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ORIGINAL PAPER

Cloning and Functional Analysis of the Promoter of a Stress-inducible Gene (ZmRXO1) in Maize

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Abstract The ZmRXO1 gene is a nucleotide-binding site leucine-rich repeat (NBS–LRR) type of R gene in maize (Zea mays). To understand the regulatory mechanism of ZmRXO1 gene expression, we isolated and characterized the ZmRXO1 promoter (PZmRXO1)—the 5′ flanking region of ZmRXO1. A series of PZmRXO1 deletion derivatives, R1–R4, from the translation start code (−1,576, −934, −829, and −582) were fused to the GUS reporter gene, and each deletion construct was analyzed by *Agrobacterium*-mediated transformation into tobacco. Sequence analysis showed that several cis-acting elements (MBS, Box-I, TGA-element and CCAAT-box) were located within the promoter. Deletion analysis of the promoter suggested that the 1,576-bp fragment upstream of ZmRXO1 gene showed a high level of GUS expression in tobacco. The promoter sequence (−582 to −1) was sufficient to improve transcription of GUS gene under hormones (MeJA, GA, ABA), drought and low temperature. Moreover, there might be repressor elements in the region (−1,576 to −934 bp) to repress ZmRXO1 gene expression under treatment with salicylic acid.

Keywords Maize · Stress-inducible · Promoter cloning · Functional analysis

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Ye Tao and Fengting Wang contributed equally to this work and are co-first authors.

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Introduction

Adverse environmental stresses such as drought, salinity, low temperature and pathogen attacks have drastic effects on plant growth and development, which significantly limit agricultural productivity. To increase crop yield under stress conditions, molecular breeding technologies relying on genetic engineering represent an important addition to conventional breeding technology. The cloning of key genes related to adverse stress is the core to solving the problem. Importantly, cloning and understanding stress-related promoters are the keys to realizing molecular breeding goals. In current applications, constitutive promoter expression, such as that from the cauliflower mosaic virus 35S (CaMV35S) and ubiquitin promoters, is crucial.

Constitutively expressed promoters, such as CaMV35S, have been used to assess the effects of transgene expression in various plant species. But the high expression of transgenes in all tissues during all developmental periods increases the metabolic burden to a great extent, leading to a loss of material and energy in the transgenic plants (Vaucheret et al. 1998; Malnoy et al. 2006). Therefore, an inducible promoter to drive gene expression in transgenic plants could minimize the negative effects on plants.

Nowadays, many inducible promoters have been identified in the search to understand the molecular mechanisms of signaling pathways. Several inducible promoters, such as the pathogen-inducible promoter Pgst1 (Malnoy et al. 2006), light-regulated promoter AtPolλ (Sujit et al. 2012), wounding and tensile stress-inducible gene promoter from *Brassica napus* (Katherine AE and Anil HS. 1998), and oxidative stress-inducible peroxidase SWPA2 promoter from sweet potato (Kee-Yeun et al. 2003), have been studied in plants. The cloning and use of these inducible promoters have contributed to the generation of high-quality transgenic plants (Rushton et al. 2002).

The ZmRXO1 gene, which involves in signaling pathways to induce basic defense reactions, could specifically activate large numbers of genes (Bingyu et al. 2005; Yong-Li et al. 2010). But there is little information about the response of its core element to some abiotic stresses, such as methyl jasmonate (MeJA), salicylic acid (SA), gibberellin (GA) and abscissic acid (ABA), as well as to drought and low temperature. To better understand how the ZmRXO1 promoter is regulated, we characterized the ZmRXO1 promoter region using β-glucuronidase (GUS) gene as a reporter gene. Our results showed that the 5′-flanking sequence of the ZmRXO1 promoter was induced by hormones (MeJA, GA, ABA), drought and low temperature. These results provide valuable insights into the role of the ZmRXO1 promoter in regulating gene expression patterns under biotic and abiotic stresses.

Materials and Methods

Plant Materials and Growth Conditions

Seeds of *Zea mays* (B73) were obtained and used as the source material for this study. Maize plants were raised in pots under greenhouse conditions (16/8 h photoperiod; 25 °C). Tobacco *Nicotiana tabacum* (NC89) plants used for tissue culture, were raised on Murashige-Skoog (MS) medium supplemented with 30 g/L sucrose, 7 g/L agar, 3 mg/L 6-BA, 0.2 mg/L NAA and pH 5.8. The plants were maintained in a growth chamber with a 16/8 h light/dark at 25 °C. The fully developed tobacco leaves were then used for genetic transformation experiments.

Promoter Cloning and Sequence Analysis

To determine the structure of the ZmRXO1 promoter, polymerase chain reaction (PCR) was performed using the primer pairs (RXO1-F/RXO1-R, Table 1) with maize genomic DNA as template. The PCR conditions were as follows: 94 °C for 5 min and 30 cycles of 94 °C for 45 s, 56 °C for 40s, 72 °C for 2 min and 72 °C for 10 min. The PCR products were cloned into the vector pMD18-T vector and the recombinant clones obtained were sequenced. Sequences analysis of the *ZmRXO1* promoter from maize was carried out using the PlantCARE database (Lescot et al. 2002).

