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Isolation and characterization of the promoter of *RGG1* using Agrobacterium-mediated transient assay

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ABSTRACT

The heterotrimeric G protein complex, comprising of G α , G β , and G γ subunits, 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 have isolated the promoter of rice G-protein γ subunit 1(RGG1) from rice genomic DNA. In silico analysis revealed that promoter of RGG1 contained a TATA, a CAAT motif and also harbors some important stress and hormone associated cis regulatory elements, including E-box, GAAAAA, GATA-box, CACATG, CCGTCC and CCACC. Functional analyses were performed by Agrobacterium-mediated transient assay in tobacco leaves. Very high level of GUS activity was observed in agro infiltrated tobacco leaves by the construct carrying the RGG1 promoter::GUS, subjected to abiotic stress and exogenous hormonal treatments. Stress-inducible nature of RGG1 promoter opens possibility for the study of the gene regulation under stress condition. Therefore, the result may be useful in the field of agriculture and biotechnology

Key words: *Cis acting elements, RGG1 promoter, Place database, Agroinfiltration, GUS activity*
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The heterotrimeric G-proteins are composed of Gá (39-52 kDa), Gâ (34-36 kDa) and Gã (7-10 kDa) subunits (Gilman 1987; Tuteja and Sopory 2008). G-proteins transduce signals from the outside environment to inside possibly through regulators (Colaneri *et al.* 2014). The 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). The 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 is found that the G protein alpha null mutation confers prolificacy potential in maize (Urano *et al.* 2015), and the 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). 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 GGC2 have been identified (Trusov *et al.* 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). Previously it has been reported that the expression of two isoforms of Gγ i.e., *RGG1* and *RGG2* is increased 10 to 20 fold by NaCl stress (Yadav *et al.* 2012).

A promoter is a DNA sequences required for appropriate spatial and temporal expression pattern of genes in transgenic plants. CaMV 35S promoter (cauliflower mosaic virus) is most widely used promoter for expression of transgenes, but sometime it may causes some undesirable effect in plants such as gene silencing, delayed growth, dwarfism and low yield (Ow *et al.* 1986; Vaucheret *et al.* 1998; Yowm *et al.* 2008; Achard *et al.* 2008; Kanneganti *et al.* 2008). The dehydration-responsive element (DRE) with the core sequence A/GCCGAC has been identified as a cis-acting promoter element in regulating gene expression in response to drought, high-salt, and cold stresses in *Arabidopsis* (Yamaguchi-Shinozaki and Shinozaki 1994). Recently, it has been reported that transgenic rice plants coexpressing both *OsOXO4* and *OsCHI11* under the control of two distinct green tissue-specific promoters have shown enhanced tolerance to sheath blight disease (Karmakar *et al.* 2016). So, inducible and tissue-specific promoters are required to study the gene regulatory networks in plant (Oettgen 2001, Huda *et al.* 2013). *Cis*-acting regulatory elements present in promoter sequence may function as molecular switch by controlling transcriptional regulation of gene activities. Previously, it was reported that promoter of helicases contained stress responsive *cis*-elements (Tran *et al.* 2010, Dang *et al.* 2011a, 2011b; Tajrishi and Tuteja 2011). So isolation of inducible promoters are essential to study the gene regulatory networks in plants (Oettgen *et al.* 2001).

In the current study, the promoter of rice G-protein α subunit 1(*RGG1*) has been isolated and charecterized in response to abiotic and hormonal treatment by A*grobacterium*-mediated transient assay. *In silico* analysis also identified that the promoter of *RGG1* harbored multiple stress responsive *cis*-acting elements. Transient assay showed that promoter of *RGG1* drives high levels of GUS expression under abiotic stress and hormonal treatment. Therefore, this promoter could be used for the study of the spatio-temporal expression pattern and development of stress tolerant transgenic crops in future.

