A leader intron and 115-bp promoter region necessary for expression of the carnation *S*-adenosylmethionine decarboxylase gene in the pollen of transgenic tobacco

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Abstract The expression of *CSDC9* encoding *S*-adenosylmethionine decarboxylase (SAMDC) is developmentally and spatially regulated in carnation. To examine the regulation of the *SAMDC* gene, we analyzed the spatial expression of *CSDC9* with a 5′-flanking β-glucuronidase fusion in transgenic tobacco plants. *GUS* was strongly expressed in flower, pollen, stem and vein of cotyledons. Expression in both anther and stigma was under developmental control; analysis of a series of mutants with deletions of the 5′-flanking region demonstrated differential activation in petal, anther, stigma and pollen grains. All the major *cis*-regulatory elements required for pollen-specific transcription were located in the upstream region between −273 and −158. This region contains four putative elements related to gibberellin induction (pyrimidine boxes, TTTTTTCC and CCTTTT) and pollen-specific expression (GTGA and AGAAA). In addition, the first 5′-leader intron was necessary for tissue-specific expression.

Keywords: *S*-Adenosylmethionine decarboxylase; Promoter analysis; β-Glucuronidase; Pollen; *cis* Element; *Nicotiana tabacum*

1. Introduction

S-Adenosylmethionine decarboxylase (EC 4.1.1.50, SAMDC) catalyzes the conversion of *S*-adenosylmethionine to decarboxylated *S*-adenosylmethionine (dcSAM), which is a donor of the aminopropyl moiety to putrescine (Put) and spermidine (Spd). Synthesis of Spd and spermine (Spm) is mainly regulated at the level of SAMDC [1–3]. Accordingly, insight into the mechanisms regulating SAMDC expression may be important for understanding how the levels of polyamines (PAs) are controlled during plant growth and development.

Expression of *SAMDC* genes is high in young and actively dividing tissues of potato and pea plants [4,5], but active cell division was not found to be responsible for the increase in *SAMDC* transcript levels in Tritordeum [6]. Expression is also induced at the onset of fruit development and in senescing pea ovaries [5]. Potato plants transgenic for a *SAMDC* antisense construct displayed a variety of stunted phenotypes, with highly branched stems, short internodes, small and chlorotic leaves and reduced root growth. In addition, the plants did not flower, and produced very small and elongated tubers [7]. Although the tissue-specific and developmental expression patterns of *SAMDC* and the results of transgenic approaches imply that SAMDC is involved in a number of physiological processes, its precise roles in plant growth and development are not yet well understood.

We previously isolated two types of *SAMDC* cDNAs (CSDC9 and CSDC16) from carnation petals [8]. Expression of the corresponding genes was high mainly in petals and stem. In flower development, *CSDC9* mRNA started to increase at anthesis, and peaked 3 day thereafter (stage E). The pattern of SAMDC activity paralleled changes in the amount of *CSDC9* transcripts, with both SAMDC activity and transcript levels peaking in stage E. Based upon these results, it was concluded that expression of *CSDC9* is under complex regulation during flower development.

In the present work, we isolated a *SAMDC* genomic clone corresponding to *CSDC9* (*gCSDC9*), and made fusion constructs with the β-glucuronidase (GUS) gene under the control of 5′-flanking deletions of *gCSDC9*. We then performed histochemical GUS assay in tobacco plants transgenic for the various constructs to analyze the upstream region involved in expression of *SAMDC* in pollen.

2. Materials and methods

2.1. Plant materials

Carnations (*Dianthus caryophyllus* L. cv. White sim) were grown under greenhouse conditions at 23 °C with a 16 h/8 h (L/D) photoperiod. Leaves were collected, frozen immediately in liquid nitrogen, and stored at −80 °C for extraction of genomic DNA. Tobacco plants (*Nicotiana tabacum*) were grown from seeds under greenhouse conditions and young leaves were harvested for *Agrobacterium*-mediated transformation.