Table 1 Primers used for polymerase chain reaction (PCR)[a]

ZmRXO1 (R1)-F	5′-CGGAATTCACAATATCGGTTCCGCTGC-3′
ZmRXO1-R	5′-CCCATGGCTTCTTATTCGATCAGAC
R2-F	5″-CGGAATTCTGAAGAGTCATTTACAGAG-3′
R3-F	5′-CGGAATTCTTAGGCCTGCAGACTGAC-3′
R4-F	5′-CGGAATTCTCTTCCTCCTATACAATAC-3′
ACTIN- F	5′-CGGAATTCACAATATCGGTTCCGCTGC-3′
ACTIN- R	5′-CCCATGGCTTCTTATTCGATCAGAC-3′
GUS-F	5′-CGGGATCCTGCCGTGATACCGACTTGA-3′
GUS-R	5′CCCATGGTTCAGCTTGCTTGTTGCTTG-3′

[a] The underlined letters represent restriction enzyme sites of *Eco*RI and *Nco*I

Construction of Expression Vectors and Genetic Transformation

In order to study the functional regions of the *ZmRXO1* promoter, 5′-end deletion analysis was carried out. A series of deletions of the *ZmRXO1* promoter was generated by PCR amplifications. Four deletion fragments were named as R1 (−1,576 bp), R2(−934 bp), R3 (−829 bp), R4 (−582 bp) with the common reverse primer (RXO1-R) and the forward primers R2-F, R3-F, R4-F, respectively (Table 1). The PCR products were cloned into the pCMBIA1301 plasmid to replace the CaMV35S promoter (Fig. 1). The recombinant plasmids were introduced into the *Agrobacterium tumefaciens* strain EHA105 for *Agrobacterium*-mediated transformation of tobacco leaves. To evaluate promoter activity, expression of the gene reporter beta-glucuronidase (GUS) was measured.

Plant Treatment

To understand the effects of different stresses on GUS expression mediated by the *ZmRXO1* promoter, plants were treated for different durations with dehydration, low temperature (4 °C), abscisic acid (ABA), methyl jasmonate (MeJA), salicylic acid (SA), gibberellin (GA) before sampling. For drought stress treatment, the roots of transgenic tobacco plants were soaked in 20 % polyethylene glycol 6000 (PEG6000). For low temperature treatment, the transgenic plants were put into a 4 °C growth chamber. For different hormone stimuli treatments, the transgenic tobacco plants were sprayed with 100 μM MeJA, 1 mM SA, 100 μM ABA and 100 μM GA, respectively. Transgenic tobacco plants of different deletion promoters and the CaMV35S (pCAMBIA1301 vector) transformants treated with water were used as negative and positive controls, respectively. The tobacco samples were treated for 1 h, 3 h, 5 h, 10 h and 24 h. After treatment, tobacco leaves were harvested, quickly frozen in liquid nitrogen, and stored at −80 °C for total RNA extraction and fluorometric GUS assay.

Total RNA Extraction and Real-Time Quantitative RT-PCR Analysis

To understand the effects of different stresses on GUS expression mediated by the *ZmRXO1* promoter, the fourth–sixth leaves of the stress-treated transgenic tobacco were used as the plant materials. Total RNA from tobacco leaves was extracted using the RNAiso Reagent (Takara, Changchun, China) and single-stranded cDNA was synthesized from total RNA with reverse

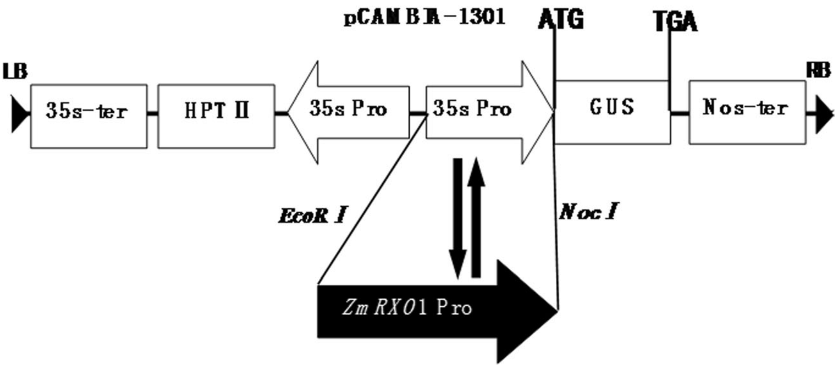


Fig. 1 Schematic representation of the P*ZmRXO1*: *GUS* construct. The insertion position of the *ZmRXO1* promoter in the vector is indicated with restriction enzyme sites (*Eco*RI and *Nco*I). *LB* Left border, *RB* right border, *35s-ter* Cauliflower Mosaic virus 35S terminator, *35s Pro* Cauliflower Mosaic virus 35S promoter, *GUS* β-glucuronidase gene, *HPTII* hygromycin phosphotransferase (II) coding region, *NOS-ter* nopaline synthase terminator, *ZmRXO1 Pro ZmRXO*1 promoter

transcriptase M-MLV and the oligo (T) 18 primer (Takara). Real-time quantitative RT-PCR (qRT-PCR) analysis was performed using an Applied Biosystems 7500 apparatus (Applied Biosystems, Foster City, CA). The *ACTIN* gene (GenBank accession No. U60491) was used as an endogenous control gene (ACTIN-F/ACTIN-R, Table 1). *GUS-F*/*GUS-R* primers (Table 1) were used for GUS gene. The PCR conditions were: 95 °C for 30s, 95 °C for 5 s, 56 °C for 40s, 40 cycles. Data from the individual runs were collated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Mean expression levels and standard deviations (SD) were calculated from the results of three repeat experiments.

