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Original Article

Cloning, characterization and analysis of the *Arabidopsis RD29A* promoter for its inducible expression in rice under salinity and drought stress

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ABSTRACT: Traditional breeding as well as genetic engineering have been utilized to improve drought and salinity tolerance of rice with the goal of increasing its productivity. Use of modern molecular biology tools for engineering stress tolerant crops is based on the expression of specific stress-related genes. So, the choice of promoter can play an important role in abiotic stress tolerance as it controls gene expression. Thus inducible expression of gene is crucial for transgenic rice research which is usually implemented by making use of inducible promoters. In this study, therefore, a stress inducible promoter *RD29A* from *Arabidopsis* was characterized in *Binnatoa* rice variety. For this characterization, *Agrobacterium* mediated transformation of *Binnatoa* rice was carried out using the *RD29A-GUS* construct. The transgene was confirmed up to T_2 generation by *RD29A* specific primer. Southern analysis indicated single-copy integration of the promoter in the rice genome. Histochemical assay was performed in rice leaves, roots and seeds to test the inducibility of *RD29A* under both drought and salt stress (100 mM and 200 mM). Salt stress was applied at both seedling and reproductive stages whereas drought stress was applied only at seedling stage. Under drought stress, no expression was visible in root samples but a very prominent expression was found in leaves at day 7, 10, 13 and 16. At 100 mM salt stress, color intensity indicated gradual *GUS* expression increase up to 24h but a decline at 48h in both leaves and roots. At 200 mM salt stress however, expression of *GUS* in leaf sample was found remarkably higher at 5, 10, 24 and 48 hours. In the seedling root however, highest color was found after 5 hours of 200 mM stress. At reproductive stage stress, at both 100 and 200 mM salt exposure 6 days after flowering, *GUS* expression was maximal in leaves and seeds at 24 hours, but no expression was detected in roots. The time required for the toxicity of the salt to take effect, particularly in photosynthesizing leaves is about 24 hours. Therefore, maximal expression of the *GUS* gene at 24 hours indicates the suitability of the *RD29A* promoter for driving transgenes suitable in conferring salt tolerance. This promoter was also found suitable for driving transgenes in leaves under drought stress.

KEYWORDS: *RD29A* (Responsive to Desiccation), *Binnatoa* cultivar, *β-glucuronidase* (*GUS*), *Agrobacterium*-mediated transformation, Histochemical assay.

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Introduction

Salinity, drought and high or low temperatures are the most frequently occurring abiotic stresses which lead to biochemical, morphological and physiological changes in plants, affecting its growth, development and productivity.[1, 2] Thus achieving abiotic stress tolerance in plants has become one of the main focuses of plant science research. Rice (*Oryza sativa*) is one of the most important global crops as it is the staple food source for more than half of the world's population.[3] Till now global rice production has been able to meet population demands but in the immediate future, the main challenges for rice production include increasing demand for rice and declining rice yield growth due to different abiotic and biotic stresses.[4] Improving abiotic stress tolerance in rice will increase agricultural productivity, thus supporting an increasing population.

Plant resistances to abiotic stresses are multigenic, and therefore more difficult to control and engineer.[5] So it has become a challenge to understand and manipulate the molecular basis of abiotic stress tolerance.[6] Of the strategies used for such a purpose, both traditional breeding and genetic engineering of rice have been utilized to improve abiotic stress tolerance or resistance. The conventional breeding programs are slow but in some cases have been successful in developing abiotic stress-tolerant lines;

however in most of these cases the tolerant plant shows an inverse relationship with yield.[7] Here genetic engineering appears to be a more effective way to develop rice cultivars with improved stress tolerance, because transgenes can be introgressed into a high-yielding genetic background.[8-11]

Genetic engineering allows control of the timing, tissue-specificity, and expression level of the introduced foreign genes for their optimal function.[12] An increasing number of stress genes have now been characterized and can be genetically transformed using the innumerous protocols that have been published.[13] For efficient genetic transformation, however, constitutive, tissue-specific as well as stress-inducible promoters are required.[12, 14]

Since promoters affect transcription both quantitatively and qualitatively, the success of gene transfer technologies, in basic research or crop improvement programs depends on their efficacious selection and use.[14] The availability of a broad spectrum of promoters that differ in their ability to regulate the temporal and spatial expression patterns of the transgene can dramatically increase the successful application of transgenic technology.[14]

It has been reported that foreign gene expression under constitutive promoter may have detrimental effect on the host plant, with increased sterility, retarded growth, yield penalty, abnormal morphology or transgene silencing.[15, 16] This problem could be solved by using inducible or tissue specific promoter which will express the gene only in the desired tissue in response to stress or at a particular developmental age.[17, 18] Tissue specific promoter *GluB-1* expresses specifically in the endosperm region of seed rather than leaf or root tissue and has been commonly used for foreign gene expression in transgenic rice seeds.[19, 20]

