Application of *Arabidopsis AGAMOUS* second intron for the engineered ablation of flower development in transgenic tobacco

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Abstract

To explore new approach to generating reproductive sterility in transgenic plants, the *barnase* gene from *Bacillus amyloliquefaciens* was placed under the control of an 1853 bp nucleotide sequence from the 3'end of the second intron of *Arabidopsis AGAMOUS* and CaMV 35S (-60) minimal promoter [*AG-I-35S (-60)::Barbase*], and was introduced into tobacco through transformation mediated by *Agrobacterium tumefaciens*. All *AG-I-35S (-60)::Barbase* transgenic plants showed normal vegetative growth and 28% of the transgenic lines were observed with complete ablation of flowering. Two transgenic lines Bar-5 and Bar-15 were 98.1% and 98.4% sterile as tested by seed production and germination. When controlled by *AG-I-35S (-60)* chimeric promoter, *barnase* mRNA was detected in the reproductive tissues of transgenic tobacco plants, but not in vegetative parts. This study presents the first application of *AG* intron sequence in the engineered ablation of sexual reproduction in plants. The *AG-I-35S (-60)::Barbase* construct is useful to diminish pollen and seed formation in plants, providing a novel bisexual sterility strategy for interception of transgene escape and it has other potentially commercial use for transgenic engineering.

Keywords *AGAMOUS* intron . *Barnase* . *Nicotiana tabacum* . Biosexual sterility

Introduction

When using genetically modified plants, there is the concern that foreign genes may transfer to related plant species through altered pollens. Such unintentionally modified plants have an impact on ecosystems (Strauss et al. 1995; Dale et al. 2002). The ability to produce sterile transgenic plants is very desirable because there is a high potential for interception of transgene escape under field conditions. The plant fertility control and its application in hybrid seed production are also very important in molecular plant breeding and other agricultural activities (Khan 2005). Therefore, engineering total male and female sterility is a highly desirable characteristic for transgenic plants to possess for gene containment and selective breeding of other beneficial traits.

Several molecular approaches have been employed to obtain male or/and female sterility in plants (Ruiz and Daniell 2005). RNA silencing through anti-sense or co-suppression was used to interfere normal reproductive organ development that resulted in plant sterility (van der Meer et al. 1992; Yui et al. 2003). Expression of a cytotoxic gene for a programmed cell death under a control of reproductive tissue-specific promoter is another strategy for plant sterility. Mariani et al. (1990) first developed a male sterility system with the *barnase* gene regulated by the tapetum-specific *TA29* promoter. *Barnase* is a cytotoxic gene, encoding an RNase originally from *Bacillus amyloliquefaciens* (Hartley 1988). As a highly toxic protein to plant cells, this enzyme degrades cellular RNA, resulting in cell death. As driven by floral tissue-specific promoters, *barnase* has been used for engineered male and

female sterility in a few plant species (Mariani et al. 1990; Goldman et al. 1994; De Block et al. 1997; Roque et al. 2007; Wei et al. 2007). Other cytotoxin genes used in plant transgene sterility include those encoding for chain A of diphtheria toxin (DT-A) (Nilsson et al. 1998), RnaseT1 (Mariani et al. 1990), ribosomal inactivating protein (RIP) (Cho et al. 2000), and stilbene synthase (Höfig et al. 2006).

Any leaked expression of a cytotoxin gene in plant tissues other than reproductive tissues would lead to aberrant vegetative development. Different so-called floral-specific promoters have been used in genetic engineered sterility in various plants. Apart from tapetal specific *TA29* promoter (Mariani et al. 1990), other similar promoters include those from floral primordial specific *LEAFY* (Nilsson et al. 1998), *BpMADS1* (Lemmetyinen et al. 2004), *BpFULL1* (Lannenpaa et al. 2005), *AtDMC* (Kobayashi et al. 2006), and others. A *LEAFY::TD-A* transgene was shown to eliminate flowering completely in transgenic *Arabidopsis* (Nilsson et al. 1998). But another research suggested that there was a substantial expression in non-reproductive tissues regulated by a polar *LEAF* promoter (Wei et al. 2007). Non-flowering plants from transgene of *PTD::DTA* also exhibited less vigorous vegetative growth, suggesting toxin expression in vegetative tissue regulated by the promoter of this MADS box gene, a homolog of *DEFICIENS* and *APETALA3* (Skinner et al. 2003). *BpMADS1 promoter::barnase* transgene can prevent plant flowering, but still displayed some undesired effects (Lemmetynen et al. 2004). Therefore, confinement of cotytoxic protein only in desired plant tissue is a practical challenge for biotechnology.