MATERIALS AND METHODS

Analysis of Promoter Sequences

DNA sequences were analysed by using DNAMAN software, while PLANT CARE (http:// bioinformatics.psb.ugent.be/ webtools/plantcare/html/, 29) and PLACE (http://www.dna. affrc.go.jp/PLACE/ , 30) were used to determine the *cis*-acting regulatory elements and to analyze the *RGG1* promoter sequences (http://rice.plantbiology.msu.edu).

Amplification of *RGG1* and Construction of Chimeric Promoter

Genomic DNA was extracted from leaves of *Oryza sativa* (IR 64) by CTAB method and used as template for PCR amplification of *RGG1* full length (-1003bp) promoter using the forward primer 5' AAAAAAAATCTATACAAAAGTTGATTG 3' and reverse primer 5' CATCTCCCCCTCACCACCACAA 3'. A single deletion (RGG1D) at 500bp was made using the forward primer 5' AAAAAAAATCTATACAAAAGTTGATTG 3' and reverse primer 5' TTCAGAATTTGATCAAGATGTGG 3'.The amplified fragments were then cloned into pGEMTeasy vector (Promega corporation, USA). Full length promoter of RGG1 and the deleted (*RGG1*D) promoter were released by *Hind*III and *Eco*R1 digestion and then cloned in a promoter less vector pCAMBIA-1391Z (Cambia, Australia) in the same restriction site. The colonies were checked by PCR, followed by restriction analysis with *Hind*III and *Eco*RI restriction enzymes. The *RGG1* promoter cloned in pCAMBIA-1391Z vector was transformed in *Agrobacterium tumefaciens* (LBA4404) and confirmed by colony PCR using *RGG1* promoter specific primers. In addition, the CaMV35S promoter was used as positive control and wild-type tobacco as negative control, in order to determine *RGG1* promoter activity.

Agrobacterium-mediated transient assays

Agro infiltration assays were performed by a previously described method (Yang *et al*. 2000). The *RGG1* promoter, transformed in *Agrobacterium tumefaciens* (LBA4404) were grown in LB medium containing 50 ìg/ml rifampicin, 50ìg/ml kanamycin and incubated overnight at 28°C. The cells were harvested by centrifugation at 3,000 g for 15 min and further resuspended in infiltration media (10 mM $MgSO_4$, 200 µM acetosyringone, 20 mM MES pH 5.6). Fully expanded leaves of tobacco (*Nicotiana tobaccum* cv USA) plants grown in greenhouse at 22°C were agro infiltrated by using a 1-ml syringe. After 48h, infiltrated leaf discs were collected.

Stress treatments

For salinity and drought stress, tobacco leaves were agro infiltrated with 200 mM NaCl and 20% PEG solution or water as a control and then collected after 24h. For hormonal stress, tobacco leaves were agro infiltrated with 10 µM naphthalene acetic acid (auxin) and 100 µM ABA respectively or water as a control and then collected after 24h.These treatments were given according to the protocol previously described by (Huda *et al.* 2013).

GUS activity detection

The leaf discs were incubated overnight at 37 °C in GUS assay solution containing 1 mg/ml X-Gluc, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.2% Triton X-100 in 100 mM sodium phosphate buffer (pH 7.4) followed by extraction of chlorophyll using 70% ethanol at room temperature.

GUS activity quantification

β-Glucuronidase activity was quantified by fluorometric GUS assays. Agro infiltrated leaves were homogenized in 1 ml extraction buffer containing 10 mM EDTA, 50 mM NaH_2PO_4 pH 7, 0.1% sodium lauryl sarcosine, 10 mM â-mercaptoethanol and 0.1% Triton X-100. The homogenate was centrifuged at 12,000 rpm for 15 min at 4°C and finally supernatant was collected. The concentration of protein was measured by Bradford method (Bradford 1976) by using bovine serum albumin (BSA) as a standard. GUS activity was performed by earlier described method (Jefferson 1987) and expressed as picomoles of 4-MU (methylumbelliferone) produced per minute per milligram of protein.

