 0.8[a,b]	14.8 ± 0.02[b]	28 ± 1.0[a]	24 ± 1.0[a,b]	28 ± 1.5[a]	21 ± 1.5[a,b]
Root dry weight (g)	2.6 ± 0.13[b]	1.2 ± 0.01[c]	2.7 ± 0.13[b]	1.5 ± 0.01[c]	2.8 ± 0.14[a]	2.1 ± 0.14[a]	3.2 ± 0.12[a]	2.6 ± 0.17[a]
Leaf area (cm^2/plant)	85 ± 2.3[a,b]	41.72 ± 2.0[c]	87 ± 2.3[a,b]	40.70 ± 2.0[c]	96 ± 1.0[a]	78 ± 1.7[a,b]	96 ± 1.0[a]	76 ± 1.4[a,b]
Net photosynthetic rate (P_N, μmol CO_2 m^{-2} s^{-1})	9.17 ± 0.8[b]	6.04 ± 0.21[c]	9.10 ± 0.8[b]	6.01 ± 0.21[c]	10.35 ± 0.6[a]	8.27 ± 0.8[a]	10.55 ± 0.1[a]	9.38 ± 0.3[a]
Stomatal conductance (gs, mmol m^{-2} s^{-1})	227 ± 11.4[a]	109.3 ± 5.3[b]	223 ± 11.4[a]	105.3 ± 5.3[b]	242 ± 10.9[a]	209 ± 11.6[a]	255 ± 10.9[a]	212 ± 10.7[a]
Intracellular CO_2 (C_i, μmol mol^{-1})	227 ± 11.3[a]	102.1 ± 4.4[b]	224 ± 11.3[a]	101.1 ± 4.4[b]	225 ± 10.2[a]	209 ± 10.2[a]	227 ± 10.5[a]	203 ± 10.2[a]
Phosphorus (%)	0.223 ± 0.014[b]	0.125 ± 0.002[c]	0.227 ± 0.014[b]	0.120 ± 0.002[c]	0.226 ± 0.010[a]	0.238 ± 0.010[a]	0.224 ± 0.011[a]	0.248 ± 0.011[a]
Potassium (%)	0.146 ± 0.002[b]	0.094 ± 0.002[c]	0.145 ± 0.002[b]	0.093 ± 0.002[c]	0.148 ± 0.002[a]	0.129 ± 0.002[a]	0.147 ± 0.002[a]	0.139 ± 0.002[a]
Sodium (%)	0.005 ± 0.001[a]	0.066 ± 0.001[a]	0.005 ± 0.001[a]	0.067 ± 0.001[a]	0.005 ± 0.001[a]	0.048 ± 0.001[a]	0.005 ± 0.001[a]	0.042 ± 0.001[a]

Yield attributes	200 mMNaCl grown T_1 *RGG1* transgenic plants					
	Line 3		Line 4		Line 5	
	0	200	0	200	0	200
Plant height (cm)	78 ± 3.1[a]	74 ± 3.1[a]	73 ± 3.2[a]	69 ± 3.2[a]	75 ± 3.1[a]	71 ± 3.3[a]
Root length (cm)	29 ± 1.3[a]	26 ± 1.0[a]	28 ± 1.0[a]	25 ± 1.1[a,b]	29 ± 1.4[a]	21 ± 1.4[a,b]
Root dry weight (g)	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)	93 ± 1.6[a]	81 ± 1.6[a,b]	95 ± 1.0[a]	84 ± 1.8[a,b]	98 ± 1.0[a]	88 ± 1.6[a,b]
Net photosynthetic rate (P_N, μmol CO_2 m^{-2} s^{-1})	10.07 ± 0.4[a]	9.17 ± 0.6[a]	10.38 ± 0.6[a]	8.29 ± 0.8[a]	10.54 ± 0.2[a]	9.37 ± 0.2[a]
Stomatal conductance (gs, mmol m^{-2} s^{-1})	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 (C_i, μmol mol^{-1})	224 ± 11.5[a]	206 ± 10.2[a]	228 ± 10.2[a]	204 ± 10.3[a]	231 ± 10.5[a]	206 ± 10.1[a]
Phosphorus (%)	0.223 ± 0.011[a]	0.263 ± 0.011[a]	0.225 ± 0.010[a]	0.237 ± 0.011[a]	0.226 ± 0.012[a]	0.247 ± 0.011[a]
Potassium (%)	0.147 ± 0.001[a]	0.148 ± 0.005[a]	0.145 ± 0.002[a]	0.139 ± 0.002[a]	0.148 ± 0.002[a]	0.136 ± 0.003[a]
Sodium (%)	0.005 ± 0.001[a]	0.047 ± 0.001[a]	0.006 ± 0.001[a]	0.043 ± 0.002[a]	0.004 ± 0.001[a]	0.049 ± 0.001[a]

