Breakthrough Technologies

# A Ligation-Independent Cloning Tobacco Rattle Virus Vector for High-Throughput Virus-Induced Gene Silencing Identifies Roles for *NbMADS4-1* and *-2* in Floral Development[1][W][OA]

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Virus-induced gene silencing (VIGS) is a widely used, powerful technique for reverse genetics. VIGS vectors derived from the *Tobacco rattle virus* (*TRV*) are among the most popular for VIGS. We have developed a TRV RNA2 vector that allows the insertion of gene silencing fragments by ligation-independent cloning (LIC). This new vector has several advantages over previous vectors, particularly for applications involving the analysis of large numbers of sequences, since TRV-LIC vectors containing the desired insert are obtained with 100% efficiency. Importantly, this vector allows the high-throughput cloning of silencing fragments without the use of costly enzymes required for recombination, as is the case with GATEWAY-based vectors. We generated a collection of silencing vectors based on 400 tomato (*Solanum lycopersicum*) expressed sequence tags in this TRV-LIC background. We have used this vector to identify roles for *SlMADS1* and its *Nicotiana benthamiana* homologs, *NbMADS4-1* and *-2* in flowering. We find that *NbMADS4-1* and *-2* act nonredundantly in floral development and silencing of either gene results in loss of organ identity. This TRV-LIC vector should be a valuable resource to the plant community.

The last decade has seen an explosion in the availability of plant gene sequences. The genomes of the model species Arabidopsis (*Arabidopsis thaliana*) and rice (*Oryza sativa*) have both been sequenced, while those of tomato (*Solanum lycopersicum*) and maize (*Zea mays*) are currently being sequenced (Mueller et al., 2005; http://www.sgn.cornell.edu/about/tomato_project/; http://www.maizesequence.org/index.html). Large collections of ESTs have also been generated for a variety of species that are widely used for research purposes. Concomitant with the availability of this sequence information, many important aspects of plant growth and development have been analyzed by DNA micro-

arrays, leading to the identification of numerous genes potentially involved in these processes. At this time then, the challenge to most plant biologists is to effectively mine this data to identify and characterize the genes and gene products that are critical to the crucial processes that have been investigated. This calls for techniques that start with a known DNA sequence and allow the determination of biological function. This approach is called reverse genetics and some of the most common methods for performing reverse genetic studies are based on RNA silencing.

Although recently discovered, RNA silencing is a well-characterized, endogenous system for monitoring RNA inside a cell and eliminating foreign molecules or inhibiting mRNA translation (for review, see Brodersen and Voinnet, 2006). It is a homology-based process that uses small RNA fragments to identify targets for destruction or inhibition. RNA silencing is also indispensable for normal plant growth and development, regulating the expression of central genes in flowering, meristem identity, and other processes (Meins et al., 2005). In plants, RNA silencing plays critical roles in viral defense, generating small interfering RNA sequences that target the foreign viral RNA for degradation. It is this capability of RNA silencing in plants that has been harnessed for use as a tool in reverse genetics. Virus-induced gene silencing (VIGS) is an efficient tool based on RNA silencing. VIGS uses viral vectors to introduce gene fragments homologous to a gene of interest into a plant host (for review, see Lu et al., 2003; Burch-Smith et al., 2004). Endogenous RNA silencing machinery then acts to eliminate viral RNA sequences and inadvertently also targets the mRNA of

the gene of interest for removal. In this way, the gene of interest is silenced, through the removal of its mRNA.

VIGS has been widely adapted for functional gene studies (Lu et al., 2003; Burch-Smith et al., 2004; Robertson, 2004). It was originally described for use in tobacco (*Nicotiana tabacum*) and its relative *Nicotiana benthamiana* (Kumagai et al., 1995; Ruiz et al., 1998), but VIGS has now been adopted for gene analysis in many dicotyledonous species including Arabidopsis (Burch-Smith et al., 2006; Wang et al., 2006), potato (*Solanum tuberosum*; Brigneti et al., 2004), tomato (Liu et al., 2002a), and pepper (*Capsicum annum*; Chung et al., 2004). Even plant species that are not core eudicots have been analyzed with VIGS, including opium poppy (*Papaver somniferum*; Hileman et al., 2005), and *Aquilega* (Gould and Kramer, 2007). VIGS has also been developed for use in monocots and it has been described in barley (*Hordeum vulgare*; Holzberg et al., 2002; Hein et al., 2005), wheat (*Triticum aestivum*; Scofield et al., 2005), and rice (Ding et al., 2006, 2007).

VIGS has become a widely used technique for reverse genetics because it is transient and does not require the generation of stable transgenics. In addition, it is rapid with phenotypes observed with 10 d to 3 weeks of silencing. Another advantage of VIGS is that it can be designed to silence either a single member or multiple members of a gene family, addressing the problem of functional redundancy. The most widely used VIGS vectors are derived from the *Tobacco rattle virus* (*TRV*). It has been used for VIGS in the Solanaceae (Ratcliff et al., 2001; Liu et al., 2002a, 2002b; Brigneti et al., 2004; Chung et al., 2004), Arabidopsis (Burch-Smith et al., 2006; Wang et al., 2006), opium poppy (Hileman et al., 2005), California poppy (*Eschscolzia californica*; Wege et al., 2007), and *Aquilega* (Gould and Kramer, 2007). TRV is a bipartite, single-stranded, positive sense RNA virus with a large host range (Hull, 2002). Some of the features of this virus that are believed to contribute to its success as a VIGS vector include its ability to enter growing parts of the plant, its toleration of DNA inserts up to about 1 kb in size, and mild symptoms of infection (Lu et al., 2003; Burch-Smith et al., 2004).

We have previously generated and described TRV-based VIGS vectors (Liu et al., 2002a, 2002b). Our first generation TRV VIGS vector used restriction digestion and ligation for cloning of inserts (Liu et al., 2002b). We then developed a second-generation vector that carried GATEWAY recombination sites (Liu et al., 2002a). However, prohibitive features of this vector include time-consuming and complicated cloning procedures. In addition, the comparatively high cost of the enzymes used for GATEWAY-based cloning and proprietary issues are also a consideration for large-scale gene cloning and VIGS screening. To overcome these limitations, we have adopted modified ligation-independent cloning (LIC; Aslanidis and de Jong, 1990; Dieckman et al., 2002) as an approach for high-throughput cloning into the TRV VIGS vector. LIC is much faster and more accurate than other cloning

strategies as only a single transformation is required. We used this new TRV-LIC vector to clone 400 tomato ESTs and we assessed its effectiveness in *N. benthamiana*. Thus, the TRV-LIC vector is an improved vector for high-throughput VIGS.