RESULTS AND DISCUSSION

Isolation of the *RGG1* promoter from rice and *in silico* analysis

Based on the annotation of rice genome, the 1003 bp fragment of *RGG1* promoter was isolated from *Oryza sativa* genomic DNA using the *RGG1* specific primer sets. The 1003bp PCR product was cloned in pCAMBIA 1391Z vector, which is a promoter less vector, in order to use it for agro infiltration assay. In order to identify the *cis*-acting elements involved in response to various stress condition, we analysed the activity of *cis* element using PLACE and PLANTCARE databases (Figure 1). The 1003 bp promoter region upstream of the *RGG1* start codon contains various putative *cis*-elements and we analyzed only *cis*-elements in boxes known to be related to abiotic stress and hormone signalling (Figure 1). Predicted *cis*-elements present in *RGG1* promoter using database analysis was shown to harbour multiple stress responsive *cis*-acting elements (Table 1). Four homologue sequences of the salt-related *cis*-acting element GT1GMSCAM4 (GAAAAA) and pathogenesis- were evidenced Two homologues of *cis* acting elements PYRIMIDINEBOXOSRAMY1A (TTTTTTCC) and PYRIMIDINEBOXHVEPB1 (TTTTTTCC) which mainly participates in sugar signal transduction (Wang *et al.* 2014) were also found. In addition, six homologues of light-responsive elements such as GT1CONSENSUS (GRWAAW), IBOX (GATAAG), IBOXCORE (GATAA), IBOXCORENT (GATAAGR) and INRNTPSADB (YTCANTYY) were present. Various elements responsible for ABA, drought and dehydration were individuated. The analysis showed that most elements present in *RGG1* promoter were mainly environmental or hormone-responsive motifs. From the *in silico* analysis, it can be predicted that *RGG1* promoter could be an inducible promoter, regulated by multiple abiotic factors and hormones.

Cloning of *RGG1* promoter in binary vector and Agrobacterium transformation

The schematic representation of full length, deleted *RGG1* promoter fragment has been shown in (Figure 2a). The schematic representation of cloning of these two fragments in pCAMBIA-1391Z vector were shown in (Figure 2b). Cloning of the *RGG1* promoter was confirmed by colony PCR and restriction analysis (data

Table 1. Prediction of *cis*-regulatory elements of *RGG1* promoter using PLACE database