All the data were collected at the matured stage of the plant. 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] Significant differences at $P > 0.05$ level as determined by Duncan's multiple range test (DMRT)

Table 3 Comparison of various yield parameters of WT, VC and T_1 generation of *RGG1* over expressing transgenic lines (line 1, line 2, line 3, line 4 and line 5) of rice (*O. sativa* L. cv. IR64) under 0 or 200 mM NaCl, respectively

Yield attributes	Control WT plants		VC plants		200 mM NaCl grown T_1 *RGG1* transgenic plants			
					Line 1		Line 2	
	0	200	0	200	0	200	0	200
Time required for flowering (days)	90 ± 2.3[a]	ND*	90 ± 2.1[a]	ND*	92 ± 3.6[a]	87 ± 2.2[a]	92 ± 3.0[a]	91 ± 2.7[a]
No. of tillers/plant	21 ± 1.0[c]	ND	20 ± 1.0[c]	ND	23 ± 0.12[ab]	22 ± 1.2[ab]	22 ± 0.15[ab]	21 ± 1.2[ab]
No. of panicle/plant	16 ± 0.5[c]	ND	18 ± 0.5[c]	ND	20 ± 0.11[ab]	21 ± 1.1[ab]	25 ± 0.13[ab]	21 ± 1.1[ab]
No. of filled grain/panicle	81 ± 3.1[b]	ND	80 ± 3.1[b]	ND	97 ± 3.2[a]	83 ± 3.2[a]	93 ± 3.23[a]	85 ± 4.1[a]
No. of chaffy grains/panicle	13 ± 0.21[a]	ND	12 ± 0.21[a]	ND	07 ± 0.12[b]	05 ± 0.11[b]	05 ± 0.06[b]	04 ± 0.22[b]
Straw dry weight (g)	54 ± 1.3[b]	ND	55 ± 1.3[b]	ND	57 ± 3.05[a]	47 ± 2.3[a]	64 ± 2.1[a]	60 ± 1.6[a]
100 grain weight	2.75 ± 0.1[a]	ND	2.72 ± 0.1[a]	ND	2.68 ± 0.12[b]	2.61 ± 0.11[a]	2.67 ± 0. 12[b]	2.66 ± 0.10[a]
Seed weight per plant	35.64 ± 1.2[a]	ND	35.53 ± 1.2[a]	ND	51.99 ± 1.4[b]	45.49 ± 0.3[b]	62.07 ± 0.5[a]	47.48 ± 1.1[b]

Yield attributes	200 mM NaCl grown T_1 *RGG1* transgenic plants					
	Line 3		Line 4		Line 5	
	0	200	0	200	0	200
Time required for flowering (days)	93 ± 2.6[a]	90 ± 2.7[a]	92 ± 3.6[a]	90 ± 2.1[a]	92 ± 3.1[a]	89 ± 2.4[a]
No. of tillers/plant	21 ± 0.12[a]	23 ± 1.0[a]	21 ± 0.12[ab]	21 ± 1.3[ab]	23 ± 0.14[ab]	21 ± 1.2[ab]
No. of panicle/plant	25 ± 0.15[a]	21 ± 1.2[a]	25 ± 0.11[ab]	20 ± 1.0[ab]	26 ± 0.13[ab]	21 ± 1.0[ab]
No. of filled grain/panicle	96 ± 3.27[a]	86 ± 3.2[a]	97 ± 3.3[a]	81 ± 3.1[a]	95 ± 3.23[a]	88 ± 4.0[a]
No. of chaffy grains/panicle	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)	62 ± 1.8[b]	55 ± 1.3[a]	58 ± 3.04[a]	49 ± 2.1[a]	63 ± 2.2[a]	61 ± 1.4[a]
100 grain weight	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	63.36 ± 1.2[b]	47.13 ± 1.1[b]	64.99 ± 1.2[b]	42.28 ± 1.1[b]	66.19 ± 1.2[b]	48.6 ± 1.3[b]

All the data were collected at the matured stage of the plant

ND no data

* Control plants did not survive 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] Significant differences at $P > 0.05$ level as determined by Duncan's multiple range test (DMRT)

activities of CAT, APX and GR due to salt treatment (200 mM NaCl) in *RGG1* transgenic plants (Fig. 4d–f). This resulted in decreased accumulation of ROS, i.e. H_2O_2 in the transgenic plant in stressful environment. The changes induced by the presence of salt in the accumulation of malondialdehyde (MDA), H_2O_2, ion leakage, relative water content (RWC), proline and antioxidant machineries in T_2 transgenic lines (L1–L5) were compared with that ones in WT and VC seedlings. The levels of MDA, H_2O_2 and ion leakage were significantly reduced while RWC and proline content were increased in *RGG1* transgenic lines as compared to WT and VC under salt stress (200 mM NaCl) (Fig. 5a–e). The increased detoxification of ROS led to reduced membrane lipid peroxidation, i.e. MDA production and membrane damage as indicated by electrolyte leakage.