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Abbreviations: SAMDC, *S*-adenosylmethionine decarboxylase; dcSAM, decarboxylated *S*-adenosylmethionine; GUS, β-glucuronidase; PAs, polyamines; 5′-UTR, 5′-untranslated region; *gCSDC9*, genomic clone corresponding to *CSDC9*; *CSDC9*, cDNA encoding carnation SAMDC9; X-GlcA, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid; Put, putrescine; Spd, spermidine; Spm, spermine; uORF, upstream open reading frame

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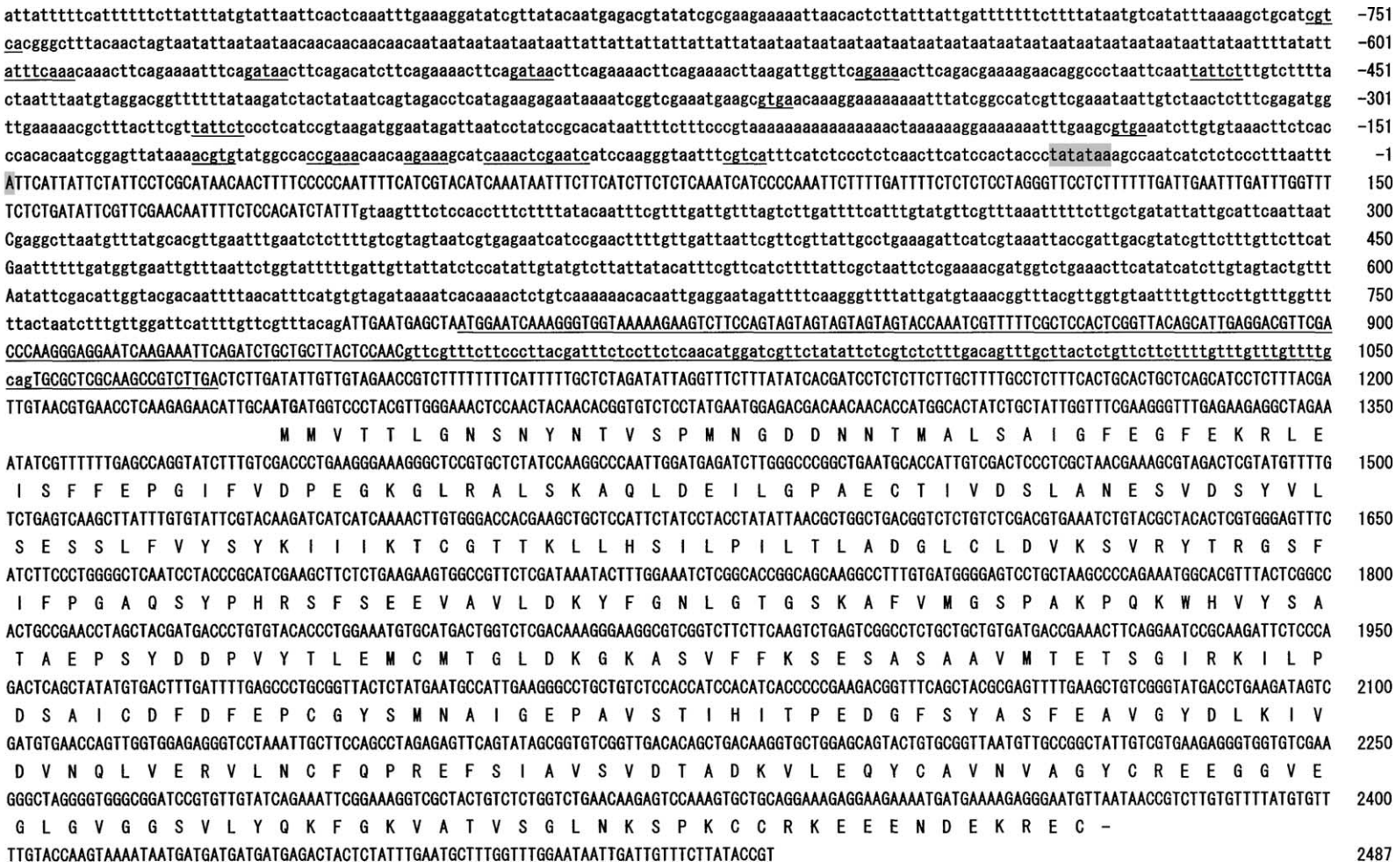