Histochemical Staining and Fluorometric GUS Assay

Histochemical staining and fluorometric GUS assay analysis for GUS activity was carried out as described by Jefferson et al. (1987). The leaves of stress-treated transgenic tobaccos were incubated in GUS reaction buffer (3 mg/mL X-gluc, 40 mM sodium phosphate pH 7, 10 mM EDTA, 0.1 % Triton X-100, 0.5 mM ferricyanatum kalium, 0.5 mM ferrocyanatum kalium and 20 % methanol). After overnight incubation at 37 °C, the stained samples were bleached with 70 % (v/v) ethanol to remove chlorophyll. Photos of those stained samples were obtained by a Nikon SMZ1000 microscope under white light. For fluorometric GUS assay, leaves of stress-treated transgenic tobaccos were used to determine GUS enzyme activity by measuring the fluorescence of 4-methylumbelliferone produced by GUS cleavage of 4-methylumbelliferyl-β-D-glucuronide (4-MUG) (Jefferson 1988). GUS activity is expressed as nanomoles of methylumbelliferone per minute per milligram of protein. Protein amount was determined using a Protein Assay kit (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard.

Results

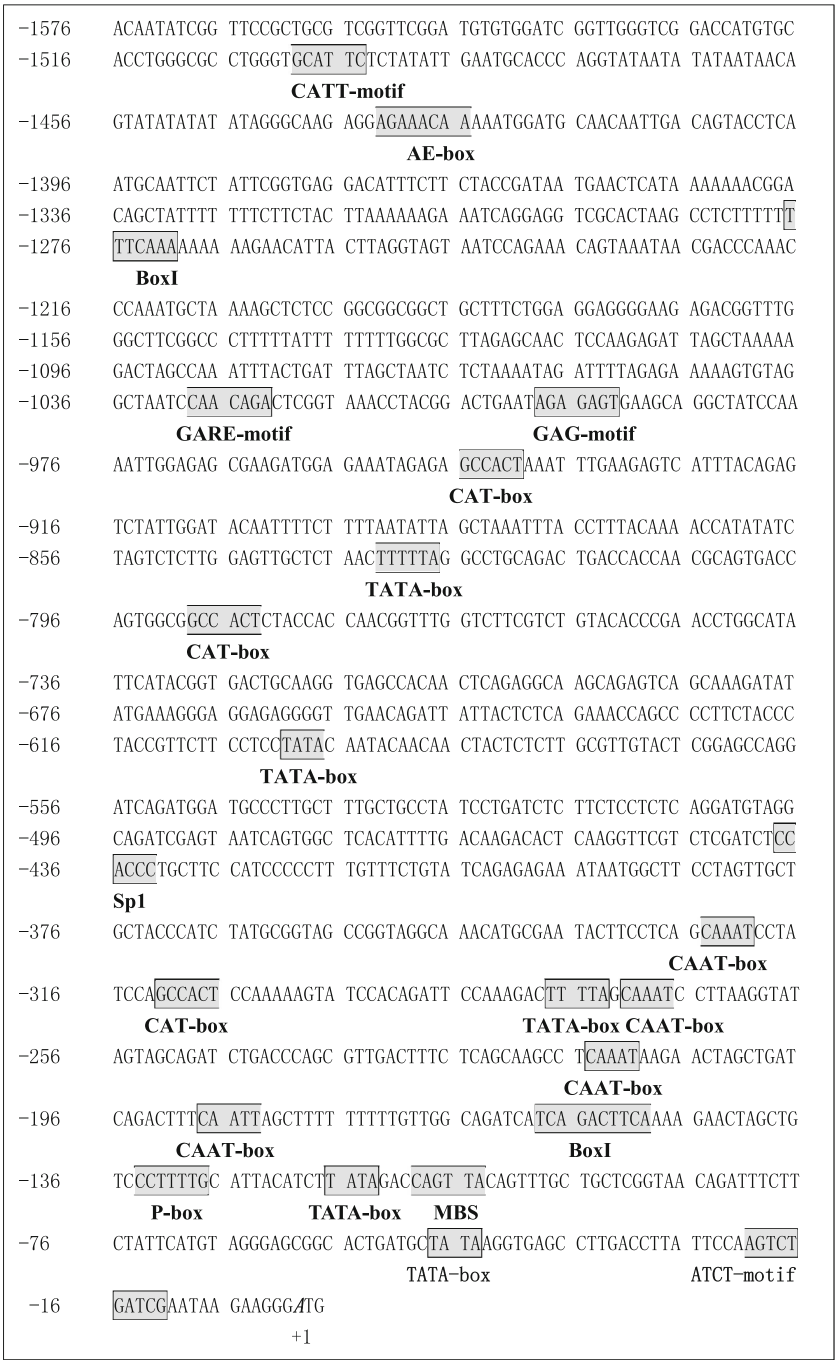
Promoter Sequence Analysis

For a more comprehensive identification of cis-acting regulatory elements, the 1,576 bp upstream sequence before the translation initiation codon (indicated with +1, Fig. 1) was analyzed using the PLACE and the PlantCARE databases. Bioinformatics analysis of *ZmRXO1* promoter detected some putative cis-acting regulatory elements known to modulate gene expression in different plant species (Fig. 2). Several core fragments were identified in the *ZmRXO1* promoter sequence (Table 2). They consisted of four CAAT-box elements, five TATA-box elements, one CATT motif (GCATTC), one AE-box (AGAAACAA), one ATCT-motif (AATCTGAT CG), one GAG-motif (AGAGAGT), one MBS (TAACTG), two gibberellin-responsive elements (P-box and GARE-motif) and many other cis-acting regulatory elements, such as CAT-box, SP1, Box-W1.

