

Genome-wide characterization of MADS-box genes and their roles in axillary bud development in tobacco

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Abstract

MADS-box transcription factors are core regulators of plant development, but their functions in axillary bud development in *Nicotiana tabacum* L. have not been systematically elucidated. In this study, 118 NtMADS-box genes were identified from the tobacco genome. Phylogenetic analysis classified them into Type I (comprising the Ma and My subfamilies) and Type II (comprising the MIKC* and MIKCC clades). Promoter analysis revealed that cis-acting elements were predominantly associated with light and hormone responses. RNA-seq analysis of axillary buds after topping identified 60 differentially expressed NtMADS-box genes, from which 12 candidate genes with significant expression changes were selected. Tissue-specific qRT-PCR revealed that seven of these genes were preferentially expressed in axillary buds, among which members of the SOC1 and SVP subfamilies accounted for the majority. Exogenous application of abscisic acid and the strigolactone analog GR24 significantly suppressed the expression of most candidate genes, including NtMADS91. Functional analysis demonstrated that overexpression of NtMADS91 significantly promoted axillary bud growth, increasing both the number and length of axillary buds. This study provides a theoretical basis for elucidating the regulatory mechanism of the NtMADS-box gene family in axillary bud development and also offers important candidate genes for research related to tobacco axillary bud development.

Key message

A total of 118 NtMADS-box genes were identified in tobacco, revealing their crucial roles in axillary bud development. Overexpression of *NtMADS91* promoted the growth and development of tobacco axillary buds.

Introduction

MADS-box genes encode a class of transcription factors that are widely present and highly conserved in eukaryotes (Gramzow and Theissen 2010; Zhang et al. 2024). This family name is derived from the initials of four genes: yeast *MCM1*, *AGAMOUS* from *Arabidopsis thaliana*, *DEFICIENS* from *Antirrhinum majus*, and human serum response factor SRF (Schwarz-Sommer et al. 1990). All members contain a highly conserved MADS domain at the N-terminus, which consists of approximately 58 amino acids (Messenguy and Dubois 2003; Theißen et al. 2016). Based on phylogenetic relationships and protein structural characteristics, plant MADS-box genes can be divided into two major types: Type I and Type II. Type I proteins have relatively simple structures, whereas Type II proteins possess more complex domain architectures (De et al. 2003; Masiero et al. 2011). In addition to the MADS domain, they sequentially contain three characteristic domains: I (intervening), K (keratin-like), and C (C-terminal), which are crucial for protein interactions, complex formation, and transcriptional activation (Immink et al. 2002).

MADS-box genes regulate multiple core processes of plant growth and development. The earliest and most classically studied function of these genes is the regulation of floral organ development. In the

context of the “ABC model” and its extended theories, different classes of MADS-box transcription factors jointly determine floral meristem identity and organ development through combinatorial action (Pelaz et al. 2000; Theißen 2001; Li et al. 2010; Heijmans et al. 2012; Abraham-Juárez et al. 2020). As research has progressed, the known functions of this gene family have expanded to encompass nearly the entire plant life cycle, including the regulation of flowering time (Seo et al. 2009; Posé et al. 2013; Jin et al. 2022), bud dormancy (Yang et al. 2020; Ma et al. 2024; Demei et al. 2025), fruit ripening (Vrebalov et al. 2002, 2009; Dong et al. 2013; Fujisawa et al. 2014), endosperm development (Xu et al. 2016; Batista et al. 2019; Tonosaki et al. 2021; Zhang et al. 2024), and root development (Yu et al. 2014; Sun et al. 2018; Alvarez-Buylla et al. 2019). Furthermore, recent studies indicate that MADS-box genes are also involved in plant responses to abiotic stresses, including drought (Khong et al. 2015; Zhao et al. 2020), salt (Chen et al. 2019; Zhao et al. 2021), and low temperature (Chen et al. 2019; Voogd et al. 2022). Besides their roles in processes ranging from floral organ development to stress responses, the functions of MADS-box genes in axillary bud development have also gradually attracted attention.

The maintenance of dormancy, initiation of outgrowth and elongation of axillary buds are key processes that determine plant branching characteristics (Domagalska and Leyser 2011; Wang et al. 2018; Barbier et al. 2019). These processes are synergistically regulated by various transcription factors, among which the MADS-box gene family plays critical and diverse roles. Studies have shown that some genes mainly participate in maintaining axillary bud dormancy or inhibiting growth. For example, *Arabidopsis AGL79* has been identified as a key factor inhibiting axillary bud growth (Gao et al. 2018); potato *POTM1* maintains dormancy by suppressing cytokinin accumulation in axillary buds (Rosin et al. 2003). In contrast, other genes mainly promote axillary bud outgrowth and elongation. For instance, tomato *SIMBP11* may indirectly promote axillary bud outgrowth by repressing strigolactone biosynthesis (Guo et al. 2017); cucumber *CsAGL16* reduces ABA levels in axillary buds by activating the ABA catabolic gene *CsCYP707A4*, thereby releasing dormancy and promoting outgrowth and elongation (Chen et al. 2024, 2025). Although previous studies have shown that individual MADS-box genes are involved in regulating axillary bud growth and development, the systematic members, expression patterns, and key regulatory factors of this family in tobacco axillary buds remain largely unclear.

In tobacco production, axillary bud growth consumes plant nutrients, reduces leaf yield and quality, and increases cultivation and management costs such as topping, directly affecting production efficiency. To this end, this study performed genome-wide identification, phylogenetic analysis, and expression pattern analysis of the MADS-box gene family based on the ‘K326’ reference genome, screened key candidate genes associated with axillary bud development, clarified their tissue-specific expression patterns and hormone response characteristics, and further performed functional verification via overexpression of the *NtMADS91* gene. The results of this study will help elucidate the molecular mechanisms by which MADS-box genes regulate axillary bud development in tobacco and provide important genetic resources and theoretical support for breeding new varieties with low branching traits.

