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ORIGINAL PAPER

Inducibility of three salinity/abscisic acid-regulated promoters in transgenic rice with *gusA* reporter gene

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Abstract The present study evaluates the pattern of stress inducibility of one natural promoter (from rice *Rab16A*) and two synthetically designed promoters, viz., 4X ABRE (abscisic acid-responsive element, having four tandem repeats of ABRE) and 2X ABRC (abscisic acid-responsive complex, having two tandem repeats of ABRE and two copies of coupling elements), in response to varying concentrations of NaCl and abscisic acid (ABA). Each promoter, independently linked to *gusA* (that encodes β glucuronidase, GUS), was introduced into rice (cv. Khitish) through particle bombardment. The T_2 progenies showed integration of *gusA* in their genome. The accumulation of *gusA* transcript, driven by each promoter in T_2 transgenics, increased with increasing salt/ABA concentration, with ABA being the better activator of each promoter. Induction in GUS expression, driven by different promoters, was noted on exogenous salt/ABA treatments in a concentration-dependent manner. The maximum induction was observed with 2X ABRC promoter. All the three promoters could drive stress-inducible GUS expression in both vegetative and floral organs. However, prominent GUS expression was noted in the whole seed (both embryo and aleurone layer of endosperm) only by 2X ABRC, whereas it was localized only in the embryo for the other two promoters. Thus, our observation characterizes three efficient salinity/ABA-inducible promoters that have the potentiality in crop biotechnology to drive transgene expression for stress tolerance, whenever abiotic stress is encountered.

Keywords Abiotic stress · Abscisic acid · β-Glucuronidase · Salt stress · Stress-inducible promoters · Transgenic rice

Abbreviations

ABA	Abscisic acid
ABRC	Abscisic acid-responsive complex
ABRE	Abscisic acid-responsive element
CE	Coupling element
GUS	β Glucuronidase
MS	Murashige and Skoog medium
4MU	4, Methyl umbelliferone
Rab p	Rab promoter
WT	Wild type

Introduction

High soil salinity constitutes one of the foremost abiotic stresses that imposes a serious threat to the productivity

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and sustainability of rice worldwide. Salinity stress triggers the production of the phytohormone abscisic acid (ABA), which activates different pathways in plants for stress tolerance. ABA also plays a key role in the accumulation of storage proteins for plant survival. It arrests embryonic development by enforcing a phase of seed dormancy and also functions during seed maturation (Bartels and Sunkar 2005; Seki et al. 2007). Cloning and characterization of several ABA-inducible genes, such as *Em* from wheat (Marcotte et al. 1989), *Osem*, *Rab16A-D* and *SalT* from rice (Mundy and Chua 1988; Claes et al. 1990; Hattori et al. 1995), *Rab17* from maize (Villardell et al. 1990), and *HVA1* and *HVA22* from barley (Shen and Ho 1995; Shen et al. 1996) have been demonstrated earlier. These genes are induced during salinity or drought stress when the endogenous ABA level becomes high or during exogenous application of ABA. Most of these ABA-inducible genes contain a conserved cis-acting element called abscisic acid-responsive element (ABRE, C/GACGTGGC) in their promoter regions that interacts with basic leucine zipper (bZIP) group of trans-acting factors, such as wheat EmBP-1; rice RITA-1, OSBZ8, osZIP-1a, and TRAB-1; tobacco TAF-1, etc., and hence upregulate the concerned gene expression. The wheat *Em* promoter is represented by box Em 1 (ABRE), which is repeated as Em1a and Em1b and box Em2 (non-ACGT sequence), both conferring ABA inducibility. The promoter of *Rab16A* contains a conserved, ACGT-containing ABRE called motif I, along with certain non-ACGT, GC-rich sequences called motif II, which are duplicated (motif IIa and motif IIb) (Yamaguchi-Shinozaki et al. 1990). Roychoudhury et al. (2008) proposed that motif IIa acts as a coupling element (CE) for *Rab16A*, which also binds to the bZIP factor, OSBZ8, similar to motif I. An ABRE, thus, often functions with a GC-rich second sequence called CE such as the CE1 in barley *HVA22*, CE3 in barley *HVA1* and rice *Osem* and motif III in *Rab16B*. The two elements ABRE and CE together constitute an ABA-responsive complex (ABRC), which in conjunction can activate gene expression in response to osmotic stress and/or ABA.

Multiple copies of ABREs can confer ABA response to a stress-inducible promoter (Ishige et al. 1999). A hexamer of motif I, when fused with 90 bp of CaMV35S promoter, conferred ABA-inducible gene expression (Skriver et al. 1991). Similarly, wheat *Em* promoter could drive reporter gene expression in embryo and aleurone of transgenic barley and rice (Furtado and Henry 2005). The promoter analysis of rice *Rab16A*, fused to *gusA* reporter, was earlier performed in tobacco (Yamaguchi-Shinozaki et al. 1990) and rice (Ono et al. 1996). Su et al. (1998) used separately one and four copies of a 49-bp ABRC1 from *HVA22* promoter, fused with rice *Act1* (actin) promoter, and reported three- to eightfold induction during stress; the four ABRC1 repeats showed higher induction. The promoters from *Arabidopsis rd29A* (Kasuga et al. 1999) and *Rab18* (Karim et al. 2007) have also been used in rice to induce the expression of transgenes under abiotic stress conditions. The promoter from *HVA1*-like gene, namely, *OsLEA3* from rice, rendered tenfold increase in GUS expression (Xiao et al. 2007) in transgenic rice.