Fig. 1. DNA sequences of *gCSDC9* from carnation (*D. caryophyllus* L.). The nucleotide at position +1 (bold A in the gray box) is the transcription start site. Nucleotides are number upstream from +1. The sequence of the transcribed mRNA is shown in capital letters and the remainder of the sequence in small letters. The derived amino acid sequence is in one-letter code below the DNA sequence. The TATA box is indicated by the gray box, and putative *cis*-regulatory elements and uORF are underlined.

2.2. Construction and screening of a genomic library

Genomic DNA was isolated from carnation leaves by the procedure of Dellaporta et al. [9]. 100 μg of genomic DNA was used to construct a genomic library with a lambda DASH II/*Bam*HI vector kit (Stratagene, USA). About 9×10^4 recombinant phage from the genomic library were screened by plaque hybridization with a 359 bp *Eco*RI/*Xba*I fragment of *CSDC9* previously labeled with ^{32}P by means of a random-primed DNA labeling kit (Roche, Germany). Plaque lifts (Hybond-N+, Amersham, England) were prehybridized at 42 °C for 4 h in a solution containing 50% formamide, 6× SSPE, 5× Denhardt's solution, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA. Hybridization with denatured ^{32}P-labeled probes was carried out overnight in the same solution at 42 °C. Membranes were washed twice in 2× SSPE and 0.1% SDS at 50 °C for 15 min and twice in 25 mM NaH_2PO_4, 1 mM EDTA and 0.1% SDS at 50 °C for 15 min, and exposed to X-ray film with an intensifying screen at −80 °C for 24 h. Nine positive plaques were isolated in three rounds of screening. Of these, two contained genomic clones corresponding to *CSDS16* and seven corresponding to *CSDC9*. The latter were approximately 5.6 kb in size. Both strands of the DNA were sequenced by the dideoxy chain termination method with a Sequenase 2.0 DNA sequencing kit (US Biochemicals, USA).

2.3. Primer extension

To determine the exact position of the 5′ end of the mRNA, we performed primer extension. Two oligonucleotide primers (primer 1: 5′-TTTGATGTACGATGAAAATTGGGGG-3′ and primer 2: 5′-AAAGAGGAACCCTAGGAGAGAGAAAATC-3′) complementary to the sequences between nt +35 and +59, and +100 and +127, respectively, were used in a reverse transcription assay as primers with poly(A)$^+$ RNA (0.5–1 μg) extracted from carnation stems. The oligonucleotides were 5′-end-labeled with T4 polynucleotide kinase (Promega, USA) and [γ-^{32}P]ATP, annealed to the mRNA at 60 °C for 20 min and extended with MMLV-reverse transcriptase (Promega, USA) at 42 °C for 1 h. The products were analyzed on a urea/polyacrylamide gel. The same oligonucleotides were used as primers in dideoxy-sequencing reactions performed with *gCSDC9* and a T7 Sequencing kit (Amersham, England).

2.4. Plasmid construction

To construct 5′ deletion mutants of the gCSDC9 5′-flanking region, PCR primers were designed to amplify portions of the region using plasmid 1821+5′-UTR (see below) as template, and unique restriction sites were introduced (*Hin*dIII at the 5′ and *Xba*I at the 3′ end, respectively). The PCR products were digested with *Hin*dIII/*Xba*I and cloned into pBI121 lacking the 35S promoter (Clontech, USA). All PCR fragments used to make constructs were sequenced in their entirety with a T7 Sequencing kit (Amersham, England).

2.5. Plant transformation

Binary vectors were transformed into *Agrobacterium tumefaciens* strain LBA4404 by the freeze–thaw method, and tobacco leaf discs were transformed with *A. tumefaciens* as described by Horsch et al. [10]. Shoots were rooted in solid MS medium containing 100 mg/l kanamycin sulfate, transferred to soil and grown to maturity in growth chambers. Primary transformants (T_0) were self-fertilized and seeds were collected.