The normal floral development, from inflorescence initiation to floral organ formation, depends on spatial and temporal expression control of a series of genes. The floral homeotic gene *AGAMOUS* (*AG*) of *Arabidopsis* is required to confer determinacy to the floral meristem and to specify the identity of sexual organs (stamens and carpels) in floral development (Yanofsky et al. 1990; Dinneny and Yanofsky 2004). Expression of *AG* mRNA was detected early in the central region of the floral meristem that develops into the sexual organs (Drews et al. 1991). In mature flowers, *AG* transcript is expressed in the connective tissue of stamens and in the stigma and ovules of carpels (Bowman et al. 19991).

The *AG* expression pattern requires a 3.8 Kb sequence located in the second intron of this gene (Sieburth and Meyerowitz 1997; Busch et al. 1999; Deyholos and Sieburth 2000). Sieburth and Meyerowitz (1997) have first found that DNA sequences controlling proper *AG* expression lie within the 3.8 Kb intragenic region by characterizing *AG::β-glucuronidase* (*GUS*) gene fusion. By contrast, the GUS activity driven by the *AG* 5'-untranscribed region is significantly different from *in vivo AG* expression. A further investigation on this *AG* 3.8 Kb intragenic region with a series of *AG::reporter* gene constructs identified an 1653 bp DNA fragment from the 3'-end of the second intron that confers a normal *AG* expression pattern (Deyholos and Sieburth 2000). Phylogenetic footprinting and shadowing analysis revealed *cis*-regulatory elements responsible for *AG* expression localized in this intron (Hong et al. 2003).

In this study, we explored to incorporate *Arabidopsis AG* second intron in

genetic engineering of plant sterility using model plant species tobacco. With *barnase* expression under control of the second intron fragment of the *Arabidopsis AG* gene, we obtained transgenic plant lines with non-flowering and abnormal flowering phenotypes.

Materials and Methods

Cloning of *Barnase* and *AG* second intron

Barnase coding region (Paddon and Hartley 1985) was amplified from *Bacillus amyloliquefaciens* genomic DNA. Based on *barnase* sequence (GeneBank accession No. M14442), two primers were designed for polymerase chain reaction (PCR) as Bar-5 (5'-T ACC ATG GTA CCG GTT ATC AAC ACG TTT GAC GG-3') and Bar-3 (5'-TAG GATC C TTA TCT GAT TTT TGT AAA GGT CTG-3') with restriction sites *Nco* I and *Bam*H I respectively.

Arabidopisis thaliana (WS) was used for the cloning of the second *AG* intron. Two PCR primers were designed as below according to the gene sequence (GenBank accession No. AL021711): AG-I5 (5'-GGA AGC TTC GAA CTA CAT TAC TCA AAC TA G-3') and AG-I3 (5'-GGG GTA CCC TTC TTG TAC CTC TCA ATA GT-3'), which had restriction sites *Hind* III and *Kpn* I respectively for further molecular manipulation. PCR was carried out at 94ºC for 5 min, followed by 30 cycles of denaturing at 94ºC for 30 s, annealing at 53ºC for 30 s, and extension at 72ºC for 2 min, and a final extension at 72ºC for 10 min.

Vector construction

The *barnase* gene construct was generated by placing *AG* intron (*AG-I*) DNA fragment upstream of a minimal region (-60) of the cauliflower mosaic virus (CaMV) 35S promoter (Fang et al. 1989; Deyholos and Sieburth 2000). PCR products of 328 bp *barnase* coding region and 1869 bp AG-I were separated on 0.8% agarose gels, and cloned into pUC-T vector (Shanghai Biotech Inc., Shanghai, China), producing recombinant plasmids pUC-T-bar and pUC-T-AGI. Following full-length sequence analysis of both PCR fragments, the *barnase* fragment was digested with *Nco* I/*Bam* HI, and ligated into the corresponding sites of pCaMV-60 , a generous gift from Prof. Z. Meng (Institute of Botany, Chinese Academy of Sciences), resulting in a recombinant plasmid pCaMV-60-Barnase with *barnase* coding sequence behind minimal CaMV 35S (-60) promoter. After restriction enzyme digestion, *AG-I* fragment was ligated into *Hind* III/*Kpn* I sites of pCaMV-60-Barnase, producing a recombinant plasmid pAG-I-CaMV-60-Bar where *AG-I* fragment was localized upstream of -60 bp of minimal CaMV 35S promoter. The expression cassette of AG-I-35S (-60)-Barnase-Nos terminator was excised with *Hind* III/*Sac* I, and inserted into the corresponding sites of pBI 121 (Jefferson et al. 1987), resulting in a binary vector pAG-I-35S (-60)-Bar (Fig. 1). All DNA manipulations were performed using standard protocols (Sambrook and Russellet 2001).