GUS Expression Under the Control of the *ZmRXO1* Promoter in Response to Different Stimuli

To determine the regulatory mechanism controlling expression of the *ZmRXO1* gene, the 1,576 bp full-length *ZmRXO1* promoter was fused to the GUS reporter gene in a plant expression vector and transferred into tobacco plants. The GUS gene expression levels in the transgenic tobacco by histochemical GUS staining clearly revealed the inducible activity of the *ZmRXO1* promoter. The results indicated that expression of the GUS gene was decreased after SA treatment (Fig. 3a, c, e), but increased after the other treatments (Fig. 3a, c, d, f–i). From Fig. 3a–c, we can see that the untreated transgenic tobacco showed a certain degree of GUS expression, which was apparently lower than that of the CaMV35S transformant tobacco plants. Moreover, GUS gene expression

Fig. 2 Analysis of *ZmRXO1* promoter region. Putative cis-acting regulatory elements, detected in the promoter fragment using PlantCARE database are indicated within *grey shaded boxes*. The transcription initiation site is indicated with +*1*



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-1576   ACAATATCGG TTCCGCTGCG TCGGTTCGGA TGTGTGGATC GGTTGGGTCG GACCATGTGC
-1516   ACCTGGGCGC CTGGGTGCAT TCTCTATATT GAATGCACCC AGGTATAATA TATAATAACA
                         CATT-motif
-1456   GTATATATAT ATAGGGCAAG AGGAGAAACA AAAATGGATG CAACAATTGA CAGTACCTCA
                                  AE-box
-1396   ATGCAATTCT ATTCGGTGAG GACATTTCTT CTACCGATAA TGAACTCATA AAAAAACGGA
-1336   CAGCTATTTT TTTCTTCTAC TTAAAAAAGA AATCAGGAGG TCGCACTAAG CCTCTTTTTT
-1276   TTCAAAAAAA AAGAACATTA CTTAGGTAGT AATCCAGAAA CAGTAAATAA CGACCCAAAC
         BoxI
-1216   CCAAATGCTA AAAGCTCTCC GGCGGCGGCT GCTTTCTGGA GGAGGGGAAG AGACGGTTTG
-1156   GGCTTCGGCC CTTTTTATTT TTTTTGGCGC TTAGAGCAAC TCCAAGAGAT TAGCTAAAAA
-1096   GACTAGCCAA ATTTACTGAT TTAGCTAATC TCTAAAATAG ATTTTAGAGA AAAAGTGTAG
-1036   GCTAATCCAA CAGACTCGGT AAACCTACGG ACTGAATAGA GAGTGAAGCA GGCTATCCAA
             GARE-motif                        GAG-motif
-976    AATTGGAGAG CGAAGATGGA GAAATAGAGA GCCACTAAAT TTGAAGAGTC ATTTACAGAG
                                         CAT-box
-916    TCTATTGGAT ACAATTTTCT TTTAATATTA GCTAAATTTA CCTTTACAAA ACCATATATC
-856    TAGTCTCTTG GAGTTGCTCT AACTTTTTAG GCCTGCAGAC TGACCACCAA CGCAGTGACC
                                TATA-box
-796    AGTGGCGGCC ACTCTACCAC CAACGGTTTG GTCTTCGTCT GTACACCCGA ACCTGGCATA
              CAT-box
-736    TTCATACGGT GACTGCAAGG TGAGCCACAA CTCAGAGGCA AGCAGAGTCA GCAAAGATAT
-676    ATGAAAGGGA GGAGAGGGGT TGAACAGATT ATTACTCTCA GAAACCAGCC CCTTCTACCC
-616    TACCGTTCTT CCTCCTATAC AATACAACAA CTACTCTCTT GCGTTGTACT CGGAGCCAGG
                   TATA-box
-556    ATCAGATGGA TGCCCTTGCT TTGCTGCCTA TCCTGATCTC TTCTCCTCTC AGGATGTAGG
-496    CAGATCGAGT AATCAGTGGC TCACATTTTG ACAAGACACT CAAGGTTCGT CTCGATCTCC
-436    ACCCTGCTTC CATCCCCCTT TGTTTCTGTA TCAGAGAGAA ATAATGGCTT CCTAGTTGCT
        Sp1
-376    GCTACCCATC TATGCGGTAG CCGGTAGGCA AACATGCGAA TACTTCCTCA GCAAATCCTA
                                                             CAAT-box
-316    TCCAGCCACT CCAAAAAGTA TCCACAGATT CCAAAGACTT TTAGCAAATC CTTAAGGTAT
          CAT-box                                TATA-box CAAT-box
-256    AGTAGCAGAT CTGACCCAGC GTTGACTTTC TCAGCAAGCC TCAAATAAGA ACTAGCTGAT
                                                    CAAT-box
-196    CAGACTTTCA ATTAGCTTTT TTTTTGTTGG CAGATCATCA GACTTCAAAA GAACTAGCTG
             CAAT-box                                 BoxI
-136    TCCCTTTTGC ATTACATCTT ATAGACCAGT TACAGTTTGC TGCTCGGTAA CAGATTTCTT
          P-box           TATA-box    MBS
-76     CTATTCATGT AGGGAGCGGC ACTGATGCTA TAAGGTGAGC CTTGACCTTA TTCCAAGTCT
                                     TATA-box                ATCT-motif
 -16    GATCGAATAA GAAGGGATG
                          +1
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occurred mainly in the aerial parts of the plants and there was hardly expression in roots.