Specific directions of targeted gene expression make stress inducible promoters potentially useful for the engineering of resistance genes and thus have the potential to protect the whole plant from the applied stress only when needed. Numerous inducible promoters (Hsp1 and 2, ThGLP, PvSR2 etc.) have been isolated from a wide variety of plants so far.[21-23] A strong oxidative stress-inducible peroxidase (POD) promoter from sweet potato (*Ipomoea batatas*) was characterized in transgenic tobacco which showed strong inducibility in response to environmental stresses including hydrogen peroxide, wounding and UV treatment.[24] Another inducible promoter *Alcohol dehydrogenase* (*Adh*) was found to be induced by abiotic stresses such as hypoxia, drought, and cold stresses and expressed mainly in roots.[25, 26]

Among various stress-inducible promoters, *RD29A* has been widely preferred and already used to drive transgene expression in a inducible pattern that also minimized the negative effect on plant growth.[27-30] In transgenic tomato (*Solanum lycopersicum*), *miR399d* gene expression under the control of promoter *RD29A* showed the potential to improve growth when it was exposed to abiotic stresses such as salinity and drought.[31] The transgenic tobacco plants with the *IPT* (isopentyl transferase) gene driven by *RD29A* grew normally with no obvious adverse effect on growth and development in the presence of 150 mmol L−1 NaCl.[32] In potato (*S. tuberosum*), *RD29A* drove the ectopic expression of the *Arabidopsis AtCBF1-3* genes and improved freezing tolerance while minimizing negative effects on tuber yield.[18]
Promoter *RD29*A (Responsive to desiccation) contain two major *cis*-acting elements, the ABA-responsive element (ABRE) and the dehydration-responsive element (DRE)/C repeat (CRT), both are involved in stress-inducible gene expression.[33] In previous studies, inducibility of *RD29A* was tested either under salt or drought conditions in various crops. But in rice, study of *RD29A* promoter under both stresses has not been reported yet. In this study, *RD29A* promoter was cloned from *Arabidopsis* and transferred into Bangladeshi rice variety *Binnatoa* by *Agrobacterium* mediated tissue culture method. Inducibility of this promoter was analyzed by histochemical *GUS* assay under drought stress in seedling stage and salinity (100 mM and 200 mM) stress both in seedling and reproductive stage.

Methods and materials

Construction of pENTR_RD29A vector

DNA was extracted from *Arabidopsis* using the CTAB method[34] and quantified by Nanodrop® spectrophotometer ND-1000 (Thermo Fisher Scientific Inc. Waltham, MA, USA). Later the upstream region (597 bp) of *RD29A* gene (*RD29A* promoter; Accession no: AY973635.1) was amplified by polymerase chain reaction (PCR) with target specific primers (Table 1). To ensure primer compatibility with *pENTR/D- TOPO* vector, CACC overhang was added to the designed forward primer. PCR reaction program was optimized as follows to amplify *RD29A* promoter: Initial denaturation was at 95°C for 5 min and 35 cycles of denaturation at 95°C for 1 min, annealing at 63°C for 1 min, extension at 72°C for 1 min followed by a final extension at 72°C for 7 min. A final concentration of 2.3 mM $MgCl_2$, 0.1 mM dNTPs, 0.3 µM of each primer and 1 unit of recombinant Taq polymerase (Invitrogen, Carlsbad, CA, USA) was used. The desired *RD29A* promoter of 597 bp was extracted from the gel using Qiaquick Gel extraction kit (Qiagen, Hilden, Germany) and then quantified as above.

Following the manufacturer's protocol, the cloning reaction was initiated into *pENTR/D- TOPO* vector (Invitrogen, Carlsbad, CA, USA). Employing standard protocol of heat shock method, *E. coli DH5α* competent cells were transformed by the *pENTR_RD29A* plasmid construct.[35] Successful cloning into *pENTR/D- TOPO* was confirmed by PCR using *RD29A* specific primers and restriction digestion with enzyme *EcoRV* (NEBrinc, Ipswich, MA, USA). Final confirmation of the *pENTR_RD29A* plasmid construct was done with gene specific primers by direct sequencing.

Construction of *pHGWFS7.0_RD29A_GUS* vector and transformed into *Agrobacterium*

The Gateway® LR recombination reaction (Invitrogen, Carlsbad, CA, USA), *pENTR_RD29A* vector was used to recombine *RD29A* into the destination vector (*pHGWFS7.0*) according to manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Vector *pHGWFS7.0* is efficient for analysis of promoter expression.[36] Gateway system contains the site specific recombination properties which allow recombination between *RD29A* promoter from the *pENTR_RD29A* and the target destination vector (*pHGWFS7.0*). Successful recombination would place *RD29A* promoter immediate upstream of the *GUS* gene. Positive colonies were confirmed by PCR with *RD29A* specific primers and restriction digestion with *NdeI* (NEBrinc, Ipswich, MA, USA). Then *Agrobacterium tumefaciens* (*LBA4404*) was electroporated with the cloned destination vector *pHGWFS7.0_RD29A_GUS* using standard protocols.[37] Finally positive colonies which contain desired *pHGWFS7.0_RD29A_GUS* vector were confirmed by PCR reactions with *RD29A* promoter specific primers.

