Green Tissue-Specific Analysis of a Cloned *rbcS* Promoter from *Lemna gibba*

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Abstract

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Many plant genetic engineering taskss require the spatial expression of genes which in turn depends upon the availability of specific promoters. The present paper analyses the green-tissue characteristics of a new *L. gibba rbcS* promoter driving the expression of the *gus* gene in transgenic tobacco. A 1491 bp *rbcS* (small subunit of ribulose bisphosphate carboxylase) promoter was isolated from *Lemna gibba*. The sequence analysis revealed that this promoter is different from the previously reported *rbcS* promoter and is named *SSU5C*. A 1438 bp fragment of the *SSU5C* promoter was fused with the gus gene and transgenic tobacco plants were generated. The analysis of T_1 tobacco *p1438-gus* revealed that GUS expression driven by the *SSU5C* promoter was detected in the green part of vegetative organs. The promoter deletion analysis confirmed a region from position –152 to –49 relative to the start of transcription containing boxes X, Y and Z, while a positive regulatory region conferred green tissue-specific expression. Further functional analysis of constructs of box-X, Y, Z, which was fused with the basal *SSU5C* promoter, confirmed that the boxes X, Y and Z represent the new minimized functional promoter, respectively, and are able to direct green tissue-specific expression. This promoter may be used for gene expression in a tissue-specific manner in plant molecular breeding.

Keywords: *Lemna gibba*; *rbcS*; *SSU5C* promoter; tissue characterization

So far, constitutive promoters, in particular the cauliflower mosaic virus (CaMV) 35S promoter, have been widely used in plant genetic engineering. It has been accepted, however, that the constitutive expression of foreign proteins may impose extra metabolic burden as well as other adverse effects on transgenic plants, finally resulting in abnormal development (Anisimov *et al.* 2007). One possible approach to overcome the aforementioned obstacles is to use a strong tissue-specific promoter that is not constitutively active throughout the entire life cycle of the plant, but active only during definite stages of plant development (Anisimov *et al*. 2007).

Although a large number of tissue-specific promoters have been identified, the regulatory mechanism of tissue-specific gene expression remains unclear, the transcription factors that interact with these identified promoter elements are yet to be identified. The transcriptional factors and sequence elements involved in the tissue-specific regulation of *rbcS* promoter still remain elusive (Arguello-Astorga *et al.* 1996; Arguello-Astorga 1998; Gray *et al*. 2003; Walker *et al*. 2005; Anisimov *et al*. 2007). The RbcS gene exists in numerous plants and autotrophic bacteria, and the RbcS protein plays a major role in the assimilation and conversion of atmospheric carbon dioxide during photosynthesis (Walker *et al.* 2005). Ribulose-1,5-bisphosphate carboxylase is the most abundant soluble protein in leaves, accounting for approximately 50–60% of the total soluble protein (Donald & Cashmore 1990; Arguello-Astorga *et al*. 1996; Arguello-Astorga 1998; Gray *et al*. 2003; Walker *et al*. 2005; Anisimov *et al*. 2007). Since RbcS is highly expressed, its promoter is commonly used in transgenic plants (Wang *et al*. 2013).

Three *rbcS* (the small subunit of ribulose-1,5-bisphosphate carboxylase) promoters from *L. gibba*

have been isolated, namely *SSU13*, *SSU5A* and *SSU5B*. 5' deletion analysis of *SSU5B* promoter in a transient assay system defined a region from position –205 to position –83 relative to the start of transcription as necessary to observe the phytochrome response (Buzby *et al*. 1990; Rolfe 1991), but the tissue characterization of *SSU5B, SSU13, SSU5A* promoter was not intensively studied (Stiekema *et al*. 1983; Silverthorne *et al*. 1990).

