BIOLOGIA PLANTARUM 56 (2): 261-268, 2012

Isolation and functional analysis of 4-coumarate:coenzyme A ligase gene promoters from *Salvia miltiorrhiza*

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Abstract

The enzyme 4-coumarate:coenzyme A ligase (4CL) plays an important role in phenylpropanoid metabolism. The 5'-upstream regions of two *Sm4CL* genes were isolated from danshen (*Salvia miltiorrhiza* Bunge) and their functions were characterized by promoter-directed *GUS* gene expression assay in transgenic *Arabidopsis*. Seedlings containing pSm4CL1 promoter:GUS fusions showed apparent GUS staining in hypocotyl and those harboring pSm4CL2 promoter:GUS fusions were clearly stained in cotyledon vasculars and roots. Mature *Arabidopsis* transformed with pSm4CL1 promoter:GUS exhibited GUS expression which was weak in the shoots and scarcely in roots and those modified with pSm4CL2 promoter:GUS displayed obvious GUS staining in roots, stigmatic papillae, stamens and sepal veins. Semi-quantitative RT-PCR revealed that *Sm4CL2* was transcribed at the highest level in roots which was also shown to be the major accumulation site of salvianolic acid B. The results suggested that *Sm4CL2* rather than *Sm4CL1* might be responsible for the biosynthesis of salvianolic acid B in danshen roots.

Additional key words: danshen, GUS staining, HPLC, salvianolic acid B.

Introduction

Phenylpropanoid metabolites are important natural products not only because they are essential for higher plant surviving in the ecosystem but also valuable chemicals in medicine and food industry (Douglas *et al.* 1992). Water-soluble phenolic acids in danshen (*Salvia miltiorrhiza* Bunge), a famous traditional Chinese medicinal herb, are such metabolites with distinct pharmaceutical effects (Durairajan *et al.* 2008). Salvianolic acid B (SAB; also called lithospermic acid B) is the major water-soluble bioactive constituent in danshen and is presumed to be synthesized from rosmarinic acid (RA) *via* phenylpropanoid pathway (Yamamoto *et al.* 2002).

4-Coumarate:coenzyme A ligase (4CL) (EC 6.2.1.12) plays an important role in the general phenylpropanoid pathway by catalyzing a series of aromatic substrates to form their corresponding hydroxycinnamoyl-CoA esters, key precursors in the biosynthesis of numerous phenylpropanoid derivatives including RA (Yamamoto *et al.* 2002, Kim *et al.* 2004). Genes encoding 4CL have been cloned from a number of plant species, where they exist in small multigene families. In some species, *e.g.* potato (Becker-André *et al.* 1991) and tobacco (Lee and Douglas 1996), 4CL isoenzymes have similar molecular properties and possess identical or nearly identical substrate utilization profiles. In the others, 4CL isoforms frequently exhibit distinct substrate affinities and cell- or tissue-specific expression profiles, which are supposed to

Received 20 September 2010, *accepted* 20 December 2010.

Abbreviations: 4CL - 4-coumarate:coenzyme A ligase; GUS - β-glucuronidase; HPLC - high performance liquid chromatography; MeJA - methyl jasmonate; MS - Murashige and Skoog; RA - rosmarinic acid; RT-PCR - reverse transcription polymerase chain reaction; SAB - salvianolic acid B; X-gluc - 5-bromo-4-chloro-3-indolyl-β-D-glucuronide.

Acknowledgement: This work was financially supported by National Natural Science Foundation of China (No. 30300447, No. 30973878) and Shanghai Shuguang Project (No. 06SG44). We would like to thank Professor Shouming Zhong for modifying the article, Prof. Yuke He for providing *Agrobacterium tumefaciens* GV3101 and *Arabidopsis* (Columbia) and Dr. Gonghao Jiang and Mr. Guixuan Song for technical assistance. The nucleotide sequence data reported have been submitted at the Genbank database under the accession numbers EF458149 (*Sm4CL1* 5' upstream region) and EF458150 (*Sm4CL2* 5' upstream region).