Generation of transgenic rice by tissue culture method

Induction, pre-incubation and co-cultivation of callus:

Binnatoa rice variety was used for transformation with desired *pHGWFS7.0_RD29A_GUS* vector. Dehusked mature seeds were surface sterilized and plated directly on MS callus induction medium. After 21-30 days, calli were pre-incubated for 72 h in semisolid explants pre-incubation medium[38]

(Khanna and Raina 1999). Then the calli were immersed in bacterial suspension containing desired *pHGWFS7.0_RD29A_GUS* vector for 10 min. Excess bacterial suspension was removed by placing them on sterile filter paper before transferring to semi solid co-cultivation medium. The cultures were incubated at 25°C for three days in the dark. After three days the calli were removed and transferred to callus selection media.

Transformed callus regeneration:

After co-cultivation, calli were incubated in the selection media with hygromycin. After 3-4 weeks, proliferating hygromycin- resistant micro calli were subcultured on plant regeneration medium. Regenerants were transferred to rooting medium. After emergence of both shoot and root, seedlings were transferred to a hydroponic system of Yoshida solution[39] for hardening and finally transferred to soil. All the media composition used here were exactly same as followed in Sarker S et al (2015).The transgenic plants were kept at a confined area in a net house. By PCR analysis with *RD29A* specific primers, positively transformed plants were confirmed under conditions as above. After molecular confirmation, T_0 plants were advanced to T_2 generation.

Table 1: Primers used in the study

Primers	Sequence
RD29A_pENTR_F	**5′-** CACCTGAGGAATATTCTCTAGTAAGATCA**-3′**
RD29A_pENTR_R	**5′**-GTAATCAAACCCTTTATTCCTGATGATTG**-3′**
GUS_F	**5′**-GTCACAGCCAAAAGCCAGAC**-3′**
GUS_R	**5′**-GGCACAGCACATCMAGAGA**-3′**

Southern blot hybridization

20 µg of Genomic DNA from the transgenic PCR positive T_2 plants were digested with restriction enzyme *BamH1*. The enzyme was strategically chosen to cut once within the insert so that a band above 3kb would indicate an insertion of the transgene in the putatively transformed plants. The number of bands obtained should depict the number of copies inserted in the genome. As positive control the whole plasmid *pHGWFS7_RD29A* from the first construct was used after a single cut with *BamH1* and the plasmid was diluted to the amount of a single copy before loading into the gel. After electrophoresis, the DNAs were blotted onto a nylon membrane and probed using DIG-labeled PCR amplified product 806 bp using *GUS* gene specific primers (Table 1) following standard protocol (Roche Diagnostics Inc., Mannheim, Germany).

Characterization of *RD29A* under salt and drought stress:

For testing *RD29A* promoter efficacy under salt stress, T_2 *RD29A_GUS* transgenic plants were transferred to two separate sets (one for control and the other for salt) of netted, floating Styrofoam and arranged in a completely randomized way. Each tray contained nine seedlings from each positive transgenic plant. The seedlings were allowed to grow in Yoshida (Yoshida et al. 1976) culture solution until they reached the four-leaf stage (14–18 days after germination). Then, 100 mM and 200 mM NaCl stress was applied. After giving salt stress, rice leaf and root samples were collected from both the stressed conditions at four different time points (5, 10, 24 and 48 hours) for further assay. Similarly *RD29A_GUS* transformed T_2 plants were given 100 mM and 200 mM salinity stress at the reproductive stage. Leaf and root samples were collected after 24, 48, 72 and 96 hours.

Inducibility of promoter under drought stress was also tested in transgenic and wild-type plants at vegetative stage. Transgenic and wild-type seedlings were grown in the same pot until they grew up to four-leaf stage. Then, drought stress was applied by totally withholding water for 16 days. Rice leaf and shoot sample were collected at day 7, 10, 13 and 16 after withdrawal of water.

***GUS* assay in transformed plants:**

Shoot, root and seed samples collected after giving salinity and drought stresses from *RD29A_GUS* transgenic (T_2) plants were assayed for beta-glucuronidase (*GUS*)[40] using the indigogenic substrate X-gluc (5-bromo-4-chloro-3-indolyl b-D-glucuronide). Here wild type *Binnatoa* was used as control. At first tissue segments were fixed at pH 5.6 by fixation solution containing 10mM MES, 0.3% formaldehyde and 0.3 M mannitol, and vacuum infiltrated for 5–6 min. The tissues were then washed several times in 50 mM phosphate buffer (pH 7.0). After washing samples were immersed in ~200 µL X- gluc solution (0.1 M $NaPO_4$ pH 7.0, 0.5 mM $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 10mM EDTA, 5 bromo-4-chloro-3-indolyl-â-glucuronide; X-gluc) for 16–72 h at 37°C for color development. 10 mg X-gluc was dissolved in 1 mL methanol and made up to a final concentration of 1 mg/mL with 50 mM phosphate buffer at pH 7.0. After staining, sections were washed with 70% ethanol and then stored in 70% ethanol.

