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Isolation of a maize *ZmCI-1B* promoter and characterization of its activity in transgenic maize and tobacco

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Abstract

Key message **The 2-kb *ZmCI-1B* promoter is active in the root and embryo and induced by wounding in maize and the 220-bp 5′-deleted segment maybe the minimal promoter.**

Abstract The subtilisin–chymotrypsin inhibitor gene, CI-1B of *Zea mays* (*ZmCI-1B*), has been suggested to induce the maize defense system to resist insect attack. Real-time RT-PCR showed that *ZmCI-1B* gene exhibited especially high expression in roots and embryos. The 2-kb full-length promoter of *ZmCI-1B* gene was isolated from the maize genome and used to drive expression of a beta-glucuronidase (*GUS*) reporter gene for transient expression and stable expression analysis in maize. The results of GUS histochemical staining in transgenic maize plants revealed that the *ZmCI-1B* promoter induced *GUS* expression preferentially in roots and embryos and in response to wounding. A series of 5′-deleted segments of the *ZmCI-1B* promoter were cloned individually to drive *GUS* expression for further analysis. Deletion analysis combined with the histochemical staining of transgenic tobacco plants revealed 220-bp segment could drive *GUS* in a tissue-specific and wounding-induced expression in tobacco; thus, it maybe the minimally active promoter of *ZmCI-1B* gene. Furthermore, it revealed that the *ZmCI-1B* promoter contained tissue-specific and wounding-induced elements.

Keywords Tissue-specific · Root and embryo promoter · Protease inhibitor · Wound-induced · GUS

Abbreviations

CaMV   Cauliflower mosaic virus
DAP   Days after pollination
DAG   Days after germination
ppt   Phosphinothricin

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Introduction

Promoters are of great value for gene function studies and research into the regulation of plant development. Constitutive promoters—such as the cauliflower mosaic virus (*CaMV*) *35S* promoter and the maize ubiquitin promoter—have been characterized and used widely to promote exogenous gene expression (Christensen et al. 1992; Cornejo et al. 1993; Odell et al. 1985). Although they can highly constitutively express a foreign gene in host plants, they result in expenditure of unnecessary material and energy and induce the host plants to produce many useless

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compounds. As a result, overexpression of an exogenous gene in all tissues harms the transformed plants during growth and development (Kasuga et al. 2004; Pino et al. 2007). Tissue-specific or inducible promoters may overcome the shortcomings of constitutive promoters, as they can fine tune the expression of a target gene to particular tissues or under the presence of a special inducer; hence, expression of a foreign gene can be accurately controlled in a plant to avoid the undesirable effects of constitutive promoters. Because of their advantages, a variety of tissue-specific or inducible promoters have been characterized and used to drive targeted gene expression in various species (Potenza et al. 2004). In previous research, a genome-scale microarray-based approach was used to identify embryo-specific genes and promoters in maize (Liu et al. 2014). After further analysis by the UniGene database and by real-time RT-PCR, twenty-eight candidate embryo-preferred genes were confirmed. Of these, the subtilisin–chymotrypsin inhibitor CI-1B (*ZmCI-1B*) gene which expressed most highly in roots and embryos was discovered in maize.

Both roots and embryos are important tissues for plants. Roots are responsible for absorption of water, oxygen, and micronutrients during plant growth and development. Many studies have been conducted on root-specific promoters, such as the *rolD* promoter from *Agrobacterium rhizogenes* (Leach and Aoyagi 1991), the *TobRB7* promoter from tobacco (Yamamoto et al. 1991), the *pyk10* promoter from *Arabidopsis thaliana* (Nitz et al. 2001), and the *Os02g37190* and *Os03g01700* promoters from rice (Li et al. 2013). Embryo in plant functions as an important nutrient source for later stages of seedling growth because plants accumulate storage proteins, carbohydrates, and oils in their embryonic cells. Although few embryo-specific promoters have been reported, tissue-specific promoters that drive transgene expression in embryos have been a focus of attention for their special use in studying embryo growth and development, in addition to accumulate seed oil or recombinant protein in monocotyledon (José-Estanyol et al. 2005; Kim et al. 2011).

Plant protease inhibitors (PIs) mainly consist of four families: serine protease inhibitors, cysteine protease inhibitors, metallo-protease inhibitors and aspartic protease inhibitors (Koiwa et al. 1997). PIs bind to and disrupt the function of proteases and play an important role in physiological and developmental regulation (Dombrowski 2003; Farmer and Ryan 1992; Kawasaki et al. 2001; Wang et al. 2008). Genes encoding PIs are induced at wound sites and have elicited interest for researchers studying plant responsiveness to mechanical damage or insect biting (Enyedi et al. 1992). Serine protease inhibitors constitute by far the largest part of plant PIs, and they usually accumulate in seeds and tubers of plants where PIs are

synthesized and stored as an indicator of defense function (Koiwa et al. 1997). The subtilisin–chymotrypsin PIs are among the serine PIs and so far, a great number of their genes have been isolated and identified in dicotyledonous plants such as potato, tobacco, and winged beans (Dunse et al. 2010; Telang et al. 2009; Valueva et al. 2012). As for monocotyledons such as wheat, barley, and rice, relevant researches were also investigated in this respect (Di Gennaro et al. 2005; Hejgaard 1981; Mur et al. 2004; Ohtsubo and Richardson 1992). The expression of two subtilisin–chymotrypsin PI genes *OCPI1* and *OCPI2* in rice was induced by a variety of stressors, including mechanical injury and low temperature (Singh et al. 2009). A PI gene from the grass *Brachypodium distachyon* (*Bdpin1*) was induced by wounding (Mur et al. 2004). The gene encoding a wheat PI active against subtilisin and chymotrypsin has been reported to respond to biotic stress caused by pests (Di Gennaro et al. 2005). The mRNA accumulation of the proteinase inhibitor protein (*MPI*) in maize was induced by wounding, abscisic acid and fungal infection (Cordero et al. 1994). These reports suggest that the presentation of serine PIs in plants show somewhat tissue-specific and involved in the response to injury, biotic or abiotic stress.

In maize, CI-1B belongs to the Potato inhibitor I family of serine protease inhibitors, and was suggested to highly express in maize roots and embryos and maybe related to some environmental stress. We investigated the spatial and temporal *ZmCI-1B* expression pattern and isolated its promoter. We bombarded the constructed plasmid into maize embryos for transient expression analysis; *Agrobacterium*-mediated maize transformation was conducted by combining the *ZmCI-1B* promoter with a *GUS* reporter gene. A series of 5′-deleted segments fused with *GUS* gene were also constructed and further transformed into tobacco to investigate the putative regulatory cis-elements of the promoter.

Materials and methods

Plant material and growth conditions

The maize (*Zea mays*) inbred-line B73, Zheng 58, wide-type Hi-II maize and transgenic maize plants were grown in a greenhouse under an 18-h light: 6-h dark photoperiod at average temperatures of 22 °C during the night and 28 °C during the day. Tissues of roots, stems, immature leaves, mature leaves, leaf sheaths from the flare-opening period; immature ears, husks, silks, cobs, immature tassels, mature tassels, glumes, anthers, pollens; seeds of 10 days after pollination (DAP) from B73 plants were collected individually. Embryos and endosperms were also collected at 15 and 20 DAP. All the above samples were frozen and

Fig. 1 Nucleotide sequences of the 2-kb upstream region of the ▶ *ZmCI-1B* gene, the primers and putative cis-acting elements. The nucleotide number with the translation initiation site ATG designated as +1 was shown on the *left*. *Underlines* showed the sequences of the 10 forward primers and 1 common reverse primer for the 10 5′-deletion constructs, with labels shown *beneath*. The putative intron sequences were in *bold*. The putative TATABOX1, CAATBOX1, and several other important putative cis-acting elements were *boxed* and *labeled below* or *above*

ground into powder with liquid nitrogen and stored at −80 °C before use. Wild-type tobacco (*Nicotiana benthamiana*) and all the transgenic tobacco plants were grown in the same greenhouse at the same condition.