To improve stress tolerance of crop plants, several genes including transcription factors can be overexpressed in transgenic plants, using either constitutive or stress-inducible promoters. However, strong constitutive overexpression of transgene hampers the growth of plants, which leads to reduced productivity and altered phenotypes like low reproductive yield, varying degree of growth retardation, abnormal development and reduced seed production (Romero et al. 1997; Capell et al. 1998; Kasuga et al. 1999; Hsieh et al. 2002; Karim et al. 2007; Nakashima et al. 2007). The use of a synthetically designed promoter, with inducible regulatory sequences, proves invaluable in genetic engineering programs (Venter 2007). The multimerization of conserved sequences or combinations of different conserved motifs found to function synergistically and producing enhancement in reporter activity were used to create synthetic promoters (Cazzonelli and Velten 2008). The homology-dependent gene silencing often occurs in transgenic plants with multiple copies of the same promoter (Matzke et al. 1994; Bhullar et al. 2003). Hence, to avoid such silencing phenomena, stacking of several transgenes for abiotic stress tolerance within a single construct requires a cluster or a battery of several functional stress-inducible promoters. Many such stress-inducible promoters have been characterized including *OsABA2* (Rai et al. 2009) and *Arabidopsis rd29A* (Yamaguchi-Shinozaki et al. 1990). Despite considerable efforts to functionally characterize stress-inducible promoters, limited studies have focused on their responses to stresses to varying concentrations of different elicitors such as NaCl or ABA. Thus, in our current study, we selected the three stress-inducible promoters, namely 4X ABRE (containing four tandem repeats of ABRE), 2X ABRC (containing two tandem repeats of ABRE with two copies of CE) and rice *Rab16A* promoter (containing motif I as ABRE and motif IIa and IIb as CE-like sequences). The pattern of inducibility or induction level driven by these promoters was characterized in transgenic rice plants by monitoring the expression of *gusA* reporter gene, upon exposure of these plants to varying concentrations of NaCl and ABA. In addition, the promoter activity was also assessed through fluorometric estimation of GUS expression in transgenic plants.

Materials and methods

Preparation of promoter constructs

The two synthetic promoters, containing either four tandem copies of ABRE (CGAAAGCTTGCCACGTGGCGCCACGTGGCGCCACGTGGCGCCACGTGGCACCCTTCCTT) of Em1a sequence of wheat *Em* (considered as the strongest G-box element) or two copies of ABRC (GTCAGAAAGCTTGCCACGTGGCTGCAGTGCCATTGCCACCGGATCAGCCACGTGGCTCCAGTGCCATTGCCACCGGATGACGCACAATCCCAC), having two tandem repeats of *Em* ABRE, with two copies of CE, namely, CE1 of barley *HVA22,* each fused with common 3′ ends, providing the basal promoter up to −70 from CaMV35S promoter and supplying CAAT and TATA boxes, were designed. Since the basal transcription factors are required to assist RNA polymerase II to initiate transcription from the start site, CAAT and TATA boxes were included in the downstream of these two synthetic promoters. The amplification of 4X ABRE and 2X ABRC promoters and their subcloning separately in the binary vector pBI121 at *Hin*dIII and *Bam*HI sites were performed as described earlier (Roychoudhury and Sengupta 2009; Sambrook and Russell 2001). The 300-bp promoter of *Rab16A* (Rab p) was amplified from the genomic DNA of the salt-tolerant rice variety, Nonabokra, using Rab21-5 (5′-CGACAAGCTTCACGCGCACAGTACACACACAT-3′) and Rab21-3A (5′-TAGAGCTCGGATCCTCTAGACTGCAGTCATGCTGTGGTGTGCAAGCA GG-3′) primer pairs and subcloned in pBI121 at *Hin*dIII and *Bam*HI sites, as described earlier (Roychoudhury et al. 2007).