A small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcS*) gene promoter was cloned from *L. gibba*. Sequence analysis revealed that this promoter is different from the previously reported rbcs promoters and is named *SSU5C*. Analysis of T_1 transgenic tobacco plants with a reporter gene under the control of the *SSU5C* promoter revealed that this promoter is tissue-specific and is positively regulated by red light. Promoter deletion analysis confirmed a region from position –152 to –49 relative to the start of transcription containing boxes X, Y and Z, and is identified to be critical for phytochrome responses. Further functional analysis of constructs of box-X, Y, Z, which was respectively fused with the basal *SSU5C* promoter, defined that boxes X, Y and Z alone are able to direct phytochrome-regulated expression (Wang *et al*. 2011).

By 5' deletion of *SSU5C* promoter, we determined that a 152-bp short sequence of *SSU5C* promoter was sufficient to confer a transcriptional response to green organ-specific cues. Further deletion analysis of *SSU5C* promoter defined that boxes X, Y and Z of *SSU5C* promoter are alone able to direct green tissue-specific expression.

MATERIAL AND METHODS

Plant materials and growth conditions. Sterile seeds of tobacco (*Nicotiana tabacum* cv. Xanthi) were germinated and seedlings were grown on MS basal medium (Murashige & Skoog 1962) for 2 months for *Agrobacterium*-mediated transformation. The cultures and stable transgenic tobacco lines were maintained in a growth chamber at 25°C under cool white fluorescent lighting (90–100 μmol photons per m^2/s) in a 16/8 h (light/dark) photoperiod.

Sequence analysis of the *cloning rbcS* promoter from *Lemna gibba*. Genomic DNA was isolated from fronds of *L. gibba* using the modified CTAB extraction method (Luo *et al*. 2006). Based on the known *L. gibba rbcS* gene sequences, a new *rbcS* gene with the size of 1346 bp was obtained by PCR (data unpublished), *L. gibba* genomic DNA (3–5 μg) was completely digested with 30 units of isocaudamers (e.g. *Bam* HI and *Bgl*II) in a total volume of 50 μl at 37°C for 2–4 h. The digested DNA fragments between 2 kb and 4 kb were purified and self-ligated at a concentration of 0.3–0.5 ng/μl in the presence of 3 U/ml T_4 DNA ligase (Promega) overnight at 15°C. The ligation mixture was extracted with phenol/chloroform, precipitated with ethanol, and resuspended in sterile distilled H_2O to a concentration of 20 ng/μl. The total DNA of *L. gibba* without enzyme digestion was used as a control template for I-PCR. The 5' region of *L. gibba rbcS* gene was obtained by the first round of PCR and the second round nested PCR using the ligation mixture as a template through the primers (Table 1). Nested PCR products were cloned into T-vector (Takara, Dalian,China) and sequenced. The identified *SSU5C* promoter was aligned to the known *rbcS* gene promoters from *L. gibba* using MegAlign 5.0 (DNASTAR, Madison, USA) and subsequently manually improved. The location and distribution of cis-regulatory sequence elements in the *SSU5C* promoter were analysed by a signal scan search in the PLACE database (http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html). The closest homologues to the *rbcS* promoter were identified by a homology-based search in the PLACE database.

Construction of vectors and *Agrobacterium* **transformation**. *SSU5C* promoter fragments were

Table 1. Primers used for the construction of the *SSU5C* promoter deletion plasmids

ePrimers	Primers sequence (5' to 3')	Constructs
PF1 (–700)	ttt*ggatcc*GCTATTGCTTGTGGAAACCAACT	p700-GUS
PF2 (–510)	ttt*ggatcc* ACTATGCCCCCCTAACAAAACACAC	p510-GUS
PF3 (–310)	ttt*ggatcc* ACCACTTATCCCATCCCACCTAACA	p310-GUS
PF4 (–152)	ttt*ggatcc* CTCCATAAAAGTGTAGCCCCTTGAG	p152-GUS
PF5 (–49)	ttt*ggatcc* CCCTAAGACAGTGACTGTCCCTT	p49-GUS
PR (–1)	*ttt**aagctt*** TGAGAGAGCTCTTGGTCTGATTAAAT	