In addition, we observed several interesting phenotypes and identified *N. benthamiana* MADS-box genes with important, nonredundant roles in flowering. *MADS*-box genes represent a large family of transcription factors with critical roles in floral and general development. In particular, they function in conferring floral organ identity, floral determinancy, and the timing of the vegetative-floral transition (Ng and Yanofsky, 2001; Henderson and Dean, 2004). We used our TRV-LIC vector to silence *NbMADS4-1* and *NbMADS4-2* simultaneously or individually. In all instances we observed a loss of floral organ identity and determinancy, resulting in green, bushy flowers. These data suggest that *NbMADS4-1* and -2, despite their high homology, have critical, nonredundant roles in floral development.

## RESULTS

### Development of a LIC Strategy for TRV-Based VIGS

One limitation of currently available VIGS vectors is the multiple steps involved in the cloning of the silencing fragment into the vector. This can become quite expensive when using GATEWAY-based recombination vectors. To overcome these limitations, we adopted a modified LIC strategy (Aslanidis and de Jong, 1990; Dieckman et al., 2002) for the high-throughput cloning of inserts into our TRV VIGS vector. To do this we added two LIC adaptors to the TRV2 vector, pYL170 (Burch-Smith et al., 2006), to generate pYY12. These adaptors consist of 15 bp sequences containing central *Pst*I restriction sites (Fig. 1A). We then inserted a *ccdB* gene between the two LIC sites by *Pst*I digestion to create pYY13, referred to as TRV2-LIC (Fig. 1A). The *ccdB* gene allows us to screen putative recombinant colonies rapidly and accurately, a strategy employed by the widely used GATEWAY technology. Digestion of TRV2-LIC with *Pst*I linearizes the vector, and subsequent treatment with T4 DNA polymerase generates sticky ends. PCR products carrying ends homologous to the adaptors and similarly treated with T4 DNA polymerase can form covalent bonds with ends of the linearized vector (see "Materials and Methods" for details). Our new TRV2 vector, TRV2-LIC, can thus be used for high-throughput cloning since any number of sequences carrying compatible ends can be combined with TRV2-LIC.

We confirmed that the changes we had made to TRV RNA2 in generating TRV2-LIC did not compromise its infectivity by examining whether the virus was able to spread to the upper leaves of plants. We infected *N. benthamiana* plants with the TRV RNA1 and TRV2-LIC vectors only and collected upper leaves and inflorescence tissue 20 d later. The presence of TRV RNA2 in
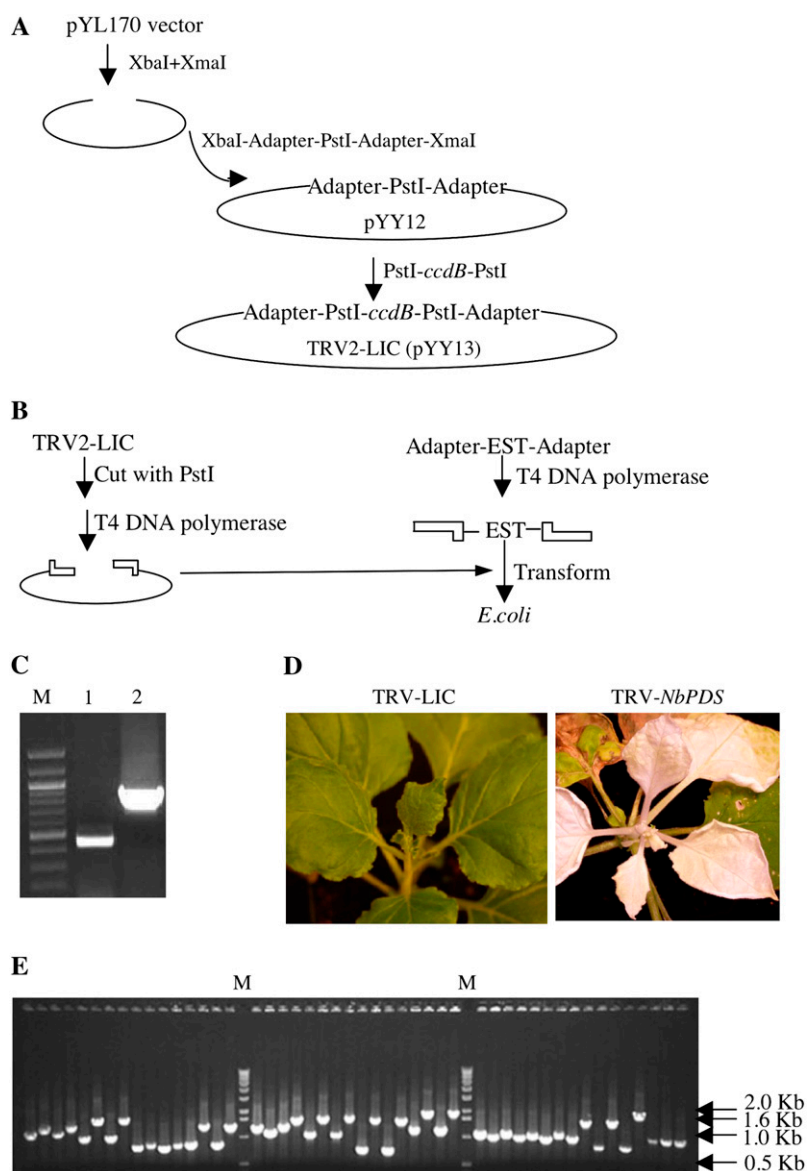
**Figure 1.** TRV2-LIC vector. A, Construction of the TRV2-LIC vector. The TRV2 vector, pYL170, was used to generate the new TRV2-LIC vector by inserting a cassette containing adapters and two *PstI* sites in two digestion and ligation reactions. B, LIC cloning of inserts into TRV2-LIC. Briefly, the TRV2-LIC vector is digested with *PstI* and treated with T4 DNA polymerase. The EST carrying the relevant adapter sequences is generated by PCR and also treated with T4 DNA polymerase. The vector and insert are then mixed and used to transform *Escherichia coli* cells. C, TRV-LIC does not interfere with viral replication and spread as shown by the presence of TRV RNA1 (lane 1) and modified RNA2 (lane 2) in upper leaves of *N. benthamiana* plant. Lane M contains DNA size marker. D, TRV2-LIC allows efficient silencing of *NbPDS* as shown by the photobleaching of silenced plant (right). Control plant infiltrated with empty TRV-LIC vector shown for comparison (left). E, EST cloning efficiency into TRV-LIC is 100% as shown by PCR on colonies obtained from transformation with the vector and PCR product mixture. All 48 of the colonies tested here contain insert. Lane M contains DNA size marker.