Materials and methods

Plant materials

Nicotiana tabacum L. variety 'Yunyan 87' was used in this study to analyze the expression patterns of the MADS-box gene family. The flue-cured variety 'K326' was used as the control material for genetic transformation and the generation of overexpression lines. All materials were obtained from the Guizhou Provincial Key Laboratory for Tobacco Quality Improvement and Efficiency Enhancement, Guizhou, China. The seeds were sown in 12-cell seedling trays filled with a cultivation substrate (72 × 49 × 62 mm per cell), with one seedling per cell, and cultured in an artificial climate incubator. The environmental conditions were as follows: 25°C/15°C (day/night), 16 h photoperiod, 10,000 lx light intensity, and 75% relative humidity. When the seedlings reached the five-leaf stage, uniformly growing individuals were selected and transplanted into plastic pots (top diameter: 312 mm, height: 195 mm), with one plant per pot. After transplantation, all materials were subjected to the same standard management practices for irrigation, fertilization, pest, and disease control.

Identification of MADS-box genes in tobacco

The *Nicotiana tabacum* genome data were downloaded from http://solgenomics.net/organism/Nicotiana_tabacum/genome/Edward2017. The Hidden Markov Model (HMM) file PF00319 was downloaded from the Pfam website (<http://pfam.xfam.org/>) and the hmmsearch program was used to perform an initial screening of tobacco protein sequences, with parameters set to E-value $\leq 10^{-5}$, while excluding sequences with low match scores. NCBI BLAST was used to further verify the obtained protein sequences to identify NtMADS-box family genes. Based on the *Nicotiana tabacum* genome annotation GFF file, the CDS lengths of the tobacco MADS-box genes were analyzed. The ExPASy online tool (<http://web.expasy.org/protparam/>) was used to determine the physicochemical properties of the encoded MADS-box proteins (Duvaud et al. 2021).

Phylogenetic analysis of the MADS-box proteins

Arabidopsis genome data were downloaded from the TAIR database (<https://www.arabidopsis.org/>), *S. lycopersicum* genome data from the SGN (<https://solgenomics.net/>), and *Capsicum annuum* genome data from the National Gene Bank Life Big Data Platform (<https://db.cngb.org/>). The phylogenetic tree was constructed using the neighbor-joining (NJ) method in MEGA-12 with 1000 bootstrap replicates and default parameters (Kumar et al. 2024; Saitou and Nei 1987). Finally, the evolutionary tree was visualized and annotated using the iTOL v7 online tool (<https://itol.embl.de/>). In addition, the functions of rice *OsMADS5*, tomato *STM3*, and *Brassica juncea* *BjuVAR20_SOC1* have been described previously (Zhu et al. 2022; Wang et al. 2021, 2023; Sun et al. 2023; Tyagi et al. 2019).

Chromosome localization, motif and gene structure analysis

Based on the tobacco genome annotation GFF file, the identified NtMADS-box genes were subjected to chromosomal localization analysis using the “Gene Location Visualize from GFF/GTF” program in TBtools software and numbered according to their positions (Chen et al. 2023). The conserved domains of NtMADS-box proteins were analyzed using MEME online software (<http://meme-suite.org/>) with the maximum number of conserved motifs set to 15 (Bailey et al. 2009). The obtained conserved motifs were functionally annotated using the InterPro online software (<http://www.ebi.ac.uk/interpro>). Exon and intron information of NtMADS-box genes was extracted from the tobacco whole-genome GFF file. The TBtools software was used to visualize the conserved domains and gene structures of NtMADS-box proteins.

Analysis of cis-acting elements in promoters

The 2000 bp sequence upstream of the transcription start site of NtMADS-box genes was extracted, and the number and types of cis-acting elements were predicted using the online tool PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) (Lescot et al. 2002) and visualized using TBtools.

NtMADS-box gene expression pattern analysis

The transcriptome data used for the gene expression pattern analysis under topping treatment was derived from the NCBI GEO database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE233880>) (Tian et al. 2023). The whole genome of ‘K326’ was used as the reference genome, and gene expression levels were normalized using FPKM values. Gene expression heatmaps were generated using the TBtools software.

Hormone treatment experiments were performed 50 days after transplantation (DAT) and consisted of two separate treatments: ABA and GR24. For ABA treatment, two subgroups were established: a decapitated control and an exogenous application of 300 $\mu\text{mol}\cdot\text{L}^{-1}$ ABA supplemented with 0.2% Tween-20. After topping, 100 μL of ABA solution was pipetted directly onto the axillary bud region daily for seven consecutive days, and axillary buds at the first node below the cut were collected at 0, 3, 5, 7, and 10 days after treatment. For GR24 treatment, two subgroups were set: a decapitated control and an exogenous application of 10 $\mu\text{mol}\cdot\text{L}^{-1}$ GR24 containing 0.2% Tween-20. After topping, 100 μL of GR24 solution was pipetted directly onto the axillary bud region every 2 days, and axillary buds at the first node below the cut were collected at 0, 3, 5, 7, 9, 11, and 13 days after treatment. Each treatment included three biological replicates, with 30 plants per replicate, resulting in a total of 90 plants per treatment group.

For tissue-specific expression analysis, non-decapped plants were sampled at 60 DAT. Tissues were collected from white fibrous roots, apical flower buds, axillary buds at the 5th node, stem phloem at the 5th node, and leaves at the 5th node. All samples were immediately frozen in liquid nitrogen and stored at -80°C until further use.