To further verify the above findings, GUS expression from the *ZmRXO1* promoter was analyzed by qRT-PCR and fluorometric GUS assay (Figs. 4, 5). The GUS gene transcript level could be induced substantially by ABA and PEG with a maximal level at 10 h. GUS gene transcription in plants treated with MeJA, GA or low temperature also increased significantly at 1 h, 3 h or 5 h, respectively. However, the GUS expression level of SA-treated plants was lower than that of untreated plants (0 h). From Fig. 5, we found the same phenomenon as that shown in Fig. 4. *ZmRXO1* promoter-mediated GUS activity increased under dehydration, cold stresses, MeJA, GA, ABA treatment, but declined under SA treatment.

Activity Analysis of *ZmRXO1* Deletion Promoters

To further study the stress-inducible expression of the gene promoter, a series of 5′ end deletion promoter:: GUS

Table 2 Putative cis-acting elements and their positions in the *ZmRXO1* promoter

Cis element	Sequence	Position	Function
Box-W1	TTGACC	-39	Fungal elicitor responsive element
MBS	TAACTG	-110	MYB Binding Site
Box-I	TTTGAAA	-159	Part of a light responsive element
GAG-motif	AGAGAGT	-999	Part of a light responsive element
CAT-box	GCCACT	-312,-789,-946	cis-acting regulatory element related to meristem expression
CCAAT-box	CAACGG	-877	MYBHv1 binding site
GARE-motif	TCTGTTG	-1029	Gibberellin-responsive element
P-box	CCTTTTG	-134	Gibberellin-responsive element
Sp1	CC (G/A) CCC	-438	Light responsive element
TGA-element	AACGAC	-1,327	Auxin-responsive element
AE-box	AGAAACAA	-1,433	Part of a module for light response
CATT-motif	GCATTC	-1,500	Part of a module for light response

constructs was also produced, and then transferred to tobacco plants for analysis of transient expression. The deletion promoters were named as R1 (−1,576 to −1), R2 (−934 to −1), R3 (−829 to −1) and R4 (−582 to −1), respectively. Leaves of stress-treated transgenic tobaccos were subjected to the fluorometric GUS activity assay. The results indicated that the

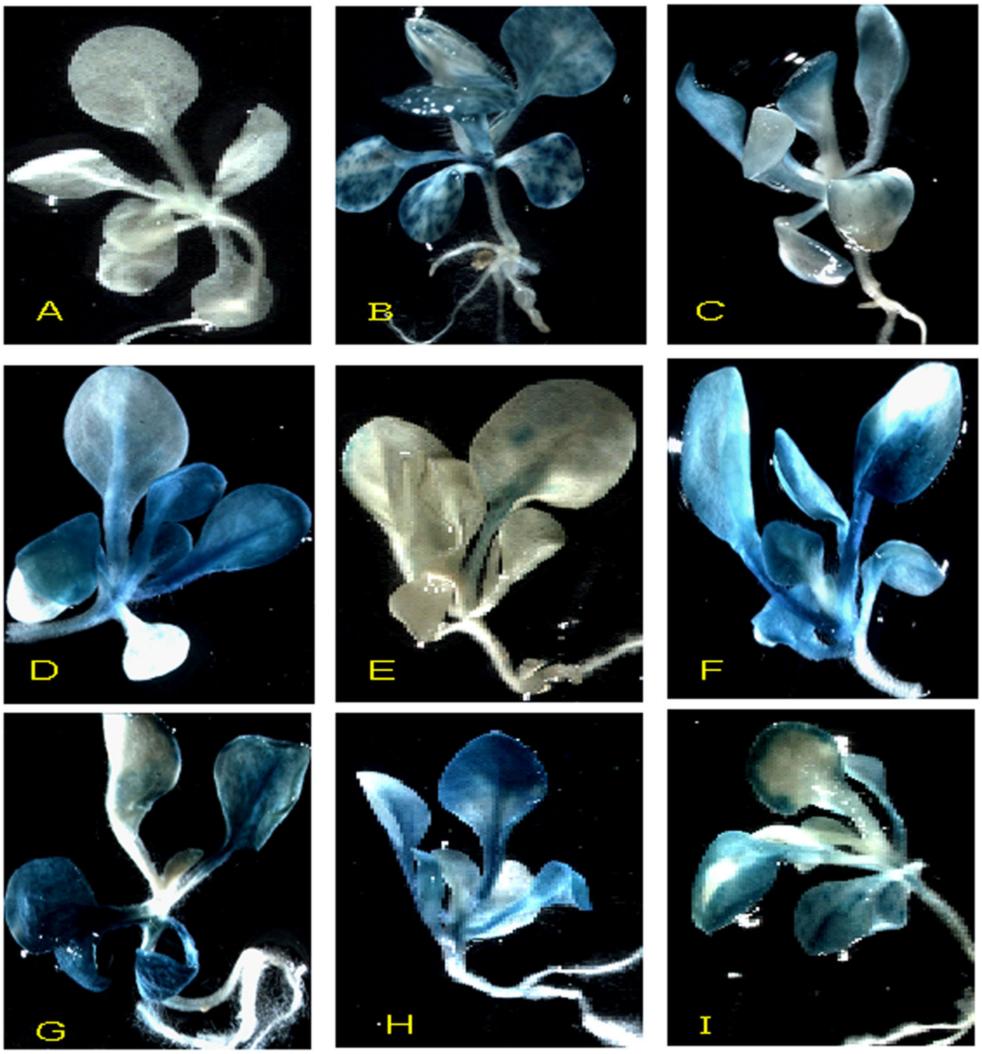


Fig. 3 Histochemical staining of GUS activity in six-week-old transgenic tobacco plants. **a**, **b** Beta-glucuronidase (GUS) expression in wild type (**a**) and CaMV35S-transformed (**b**) tobacco plants. **c** Untreated transgenic tobacco plants. **d**–**l** Transformed tobacco plants were treated with 100 μM methyl jasmonate (MeJA), 1 mM salicylic acid (SA), 100 μM abscisic acid (ABA), 20 % polyethylene glycol (PEG), 100 μM gibberellin (GA) and low temperature treatment (4 °C), respectively