RNA isolation and real-time RT-PCR

Total RNA from the above tissues were extracted using TRIzol reagent (Takara Bio, Shiga, Japan). For cDNA synthesis, approximately 4 μg of total RNA was used in 20 μL reaction using Oligo (dT) primer, TranScript RT/RI Enzyme Mix, TS reaction mix and gDNA remover (TransGen Biotech, Beijing, China). Real-time RT-PCR amplification was performed in a total volume of 20 μL with 5 μL 5× or 10× diluted cDNA, 0.2 μM gene-specific primers, 0.4 μL 50× Passive Reference Dye, 10 μL 2× TransStart Tip Green qPCR SuperMix. The PCR reaction was run under the following procedure: initial denaturation at 94 °C for 30 s, followed by 40 cycles composed of denaturation at 94 °C for 5 s and annealing/extension at 60 °C for 34 s. The *ZmCI-1B* gene-specific primers were as follows: forward primer 5′-TGGAAGAAGCCAAGAGGG TGAT-3′, reverse primer 5′-TTAGCCGATGTGGGGTG CC-3′. ABI 7500 real-time PCR system was used for the segment amplification and data analysis. The $\Delta\Delta C_t$ method was used to calculate changes in transcript levels (Yuan et al. 2006). For the real-time RT-PCR analysis, three biological replicates and four technical replicates per each biological replicate were performed. The *β-actin* gene was amplified as the internal control using the forward primer: 5′-ATGTTTCCTGGGATTGCCGAT-3′ and reverse primer: 5′-CCAGTTTCGTCATACTCTCCCTTG-3′.

Construction of the putative full-length *ZmCI-1B* promoter and series of 5′-deleted segments

The putative full-length *ZmCI-1B* promoter and a series of 5′-deleted segments were constructed. The 2-kb upstream region from ATG (+1) (Fig. 1) of the *ZmCI-1B* gene was cloned from the genome of maize B73. Then, it was firstly cloned into the cloning vector pEASY-Blunt (TransGen Biotech, Beijing, China) and then sequenced and designated as the putative full-length *ZmCI-1B* promoter, and named P0 (−2000). Based on bioinformatic analysis of the
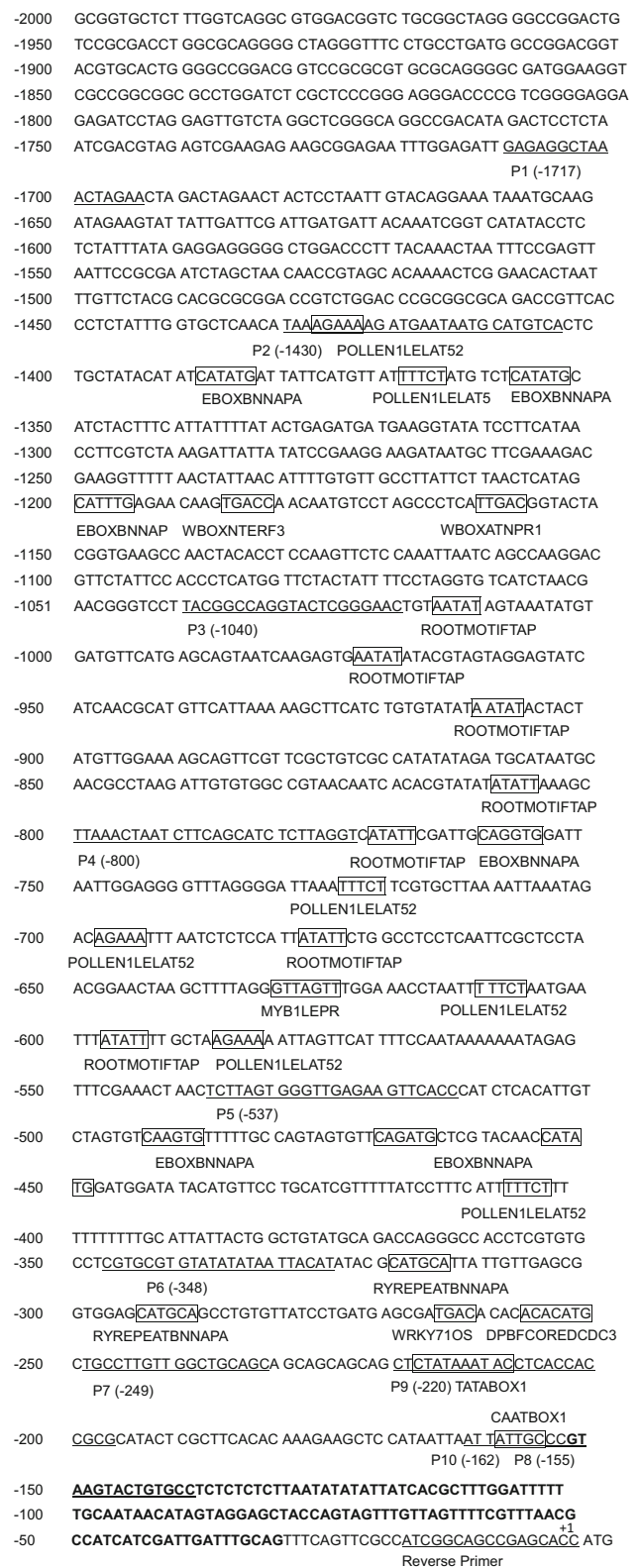
implied cis-acting elements, ten 5′-deleted segments: P1 (−1717), P2 (−1430), P3 (−1040), P4 (−800), P5 (−537), P6 (−348), P7 (−249), P8 (−155), P9 (−220), and P10 (−162) were cloned individually by PCR amplification.

The 5′-end primers were named FP0–FP10; RP was the common 3′-end primer (Supplementary Table 1). Each 5′-end primer was added to a *BamH*I restriction site and the 3′-end was added to a *Nco*I restriction site. The full-length promoter and ten deleted segments were digested with *BamH*I and *Nco*I and then cloned into the pCAMBIA3301 binary vector. The native *CaMV35S* promoter of the vector was cut and replaced by the cloned sequences followed by the *GUS* gene. Each of the eleven constructed plasmids was introduced into *Agrobacterium tumefaciens* strain EHA105.

Maize stable transformation

Immature embryos of Hi-II maize were used as the starting material. Regenerated plants with the putative full-length *ZmCI-1B* promoter-*GUS* (P0 (−2000):*GUS*) construct was obtained using *Agrobacterium*-mediated maize transformation (Zhao et al. 2002). Callus was selected under the treatment of 3 mg/L bialaphos. Transgenic maize plants were further identified by painting herbicide and PCR analysis. T_0 plants were self-crossed to generate T_1 seeds.

Tobacco transformation

Tobacco (*Nicotiana benthamiana*) sterilized plantlets cultured on MS medium (Murashige and Skoog 1962) were used for transformation. The tobacco transformation was carried out according to the leaf-disc method (McCormick et al. 1986). Primary shoots were selected on MS medium containing 4 mg/L phosphinothricin (ppt) and were induced for rooting on 1/2 MS with 3 mg/L ppt. Regenerated tobacco plants were first identified by PCR analysis and then cultivated in an illumination incubator at 28 °C under a photoperiod of 18-h light: 8-h dark. The primary transgenic plantlets were transplanted into pots and grown in greenhouse.

Transient expression analysis in maize embryos

At about 20-DAP, the embryos were isolated from maize Zheng 58 grown in the greenhouse. The 20-DAP ears were first soaked in 5 % hypochlorite solution for half an hour and then washed three times with sterile water. The embryos were isolated and put into sterile liquid MS medium and then transferred into solid hypertonic MS medium (twelve embryos per dish) before use. The embryos were bombarded using a helium pressure of 1350 psi with the Bio-Rad PDS-1000/He system (Hercules, CA, USA). The details of the procedure have been described previously (Taylor et al. 1993). After bombardment, the embryos were transferred to solid MS resting medium incubated at 28 °C in dark for 2 days. Then, they were subjected to GUS histochemical assay.