Italics indicates restriction sites of *Bam*HI and *Hind*III nucleases; underlined *ggatcc* are *Bam*HI site, and underlined *aagctt* is *Hind*III site

Table 2. Primers used for constructs of box-X, Y, Z fused with the basal *SSU5C* promoter

Primers	Primers sequence (5' to 3')	Constructs
PF6 (box X)	ttt*ggatcc* ATAAAAGTGTAGCCAACCCTAAGACAGTGACTG	*p*X-IGUS
PF7 (box Y)	ttt*ggatcc*TGGAGGAGCGGGTGGATTGTCAACCCTAAGACAGTGACTG	*p*Y-IGUS
PF8 (box Z)	ttt*ggatcc* GATCGGACCATGGCAGCCATAGAATACCCAACCCTAAGACAGTGACTG	*p*Z-IGUS
PF9 (box XY)	ttt*ggatcc***ATAAAAGTGTAGCC(box-X)**CCTTGAGATAAGACAGTTG**TGGAG-GAGCGGGTGGATTGT (Box-Y)**ACCCTAAGACAGTGACTGT	*p*XY-IGUS
PF10(box YZ)	ttt*ggatcc*G**TGGAGGAGCGGGTGGATTGTC(box-Y)**CCCCCTTGCAGAA**GATCGGACC ATGGCAGCCATAGAATACCC(box-Z)**AAAACCCTAAGACAGTGACTGT	*p*YZ-IGUS
PF11(box XZ)	ttt*ggatcc***ATAAAAGTGTAGCC(box-X)**CCTTGAGAGAA**GATCGGACCATGGCAGCCAT AGAATACCC(box-Z)**AAAACCCTAAGACAGTGACTGT	*p*XZ-IGUS
PR (–1)	*ttt**aagctt*** TGAGAGAGCTCTTGGTCTGATTAAAT	

Italics indicates restriction sites of *Bam*HI and *Hind*III nucleases; underlined *ggatcc* are *Bam*HI site, and underlined *aagctt* is *Hind*III site

amplified by PCR with a pair of primers (PF: ttt*ggatcc*CGTAAGCCAGTAATGACGAATC, PR: *tttaagctt*TGAGAGGCTCTATGGTCTGATTAAAT) carrying a *Bam*HI(*ggatcc*) and *Hind*III(*aagctt*) restriction site performed for 30 cycles. The PCR products obtained were sequenced and cleaved with *Bam*HI and *Hind*III, then ligated into the vector pCAMBIA1381 digested with *Bam*HI and *Hind*III to construct 5' deletion series of *SSU5C* promoter-*gus* constructs (Tables 1 and 2). *35S-gus* was constructed in the same way. The constructs were transferred into *Agrobacterium tumefaciens* EHA105 and used for plant transformation. A leaf disc transformation method was employed for tobacco transformation (Horsch *et al*. 1985). The rooted T_0 seedlings were transferred to soil and grown. Independently derived T_1 transgenic tobacco lines were selected for further study.

GUS assay in transgenic tobacco. GUS histochemical analysis was performed as described by Acevedo-Hernandez *et al*. (2005). The GUS enzymatic activity measurements of different tissues were conducted as previously described by Jefferson (1987). GUS activity was reported as picomoles of 4-methylumbelliferone per microgram of protein per minute, three replicates were performed for each sample.