RESULT

Cloning of *RD29A* promoter into *pHGWFS7.0* vector:

The *RD29A* promoter (597 bp) was amplified using polymerase chain reaction (PCR) with specific primers from *Arabidopsis* genomic DNA. Later it was cloned in the *pENTR/D-TOPO* cloning vector according to the manufacturer's instructions and transformed into *E. coli DH5α* by heat shock method. Out of 21, only three positive colonies were confirmed by restriction digestion with *EcoRV and* polymerase chain reaction with *RD29A* specific primers (Figure 1c).

Later three *pENTR_RD29A* plasmids were further confirmed by sequencing with M13 primers and *RD29A* specific primers sets (Table and blast hit of sequencing results indicated 100% match with the desired *RD29A* promoter sequence).

From the entry clone *pENTR_RD29A* the promoter was cloned into the destination vector *pHGWFS7.0* using Gateway LR recombination reaction according to manufacturer's instructions. Gateway system allows site specific recombination of *RD29A* promoter from the *pENTR* clone to immediate upstream of the *GUS* gene in *pHGWFS7.0*. Two

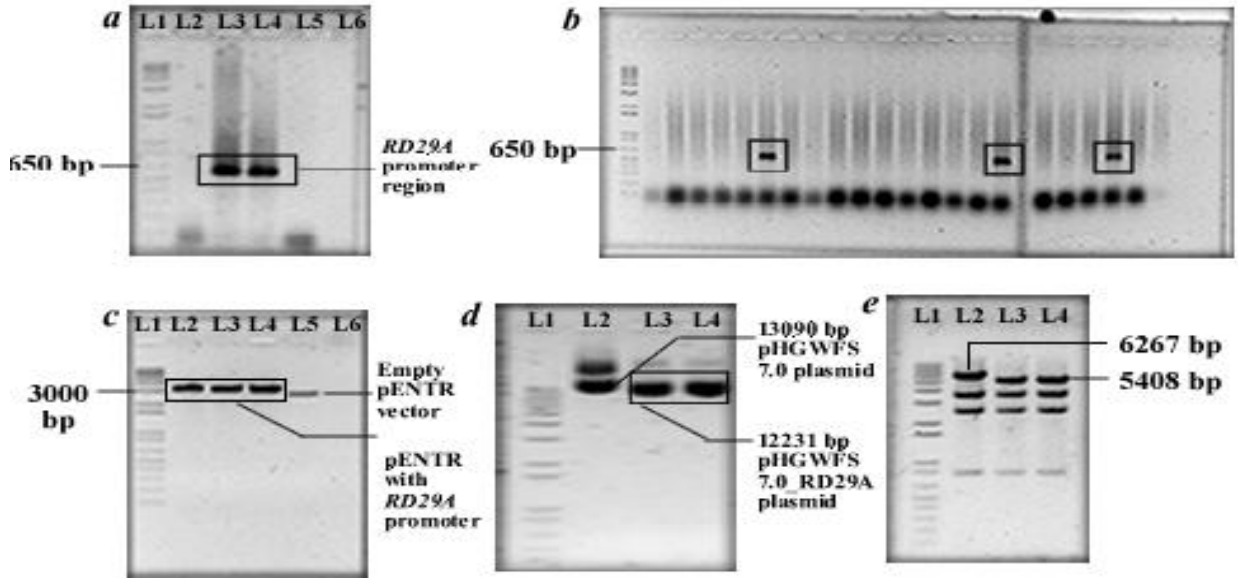

Fig 1: Cloning of *RD29A* promoter into destination vector *pHGWFS7.0* from *Arabidopsis*. **a)** PCR product analysis in 0.8% agarose gel **b)** Lysate PCR of positive colonies. Three positive colonies found (squared 1, 2 and 3) **c)** Restriction Digestion of *pENTR* plasmid with *EcoRV* (0.8% Agarose Gel). **d)** Plasmid isolation of positive colonies. The gel image shows clear indications of size difference due to LR recombination (0.8% Agarose gel). **e)** Confirmation of *pHGWFS7.0_RD29A* by restriction digestion with *NdeI*

