

The cauliflower mosaic virus (CaMV) 35S promoter sequence alters the level and patterns of activity of adjacent tissue- and organ-specific gene promoters

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Abstract Here we report the effect of the 35S promoter sequence on activities of the tissue- and organ-specific gene promoters in tobacco plants. In the absence of the 35S promoter sequence the *AAP2* promoter is active only in vascular tissues as indicated by expression of the *AAP2:GUS* gene. With the 35S promoter sequence in the same T-plasmid, transgenic plants exhibit twofold to fivefold increase in *AAP2* promoter activity and the promoter becomes active in all tissue types. Transgenic plants hosting the ovary-specific *AGL5:iaaM* gene (*iaaM* coding an auxin

biosynthetic gene) showed a wild-type phenotype except production of seedless fruits, whereas plants hosting the *AGL5:iaaM* gene along with the 35S promoter sequence showed drastic morphological alterations. RT-PCR analysis confirms that the phenotype was caused by activation of the *AGL5:iaaM* gene in non-ovary organs including roots, stems and flowers. When the pollen-, ovule- and early embryo-specific *PAB5:barnase* gene (*barnase* coding a RNase gene) was transformed, the presence of 35S promoter sequence drastically reduced transformation efficiencies. However, the transformation efficiencies were restored in the absence of 35S promoter, indicating that the 35S promoter might activate the expression of *PAB5:barnase* in non-reproductive organs such as calli and shoot primordia. Furthermore, if the 35S promoter sequence was replaced with the *NOS* promoter sequence, no alteration in *AAP2*, *AGL5* or *PAB5* promoter activities was observed. Our results demonstrate that the 35S promoter sequence can convert an adjacent tissue- and organ-specific gene promoter into a globally active promoter.

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Barnase

Abbreviations

AAP2 H⁺/amino acid permease gene 2
AGL5 AGAMOUS-like MADS box protein 5
PAB5 Poly (A) binding protein 5
iaaM IAA monooxygenase
CaMV 35S Cauliflower mosaic virus 35S promoter
GUS β-Glucuronidase

<i>NOS</i>	Nopaline synthase
<i>nptII</i>	Neomycin phosphotransferase
<i>BA</i>	Benzylaminopurine
<i>NAA</i>	Naphthaleneacetic acid
<i>X-Gluc</i>	5-Bromo-4-chloro-3-indolyl- β -D-glucuronic acid
<i>MU</i>	4-Methyl umbelliferone

Introduction

Transgenic plant technology provides an indispensable and powerful tool for analysis of gene functions and stably expression of foreign genes (Daniell et al. 2001; Fischer et al. 1999; Wilke 1999). In many cases, tissue/organ- or developmental stage-specific expression of transgenes in plants is highly desirable. In recent years, a large number of tissue/organ- or stage-specific promoter sequences have been cloned and characterized (Carolina 2003). On the other hand, for a number of transformation vectors used for dicot and monocot plants, such as pCAMBIA, pPZP, pINDEX1, pGKB5, pGA and Gateway's pGWB, the 35S gene promoter from cauliflower mosaic virus (CaMV) has been used to drive the selective marker genes or reporter genes (Battraw and Hall 1990; Bouchez et al. 1993; Curtis and Grossniklaus 2003; Franck et al. 1980; Hajdukiewicz et al. 1994; Ho et al. 1999; Ouwerkerk et al. 2001).

In the early 1980s, some of the first plant transformation vectors were designed, using the *NOS* promoter, not the 35S promoter, to control the expression of selection marker genes such as *nptII* or *APHII* and *DHFR* (Bevan et al. 1983a, b; Bevan 1984; Herrera-Estrella et al. 1983). Kay et al. 1987 reported that duplicated CaMV 35S promoter sequence increased the transcript activity of an adjacent octopine-type T-DNA gene 7 more than 50-fold. In addition, the transcription initiation of another octopine-type T-DNA gene 5 which located approximately 2,000 bp downstream of 35S promoter was also greatly increased. Later, it was further shown that the CaMV 35S promoter could function as an enhancer in either orientation to increase transcription of nearby genes (Benfey et al. 1989, 1990a, b; Fang et al. 1989; Ohtsuki et al. 1998). Because of this effect, the 35S promoter sequence was used to create insertional mutations in higher plants (Hayashi et al. 1992; Jeong et al. 2002; Long et al. 1993; Weigel et al. 2000; Wilson et al. 1996).

More recently, the effect of the 35S promoter sequence on the expression of the nearby genes in transgenic plants was further studied by Yoo et al.