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coincide with specific metabolic functions (Ehlting *et al.* 1999, Hamberger and Hahlbrock 2004). For example, in *Populus tremuloides*, Pt4CL1 prefers to use ferulic and 5-hydroxyferulic acids and is associated with lignin biosynthesis in developing xylem of woody stems, Pt4CL2 takes part in the biosynthesis of phenylpropanoids other than lignin in epidermal cells of the stem and the leaf (Hu *et al.* 1998).

We have also cloned two *4CL* genes, *Sm4CL1* and *Sm4CL2* (GenBank accession number: AY237163 and AY237164) from danshen. They shared 62.7 % homology and 80 % similarity at the amino acid level. Enzymatic kinetic assays with recombinant proteins showed that Sm4CL2 possessed much higher affinities to *p*-coumaric acid, caffeic acid, ferulic acid, and trans-cinnamic acid than Sm4CL1, which indicated that Sm4CL2 was more favorable to utilize most of the substrates in phenylpropanoid pathway (Zhao *et al.* 2006). Phylogenetic tree inferred with *CLUSTAL* method indicated that both genes belonged to class I cluster (Zhao *et al.* 2006), which represented the majority of the 4CLs and functioned in the biosynthesis of most of the phenylpropanoids (Ehlting *et al.* 1999, Hamberger and Hahlbrock 2004). However, it is still unclear what is the specific function of each *Sm4CL* gene in phenylpropanoid pathway, especially in the biosynthesis of water-soluble phenolic acids in danshen. To make it clear, research on the regulation profiles of *Sm4CL* genes is required.

Genes in general phenylpropanoid pathway are largely controlled at the transcriptional level and can be activated by stresses such as wounding, UV radiation, or pathogen attack (Bauer *et al*. 2009, Sullivan 2009, Gutiérrez-Carbajal *et al*. 2010). However, the response of individual gene was different. *Cis*-elements in the promoters were reported to contribute to the difference (Soltani *et al.* 2006, Osakabe *et al.* 2009). Promoter directed reporter gene expression in transgenic plant is a typical method to characterize promoter activities (Hauffe *et al.* 1991, Neustaedter *et al.*1999, Rusak *et al.* 2010). In this way, the *Pc4CL-1* promoter fragment directed specific complex expression pattern was elucidated and *cis*-acting elements responding to developmental signals were identified (Hauffe *et al.* 1991, Neustaedter *et al.* 1999). Similar work had been carried out on *At4CL2* and *Pta4CLα* promoters (Soltani *et al.* 2006, Osakabe *et al.* 2009).

We report herewith the isolation and characterization of *Sm4CL1* and *Sm4CL2* promoters with the aim to elucidate their distinct transcriptional regulation profiles.

Materials and methods

Seeds of wild type *Arabidopsis thaliana* (ecotype Columbia) were sterilized with 0.1 % $HgCl_2$ for 10 min, rinsed with sterilized water 4 times, and then inoculated on Murashige and Skoog (MS) medium with 0.6 % agar. Plates were initially kept at 4 °C for 2 d and then moved into the growth chamber at 22 °C with a 16-h photoperiod (irradiance of 80 - 100 μmol m^{-2} s^{-1}). Two to three weeks old seedlings were then transplanted into soil in a greenhouse at the same conditions. Danshen plants were cultivated in the outdoor garden of the University.

The 5'-upstream regions of *Sm4CL1* and *Sm4CL2* were cloned using a PCR-based 5'-DNA walking strategy according to the protocol provided with the *GenomeWalker* universal kit (*Clontech*, Palo Alto, CA, USA). Genomic DNA was isolated from danshen leaves according to Sambrook and Russell (2001) and digested with four restriction enzymes. Genomic libraries were generated after ligating the digested fragments with the adaptors. Two rounds of PCR were performed using adaptor primers (AP1 and AP2) and gene-specific primers (GSP1 and GSP2) which were designed according to the coding sequence of *Sm4CL* genes. Fragments were cloned into pMD18-T vector (*Takara*, Dalian, P.R. China) and sequenced.