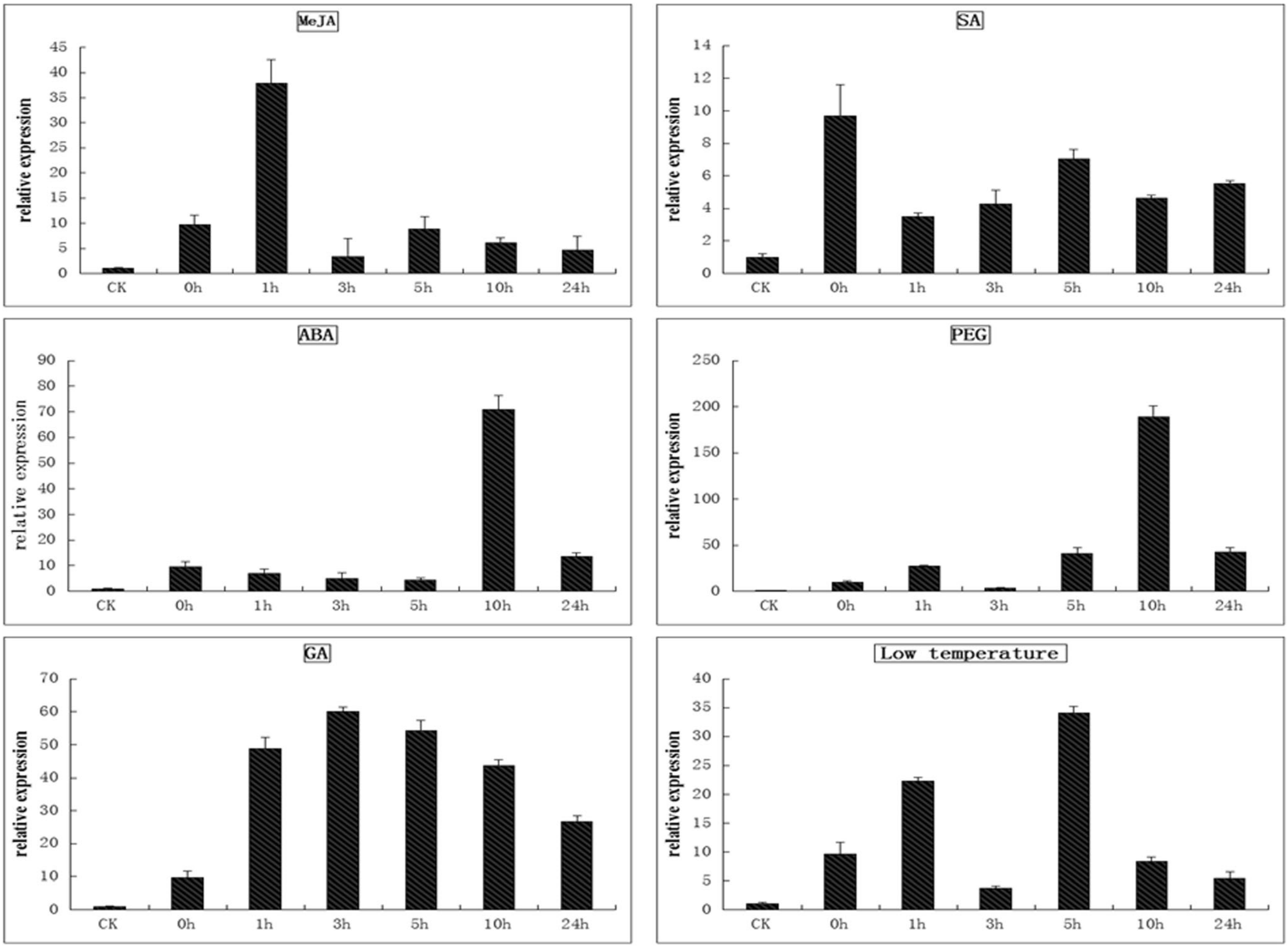
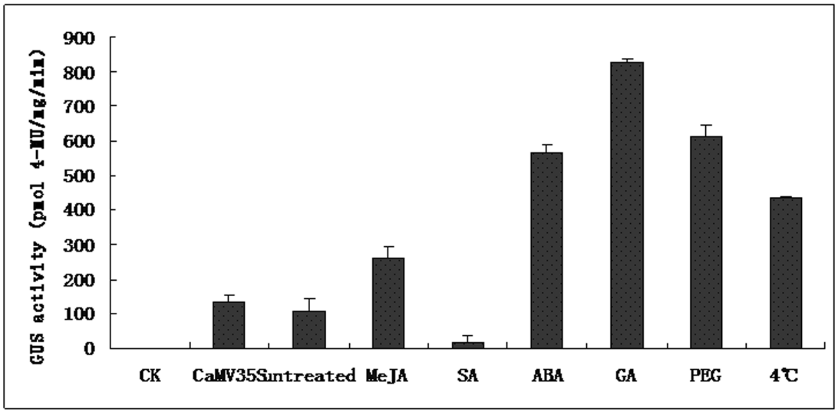


Fig. 4 Time course of the *ZmRXO1* transcript levels in the leaves of the transgenic tobacco under methyl jasmonate (MeJA), salicylic acid (SA), abscisic acid (ABA), polyethylene glycol (PEG), gibberellin (GA) and low temperature (4 °C) treatment. CK (wild-type) and 0 h-treated tobacco plants were left untreated as controls. At least three independent determinations were performed for each sample