(2005). These authors reported that the 35S promoter sequence affected the expression pattern of adjacent *FT* gene, which acted as a floral inducer. The *LRPI::FT* chimeric gene constructed in pPZP212 vector caused precocious flowering in *Arabidopsis* and Northern blot hybridization demonstrated that the transgene transcripts unexpectedly accumulated in all tissues of the transgenic plants. The effect of the 35S promoter sequence on expression level and pattern of adjacent tissue- and organ-specific genes may therefore lead to misinterpretations of experimental results or make the tissue and organ specific promoters less of value.

To further investigate the effect of the 35S promoter on the expression of tissue-specific transgenes, we studied the influence of the 35S promoter on expression of a GUS reporter gene that was under the control of (1) a vascular tissue-specific *AAP2* promoter (Hirner et al. 1998), (2) on expression of an *iaaM* gene (an auxin biosynthesis gene from *Agrobacterium tumefaciens*) under the control of an ovary-specific *AGL5* promoter (Savidge et al. 1995), and (3) on expression of a cytotoxic gene *barnase* under the control of sexual reproduction and early embryogenesis specific *PAB5* gene promoter (Belostotsky and Meagher 1996). In this manuscript, we report that the 35S promoter sequence can convert tissue/organ-specific gene promoters into constitutive gene promoters with an expression pattern similar to that of the 35S gene promoter, using three different genes: *GUS*, *iaaM* and *barnase*. We also show that the 35S promoter sequence-mediated increases in activities of the adjacent gene promoters vary from organ to organ. We finally demonstrate that *NOS* gene promoter of *Agrobacterium* can be used to replace the 35S promoter to control selection marker or reporter gene expression.

Materials and methods

Construction of gene cassettes

The *AAP2* promoter (EMBL databank accession no: X95623) was amplified by PCR from *Arabidopsis thaliana* ecotype Columbia using the primers: *AAP2F* (5'-GCAAGCTTCTCAAAGCTCCAGTCTTAGA TAA-3') and *AAP2R* (5'-GCGGATCCTATGAACT AGGAATCCTAAGGAGAT-3'). The *Hind*III and *Bam*HI restriction sites are underlined, respectively. PCR was performed under the following condition: genomic DNA was denatured at 94°C for 5 min, followed by 25 cycles of amplification (94°C for 1 min, 50°C for 1 min and 72°C for 2 min) and 7 min at 72°C.

The *AAP2* promoter sequence was subcloned into the *HindIII/BamHI* site of pBI121 (Clontech Inc.), resulting in p*AAP2:GUS* plasmid. Likewise, the 35S promoter sequence was amplified from pBI121 plasmid with a pair of primers, 35SF (5'-GCAAGCTTCCC ACAGATGGTTAGAGA-3') and 35SR (5'-GCAAGCTTCCGTGTTCTCTCCAAATGA-3'), in which the restriction sites of *HindIII* are underlined. PCR amplification was carried out under the following condition: 1 cycle of 94°C for 5 min; 25 cycles of 94°C for 30 s, 50°C for 1 min, 72°C for 1 min; 72°C for 10 min. The 35S promoter sequence was subcloned into *HindIII* site of p*AAP2:GUS* and produced p35S-*AAP2:GUS*.

To construct the fusion gene cassettes, *AGL5:iaaM* chimeric gene, which was derived from the pBin19-*AGL5:iaaM* (Li 1998), was digested with *SalI* and *EcoRI* and inserted into the plant transformation vector pBin19 to produce p*AGL5:iaaM* vector. Subsequently, the *AGL5:iaaM* fragment was produced by partial digestion with *SalI* and *EcoRI* and subcloned into pCambia1305.1. The resulting construct was renamed p35S-*AGL5:iaaM*.

In order to modify pCambia1305.1, the *NOS* promoter sequence was amplified from pBI101 (Clontech Inc.) with the following primers: NOS-F (5'-GCAAGCTTGATCATGAGCGGAGAATTAAG-3') and NOS-R (5'-CGCCATGGAGATCCGGTGCAGATTATTTG-3'). The synthetic restriction sites (*HindIII* and *NcoI*) are underlined. PCR was carried out under the following condition: plasmid DNA was denatured at 94°C for 5 min, followed by 25 cycles of amplification (94°C for 30 s, 54°C for 30 s and 72°C for 1 min) and finally 7 min at 72°C. The *NOS* promoter was subcloned into the *HindIII/NcoI* site of pCambia1305.1. The *AGL5:iaaM* gene fragment was ligated into *EcoRI/XhoI* sites of modified pCambia1305.1, generating p*NOS-AGL5:iaaM* plasmid.