Plant transformation

Biolistic transformation was carried out following the protocol described by Datta et al. (1998) with some modifications. The immature embryos of an elite indica rice variety Khitish were procured from Chinsurah Rice Research Station, Hooghly, West Bengal. They were separately bombarded with the following three constructs: pBI: 4X ABRE: *gusA*, pBI: 2X ABRC: *gusA* and pBI: Rab p: *gusA* using Biolistic PDS-1000/He (Biorad). Six bombardments into immature embryo were made for each construct. The immature embryos were co-transformed with pGL2 vector, containing the selectable marker hygromycin phosphotransferase (*hpt*). Following bombardment, the immature embryos were transferred to the callus induction medium (MS with 30 g l^{-1} sucrose, 2 mg l^{-1} 2, 4-D and 8 g l^{-1} agar), supplemented with 50 mg l^{-1} of hygromycin B and maintained in the dark for 45 days. It was passed through three successive selection cycles of 2 weeks each. The calli were transferred to the regeneration medium (MS with 30 g l^{-1} sucrose, 2 mg l^{-1} kinetin, 0.5 mg l^{-1} NAA and 8 g l^{-1} agar) and maintained in light for 20 days. The regenerated plants were then transferred to the rooting medium (MS without hormone) for 15 days and finally grown to maturity. All the plants were fertile with normal phenotype. The T_0 transgenic rice plants grown in the greenhouse were confirmed for the presence of *gusA* (data not shown). Subsequently, T_1 and T_2 generation of transgenic plants were raised. About 34 T_0 transgenic plants for each of the promoter constructs were derived and grown up to T_2 generation. Four T_2 transgenic lines for each promoter were considered for further molecular analysis for promoter characterization.

Polymerase chain reaction (PCR)

The T_2 transgenic plants, for each of the promoters, were verified through PCR, using genomic DNA extracted from young leaves (0.5 g) of healthy plants (Dellaporta et al. 1983). The PCR amplification of the integrated *gusA* gene, in each of the transgenics, was performed with 150–200 ng of genomic DNA, using GUS5A (5′ATGTTACGTCCTGTGGAAACC3′) and GUS3 (5′TTCATTGTTTGCCTCCCTGCT3′) primer pairs. The PCR cycle was as follows: 94°C for 60 s, 54°C for 60 s and 72°C for 150 s. The amplification was done for 35 cycles.

ABA and NaCl treatment of transgenic rice

The 21-day-old T_2 transgenic plants, for each of the promoters, were used for promoter characterization. The seedlings were grown on modified Yoshida solution supplemented with different concentrations of NaCl (0 mM, 100 mM, 150 mM) or ABA (0 μM, 50 μM, 100 μM) for 16 h at 32°C. The plants maintained in 0 mM NaCl or 0 μM ABA served as experimental control.

Reverse transcriptase (RT)-PCR

Total RNA was extracted from the leaves of untreated and stressed T_2 transgenic plants, using Trizol reagent (Invitrogen, USA) following the manufacturer's protocol, followed by DNase I (Roche, Switzerland) digestion. About 5 μg of total RNA was used for first-strand cDNA synthesis using Invitrogen two-step RT-PCR kit. PCR was carried out with GUS5A and GUS3 primer pairs as described above.

Histochemical GUS assay

Histochemical GUS staining was performed as described by Jefferson et al. (1987). The GUS expression in transgenic plants was visualized in leaves, spikelets and seeds,

under non-treated and stressed conditions. The GUS reaction mixture consisted of the solution containing sodium phosphate (pH 7.0), chloramphenicol, Triton-X-100, methanol and 5-bromo-4-chloro-3-indolyl- β-D-glucuronide (X-gluc). The samples were incubated in the solution at 37°C for 24 h. The reaction was stopped by adding 70% (v/v) ethanol and the pigments and chlorophylls were removed by repeated ethanol treatment.

Quantitative RT-PCR (qRT-PCR) analysis

Preparation of total RNA from the leaves and cDNA synthesis was performed as described above (in RT-PCR). The qRT-PCR was performed with *gusA* specific primers and the cycle was as follows: 95°C for 30 s, 56°C for 30 s, 72°C for 30 s. The procedure was according to the manufacturer's instructions (CFX 96 Real time system, Biorad). The quantitative variation between different samples was evaluated by the ΔΔCt method, and the amplification of *tubulin* gene, using TF (5′ATGCGTGAGATTCTTC ACATCC3′) and TR (3′TGGGTACTCTTCACGGACTT TAG 5′) primer pairs, was performed to serve as internal control to normalize all data. To validate the qRT-PCR results, the experiments were repeated three times. The mean values for the expression levels of the examined genes were calculated from three independent experiments. The relative expression level was calculated as $2^{-\Delta\Delta CT}$. The $2^{-\Delta\Delta CT}$ value for the untreated transgenics was normalized to 1.

Quantitative GUS assay

Total protein was extracted from 21-day-old leaves (1 g) of T_2 transgenic rice plants. Fluorometric GUS assay, using 4-methyl umbelliferyl-β-D-glucuronide (MUG) substrate, was performed according to Jefferson et al. (1987). The samples were placed in the reaction mixture containing 50 mM phosphate buffer (pH 7.0), 10 mM EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton-X-100 and 10 mM β mercaptoethanol. It was incubated with MUG for 1 h. The reaction was stopped with stop solution containing sodium carbonate (0.2 M Na_2CO_3). The reaction product 4-methyl umbelliferone (4-MU) was measured with a fluorometer (Jasco, Japan) with an emission at 455 nm and excitation at 365 nm. The GUS enzyme activity was expressed as picomoles of 4 MU produced per hour per microgram of total protein against the standard curve made with 4 MU.

Statistical analyses

The experimental data values were mean value from three independent series, each done with three replicates, and the results presented as means ± standard error (SE), based on three replications. The statistical significance at $p \leq 0.05$ was calculated.