2.6. In vitro pollen germination

Samples of dry mature pollen obtained after dehiscence of the anther in transgenic tobacco were placed in germination medium GK [11] modified to contain 3.23 mM H_3BO_3 [12]. The pollen grains were germinated for 2 h at 25 °C and assayed histochemically with GUS staining solution.

2.7. Histochemical localization of GUS activity

Tissue samples of T_1 transgenic tobacco plants were incubated overnight at 37 °C in the presence of 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-GlcA) and 0.5 mM potassium ferricyanide. Photographs were taken under low magnification with a light microscope.

3. Results and discussion

3.1. Cloning of a carnation SAMDC genomic clone corresponding to CSDC9 from carnation leaves, and determination of the transcription initiation site

As a first step in investigating the mechanism of developmental and tissue-specific expression of the SAMDC gene, *CSDC9*, we isolated and characterized genomic clones from a carnation genomic library (Fig. 1). The genomic clone corresponding to *CSDC9*, *gCSDC9* (GenBank Accession No. U94786) consists of three exons interrupted by two introns of 598 and 108 bp, whose junctions agree with the consensus intron/exon borders of plant genes [13]. The coding region of *gCSDC9* comprises 1143 bp and encodes a protein of 381 amino acids with a predicted molecular mass of 40.2 kDa (Fig. 1). The coding sequence matched completely the previously reported sequence of *CSDC9* [8]. Interestingly, both of the introns are located in the 5′-untranslated region (UTR). The second intron is in an upstream open reading frame (uORF) of 54-amino acids (Fig. 1), and the intron is positioned between asparagine and cysteine of the uORF (Fig. 1). To determine the exact position of the 5′ end of *CSDC* transcripts, we performed primer extension analysis using two kinds of primers. Using primer 1, we identified three distinct primer extension products corresponding to the three adenine residues located 1280, 1283, and 1287 nt upstream of the ATG start codon. The major site located 1287 bp upstream from the first ATG was defined as position +1. The position of the transcription start site determined with primer 2 was in accordance with the position determined with primer 1 (data not shown).

3.2. Analysis of putative regulatory elements

We analyzed the *gCSDC9* promoter region as far as 1821 nt upstream of the transcriptional start site with the PLACE database [14]. The 5′-UTR of *gCSDC9* is 1287 nt long, and putative CAAT (CAAT) and TATA (TATATAAA) boxes are located 145 and 32 nt upstream of the transcriptional start site, respectively (Fig. 1). The TATA box at −32 bp has the standard sequence described for other plant genes and usually found at position −32 (±7) [15]. As shown in Table 1, the promoter region contains various potential motifs such as SA, auxin, abiotic and biotic stress-response elements (TGACG) [16–22], a circadian rhythm-related element (CAAACTC-

Table 1
Analysis of putative regulatory *cis*-elements present in *gCSDC9* promoter using PLACE database

Putative *cis*-element	Function	Location
TATTCT	Blue, white, UV-A responsive	−279, −466
ACGTG	ABA responsive	−129
CAACTC	GA responsive	−1057, −1112, −1173
CATATG	Auxin responsive	−1629
AAAAATCT	Phytochrome regulated	−1359
CAACTTCATC	Circadian rhythm-regulated	−50
ATTTCAAA	Ethylene responsive	−600
GATA	Light-regulated	Several
GRWAAW	Light-regulated	Several
GTGA	Late pollen gene (g10)	−175, −365
TAATMATTA	Homeobox gene binding	Several
AGAAA	Pollen-specific activation	Several
AACGTGT	Wounding, tensile stress	−130
TGACG (−)	Biotic and abiotic stress	−69, −753