The *PAB5* gene promoter was amplified using PCR from *A. thaliana* ecotype Columbia with a pair of primers: PAB5F (5'-CGAAGCTTCTCGAGCTA GAAAGAGAACC-3') and PAB5R (5'-GCGGATCCGGCCGTCGTCGGGGCAATTCC-3'). The restriction sites *HindIII* and *BamHI*, sites are underlined, respectively. PCR was performed under the following condition: genomic DNA was denatured at 94°C for 5 min, followed by 30 cycles of amplification (94°C for 1 min, 56°C for 30 s and 72°C for 2 min) and 7 min at 72°C. The *PAB5* promoter sequence was subcloned into the *HindIII/BamHI* site of pCambia1305.1. A 330 bp *barnase* gene fragment was amplified from pMT1002 plasmid (provided by Dr. Robert W Hartley)

with primers: PMT5 (5'-GCGGATCCATGGCTCAGGTTATCAACAC-3') and PMT3 (5'-GCGAGCTCTCAACGGATCTTAGTAAAGGT-3'). The restriction sites, *BamHI* and *SacI*, are underlined, respectively. PCR was performed at the following condition: pMT1002 plasmid DNA was denatured at 94°C for 5 min, followed by 25 cycles of amplification (94°C for 30 s, 56°C for 30 s and 72°C for 30 s) and 7 min at 72°C. The 330 bp *barnase* gene coding sequence was subcloned into the *BamHI/SacI* sites of pCambia1305.1-PAB5, resulting in p35S-*PAB5:barnase* plasmid. The *HindIII/EcoRI* digested *PAB5:barnase* fragment replaced the *AGL5:iaaM* of p*NOS-AGL5:iaaM* construct to produce p*NOS-PAB5:barnase* plasmid.

All the genes and promoters were confirmed by DNA sequencing.

Plant transformation and growth conditions

Binary Ti plasmids were electroporated into *Agrobacterium tumefaciens* strain EHA105 and the resulting bacteria were used to transform *Nicotiana tabacum* cv. Xanthi and Petit Havana Ottawa (Horsch et al. 1985). Tobacco was grown in a greenhouse at 25°C and under an 18 h light/6 h dark photoperiod. Leaf discs, approximately 0.5 × 0.5 cm, were incubated with *A. tumefaciens* (OD₆₃₀ = 0.5) containing binary vector for 5 min, and then transferred onto sterile filter paper discs to remove excess liquid and bacteria. After 3 days of co-cultivation at 28°C in dark, infected discs were transferred to MS medium (Murashige and Skoog 1962) containing 1 mg l⁻¹ BA, 0.1 mg l⁻¹ NAA, 100 mg l⁻¹ kanamycin, 8 g l⁻¹ agar, 30 g l⁻¹ sucrose, 150 mg l⁻¹ cefotaxime for selection and shooting. More than 15 kanamycin-resistant putative transformants were obtained for each construct. Putative transgenic shoots were transferred to 1/2 MS medium supplemented with 100 mg l⁻¹ kanamycin for rooting. The plantlets were transplanted in soil and grown in the greenhouse.

RNA isolation and RT-PCR

For reverse transcription-polymerase chain reactions (RT-PCR), 20 µg of total RNA isolated from various tissues of tobacco plants was used. The RNA extraction protocol was followed as molecular cloning: a laboratory manual (Sambrook and Russell 2000) described. For RT-PCR analysis, DNase (DNA-free™, Ambion, Inc.) treatment was used to eliminate DNA

contamination from RNA samples. First strand cDNA was synthesized from 1 µg of total RNA using cDNA synthesis kit (ImProm-II™ Reverse Transcription System, Promega Co.). One twentieth of volume of the first strand cDNA reaction was used for PCR reactions to amplify the *iaaM* and *TAC9* gene cDNAs (Thangavelu et al. 1993). The primers, IAAM1 (5'-AAGGTAGCAGTTCTCTCCGC-3') and IAAM2 (5'-TCGGCTTAGGAACATCCTCC-3') were used to amplify the *iaaM* cDNA. The PCR condition was 94°C for 30 s, 56°C for 30 s and 72°C for 30 s, 30 cycles in total. T1 (5'-ATGCCCTCCCACATGCTATTC-3') and T2 (5'-AACATGGTAGAGCCACCACTA-3') primers were used to amplify the *TAC9* gene used for an internal control. The PCR condition was 94°C for 30 s, 56°C for 30 s and 72°C for 30 s, 25 cycles in total.

Histochemical staining and fluorometric assay of GUS activity

Histochemical staining for β -glucuronidase (GUS) activity was performed as described by Jefferson et al. (1987). Sections of various tissues of transgenic plants were incubated in 1 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (X-Gluc) in 50 mM phosphate buffer, pH 7.0 at 37°C for 24 h. After staining, sections were rinsed in 75% (v/v) ethanol for at least 1 h, and then mounted for microscopy.