Element name	Sequence	Function
ABRECE1HVA22	TGCCACCGG	ABA responsive complex consists of a G-box
ACGTATERD1	ACGT	Required for etiolation-induced expression of erd1 (early responsive to dehydration) in Arabidopsis
ARR1AT	NGATT	ARR1 is a response regulator
BOXIINTPATPB	ATAGAA	Plastid atpB gene promoter, Conserved in several NCII. Important for the activity of this NCII promoter
CAATBOX1	CAAT	CAAT promoter consensus sequence" found in legA gene of pea
CCA1ATLHCB1	AAMAATCT	CCA1 binding site, Related to regulation by phytochrome
DOFCOREZM	AAAG	Dof proteins are DNA binding proteins, with presumably only one zinc finger, and are unique to plants
EBOXBNNAPA	CANNTG	E-box of napA storage-protein gene of Brassica napus
GATABOX	GATA	GATA motif in CaMV 35S promoter; Binding with ASF-2;Three GATA box repeats were found in the promoter of Petunia (P.h.)chlorophyll a/b binding protein, Cab22 gene; Required for high level, light regulated, and tissue specific expression
GT1CONSENSUS	GRWAAW	Consensus GT-1 binding site in many light-regulated genes
GT1GMSCAM4	GAAAAA	GT-1 motif" found in the promoter of soybean (Glycine max) CaM isoform, SCaM-4; Plays a role in pathogen- and salt-induced SCaM-4 gene expression
IBOX	GATAAG	"I box"; "I-box"; Conserved sequence upstream of light-regulated genes; Sequence found in the promoterregion of rbcS of tomato and Arabidopsis
LECPLEACS2	TAAAATAT	Core element in LeCp (tomato Cys protease) binding cis-element (from -715 to -675) in LeAcs2 gene
MYB1AT	WAACCA	MYB recognition site found in the promoters of the dehydration-responsive gene rd22 and many other genes inArabidopsis; W=A/T
MYCATRD22	CACATG	Binding site for MYC (rd22BP1) in Arabidopsis (A.t.)dehydration-resposive gene
PYRIMIDINEBOXOSRAMY1A	TTTTTTCC	Pyrimidine box found in rice (O.s.) alpha amylase (RAmy1A) gene; Gibberellin-respons cis-element of GARE and pyrimidine box arepartially involved in sugar repression; Found in the promoter of barley alpha-amylase (Amy2/32b) gene which is induced in thealeurone layers in response to GA
S1FBOXSORPS1L21	ATGGTA	S1F box" conserved both in spinach (S.o.) RPS1 and RPL21 genes encoding the plastid ribosomal protein S1 and L21, respectively; Negative element; Might play a role in downregulating RPS1 andRPL21 promoter activity
TAAAGSTKST1	TAAAG	TAAAG motif found in promoter of Solanum tuberosum (S.t.) KST1 gene; Target site for trans-acting StDof1 protein controlling guard cell-specific gene expression
WRKY71OS	TGAC	A core of TGAC-containing W-box" of, e.g., Amy32b promoter; Binding site of rice WRKY71, a transcriptional repressor of the gibberellin

not shown). The fusion construct containing *RGG1* promoter-GUS (α -glucuronidase) in pCAMBIA-1391Z was further transformed *Agrobacterium tumefaciens* strain (LBA4404) and verified by colony PCR using promoter specific primers.

Regulation of *RGG1* promoter activities

To compare the regulation of *RGG1* promoter activity, we used transient expression by agro infiltration in the tobacco leaves (Yang *et al.* 2000). This method was selected to avoid long-time regeneration protocol. The

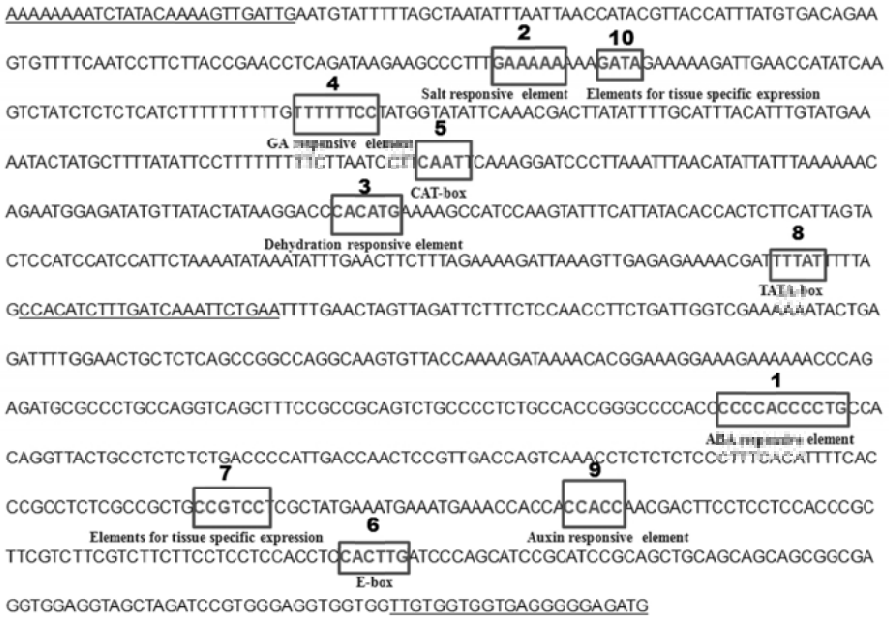

Fig. 1. A schematic representation showing various *cis*-elements present in the upstream region of *RGG1* promoter sequence as determined by PLACE program.