GUS activity of R1-promoter plants was higher than that of R2-, R3- and R4-promoter plants (Fig. 6). The deletion promoters were subjected to abiotic stress and phytohormones (MeJA, GA, ABA); GUS activity of the R1, and R4 promoters increased by 1.88-fold and 2.67-fold (compared with the untreated control), respectively, after GA treatment. The two gibberellin-responsive elements (GARE-motif and P-box) might play an important role in improving the GUS activity of R1 and R4. However, R1-mediated GUS activity was reduced significantly (about 0.48-fold) by SA treatment, and

Fig. 5 GUS activity of the ZmRXO1 promoter in response to methyl jasmonate (MeJA), salicylic acid (SA), abscisic acid (ABA), polyethylene glycol (PEG), gibberellin (GA) and low temperature (4 °C) treatments. GUS activity from the CaMV35S (pCAMBIA1301 vector) transformants, wild type and untreated transformants served as controls



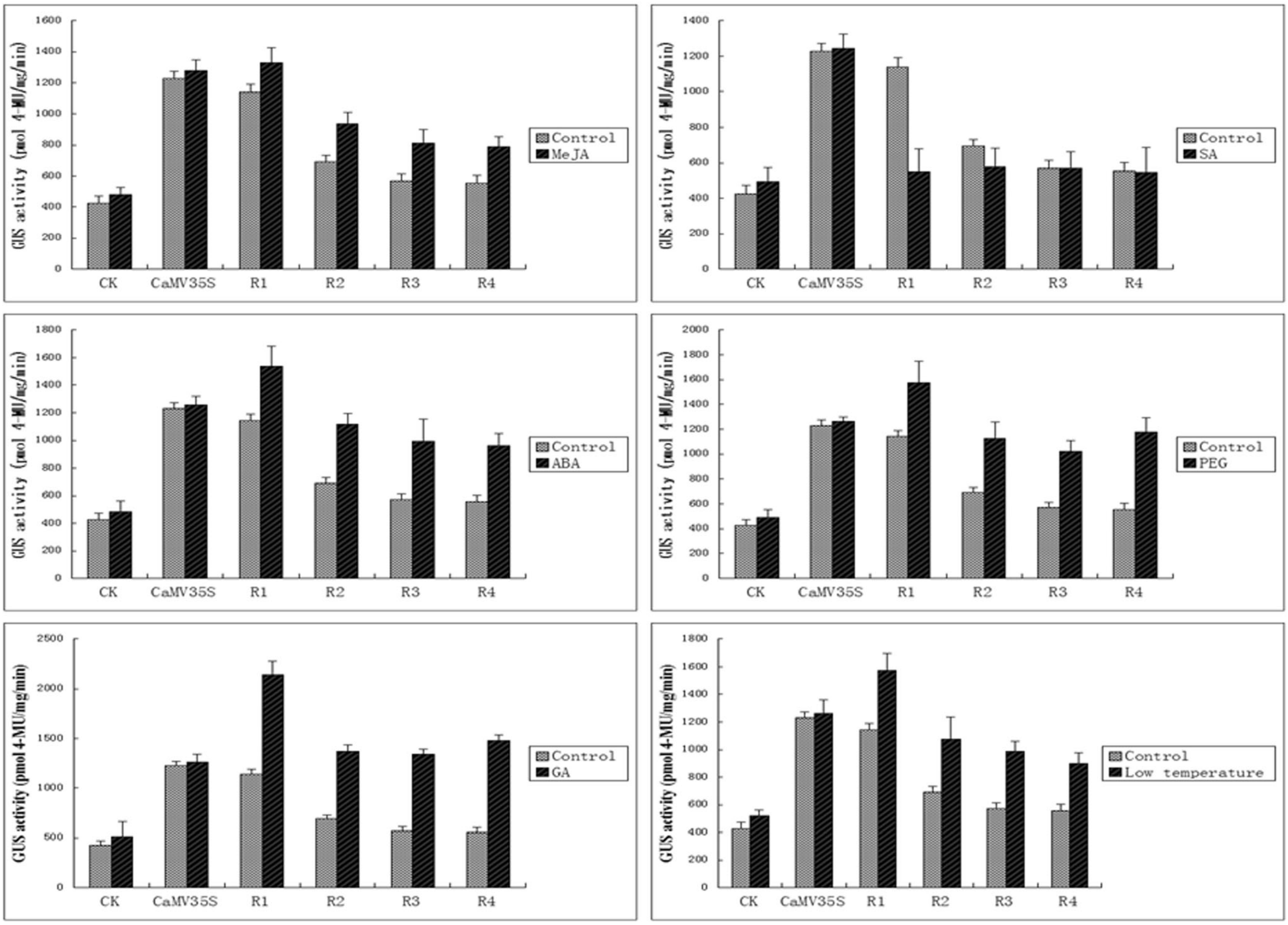


Fig. 6 Analysis of *ZmRXO1* promoter deletion mutants. GUS activity in methyl jasmonate (MeJA), salicylic acid (SA), abscisic acid (ABA), polyethylene glycol (PEG), gibberellin (GA) and low temperature (4 °C) treatments of plants carrying the *ZmRXO1* promoter deletion series is shown. GUS activity from the CaMV35S (pCAMBIA1301 vector) transformants, wild type and untreated transformants served as controls

while that mediated by R2, R3, and R4 showed no differences from that of untreated plants.