these tissues was confirmed by reverse transcription (RT)-PCR (Fig. 1C). Thus, the modifications made to TRV RNA2 to produce the TRV2-LIC plasmid does not hinder the ability of the virus to replicate and spread in plants. We also confirmed the ability of the new TRV-LIC vector to initiate gene silencing by inserting a fragment of the *NbPHYTOENE DESATURASE* (*NbPDS*) gene into this vector. We performed *PDS* silencing experiments according to standard protocols (Dinesh-Kumar et al., 2003) and found that after 10 d, we observed photobleaching of upper leaves in presumably silenced plants (Fig. 1D). The timing and appearance of photobleaching was indistinguishable from that observed with earlier versions of our TRV VIGS vectors (data not shown). Thus, our TRV2-LIC vector functions in VIGS in a manner similar to other TRV-based VIGS vectors.

## High-Throughput Cloning of Tomato ESTs

While *N. benthamiana* has become extremely popular for gene studies, the limited availability of sequence collections hinders the study of gene function. In contrast, large EST collections from tomato are readily accessible for large-scale gene function analyses (D'Agostino et al., 2007). Tomato and *N. benthamiana* are both members of the Solanaceae family and there is usually a high degree of similarity in gene coding sequences. Indeed, tomato gene fragments have been successfully used to silence homologs in *N. benthamiana* (Liu et al., 2004b) or vice versa (Ekengren et al., 2003). To demonstrate the efficacy of the TRV-LIC vector for high-throughput screening for gene function, we cloned 400 tomato ESTs into this vector (Supplemental Table S3; see "Materials and Methods"). These ESTs predominantly belonged to one of the following three

**Figure 2.** Selected phenotypes observed with silencing of tomato ESTs. A, Silencing of SlRibosomal protein L11 like, Slα-tubulin, SlHSP70, and SlPolyubiquitin causes death. B, Silencing *SlMAP3K epsilon* disrupts leaf development resulting in severe crinkling. C, Reduction in *SlGeranylgeranyl reductase* levels causes yellowing of leaves. D, Reduced *SlPlastidic aldolase* causes photobleaching and a variegated appearance. E, Decreased *SlCyclin-dependent protein kinase, p34cdc2*, levels cause severe stunting. F, Homeotic transformations of floral tissue in *SlTAG1*-silenced plants. G, mRNA levels in control (left) versus silenced (right) tissue indicate effective silencing of genes indicated. Number of PCR cycles is indicated above lanes. Lane M is the DNA size marker. C is no RT control. Actin is a loading control.
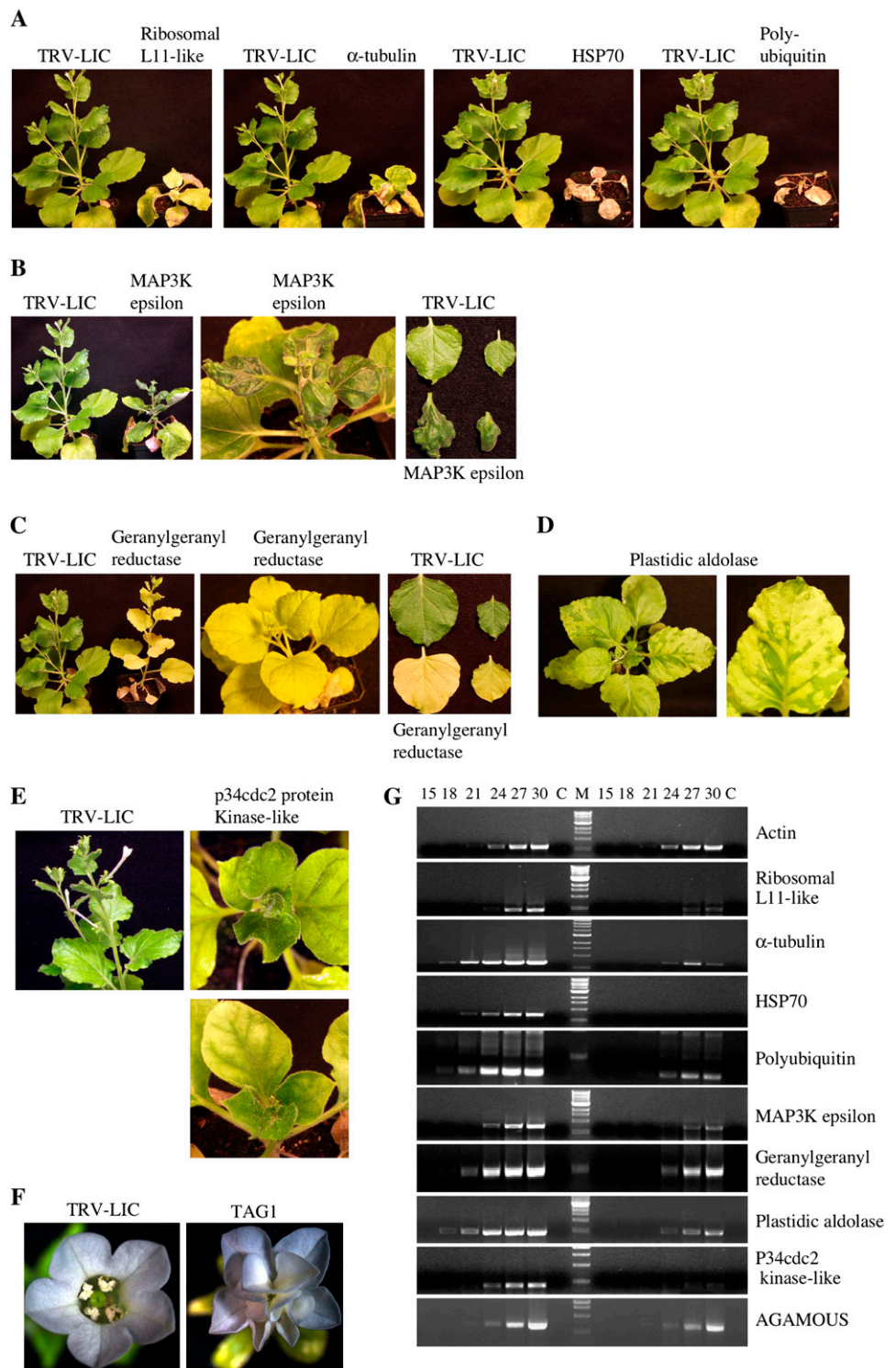


classes: kinases, transcription factors, and phosphatases. The efficiency of this large-scale cloning approach was confirmed by the presence of inserts in the vector (representative gel is shown in Fig. 1E). The presence of the desired insert was detected in 100% of all clones examined.