RGG1 promoter both full length (F) and deletion (RGG1D) was fused with GUS reporter gene in pCAMBIA-1391Z vector and infected into the leaves of tobacco by Agrobacterium infiltration. The CaMV35S promoter fused with GUS and WT tobacco plants used was as positive and negative control respectively, in order to determine *RGG1* promoter activity (Figure 2c).

Activities of *RGG1* promoter in tobacco leaves

To check whether the isolated regions of *RGG1* genes have promoter activity, the constructs containing promoter of *RGG1*::GUS and CaMV35S::GUS were agro infiltrated into the leaves tobacco. Both the constructs drove strong levels of GUS expression, but a higher level of GUS gene expression was found to be driven by the CaMV35S promoter than the *RGG1* (Figure 2c). No GUS expression was observed for the negative control. These results indicated that the promoter sequence isolated from the upstream of the *RGG1* gene was functional in tobacco leaves.

Hormone–induced and abiotic stress and activities of *RGG1* full length promoter

In the agro infiltrated leaves of the tobacco, the *RGG1* promoter construct showed GUS positive expression in response to hormonal (Auxin and ABA) treatments (Figure 3 a & b). High GUS expression was observed in response to ABA followed by Auxin treatment (Figure 3 a & b). To quantify the GUS expression, equal amounts of protein was isolated from agro infiltrated leaves and assayed for fluorescence. GUS activity increased by~13 and ~9 folds respectively by application of Auxin and ABA treatments (Figure 5a). The variation in the activity may be due to presence of hormone-induced *cis*-acting elements in different position of *RGG1* promoter. The impact of abiotic stresses (salt and PEG) on the activities of *RGG1* promoter was verified by transient assay in the tobacco leaves (Figure 3c & d). Abiotic stress treatments applied on agro infiltrated leaves increases the expression of GUS activity (Figure 3c & d). The effect of abiotic stress was varied for the *RGG1* promoter. GUS activity was increased ~19 - folds in response to salt and ~15- folds upon PEG treatment respectively (Figure 5a). The CaMV35S promoter also displayed highest GUS activity levels. These results indicated that the promoter of *RGG1* is a stress inducible promoter.

Abiotic stress activities of *RGG1*D promoter

The impact of abiotic stress (salt and PEG) on the activities of RGG1D promoter was verified by transient assay in the tobacco leaves (Figure 4a & b). Abiotic stress treatment applied on agro infiltrated leaves