Tobacco transformation

The construct pAG-I-35S (-60)-Bar was introduced into *Agrobacterium*

tumefacine GV3101 by electroporation. *Nicotiana tabacum cv.* Huangmiaoyu, provided by Prof. C.-S. Sun (Rice Research Institute of China), was used for gene transformation. Tobacco leaf discs were transformed as described (Horsch et al. 1985) with slight modification. *A. tumefacine* clones were directly picked up from solid medium and resuspended in MS liquid medium (Murashige and Skoog 1962) containing 1.0 mg l^{-1} 6-benzylaminopurine (6-BA) and 100 μmol l^{-1} acetosyringone (AS). Leaf discs were incubated in *A. tumefacine* solution for 10 min with gentle shaking. After removing excessive bacterial solution, leaf discs were transferred in MS medium containing 1.0 mg l^{-1} 6-BA, and co-cultured in dark at 27ºC for 3 days. The selection of transformed tissues was performed in shoot-induction medium containing 50 mg l^{-1} kanamycin and 300 mg l^{-1} cefotaxime sodium. Kanamycin-resistant shoots were cultured in MS medium supplemented with 3 g l^{-1} sucrose, 7 g l^{-1} glucose, 50 mg l^{-1} kanamycin, and 300 mg l^{-1} cefotaxime sodium for root induction. Plantlets were moved to soil in a green house for further plant growth and development. Nodes from some transgenic lines were excised for clonal propagation.

PCR and Southern blot analyses of *AG-I-35S (-60)::barnase* transgene

To confirm transgene insertion in transgenic tobacco plants, genomic DNA extraction from the transformants and PCR detection were conducted as described previously (Liu and Ekramoddoullah 2003) to test transgene insertion in transgenic tobacco plants. Two primers were designed to target the internal regions of *AG* intron

and *barnase* respectively: AB-5 (5'-ATC TAC GGT CAT GAT CTC TCC-3') and AB-3 (5'-GTA AAT CAG CCA GTC GCT TGA-3'). Using tobacco genomic DNA as templates, PCR was performed with conditions as a pre-denaturing at 94ºC for 5 min, 30 cycles of denaturing at 94ºC for 30 s, annealing at 55ºC for 30 s, and extension at 72ºC for 1 min, and an ending extension at 72ºC for 10 min.

For Southern blot analysis, genomic DNA (10 μg) was digested with *Eco*R V. Because *barnase* sequence does not contain *Eco*R V restriction site, the copy numbers of transgene insertions could be estimated from the Southern hybridization pattern. After separated on 0.8% (w/v) agarose gels, digested genomic DNA fragments were transferred onto Hybond N^+ membranes (Amersham). A *Barnase* DNA fragment was excised from pUC-T-bar plasmid with *Nco* I/*Bam* H I and labeled with ^{33}P-dCTP as the probe. Southern DNA hybridization and probe washing were performed as previously described (Liu et al. 2004).

Plant RNA isolation and reverse-transcriptase (RT)-PCR analysis of *barnase* expression in transgenic plants

Total plant RNA was extracted using a SV Total RNA Isolation System (Promega, Madison, WI, USA). After removal of residual genomic DNA with a DNA-free Kit (Ambion, Austin, TX, USA), the total RNA was quantified spectrophotometrically and by agarose gel electrophoresis. Two micrograms of total RNA was reverse-transcribed into first-strand cDNA using a Superscript First-strand Synthesis system (Invitrogen, Carlsbad, CA, USA).