Results

Confirmation of the T_2 transgenics through PCR

The three promoter constructs, pBI: Rab p: *gusA* (Fig. 1a), pBI: 4X ABRE: *gusA* (Fig. 1b) and pBI: 2X ABRC: *gusA* (Fig. 1c) are shown schematically. PCR confirmation (Fig. 2) of the T_2 transgenics (containing each promoter construct) showed the integration of 1.8-Kbp band of *gusA* in each case (lanes 2–13). The band was absent in the WT plant (lane 14).

RT-PCR analyses

The *gusA* transcript accumulation, driven by individual promoter, i.e., Rab p (Fig. 3a), 4X ABRE (Fig. 3b) and 2X ABRC (Fig. 3c), under varying concentrations of NaCl and

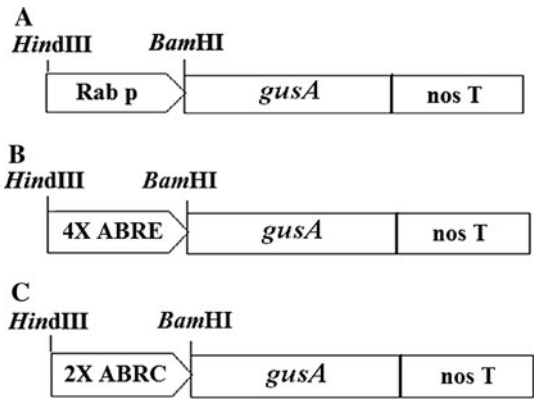

Fig. 1 Schematic representation of the constructs, pBI: Rab p: *gusA* (**a**), pBI: 4X ABRE: *gusA* (**b**) and pBI: 2X ABRC: *gusA* (**c**)

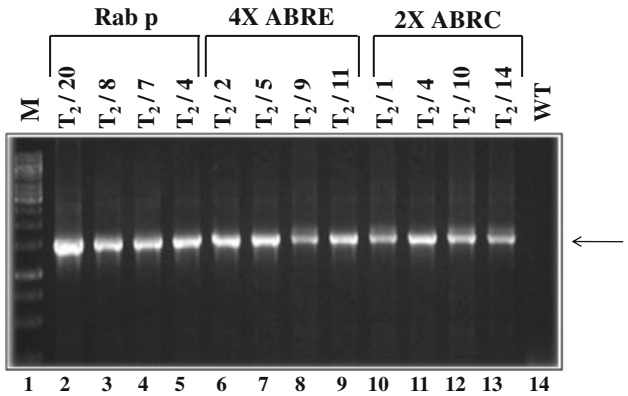

Fig. 2 PCR confirmation showing the integration of 1.8 Kbp *gusA*, *M* represents DNA molecular weight marker

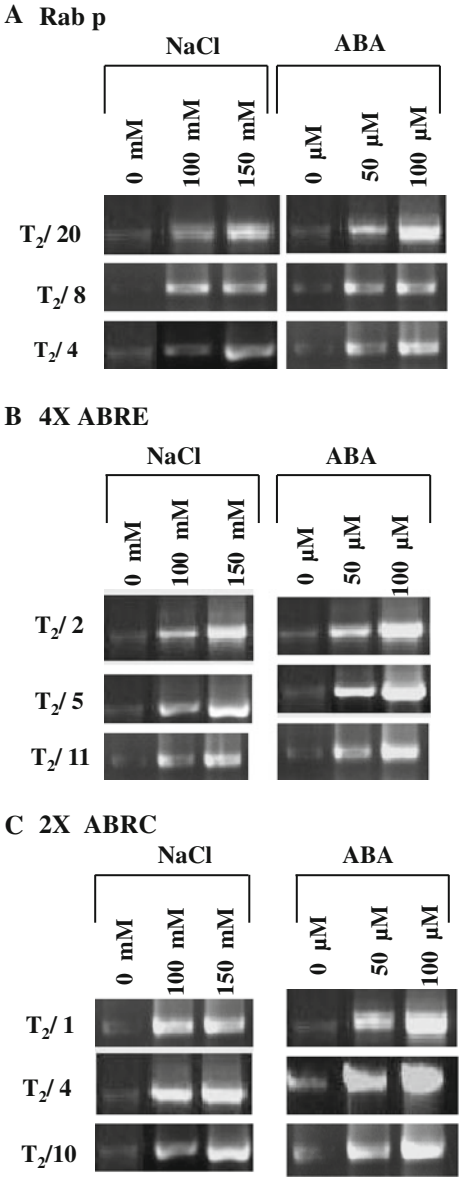

Fig. 3 RT-PCR showing *gusA* expression driven by stress-inducible promoters Rab p (**a**), 4X ABRE (**b**) and 2X ABRC (**c**) in T_2 transgenics, induced with different concentrations of NaCl and ABA

ABA, showed that for each promoter set, the transcript level increased with the increase in NaCl or ABA concentration and peaked at 150 mM NaCl or 100 µM ABA. The WT plants showed no *gusA* expression, even with exogenous stress treatment, proving that there is no endogenous *gusA* expression (data not shown). It is noteworthy that the inducibility of promoters for *gusA* expression was more pronounced with ABA, compared to NaCl, for all the promoters, particularly 4X ABRE and 2X ABRC. Though the *gusA* transcript was detectable at the background level, i.e., untreated conditions (0 mM NaCl or 0 µM ABA) of the transgenics, its level was much lower as compared to the induced conditions.