RNA extraction, reverse transcription, and qRT-PCR

RNA extraction, reverse transcription, and qRT-PCR

Total RNA was extracted from plant samples using the RNA Easy Fast Plant Tissue Kit (Tiangen, China), and cDNA was synthesized according to the instructions of FastKing gDNA Dispelling RT SuperMix (Tiangen). The quantitative real-time PCR (qRT-PCR) reaction system was 20 μL , containing 10 μL of 2 $\times$ Talent qPCR PreMix, 0.6 μL of each forward and reverse primer ($10 \mu\text{mol}\cdot\text{L}^{-1}$), 2 μL of cDNA template, and 6.8 μL of RNase-free ddH₂O. The reaction program was as follows: pre-denaturation at 94°C for 3 min; then 95°C for 5 s, 50–60°C annealing/extension for 10 s, for 40 cycles, followed by extension at 74°C for 15 s. Gene expression levels were calculated using the $2^{-\Delta\Delta\text{CT}}$ method (Livak and Schmittgen 2001), with *L25* as the internal reference gene (Table S1). All experiments were performed in triplicate. Expression heatmaps were drawn using TBtools software, and dynamic trends in gene expression after hormone treatment were plotted using Excel.

Creation and phenotypic identification of *NtMADS91* overexpression lines

The CDS sequence of *NtMADS91* (Nitab4.5_0001127g0080) was amplified by PCR. The CDS sequence of *NtMADS91* was fused with the *egfp* gene via a linker and inserted into the plant overexpression vector pBWA(V)HS to construct the 35S::*NtMADS91-EGFP* fusion expression vector. After verification by enzyme digestion and sequencing, the recombinant plasmid was transformed into *Agrobacterium tumefaciens* GV3101. Leaves of aseptically cultured 'K326' were infected using the leaf disc method, and after co-cultivation, they were transferred to screening and differentiation medium containing 25 mg/L hygromycin to induce adventitious bud differentiation. The regenerated shoots were transferred to rooting medium for culture to obtain T₀ generation transgenic plants. Genomic DNA was extracted from the leaves of transgenic lines and wild-type plants, and PCR amplification and sequencing were performed to verify and identify transgenic positive lines. The relative expression level of *NtMADS91* in positive lines was detected by qRT-PCR, and lines with significantly higher expression than the wild type were selected as overexpression lines for subsequent phenotypic identification.

Data and statistical analysis

Data were processed and plotted using Excel. One-way ANOVA was performed, and multiple comparisons were conducted using Duncan's multiple range test at the significance level of $P < 0.05$. Each treatment included three biological replicates, and the results were expressed as mean $\pm$ standard error (mean $\pm$ SE, $n = 3$).

Results

Identification of MADS-box genes in tobacco

To identify members of the MADS-box gene family in tobacco, we first performed sequence alignment on candidate genes derived from preliminary screening. After removing redundant sequences, a total of 118 MADS-box genes were identified from the whole genome of 'K326' using the HMM approach. These genes were sequentially designated *NtMADS1* to *NtMADS118* based on their chromosomal distribution. The 118 genes were unevenly distributed across 23 tobacco chromosomes, with no gene detected on chromosome 18 (Fig. 1). Chromosome 19 harbored the highest number of members (21), followed by chromosome 15 (10). In contrast, only one gene was localized on each of chromosomes 5, 6, 8, and 10. This non-uniform distribution highlights the diversity and complexity of the NtMADS-box gene family in tobacco.

The physicochemical properties of all identified NtMADS-box genes were further analyzed (Table S2), including coding sequence (CDS) length, number of amino acids (AA), protein molecular weight (MW), theoretical isoelectric point (pI), grand average of hydropathicity (GRAVY), and instability index (II). The CDS lengths of these NtMADS-box genes ranged from 189 bp (*NtMADS92*) to 1830 bp (*NtMADS108*), encoding proteins of 62 to 609 amino acids with molecular weights from 7.00 to 68.04 kDa. The theoretical pI values varied from 4.56 (*NtMADS109*) to 11.30 (*NtMADS111*), GRAVY values ranged from -0.956 (*NtMADS116*) to 0.84 (*NtMADS35*), and instability index values ranged from 3.17 (*NtMADS45*) to 70.51 (*NtMADS36*), with 76.27% of the NtMADS-box proteins predicted to be unstable.

Phylogenetic analysis and classification of NtMADS-box genes

To elucidate the evolutionary relationships among members of the tobacco MADS-box family, a phylogenetic tree was constructed using protein sequences from *Arabidopsis* (AtAGL), *S. lycopersicum* (SIMADS), and *C. annuum* (CaMADS). Based on sequence alignment with *Arabidopsis* MADS-box proteins, the 118 identified NtMADS-box proteins were classified into two major clades: Type I (64 members) and Type II (54 members) (Fig. 2A). Type I proteins were further subdivided into the Ma and My subfamilies, and no M β and M δ subfamilies were identified. Comparative analyses with MADS-box proteins from tomato and pepper revealed 50 members in the Ma subfamily and 14 in the My subfamily (Fig. 2B), with no M β or M δ subfamilies detected in any of the analyzed species. Type II proteins were categorized into two subtypes: MIKC* and MIKC^C. The MIKC^C subtype included 11 distinct subfamilies (Fig. 2C), namely FLC (9 members), SEP and SOC1 (7 members each), AGL17 (6 members), SVP (5 members), AG, AP1/FUL, and AP3/PI (4 members each), as well as AGL15, BS, and AGL12 (1 member each).

Gene structure and conserved motifs of NtMADS-box

We analyzed the gene structure and conserved protein motifs of the NtMADS-box family (Fig. 3). Gene structure analysis revealed remarkable differences between Type I and Type II members. Type I genes contained 1–5 exons, among which 30 genes possessed introns (1–4 introns per gene). In contrast, for Type II genes, all members except *NtMADS52*, *NtMADS53*, *NtMADS55*, *NtMADS102*, and *NtMADS114*

contained introns, with the number of introns ranging from 2 to 12. The number of exons in Type II genes varied from 1 to 13.