## Developmental Phenotypes Revealed by Silencing 400 Tomato ESTs

Our TRV-LIC EST clone collection was then screened for silencing phenotypes in *N. benthamiana* plants. For this, we infiltrated four-leaf stage plants with a 1:1
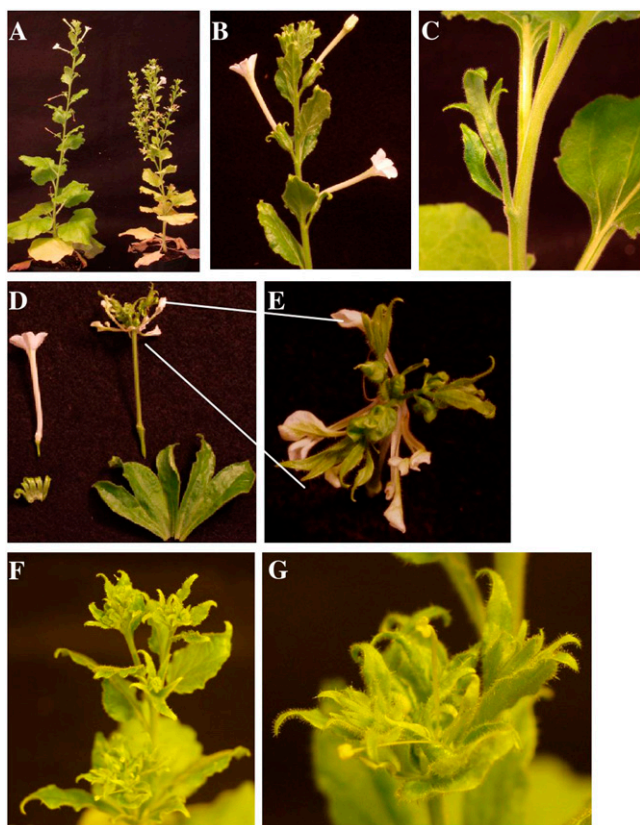
**Figure 3.** Silencing *SlMADS1*. A, *SlMADS1*-silenced plants (right) show loss of apical dominance compared to control empty vector-treated plants (left). B, Flowers on control empty vector-treated plants show distinct white petals surrounded by sepals. C, Flowers on *SlMADS1*-silenced plants have enlarged sepals and white petals are largely absent. D, Sepals separated from inner whorls from control (left) and *SlMADS1*-silenced (right) flowers. E, Magnified view of inner whorls of flower dissected in D. Note reduced white petals and the presence of flower-like organs. F, Indeterminate flowers of *SlMADS1*-silenced plants. G, Magnified view of one of the flowers in F.

mixture of TRV1 and TRV2-LIC-EST fragment for each of the clones in our collection, and monitored the infiltrated plants for 8 weeks. Eventually, we observed several developmental phenotypes in our silenced plants, and some examples are shown (Fig. 2). For each EST, we infiltrated four plants per trial and did at least three trials.

One of the most common phenotypes resulting from silencing was lethality (Fig. 2A). This is exemplified by the silencing of *SlRibosomal protein L11 like*, *Slα-tubulin*, *SlHSP70*, and *SlPolyubiquitin* (Fig. 2A). Another phenotype we observed was the deformation of the upper, presumably silenced, leaves as shown for *SlMAP3K epsilon* (Fig. 2B). These plants were ultimately much shorter than control plants. Interestingly, *SlMAP3K epsilon*-silenced plants did not produce inflorescences, suggesting severe disruption of development beyond leaf structure (Fig. 2B, center section). The silencing of several constructs led to loss of pigmentation or chlo-

rosis, as shown for silencing with *SlGeranylgeranyl reductase* (Fig. 2C) and *SlPlastidic aldolase* (Fig. 2D). The phenotypes that we observed in these two cases are in close agreement with previous reports on the effects of reduction of the levels of these gene products (Boldt et al., 1992; Tanaka et al., 1999).

Interestingly, silencing of some clones led to developmental defects in the meristems (Fig. 2E) or inflorescences (Fig. 2F) of plants. Silencing *SlCyclin-dependent protein kinase*, *p34cdc2* (Fig. 2E), resulted in an attenuation of apical growth and severe stunting. The leaves of silenced plants also showed chlorosis. Silencing initiated by the tomato *AGAMOUS* homolog, *SlAG1*, produced phenotypes previously described for knock down in tomato (Pnueli et al., 1994). As in tomato, we observed the homeotic transformation of the third whorl stamens into petals, and the replacement of fourth whorl carpels with other flowers or inflorescences (Fig. 2F). These results reinforce our previous conclusion that TRV-mediated VIGS is an effective tool for assessing the functions of genes in the reproductive tissues of plants (Chen et al., 2004; Liu et al., 2004a).

For 21 EST clones representing 18 unique genes that showed interesting developmental phenotypes when silenced, we attempted to clone their *N. benthamiana* homologs into the TRV2-LIC silencing vector (Supplemental Table S1). We failed to obtain *N. benthamiana* homologs corresponding to four tomato ESTs. We repeated the silencing using 14 *N. benthamiana* sequences and observed similar developmental phenotypes as
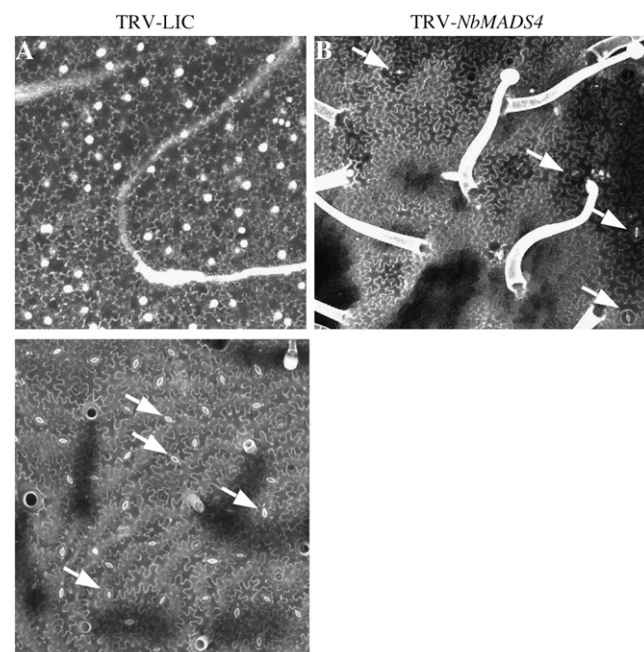


**Figure 4.** *NbMADS4*-silenced petals compared to controls. A, Abaxial surface of control *N. benthamiana* petal. Note absence of trichomes. B, Abaxial surface of *SlMADS1*-silenced petal contains both trichomes and stomata (arrows). C, Abaxial surface of control *N. benthamiana* leaf contains many stomata. Scale bar represents 50 $\mu$m.