RESULTS

Sequence analysis of the isolated *SSU5C* promoter from *Lemna gibba*. A novel *rbcS* gene with the size of 1346 bp was cloned from *L. gibba* genomic DNA (data unpublished). A 1491 bp 5' flanking region of *lgrbcS* gene (named *SSU5C* promoter) was isolated by 5'-walking technology (Figure 1). A putative TATA box, which served as basal promoter elements for the transcription of eukaryotic genes, was found in *SSU5C* promoter. Using PLACE (http://dna.affrc.go.jp/PLACE), a number of putative regulatory elements corresponding to the known cis-elements of

AAATTTGTCTTTAAGGATGGGGTGGCTGGACTTCAATGCTTAAGGTGAAAATTTTTCAA
CATCCTGCACAAGTTCCTCTCTCTCTCTCCATCTCTCTTGGTGAAAAATATTTTTTTG
AACCTTATATATCTCTTCTTACAAAAACTTAATTTTTAGCAAAGCACTTCGTTCTCTTTCT
CACTCAGACACAAACAAAAAGATAACTTTTAGCAAACCACCTCATCTCCCTTTCTCTAA
ATCTTCATCAAAAACTCAATTTTTGGTTGAATCCCTCTATCTATATTTGAAGGAAACCTA
TTTCAATCAAATATCTCACATTTCACAGAAATTGGTATTGATTATTGAATTCAATATCGTA
AGCCAGTAATTTTTGACGAATCCCTCTCTCTATATTTGTAGGAAACCCTATTTTTGTCAA
ATCCCTCACATTTCACAAATTTGGTATTGATTATTGAATTGGGTACTTCGAATATGTAATT
GAGTCAAAATGTCCATCCACGTCCCTTAGGAAGTCAATGCATGGTACAACTATTGGGCA
CCAATTCATTAACAATGCTCAATAGAAAATTCATGCCTAATATTTAACTAGATATCACTTT
CTCCCTTCATGGGGTATCAGTGTGCGTGTCACCAGGATCAGGGCAAGGATCGAACGAC
GAACACTAGTTGGAATTTATATAGAGCCTTTGTTTAATGAGGCATTTCATGTCTTCTTTG
GAACACAAACTAGTTGTCACTT ATCTCATATCTTCGTTGGAAAACAAACCAATATCTCA
CTAATCACGCC**GCTATTGCTTGTGGAAACCAACT**AATGACCACTCCTTTAATCCTTAG
PF1
GCCAAATCAACATTGCCTCCTGGAAGAAGTCACCAAGCCTCTCCCACTATCAACATTA
CCCTTGTATGCCATCAAAATTCACAGCAAATTCCACCTTCTCCCACCATGTGTTCTTAGC
TACATGAACCTTAAGTGTCAATTAT**ACTATGCCCCCCTAACAAAACACAC**CCAAGAC
PF2
AATAATTGGATTTGAATAGGTGAGTTTAATTTATTTTGAAATATTTAAATCCACCTGACTC
CAATAATATCTCACTCTATCACAACAAAGACAAAATCTTCTTATTTTTTTAAGTGTATAA
TGAAAGTGAAGCAAGCAAATAAACTTATCCCTAGATAGTAATTCCAT**ACCACTTATCCC**
ATCCCACCTAACACAAGTATCATCCCCTACTCACTCTCCCCTCCAATAAAACCACTCAA
PF3
ATCCATCAAAACCATACATCTCAAGATAAAGTAATGCAACCCTAAACCACATATCCCTA
GGCTTTGTCCTTTTCCCACAGATAGCCT**CTCC**ATAAAAGTGTAGCC**CCTTGAG**ATAA
Box X PF4
GACAGTTGTGGAGGAGCGGGTGGATTGTCCCCCCTTGCAGAAGATCGGACCATGG
BoxY Box Z
CAGCCATAGAATACCCAAA**CCCTAAGACAGTGACTGTCCCTTA**T**T**TAAT**T**CA
PF5 /PF6/ PF7/ PF8/ PF9/ PF10 TATA box
GACCAAGAGCTCTCTCAGAGACCCCAGAGCTTCCGAGAAGAAGAGGAAGAGAGAGA

GAAGGAGAGTG ACCatg

Figure 1. Sequence of *SSU5C* promoter from *Lemna gibba* PF1-PF5 represent the primers for the construction of deletion promoters fused with the GUS coding sequence, PF6-PF10 represent the primers fused with different conserved boxes to construct vectors harbouring the basal *SSU5C* promoter; box X, box Y and box Z are predicted conserved regions of *SSU5C* promoter