Using MEME software, we identified 15 conserved motifs. Among these, motifs 1, 2, and 3 together constituted the MADS domain, while motif 4 corresponded to the K-box domain (Table S3). The number of motifs per protein ranged from 1 to 8, with all NtMADS-box proteins containing the conserved MADS domain. Notably, approximately 52% of Type II proteins harbored motif 4 (the K-box domain), as did the Type I proteins *NtMADS17*, *NtMADS18*, and *NtMADS19*. Overall, the number of conserved motifs was comparable between Type I and Type II proteins.

The cis-acting elements in the promoter of NtMADS-box gene

To identify cis-acting elements in NtMADS-box genes, the 2000 bp promoter sequences upstream of each gene were extracted. As illustrated in Fig. 4, a total of 35 cis-acting elements were identified and further classified into four functional groups: development-related (5%), environmental stress-responsive (19%), hormone-responsive (28%), and light-responsive (48%). The development-related elements included the CAT-box, MSA-like, NON-box, and MBSI motifs, which are implicated in meristem expression, cell cycle regulation, meristem-specific activation, and the regulation of flavonoid biosynthetic genes, respectively. Among these elements, the CAT-box was the most abundant, detected in 34 genes (28.8%), suggesting its potential role in regulating meristem expression. The hormone-responsive elements encompassed five main types associated with responses to methyl jasmonate (CGTCA-motif and TGACG-motif), auxin (TGA-element, AuxRE, and AuxRR-core), gibberellin (TATC-box, GARE-motif, and P-box), abscisic acid (ABRE), and salicylic acid (TCA-element and SARE). Notably, ABRE, CGTCA-motif, and TGACG-motif were highly prevalent, detected in 86 (72.9%), 79 (66.9%), and 79 (66.9%) of the NtMADS-box genes, respectively. Collectively, this promoter analysis revealed that NtMADS-box family genes are extensively involved in mediating plant hormonal responses, potentially through abscisic acid and methyl jasmonate signaling pathways.

Expression pattern of NtMADS-box genes in axillary buds after topping

To investigate the expression dynamics of NtMADS-box family genes during axillary bud break and development following tobacco topping, transcriptome-based expression profiling of 118 family members was performed at 0, 2, and 6 days after topping. The results showed that a total of 58 genes were barely expressed. These genes were unevenly distributed between Type I (42 genes) and Type II (16 genes), with Type I genes accounting for the majority. After filtering out these non-expressed genes, the remaining 60 genes displayed significant expression changes.

Among the differentially expressed genes, 22 belonged to Type I (Fig. 5A), including 14 of the Ma subfamily and 8 of the My subfamily. These genes exhibited three distinct expression trends during

axillary bud break and development: five genes, including *NtMADS90* and *NtMADS49*, showed the highest expression levels at 0 days after topping, followed by a gradual decrease; seven genes, including *NtMADS70* and *NtMADS31*, peaked at 2 days after topping, displaying an initial increase followed by a decrease; and 10 genes, including *NtMADS66* and *NtMADS37*, showed continuously increasing expression, reaching maximum levels at 6 days after topping.

For Type II, 38 genes were differentially expressed (Fig. 5B), with members from the SEP, SOC1, FLC, and SVP subfamilies accounting for a relatively high proportion. Their expression patterns fell into four categories: eight genes, including *NtMADS107* and *NtMADS15*, continuously decreased with axillary bud break and development; eight genes, including *NtMADS42* and *NtMADS108*, showed an increase-then-decrease pattern; 12 genes, including *NtMADS75* and *NtMADS111*, displayed a decrease-then-increase pattern; and 10 genes, including *NtMADS40* and *NtMADS98*, were persistently up-regulated during axillary bud development.

These results indicate marked differences in the expression profiles of distinct NtMADS-box types and subfamilies during axillary bud development after topping. The specific expression characteristics of these subfamilies suggest that different NtMADS-box members may play distinct regulatory roles in this process. Among them, the Type II subfamilies SEP and SOC1 may act as key regulators, due to their high proportion of differentially expressed genes and diverse expression patterns.

Expression patterns of NtMADS-box genes in different tissues

Twelve NtMADS-box genes, including *NtMADS37*, *NtMADS93*, and *NtMADS118*, were identified from transcriptome data, and their relative expression levels in leaves, axillary buds, roots, stems, and apical buds were examined by qRT-PCR (Fig. 6). Tissue-specific expression patterns were observed among different subfamilies. For instance, *NtMADS93* and *NtMADS118* were highly expressed in apical buds, whereas *NtMADS41*, a member of the Ma subfamily, showed predominant expression in roots. Notably, seven NtMADS-box genes exhibited relatively high transcript levels in axillary buds, including members of the SOC1 (*NtMADS91*, *NtMADS100*), SVP (*NtMADS30*, *NtMADS42*, *NtMADS48*, *NtMADS113*), and My (*NtMADS37*) subfamilies. In addition, *NtMADS57* (SOC1) and *NtMADS21* (AGL12) showed the second-highest expression levels in axillary buds. These findings reveal distinct tissue-specific expression profiles among NtMADS-box subfamily genes. The preferential enrichment of SOC1 and SVP genes in axillary buds suggests their potential involvement in regulating axillary bud growth and development in tobacco.