**Figure 5.** Silencing *NbMADS4-1* and *NbMADS4-2*. A, Control *N. benthamiana* flower. B and C, Silencing *NbMADS4-1* using fragment 1 (B) or fragment 2 (C) causes enlargement of sepals and the loss of large white sepals. D and E, Silencing *NbMADS4-2* causes identical phenotypes to silencing with either *NbMADS4-1* fragment. F, The reduction of NbMADS4-1 transcript levels was confirmed by semiquantitative RT-PCR in tissue that had been silenced with fragment 1 (row 2) or fragment 2 (row 3). The levels of *NbMADS4-2* mRNA were not altered in *NbMADS4-1* fragment 1 silenced plants (row 4) and *NbMADS4-1* fragment 2 silenced plants (row 5). The transcript levels of the related genes *NbMADS5* and *NbMADS11* were also examined to confirm specificity of silencing (rows 6 and 7, respectively). The phenotypes shown are not due to the silencing of another MADS-box transcription factor *NbAG1* (row 8). Lanes to the left of the marker (M) contain control tissue and those on the right contain tissue from silenced tissue. C is no RT control. Numbers at the top of each lane indicate number of PCR cycles.
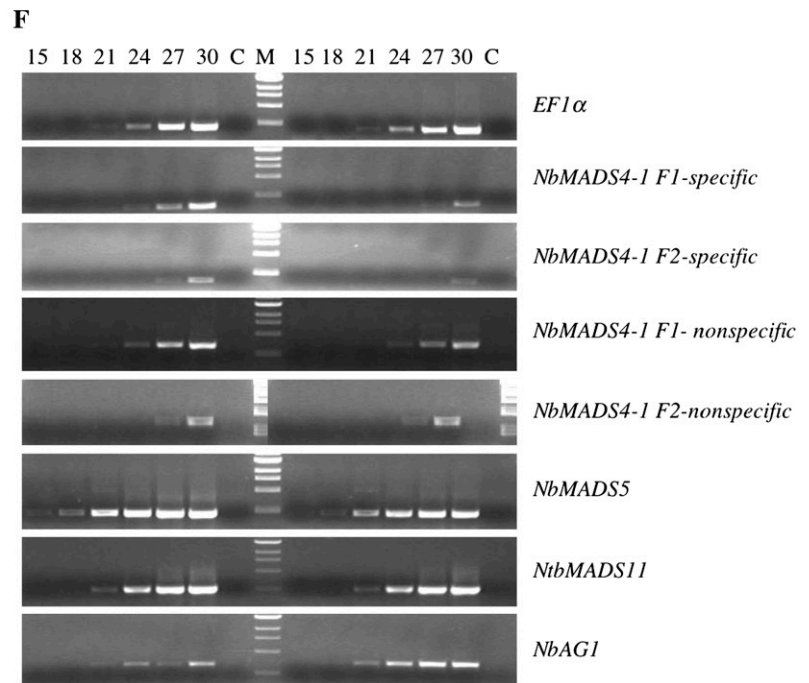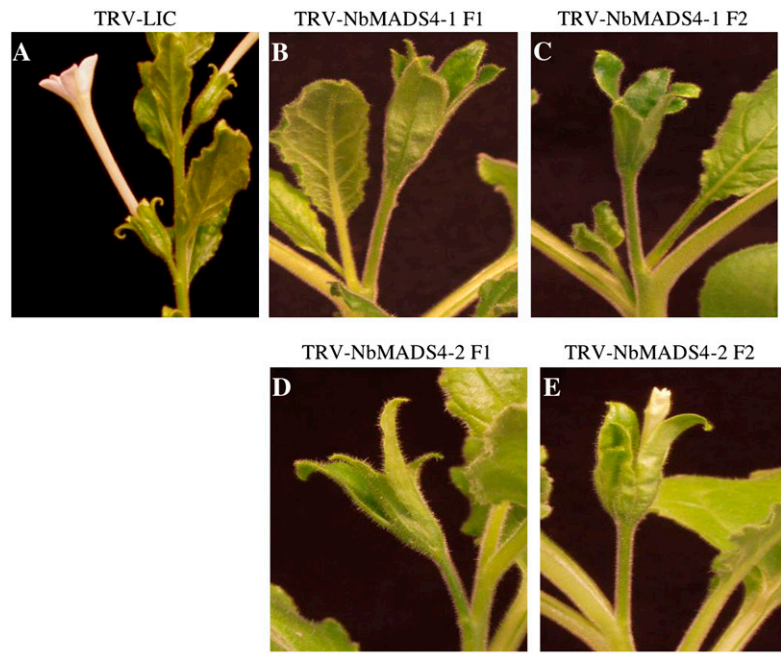


had been obtained with the tomato sequences (data not shown). We then determined the degree of silencing of each of the 14 genes by RT followed by semiquantitative RT-PCR using primers annealing outside the sequence used for silencing (Fig. 2G; Supplemental Table S2; "Materials and Methods"). In all cases we observed a greater than 60% reduction in transcript levels in the silenced plants. Thus, the approach used for the high-throughput cloning and silencing of tomato ESTs to assess their function using *N. benthamiana* as a heterologous system was successful.

## Silencing *SlMADS1* Produces Homeotic and Other Transformations in Flowers

Plants infiltrated with TRV carrying a fragment of *SlMADS1* displayed very interesting developmental phenotypes, particularly in the floral inflorescences and individual flowers (Fig. 3). The altered development is first manifested as increased branching, first of the primary stem and then of the secondary stems, giving rise to a bushy plant compared to the control plants (Fig. 3A). Most of the branches of the *SlMADS1*-silenced

plants went on to produce inflorescences, such that the final number of flowers in the silenced plants is almost double that of the control plants (Fig. 3A; data not shown). The timing of the floral transition did not differ between control and *SlMADS1*-silenced plants.