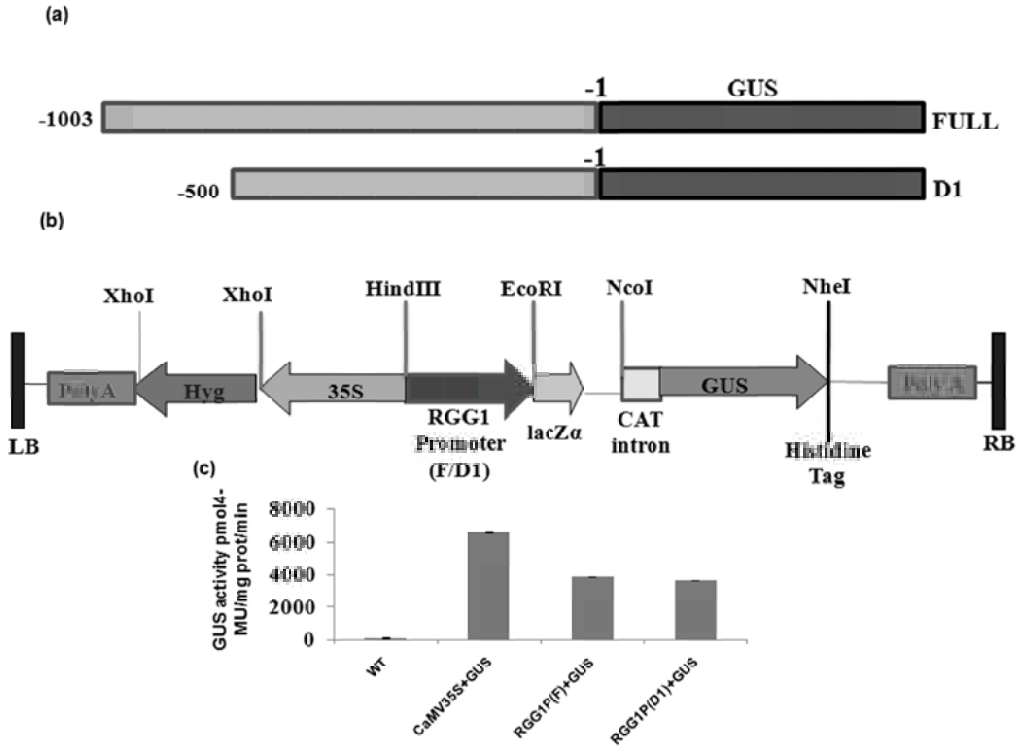

Fig. 2. *RGG1* promoter deletion analysis and schematic representation of different constructs. (a) *RGG1* promoter deletion analysis: F, full-length PM *RGG1*promoter; D1, deletion of 500 bp. (b) Schematic representation *RGG1* promoter in pCAMBIA 1391Z (a promoter less vector). (c) GUS activity was determined 48h after infiltration of tobacco leaves with Agrobacterium (OD 0.7) containing *RGG1* promoter::GUS and CaMV35S::GUS constructs, or WT (negative control).

increases the expression of GUS activity (Figure 4a & b). The effect of abiotic stress was varied for the *RGG1*D promoter; GUS activity increased ~15 - folds in response to salt stress but upon PEG treatment the expression ~12- folds respectively (Figure 5b). The CaMV35S promoter also displayed highest GUS activity levels. These results indicated that *RGG1*D is a stress inducible promoter.

Constitutive promoters such as CaMV35S or inducible-promoters are required for expression of genes in most plant tissues. However, in case of the transgenes driven by constitutive promoters may result in homology-dependent gene silencing, particularly when the promoter is highly active (Vaucheret *et al.* 1998). These promoters are very powerful tool for improving plant resistance to abiotic and biotic stresses. *Cis*- regulatory elements present in the promoters of stress responsive genes controlled many essential biological processes including abiotic stress responses, hormone responses and developmental processes. In plants, a number of *cis*-regulatory elements have shown to be essential for the transcription of stress-responsive genes (Yadav *et al*. 2014). A chromatin immunoprecipitation study identified that ETHYLENE RESPONSE FACTOR1 bind with stress-specific GCC or DRE/CRT elements and upregulates specific suites of genes in response to abiotic stresses (Cheng *et al* 2013). Hence, to analyze the effects of transgene expression and produce transgenic plants with resistance to various abiotic stresses, inducible promoters are preferred as experimental tools.

The current study demonstrated that *RGG1* promoter sequence in rice harbored multiple stress responsive *cis*-acting elements. It has been reported that drought-induced elements usually exist upstream of genes induced by drought stress (Guiltinan *et al.* 1990; Mundy *et al*. 1990). Additionally, several transcription binding factors which take part in abiotic stress response can contain drought responsive elements, *cis*-elements like ABA responsive element