Phylogenetic relationship between NtMADS-box genes and reference genes

Phylogenetic analysis showed that the 12 tobacco NtMADS-box genes were distributed across multiple clades (Fig. 7). Among them, tobacco *NtMADS93* clustered with rice *OsMADS5* with a bootstrap value of

99%; tobacco *NtMADS91* showed a close phylogenetic relationship with tomato *STM3* with a bootstrap value of 98%; and tobacco *NtMADS57* and *NtMADS100* clustered with *B. juncea* *BjuVAR20_SOC1* on the phylogenetic tree with a bootstrap value of 85%.

Expression patterns of NtMADS-box genes in axillary buds after ABA and GR24 treatment

To explore the expression patterns of the 12 NtMADS-box genes in response to abscisic acid (ABA) and GR24, qRT-PCR was performed to analyze the changes in the relative expression levels of these family genes in tobacco axillary buds treated with the two hormones after topping.

To analyze the impact of ABA treatment on the expression of NtMADS-box family genes, this study compared the expression dynamics under topping combined with exogenous ABA versus topping alone (Fig. 8). The results showed that under topping-alone conditions, the expression levels of seven genes generally exhibited an increasing trend during axillary bud outgrowth, including three SVP subfamily members (*NtMADS30*, *NtMADS48*, and *NtMADS113*), one SOC1 member (*NtMADS100*), two SEP members (*NtMADS93* and *NtMADS118*), and one My member (*NtMADS37*). *NtMADS42*, also belonging to the SVP subfamily, also showed an upward trend overall, although its expression began to decrease on day 7. The expression levels of *NtMADS57* (SOC1) and *NtMADS21* (AGL12) first decreased and then increased. The expression level of *NtMADS91* (SOC1) increased, peaked on day 5, and then decreased, whereas *NtMADS41* (Ma) showed a general downward trend. Following exogenous ABA treatment, except for *NtMADS21* and *NtMADS41*, whose relative expression changes were generally consistent with those in the control; the expression levels of the remaining genes were all lower than those of the control, and most of them showed a response trend of first decreasing and then increasing. These results indicate that ABA treatment can significantly regulate the expression of multiple NtMADS-box genes, especially exerting an inhibitory effect on members of the SVP, SEP, and SOC1 subfamilies, which may play an important role in hormone-mediated regulation of axillary bud outgrowth and development.

To investigate the role of strigolactone signaling in regulating the expression of NtMADS-box family genes, this study examined their expression dynamics following topping with or without exogenous GR24 application (Fig. 9). Under topping alone, most genes displayed dynamic expression patterns during axillary bud growth. Specifically, seven genes, including *NtMADS30*, *NtMADS42* and *NtMADS113* from the SVP subfamily, *NtMADS91* from the SOC1 subfamily, *NtMADS93* and *NtMADS118* from the SEP subfamily, and *NtMADS37* from the My subfamily, exhibited an initial upregulation followed by downregulation. In contrast, *NtMADS48* (SVP subfamily) and *NtMADS100* (SOC1 subfamily) showed a progressive increase in transcript levels, whereas *NtMADS57* (SOC1 subfamily), *NtMADS21* (AGL12 subfamily) and *NtMADS41* (Ma subfamily) displayed a general downward trend.

Following exogenous GR24 treatment, seven genes, including *NtMADS42*, *NtMADS113* and *NtMADS57*, exhibited an initial upregulation followed by downregulation. Meanwhile, the transcript levels of *NtMADS30*, *NtMADS21* and *NtMADS41* generally decreased, whereas those of *NtMADS48* and

NtMADS100 continued to increase progressively. Compared with the topping-only control, the expression trends of *NtMADS100* and *NtMADS21* remained largely consistent with those in the control; the expression levels of *NtMADS57* and *NtMADS37* were higher than those in the control, while the expression of the remaining eight genes, including *NtMADS91*, was suppressed.

Effects of *NtMADS91* overexpression on axillary bud development in Tobacco

Based on tissue specificity, phylogenetic relationships, and expression profiles in response to hormone treatments, *NtMADS91* was selected for further functional analysis. To investigate its role in regulating axillary bud growth in tobacco, we generated *NtMADS91* overexpression lines via *Agrobacterium*-mediated genetic transformation. Three independent T₀ transgenic lines with high transcript levels were chosen for phenotypic evaluation (Fig. S1). As illustrated in Fig. 10, the overexpression lines displayed increased plant height and a greater number of axillary buds compared with WT plants at both 30 and 60 DAT. Furthermore, at 75 DAT, the elongation of axillary buds at the first to third nodes was notably promoted in all three OE lines compared with WT, with the most pronounced difference observed in the OE-7 line, followed by the OE-5 line.

Discussion

MADS-box transcription factors play a central regulatory role in plant growth and development and are widely involved in key processes such as floral organ formation and flowering time control (Smaczniak et al. 2012). In this study, 118 *NtMADS*-box genes were identified from the whole genome of tobacco cultivar 'K326'. Their number was similar to that in other Solanaceae plants such as tomato and pepper, but differed from the 168 members reported by Bai et al. (2019), which may be mainly due to differences in the genome assembly quality of the tobacco materials used. Phylogenetic analysis classified them into 64 Type I and 54 Type II genes, consistent with the classic classification (Alvarez-Buylla et al. 2000; Gramzow and Theissen 2010). No M β or M δ subfamily members were detected in Type I, which is consistent with the findings of Bai et al. (2019) in tobacco, and may reflect that the distribution characteristics of this family in tobacco differ from those in other species. Chromosomal localization showed that this family was relatively concentrated on chromosome 19, containing a total of 21 members; this non-random distribution pattern may be related to their coordinated expression or functional association.