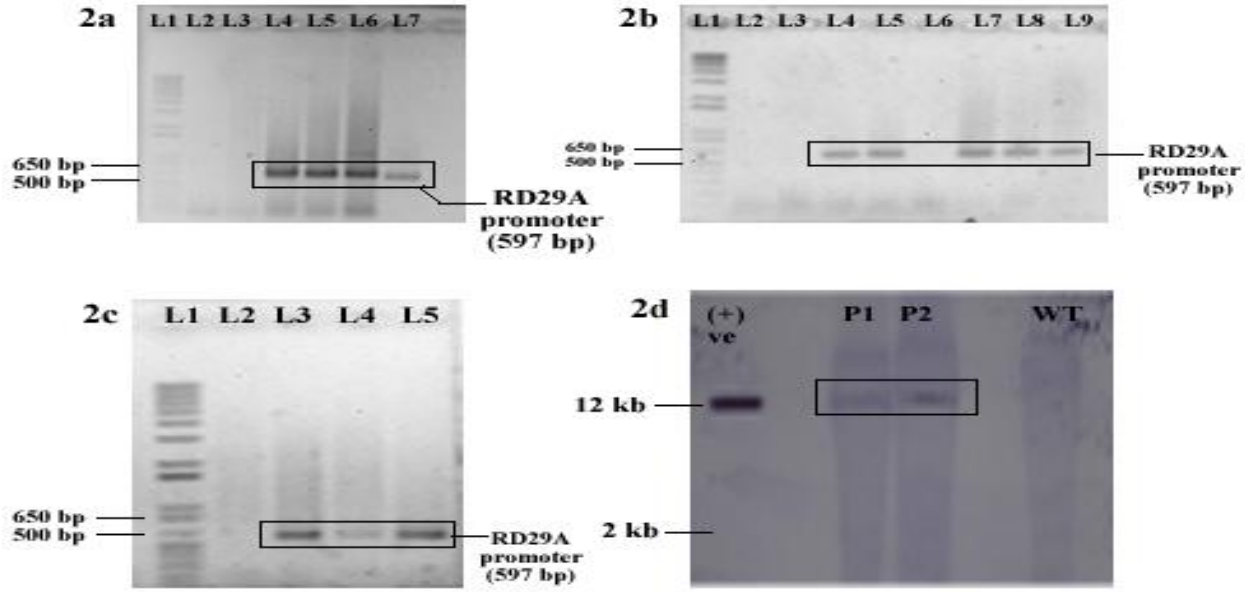

Fig 2: Detection of promoter *RD29A* at T_0 (2a), T_1 (2b) and T_2 (2c) generation. L1 is $1kb^+$ ladder for 2a, 2b, 2c; Positive control: L7 (2a), L9 (2b) and L5 (2c). The transformed samples showed the correct band size (597 bp). (2d) Southern blot hybridization analysis of T_2 generation. Rice genomic DNA and the binary vector *pHGWFS7.0_RD29A* (positive control) were completely digested with *BamH1* and the *GUS* gene was used as probe. Transgenic lines (P-1 and P-2) showed one band. No band was observed in wild type BA plant.

positive colonies were determined by PCR with *RD29A* promoter specific primers and restriction digestions with *NdeI* (NEBr inc., Ipswich, MA, USA) (Fig 1e).

The positive *pHGWFS7.0_ RD29A _GUS* construct was electroporated into *Agrobacterium tumefaciens (LBA4404)* following standard protocols (Sambrook et al. 1989) and used for rice transformation. In six individual experiments, ~1000 calli were infected and after hygromycin (50 mg/L) selection three successfully transformed T_0 plants were obtained via tissue culture method. T_0 generation was advanced up to T_2 and transformants were confirmed by PCR using *RD29A* specific primers and the precise band of 597 kb was observed (Fig 2a, 2b, 2c).

Transgene inheritance:

Successful integration of transgene into whole genome was confirmed by Southern blot hybridization at T_2 generation for both the transgenic lines (Fig 2d). In the T_2 generation, integration of the transgene by Southern hybridization was

confirmed from pooled DNA. Both lines showed single copy of the gene.

Drought stress screening of *BA_RD29A_GUS* transgenic in T_2 generation in seedling stage:
The seedlings of the *RD29A_GUS* transgenic line were tested under drought stress by simply withdrawing water. Samples were collected after 7, 10, 13 and 16 days of stress for histochemical *GUS* assay. After performing the assay, it was found that in compared to normal condition *GUS* gene expression was significantly higher in *RD29A_GUS* transgenic. Highest expression was observed in Day 7 and continues up to day 13 (Fig 3). But expression level decreases at day 16 which may be caused due to damage of plant during drought stress. In root no expression of *GUS* gene was found under drought condition.

Salinity screening in seedling stage at T_2 generation:
T_2 seedlings of *RD29A_GUS* transgenic were kept at 100 and 200 mM salt stress (NaCl) for 5, 10, 24 and 48 hours. After histochemical assay performed on the leaf and root samples, significant color was found in both the stressed conditions compared to the control one.

In leaf samples, at 100 mM salt stress, color intensity that indicates *GUS* expression, increases with time and was found maximum at 48 hours (Fig 4). But at 200 mM salt, *GUS* expression was higher at the early time points (5 and 10 hours) after giving stress and slowly decreased yet remained very strong up to 48 hours. *GUS* expression was also found in root

but not as significant as in leaves. Highest expression was found at three different time points in two stressed conditions (100 mM 24 h, 200 mM 5 h and 10 hours).