Wild-type *N. benthamiana* flowers typically possess a first whorl of five green sepals and a second whorl of five white petals that fuse to form a tube that surrounds the reproductive organs (Fig. 3B). The third whorl usually consists of four stamens that surround the central gynoecium of the fourth whorl (Fig. 2F, left section). *SlMADS1*-silenced flowers carried enlarged sepals that were about 5-times bigger than the sepals of control flowers (Fig. 3, C and in D compare left and right sections). Further, petals and stamens converted into green leaf-like structures (Fig. 3, C–E). Interestingly, in some cases the fourth whorl carpel was replaced by another flower or even inflorescence (Fig. 3, D–G), resulting in indeterminate flowers.

We examined the ultrastructure of these modified petals and stamens by confocal microscopy. Wild-type petals and stamens normally do not possess stomata and trichomes on their abaxial surface (Fig. 4, left section). Both these structures were present in the leaf-like structures of *SlMADS1*-silenced flowers (Fig. 4, right section). Thus, the cells on the leaf-like petals and stamens in *SlMADS1*-silenced plants looked very similar to pavement cells of leaf epidermis.

### *NbMADS4-1* and *4-2* Have Nonredundant, Critical Roles in Floral Development

To further characterize *SlMADS1*'s role in plant development, we cloned the *N. benthamiana* sequences showing highest similarity to *SlMADS1*. A search of the GenBank database revealed that the tobacco sequence, *NtMADS4*, showed highest similarity (85%) to *SlMADS1*at the amino acid level (Supplemental Fig. S1). Using RT-PCR with primers based on *NtMADS4*, we obtained two sequences from *N. benthamiana* that we named *NbMADS4-1* and *NbMADS4*-2 (Supplemental Fig. S2). These two genes are 82% identical to each other at the nucleotide and amino acid levels. NbMADS4-1 and NbMADS4-2 show 83% and 97% identity to NtMADS4, respectively (Supplemental Fig. S1).

We then cloned two different fragments of each of these genes separately into the TRV2-LIC vector for silencing (Supplemental Figs. S3 and S4). The phenotypes obtained on silencing *NbMADS4-1* and *NbMADS4-2*, each with either fragment, were identical to those observed on silencing *SlMADS1* (Figs. 3 and 5). This suggests that *NbMADS4-1* and *NbMADS4-2* have closely related functions in flower development. To determine whether *NbMADS4-1* and *NbMADS4-2* were functionally redundant, we examined the mRNA levels of these genes in plants where silencing using the alternate gene had been initiated. In *NbMADS4-1*-silenced plants, *NbMADS4-1* RNA transcript levels decreased by about 90% (Fig. 5F), while levels of *NbMADS4-2* mRNA were virtually unaffected (Fig. 5F).

Similarly, while *NbMADS4-2* silencing produced a drastic reduction in *NbMADS4-2* transcript levels, *NbMADS4-1* was unaffected (Fig. 5F). We confirmed that the expression of other *MADS*-box genes was not affected when silencing *NbMADS4-1* and -2 by monitoring levels of two closely related genes, *NbMADS5* and *NbMADS11* (Fig. 5F). We also confirmed that other genes involved in floral organ identity were not disrupted in *NdMADS4-1* or -2-silenced tissue by determining that levels of *NbAG1*, the homolog of *AtAGAMOUS*, were unchanged (Fig. 5F). These data suggest that *NbMADS4-1* and *NbMADS4-2* have important, nonredundant roles in floral development in *N. benthamiana*.

Given the effects on floral development we observed, we examined the expression of *NbMADS4-1* and *NbMADS4-2* in different organs. Both genes were expressed in all whorls of floral organs (Fig. 6), while neither was highly expressed in any of the vegetative organs sampled (Fig. 6).

### DISCUSSION

We report here the construction of a TRV-LIC vector that facilitates the high-throughput cloning of silencing fragments. This vector allows rapid, efficient cloning of inserts and has the added advantage of doing so at markedly reduced costs. While gene-specific primers are still required for each insert of interest, these are shorter than those used with other systems like GATEWAY, which have been adopted for cloning large numbers of inserts (Liu et al., 2002a). Further, there are fewer cloning steps and neither ligases nor recombinases are required, further reducing the tediousness and cost of the procedure. In addition, we have demonstrated that for the 400 tomato ESTs cloned here, cloning efficiency approaches 100%; thus, there is less need for screening colonies obtained after the cloning process to confirm the presence of inserts.

The TRV-LIC vector achieves silencing of target genes with the same timing and efficiency as previous
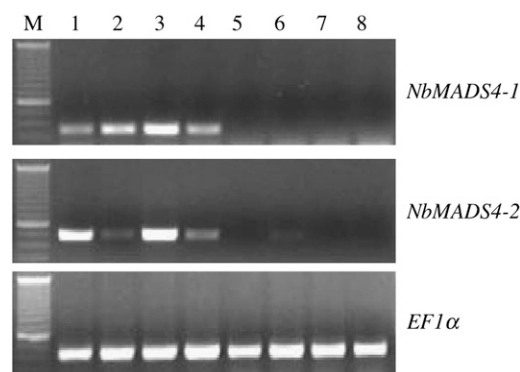


**Figure 6.** Analysis of *NbMADS4* tissue-specific expression. *NbMADS4-1* and *NbMADS4-2* transcripts were detected by semiquantitative RT-PCR. Lane 1 is tissue from sepals, 2 is petals, 3 is petioles, 4 is carpels, 5 is roots, 6 is stems, 7 is leaves, and 8 is seedlings. Lane M is the size marker. *EF1α* was used as a loading control.

TRV-based VIGS vectors. We observed greater than 60% reduction in target transcript levels in all cases examined. The TRV-LIC vector is also able to silence genes expressed in the growing parts of the plant as demonstrated by the flowering defects we observed in several instances (Figs. 2 and 3). This is consistent with other reports of VIGS of meristematic and flowering genes (Ratcliff et al., 2001; Chen et al., 2004; Liu et al., 2004a; Hileman et al., 2005; Gould and Kramer, 2007; Wege et al., 2007).

We have used the TRV-LIC vector to generate a collection based on 400 tomato ESTs. This collection includes kinases, phosphstases, and transcription factors. It should therefore prove to be a useful collection for researchers interested in investigating the function of members of these protein families. TRV-VIGS is effective in several *Solanaceous* species and our tomato collection could be used for VIGS in tomato, potato, pepper, or *N. benthamiana*. Here, we used tomato clones to silence several *N. benthamiana* genes. Based on the tomato sequence, we cloned the corresponding *N. benthamiana* sequence and examined whether the phenotypes we obtained with the tomato clone were an accurate representation of the endogenous gene function. In all cloned 14 homologs of *N. benthamiana*, we found that the results from tomato clones held true for the *N. benthamiana* sequence.