Promoter fragments, -2068/+29bp and -556/+29bp of *Sm4CL1* (named Sm4CL1-NH and Sm4CL1-NE), -750/+95bp and -379/+95bp of *Sm4CL2* (named Sm4CL2-NP and Sm4CL2-NX), were subcloned. A modified pCAMBIAGUS vector was created by replacing *GFP* in pCAMBIA1302 (http://www.cambia.org) with a *GUS* gene. *Sm4CL* promoter: GUS vectors were generated by replacing the 35S promoter cassette with certain promoter fragment, designated as pSm4CL1-NH:GUS, pSm4CL1-NE:GUS, pSm4CL2-NP:GUS, and pSm4CL2-NX:GUS, respectively. Each fusion was verified by DNA-sequencing. Plasmid was introduced into *Agrobacterium tumefaciens* GV3101 and transformation was carried out using floral dip method (Clough and Bent 1998). Transgenic *Arabidopsis* was selected on MS media containing 25 μg cm^{-3} of hygromycin B and further confirmed with PCR amplification.

Histochemical GUS assay was performed by vacuum infiltration of whole seedlings or different organs or tissues of mature plants in 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) solution, containing 1 mM of X-gluc, and incubated at 37 °C overnight, followed by a 95 % ethanol washing step (Jefferson *et al*. 1987).

For the analysis of wound-induced GUS expression, leaves from 40-d-old *Arabidopsis* were punctured 1 - 2 times and harvested 15 min later for GUS staining. For MeJA-induction assay, 100 μM of MeJA (*Sigma-Aldrich*, St. Louis, USA) was sprinkled to the mature transgenic *Arabidopsis* and the leaves were cut down 2 h later. To do the light-responding tests, sterilized seeds were incubated on MS media at 4 °C for 2 d in darkness. Then half of vessels was covered with dark paper and

grown in the chamber for 3 d before GUS staining. Seeds uncovered and cultivated under 16-h photoperiod were used as the control.

Total RNA was isolated from leaves, stems, and roots using method of Sambrook and Russell (2001). cDNA was obtained using 2 μg of total RNA per 0.025 cm^3 reaction mixture and calibrated with *Smactin* gene before the semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) assay according to Soltani *et al.* (2006). Optimal PCR cycle numbers were 25 cycles for *Smactin*, 34 cycles for *Sm4CL1*, and 30 cycles for *Sm4CL2*, respectively. Samples were separated and visualized on 1 % agarose gels containing ethidium bromide. Each assay was performed with at least three independent samples.

The flowers, leaf blades, leaf petioles, stems, and roots were harvested and lyophilized. Each sample (100 mg) was powdered, transferred into a 50 cm^3 centrifuge tube and extracted in 10 cm^3 of 70 % methanol with an ultrasonic apparatus for 40 min. The extracts were centrifuged at 4 000 *g* for 5 min and the supernatant was filtrated through a 0.45 μm membrane before being applied to HPLC analysis (Liu *et al*. 2007). Reference standard solutions were prepared by accurately weighting *SAB* or *RA* standards (*Medicine Inspection Office*, Shanghai, China) dissolved in 5 cm^3 of 70 % methanol.

Results

The 5'-flanking promoter fragments, 2.1 kb of *Sm4CL1* and 0.8 kb of *Sm4CL2*, were isolated and sequenced. Both promoters had a typical TATA box, present at -39 bp upstream of the transcriptional start site (TSS) in *Sm4CL1* and -27 bp upstream of the TSS in *Sm4CL2*, respectively. Analysis with *PlantCARE* (Lescot *et al.* 2000) showed that a number of potential *cis*-acting elements scattering in both promoter regions (Tables 1, 2). Those elements are related either with tissue-specific or with light- and elicitor-responsive gene expression. This suggested that both *Sm4CL* promoters had typical characteristics of phenylpropanoid genes.

Histochemical GUS staining was performed on 8 to 11 transgenic homozygous lines for each construct. Seedlings containing pSm4CL1-NH:GUS and pSm4CL1-NE:GUS constructs showed identical GUS staining patterns, strong in cotyledon apex, hypocotyl, and stem, weak in the veins of cotyledon and scarcely in roots (Fig. 1*A*,*B*). Mature plants harboring either pSm4CL1-NH:GUS or pSm4CL1-NE:GUS fusions showed GUS activity in leaf apex, anthers and veins on sepals (Fig. 1*A*,*B*), but not in roots and siliques. The results suggested that the deletion of -2068/-556 bp fragment hardly affected the activity of the promoter and indicated that *Sm4CL1* -556 to +29 bp fragment had the basic tissue-specific expression activity.