Semiquantitative RT-PCR analyses were carried out as described by Liu et al. (2005). RT-PCR amplifications were performed three times for each RNA sample using primers Bar-5 and Bar-3. To verify the quality of cDNAs from different tissue samples, *actin* cDNA was amplified as internal control using primer P1 (5'-ATG GCG GAT GGG GAG GAC ATT-3') and P2 (5'-TTA GAA GCA TTT GCG GTG GAC-3') based on *N. tabacum actin* mRNA (GenBank accession No. AB158612.1). RT-PCR amplification conditions consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 54°C, and 1 min at 72°C, with a final 10-min extension at 72°C.

Microscopy

To test pollen viability, anthers were taken individually from flowers. Pollen grains were deposited onto a glass slide, stained with 0.1% I-KI solution, and observed immediately under a light microscope.

Scanning electric microscopy (SEM) was performed as described by Guo et al. (2005). Pollen samples were gold coated and SEM images were produced using the scanning electron microscope model JSM-6400F (JEOL, Peabody, MA, USA).

Results

DNA sequences of the *Barnase* gene and the *AG* second intron

A 328 bp fragment was amplified from *B. amyloliquefaciens* genomic DNA using Bar-5 and Bar-3 primers. Sequence analysis showed that its nucleotide

sequence was identical to that of GenBank accession number M14442 in the corresponding region. PCR primers AG-I5 and AG-I3 amplified a DNA fragment of 1869 bp from *Arabidopsis* genomic DNA. Only two nucleotides were found different from the GenBank AL021711 sequence, probably due to intron polymorphisms in *Arabidopsis* populations or by PCR mismatching. This *Arabidopsis* DNA fragment covered the 1653 bp from the 3' end of the *AG* second intron, the smallest *AG* second intron DNA fragment that was tested to confer a normal AG expression pattern (Deyholos and Sieburth 2000).

Regeneration of transgenic tobacco plants

Agrobacterium-mediated transformation is a well-accepted method for transgene production. In the present study we used a modified protocol for tobacco leaf disc transformation. Single colonies were used directly to prepare *Agrobacterium* suspension for leaf disc infection. A similar method was described recently by Logemann et al. (2006) for transformation of *Arabidopsis*. Two regular steps, *Agrobacterium* growth in liquid medium overnight and bacterial cell collection by centrifugation, were purposely omitted to reduce experimental time and risk of contamination. In addition, following *Agrobacterium* infection, filter papers were used to remove excessive bacterial solution from leaf discs instead of repeated rinsing of leaf discs with sterile water. Kanamycin-resistant shoots formed in differentiation medium in about twenty days. The construct pAG-I-35S (-60)-Bar showed similar transformation frequencies as compared to control plant transformation with pBI121

blank vector.

From thirty independent transgenic lines, twenty-five were selected after confirmation of transgene incorporation in plant genome by PCR. In these plants, a genomic DNA fragment with the expected size of about 1.2 Kb was amplified using *AG* intron primer AB-5 and *barnase* primer AB-3 (Fig. 2A). A 328 bp fragment of *barnase* gene probe was used to hybridize genomic DNA following *EcoR* V digestion. Southern blot analysis confirmed the integrations of the target gene in the tobacco genome (Fig. 2B). The transgene copy numbers ranged from one to three in nine tested transgenic lines. These transgenic tobacco lines were grown to the stage of flowering for phenotype assessment. We found that the copy numbers of the transgene were independent of the transgenic phenotype development. Because transgenic lines were sterile, phenotypes were analyzed with the primary (T_0) transgenic plants.

Based on observation of flower development, the transgenic plants can be divided in three groups. Group I, normal flowering phenotype, contains total 16 transgenic lines, which had a phenotype similar to wild-type control plants (Fig. 3-a). Group II, abnormal flowering phenotype, has two transgenic lines, Bar-5 and Bar-15, which showed abnormal reproductive development (Fig. 3-b). Group III, non-flowering phenotype, includes seven transgenic lines, Bar-4, -12, -19, -20, -25, -26, and -27, which lacked any flower development (Fig.3-c), while non-transgenic wild-type plants finished their life cycles under the same growth conditions during the same time.

AG-I-35S (-60) mediated mRNA expression of the *barnase* gene in transgenic tobacco plants

Semi-quantitative RT-PCR was used to examine the *barnase* mRNA expression regulated by *Arabidopsis AG-I-35S (-60)* sequence. *Barnase* expression was detected in the inflorescence primordia in both non-flowering and abnormal flowering phenotypes of transgenic tobacco plants (Fig. 4-A). For abnormal flowering type plants, we purified total RNA from different flower organs, and found that the *barnase* mRNA was expressed in only carpels and anthers, but not in sepals, petals, or vegetative leaf tissues (Fig. 4-B).