Endogenous soluble sugars and hormone content of T_2 *RGG1* transgenic plants

The T_2 *RGG1* transgenic plants (L1–L5) accumulated almost twice the amount of glucose and 3.5-fold more fructose in both their roots and shoots as weighed against the WT and VC plants during salinity stress (Fig. 6a–d). The T_2 *RGG1* transgenic lines L1–L5 illustrated high endogenous content of plant hormones as compared with the WT and VC plants (Fig. 6e–g). In this generation, GA3 content in the shoots of *RGG1* transgenic rice lines (L1–L5) was relatively higher (ranging from 2.5 to 3.2 μg/gFW) as compared to the WT (1.49 μg/gFW) and VC (1.46 μg/g FW) plants. GA3 content ranged from 1.6 to 2.2 μg/gFW in the roots and 0.3–0.5 μg/gFW in the leaves of *RGG1* lines as compared to roots (0.9 μg/gFW) and leaves

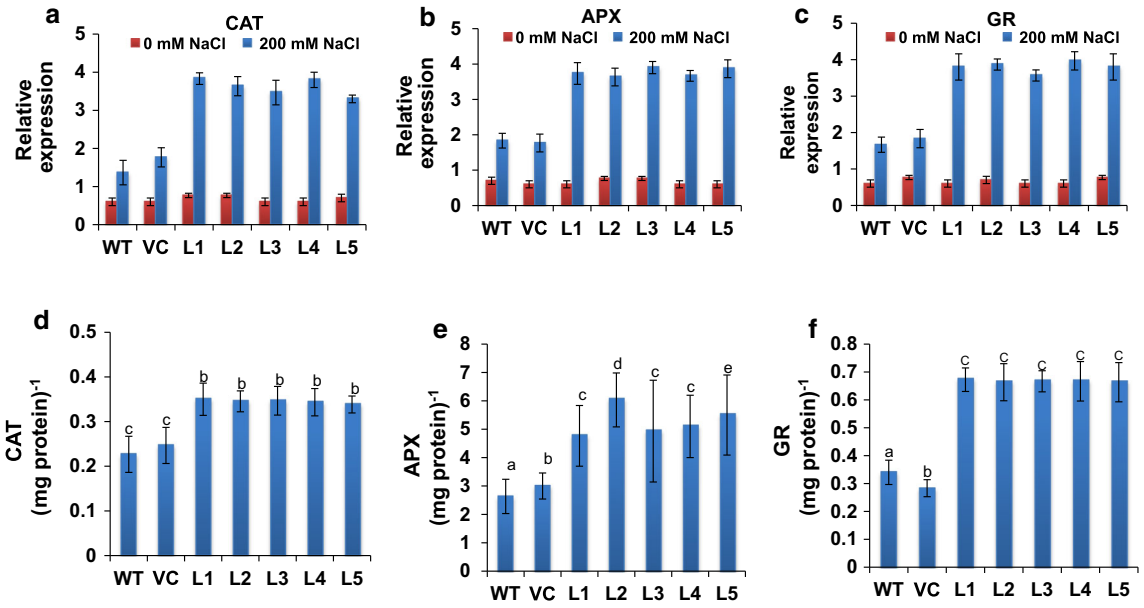

Fig. 4 Relative gene expression and antioxidant activities of T_1 plants. **a** Relative gene expression of *CAT* in WT, VC and different transgenic lines (L1–L5) in H_2O as well as in 200 mM NaCl stress. **b** Relative gene expression of *APX* in WT, VC and different transgenic lines (L1–L5) in H_2O as well as in 200 mM NaCl stress. **c** Relative gene expression of *GR* in WT, VC and different transgenic lines (L1–L5) in H_2O as well as in 200 mM NaCl stress. **d** Catalase (CAT) activity, one unit of enzyme activity defined as 1 µmol H_2O_2 oxidised min^{-1}. **e** Ascorbate peroxidase (APX) activity, one unit of enzyme activity defined as 1 µmol of ascorbate oxidised min^{-1}. **f** Glutathione reductase (GR) activity, one unit of enzyme activity is defined as 1 µmol of GS-TNB formed min^{-1} due to reduction of DTNB