Histochemical localization of GUS activity

The localization and induction of GUS expression was prominently visualized in all the tissues, viz., leaves (Fig. 4a) and floral organs, i.e., spikelets (Fig. 4b) or seeds (Fig. 4c), in response to 150 mM NaCl and 100 µM ABA treatments for 16 h. Unlike 2X ABRC promoter, which induced GUS expression both in the embryo and endosperm of seeds, the 4X ABRE promoter and Rab p could drive the expression only in embryo but not in the endosperm.

qRT-PCR analyses

The qRT-PCR analyses, performed with three T_2 transgenic plants from each promoter set (those tested in RT-PCR analysis), showed that the *gusA* mRNA levels were progressively increased with increase in NaCl and ABA concentration. The fold-induction levels of *gusA* mRNA in stressed leaves over untreated conditions varied widely depending on the promoter used. The maximum activation of Rab p was noted in the transgenic line T_2/20, which showed 6.5- and 8.2-fold induction over control upon 100 and 150 mM NaCl treatments, respectively (Fig. 5a), and 7.1- and 10-fold induction at 50 and 100 µM ABA treatments, respectively (Fig. 5b). Likewise, in the line T_2/2 for 4X ABRE promoter, the maximum induction was noted, which was 13.8- and 16-fold upon 100 and 150 mM NaCl treatments (Fig. 5c), and 15- and 20.8-fold in case of 50 and 100 µM ABA treatments, respectively (Fig. 5d). Similarly, the line T_2/4 for 2X ABRC promoter showed maximum promoter induction, i.e., 20- and 22.6-fold induction over control upon 100 and 150 mM NaCl treatments (Fig. 5e), and 22.8- and 31.2-fold induction upon treatment with 50 and 100 µM of ABA, respectively (Fig. 5f).

Quantitative expression of GUS

Quantification of GUS activity using MUG assay, in 21-day-old T_2 transgenics, treated with different concentrations of NaCl and ABA for 16 h, revealed that the expression was much higher on application of 150 mM NaCl and 100 µM ABA in case of all the promoters. The maximum GUS expression with Rab p was noted in the transgenic line T_2/20, which was 46 and 92 pmol h^{-1} μg^{-1} total protein upon 100 mM and 150 mM NaCl treatments, respectively (Fig. 6a), and 54 and 100 pmol h^{-1} μg^{-1} upon 50 µM and 100 µM ABA treatments, respectively (Fig. 6b). Likewise, in the line T_2/2 for 4X ABRE promoter, the GUS activity was found to be maximum, which was 64 and 116 pmol h^{-1} μg^{-1} total protein on treatment with 100 mM and 150 mM NaCl (Fig. 6c), and 85 and