Phenotypic analysis of transgenic tobacco plants

Compared to wild type plants, no transgenic lines, including seven transgenic lines with non-flowering phenotype (Group III), showed any significant deleterious phenotype throughout the vegetative growth phase (Fig. 3-a, -b, -c).

The two transgenic lines Bar-5 and Bar-15 from the group II abnormal flowering phenotype grew normally until control plants began to flower. They can develop flowers. However, the flower number was dramatically reduced and the flower buds were significantly smaller compared to the same flower development stage of wild-type plants. Most flower buds developed abnormally in those transgenic lines, and stop growth at early stages just as described previously by Koltunow et al. (1990). Eventually those flower buds turned yellow and finally dropped. Only a few of flowers survived and matured. However, they showed

abnormal reproductive organ structures. Their sepals and petals displayed normal morphology (Fig. 3-e) while pistils and stamens exhibited aberrant development (Fig. 3-g and –i). Their phenotypes were characterized by petal-like filaments and with elongated and distorted carpels with a shorter style.

Sterility of abnormal flowering transgenic plants

The seven transgenic lines of group III non-flowering phenotype showed completely sterile. Pollens from the group II abnormal flowering phenotype (lines Bar-5 and Bar-15) and from non-transgenic wild type plants were stained with I_2-KI and observed under light microscopic to test pollen viability. Wild-type anthers contained many mature pollen grains that were darkly stained as a positive test for starch (Fig. 5-A). By contrast, the abnormal flowering transgenic lines contained less pollen and most of pollen grains (95.1% and 97.8%) were stained red and identified as the stained abortion type (Fig. 5-B). The I_2/KI staining also revealed that there were two other types of pollen sterility as typical and spherical abortion, but both pollen abortion types occurred rarely (<1%) in both transgenic lines. The transgenic lines Bar-5 and Bar-15 only produced 3.8% and 1.3% normal pollen grains that were morphologically identical to those from wild-type plants, while 71.2% wild-type pollens were normal and considered to be fertile. The ratios of viable pollen production were statistically different between wild-type plants and the two selected transgenic lines (X^2 test $p<0.001$ for both cases) (Fig.5-E).

Scanning electric microscopy (SEM) was performed on pollens from wild-type as

well as abnormal flowering anthers to further characterize male sterility in transgenic lines Bar-5 and Bar-15. SEM observation revealed that the pollen grains from wild-type plants had uniform size and were filled with starch (Fig. 5-C). In contrast, pollen grains from transgenic anthers displayed different shapes and sizes with a distorted, shrunken, or collapsed morphology (Fig. 5-D). An obvious lack of turgidity of the pollen grains may result from insufficient intracellular materials, such as starch as evidenced by I_2-KI staining. The results revealed that pollen grains of the stained abortion type in both Bar-5 and Bar-15 may be directly related to the diminution of intracellular substances for metabolism.

Addition to pollen abortion was the female infertility characterized by abnormal carpel development. Because of *barnase* expression, the shapes of the carpel in plants Bar-5 and Bar-15 became distorted (Fig. 3-i). After pollination, most young fruits failed to develop further and fell off. A shrieked shape indicated abnormal seed development inside (Fig. 3-k). A few fruit capsules grew to maturity and were mostly empty. The total number of seeds produced per plant was significantly reduced in transgenic lines Bar-5 and Bar-15. Each wild type plants averagely produced 1562 seeds, and the seed germination rate was 94.6%. In contrast, only 151 and 236 seeds were produced from Bar-5 and Bar-15 respectively, and their seed germination rates were only 15.8% and 12.4%, respectively (Fig. 6). The offspring production frequencies of abnormal flowering transgenic lines were only 1.6% and 1.9% of those of wild-type plants. The difference in reproduction between transgenic lines and wild-type plants was significant statistically (X^2 test $p<0.001$).

Discussion

Transgene flow and escape is a major public concern regarding the investigation and application of transgenic crops. Complete ablation of reproductive organ development *via* genetic engineering strategies would prevent such undesired gene flows and reduce public concerns about gene modified organisms. Various floral tissue-specific promoters and cytotoxic genes were investigated for their usages in genetic engineered sterility. However, most promoters of genes functioning in floral development are not exclusively active in reproductive tissues (Wei et al. 2007).