In the course of our studies with this silencing collection we observed a range of developmental phenotypes. Of particular interest was that generated by silencing *SlMADS1*. Sequence analysis shows that *SlMADS1* belongs to the MADS transcription factor family. Members of this family have critical roles in flowering time and floral organ identity, in addition to roles in other important aspects of plant development (Ng and Yanofsky, 2001; Henderson and Dean, 2004). *SlMADS1* belongs to the MIKC class of MADS transcription factors and its domain architecture consists of the MADS box (M) that is important for DNA binding and dimerization, the Intervening (I) domain for functional specialization, the Keratin-like (K) domain, and the C-terminal (C) domain for transcriptional activation (Kaufmann et al., 2005). The flowers of *SlMADS1*-silenced plants displayed enlarged sepals, the conversion of second-whorl petals to leaves in some cases, and the replacement of the fourth-whorl carpel with other flowers that also showed larger-than-normal sepals and petal conversion. Some of these phenotypes are reminiscent of the *sepallata1* (*sep1*) *sep2 sep3* triple mutant (Pelaz et al., 2000). However, *SlMADS1*-silenced plants also displayed a loss of apical dominance and the production of many secondary branches. *SlMADS1* is most similar to *NtMADS4*, a member of the AGAMOUS-like2 subfamily of MADS transcription factors (Jang et al., 2002). *NtMADS4* is known to have a role in flowering in tobacco, where ectopic overexpression leads to early flowering, although the flowers showed no defects in organization or tissue identity (Jang et al., 2002).

We cloned the *N. benthamiana* homolog of *SlMADS1* and found two very similar sequences, *NbMADS4-1*

and *NbMADS4-2*. Both *NbMADS4-1* and *NbMADS4-2* show typical MIKC-type MADS transcription factor domain architecture (Supplemental Fig. S1) and they are highly similar to other MADS proteins, including Arabidopsis SEP proteins. Similar to *NtMADS4* and other AGL2 family members, *NbMADS4-1* and -2 were expressed almost exclusively in floral tissue (Jang et al., 2002). We used the TRV2-LIC vector to silence both *NbMADS4-1* and *NbMADS4-2*, either simultaneously or individually. It should be noted that the sequences we used for specific silencing contained one or two regions of greater than 21 bp of homology between NbMADS4-1 and -2 (Supplemental Figs. S3 and S4). Despite this, we were able to generate specific silencing, as shown by our RT-PCR analysis. One possible explanation for this is that insufficient small interfering RNAs were derived from these regions to initiate silencing of the homolog not being targeted.

Silencing *NbMADS4-1* and *NbMADS4-2*, either simultaneously or separately, resulted in a loss of apical dominance and defects in floral organ identity. However, we did not observe a change in the timing of floral initiation. Interestingly, a loss of apical dominance was also observed when *NtMADS4* was overexpressed (Jang et al., 2002). Together, these phenotypes point to an important role for *NtMADS4/NbMADS4-1/2* in flowering. Interestingly, *NbMADS4-1* and -2 genes are not redundant as specific silencing of either homolog produced all the phenotypes observed when *SlMADS1* was silenced. This is quite different from the situation with the *AtSEP* genes, which are functionally redundant (Pelaz et al., 2000). Thus while *NbMADS4-1* and *NbMADS4-2* function in the same process, they appear to have independent, essential roles. MADS proteins are known to function as dimers (Kaufmann et al., 2005). We speculate the NbMADS4-1 and -2 may act together as a dimer and loss of either protein results in loss of activity.

## MATERIALS AND METHODS

### Plasmid Construction

The VIGS vectors pTRV1 (pYL192) and pTRV2 (pYL170) have been described previously (Liu et al., 2002b; Burch-Smith et al., 2006). The TRV2-LIC vector (pYY13) was derived from pYL170 (Burch-Smith et al., 2006). Two adaptors with *Pst*I restriction sites were inserted into pYL170 by *Xba*I and *Xma*I digestion and ligation to produce pYY12. The *ccdB* gene was amplified by primers 5'-GCTAAGGAAGCTAAACTTTTGCTGACGAGAACAGGGACTGG-3' and 5'-CGCCTGCAGCTCGAGCAGACTGGCTGTGTATAAGGGAGCCTG-3', and inserted into pYY12 at the *Pst*I site to yield TRV2-LIC (pYY13; Fig. 1A).

### Cloning Tomato EST VIGS Collection

Four hundred tomato (*Solanum lycopersicum*) ESTs were amplified with primers: 5'-CGACGACAAGACCCT-plasmid-specific sequence-3' and 5'-GAG-GAGAAGAGCCCT-plasmid-specific sequence-3'. The PCR products were purified with polyethylene glycol/MgCl₂ to remove any nonspecific PCR products and primers. A total of 50 ng of purified PCR product was treated with T4 DNA polymerase (New England Biolabs) in 1× reaction buffer containing 5 mM dATP and dithiothreitol at 22°C for 30 min followed by 20 min of inactivation of T4 DNA polymerase at 70°C. The TRV2-LIC vector was then digested with *Pst*I and similarly treated with T4 DNA polymerase

but dTTP replaced dATP. A total of 50 ng of treated PCR product and TRV2-LIC vector were mixed and incubated at 65°C for 2 min and then 22°C for 10 min. Then 6 $\mu$L of the mixture was transformed into *Escherichia coli* DH10B competent cells (Fig. 1B). Transformants were tested by PCR amplification using primers 5′-TGTTACTCAAGGAAGCACGATGAGCT-3′ and 5′-GAG-GAGAAGAGCCCTGCCGCTCTAGAACTAGTGGATCC-3′. Plasmids from positive clones were purified and sequenced. To generate TRV2-LIC-*NbPDS*, *NbPDS* was amplified from *Nicotiana benthamiana* genomic DNA using primers 5′-CGACGACAAGACCCTCGGTCTAGAGGCACTCAACTTTATAAACC-3′ and 5′-GAGGAGAAGAGCCCTTCCCTTCAGTTTTCTGTCAAACC-3′.