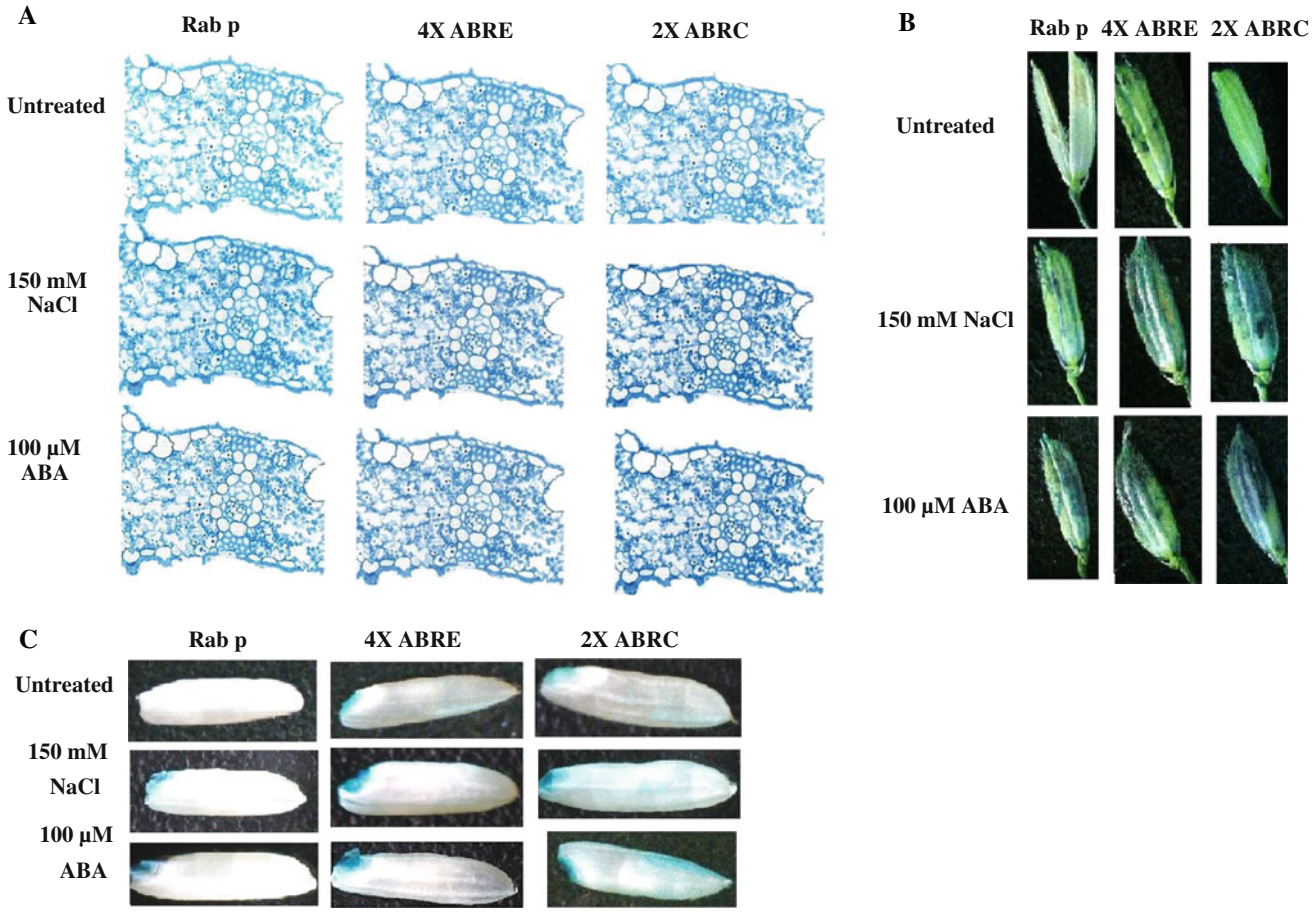

Fig. 4 Histochemical localization of GUS activity observed in leaves (**a**), spikelets (**b**) and seeds (**c**) of Rab p, 4X ABRE and 2X ABRC transgenic plants of T_2 generation, in response to 150 mM NaCl and 100 µM ABA treatment for 16 h. The untreated transgenics served as experimental control

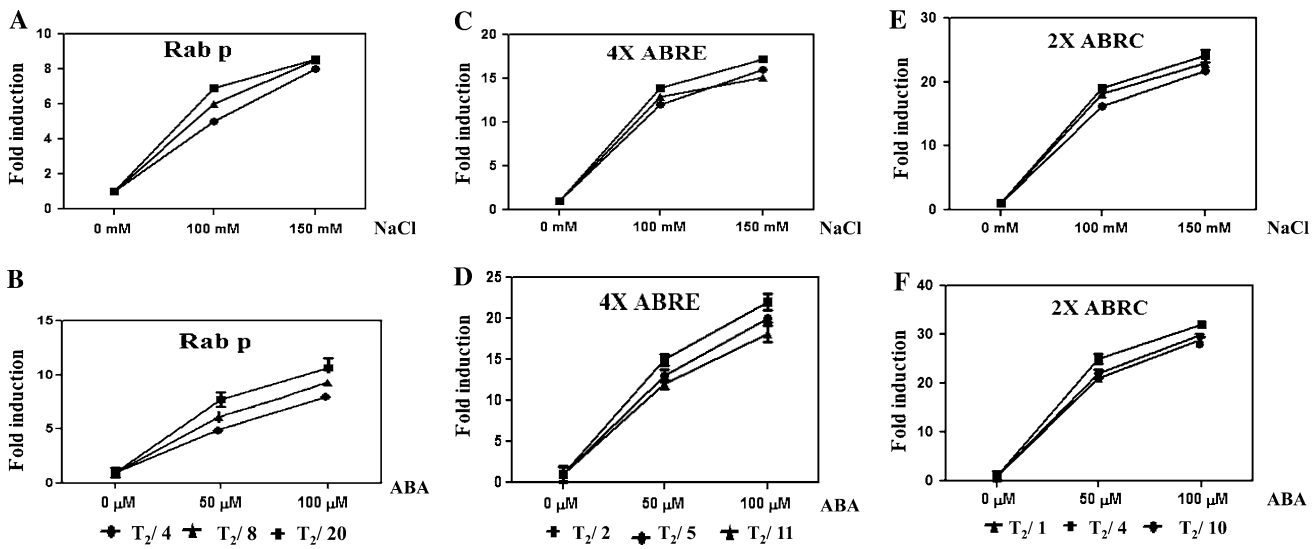

Fig. 5 qRT-PCR analyses showing the fold induction of *gusA* expression in response to varying concentrations of NaCl or ABA, in the leaves of Rab p (**a**, **b**), 4X ABRE (**c**, **d**) and 2X ABRC (**e**, **f**) transgenics of T_2 generation. The expression of genes was determined using *tubulin* as an internal control. The values were means from three independent series, and the results are represented as means ± SE, based on three replicates. The statistical differences at $p \leq 0.05$ have been calculated