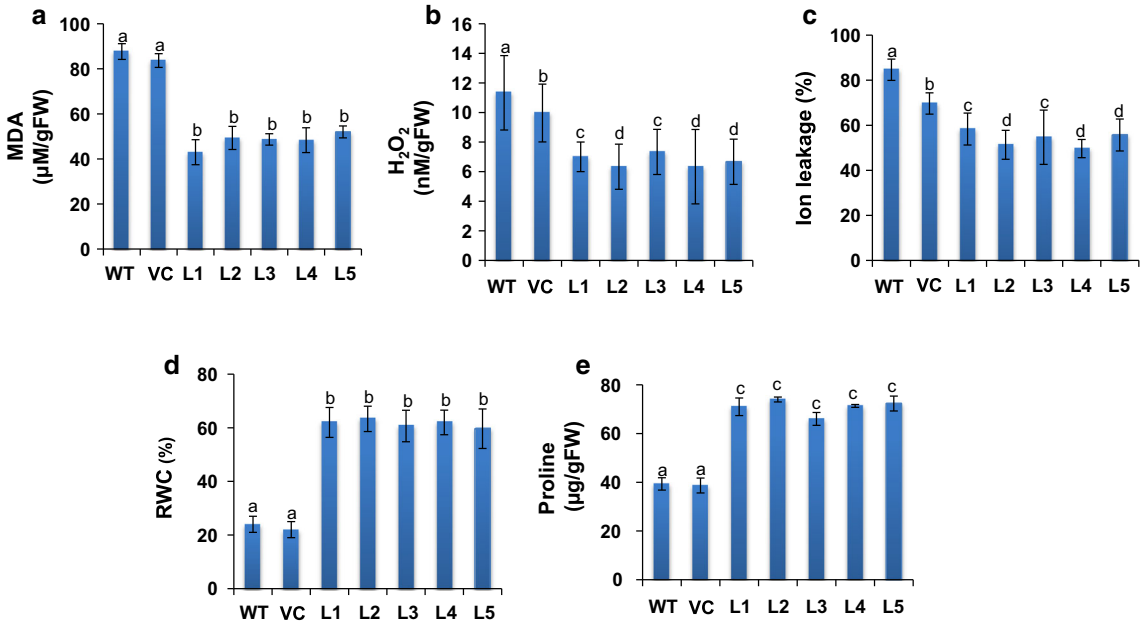

Fig. 5 Biochemical analysis of *RGG1* over expressing T_1-transgenic lines (L1–L5), WT and empty vector control (VC) plants exposed to 24 h salinity stress (200 mM NaCl). **a** Lipid peroxidation expressed in terms of malondialdehyde (MDA) content. **b** Hydrogen peroxide (H_2O_2) content. **c** Percent electrolytic leakage. **d** Percent relative water content (RWC). **e** Level of proline accumulation. *Error bars* indicate the standard errors (SEs) calculated from three independent experiments

(0.1 µg/gFW) of WT and VC plants (Fig. 6e). In transgenic plants, the value of zeatin averaged between 0.8–1.5 µg/gFW, while that of WT and VC plants contained 0.3–0.7 µg/gFW of zeatin in roots, shoots and leaves (Fig. 6f). The endogenous amount of IAA in leaves, shoots and roots of transgenic lines ranged between 2–3.2 µg/

gFW against the values of 1.5–1.7 µg/gFW in WT and VC plants (Fig. 6g).

Isolation of interacting partners of RGG1 protein through yeast two-hybrid system

The interacting partners of RGG1 were isolated by yeast two-hybrid (Y2H) screening assay. About one hundred positive colonies were selected randomly and transferred to 3 DO-SD media and those which evidenced better growth even after 16 days were moved onto 3 DO-SD + 5 mM 3-AT (3-amino-1,2,4-triazole) media. Ten interacting partners were isolated and their putative functions have been presented (Table 4).

Validation of yeast two-hybrid interaction candidates using bi-molecular fluorescence complementation (BiFC) assay

The interacting proteins of RGG1 identified in yeast two-hybrid screening technique were further validated by BiFC assay. No signals were detected in the onion epidermal cells when the cells were co-bombarded with pSY728 and pSY738 and also when bombarded with pSY728- RGG1 and pSY738; pSY728 and pSY738-RGG1 and were used as negative control (Fig. 7). The BiFC signals were found for all the interacting proteins. The expression of YFP fusion protein was distinguished in the plasma membrane system and cytoplasm of cells, signifying the interaction of the candidate interacting partner with RGG1 protein (Fig. 7).