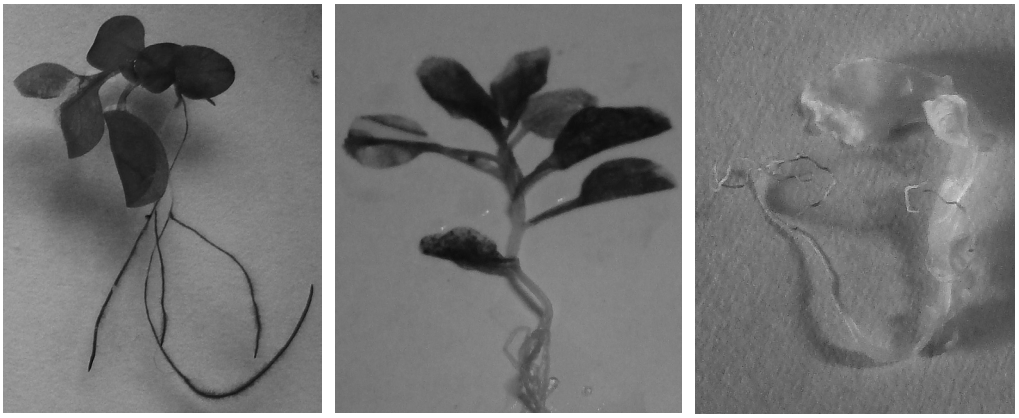

Figure 2. GUS assay of vegetative organs from T_1 tobacco plants driven by the *SSU5C* promoter
Histochemical GUS assay of vegetative organs from T_1 tobacco plants at the age of 1 month driven by the *SSU5C* promoter; C2-*gus* is the promoterless-*gus* construct

L. gibba rbcS gene promoters were present, such as box X, Y, Z elements (Figure 1).

***SSU5C* promoter directs green tissue-specific gene expression**. To analyse the tissue characterization of *SSU5C* promoter, 15 independent T_1 transgenic tobacco lines carrying *p1438-gus* were analysed by a GUS biochemical assay experiment. Histochemical staining revealed that GUS expression was observed in green tissue parts, but not in roots (Figure 2). *35S-gus* and C2-*gus* (promoterless-gus construct) constructs were used as control (Figure 2). Our results verified that *SSU5C* was a green tissue-specific promoter.

Deletion analysis of the *SSU5C* promoter in transgenic tobacco. To investigate the cis-acting regulatory elements which were sufficient for green tissue-specific expression in *SSU5C* promoter, a series of truncated *SSU5C* promoter (Figure 3) was fused with the *gus* reporter gene and transformed into tobacco plant. Deletion series of *SSU5C* promoter were evaluated by the analysis of GUS expression levels from T_1 stable independent transgenic plants. A promoterless construct (C2-*gus*) was used as a negative control and 35S-*gus* as a positive control. Figure 4 shows that deletion of the *SSU5C* promoter to position –152 from the start of transcription had no significant effect on GUS expression in leaves. Further deletion to –49 in the *SSU5C* promoter resulted in a failure in GUS activity in transgenic leaves. These results indicated that the *SSU5C* promoter region from –152 to –49 not only contributed to green tissue-specific expression, but also functioned as a positive cis-regulatory region. Sequence analysis showed that the *SSU5C* promoter region between

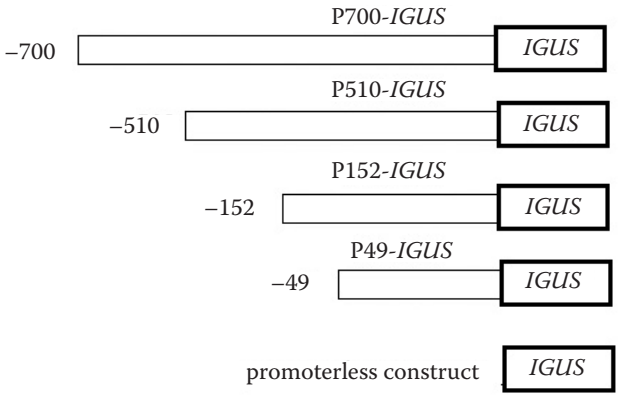

Figure 3. Schematic representation of constructs carrying deletion promoters fused with the GUS coding sequence