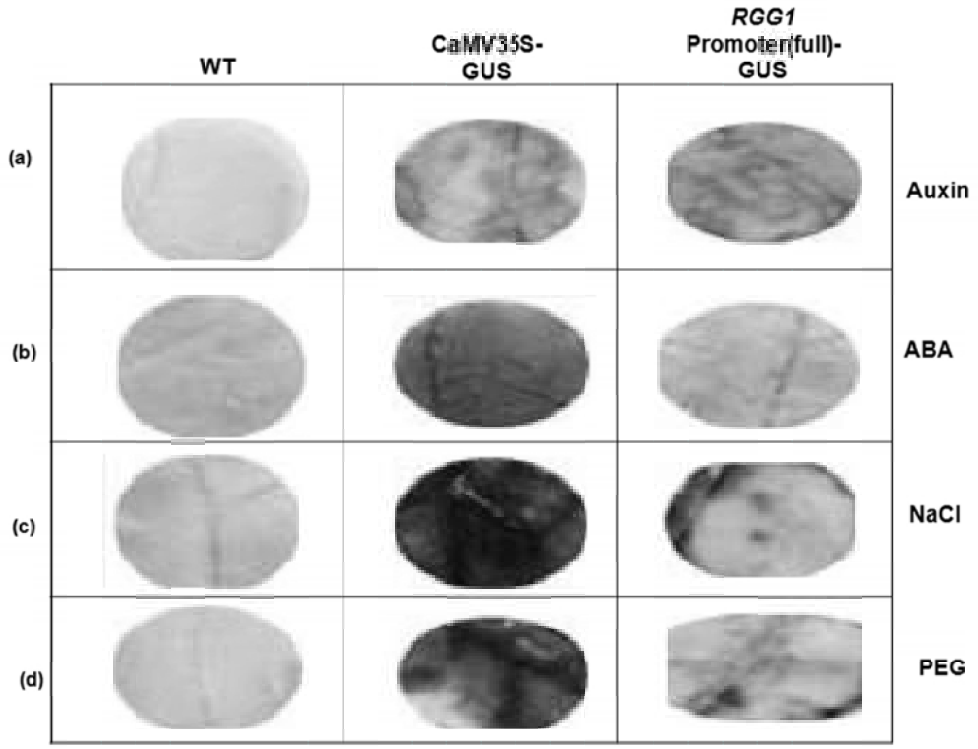

Fig. 3. *RGG1* promoter (F)-GUS analysis in response to abiotic stress and hormonal treatment(a) GUS was detected in X-Gluc solution followed by 10 μM Auxin treatment. (b) GUS was detected in X-Gluc solution followed by 100 μM ABA treatment. (c) GUS was detected in X-Gluc solution followed by 200mM NaCl treatment. (d) GUS was detected in X-Gluc solution followed by 20% PEG treatment. Water treated infiltrated leaves of WT used as negative control. Data represent the mean and SD of 4 independent experiments.

(ABRE), C-repeat elements (CRT), or MYC and MYB recognition sites (Jiang *et al.* 1999; Abe *et al.* 1997). ABRE elements often occur in multiple copies, thereby providing a quantitative effect on stress response. Response to abiotic stress can be regulated by ABA-dependent and ABA independent pathways (Shinozaki *et al.* 2003 Yamaguchi-Shinozaki and Shinozaki 2005) which overlap each other, and play a major role in response to cold, drought and salinity stress. This result implies that *RGG1* promoter could be induced by drought and salt by activating different signal transduction pathways. Several putative motifs within *cis*-acting elements, such as GAAAAA, TTTTTCC, ACGT-box, W-box and I-box, were evidenced by computational analysis in the *RGG1* promoter and they might play important role in gene expression regulation in response to salt stress. The presence of the GT-1 element suggests that it may function in RGG1 promoter activation in response to bacterial infection and salt stress. GA in the GT-1 *cis*-element (59- GAAAAA-39) is required for binding to nuclear factor (s) in response to pathogen or salt-induced stress (Park *et al*. 2004).