## Plant Growth and Agroinfiltration

*N. benthamiana* plants were grown in pots at 25°C on light carts under continuous light. For VIGS TRV1 (Liu et al., 2002b), TRV2 or TRV2-LIC and its derivatives were introduced into *Agrobacterium tumefaciens* strain GV2260 by heat shock. For screening the TRV2-LIC tomato EST collection, 2 mL overnight cultures were grown at 28°C in the appropriate antibiotic selection medium in a 96-well culture box. The next day, cultures were spun down and cells were resuspended in infiltration medium (10 mM MES, 10 mM MgCl$_2$, 200 $\mu$M acetosyringone), adjusted to OD$_{600}$ of 1, and incubated at room temperature for 3 h. *N. benthamiana* infiltration was performed as previously reported (Liu et al., 2002b). Infiltrated plants were observed for 8 weeks.

## RNA Extraction and RT-PCR Analysis

Total RNA was extracted from leaves or flowers of wild-type *N. benthamiana* plants using RNeasy plant minikit (Qiagen). First-strand cDNA was synthesized using 1 $\mu$g of total RNA, gene-specific primers, and SuperScript reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Primers are listed in Supplemental Table S1. RT-PCR products were cloned into TRV-LIC vector (pYY13) and performed VIGS in *N. benthamiana*. The expression levels of 14 genes whose silencing resulted in severe developmental phenotypes were monitored by semiquantitative RT-PCR using gene-specific primers that anneal outside the region targeted for silencing (Supplemental Table S2).

In *NbMADS4*-silenced plants, expression levels of *LeMADS5*, *NtMADS11*, and *NAG1* were examined by semiquantitative RT-PCR using primers 5′-ACC-GAATATATCAACACGAGAAGCACTG-3′ and 5′-CTAGGCCCTGCTCCTC-CTACTGTAATTG-3′, 5′-CTCATATGCTGAGAGGCAGCTTACTGCT-3′ and 5′-GATGGCGAAGCATCCATGGCGGCATTAC-3′, and 5′-CTCTCCACAAA-GGAAACTGGGAAGAG-3′ and 5′-GACTAGTTGAAGAGATGGTTGGTC-3′, respectively.

## Cloning NbMADS4-1 and NbMADS4-2 from *N. benthamiana*

*NbMADS4-1* was amplified by RT-PCR from *N. benthamiana* flower RNA using primers 5′-CGACGACAAGACCCTGGGAAGAGGAAGAGTTGAAC-TTAAG-3′ and 5′-GACCACTTTGTACAAGAAAGCTGGG(T)$_{25}$V. *NbMADS4-2* was amplified by RT-PCR using primers 5′-ACGACAAGACCCTCTTTC-CTTCTTCTGTATCTGTGAGAGAAAAGAAAG-3′ and 5′-GAGGAGAAGA-GCCCTCATCATCGTCGTATTAGTTCATACAAGTAG-3′. RT-PCR products were cloned into pCR2.1-TOPO (Invitrogen). The cDNA sequences were aligned by ClustalW.

## *NbMADS4* Expression Analysis in Different Tissues

Total RNA was purified from sepals, petioles, petals, carpels, roots, stems, leaves, and seedlings of wild-type *N. benthamiana*, and 2 $\mu$g RNA was used for RT-PCR. Primers used to amplify *NbMADS4-1* were 5′-CGACGACAAGA-CCCTGAAGATTTGGGGACATTAAGTAC-3′ and 5′-GAGGAGAAGAGCC-CTCACCCATGGGACTATATCCAAATTGAGG-3′. Primers 5′-CAATGCAG-CTACGCCTCTTTGGACCCAATG-3′ and 5′-CAGTTGCTGCTGCATTAAC-CTCGTTTCCAC-3′ were used for *NbMADS4-2*.

## Silencing Specificity

Two cDNA fragments were amplified for each *NbMADS4* gene by PCR. Primers for fragment 1 of *NbMADS4-1* were 5′-CGACGACAAGACCCTGG-

GAAGAGGAAGAGTTGAACTTAAG-3′ and 5′-GAGGAGAAGAGCCCTC-TTCCTTGACCTGATTTGCTTCAAGG-3′. Primers for fragment 2 were 5′-CGA-CGACAAGACCCTGAAGATTTGGGGACATTAAGTAC-3′ and 5′-GAGGA-GAAGAGCCCTCACCCATGGGACTATATCCAAATTGAGG-3′. Primers for fragment 1 of *NbMADS4-2* were 5′-CGACGACAAGACCCTATGGGAAGAG-GAAGAGTTGAACTAAAG-3′ and 5′-GAGGAGAAGAGCCCTGTGTGCC-CAAGTCCTCCCCAAGAAG-3′. Primers for fragment 2 were 5′-CGACGA-CAAGACCCTTCCAAGGAACTTGAGCATCTTGAG-3′ and 5′-GAGGAGA-AGAGCCCTTTACAGCATCCATCCTGGAATAAATC-3′. cDNA fragments were cloned into TRV2-LIC vector. Gene expression levels were monitored by semiquantitative RT-PCR using specific primers that anneal outside the region targeted for silencing. In addition, *NbMADS4-1*-silenced plants were also tested with *NbMADS4-2*-specific primers 5′-CGACGACAAGACCCTG-GGAAGAGGAAGAGTTGAACTTAAG-3′ and 5′-GAGGAGAAGAGCCCT-CACCCATGGGACTATATCCAAATTGAGG-3′.

## Microscopy

Tissue was vacuum infiltrated with 0.1 mg/mL propidium iodide and incubated for 30 min at 4°C in the dark. Confocal images were acquired on a Zeiss Axiovert 200 M light microscope equipped with a Zeiss LSM 510 NLO laser scanning microscope using a 10× C-Apochromat (NA 1.2) water-corrected objective lens. Scale bar is 50 $\mu$m.

## Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Alignment of amino acids of *N. benthamiana* MADS4, and their tobacco and tomato homologs.

**Supplemental Figure S2.** Alignment of cDNAs of *NbMADS4-1* and *NbMADS4-2*.

**Supplemental Figure S3.** Nucleotide sequence comparison of fragment 1 of *NbMADS4-1* (*NbMADS4-1F1*) and fragment 1 from *NbMADS4-2* (*NbMADS4-2F1*) used for silencing.

**Supplemental Figure S4.** Nucleotide sequence comparison of fragment 2 of *NbMADS4-1* (*NbMADS4-1F2*) and fragment 2 from *NbMADS4-2* (*NbMADS4-2F2*) used for silencing.

**Supplemental Table S1.** Primers to amplify *N. benthamiana* cDNAs.

**Supplemental Table S2.** Primers for RT-PCR.

**Supplemental Table S3.** EST clones.

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