In terms of gene structure and conserved motifs, Type I genes exhibited a relatively simple structure with fewer introns (Nam et al. 2004; Masiero et al. 2011), whereas Type II genes, especially MIKC^C members, possessed more complex exon-intron structures, consistent with their roles in regulating complex developmental processes (Kaufmann et al. 2005; Theissen et al. 2016). In addition, three Type I members, *NtMADS17*, *NtMADS18* and *NtMADS19*, were predicted to contain K domain-like sequences. In the classical functional model of MADS-box proteins, the K domain is almost exclusively present in Type II members and is extremely rare in Type I (Alvarez-Buylla et al. 2000; Kaufmann et al. 2005; Theissen et al.

2016). We speculate that these genes may have molecular functions or interaction properties distinct from typical Type I members, and their functional significance warrants further investigation.

Analysis of promoter cis-acting elements suggests that NtMADS-box genes may serve as key integrators of environmental and endogenous signals (Lau and Deng 2010). In addition to light-responsive elements, abscisic acid-responsive elements (ABREs) are present in 72.9% of the promoters in this gene family, indicating a potential link with the ABA signaling pathway (Wang et al. 2019). Both ABA and strigolactones are key inhibitory hormones involved in regulating axillary bud dormancy and outgrowth (Dun et al. 2009; Yao and Finlayson 2015; Tian et al. 2023). Recent studies have further demonstrated that light signals can directly modulate ABA homeostasis in axillary buds via specific transcriptional modules (Chen et al. 2024; Liu et al. 2025). Based on these findings, we speculate that NtMADS-box genes may be involved in regulating axillary bud dormancy and outgrowth by responding to ABA and strigolactone signals, thereby providing a theoretical basis for future in-depth investigations into their functional mechanisms in hormone signaling pathways and axillary bud development.

Topping is a key cultivation practice that induces the outgrowth of tobacco axillary buds (Wang et al. 2022; Xu et al. 2025). Transcriptome analysis revealed that approximately 49% of the 118 NtMADS-box family genes were barely expressed in axillary bud tissues after topping, indicating that only a subset of members is activated during the critical period when axillary buds respond to topping signals and initiate development, while the rest remain transcriptionally silent. Thus, not all NtMADS-box genes are involved in regulating axillary bud development, reflecting the specific responsiveness of gene expression to topping signals (Xu et al. 2025; Wang et al. 2022). Furthermore, the 58 non-expressed genes were extremely unevenly distributed between Type I (42 genes) and Type II (16 genes), with Type I non-expressed genes accounting for approximately 72% of the non-expressed genes, far more than in Type II. This indicates that most Type I genes do not participate in topping-induced axillary bud outgrowth, whereas Type II genes may serve as core regulators (Smaczniak et al. 2012; Theißen et al. 2016; Zhang et al. 2024).

After excluding non-expressed genes, we identified 60 genes significantly expressed in axillary buds. Type I and Type II genes exhibited completely distinct expression patterns, and genes from different subfamilies also showed specific expression at different developmental stages. Among the 22 differentially expressed Type I genes, members of the Ma and My subfamilies were mainly upregulated, with their expression levels gradually increasing or first increasing and then decreasing with days after topping. These patterns were positively correlated with the developmental transition of axillary buds from dormancy to growth, suggesting that these genes may be involved in the sustained growth of axillary buds after germination. A small number of genes showed gradually decreased expression after topping, possibly functioning in the maintenance of axillary bud dormancy.

Among the 38 differentially expressed Type II genes, few members of the AGL15, AGL12, MIKC*, BS, and AG subfamilies were involved, with relatively simple expression trends. In contrast, more genes from the SEP, SOC1, FLC, and SVP subfamilies were involved, with more complex and diverse expression patterns.

The SOC1 and SVP subfamilies were mainly upregulated, consistent with the transition of axillary buds from dormancy to germination after topping. These two subfamilies have been reported in Arabidopsis to regulate flowering time and floral meristem activity (Lee et al. 2008; Gregis et al. 2009). Their high expression in tobacco axillary buds and response to topping signals suggest that these genes may have broader functions in regulating axillary bud development. FLC subfamily genes showed diverse expression patterns, indicating that different members may play distinct roles at different stages of axillary bud development. SEP subfamily genes were predominantly downregulated, showing an opposite trend to upregulated genes such as *SOC1* and *SVP*, implying that different Type II genes may exert divergent regulatory roles in axillary bud development.

Tissue-specific expression analysis was further performed on 12 NtMADS-box genes with significantly differential expression to explore their tissue expression patterns. These genes exhibited obvious tissue-specific expression, with significant differences in expression distribution among members of different subfamilies. The largest number of genes was highly expressed in axillary buds, covering multiple subfamilies including SOC1, SVP, and My, indicating that axillary buds may be an important tissue site for the functional enrichment of NtMADS-box genes. *NtMADS113*, *NtMADS42*, *NtMADS48*, and *NtMADS30* from the SVP subfamily, as well as *NtMADS91* and *NtMADS100* from the SOC1 subfamily, were particularly highly expressed in axillary buds. Members of the SVP subfamily are known in Arabidopsis to be involved in the regulation of meristem activity and developmental stage transitions (Lee et al. 2008; Gregis et al. 2009), suggesting that they may play a similar role during tobacco axillary bud growth and development. Although *SOC1* is generally considered to be mainly involved in floral transition, its specific function in tobacco axillary buds requires further verification.