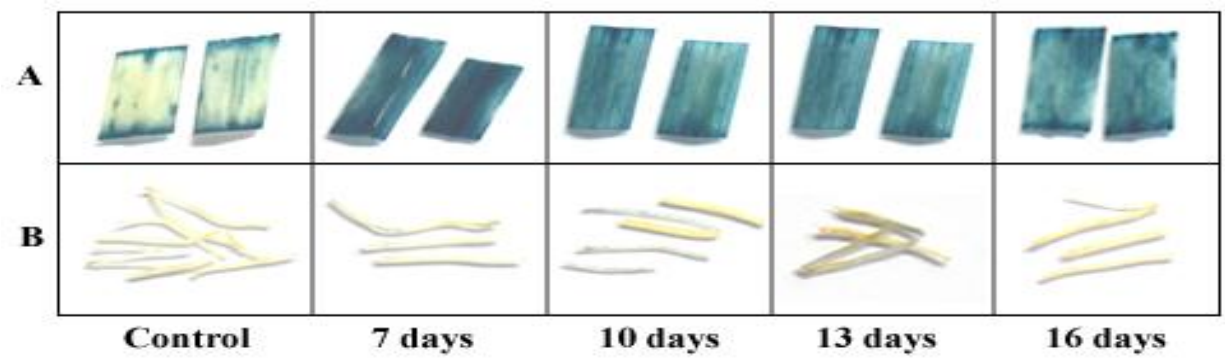

Fig 3: Histochemical assay in seedling stage of *RD29A_GUS* transgenic in T_2 generation under drought stress at different time period (day 7, 10, 13 and 16). It is to be noted that in leaf, maximum expression was found at day 7, day 10 and day 13. No *GUS* gene expression was visible in root.

Salinity screening in reproductive stage at T_2 generation:
RD29A_GUS T_2 plants were given salt stress (100 mM and 200 mM) at reproductive stage. Here no *GUS* expression was found in root. But in leaves, a prominent *GUS* expression was visible and easily distinguishable between control and different stress conditions. In leaves, at 100 mM salt stress, maximum expression was found in 24 hours and 72 hours, whereas expression was a little lower in 48 h and 96 h (Fig 5)

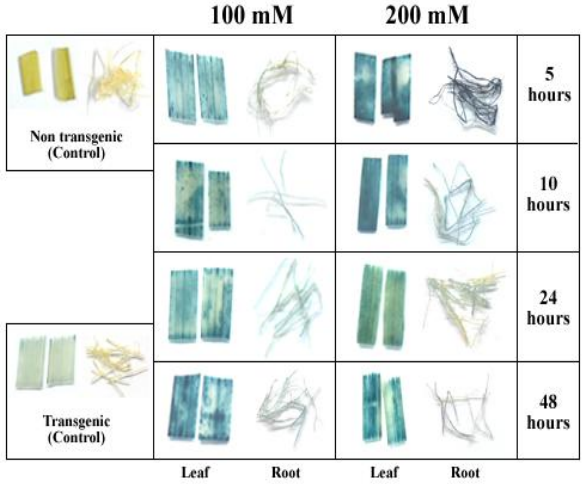

Fig 4: Hitochemical assay in leaf and root tissue of *RD29A_GUS* T_2 seedlings under salt stress (100 mM and 200 mM) for different time period (5, 10, 24 and 48 hours).

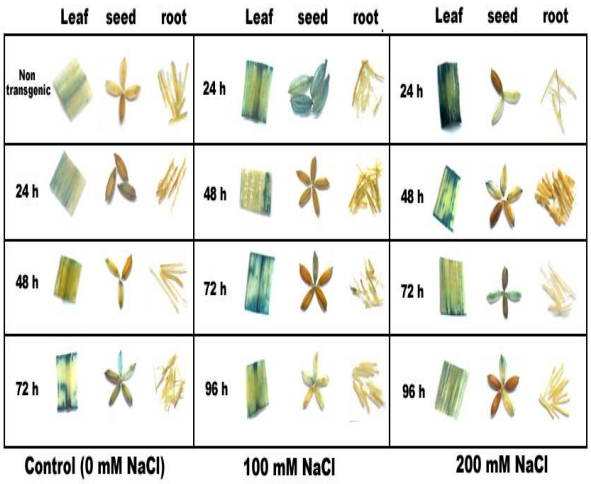

Fig 5: Hitochemical assay in leaf and root tissue of *RD29A_GUS* T_2 plants at reproductive stage under salt stress (100 mM and 200 mM) for different time period (24, 48, 72 and 96 hours).

But under 200 mM salt stress maximum expression was found at 24 and 48 hours. Gradually expression of *GUS* became lower in the following 72 h and 96 h. It may be caused due to leaf damage under such a high saline condition for so long

period. Though *GUS* expression was also seen in seeds but the expression pattern was not specific in relation with stress condition and time period. Maximum expression in seed was found at 100 mM stress at 24 hours. In other conditions, expressions of *GUS* in seeds were not significant at all.

DISCUSSION

In transgenic research, identification and characterization of specialized promoters is regarded as a key tool because it regulates transgene expression both quantitatively and qualitatively. The promoter is the central processor of regulation of a gene, since it contains binding sites for RNA polymerase and general transcription factors responsible for gene expression.[13] So, study of promoters is important as it allows proper genetic manipulation of transgenes of potential interest.[14]

Some transgenic plants over expressing stress-tolerance genes under constitutive promoter showed growth defects with improved tolerance against multiple environmental stresses.[27, 41] Therefore, stress-responsive promoters could help to avoid the adverse effects of such factors in transgenic plants.