Mechanical injury

The T_1 generation of transgenic maize matured seeds were harvested and cultured on wet napkins in dark for 3 days at room temperature under aseptic conditions, and the shoots were poked using a needle. Seeds were grown in greenhouse for various periods of time. When the transgenic maize plants reached the trefoil stage or the flare-opening period, their leaves and stems were knife-cut from the living plants. Also, leaves were needled on the surface for mechanical injury treatment. As for tobacco, leaves and stems were cut from 2-month-old transgenic tobacco plants. The tissue segments were then subjected to GUS histochemical staining assay. Wild-type Hi-II maize or tobacco were also sown and treated accordingly as controls.

GUS histochemical staining

Histochemical staining for GUS activity was conducted as described previously (Jefferson et al. 1987). Samples were immersed in GUS staining solution containing 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide), 50 mM Na_2HPO_4, 10 mM EDTA, 1 mM potassium ferrocyanide, and 0.5 % (v/v) Triton X-100 (pH 7.0). Samples were then incubated for 4–8 h at 37 °C. Green tissue samples were then immersed in 95 % ethanol for 1–2 days to remove chlorophyll. As for the tobacco seeds and embryo, after GUS staining, they were cleared in Hoyer's medium following the methods described previously (Hu et al. 2008). A Leica M165FC stereomicroscope (Leica, Wetzlar, Germany) was used to visualize GUS staining for leaves, stems, flowers, seeds and embryos of tobacco.

Results

ZmCI-1B gene expression pattern in maize tissues

In our previous report, *ZmCI-1B* gene (UniGene NO: Zm.121792) exhibited expressing preferentially in maize embryos and roots (Liu et al. 2014). Further and more detailed information of *ZmCI-1B* gene about its accurate expression profiling was unavailable which led us to investigate its expression pattern in more tissues such as glume, anther, pollen, cob, husk by real-time RT-PCR. As shown in Fig. 2, *ZmCI-1B* transcripts in 20-DAP embryo was about 2907-fold higher than it was in mature leaf (m); in 15-DAP embryo, it was about 943-fold; and in root, it was about 816-fold. While the *ZmCI-1B* gene expression in immature leaf, glume, pollen and leaf sheath was weak: in immature leaf, *ZmCI-1B* transcript level was about 173-fold; in glume, it was about 80-fold; in pollen, it was

about 72-fold; and in leaf sheath, it was about 33-fold. Fewer transcripts were detected in stem, immature tassel, mature tassel, anther, immature ear, husk, silk, cob, 10-DAP seeds or 15-, 20-DAP endosperm. The result showed that the transcript level of *ZmCI-1B* gene in embryos and roots was relatively higher than it was in other tissues which was coincident with the previous conclusion that the *ZmCI-1B* gene expression showed somewhat root- and embryo-preferred in maize.

Isolation of the *ZmCI-1B* promoter and its evaluation in a transient expression system

A 2-kb sequence upstream of the ATG codon of the *ZmCI-1B* gene including a 123-bp intron from −152 to −30 bp (bolded) was cloned from the genome of maize B73 (Fig. 1). It was then sequenced and designated as the putative full-length promoter. Subsequently, transient expression analysis followed by histochemical GUS staining assay was conducted to evaluate the promoter activity. Strong GUS staining presented in the bombarded embryos compared to the control suggesting that the 2-kb segment is a functioning *ZmCI-1B* promoter (Fig. 3).

Analysis of the *ZmCI-1B* promoter in transgenic maize plants

Agrobacterium-mediated maize stable transformation was performed to investigate the spatial and temporal expression patterns of the *GUS* gene driven by the *ZmCI-1B* promoter. We got three independent transgenic lines for the *ZmCI-1B* promoter-*GUS* construct. At least three typical *bar*-positive independent lines for each construct were chosen for further analysis. Various transgenic maize tissues at different growth and developmental stages were examined by histochemical GUS staining. To identify GUS activity driven by the *ZmCI-1B* promoter during maize seed development, transgenic maize kernels collected at four stages were harvested and the embryos were removed from the endosperm. No GUS staining was detected in 10-DAP kernel, embryo or endosperm (Fig. 4a–c), but was evident in 15-DAP embryos (Fig. 4d, e), increased in 20-DAP embryos (Fig. 4g, h), and peaked in 25-DAP embryos (Fig. 4j, k). No blue dots were found in endosperm from any stages (Fig. 4c, f, i, and l). These results indicate that GUS activity was limited to embryos and that the activity increased as the embryos developed.

Histochemical GUS staining of transgenic maize plants from different development stages was also performed. As shown in Fig. 4m, n, roots and embryos from transgenic maize seedlings on 3 days after germination (DAG) exhibited marked blue staining, whereas the shoots and endosperms were not stained. The roots from trefoil-stage transgenic maize seedlings and the flare-opening period showed strong GUS activity (Fig. 4p, r), whereas no GUS activity was observed in the stem, leaves, or sheath (shown in the next result). All these results were in agreement with the high *ZmCI-1B* expression level in roots and later-stages embryos indicated by real-time RT-PCR.

Various tissues from different stages of transgenic maize plants were collected, subjected to mechanical injury, and immersed in GUS staining solution. Blue staining was
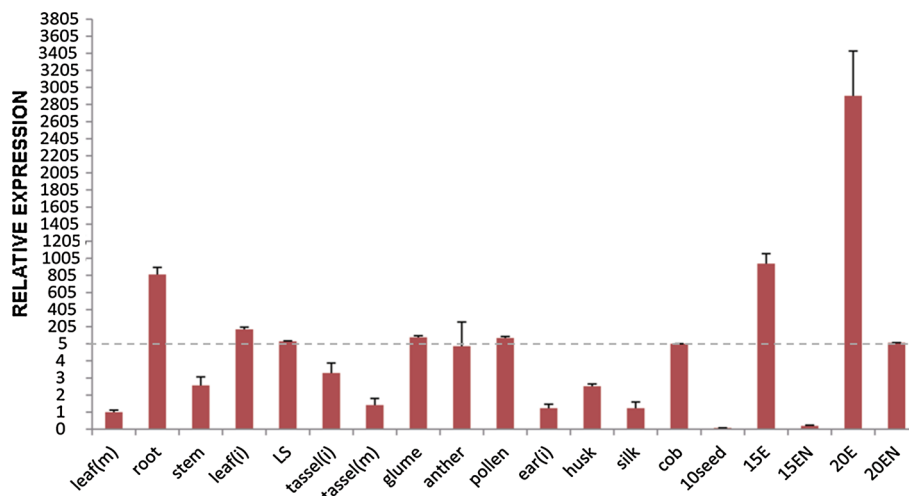

Fig. 2 Expression pattern of the *ZmCI-1B* gene in the maize plant. Expression pattern of the *ZmCI-1B* was established by real-time RT-PCR in various maize tissues during different development stages. *X-axis* shows different tissues. *LS* leaf sheath, *EM* embryo, *EN* endosperm, *i* immature, *m* mature, *10seed* seed at 10-DAP, *15 (20) E/EN* embryo/endosperm at 15 (20)-DAP. *Y-axis* shows relative expression of the *ZmCI-1B* gene. Relative mRNA abundance of the *ZmCI-1B* gene was normalized to that of the maize β-actin gene. Real-time RT-PCR data were analyzed according to the $2^{-\Delta\Delta Ct}$ method. The value in mature leaves [leaf (m)] was the control, to which all other values were normalized. *Error bars* indicate standard deviations