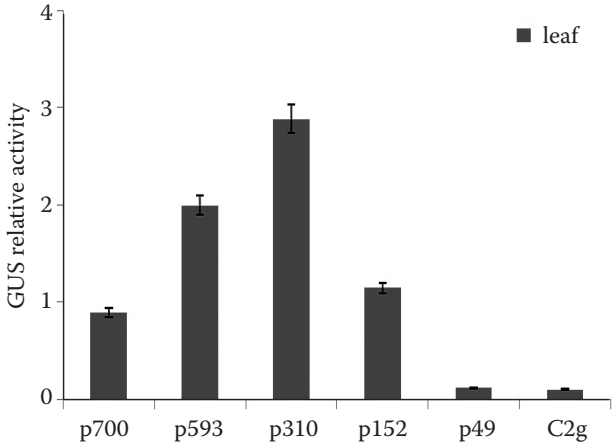

Figure 4. Deletion analysis of the *SSU5C* promoter to confirm the regions involved in green tissue-specificity
5' deletion constructs were assayed for GUS activity in T_1 transgenic tobacco plant; the numbers refer to the 5' end of the promoter relative to the start of transcription; GUS relative activity is presented, considering the activity of *p1438-gus* in leaves as the unit; the bars are 5% confidence interval (of 15 independent experiments)