In the present study we chose the second intron sequence of *Arabidopsis AG* gene to regulate *barnase* expression for transgene sterility in tobacco plants because this intron is required for normal *AG* activities and confers *AG* carpel- and stamen-specific expression patterns (Sieburth and Meyerowitz, 1997; Deyholos and Sieburth 2000; Hong et al. 2003). It has revealed that *AG* expression is determined by *cis*-regulatory elements localized in this second intron (Hong et al. 2003). A pine *AG* ortholog was also investigated for its application for the modification of plant reproduction (Liu et al. 2003). Our current transgene investigation proved that expression of *Barnase* regulated by the second *AG* intron resulted in both female and male sterility in transgenic tobacco plants. *AG* is a class C organ identity gene that is first expressed in the central apical region of early stage floral meristem (Yanofsky et al., 1990). In this study, we found *barnase* expression was first detected in the inflorescence apex in *AG-I*-35S (-60)::*Barnase* transgenic tobacco, suggesting that

barnase enzymatic activity was expressed at the early stages of inflorescence development, leading to a complete ablation of floral development in 28% of the transgenic tobacco lines.

During *Arabidopsis* floral organ initiation, *AG* mRNA is present throughout the third- and forth-whorl organ primordia that grow into stamens and carpels, but it is absent in the first- and second-whorl that give rise to sepals and petals. During floral organ development, *AG* mRNA is continuously expressed in the third- and fourth-whorl (Bowman et al. 1991). Consistent with this *AG* expression pattern in *Arabidopsis*, *barnase* mRNA was not detectable in sepals and petals and both non-reproductive floral organs developed normally in transgenic tobacco lines Bar-5 and Bar-15. In contrast, the presence of *barnase* mRNA in both male and female floral organs resulted in their distorted development and early flower abortion.

We observed petal-like filaments in transgenic line Bar-5 and Bar-15. This kind homeotic conversion of stamens into petals resembles the *Arabidopsis agamous* mutant phenotype (Yanofsky et al. 1990). When expressing *NAG1* antisense mRNA, a tobacco *AG* ortholog, transgenic tobacco plants also display conversions of stamens into petals (Kempin et al. 1993). Our result suggests that the enzymatic activity of *barnase* may interfere with *NAG1* expression in transgenic lines with abnormal flowering phenotypes. Further investigation is needed to determine whether the *NAG1* expression level was affected in the flowers of Bar-5 and Bar-15 plants.

Although engineered sterility has been explored using various mechanisms

(Perez-Prat and van Lookeren Campagne 2002), severe phenotypic alternations from harmful effects on basic metabolism and general development have prevented their application in agriculture (Goetz et al. 2001). In a few previous studies, when *barnase* gene was used for plant engineered sterility, some unfavorable characteristics were usually observed in transgenic plants (Lemmetyinen et al. 2004; Wei et al. 2007). These kinds of unfavorable transgene traits are caused by leaky expression of promoters or the position effect of chromosome site for T-DNA integration. We performed Sothern blotting to check the transgene copy number and found one to three copies of *barnase* gene integrated in the genome of transgenic tobacco lines. The present study showed that the transgene copy numbers and positions appeared not to be related to the phenotype developments of the selected transgenic lines.

A recent report (Zheng et al. 2007) demonstrated that CaMV 35S promoter alters the tissue-specific promoter activity in adjacent tissues and suggested that CaMV 35S promoter should not be used when a cell toxin is to be expressed in specific plant organs/tissues. In the present study, we only used minimal CaMV 35S promoter (-60) in T-DNA region of the binary vector where the selection marker gene (*NPT-II*) was controlled by the NOS promoter rather than regular CaMV 35S promoter. The 60 bp minimal CaMV 35S promoter was selected to avoid potential influence of the *cis*-elements in a longer 35S promoter sequence. This minimal 35S promoter (-60) itself does not direct any reporter gene expression pattern and is usually used as a universal tool for enhancer-trap studies (Campisi et al. 1999). After

fusion with *AG-I*, this chimeric promoter imparted gene expression in the same pattern as *AG* in Arabidopsis (Deyholos and Siehurth 2000). This may explain why all our transgenic tobacco lines showed no abnormal development in vegetative growth.