Seedlings harboring pSm4CL2-NP:GUS fusion exhibited GUS staining strong in vascular bundles of cotyledon and roots and weak in hypocotyl (Fig. 1*C*). Adult plants containing pSm4CL2-NP:GUS fusion displayed GUS staining significantly in roots and apexes of expanded leaves besides the sepal veins, stigmas,

Table 1. Putative *cis*-acting elements in the *Sm4CL1* promoter.

Name	Sequence	Position	Function
Box I	TTTCAAA	-1911/-1905 (+) -1393/-1387 (+)	light responsive element
rbcS-CMA7a	CCTTATCGTC	-1833/-1824(-)	part of a light responsive element
MBS	CGGTCA	-1742/-1736(+)	MYB binding site
W box	TTGACC	-1741/-1736(-)	fungal elicitor responsive element
CCAAT-box	CAACGG	-1706/-1701(+)	MYBHv1 binding site
Box 4	ATTAAT	-1541/-1536(+)	part of a conserved DNA module involved in light responsiveness
GT1-motif	GGTTAAT	-1242/-1236(+)	light responsive element
LTR	CCGAAA	-1155/-1150(-) -64/-59(+)	*cis*-acting element involved in low-temperature responsiveness
TCT-motif	TCTTAC	-1012/-1007(+)	part of a light responsive element
A box or CCGTCC-motif	CCGTCC	-953/-948(-)	*cis*-acting regulatory element *cis*-acting regulatory element related to meristem specific activation
MRE	AACCTAA	-852/-846 (-)	MYB binding site involved in light responsiveness
Skn-1 motif	GTCAT	-918/-914(+)	*cis*-acting regulatory element required for endosperm expression
ARE	TGGTTT	-487/-482(+)	*cis*-acting regulatory element essential for the anaerobic induction
ABRE	GTACGTG	-334/-327(+)	*cis*-acting element involved in the abscisic acid responsiveness
G-box	TACGTG	-333/-327(+)	*cis*-acting regulatory element involved in light responsiveness
ATC-motif	AGTAATC	-154/-148(+)	part of a conserved DNA module involved in light responsiveness
Py-rich stretch	TTTCTTCTTT	-16/-7(-)	5'UTR *cis*-acting element conferring high transcription levels

Table 2. Putative *cis*-acting elements in the *Sm4CL2* promoter.

Name	Sequence	Position	Function
CGTCA-motif	CGTCA	-749/-745(+), -246/-242 (+)	*cis*-acting regulatory element involved in the MeJA-responsiveness
EIRE	TTCGACC	-698/-691 (+)	elicitor-responsive element
GT1-motif	GGTTAA	-637/-632 (-)	light responsive element
I-box	GATATGG	-627/-621(-)	part of a light responsive element
TC-rich repeat	AATTCTCTATC	-433/-423 (-)	*cis*-acting element involved in defense and stress responsiveness
G-Box	CACGTT	-309/-304(+), -303/-298 (+)	*cis*-acting regulatory element involved in light responsiveness
Sp1	CC(G/A)CCC	-162/-153(+)	light responsive element
L-box or	TCTCACCAACC	-150/-140 (+)	light responsive element
4cl-CMA2b or AC-I			light responsive element negative regulation of phloem expression. Responsible for restricting vascular expression to the xylem
AC-II	TCCACCAACTCC	-61/-50 (+)	element involved in negative regulation on phloem expression; and responsible for restricting vascular expression to the xylem
Box E	ACCCATCAAG	-48/-39 (+)	*cis*-element for induction upon fungal elicitation

anthers, filaments in flowers, and the abscission zone and stigmatic papillae in silique (Fig. 1*C*). pSm4CL2-NX:GUS construct showed the similar GUS expression patterns to pSm4CL2-NP:GUS in most of the organs and tissues except no detectable stain in hypocotyl and stigmas, anthers, and filaments in flowers (Fig. 1*D*). These results indicated that hypocotyl-, stigma-, anther-, and filament-specific domains might exist between -750 and -379 bp regions of *Sm4CL2* promoter.