Fluorometric assay of GUS activity was measured as described by Jefferson et al. (1987). Three lines of wild-type tobacco plants, five *AAP2:GUS* fusion transgenic lines, six *35S-AAP2:GUS* fusion transgenic lines and five *35S:GUS* positive control transgenic lines were used. Samples of five different tissues were analyzed including root, stem, leaf, flower and seedlings. Tissues were lysed in extraction buffer (50 mM phosphate buffer, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.1% sodium lauryl sarcosine, and 10 mM β -mercaptoethanol) by freezing with liquid nitrogen and grinding with mortar and pestle with silicon. Aliquots of the extracts (100 µl) were added to 1 ml of assay buffer (extraction buffer containing 1 mM MU), prewarmed and incubated at 37°C. After 0, 5 and 20 min of incubation, 100 µl samples were removed and placed in 1.9 ml stop buffer (200 µM sodium carbonate). Fluorescence was measured using a Multi-Detection Microplate Reader (Bio-TEK® Synergy™ HT). Protein concentration was determined by Bradford assay (Bradford 1976). GUS activity was expressed as picomole 4-MU per minute per milligram protein with data from five independent lines. Three repeats were performed for each sample.

Results and discussion

The effect of the 35S promoter on the expression pattern and level of the vascular specific *AAP2:GUS* gene

To determine the effect of 35S promoter sequence on the pattern and level of vascular specific *AAP2* gene promoter activity, the *AAP2* promoter sequence cloned from *A. thaliana* was used to control the GUS gene expression with or without 35S gene promoter in the T-DNA region of the Ti-plasmid vector. Two plant expression vectors, *pAAP2:GUS* (without 35S promoter) and *p35S-AAP2:GUS* (with 35S promoter) (Fig. 1) was used for production of transgenic tobacco plants via *Agrobacterium*-mediated transformation. The *p35S:GUS* was introduced into tobacco plants as a control. All putative transgenic plants were initially screened by RT-PCR and histochemical staining of GUS activity.

Similar to previous reports (Benfey et al. 1989), the GUS gene under the control of the 35S promoter is active in most tissues and organs throughout the plant (marked as 35S in Fig. 2), confirming that the 35S

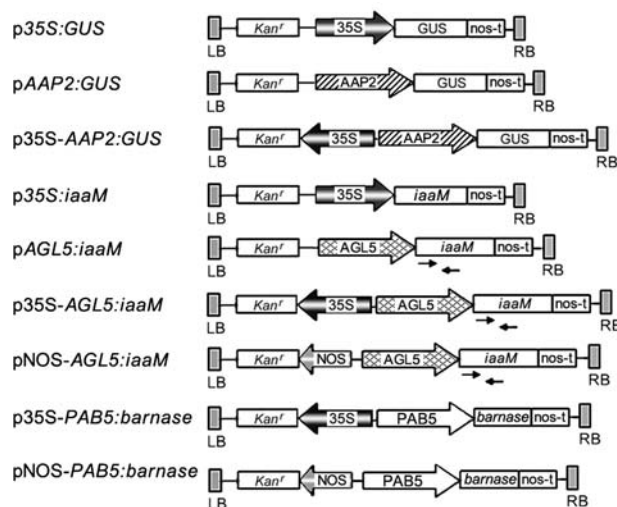
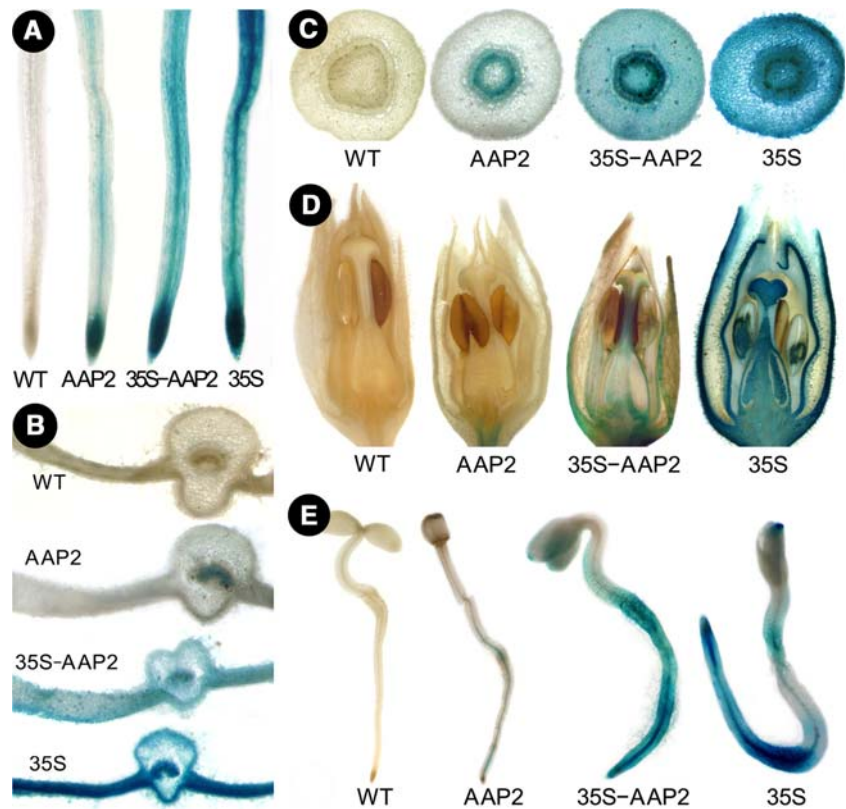