GAATC) [23], a pollen-specific activation-related element (AGAAA) [24], a low temperature response element (CCGAAA) [25], an ABA response element (ACGTG) [26], a late pollen gene g10-related element (GTGA) [27], a blue, white and UV-A light-related element (TATTCT) [28], a light regulated element (GATAA) [17] and an ethylene response element (ATTTCAAA) [29,30]. The presence of several light response elements is consistent with reports that the level of mRNA for SAMDC increases in leaves of *Pharbitis nil* exposed to visible [31] and UV light [32,33]. Identification of the regulatory element necessary for circadian expression (CAANNNNATC) provided the key to understanding the mechanism of circadian clock controlled-expression of *SAMDC* in *Tritordeum* and *P. nil* [6,33]. Since hormone and/or stress-response elements are present in the *gCSDC9* promoter region it is possible that SAMDC expression may be important for plant development and defense [34].

3.3. Spatial and temporal expression of a 5′-flanking region-GUS fusion in transgenic tobacco

We have reported that *CSDC9* mRNA is present at a high level in petal and stem of carnation [8]. To further investigate the tissue-specific expression of *gCSDC9*, we made a construct containing the 1821-bp promoter and 1179-bp 5′-UTR of *gCSDC9* fused to the *GUS* gene (p1821+5′-UTR), and assayed GUS in tobacco plants transformed with this construct. In the vegetative tissues of the transgenic plants, *GUS* was mainly expressed in the stems of young seedlings and veins of cotyledons (Fig. 2B and C). We divided flower buds into five stages and detected GUS expression in stigma and anther at several stages [35]. It was not expressed in stigma and anther in stage I, in which anthers and pistils are fully differentiated and green (Suppl. 1a and f). Weak expression began in the stigma but not in the anther of flowers at stage II, in which the corolla tube bulges out at the tip of the calyx (Suppl. 1b and g), and moderately strong expression was detected in stigma, and weak expression in anther, of flowers at stage III, when the corolla limb is half open and stigma and anthers are visible (Suppl. 1c and h). *GUS* was strongly expressed in stigma and anthers of flowers at stage IV characterized by open flowers, dehisced anthers, and a fully expanded and deep pink corolla limb (Suppl. 1d and i). This strong expression persisted in stigma, but decreased in anther, at flower stage V, when petals are wilted and ovaries enlarged (Suppl. 1e and j). GUS expression was also strong in petals and dehisced pollen, and moderate in ovary at flower stage IV (Suppl. 1a). However, expression was weak in petals in stages III and V (Suppl. 1b and c) whereas strong expression persisted in dehisced pollen (Suppl. 1h). Expression of GUS in the stems of young seedlings is in agreement with the pattern of *CSDC9* transcripts in carnation stems, and with the stunted phenotype of antisense *SAMDC* transgenic potato plants [7,8]. The presence of regulatory elements related to pollen-specific activation (AGAAA), and the element present in the late pollen gene, g10 (GTGA), and strong GUS expression in mature and germinated pollen, together support the view that increased activity of SAMDC and a resulting increase in Spd and Spm content are essential for pollen germination and tube growth [36,37]. The increased levels of Spd and Spm during pollen germination in tobacco plants may result from strong expression of *SAMDC* in mature and germinated pollen [38].

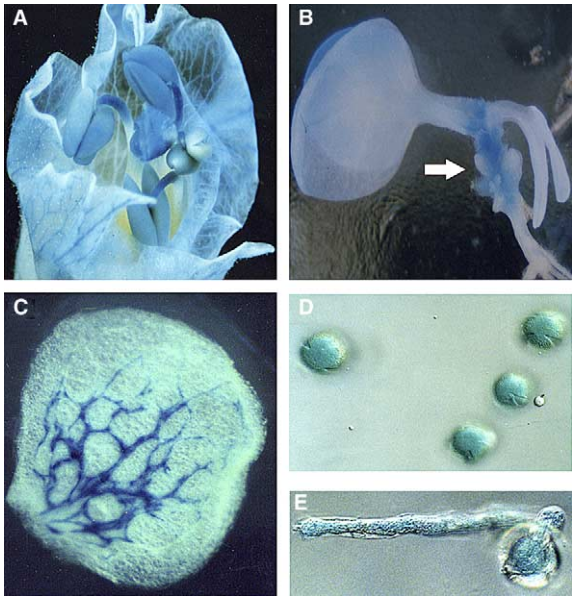

Fig. 2. *GUS* under the control of the *SAMDC* 5′-flanking region is expressed tissue-specifically in transgenic tobacco. There is strong expression in anthers, stigma and petals of flowers at stage IV (A), in the stem of young seedlings (B), veins of cotyledon (C), mature pollen (D) and germinated pollen (E). The white arrow indicates the stem of a young seedling.