Both pSm4CL2-NP:GUS and pSm4CL2-NX:GUS fusions showed obvious GUS staining at the wounded site of the leaves (Fig. 2*B*,*C*), although the stain in the latter was weaker, which proved that *Sm4CL2* promoter could respond to wound signal and that wound-responsiveness *cis*-elements scattering over this region were workable in this reaction. Interestingly, pSm4CL1-NH:GUS was not stimulated by wound (data not shown), but pSm4CL1-NE:GUS was (Fig. 2*A*). It was likely that putative negative elements responsible for wound-response were present between -2068 and -556 bp *Sm4CL1* promoter regions. In MeJA-induction assay, only pSm4CL1-NE:GUS showed GUS staining at the vascular system in the treated leaves (Fig. 2*D*).

In light-responding tests, pSm4CL1-NH:GUS fusion showed no GUS staining either in light or in darkness, while pSm4CL1-NE:GUS was induced to express in hypocotyl in light (Fig. 2*E,F*). Light-responsive *cis*-elements between -2068 and -556 bp might contribute to the difference. *Sm4CL1* promoter indeed regulated *GUS* gene expression in two-week-old seedlings (Fig. 1*A*,*B*). Thus we think that *Sm4CL1* -2068/+29 bp fragment probably did not function at early stage in the seedling development. Differently from *Sm4CL1*, pSm4CL2-NP:GUS fusions exhibited GUS staining in cotyledon, hypocotyl, and radicle whether in light or in darkness. Similar results were observed in pSm4CL2-NX:GUS fusions except no GUS staining in hypocotyl. It appeared that the *Sm4CL2* promoter could regulate *GUS* gene to express even without light signals in the seedling development and that the deletion of *Sm4CL2* -750/-379 bp fragment did not affect the activity. Moreover, the promoter: GUS assay demonstrated that the *Sm4CL2* promoter behaved more actively than *Sm4CL1* promoter did in most of the organs and tissues in the adult plant.

Taken together, those results indicated that both *Sm4CL1* and *Sm4CL2* could respond to environmental stimuli but functioned differently *in vivo*.

Semi-quantitative RT-PCR showed that both *Sm4CL1* and *Sm4CL2* were expressed in all tissues but at different levels (Fig. 3). *Sm4CL1* transcripts were high in leaves, low in stems and roots; *Sm4CL2* was expressed highly in roots and less in leaves and stems. It also revealed that the transcripts of *Sm4CL2* were much more abundant than those of *Sm4CL1* in all the tissues analyzed. In any events, both semi-quantitative RT-PCR and promoter:GUS assay suggested that *Sm4CL2* was much more active than *Sm4CL1* in danshen roots, indicating that *Sm4CL2* might play predominant role in phenylpropanoid metabolism in this organ.

Quantitative assay with HPLC showed that SAB was mainly accumulated in roots, in root cortex as well as in root xylem (Table 3). RA was chiefly deposited in leaf blades and in flowers (Table 3). In terms of the total amount of both compounds, the root cortex had the highest content (101.47 ± 3.57 mg g^{-1}), followed by leaf blades (63.10 ± 2.38 mg g^{-1}) and root xylem (48.55 ± 2.86 mg g^{-1}). The results indicated that SAB was prone to accumulating in the underground part of danshen.