Discussion

P*ZmRXO1* is a Photosynthesis Tissue-Specific Promoter

Expression of many plant genes, including structural, metabolic and many regulatory genes (Eckes et al. 1986; Simpson et al. 1986; Kaldenhoff et al. 1993; Sheen 1993), is induced by light . These genes are expressed preferentially under light conditions, but exhibit low expression or even no expression in dark conditions. Therefore, they are defined as light inducible genes and are generally expressed in leaves. In this study, the fusion construct (P*ZmRXO1*: GUS) was transformed into the tobacco genome to study the activity of this promoter. Transgenic tobacco plants exhibited blue staining in the leaves and stems, but not in the roots (Fig. 3). This result indicated that the *ZmRXO1* promoter might be regulated by light. Several types of light-responsive elements, including Sp1, GAG-motif, AE-box, CATT-motif and Box-I, were found in the *ZmRXO1* promoter sequence (Fig. 2). Box-I has been identified in nuclear extracts from tobacco and tomato leaves and cotyledons (Borello et al. 1993). LeMYBI is reported to bind specifically to Box-I and to activate transcription in plant leaves (Rose et al. 1999).

Deletion Analysis of the *ZmRXO1* Promoter

To determine the function of the cis-acting elements, deletion analysis of the *ZmRXO1* promoter was performed. The deletion promoter constructions were transformed into tobaccos using Agrobacterium-mediated leaf-disc transformation.

As shown in Fig. 6, GUS activity decreased as the *ZmRXO1* promoter length decreased from R1 to R5. The full-length *ZmRXO1* promoter (R1) had the highest activity among all the deletion promoters. This suggests that the sequence between −1,576 and −934 bp of the *ZmRXO1* promoter may contain cis-elements involved in up-regulation of GUS expression.

The *ZmRXO1* promoter exhibited inducible expression in response to different stress stimuli (ABA, drought and low temperature; Fig. 6). Sequence analysis showed that there were two cis-acting elements—MBS and the CCAAT-box (MYBHv1 binding site)—in the *ZmRXO1* promoter. We hypothesized that these two elements played an important role in increasing GUS activity in these cases. MBS is the binding site for the transcription factor MYB, which has been identified as a target of other regulators (Dubos et al. 2010). Transient expression analyses indicated that AtMYB2 could transactivate gene expression via MBS-1 by binding to the MYB recognition site MBS-1 in the AtADH1 promoter (Hoeren et al. 1998). The CCAAT box is one of the most ubiquitous of promoter elements, being present in 30 % eukaryotic promoters (Bucher 1990). Many housekeeping and inducible promoters have a CCAAT box (Roy and Lee 1995), and many DNA-binding proteins binds the CCAAT element, including CTF/NF1 (transcription factor/nuclear factor 1), C/EBP (CCAAT/enhancer binding protein), CDP (CCAAT displacement protein) (Edwards et al. 1998). In this study, we also found that GUS activity changes in transgenic tobacco under drought stress, low temperature and ABA exhibited the same pattern (Fig. 6). This may be related to ABA signal transduction. ABA is one of the most important signaling molecules in plants, and is also a cross-talking signal molecule. Under drought and low temperature conditions, various biochemical and physiological responses are triggered. The expression of many stress-inducible genes was improved and ABA accumulated in plants (Yamaguchi-Shinozaki and Shinozaki 1993). Promoters sequence analyses have revealed that ABA-responsive gene expression needs ABA-responsive elements (ABREs or CE3). This suggested that new ABA-responsive elements might exist in the *ZmRXO1* promoter.

The *ZmRXO1* gene is reported as a single dominant gene in maize that controls elicitation of the hypersensitive response to *X. oryzae* pv. *oryzicola* (Zhao et al. 2004). Plant defense responses are regulated by SA, ET-MeJA and other signaling molecules (Dong 1998; McDowell and Dangl 2000). The SA-dependent response is deployed against a biotrophic pathogen that obtains nutrients from living cells, but the ET–JA response is activated by necrotrophic pathogens that kill plant tissue. In this study, R1, R2, R3, R4-mediated GUS activity increased significantly under MeJA treatment (compared to untreated controls). However, R1-mediated GUS activity declined significantly under SA treatment (Fig. 6). This result indicated that expression of the *ZmRXO1* gene might be a MeJA-dependent response within the overall plant defense response.

The TGA-element (AACGAC) is an auxin response element. Xing reported 31 encoding ARF genes, whose products interact with the TGA-element (AACGAC) in the maize genome (Xing et al. 2011). Among these genes, 14 had auxin response elements in their promoter regions, 7 of which were not affected by exogenous auxin. Sequence analysis of the *ZmRXO1* promoter showed that one TGA-element was present in the R1 promoter. R1-mediated GUS activity declined significantly under SA treatment (compared with untreated controls). However, no significant difference in GUS activity of R2-, R3-, and R4-mediated expression was observed; the TGA-element of these deletion promoters was deleted (Fig. 6). We suspected that the TGA-element might have a negative effect on the *ZmRXO1* promoter under SA treatment. Or that there is other negative cis-element that is not found in the 642 bp fragment (−1,576 to −934).

In conclusion, GUS expression assays indicated that a 1,576-bp fragment upstream of the *ZmRXO1* gene coding sequence confers a high level of GUS expression in tobacco. The results demonstrated that elements needed for high expression might be located in a 642-bp fragment (−1,576 to −934). Also, R4 (−582) was sufficient to induce transcription of the GUS gene. Moreover, there might be repressor elements of *ZmRXO1* gene expression under SA in the region from −1,576 to −934 bp.

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