Axillary bud development is coordinately regulated by multiple hormones, including auxin (Gocal et al. 1991), cytokinin (Tan et al. 2019), strigolactone (Lin et al. 2009), abscisic acid (Yao and Finlayson 2015), and gibberellin (Katyayini et al. 2020). Among these, abscisic acid acts as a key negative regulator involved in inhibiting axillary bud outgrowth (Yao and Finlayson, 2015; Wang et al. 2025), whereas strigolactones suppress axillary bud outgrowth (Minakuchi et al. 2010; Chen et al. 2023; Tian et al. 2023). To further explore the potential roles of NtMADS-box genes in axillary bud development, this study analyzed their responses to exogenous ABA and GR24. Following exogenous ABA treatment, the transcript levels of all highly expressed NtMADS-box genes in axillary buds were downregulated compared with the control, except for *NtMADS21* (AGL12 subfamily) and *NtMADS41* (Ma subfamily). Most of these genes exhibited a response pattern of initial decrease followed by increase, predominantly members of the SVP, SEP, and SOC1 subfamilies. These results suggest that ABA participates in the regulation of tobacco axillary bud development by repressing the expression of NtMADS-box genes. After GR24 treatment, the expression of eight core genes was inhibited, whereas the transcript levels of *NtMADS57* and *NtMADS37* were elevated, and the expression patterns of *NtMADS100* and *NtMADS21* remained unchanged. This indicates that strigolactone signaling also regulates axillary bud development by modulating NtMADS-box gene expression, and different NtMADS-box genes display specific responses to GR24.

The inhibitory effects of both hormones on most axillary bud NtMADS-box genes are consistent with the known axillary bud-suppressing functions of ABA and strigolactones (Umehara et al. 2008; Cutler et al. 2010; Dun et al. 2013). This suggests that these NtMADS-box genes may act as downstream factors in hormone signaling pathways, integrating inhibitory signals to regulate axillary bud dormancy and outgrowth. These findings imply that these NtMADS-box genes may act as downstream components in hormone signaling pathways, integrating inhibitory signals to modulate axillary bud dormancy and germination. Notably, unlike the classical functions of *SVP* and *SOC1* as flowering-time regulators in *Arabidopsis thaliana* (Hartmann et al. 2000; Lee and Lee 2010), our results revealed that members of this family are coordinately highly expressed in tobacco axillary buds and respond to topping and exogenous hormone treatments. This suggests that MADS-box genes may possess more diverse functions in regulating axillary bud growth and development. In addition, the expression of *NtMADS57* and *NtMADS37* was upregulated by GR24 treatment, whereas members of the AGL12 and Ma subfamilies were insensitive to both hormones. These distinct response patterns indicate that MADS-box genes may play divergent regulatory roles in response to different hormone signals. Whether they are directly involved in the transition between axillary bud dormancy and germination warrants further investigation.

Based on the above analysis, this study selected the SOC1 subfamily gene *NtMADS91*, which is highly expressed in axillary buds and significantly repressed by both ABA and GR24, for functional validation. Overexpression of *NtMADS91* significantly increased the number and length of tobacco axillary buds, indicating that it acts as a positive regulator of axillary bud growth. Given that the promoter of this gene is rich in ABRE elements and its expression is significantly inhibited by these two hormones, we speculate that *NtMADS91* may be regulated by the inhibitory signals from these two hormones, thereby affecting axillary bud development. In addition, the function of *NtMADS91* differs significantly from those of the previously characterized tobacco SOC1 subfamily members *NtSOC1* and *NtMADS133* (Bai et al., 2019), suggesting that different members within the same subfamily may play distinct roles in axillary bud development and flowering regulation. However, the specific function of *NtMADS91* in regulating tobacco axillary bud development remains to be further investigated.

Conclusion

A total of 118 NtMADS-box genes were identified from the whole genome of tobacco cultivar 'K326' and classified into Ma, My, MIKC^{*}, and MIKC^C subfamilies. Transcriptome analysis of axillary buds after topping showed that a total of 60 genes were significantly differentially expressed, with Type I and Type II genes exhibiting distinct expression dynamics. Further screening of candidate genes revealed that members of subfamilies such as SVP and SOC1 were specifically highly expressed in axillary buds, and the expression of most of these members was inhibited by exogenous ABA and GR24 treatment. Overexpression of *NtMADS91* significantly increased the number of tobacco axillary buds and promoted axillary bud elongation, indicating that this gene acts as a positive regulator of tobacco axillary bud

development. However, the specific molecular mechanism and regulatory network by which *NtMADS91* regulates tobacco axillary bud development remain to be further elucidated.

Declarations

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

YR was responsible for experimental implementation, data analysis and manuscript drafting. MND carried out the experiment and contributed reagents, materials, and analytical tools. KLY and KHJ participated in data collection. JTP and RGW contributed to manuscript revision. GQL initiated and supervised the study. All authors read and approved the final manuscript.

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Data availability

All data generated or analyzed in the present study are included in this article and in the additional information. The RNA-seq data generated in this study are available in the Science Data Bank (ScienceDB) under accession number CSTR: 31253.11.sciencedb.36924 (DOI: <https://doi.org/10.57760/sciencedb.36924>). All other experimental data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Figures