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Figure caption:

Figure 1: Schematic representation of T-DNA region of pAG-I-35S(-60)-Bar construct. The PCR-amplified 1869-bp *AG* second intron (AG-I) of *A. thaliana* was inserted between the mini CaMV 35S promoter (-60 bp) and the terminator sequence of the nopaline synthase gene (NOS-Term). *NPT-II*, neomycin phosphotransferase II gene; NOS-Prom, promoter sequence of the nopaline synthase gene; LB, left border of the T-DNA; RB, right border of the T-DNA.

Figure 2: Identification of transgenic lines with integration of gene expression cassette of *AG-I-35S (-60)::Barnase*. (A) Genomic DNA PCR was used to detect the presence of *AG-I* and *barnase* sequences in transgenic lines, a DNA fragment of about 1.2 Kb was amplified by PCR. M, DNA molecular markers (bp); lane 1, wild-type plant as a negative control; lane2-16, fifteen independent transgenic lines. (B) Southern blot analysis of *barnase* gene integration in transgenic tobacco genome. Ten micrograms of genomic DNA was digested with *EcoR V* and probed with a 328 bp DNA fragment of barnase gene coding region. Transgenic lines Bar-4, Bar-15, Bar-19, and Bar-25 represent hybridization profiles of a single-copy integration of the T-DNA transgene. The estimated marker positions were shown at left (Kb).

Figure 3. Phenotypic characterization of transgenic tobacco plants expressing *barnase* under control of *AG-I-CaMV 35S (-60)*. Whole plant morphologies of a non-transgenic plant (wild-type) as a control (a), a representative transgenic plant

with abnormal flowering phenotype (b), and a representative transgenic plant with non-flowering phenotype (c). Flower morphology of wild-type plant (d) and transgenic plant with abnormal flowering phenotype (e), their sepals and petal are well-developed with no difference from wild-type. Stamen dissected from flower of wild-type (f) and transgenic lines (g). Petaloid tissues were attached to filament and the filament bottom parts were fused together in transgenic lines. Pistils dissected from flowers of wild-type (h) and transgenic line (i). Distorted ovary and stigma, and short style were observed in transgenic lines. Morphology of fruits at early development stage from wild-type (j) and transgenic plant with abnormal flowering phenotype (k) where sepals were removed.

Figure 4. Semi-quantative RT-PCR analysis of the *barnase* transcript distributions in plant tissues. cDNA was synthesized from total RNA extracted from different tissues. (A) Detection of *barnase* mRNA in inflorescence apices from three representative transgenic lines (Bar-4, -5, -12). Wild-type (WT) plant was used as a control. (B) Detection of *barnase* mRNA in the floral organs anther, carpel, sepal, petal, and vegetative leaves from transgenic line Bar-5. A similar result was obtained from transgenic line Bar-15. Transcripts levels of *actin* expression were detected as an internal control in each plant tissues.

Figure 5. Pollen abortions as a result of *AG-I-35S (-60):: Barnase* expression. (A) Pollen grains from anthers of wild-type plants, most of them were stained dark

using I_2-KI solution. (B) Pollen grains from anthers of transgenic line with abnormal flowering phenotype, most of them were revealed as the stained abortion type as stained red using I_2-KI solution. Scanning electron microscopy (SEM) of pollen grains from wild-type plant (C) and from transgenic line with abnormal flowing phenotype (D). The bars in the SEM images equal 20 μm. (E) Percentages of normal, typical abortion, spherical abortion, and stained abortion pollen grains in wild-type and transgenic lines Bar-5 and Bar-15. Each bar represents the average pollen count of six individual anthers from three different plants. Error bars represent standard deviations.

Figure 6. Seed production rate and seed germination rate in transgenic lines with abnormal flowering phenotype containing *AG-I-35S (-60)::Barnase* gene. Seed production rates from transgenic line Bar-5 and Bar-15 were calculated as compared with those in wild-type (100%).