RD29A is a stress inducible gene characterized from *Arabidopsis thaliana*. No close homologs are identified in relevant crop species, such as maize, rice, sorghum, and soybean. *RD29A* was reported to be quickly and strongly induced by drought and salt stresses. Analyses of this promoter have shown that a 9-bp conserved sequence, named the *DRE*, is an essential *cis*-element for regulating *RD29A* induction in the ABA-independent response to dehydration and cold.[42] The promoter region of *RD29A* with several *DRE*s (TACCGACAT) and one *ABRE* (ACGTGG/TC) is mainly induced through the ABA-independent pathway.

RD29A promoter was found useful to over-express transgenes for improving drought, salt and freezing stress tolerance in various types of transgenic plants [28, 29] including rice [43, 44].Transgenic over expression of *OsDREB2A* under stress-inducible *RD29A* promoter did not show any phenotypic abnormalities either in stress or non stress growth conditions in the transgenic rice.[45] However, no detailed analysis of the gene expression pattern with respect to time and intensity controlled by *RD29A* has been reported before.

In this study, the *RD29A* promoter region (597 kb) was amplified from *Arabidopsis* and successfully cloned upstream of *GUS* gene into destination vector *pHGWFS7.0* which was efficient for promoter expression analysis.[36]

The aim of the study was to check the inducibility of *RD29A* under drought and salt stresses in two different stages of rice growth. Histochemical *GUS* assay was performed here as this sensitive and simple technique was widely used for promoter expression studies to better understand promoter-specified gene expression patterns.

In both drought and salt stresses *RD29A* was found to be induced mainly in leaves. Though under both stresses significant color was observed in leaf sample but color intensity in drought sample was higher than in salt. In the seedling stage drought sample, *GUS* expression remained higher and continued up to 16 days. In both seedling and reproductive stage stress at 100 mM salt stress, maximal *GUS* expression was observed at 24 hours, whereas under high salt stress condition *GUS* expression was found relatively higher during the early time points, even though it increased with time.

But no expression was found in seedling stage root sample under drought condition. Even under salt stress, expression was completely absent in root at reproductive stage. Expression was found in seedling stage root sample only under high salt stress. High color intensity in leaf samples clearly indicates that under stressed condition *RD29A* mainly express in leaves other than roots and drive gene expression in an inducible pattern. At both seedling and reproductive stages, very good *GUS* expression was also seen in leaves and rice seeds after 24 hours under salt stress. This timing is crucial as it takes about 24 hours for the toxic effects of salt to reach the leaves. Therefore it can be concluded that the *RD29A* promoter is most suitable for driving transgenes for conferring salt tolerance. For drought stress, the maximum expression was observed at 7 days. This timing will also need to be matched with genes whose expression at least 7 days after drought can lead to a measure of tolerance.

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REFERENCES

1. J. P. Moore, N. T. Le, W. F. Brandt, A. Driouich and J. M. Farrant, Trends in plant science **14** (2), 110-117 (2009).
2. M. Reynolds and R. Tuberosa, Curr. Opin. Plant Biol. **11** (2), 171-179 (2008).
3. T. Sasaki and B. Burr, Curr. Opin. Plant Biol. **3** (2), 138-142 (2000).
4. N. Van Nguyen and A. Ferrero, Paddy and Water Environment **4** (1), 1-9 (2006).
5. B. Vinocur and A. Altman, Current opinion in biotechnology **16** (2), 123-132 (2005).
6. P. K. Agarwal, P. Agarwal, M. Reddy and S. K. Sopory, Plant cell reports **25** (12), 1263-1274 (2006).
7. S. Ceccarelli, Euphytica **36** (1), 265-273 (1987).
8. W. Wang, B. Vinocur and A. Altman, Planta **218** (1), 1-14 (2003).
9. J. M. Dunwell, Journal of Experimental Botany **51** (suppl 1), 487-496 (2000).
10. K. Nakashima, Y. Ito and K. Yamaguchi-Shinozaki, Plant Physiol. **149** (1), 88-95 (2009).
11. T. Hadiarto and L.-S. P. Tran, Plant cell reports **30** (3), 297-310 (2011).
12. L. Q. Qu and F. Takaiwa, Plant Biotechnology Journal **2** (2), 113-125 (2004).
13. R. L. Grunennvaldt, J. Degenhardt-Goldbach, I. R. Gerhardt, M. Quoirin and D. P. Vegetal-UFPR, Research Journal of Biological Sciences **10** (1-2), 1-9 (2015).
14. C. Potenza, L. Aleman and C. Sengupta-Gopalan, In Vitro Cellular & Developmental Biology-Plant **40** (1), 1-22 (2004).
15. N. Sinha, R. Williams and S. Hake, Genes Dev. **7** (5), 787-795 (1993).