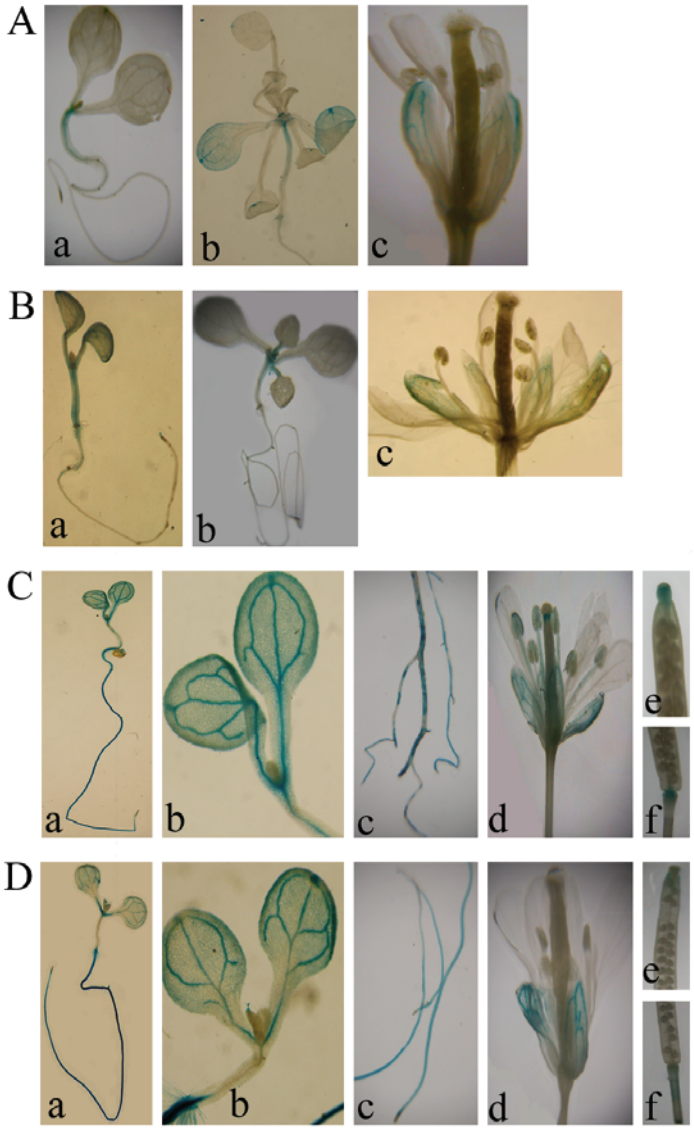

Fig. 1. Histochemical localization of GUS expression regulated by *Sm4CL* promoter fragments in transgenic *Arabidopsis*. *A*: pSm4CL1-NH:GUS; *B*: pSm4CL1-NE:GUS; *C*: pSm4CL2-NP:GUS; *D*: pSm4CL2-NX:GUS. *A* and *B*: 2-week-old seedlings (*a*), three-week-old seedlings (*b*), flowers (*c*). C and D: 10-d-old seedlings (*a*, *b*), 40-d-old roots (*c*), flowers (*d*), siliques (*e*, *f*).

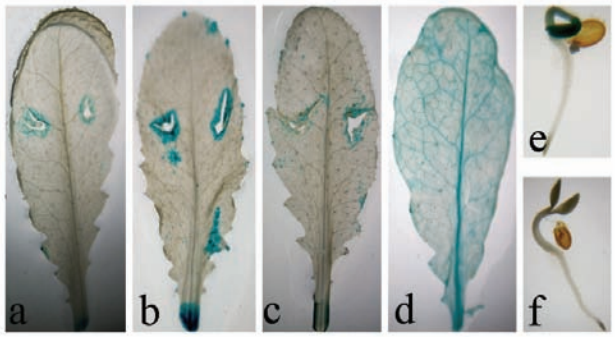

Fig. 2. Histochemical assays of *Sm4CL* promoter:GUS activity responding to wound (*a*,*b*,*c*), MeJA (*d*), and light (*e*,*f*). *a* - pSm4CL1-NE:GUS; *b* - pSm4CL2-NP:GUS; *c* - pSm4CL2-NX:GUS. *d* - pSm4CL1-NE:GUS; *e*,*f*: -seedlings containing pSm4CL1-NE:GUS fusion cultivated under light (*e*) and in darkness (*f*).

Discussion

Analysis with *PlantCARE* revealed that both *Sm4CL* promoters contained cell- or tissue-specific *cis*-acting regulatory elements. Skn-1 motif [-918/-914(+)] is an element required for endosperm expression, predicting

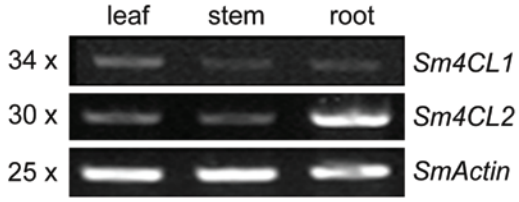

Fig. 3. Semi-quantitative RT-PCR results of *Sm4CL1* and *Sm4CL2* in danshen.