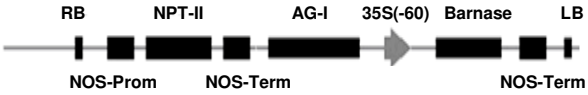

Figure 1

1

2

3

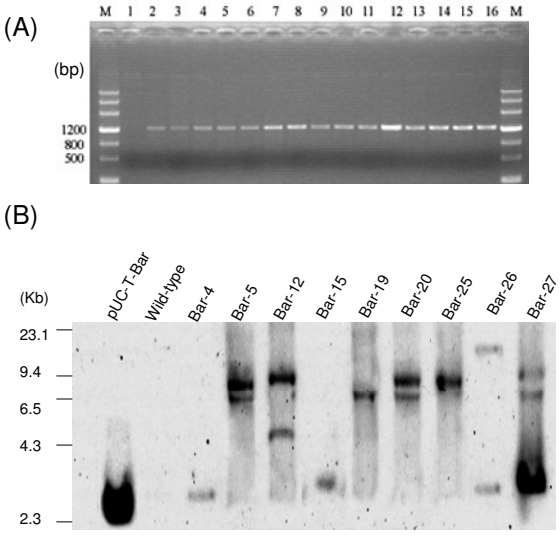

Figure 2

4

Figure 3

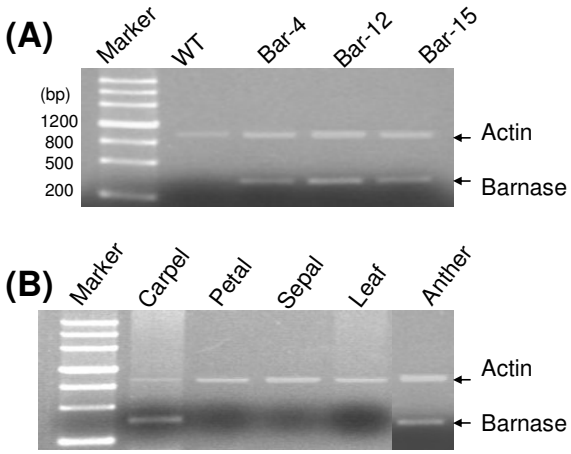
(A)
Marker
WT
Bar-4
Bar-12
Bar-15
(bp)
1200
800
500
200
Actin
Barnase
(B)
Marker
Carpel
Petal
Sepal
Leaf
Anther
Actin
Barnase

Figure 4

1

2

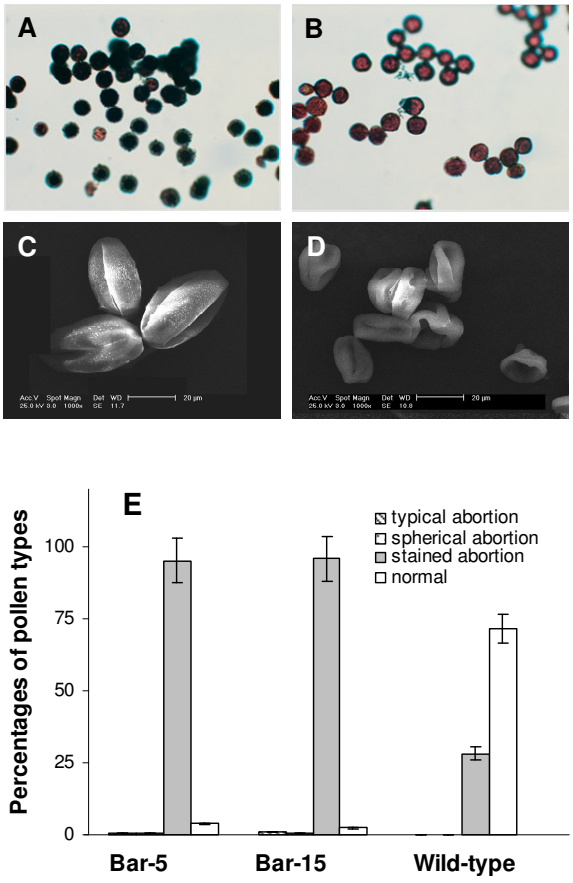
A
B
C
D
E
Percentages of pollen types
100
75
50
25
0
typical abortion
spherical abortion
stained abortion
normal
Bar-5
Bar-15
Wild-type

Figure 5

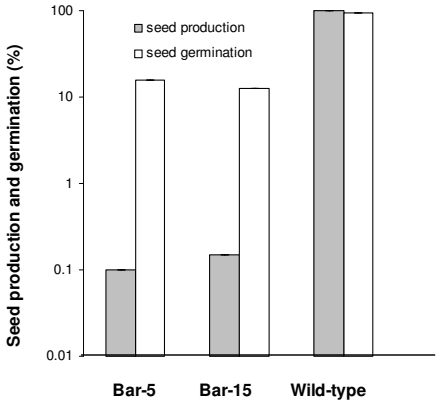
100
10
1
0.1
0.01
seed production
seed germination
Seed production and germination (%)
Bar-5
Bar-15
Wild-type

Figure 6

1