16. R. Xu, H. Zhao, R. D. Dinkins, X. Cheng, G. Carberry and Q. Q. Li, Plant Mol. Biol. **61** (4-5), 799-815 (2006).
17. S. Karim, H. Aronsson, H. Ericson, M. Pirhonen, B. Leyman, B. Welin, E. Mäntylä, E. T. Palva, P. Van Dijck and K.-O. Holmström, Plant Mol. Biol. **64** (4), 371-386 (2007).
18. M. T. Pino, J. S. Skinner, E. J. Park, Z. Jeknić, P. M. Hayes, M. F. Thomashow and T. H. Chen, Plant Biotechnology Journal **5** (5), 591-604 (2007).
19. L. Q. Qu, Y. P. Xing, W. X. Liu, X. P. Xu and Y. R. Song, Journal of Experimental Botany **59** (9), 2417-2424 (2008).
20. S. Sarker, R. Tammi, S. Biswas, S. Zaman and Z. Seraj, Biores Comm **1** (2), 69-75 (2015).
21. T. Takahashi, S. Naito and Y. Komeda, Plant Physiology **99** (2), 383-390 (1992).
22. H.-y. Li, J. Jiang, S. Wang and F.-f. Liu, Journal of Forestry Research **21** (3), 323-330 (2010).
23. X.-T. Qi, Y.-X. Zhang and T.-Y. Chai, Biochemical and biophysical research communications **356** (1), 273-278 (2007).
24. K.-Y. Kim, S.-Y. Kwon, H.-S. Lee, Y. Hur, J.-W. Bang and S.-S. Kwak, Plant Molecular Biology **51** (6), 831-838 (2003).
25. R. Dolferus, M. Jacobs, W. J. Peacock and E. S. Dennis, Plant Physiology **105** (4), 1075-1087 (1994).
26. G. S. Hossain, R. Waditee, T. Hibino, Y. Tanaka and T. Takabe, Plant Biotechnology **23** (3), 275-281 (2006).
27. M. Kasuga, Q. Liu, S. Miura, K. Yamaguchi-Shinozaki and K. Shinozaki, Nature biotechnology **17** (3), 287-291 (1999).
28. M. Kasuga, S. Miura, K. Shinozaki and K. Yamaguchi-Shinozaki, Plant and Cell Physiology **45** (3), 346-350 (2004).
29. B. Behnam, A. Kikuchi, F. Celebi-Toprak, S. Yamanaka, M. Kasuga, K. Yamaguchi-Shinozaki and K. N. Watanabe, Plant Biotechnology **23** (2), 169-177 (2006).
30. Y. Wu, H. Zhou, Y.-X. Que, R.-K. Chen and M.-Q. Zhang, Sugar Tech **10** (1), 36-41 (2008).
31. N. Gao, X. Qiang, B. Zhai, J. Min and W. Shi, Russian Journal of Plant Physiology **62** (3), 360-366 (2015).
32. W. Qiu, M. Liu, G. Qiao, J. Jiang, L. Xie and R. Zhuo, Plant Mol. Biol. Rep. **30** (3), 519-528 (2012).
33. K. Yamaguchi-Shinozaki and K. Shinozaki, Molecular and General Genetics MGG **236** (2-3), 331-340 (1993).
34. C. N. Stewart Jr and L. E. Via, Biotechniques **14** (5), 748-750 (1993).
35. J. Sambrook, E. F. Fritsch and T. Maniatis, *Molecular cloning*. (Cold spring harbor laboratory press New York, 1989).
36. M. Karimi, D. Inzé and A. Depicker, Trends in plant science **7** (5), 193-195 (2002).
37. J. Sambrook and W. Russell David, *Molecular cloning: a laboratory manual. Vol. 3*. (Cold spring harbor laboratory press, 1989).
38. H. K. Khanna and S. K. Raina, Functional Plant Biology **26** (4), 311-324 (1999).
39. S. Yoshida, D. Forno, J. Cock and K. Gomez, Laboratory manual for physiological studies of rice, 61-66 (1976).
40. R. A. Jefferson, T. A. Kavanagh and M. W. Bevan, The EMBO journal **6** (13), 3901 (1987).
41. K. Nakashima, L. S. P. Tran, D. Van Nguyen, M. Fujita, K. Maruyama, D. Todaka, Y. Ito, N. Hayashi, K. Shinozaki and K. Yamaguchi-Shinozaki, The Plant Journal **51** (4), 617-630 (2007).
42. K. Yamaguchi-Shinozaki and K. Shinozaki, The Plant Cell **6** (2), 251-264 (1994).
43. K. Datta, N. Baisakh, M. Ganguly, S. Krishnan, K. Yamaguchi Shinozaki and S. K. Datta, Plant Biotechnology Journal **10** (5), 579-586 (2012).
44. P. BIHANI, B. CHAR and S. BHARGAVA, The Journal of Agricultural Science **149** (01), 95-101 (2011).
45. G. Mallikarjuna, K. Mallikarjuna, M. K. Reddy and T. Kaul, Biotechnol Lett **33** (8), 1689-1697 (2011).