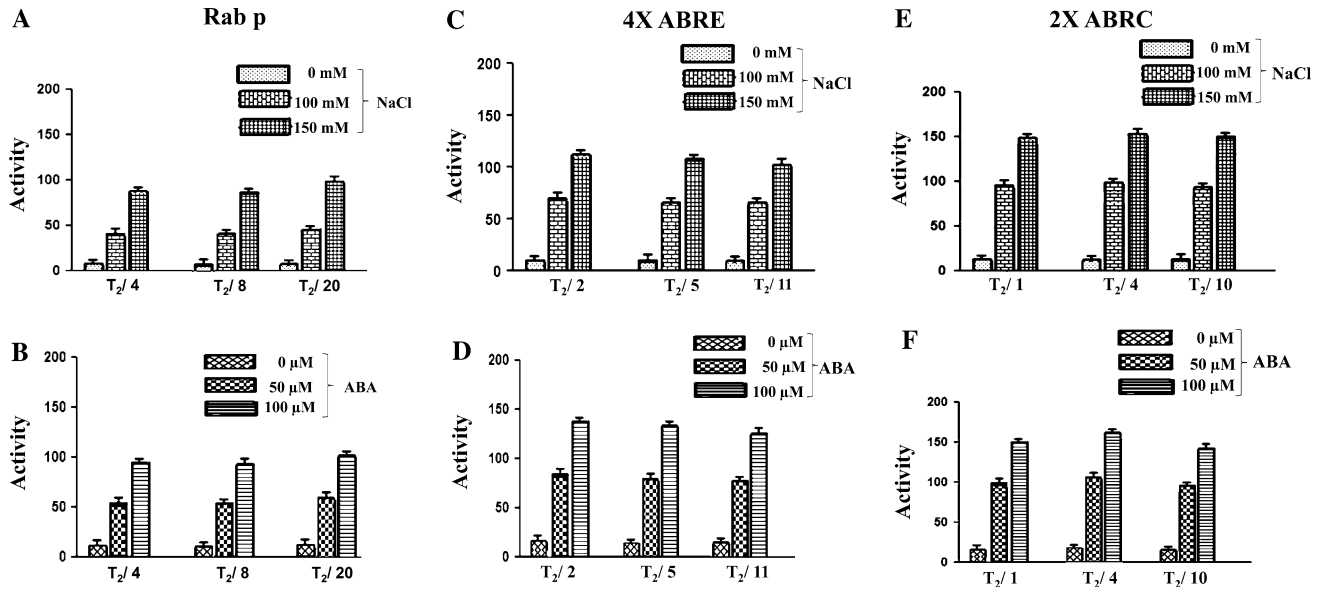

Fig. 6 Quantitative fluorometric assay of GUS activity in response to NaCl (0 mM, 100 mM and 150 mM) or ABA (0 µM, 50 µM and 100 µM) in Rab p (**a**, **b**), 4X ABRE (**c**, **d**) and 2X ABRC (**e**, **f**) transgenics of T_2 generation. The GUS activity was expressed in pmol h^{-1} μg^{-1} total protein. The values represented graphically in the form of a histogram are the mean ± SE ($n = 3$). The *vertical lines* on *top* of *bars* represent the SE. The statistical differences at $p \leq 0.05$ have been calculated

142 pmol h^{-1} μg^{-1} total protein in case of 50 and 100 µM ABA treatments, respectively (Fig. 6d). Similarly, the GUS expression was maximum in line T_2/4 for 2X ABRC promoter, viz., 99 and 150 pmol h^{-1} μg^{-1} total protein on treatment with 100 and 150 mM NaCl (Fig. 6e), and 105 and 161 pmol h^{-1} μg^{-1} total protein with 50 and 100 µM ABA treatments (Fig. 6f).

Discussion

Although the constitutively active CaMV35S promoter and its derivatives can drive high levels of transgene expression in monocot and dicot plants, their activities are substantially lower in monocots than in dicots (Gupta et al. 2001). Constitutively active promoters are not always desirable for plant genetic engineering, because constitutive overexpression of functional genes and/or transcription factors may compete for the building blocks required for plant growth under optimal conditions and hence the transgenics show undesirable phenotypes. It is therefore desirable to generate transgenic plants that accumulate transgenic products only under stress conditions. The stress-inducible promoters are considered to be ideal for driving the expression of candidate genes for abiotic stress tolerance, which has proven to be an alternative strategy to constitutive transgene expression. For instance, there were no morphological abnormalities in abiotic stress-tolerant transgenic rice plants when an ABA-inducible promoter was employed to express the *PgNHX1* gene (Verma et al. 2007). The rice stress-inducible promoters *LIP9* and *OsNAC6* have also been effectively used to overexpress *OsNAC6* gene in transgenic rice for improving stress tolerance without causing growth retardation (Nakashima et al. 2007).