Fig. 1 Schematic representation of the T-DNA regions of transformation vectors used in this study. *RB* right border, *LB* left border, *Km^r* kanamycin selection marker, 35S CaMV 35S promoter, *GUS* β -glucuronidase gene, *nos-t* the terminator of the nopaline synthase gene, *AAP2* the promoter of the *AAP2* gene (*H⁺*/amino acid permease gene 2), *iaaM* gene that encodes a tryptophan-2-monooxygenase for the production of IAM (indole-3-acetamide) in IAA biosynthesis, *AGL5* the promoter sequence of the *AGL5* gene (AGAMOUS-like MADS box protein 5), *PAB5* the promoter sequence of the *PAB5* gene [poly(A) binding protein 5], *barnase* a ribonuclease from *Bacillus amyloliquefaciens*. The positions of the primers used for RT-PCR analysis are indicated by arrows

Fig. 2 Histochemical staining of GUS activity of transgenic tobacco plants. **a–d** GUS activity was detected in roots, leaves, stems and flowers of transgenic plants transformed with p*AAP2*:*GUS*, p35S-*AAP2*:*GUS* and p35S:*GUS*. GUS activity in all tissues of p35S-*AAP2*:*GUS* plants was weaker than in the 35S:*GUS* plants but much higher than in p*AAP2*:*GUS* plants. **e** The similar result of GUS staining was also obtained in T₁ seedlings of transgenic plants. WT wild-type tobacco plants, *AAP2* transgenic plants carrying the *AAP2*:*GUS* gene, 35S-*AAP2* transgenic plants carrying 35S-*AAP2*:*GUS* gene, 35S transgenic plants carrying the 35S:*GUS* gene



promoter sequence used in our study is constitutively and globally active. Also, consistent with the results reported by Hirner et al. (1998), the *AAP2* gene promoter was active specifically in vascular tissues of roots, stems and leaves in the absence of the 35S promoter sequence (marked as *AAP2* in Fig. 2). However, the *AAP2* gene promoter if adjacent to the 35S promoter sequence exhibits an expression pattern similar to that of the 35S promoter at the tissue level (Fig. 2). Fluorometric assay of GUS activity was carried out to quantify changes in GUS activity in roots, stems, leaves, flowers and young seedlings (Fig. 3). With the 35S promoter present nearby, the *AAP2* promoter activity was enhanced by twofold to fivefold in all organs and entire young seedlings. The highest increase, about fivefold, in GUS activity was observed in leaves and young seedlings. However, relatively less increases, approximately twofold to threefold, were seen in flowers, roots and stems (Fig. 3). The variations in the level of the 35S promoter-mediated increase in the *AAP2* promoter activity in different organs demonstrate that the effect of the 35S promoter can vary among organs, suggesting that the effect of the 35S promoter on adjacent promoters at the organ level can be complex and may be difficult to predict.

The effects of the 35S promoter on expression pattern and level of the ovary-specific *AGL5:iaaM* gene

Previously, it has been shown that over-expression of the *iaaM* gene under control of 35S promoter produces many changes in phenotype such as leaf epinasty, adventitious root formation from unwounded stem and leaf tissues, stronger apical dominance and seedless fruits (Klee et al. 1987; Sitbon et al. 1992). To further examine the effect of the 35S promoter sequence on tissue specific promoters, we used a carpel and ovule specific *AGL5* promoter and the *iaaM* gene that encodes a tryptophan-2-monooxygenase for the production of IAM (indole-3-acetamide) in IAA biosynthesis (Klee et al. 1987). The *AGL5:iaaM* gene constructs with or without the 35S promoter sequence was shown in Fig. 1 (p*AGL5:iaaM* and p35S-*AGL5:iaaM*). The 35S:*iaaM* gene (p35S:*iaaM* in Fig. 1) was used as a control for the 35S-*AGL5:iaaM* plants.