Table 3. Distribution of salvianolic acid B (SAB) and rosmarinic acid (RA) [mg g^{-1}(d.m.)] in danshen. Means ± SE, n = 3.

Tissue	SAB	RA
Flower	12.48 ± 1.82	28.66 ± 2.12
Leaf blade	20.71 ± 1.40	42.39 ± 1.06
Leaf stalk	12.20 ± 2.12	12.65 ± 2.34
Stem	9.22 ± 1.28	11.48 ± 1.25
Root xylem	38.78 ± 1.32	9.77 ± 1.61
Root cortex	86.65 ± 2.16	14.82 ± 1.70
Root	74.32 ± 1.53	11.90 ± 2.05

that *Sm4CL1* may function in the seed development. CCGTCC-motif [-953/-948(-)] is related to meristem-specific activation. Lacking this motif, *Sm4CL1* -556/29 bp fragment reduced GUS expression in the stem meristem (Fig. 1*A*,*B*). Both AC-I and AC-II are the binding sites for the MYB trans-activating factor ACBF (Gómez-Maldonado *et al.* 2004) and responsible for restricting vascular expression (Séguin *et al.* 1997), thus may contribute to the vascular-specific expression of *Sm4CL2* promoter (Fig. 1*C*,*D*). Mutation experiments with bean *PAL2* promoter also suggested that AC-I and AC-II, in the context of -254 bp promoter fragment, were able to specify petal expression reciprocally and individually (Hatton *et al.* 1995). However, *Sm4CL2* -379/+95 bp fragment, containing AC-I and AC-II, could not regulate GUS to express in petals (Fig. 1*D*). Even this fragment contains two G-box [-309/-304, -303/-298 (+)], which is benefit for AC-I and AC-II in directing petal-specific expression (Hatton *et al.* 1995). Therefore, it could be speculated that AC-I and AC-II elements are necessary but not sufficient to specify petal expression.

It is generally believed that light-response is a complicated process relying on *cis*-regulatory elements and the context regions cooperating with each other to confer negative or positive light responsiveness (Kuhlemeier *et al.* 1987, Loake *et al.* 1992, Logemann *et al.* 1995). In this study, *Sm4CL2* promoter exhibited light-independent biological activity, and the *Sm4CL1* -2068/+29 bp fragment was not light-inducible but the *Sm4CL1* -556/+29 bp fragment was. *PlantCARE* analysis showed that *Sm4CL1* -556/+29 bp region contained one G-box [TACGTG, -333/-327(+)] and an ATC-motif [AGTAATC, -154/148(+)], part of a conserved DNA module involved in light responsiveness. It was unclear whether the two motifs resulted in light-responsiveness or not. In any case, those results provided certain information about the mechanism of light induced gene transcription.

Two typical MeJA-responsive regulatory elements, GACG/CGTCA-motifs [-749/-745(+), -246/-242 (+)], were present in *Sm4CL2* promoter. It was reported that jasmonic acid played a central role in rapid localized and systematic wound responses in plants (Boter *et al.* 2004). Both *Sm4CL2* -750/+95bp and -379/+95bp fragments: GUS fusions showed strong GUS staining at the wounded sites of the leaves and the staining in the former was much stronger than in the latter, suggesting that more TGACG/CGTCA-motifs might be conducive to wound-response. A TC-repeat element [-433/-423 (-)] and an EIRE motif [-698/-691(+)] also existed in *Sm4CL2* promoter. The TC-repeat element was involved in defense and stress responsiveness and EIRE motif was concerned with elicitor-response. Those motifs might also contribute to the wound stress response. Differently, the *Sm4CL1* -2068/+29 bp promoter could not be activated by wounding instantly, but the 5'-trunctated fragment could. A G-box like element (TACGTG) was found at -333/-327 bp(+). G-box was reported responsible for the wound-response (Loake *et al.* 1992, Boter *et al.* 2004), thus the G-box-like element was probably involved in the wound-induced activity of *Sm4CL1* -556/+29 bp fragment. This fragment also showed MeJA- and light-inducible abilities. It would be interesting to investigate the activity of this fragment to better understand the function of certain *cis*-elements.