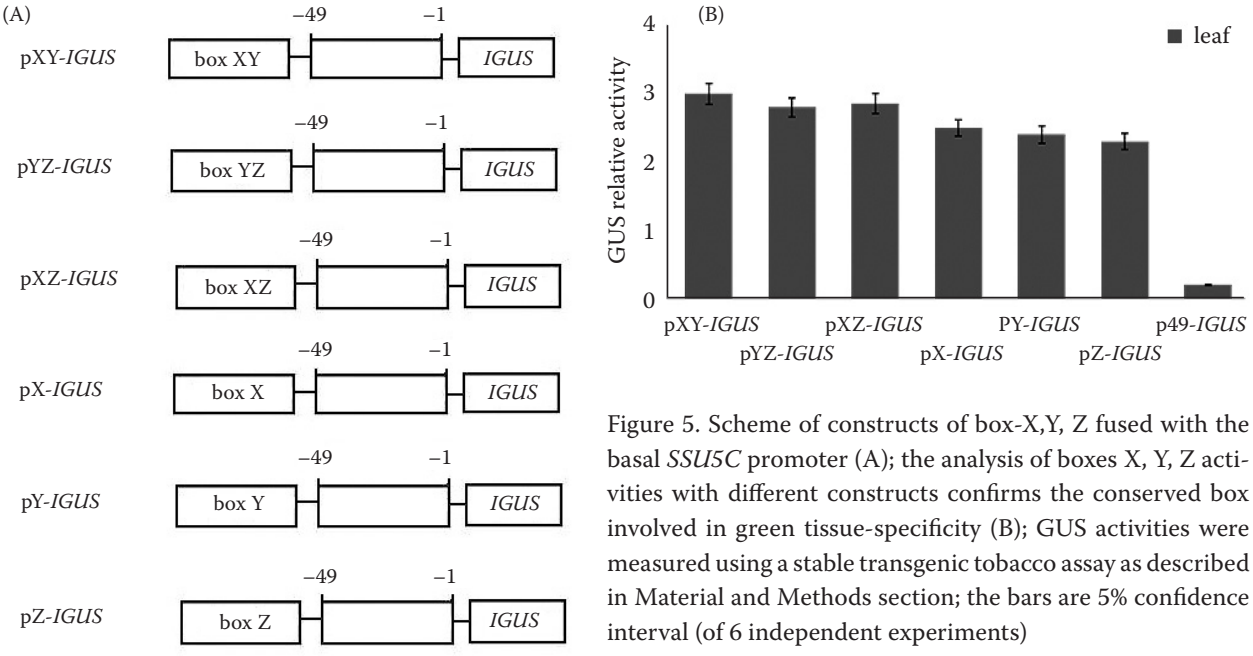

Figure 5. Scheme of constructs of box-X,Y, Z fused with the basal *SSU5C* promoter (A); the analysis of boxes X, Y, Z activities with different constructs confirms the conserved box involved in green tissue-specificity (B); GUS activities were measured using a stable transgenic tobacco assay as described in Material and Methods section; the bars are 5% confidence interval (of 6 independent experiments)

–152 and –49, which was involved in green-tissue specific expression, contained boxes X, Y and Z (Figure 4). Further fusion of boxes X, Y and Z to the basal *SS5UC* promoter was implemented (Figure 5A). The GUS activity assay showed that these boxes were alone able to confer green-tissue specific expression, respectively (Figure 5B). In conclusion, all the results described here demonstrate that boxes X, Y and Z are alone responsible for the basal activity of *SSU5C* promoter in green tissues. Furthermore, the three elements will serve as useful tools in a "green tissue-specific promoter design".

DISCUSSION

In this study, a 1491-bp fragment upstream of the initiation codon of the *rbcS* gene from duckweed was isolated and the sequence analysis revealed that the 5' upstream region of *rbcS* gene (designed as *SSU5C* promoter) is a novel promoter. The GUS activity assay performed with transgenic tobacco plants revealed that deletion of *SSU5C* promoter to position –510 enhanced to the highest level of GUS expression in leaves. Deletion from position –510 to position –152 resulted in a gradual decrease in the level of GUS expression. These data could be explained by the presence of a silencer element between –700 and –510 and an UPE (upstream promoting element) between –310 and –152. Although the level of GUS expression was modified by the presence of the UPE and the silencer, all constructs that included sequence 152 nucleotides upstream of the transcription start site showed green-tissue specificity, indicating that elements upstream of position –152 were not required for green-tissue specificity regulation. Further GUS activity assay revealed that *SSU5C* promoter ranging from nt –152 to –49 was able to drive GUS expression throughout the leaves. Thus, we conclude that elements located between positions –152 and –49, which contained boxes X, Y and Z, were an activator for conferring green-tissue specific expression (Figure 4). Further functional analysis of constructs of box-X, Y, Z defined that boxes X, Y and Z are alone able to direct green tissue-specific expression (Figure 5B). Overall, our data confirmed that boxes X, Y and Z were alone able to serve as the new minimized functional promoter to direct green tissue-specific expression. Previous reporter confirmed functional analysis of constructs of box-X, Y, Z defined that boxes X, Y and Z alone are able to direct phytochrome-regulated expression (Wang *et al.* 2011). RBCS transcript was reported to be abundant in green tissues of plant, especially in leaves, and little is known about how the green tissue-specific expression of *rbcS* gene was driven by *rbcS* promoter (Peters & Silverthorne 1995). Boxes X, Y and Z may be a useful cis-acting element to investigate the green tissue characterization of *SSU5C* promoter and the roles of boxes X, Y and Z in the regulation of green tissue characterization remained to be investigated.

In summary, the green tissue-specific activity of the *SSU5C* promoter could be conferred by only the 152-bp short fragment proximal to the 5' end of the *SSU5C*. Boxes X, Y and Z are alone able to direct

green tissue-specific expression. Taken together, our results strongly suggest that the identified promoter, *SSU5C*, and its cis-regulatory elements, boxes X, Y and Z, are potentially useful in the field of crop transgenic breeding.

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