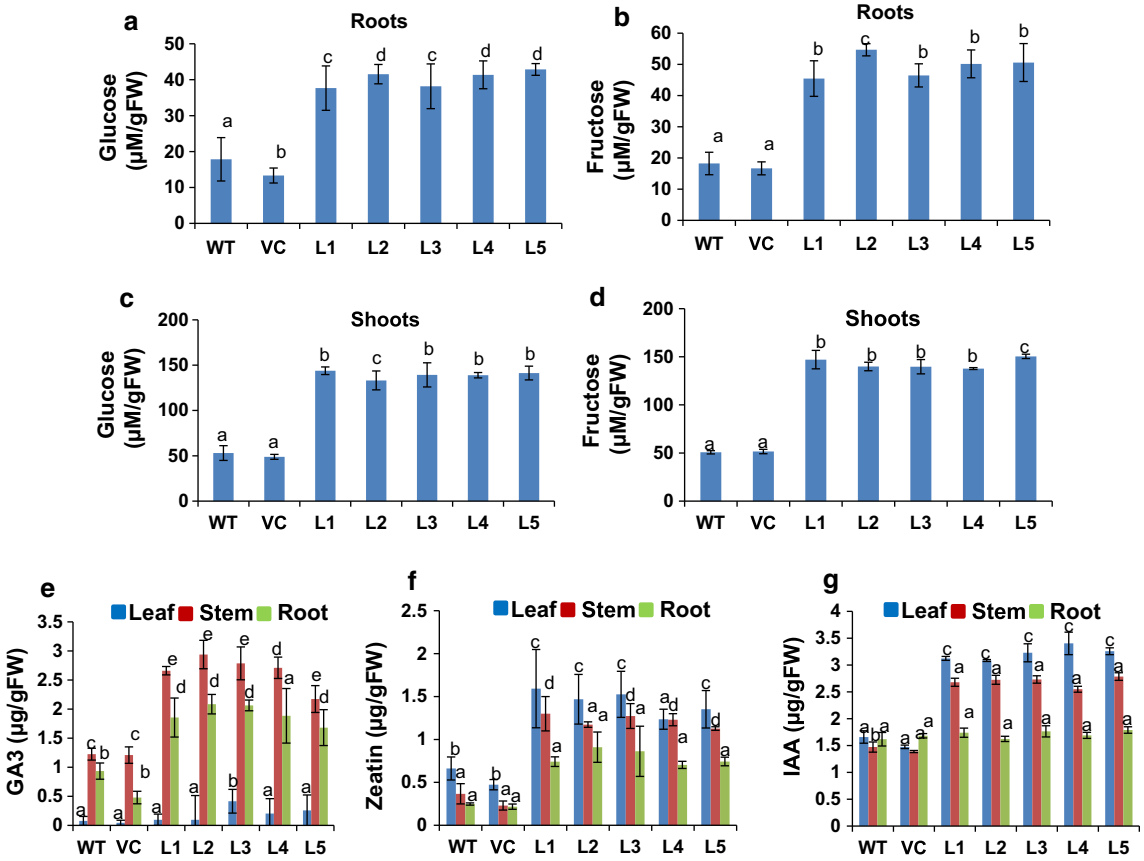

Fig. 6 Soluble sugar content in roots and shoots of *RGG1* over expressing rice T_2-transgenic lines (L1–L5) compared to WT and empty vector control (VC) plants exposed to 24 h salinity stress (200 mM NaCl). **a** Glucose content in roots. **b** Fructose content in roots. **c** Glucose content in shoots. **d** Fructose content in shoots. **e** Endogenous content (µg/g FW) of GA3 in leaf, stem and root of WT and *RGG1* T_2 transgenic lines (L1–L5) under 200 mM NaCl stress. **f** Endogenous content (µg/g FW) of zeatin in leaf, stem and root of WT and *RGG1* T_2 transgenic lines (L1–L5) under 200 mM NaCl stress. **g** Endogenous content (µg/g FW) of IAA in leaf, stem and root of WT and *RGG1* T_2 transgenic rice lines (L1–L5) under 200 mM NaCl stress. The significant difference between the mean values ($n = 3$) of rice plants WT and *RGG1* over expressing transgenic rice lines (L1–L5) was determined by one-way analysis of variance (ANOVA) using SPSS 10.0 (SPSS, Inc., now IBM, http://www-01.ibm.com/software/analytics/spss). The WT and transgenic lines at $P < 0.05$ and $P < 0.001$ were considered statistically significant

Table 4 Putative interacting partners of RGG1 protein identified by yeast two-hybrid screening

GENE NAME	PUTATIVE FUNCTION	LOCUS ID	β-Gal assay confirmation of RGG1 protein interacting partners
Rhodanese like domain containing protein	Cell cycle control	LOC_Os12g24020	
Enolase	Response to abiotic stress	LOC_Os10g08550	
G-Beta	Signal transduction, cellular process	LOC_Os03g0669200	
Glyceraldehyde 3 phosphate dehydrogenase	Response to abiotic stress	LOC_Os08g0126300	
Receptor protein kinase	Signal transduction	LOC_Os04g0201900	
Plasma membrane Ca ATPase (PMCA)	Response to abiotic stress	LOC_Os03g17310	
Calcium dependant protein kinase	Response to abiotic stress	LOC_Os02g057157	
Peptidyl prolyl isomerase	Response to abiotic stress	LOC_Os01g18210.1	
Pyridoxal dependant decarboxylase	Response to both biotic and abiotic stress	LOC_Os10g26110	
Cytochrome P450	Signal transduction, response to endogenous stimuli	LOC_Os03g40540	

Discussion

The rice Gγ subunits are potential targets for crop improvement but their complete functions are still to be elucidated (Botella 2012). *RGG1* and *RGG2* are up-regulated in response to salinity, harsh temperature and ABA treatments (Yadav et al. 2012). *AGG1* and *AGG2* are found to be involved in osmotic stress during germination and auxin control of root development, and *AGG3* in ABA signalling pathways (Chakravorty et al. 2011; Thung et al. 2013). Similarly, the soybean GγIII subunit is involved in ABA-dependent lateral root development (Roy Choudhury and Pandey 2013). Here, we have demonstrated that overexpression of *RGG1* promotes tolerance of rice plants to salinity stress. It is unravelled that RGG1 interacts with stress-responsive proteins that confer salinity tolerance in rice via detoxification of stress-induced ROS.