3.4. Role of the 5′-flanking region in tissue-specific expression of *gCSDC9*

All reported SAMDC genes in carnation, morning glory, potato, and *Arabidopsis* have at least two introns and a uORF in their 5′-UTR [39,40]. To examine the contribution of introns and uORF, respectively, to the tissue-specific expression of *gCSDC9*, we made a construct containing the 5′-flanking region without the uORF (p1821-uORF) and another with the complete 1821-bp promoter region up to the transcription start site (p1821-5′-UTR) (Fig. 3A). Transgenic tobacco carrying p1821-uORF showed the same GUS expression patterns in petals, stigma, anthers and pollen as plants carrying p1821+5′-UTR (Fig. 3B). However, expression in the former was stronger in the petals, stigma, anthers and pollen of stage IV flowers. At this stage there was no longer expression in the petals, anthers and pollen of plants harboring p1821-5′-UTR but strong GUS expression in stigma (Fig. 3B). Thus, the uORF and first intron are necessary for the extent and pattern of *gCSDC9* expression. Because qualitative and quantitative expression was altered when the first intron and uORF of *gCSDC9* were deleted we designed constructs with various deletions involving these regions (Fig. 3A). Transgenic plant with p273+5′-UTR had similar expression patterns to plants with p1821+5′-UTR. However, in plants with p158+5′-UTR there was no expression in pollen grains and reduced expression in petals, anthers and stigma (Fig. 3B). An analysis with the PLACE database revealed two putative regulatory elements related to pollen-specific expression and two pyrimidine boxes related to GA responses. The sequence (−)AGAAA, a *cis*-regulatory element required for pollen-specific transcription [27], is located at −228 relative to the transcription start site, and pyrimidine boxes, (−)CCTTTT [41,42] and (−)TTTTTTCC [43], related