Transgenic plants that expressed the *AGL5:iaaM* gene without 35S promoter exhibited no visible phenotype when compared to wild-type controls except seedless fruits (marked as *AGL5* in Fig. 4). In contrast, transgenic plants (marked as 35S-*AGL5*) that expressed the *AGL5:iaaM* gene with the 35S promoter

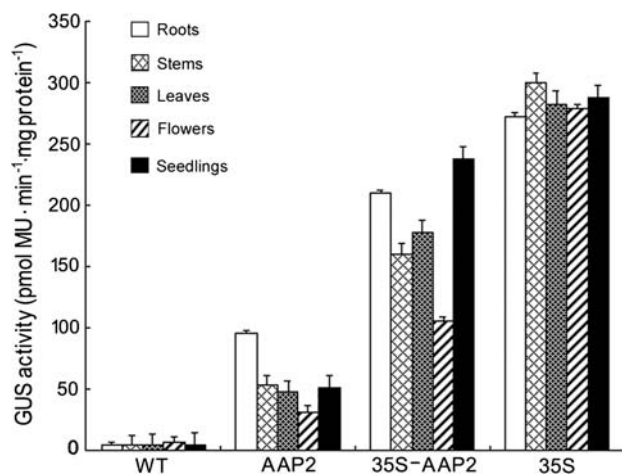


Fig. 3 Quantitative measurement of the GUS enzyme activity of transgenic tobacco plants and T_1 seedlings. *WT* wild-type tobacco plants, *AAP2* transgenic plants carrying the *AAP2:GUS* gene, *35S-AAP2* transgenic plants carrying *35S-AAP2:GUS* gene fragment, *35S* transgenic plants carrying the *35S:GUS* gene. Error bars indicate SE values ($n = 3.5\text{--}300.1$)

present nearby (*pNOS-AGL5:iaaM* in Fig. 1) exhibited a wide range of phenotypic alterations: leaf epinasty, reduction in height, adventitious root formation from the stem and leaf, reduced numbers of flower buds. The phenotype is identical or highly similar to that of the *35S:iaaM* over-expressing plants (marked as *35S* in Fig. 4; Table 1). To verify the effect of the *35S* promoter sequence on the adjacent promoter sequences, we replaced the *35S* promoter sequence with the *NOS* promoter sequence cloned from *Agrobacterium* (An et al. 1988) to make *pNOS-AGL5:iaaM* (Fig. 1). The *NOS* promoter has been extensively used in plant transformation vectors to drive the expression of selectable marker genes (An et al. 1988; Bevan 1984; Hennegan and Danna 1998; Lichtenstein and Fuller 1987; Sanders et al. 1987). After transformation, plants hosting the *NOS-AGL5:iaaM* gene displayed no obvious phenotypic difference when compared with wild-type plants (marked as *NOS-AGL5* in Fig. 4; Table 1) although these plants also produced significant numbers of seedless fruits as expected (Table 1). These results show that the *NOS* gene promoter may be used to replace the *35S* gene promoter sequence to drive marker gene expression in transgenic plants in many cases.

The effect of the *35S* promoter sequence on expression level of the *AGL5:iaaM* gene in transgenic plants was further confirmed with reverse transcription-PCR (RT-PCR) technique. The levels of the *iaaM* transcript in roots, stems, leaves, flowers and ovaries of wild type and transgenic plants that hosted