The *RGG1* transgenic lines also exhibited a higher content of endogenous nutrients under saline stress. Similar findings have been reported for *OsSUV3* overexpressing rice transgenic lines (Tuteja et al. 2013). The overexpressing *RGG1* seedlings grew in the constitutive presence

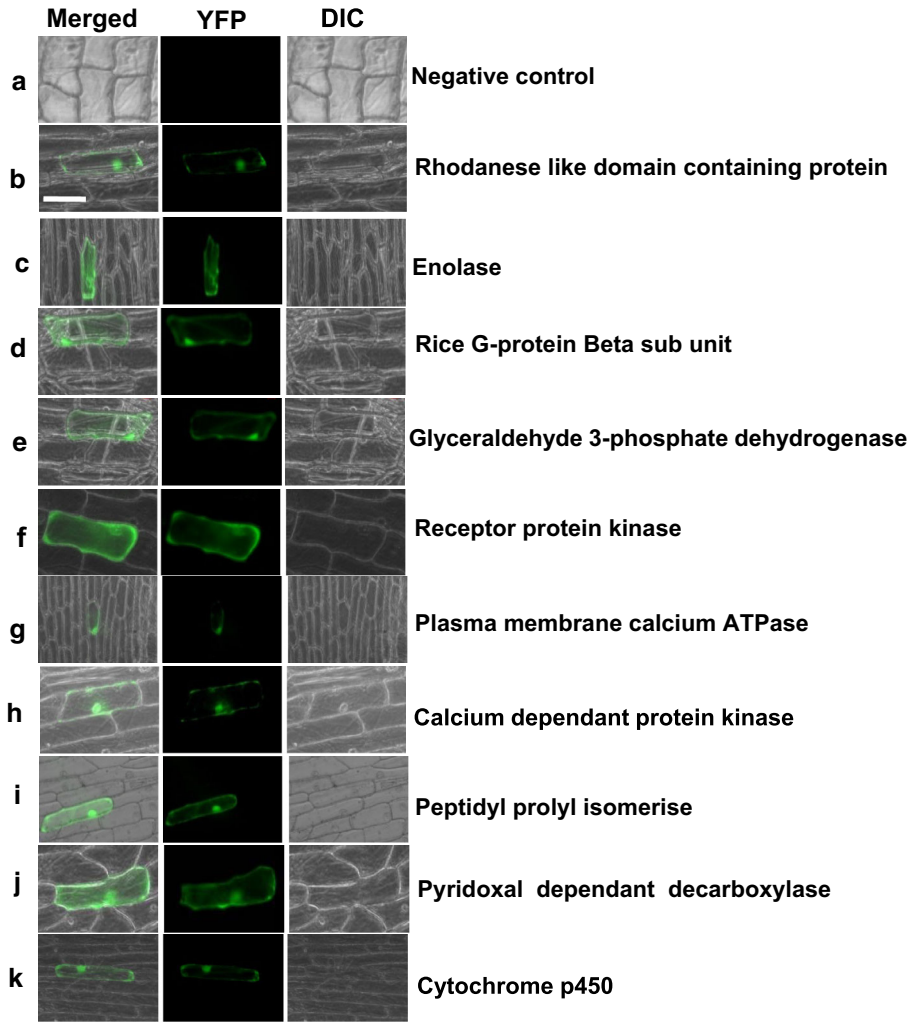

Fig. 7 BiFC-based analysis of the interaction of RGG1 with other interacting partners in transiently transformed onion epidermal cells. After bombardment, onion epidermal pieces were incubated in darkness for 24–30 h followed by a 5-min exposure to white light before observation by confocal-laser microscopy. **a** The construct pairs of pSY728 and pSY738-RGG; pSY738-RGG1 and pSY728; pSY738 and pSY728-RGG1, respectively, were used as negative controls. **b** Rhodanese-like domain containing protein. **c** Enolase. **d** G-Beta. **e** Glyceraldehyde 3 phosphate dehydrogenase (G3PDH). **f** Receptor protein kinase. **g** Plasma membrane Ca-ATPase (PMCA). **h** Calcium-dependant protein kinase (CDPK). **i** Peptidyl prolyl isomerise (PIP). **j** Pyridoxal-dependant decarboxylase. **k** Cytochrome P450 (cyt P450). All images were taken at the same magnification, *scale bars* 17 µm

of saline stress suggesting that the established trait is stable and functional in the transgenic plants. Enhanced control over the photosynthetic machinery under saline stress is perhaps due to the withholding of chlorophyll in these transgenic lines. The H_2O_2 production was significantly less in *RGG1* transgenic lines under salinity stress and these data are in agreement with the previous studies where a decreased level of H_2O_2 production under saline stress has been reported in various overexpressing rice lines such as *OsMYB2, OsPDH45* and *OsSUV3* (Yang et al. 2012; Gill et al. 2013; Tuteja et al. 2013).