Fig. 3 Histochemical staining of *GUS* gene expression driven by the *ZmCI-1B* full-length promoter in immature maize embryos after bombardment. **a** Embryos bombarded without plasmid DNA as the control. **b** Embryos bombarded with the plasmid carrying the *ZmCI-1B* full-length promoter. *Bars* 1 mm
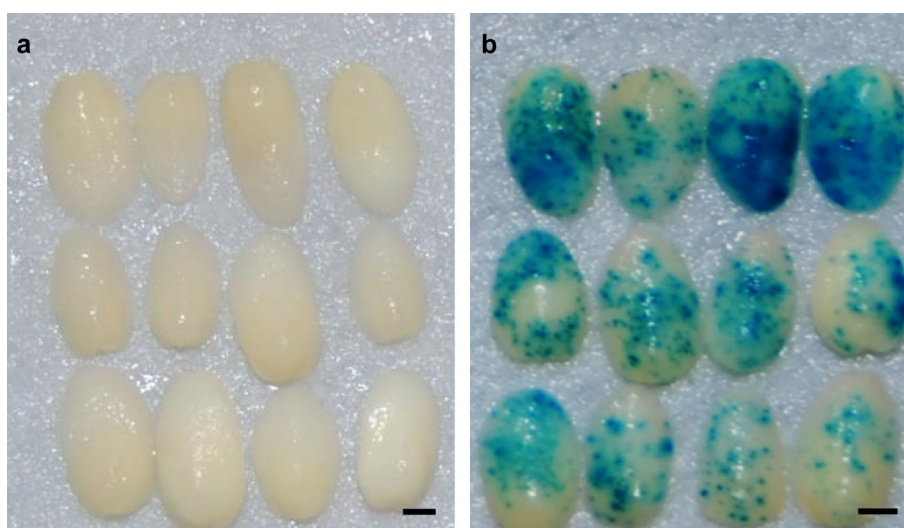
evident in only the shoot regions from maize 3-DAG seedlings poked with a needle (Fig. 5b), indicating that marked GUS activity was induced by injury. Similarly, blue spots were evident at only the edges of the leaves or sheaths cut from the living plants of trefoil-stage maize seedlings (Fig. 5d) or flare-opening period plants (Fig. 5f, j). Moreover, those pinpricks were blue, while the uninjured and inside leaf sections were not stained (Fig. 5d, f). Furthermore, strong GUS staining was seen in the transection of the transgenic maize stems (Fig. 5n). Ear and tassel tissues were also collected and immersed into GUS staining solution. The result showed that blue spots were present in injured regions of husk leaves, silks as well as glumes of the spikelet, and no GUS activity in uninjured region (Fig. 5h, l, p). Thus, the *ZmCI-1B* promoter could efficiently promote *GUS* gene expression in maize in response to mechanical injury.

Construction of 10 5′-truncated promoters and evaluation of their activity in a transient expression system

As shown in Fig. 1, many predicted cis-acting elements presented in the 2-kb promoter region according to the PLANTCARE and PLACE databases (Higo et al. 1999; Lescot et al. 2002). A hypothetical TATABOX1 (CTA-TAAATAC) was located in the −218/−209 bp region, and a putative CAATBOX1 lay between −159 and −155 bp. ROOTMOTIFTAPOX1 (ATATT): the common motifs in the *rolD* promoter and domain A of the *35S* promoter related to root-specific expression (Benfey et al. 1989; Keller and Baumgartner 1991). DPBFCOR-EDCDC3 (ACACNNG): promoted expression of the *ABI5* gene in embryos (Finkelstein and Lynch 2000). The RYREPEATBNNAPA (CATGCA) was a cis-acting element involved in seed-specific expression in dicots and

monocots (Baumlein et al. 1991, 1986; Fiedler et al. 1993). The WBOXNTERF3 (TGAC(C/T)) has been confirmed to be involved in the regulation of wound-induced gene expression (Nishiuchi et al. 2004). These elements or equivalents were all present in the region of the full-length *ZmCI-1B* promoter (Fig. 1). Many other important cis-acting elements such as EBOXBNNAPA, DOFCOREZM, MYBCORE, RHERPATEXPA7 also lie in this region (listed in Table 1).

A series of 5′-deleted segments (P1–P10) according to the cis-acting elements of each were cloned to characterize the *ZmCI-1B* promoter in great detail. Then each of the deletions was linked to a *GUS* reporter gene using the binary vector (as described previously) followed by a transient expression assay and histochemical GUS staining to evaluate the constructed 5′-deleted segments and further to determine the contributions of the cis-acting elements in the full-length *ZmCI-1B* promoter region and to localize the regions responsible for *GUS* expression. We initially constructed seven 5′-deleted segments *P1 (−1717):GUS*, *P2 (−1430):GUS*, *P3 (−1040):GUS*, *P4 (−800):GUS*, *P5 (−537):GUS*, *P6 (−348):GUS*, *P7 (−249):GUS*, which were separately bombarded into immature maize embryos using a particle gun. Histochemical GUS staining showed that all immature embryos were stained blue (Fig. 6a–g). This finding reveals that the seven truncated deletions were active in driving *GUS* gene expression in maize embryos. Subsequently, we constructed another 5′-deleted segment P8 (−155) which was 87-bp shorter than that of P7 (−249) and without the putative TATABOX1 (CTATAAATAC) to determine the core promoter sequence (Fig. 1). The *P8 (−155):GUS* plasmid was also bombarded into immature embryos for a transient expression assay. No staining was seen in the embryos bombarded with the *P8 (−155):GUS* construct (Fig. 6j). Then, we constructed another two constructs: *P9 (−220):GUS* (including TATABOX1) and
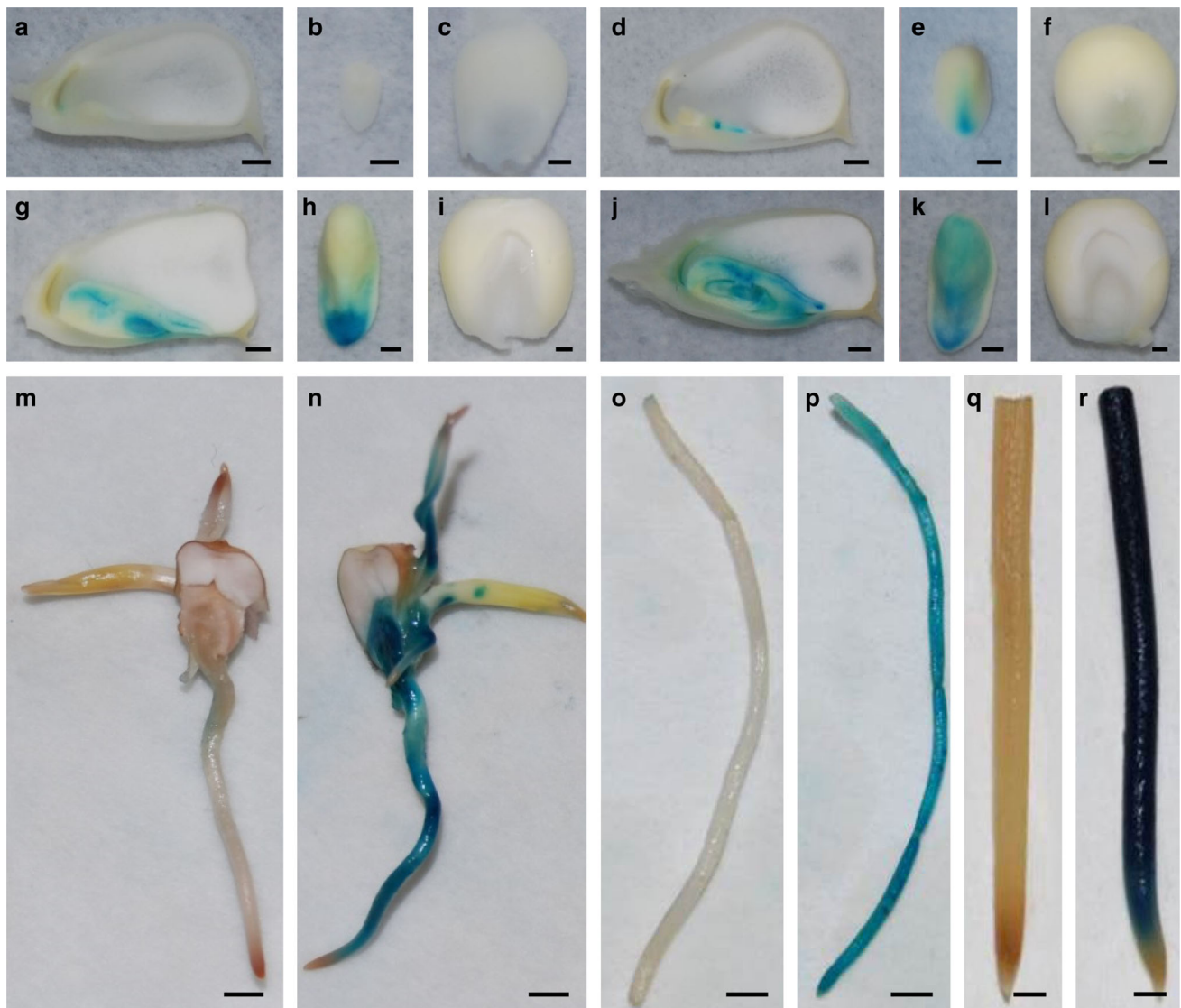
Fig. 4 Histochemical staining of *GUS* gene expression driven by the *ZmCI-1B* full-length promoter in transgenic maize plants. **a–l** Longitudinal sections of 10-, 15-, 20-, and 25-DAP kernels, isolated embryos, and individual endosperm. **a–c** 10-DAP, **d–f** 15-DAP, **g–i** 20-DAP, **j–l** 25-DAP. **m, n** Maize seedlings on 3-DAG. **o, p** Roots of maize at the trefoil-stage. **q, r** Roots of maize from the flare-opening period. **m, o, q** Controls. **a–l** *Bars* 1 mm; **m–r** *Bars* 2 mm