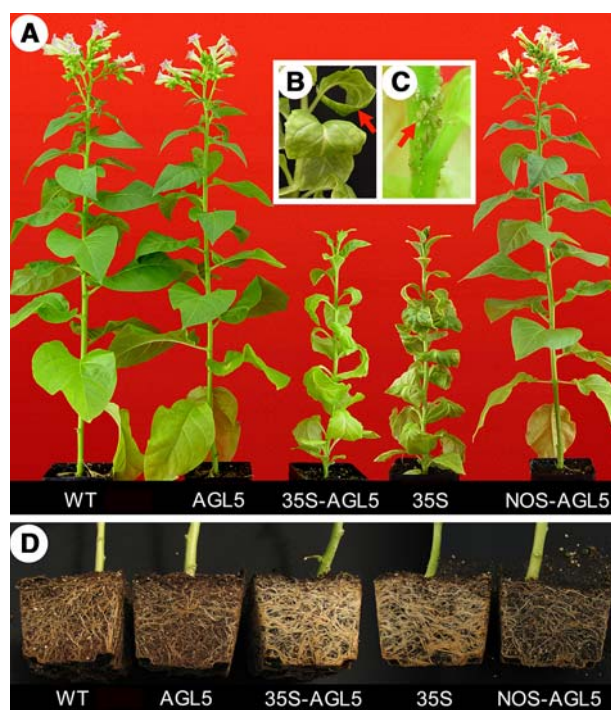


Fig. 4 Phenotypic alterations in transgenic tobacco plants carrying the *AGL5:iaaM* or *35S-AGL5:iaaM* genes **a** no obvious difference between wild-type, *AGL5:iaaM* and *NOS-AGL5:iaaM* plants were observed but *35S-AGL5:iaaM* plants and *35S:iaaM* plants showed curled leaves, enhanced root formation, adventitious roots on the stems and reduced plant size **b** and **c** curled leaves and adventitious roots on the stems of *35S-AGL5:iaaM* plants were indicated by red arrow, respectively. **d** Detailed view of root system of wild type and transgenic plants. *WT* wild-type plants, *AGL5* transgenic plants containing the *AGL5:iaaM* gene, *35S-AGL5* transgenic plants containing *35S-AGL5:iaaM* gene, *35S* transgenic plants carrying the *35S:iaaM* gene, *NOS-AGL5* transgenic plants containing *NOS-AGL5:iaaM* gene

the *AGL5:iaaM* gene with or without the *35S* promoter sequence were showed in Fig. 5. No expression of the *iaaM* gene was observed in organs of wild-type plants except in ovary and leaves. In the case of transgenic plants that hosted the *AGL5:iaaM* gene with the *35S* promoter sequence present, the *iaaM* transcript was detected in all organs examined: root, stem, leaf, flower and ovary, indicating the enhanced expression of the *iaaM* gene in these organs. However, if the *35S* promoter was replaced with the *NOS* promoter sequence, the expression pattern of the *AGL5:iaaM* gene was identical to that of the *AGL5:iaaM* plants without the *35S* promoter sequence present. These results confirm that changes in expression level and pattern of the *AGL5:iaaM* gene observed in the *pNOS-AGL5:iaaM* plants is caused by the *35S* promoter sequence (Fig. 5).

Table 1 Phenotypic characteristics of transgenic tobacco plants that express the *AGL5* promoter-*iaaM* gene with or without the 35S promoter sequence present in the same Ti vector

Constructs	Number of independent plant lines analyzed	Average height of transgenic plants lines (cm)	Average number of flower buds per line	Number of seedless lines ^a
Wild type	8	106.3 ± 10.3	69.3 ± 14.6	0
<i>pAGL5:iaaM</i>	48	104.4 ± 12.4	70.5 ± 12.4	10
<i>p35S-AGL5:iaaM</i> (low expresser) ^b	12	82.4 ± 12.5	28.7 ± 8.4	7
<i>p35S-AGL5:iaaM</i> (medium expresser) ^c	35	63.2 ± 9.4	6.3 ± 4.2	28
<i>p35S-AGL5:iaaM</i> (high expresser) ^d	10	46.5 ± 9.6	0	N/A
<i>pNOS-AGL5:iaaM</i>	26	107.3 ± 10.5	73.3 ± 15.4	7

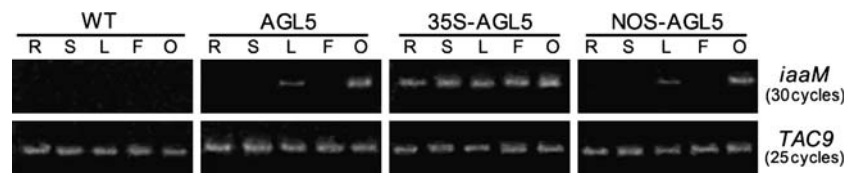
The values represent the mean ± SD

^a Number of lines producing seedless fruits

^b Transgenic plants harboring *p35S-AGL5:iaaM* construct were categorized into three levels: low, medium and high expressers. With the height over 70 cm, 12 out of 57 lines in total were defined as low expressers

^c With the height ranged between 50 and 70 cm, 35 lines were classified as medium expressers

^d The remaining 10 lines, with the height below 50 cm, were categorized as high expressers

**Fig. 5** RT-PCR analysis of *iaaM* expression in wild-type and transgenic plants. About 20 µg of total RNA isolated from different tissues were used to synthesize cDNAs. The expression of the *iaaM* gene was detected in all tissues of *35S-AGL5:iaaM*

plants. *WT* wild-type plants, *AGL5* transgenic plants containing the *AGL5:iaaM* gene, *35S-AGL5* transgenic plants containing *35S-AGL5:iaaM* gene, *NOS-AGL5* transgenic plants containing *NOS-AGL5:iaaM* gene. *R* root, *S* stem, *L* leaf, *F* flower, *O* ovule