We had cloned two divergent *Sm4CL* genes from danshen and found that Sm4CL2 had much higher affinities to 4-coumarate and the other general substrates than Sm4CL1 did *in vitro* (Zhao *et al.* 2006). In this study, *Sm4CL2* promoter could drive the *GUS* gene expression in most of the organs and tissues of the adult transgenic *Arabidopsis*; while *Sm4CL1* promoter directed *GUS* gene was restricted to express in certain tissues. Semi-quantitative RT-PCR also revealed that mRNA transcription level of *Sm4CL2* was much higher than that of *Sm4CL1* in all the organs and tissues. Those results, together with the enzymatic characteristics of Sm4CL recombinants (Zhao *et al.* 2006), helped conclude that *Sm4CL2* instead of *Sm4CL1* might have dominating

functions in responding to developmental stimuli in danshen.

It was worth noting that in the transgenic plants containing any *Sm4CL* promoters:GUS fusions, we did not observe any GUS staining in the leaves except for the part of apex, which was not consistent with semi-quantitative RT-PCR. This was probably due to that the heterologous expression of the reporter gene driven by *Salvia* promoters in *Arabidopsis* might require specific transcriptional regulation that may, under the experimental circumstances not be identical between the two species.

Another phenomenon which should be mentioned was that *Sm4CL1* was expressed higher than *Sm4CL2* in leaves in Northern blotting assay (Zhao *et al.* 2006), different from the RT-PCR results. It was well known that *4CL* was closely related to environmental signals. This difference might result from growth conditions of the plants. Materials used in present work were grown in the outside garden of Shanghai University, while plants used before were cultivated in the greenhouse of Hong Kong University. We noticed the leaves of the plants grown in Hong Kong were not pure green, but with a hint of red (data not shown). The different light and temperature conditions might lead to the differential expression levels of *Sm4CL1* and *Sm4CL2* in leaves.

To the exogenous signals such as wound, MeJA, and light, *Sm4CL* promoters exhibited different activities. *GUS* gene regulated by either *Sm4CL2* promoter fragment was instantly activated after wounding, suggesting that *Sm4CL2* promoter responded rapidly to wound signals. Light signal could activate the 5'-deleted *Sm4CL1* promoter, but could not induce the other three promoter fragments. So did in MeJA-induction assay. Those results indicated that both *Sm4CL* genes participated in plant defense but in different manners.

Most genes of phenylpropanoid pathway have cell- and tissue-specific transcription profiles. *In situ* hybridization and other studies indicated that cells and tissues where individual *4CL* mRNA accumulates are correlated with the sites where phenylpropanoid end product accumulation was regulated (Reinold and Hahlbrock 1996, Ehlting *et al.* 1999, Sullivan 2009). It is believed that 4CL is the key enzyme to form RA, intermediate in the SAB biosynthesis (Yamamoto *et al.* 2002, Kim *et al.* 2004). Thus, the transcription of *Sm4CL* is closely related to the biosynthesis of RA and SAB. In danshen, the mRNA transcription level of *Sm4CL2* was much higher than that of *Sm4CL1* in roots, which was supported by *Sm4CL* promoter directed GUS expression, semi-quantitative RT-PCR, and Northern blotting (Zhao *et al.* 2006). Total amount of RA and SAB was also the highest in roots (Table 3). Therefore, we believed that *Sm4CL2* rather than *Sm4CL1* might be responsible for the biosynthesis of water-soluble phenolic acids especially SAB in danshen roots. As for why the accumulation of RA and SAB was not consistent with each other, we speculated that it might be caused by the unknown enzymes in the biosynthetic process from RA to SAB in the plants. Similar phenomena were found in the variation of water-soluble phenolic acids induced by MeJA, *i.e.* when the accumulation of SAB increased, the accumulation of RA decreased and *vice versa* (Chen *et al*. 2010).

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