P10 (−162):GUS. Histochemical GUS staining showed that the embryos bombarded with *P10 (−162):GUS* had no stained spots (Fig. 6i) while *P9 (−220):GUS* showed (Fig. 6h), though the blue spots were considerably weaker relative to *P7 (−249):GUS*. Consequently, both P8 (−155) and P10 (−162) lost promoter function, thus, we concluded that the putative TATABOX1 (CTATAAATAC) located in the −218 to −209 bp were critical for accurate initiation. In addition, the truncated promoter P9 (−220), which was 220-bp in length and contained a TATABOX1, CAAT-BOX1, as well as a 123-bp intron, might be the promoter minimally effective in driving GUS expression in maize embryo.

Analysis of the *ZmCI-1B* promoter in transgenic tobacco plants

Considering the relatively low transformation efficiency of maize, we transferred the above 2-kb *ZmCI-1B* promoter-*GUS* and eight active truncated promoter constructs *P1 (−1717):GUS*, *P2 (−1430):GUS*, *P3 (−1040):GUS*, *P4 (−800):GUS*, *P5 (−537):GUS*, *P6 (−348):GUS*, *P7 (−249):GUS*, *P9 (−220):GUS* into tobacco to further investigate the *ZmCI-1B* promoter. We got more than nine independent transgenic lines for each construct. At least six typical *GUS*-positive independent lines for each construct were subjected to histochemical staining analysis. GUS
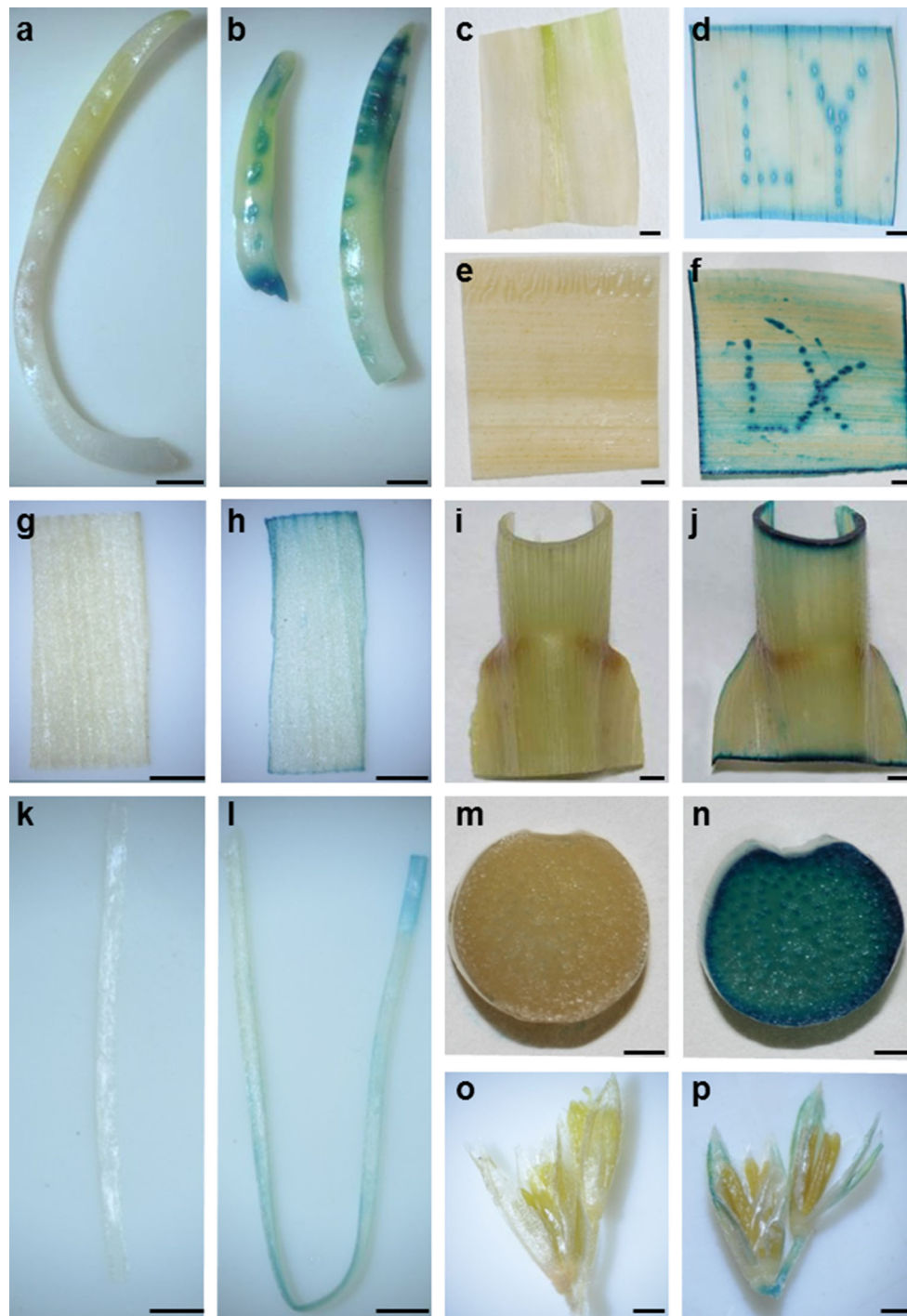
Fig. 5 Histochemical staining of *GUS* gene expression driven by the *ZmCI-1B* full-length promoter in transgenic maize after mechanical injury. **a, b** Injured shoots of maize seedlings on day 3-DAG. **c, d** Injured leaves at trefoil-stage. **e, f** Injured leaves at flare-opening period. **g, h** Husk leaves from mature ear. **i, j** Sheaths at flare-opening period. **k, l** Silks. **m, n** Transection of stems from the flare-opening period. **o, p** Tassel spikelets. **a, c, e, g, i, m, o** Controls. *Bars* 2 mm

activity was examined in leaves, stems, roots, flowers, seeds and cultured cotyledon stage embryos of T_1 transgenic tobacco plants carrying the different length promoters. As shown in Fig. 7a, b, compared to the wild-type, transgenic tobacco plants of *P0 (−2000):GUS* construct showed evident GUS staining in roots, anthers and the knife-cut edges of stems. Deleting the promoter sequence from P0 (−2000) to P1 (−1717) resulted no significant effect on *GUS* expression except that the leaves of *P1 (−1717):GUS* transgenic tobacco showed strong GUS