The effects of 35S promoter on the level and pattern of activity of pollen-, ovule- and early embryo-specific *PAB5:barnase* gene

Because *barnase*, an extracellular ribonuclease of *Bacillus amyloliquefaciens*, causes death of host cells, it may serve as an excellent reporter gene to monitor changes in promoter activity. Target expression of the *barnase* gene in the anther tapetal cell layer rendered male-sterility in transformed tobacco, corn and oilseed rape plants (Mariani et al. 1990, 1992). When we used *Agrobacterium* hosting the *p35S-PAB5:barnase* gene to

transform *Nicotiana tabacum* cv. Xanthi, we observed a 25-fold reduction in transformation efficiency (Table 2). However, if the 35S promoter was replaced with the *NOS* gene promoter, transformation efficiency was restored (Table 2). Similar results were observed when PHO (Pefit Havanna Ottawa) was used as explants for plant transformation. These results demonstrate that if a tissue/organ-specific gene promoter is used to control expression of a cell toxin gene when the 35S promoter sequence resides in the same transformation vector, the toxin gene can be activated in non-target tissues and the activation of the toxin gene may

Table 2 Effects of the *PAB5:barnase* gene on transformation efficiency of tobacco

Plant cultivar	Gene constructs	Number of explants (leaf discs) used	Number of transgenic shoots produced	Transformation events efficiency (%)
<i>Nicotiana tabacum</i> cv. Xanthi	<i>p35S:GUS</i>	100	75	75
	<i>p35S-PAB5:barnase</i>	150	5	3
	<i>pNOS-PAB5:barnase</i>	95	67	71
<i>Nicotiana tabacum</i> cv. Petit Havanna Ottawa	<i>p35S:GUS</i>	100	89	89
	<i>p35S-PAB5:barnase</i>	175	16	9
	<i>pNOS-PAB5:barnase</i>	110	96	86

The values represent the mean ± SD

subsequently reduce plant transformation efficiency (Table 2) or produce unexpected changes in phenotype (Fig. 4).

Conclusions

Yoo et al. (2005) reported that the 35S promoter used in a selectable marker gene affected the expression pattern of a root-specific transgene and the affected expression mostly disappeared in transgenic plants generated using vectors without the 35S sequences within their T-DNA region. In our current study, we have confirmed and extended their findings by using various gene constructs with three different gene promoters (*AAP2*, *AGL5* and *PAB5*) and three reporter genes (*GUS*, *iaaM* and *barnase*). Specifically, we have demonstrated that the 35S promoter sequence can:

1. Enhance overall activities of all three distinct tissue- and organ-specific gene promoters tested as reflected with the *GUS* activity, *iaaM* transcript level, auxin overproducing phenotype and *barnase*-associated phenotype. Our results confirm the previous findings reported in the literature that the 35S promoter can influence activities of adjacent gene promoters.
2. Convert tissue-specific gene promoters such as vascular-specific *AAP2* gene promoter into a functional constitutive, similar to 35S promoter, at the tissue level, which makes the *AAP2* gene promoter a globally active gene promoter although its overall activity is weaker than that of the 35S promoter. Previously, it has been shown that 35S promoter sequence can cause enhanced expression of nearby genes without affecting the expression pattern (Jeong et al. 2002; Neff et al. 1999, van der Graaff et al. 2000; Weigel et al. 2000).
3. Interfere organ-specific gene promoter in a differential manner with more profound effect on some organs than others. For instance, 35S promoter sequence exerts a stronger effect on the *AAP2* gene promoter activity in leaf than in flower. In the case of *AGL5* gene promoter, the 35S promoter appears to have stronger effect in roots, stem and flowers but relatively less in leaf and ovary. These novel findings suggest that the effect of the 35S promoter sequence on organ- and tissue-specific promoters at the organ level can vary from one promoter to another and therefore it is difficult to predict its effect on a particular gene promoter.
4. Cause detrimental effects during the process of transgenic plant production or later stages of plant

growth and development if an organ- and tissue-specific gene promoter is used to control expression of a toxin gene such as *barnase* gene in plants. Thus, the 35S promoter should not be used when a cell toxin is to be expressed in specific organs/tissues of plants.

5. Be replaced with the *NOS* gene promoter because *NOS* promoter does not interfere with adjacent gene promoters. However, because the *NOS* promoter is weaker than the 35S promoter, it won't be a good choice if strong expression is desired.

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