For protection from the injurious effects of H_2O_2, plants produce more APX from the ascorbate glutathione (AsA-GSH) cycle, where APX consumes ascorbate as the hydrogen donor and the NADPH-dependent reduction of GSSG (oxidised form) to GSH (reduced form) is catalysed by GR thus maintaining the high ratio of GSH/GSSG (Gill and Tuteja 2010). As compared to VC and WT, the gene expression of antioxidant enzymes, i.e. catalase, APX and GR, in transgenics were up-regulated by salt. It is likely that increase in the expression of antioxidative enzymes is mediated by signalling events induced by G-proteins, i.e. *RGG1*. Consequently, H_2O_2 content of salt-stressed transgenics was lower than in VC resulting in reduced damage of the plasma membrane causing minimal increase in solute leakage. Similarly, reduced membrane lipid peroxidation in transgenics caused lower accumulation of malondialdehyde (MDA). Thus, G-protein-induced signalling events that confer tolerance to salinity stress are mediated by increased gene expression and activity of the enzymes scavenging the ROS.

Sugars like glucose and fructose play a key role in the detoxification of ROS (Bohnert and Jensen 1996; Bentsink et al. 2000; Roy et al. 2005). In this study, the *RGG1* transgenic plants exhibit saline tolerance by the accumulation of sugar alcohols, amino acids and its derivatives (Rathinasabapathi 2000). All of these were found to accumulate in increased quantities in the *RGG1* transgenic rice plants thus helping them to adapt better to the changing environment. The increased glucose content

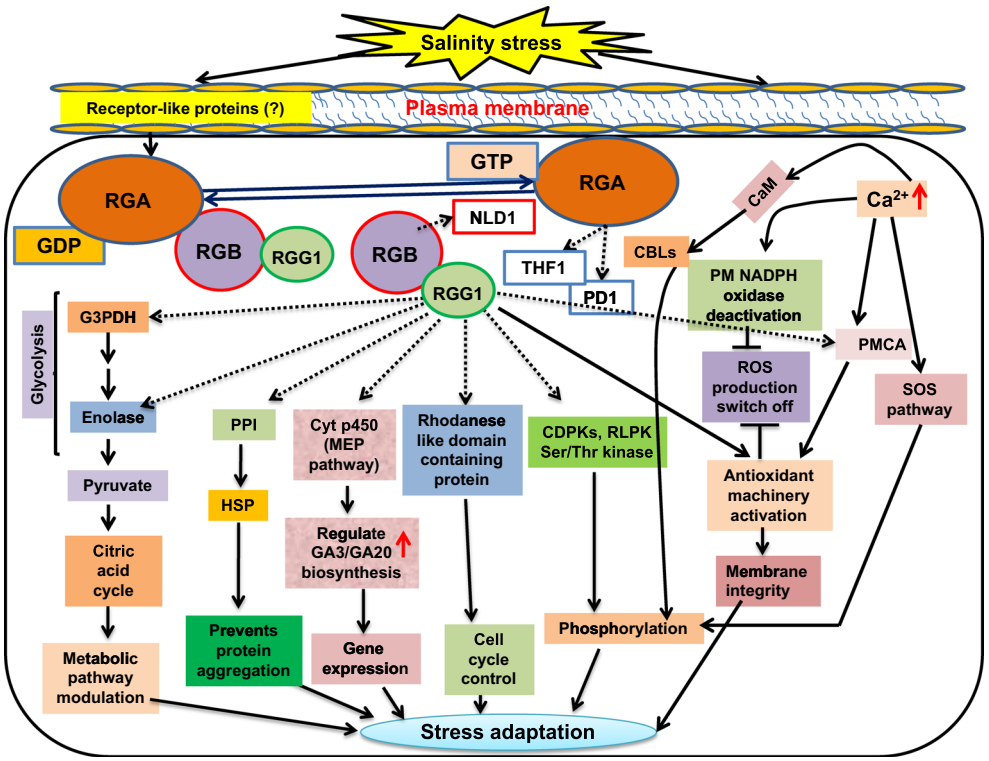

Fig. 8 Hypothetical model for the role of *RGG1* in plant stress adaptation. In response to extracellular signals including stress, the G-protein transduces the signal inside the cell resulting in the dissociation of Gα from Gβγ subunits. These Gα and Gβγ dimers can activate downstream signalling through interaction with their respective effectors. The interacting partners of RGG1 may directly or indirectly help in stress adaptation. *G3PDH* glyceraldehyde 3-phosphate dehydrogenase, *CDPK* calcium-dependant protein kinase, *RLPK* receptor-like protein kinase, *cyt p450* cytochrome p450, *PPI* peptidyl prolyl isomerise, *PMCA* plasma membrane calcium ATPase