Table 1 Cis-acting elements in the region of the 2-kb *ZmCI-1B* promoter

Element	Sequence	Element number	Function	References
AACACOREOSGLUB1	AACAAAC	1	Related to the endosperm-specific expression	Wu et al. (2000)
ACGTABOX	TACGTA	2	Responsible for sugar repression	Toyofuku et al. (1998)
ANAERO2CONSENSUS	AGCAGC	4	Responsible for anaerobic regulation	Mohanty et al. (2005)
CAATBOX1	CAAT	17	Related to tissue-preferred gene expression	Shirsat et al. (1989)
CACTFTPPCA1	YACT	29	Related to mesophyll-specific gene expression	Gowik et al. (2004)
CIACADIANLELHC	CAANNNNATC	3	Related to circadian	Piechulla et al. 1998)
CTRMCAMV35S	TCTCTCTCT	1	Enhance gene expression	Pauli et al. (2004)
DOFCOREZM	AAAG	15	Required for binding of Dof proteins in maize	Yanagisawa (2000)
DPBFCOREDCDC3	ACACNNG	5	Related to embryo-specific gene expression	Kim et al. (1997)
EBOXBNNAPA	CANNTG	20	Related to tissue-specific gene expression	Hartmann et al. (2005)
GTGANTG10	GTGA	12	Related to pollen-specific gene expression	Rogers et al. (2001)
MYB1LEPR	GTTAGTT	3	Related to defense	Chakravarthy et al. (2003)
MYBCORE	CNGTTR	2	Response to drought, flavonoid biosynthesis	Solano et al. (1995)
MYCCONSENSUSAT	CANNTG	20	Response to dehydration	Chinnusamy et al. (2004)
OSE1ROOTNODULE	AAAGAT	8	Related to activation in infected cells of root nodules	Fehlberg et al. (2005)
POLLEN1LELAT52	AGAAA	7	Related to anther-specific gene expression	Filichkin et al. (2004)
RAV1AAT	CAACA	7	Related to tissue-preferred gene expression	Kagaya et al. (1999)
RHERPATEXPA7	KCACGW	3	A root hair-specific cis-element	Kim et al. (2006)
ROOTMOTIFTAPOX1	ATATT	10	Related to root-specific gene expression	Elmayan and Tepfer (1995)
RYREPEATBNNAPA	CATGCA	3	Required for seed specific expression	Ezcurra et al. (1999)
TAAAGSTKST1	TAAAG	4	Related to cell-specific gene expression	Plesch et al. (2001)
UP2ATMSD	AAACCCTA	1	Related to axillary bud outgrowth	Tatematsu et al. (2005)
WBOXATNPR1	TTGAC	2	Related to disease resistance	Yu et al. (2001)
WBOXNTERF3	TGACY	4	Response to wounding	Nishiuchi et al. (2004)
WRKY71OS	TGAC	9	Related to pathogenesis resistance	Eulgem et al. (1999)
XYLAT	ACAAAGAA	1	Related to wood formation	Ko et al. (2006)

B = T, C or G; K = G or T; N: A, T, C or G; W = T or A; R: A or G; Y = T or C

staining in the injured edges (Fig. 7c). More interestingly, reducing the promoter sequence from P1 (−1717) to P2 (−1430) caused very low GUS staining in green tissues and no response to mechanical injury (Fig. 7d). This losing inducible promoter function suggested there must be one cis-element in response to wounding in −1717 to −1430 bp region. When further reduced to P3 (−1040), the promoter recovered GUS staining in knife-cut edges of leaves and stems (Fig. 7e) which suggested certain negative cis-element(s) in response to wounding stress must exist in the −1430 to −1040 bp region. When deleted from P3 (−1040) to P4 (−800), P4 (−800) to P5 (−537) and P5 (−537) to P6 (−348), there was no significant effect on *GUS* expression pattern, they all driven *GUS* expression in roots, anthers, embryos and the knife-cut edges of leaves and stems (Fig. 7f–h). But when the −348 to −249 bp sequences were deleted, the transgenic plants harboring *P7* (−249):*GUS* constructs only showed GUS activity in root, anther but not wounding induced (Fig. 7i). That meant the P7 (−249) truncated promoter could not response to injury

which suggested there must be an important cis-element conferring wounding-inducibility in −348 to −249 bp region. Surprisingly, the deletion from P7 (−249) to P9 (−220) recovered the GUS staining in knife-cut edges of leaves and stems (Fig. 7j) which suggested the 29 bp sequence contains a negative wounding-involved cis-element.

As for flowers, all the nine different length promoters drive strong *GUS* expression in stamen, whereas no or very low *GUS* expression was present in pistil tissues such as style, stigma, ovary. In great detail, the *GUS* expression mainly located in anthers and also in part region of sepal in transgenic plants (Fig. 7). In this case, the promoters revealed somewhat anther-preferred in flowers. Mature seeds were cultured for 2 days and the embryos were isolated. Then, they were all subjected to GUS staining solution. The result showed that the seeds of transgenic plants from all the nine different length promoters-*GUS* constructs were stained blue, in detail, the blue spots located in embryonic tissues (Fig. 8 and Supplementary Fig. 1).
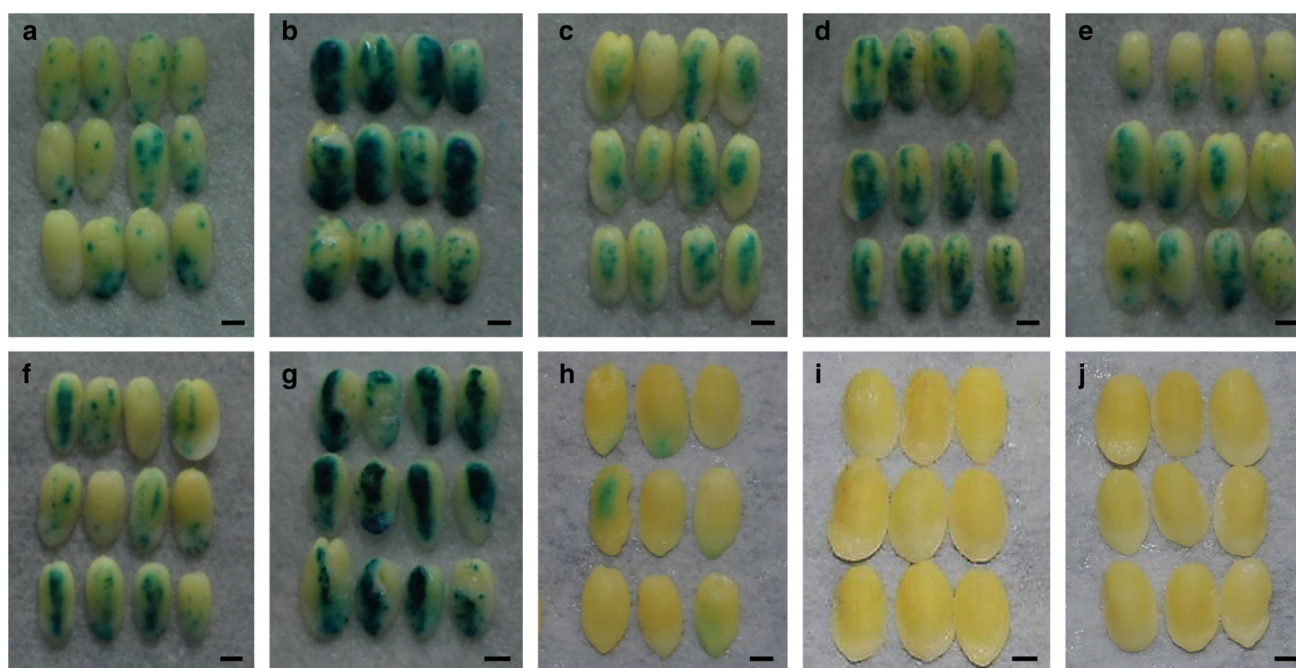
Fig. 6 Comparison of transient histochemical *GUS* gene expression in maize 20-DAP embryos driven by the 10 5′-deleted constructs after bombardment. *Blue coloration* indicates GUS activity. **a–g** Embryos transformed with the P1 (−1717) to P7 (−249) plasmids, respectively. **h** Embryos transformed with the P9 (−220) plasmid. **i, j** Embryos transformed with P8 (−155) and P10 (−162) plasmids revealed no GUS staining. *Bars* 1 mm (color figure online)

In addition, the results revealed that the P9 (−220 bp) segment could drive *GUS* expression in root, anther, embryo tissues and also in response to injury in transgenic tobacco (Fig. 7j and Supplementary Fig. 1l, p) suggesting that the −220 bp sequence containing root-, anther-, embryo-related cis-elements and wounding-involved regulatory elements. Thus, the core promoter of *ZmCI-1B* should be located within −220 bp.