Several stress-inducible promoters have been isolated and characterized, including the *kin1* and the *cor 6.6* promoters from *Arabidposis* (Wang et al. 1995), the *Wsi18* (Joshee et al. 1998) promoters from rice and the *HVA1* promoter from barley (Straub et al. 1994). Despite the availability of many stress-inducible promoters, very few of them have been analyzed and characterized in detail for their responses to abiotic stresses, especially in monocot crops. The co-operative interaction of two or three regulatory elements, placed in close proximity in such promoters, regulate gene expression at the transcriptional level. The ABRE motif shows co-operative interaction with other motifs, and the ABRE-binding factors often form a protein–protein interaction with other trans-acting factors to confer concerted gene expression. An earlier report showed high induction of transgene expression in rice protoplasts using a 290-bp *Rab16A* promoter (Mundy et al. 1990). A hexamer of motif I, when fused with −90 bp CaMV35S promoter, conferred ABA-inducible gene expression (Skriver et al. 1991). The G-box motif (GCCACGTGCC) tetramer rendered a high level of expression in seed, root, leaf, axillary bud and almost all parts of flower buds and pollen of transgenic tobacco (Ishige et al. 1999). The histochemical localization of GUS expression with *Rab16B* promoter was shown in the root,

leaf and flower of ABA/NaCl-treated or desiccated transgenic rice plants but not in untreated plants (Ono et al. 1996). A chimeric gene containing the *gusA* reporter, linked to a 646-bp 5′ fragment from wheat *Em* gene, was found to be correctly expressed in transgenic tobacco and was induced with exogenous ABA. Using a rice-transient assay, a 260-bp fragment (−168 to +92) accounted for the full ABA-specific, 15- to 20-fold increase in GUS expression (Marcotte et al. 1989). In another study, the wheat *Em* promoter was shown to drive reporter gene expression in embryo and aleurone tissues of transgenic barley and rice (Furtado and Henry 2005).

The interactions between ACGT-containing ABRE and other non-ACGT, ABA regulatory sequences called CE, arranged within an approximately short distance, constitute the so-called ABRC, which actually function co-operatively during ABA signal transduction pathways. Roychoudhury et al. (2008) have shown that in case of *Rab16A*, motif II probably acts as a CE or functions as a second copy of ABRE, which means that this gene has both ABRE and CE, the motif I (single ABRE copy) at −189 positions and the CE-like motif IIa at −171 and motif IIb at −130 positions. The presence of ABRE or ABRE-like sequences, CE in these promoters, is a significant indication that they can drive gene expression in a stress-inducible manner. In the current study, we analyzed the salt/ABA inducibility of two synthetically designed promoters, 4X ABRE and 2X ABRC, and one natural promoter from that of rice *Rab16A* gene, in transgenic rice by fusing each of them with a reporter gene *gusA*. Each of these promoters that are known to function as molecular switches in response to environmental stress signals were selected on the basis of the presence of multiple ABA-responsive cis-acting elements. We found that all the three promoters proved to be highly recommendable for use in transgene expression in monocot crops under salinity or ABA-mediated oxidative stress. All these promoters were able to drive transgene expression at very low levels under normal conditions, yet increased the level dramatically upon exposure to salt and particularly ABA in the vegetative tissues as well as in the reproductive organs. The 4X ABRE promoter and Rab p induced GUS expression only in the embryo, but not in endosperm, while the 2X ABRC promoter induced expression in both embryo and aleurone layer of endosperm of seeds. This is supported by earlier observation made by Mundy and Chua (1988), who suggested that mature rice endosperm does not show detectable *Rab16A* gene expression, whereas high level of expression is detected in mature embryo. Ono et al. (1996) also detected no GUS expression with *Rab16B* promoter in endosperm regardless of the presence of ABA. Likewise, wheat *Em* gene was also shown to be embryo specific (Furtado and Henry 2005). We consider these observations to be significant, as they suggest that these promoters can be used to drive stress-inducible tolerant transgene expression, not only in vegetative tissues, but also in floral organs, which are very sensitive tissues to stress, leading to significant yield loss due to reduced grain filling and sterility.

The transgene expression in transgenic plants is also influenced by the copy number (Rai et al. 2009), integration position of transgenes (position effect) in the genome (Bhattacharyya et al. 1994) and integrity of the transgenic insert (Kohli et al. 2003). In our study, however, the pattern of integration of transgene and its expression levels appeared not to be directly correlated. The ABA appeared to be a better elicitor of promoter activation and GUS expression than NaCl for all the promoters, and the promoter activation was purely concentration dependent, i.e., increased steadily with the increase in NaCl and ABA concentration.

Thus, in this study, we have highlighted three potential salinity/ABA-inducible promoters for use in crop biotechnology. All the three promoters seemed to be efficient for use in transgene expression in monocot crops under salinity conditions and/or ABA-mediated oxidative stress. Among the three, we found the 2X ABRC promoter as the most promising with regard to greater fold induction, both in response to salt and ABA, and exerting their function in both embryo and endosperm of seeds, apart from vegetative tissues. The Rab p and 4X ABRE promoters that we characterized can also be used under certain conditions and expression requirements. Use of alternative promoters with similar characteristics, as demonstrated in this study, will be essential for the stacking of several transgenes and driving their expression. These promoters may also be useful for transgene expression in other monocot crops such as maize, barley and wheat.

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