plays a role in improving the negative effects of the culprit Na^+ ions (Colaneri et al. 2014). The higher content of sugar in *RGG1* plants supports results reported earlier (Pattanagul and Thitisaksakul 2008; Cha-um et al. 2009; Tuteja et al. 2015), for *Triticum aestivum* (Tammam et al. 2008), *Lactuca sativa* (Hasaneen et al. 2009) and *Solanum lycopersicum* (Mohamed and Ismail 2011).

It could be possible that overexpression of *RGG1* may have pleiotropic effects and high levels of RGG1 protein in the cell may have secondary effects of sequestering all available beta molecules to form a dimer with RGG1 (beta/RGG1). The phenotypic transformations observed in this study could be mainly due to alteration in the level of the RGG1 in stres condition. In *RGG1* transgenic lines in salinity, the transcript levels of *RGG2*, *RGB, RGA, DEP1 and GS3* also increased in addition to *RGG1*.

A novel function of G-proteins in cell wall modification and its regulation has been suggested in the G-protein interactome study in *Arabidopsis* (Klopffleisch et al. 2011). The interacting partners of Gβ subunit of G-proteins in pea have a role in stress signal transduction and developmental pathways (Bhardwaj et al. 2012). The different interacting proteins of *RGG1* such as Gβ, receptor-like protein kinase, plasma membrane Ca-ATPase (PMCA), calcium/calmodulin dependent protein kinases (CDPK), cyt P450, rhodanase-like domain containing protein, peptidyl prolyl isomerase (PPI), enzymes involved in glycolysis like glyceraldehyde 3-phosphate dehydrogenase (G3PDH) and enolase are obtained from the yeast two-hybrid assay (Table 4). Earlier reports suggest that AtRGS1- associated proteins identified by yeast two-hybrid system interact with other proteins. The co-purification of enolase 2 known to interact with AGB1 indicates its multiple functions (Klopffleisch et al. 2011). These observations suggest that RGG1 interacts with various proteins having distinct biochemical properties and possibly modulates various cellular and physiological processes.

A hypothetical model unfolding the role of isolated effectors of RGG1 in stress adaptation has been proposed although there is no direct evidence for these interactions (Fig. 8). The salinity stress tolerance is guarded by intricate mechanisms involving activation of calcium signal decoders such as calmodulin-like proteins (CaM) and calcium/calmodulin-dependent protein kinases (CDPKs). In the present study, we have shown the interaction of RGG1 with CDPKs which are known to regulate the production of ROS during salinity tolerance (Kobayashi et al. 2007), thus confirming the involvement of ROS scavenging machinery, which may finally lead to stress adaptation (Fig. 8). The

increase in GA3 content is positively regulated by IAA (Dolley and Leyton 1968; Nelissen et al. 2012) and therefore it might be possible that the *RGG1* may be indirectly involved in GA3 biosynthesis through cyt P450 which may help to increase the biomass of the transgenic plants leading to stress adaptation (Fig. 8). The CDPKs and cyt P450 are involved in other important biological pathways like transcription and signalling in which RGG1 is implicated. The role of PPI is evident under salinity-induced perturbation as the protein folding process is affected and proteins usually undergo mis-folding or aggregation. Folded proteins are less prone to aggregation and are necessary in a wide array of cellular pathways (Thomas and Sheena 2007). RGG1 interacts with a wide range of enzymes involved in glucose metabolism, e.g. glyceraldehyde 3-phosphate dehydrogenase (G3PDH) and enolase, thus indicating its role in various pathways involved in cellular metabolisms which may ultimately help the transgenic plants in stress adaptation (Fig. 8). The intracellular signal transduction is probably mediated via glycolysis with the involvement of G3PDH and enolase for the compensation of the energy need under salinity stress. Thus, the energy need of the cell for various metabolic processes is met by the de novo synthesis of glucose in response to the salinity stress in the *RGG1* transgenic lines.

The exact mechanism of stress tolerance of *RGG1* is not known yet; it may be attributed to improved maintenance of photosynthesis, antioxidant machinery, increase of proline and cellular sugar levels in transgenic plants under salinity stress possibly by interacting with stress-responsive proteins. Overall, the emerging function of *RGG1* in promoting saline tolerance, without adversely influencing the yield, will help in understanding the G-protein-mediated stress tolerance in plants.

Author contribution statement NT planned the experiment, DMS, RKS and VKS performed the experiments, NT, RT and BCT analyzed the data, NT and RT contributed reagents or other essential materials, and NT and RT wrote the paper.

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