Discussion

Zea mays is one of the most important crops globally and serves as major livestock feed for animals. The elucidation of gene expression and regulation is essential for functional characterization of genes, biotechnology breeding and biofortification in maize. Promoters, including tissue-specific and inducible promoters, are important regulatory elements for research on genetic improvement of maize yield and quality; thus, they have been attracting much attention.

Plant growth and development relies on the mineral nutrients available in the environment. Mineral nutrients deficiency is one of the key constraints on crops yield and quality. Previous reports showed that iron and zinc mainly stored in the embryo scutellum and aleurone and their increase relay on uptake, long-distance transport from the root (Brinch-Pedersen et al. 2007; Ishimaru et al. 2005). Thus, we hypothesized that a root- and embryo-preferred promoter driving targeted gene expression in both roots and embryos would be more useful for plant nutrient research because this kind of promoter is promising in terms of mineral nutrients uptake, transport and homeostasis.

Previous reports indicated that expression of protease inhibitor gene was induced in vegetative tissues as a response to wounding involved in enhancing insect resistance (Koiwa et al. 1997).Our GUS histochemical assay findings regarding the *ZmCI-1B* promoter-*GUS* transgenic maize showed that the *ZmCI-1B* promoter may be a root- and embryo-preferred promoter. Cordero et al. 1994 reported that the accumulation of the *MPI* mRNA was induced in response to fungal infection in maize embryos. Serine protease inhibitors mainly accumulate in seed or tubers to facilitate storage protein accumulation (Koiwa et al. 1997). This maybe the reason for the high GUS activity in root and embryo of maize plants harboring the *ZmCI-1B* promoter-*GUS* construct. Consistent with these reports, *ZmCI-1B* promoter induced *GUS* gene expression in mechanical injured tissues such as leaves and stem. This additional characteristic makes the *ZmCI-1B* promoter more interesting, as it may also be useful for plant engineering research on environmental stress. To our knowledge, this is the first report of a maize PI gene promoter driving strong expression in roots, embryos, and mechanically injured
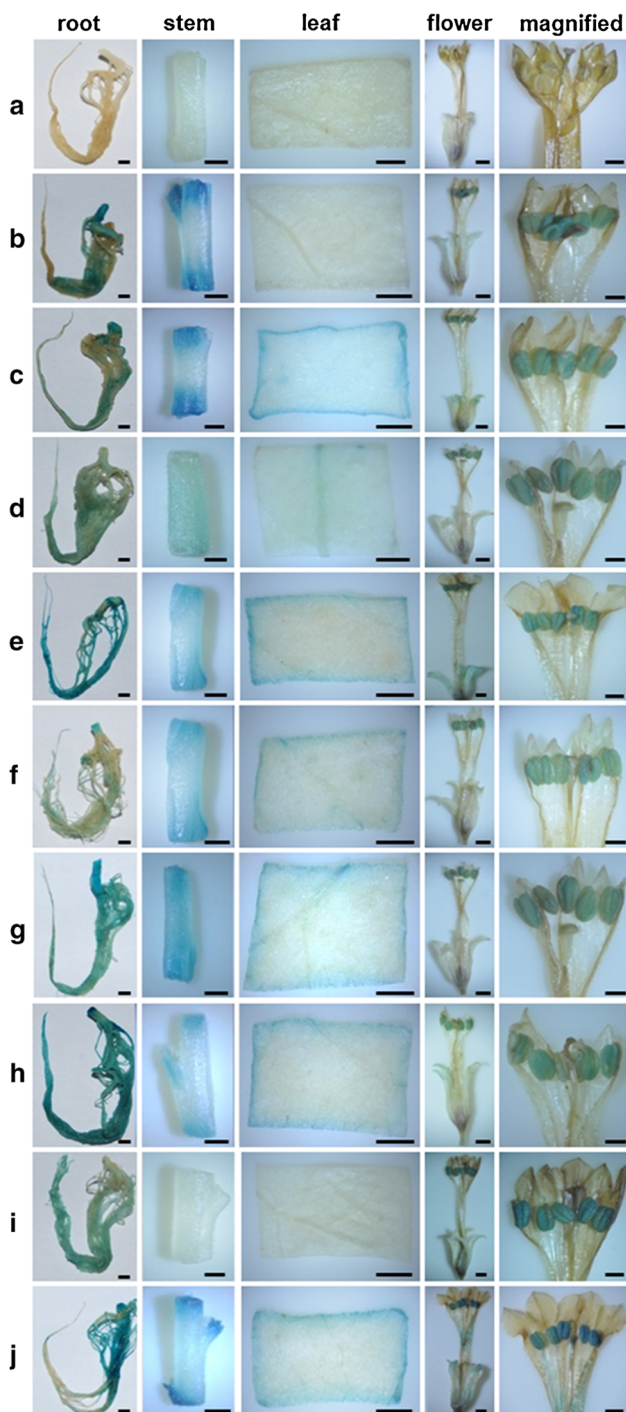
Fig. 7 Histochemical staining of *GUS* gene expression driven by the *ZmCI-1B* full-length promoter and several 5′-truncated promoters in transgenic tobacco. All the transgenic plants were from the T_1 generation. For each construct, at least six typical *GUS*-positive independent lines for each construct were subjected to histochemical staining analysis. The GUS histochemical staining was conducted in various tissues namely root, stem, mature leaf, flower of wild-type and transgenic tobacco plants. **a** wild-type; **b** *P0 (−2000):GUS*; **c** *P1 (−1717):GUS*; **d** *P2 (−1430):GUS*; **e** *P3 (−1040):GUS*; **f** *P4 (−800):GUS*; **g** *P5 (−537):GUS*; **h** *P6 (−348):GUS*; **i** *P7 (−249):GUS*; **j** *P9 (−220):GUS*. For root, stem, mature leaves, flower, *bars* 2 mm; for magnified view of the top flower, *bars* 1 mm

tissues. Thus, the *ZmCI-1B* promoter cloned here may be of great value for improvement of maize quality and responsiveness to biotic or abiotic stress.

We confirmed that *ZmCI-1B* expression could be induced by mechanical injury. Thus, the low transcript levels of the *ZmCI-1B* gene in immature leaf (Fig. 1), the limited GUS staining in shoots (Fig. 4n) and low GUS staining in sepal of P0 (−2000), P1 (−1717), P2 (−1430), P3 (−1040), P6 (−348), P9 (−220) transgenic plants (Fig. 7b, c, d, e, h, j) may be caused by careless injury during collection of tissues or during the experiment itself.

The GUS staining result showed that *ZmCI-1B* promoter expressed preferentially in maize roots and embryos, while in tobacco, it was detected in root, embryonic tissues and anthers. This revealed that the *ZmCI-1B* gene promoter could drive foreign gene in a tissue-specific and wounding-induced manner. In this case, promoters in monocotyledon may have similar function in dicot plants. However, there was strong staining in anthers of transgenic tobacco (Fig. 7), while the expression profiling of *ZmCI-1B* gene from the real-time RT-PCR data showed that very low transcript level in maize anthers (Fig. 2), consistently, the GUS histochemical assay in transgenic maize showed that no GUS activity was detected in maize anthers (Fig. 5p). This discrepancy revealed that anther-specific cis-acting elements in the *ZmCI-1B* gene promoter function normally in tobacco but not in maize, this may be due to differences in the exogenous environment of the *ZmCI-1B* promoter between maize and tobacco.