Abiotic stress tolerance in rice is a complex trait, and single transgene introduction may not be sufficient to impart stress tolerance under field conditions. Further strategies involving rice genetic transformation with multiple transgenes expressed in an inducible manner will help to improve its stress tolerance. The present study demonstrates that the promoter of *RGG1* contains drought, salt, cold, MV, ABA and MeJA related *cis*-elements, which regulates gene expression. It was also revealed that in the leaves these *cis*-elements might exist outside the region contained in the 2519 bp deletion construct. Overall, the tissue-specificity and inducible nature of *RGG1* promoter could grant wide applicability in plant biotechnology. In consequence, the promoter analyzed

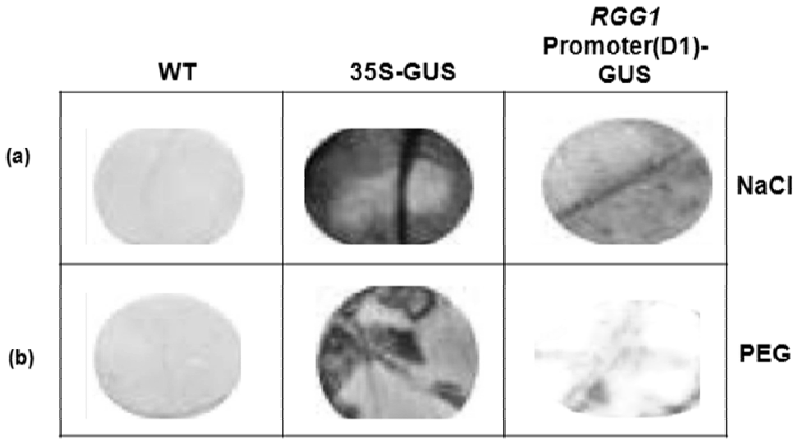

Fig. 4. *RGG1* promoter (D1)-GUS analysis in response to abiotic stress treatment/(a)GUS was detected in X-Gluc solution followed by 200mM NaCl treatment. (b) GUS was detected in X-Gluc solution followed by 20% PEG treatment.

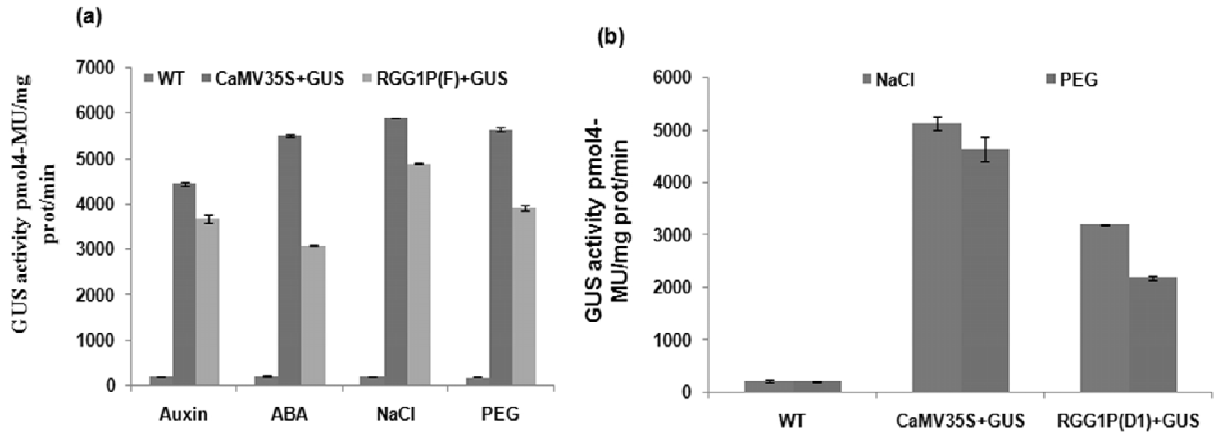

Fig. 5. Transient expression of *RGG1* promoters in agroinfiltrated tobacco leaves/(a) Effect of abiotic stress on the transient expression of *RGG1* promoter (F) in agro infiltrated tobacco leaves. (b) Effect of abiotic stress on the transient expression of *RGG1* promoter (D1) in agro infiltrated tobacco leaves.

in the present study could be of great use to drive transgenes based on expression pattern and extent of required inducibility.

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