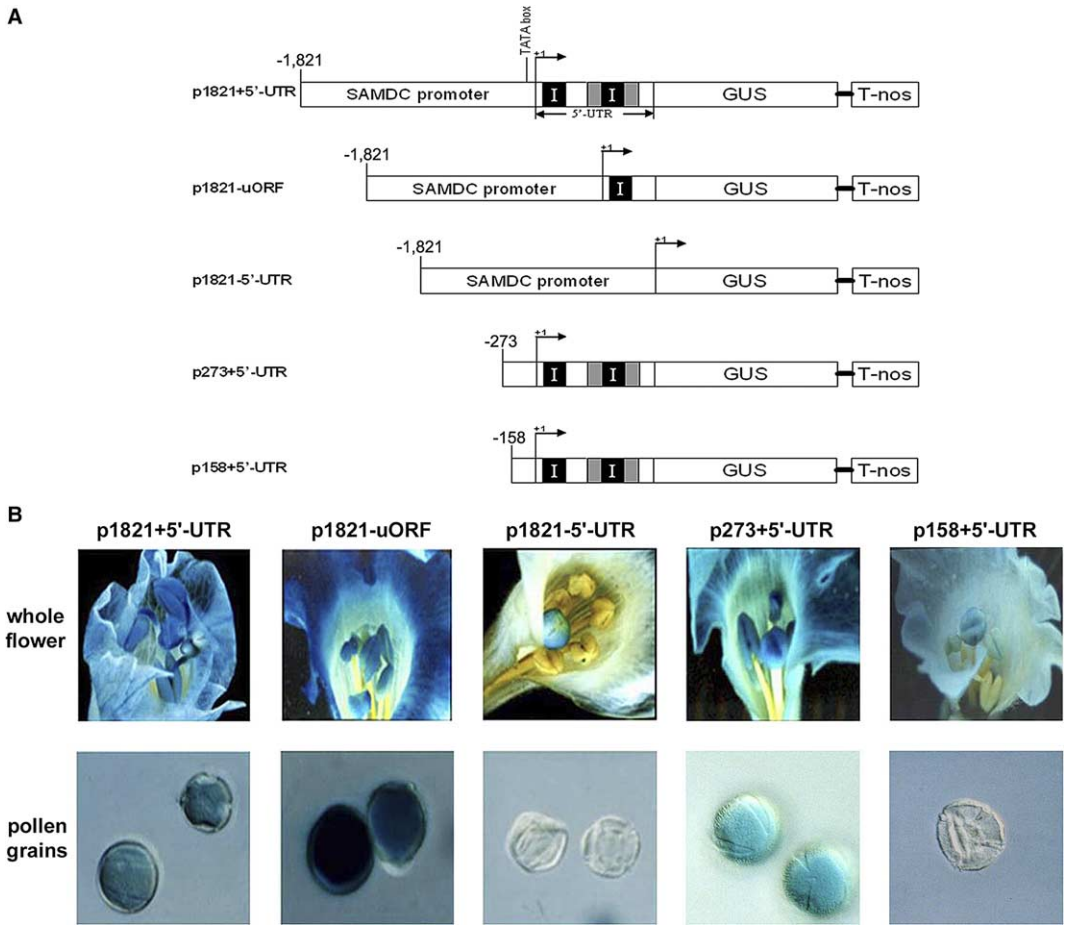

Fig. 3. Schematic diagrams of the chimeric *SAMDC* promoter-*GUS* constructs (A) and *GUS* expression from the deletion constructs in transgenic tobacco (B). (A) Schematic of the *SAMDC* 5′-flanking regions fused to *GUS*. Details of how these constructs were prepared are given in Section 2. p1821+5′-UTR contains 1821 bp of the promoter region and 1179 bp of the 5′-UTR with two introns and the uORF. p1821-uORF contains 1821 bp of promoter region and 5′-UTR without the uORF, and an intron. p1821-5′-UTR contains only 1821 bp of promoter region; p273+5′-UTR contains 273 bp of promoter region and the 5′-UTR, and p158+5′-UTR, 158 bp of promoter region and the 5′-UTR. The first and second introns (I) are in the 5-UTR (black box) and the second intron is within the uORF (shaded gray). The TATA box is located at −32 to −26, and the transcription start site (+1) is indicated by the arrow. (B) GUS expression from the five constructs in stigma, anther, petal and pollen grains of flowers at stage IV of the transgenic tobacco.

to GA induction, are located at −198 and −192, respectively. The motif (+)GTGA, which enhances pollen-specific expression [27], is located at −175 (Table 1). These elements may participate in regulation of the expression of *CSDC9* in pollen although the involvement of other elements within the 115-bp region cannot be excluded.

We also demonstrated that the expression of *CSDC9* in pollen is under the control of the 5′-leader intron and 115-bp promoter region that contain putative sequences related to pollen-specific activation (Fig. 3). A role of 5′-leader introns in *g CSDC9* expression has not previously been demonstrated in plants although a long leader intron is important for temporal and spatial expression of a sucrose synthase gene in potato [44] and maize [45–47]. Our results suggest that the 5′-leader intron may be important for tissue-specific expression of *CSDC9*. In addition the increased GUS activity caused by deletion of the uORF may be related to the inhibitory role of the uORF in translation of SAMDC in *Arabidopsis* [48].

Our results thus suggest that the GTGA and AGAAA motifs together with the 5′-leader intron may be essential for pollen-specific expression of *gCSDC*. However, the 115-bp promoter region needs to be further analyzed to identify any additional *cis*-elements necessary for pollen-specific expression.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version at doi:10.1016/j.febslet.2004.11.005.

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