Cis-acting elements in promoter region are important molecular switches for gene expression. The histochemical GUS staining showed that the deletions of P1 (−1717), P3 (−1040), P4 (−800), P5 (−537), P6 (−348) and P9 (−220) could drive *GUS* expression in root, anther, embryonic- and injured- tissues. A search from the PLACE database for possible cis-elements in the 220-bp region of *ZmCI-1B* promoter revealed the core promoter motifs TATABOX1 as expected, several potential regulatory cis-elements such as CACTFTPPCA, CGCG box, CT-rich motif, GTGANTG10 (Table 2) presented in the region as well. We speculated the existence of ROOTMOTIFTAPOX1, DOFCOREZM, GTGANTG10 may confer the *ZmCI-1B* promoter of root-, embryo- and anther-preferred traits. Furthermore, there must be a potential wounding-related element in the 220 bp sequence for the activity in response to injury which we are still not clear. When deleted from P6 (−348) to P7 (−249), the promoter resulted in no response to injury either in leaves or stems of transgenic tobacco, this indicated that some wounding-related elements may locate in the 99 bp sequence. As shown in Fig. 1, several putative tissue-specific elements such as RYREPEATBNNAPA, DPBFCOREDCDC3 and a wounding-related element WRKY71OS located in the

Fig. 8 Histochemical staining of transgenic tobacco 2-DAG seeds and the cultured cotyledon stage embryos expressing recombinant promoter. **a, b** wild-type; **c, d** *P1 (−1717):GUS*; **a, c** seed; **b, d** cultured cotyledon stage embryos. *Bars* 0.1 mm
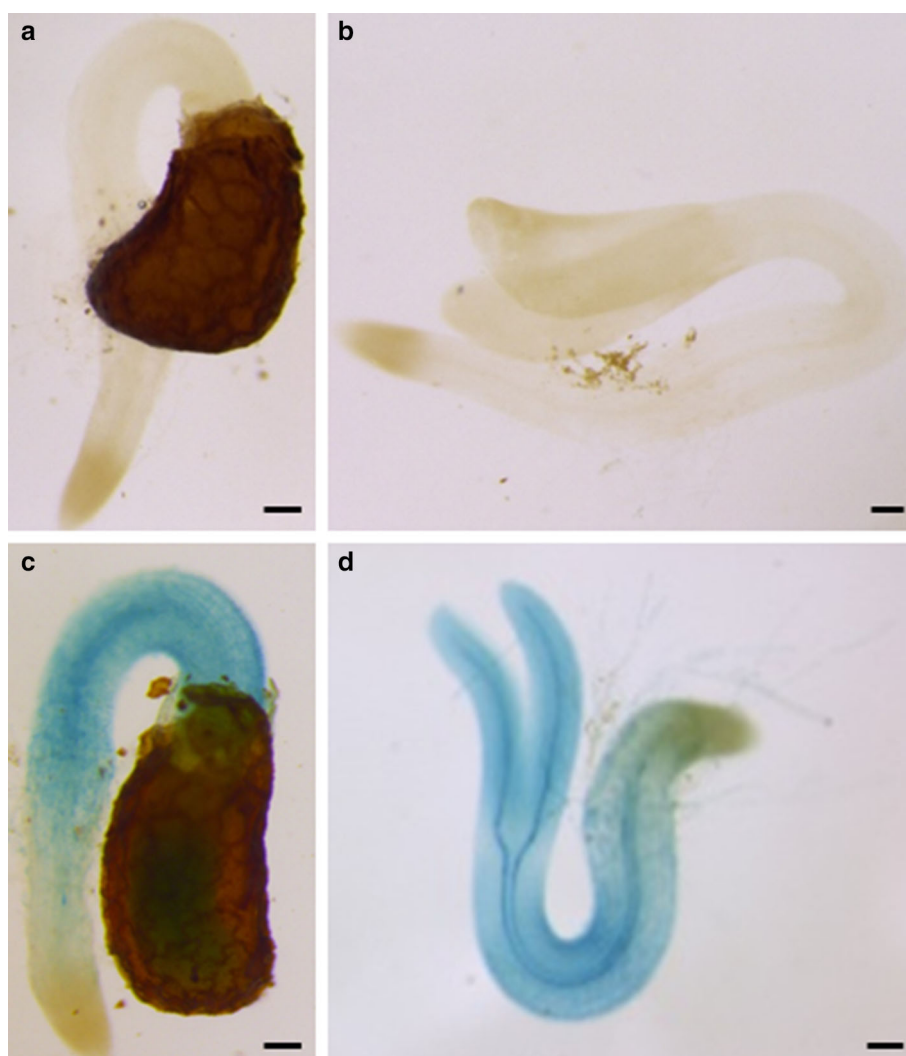

Table 2 Cis-acting elements in the region of the P9 (220-bp) truncated *ZmCI-1B* promoter

Element	Sequence	Element number	Function	References
CAATBOX1	CAAT	3	Related to tissue-specific gene expression	Shirsat et al. (1989)
CACTFTPPCA1	YACT	5	Related to mesophyll-specific gene expression	Gowik et al. (2004)
CT-rich motif	TCTCTCTCT	1	Enhancer	Pauli et al. (2004)
CURECORECR	GTAC	2	Response to copper signal	Moseley et al. (2000)
DOFCOREZM	AAAG	2	Required for binding of Dof proteins in maize	Yanagisawa (2000)
GAREAT	TAACAAR	1	Response to GA signal	Ogawa et al. 2003)
GATABOX	GTTAGTT	1	Response to light signal	Gidoni et al. (1989)
GTGANTG10	GTGA	3	Related to pollen-specific gene expression	Rogers et al. 2001)
MYB1LEPR	GTTAGTT	1	Response to stress	Singh et al. (2002)
OSE2ROOTNODULE	CTCTT	1	Related to activation in infected cells of root nodules	Stougaard et al. (1987)
ROOTMOTIFTAPOX1	ATATT	2	Related to root-specific gene expression	Elmayan and Tepfer (1995)
XYLAT	ACAAAGAA	1	Related to wood formation	Ko et al. (2006)

R: A or G; Y = T or C

−348 to −250 bp region. Transgenic tobacco plants carrying *P1 (−1717):GUS* showed strong *GUS* expression in root, embryo, anther and injured leaves or stem, when deleted from P1 (−1717) to P2 (−1430) caused to the lost activity in injured leaves and stem. And when further deleted from P2 (−1430) to P3 (−1040) resulted in the recovery of activity in injured leaves. These results suggested that there must be a binding site for wounding-induce in −1717 to −1430 bp region and some suppressor(s) for wounding-induce in −1430 to −1040 bp region. The leaves of P0 (−2000) transgenic tobacco plants showed no response to mechanical injury was a little puzzled suggesting further investigation was needed to carry out.

Conclusion

We characterized a novel maize tissue-preferred and wounding-related promoter that regulated the expression of an exogenous gene preferentially in roots, embryos, and injured tissues through cis-elements in the promoter sequence. Using 5′-deletion analysis and histochemical GUS staining in transgenic tobacco plants, we identified the 220-bp minimally effective promoter. Our results may be useful for genetic engineering approaches to investigation of maize absorption and storage of soil nutrients, as well as protection against abiotic- or biotic- stress.

Author contribution statement RMC conceived and designed the research. YL and XQL performed the bioinformatics analysis, gene cloning, real-time RT-PCR, particle bombardment, maize stable transformation and tobacco transformation. JL, GYC, SZL, XJZ and WZY participated in molecular analysis. YL drafted the manuscript, and RMC contributed to revisions of the manuscript. All authors read and approved the final manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

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