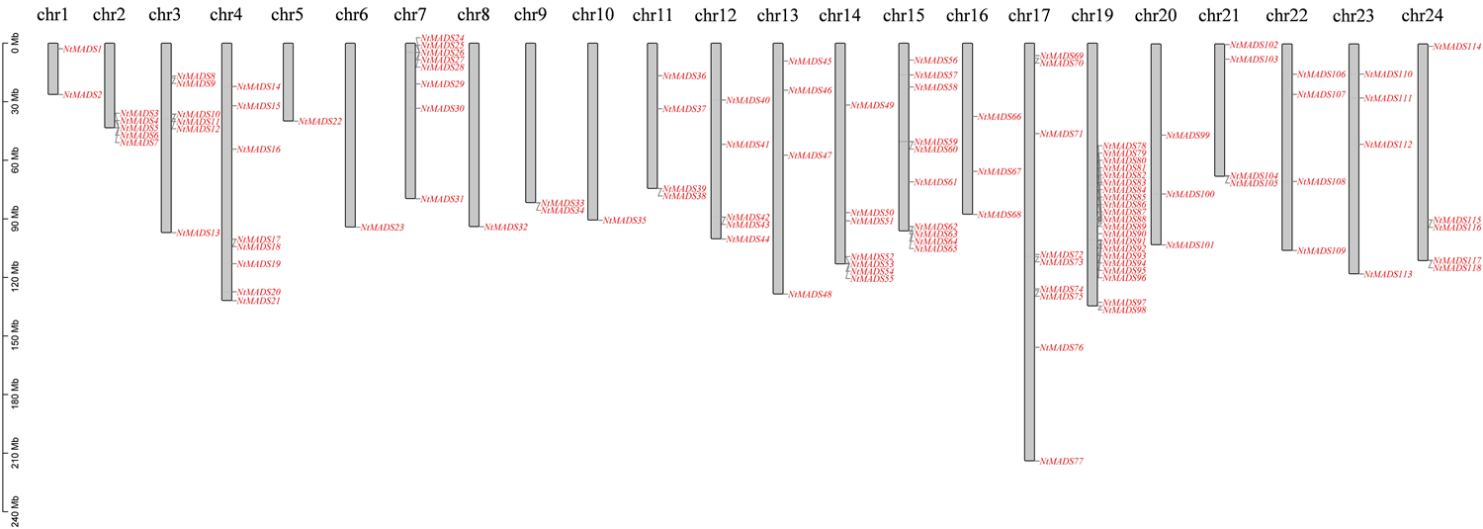


Figure 1

Chromosome localization distribution of NtMADS-box genes in tobacco

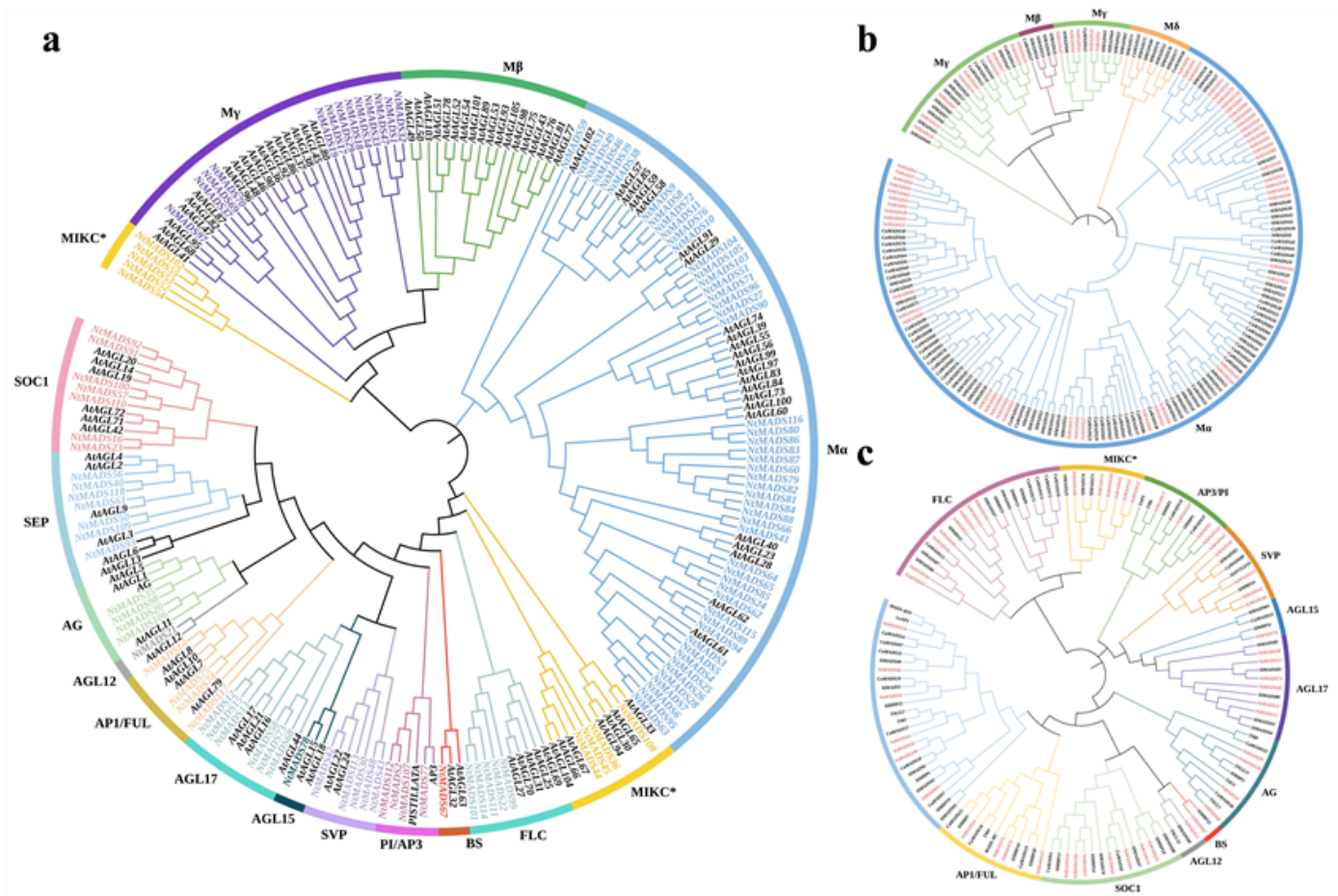


Figure 2

Phylogenetic relationships of NtMADS-box proteins. **a** The phylogenetic tree in compares MADS-box proteins from tobacco and Arabidopsis. Trees in **(b)** and **(c)** detail the evolutionary relationships within Type I and Type II clades, respectively, among tobacco, tomato, and pepper. NtMADS-box proteins are highlighted in different colors for easy identification

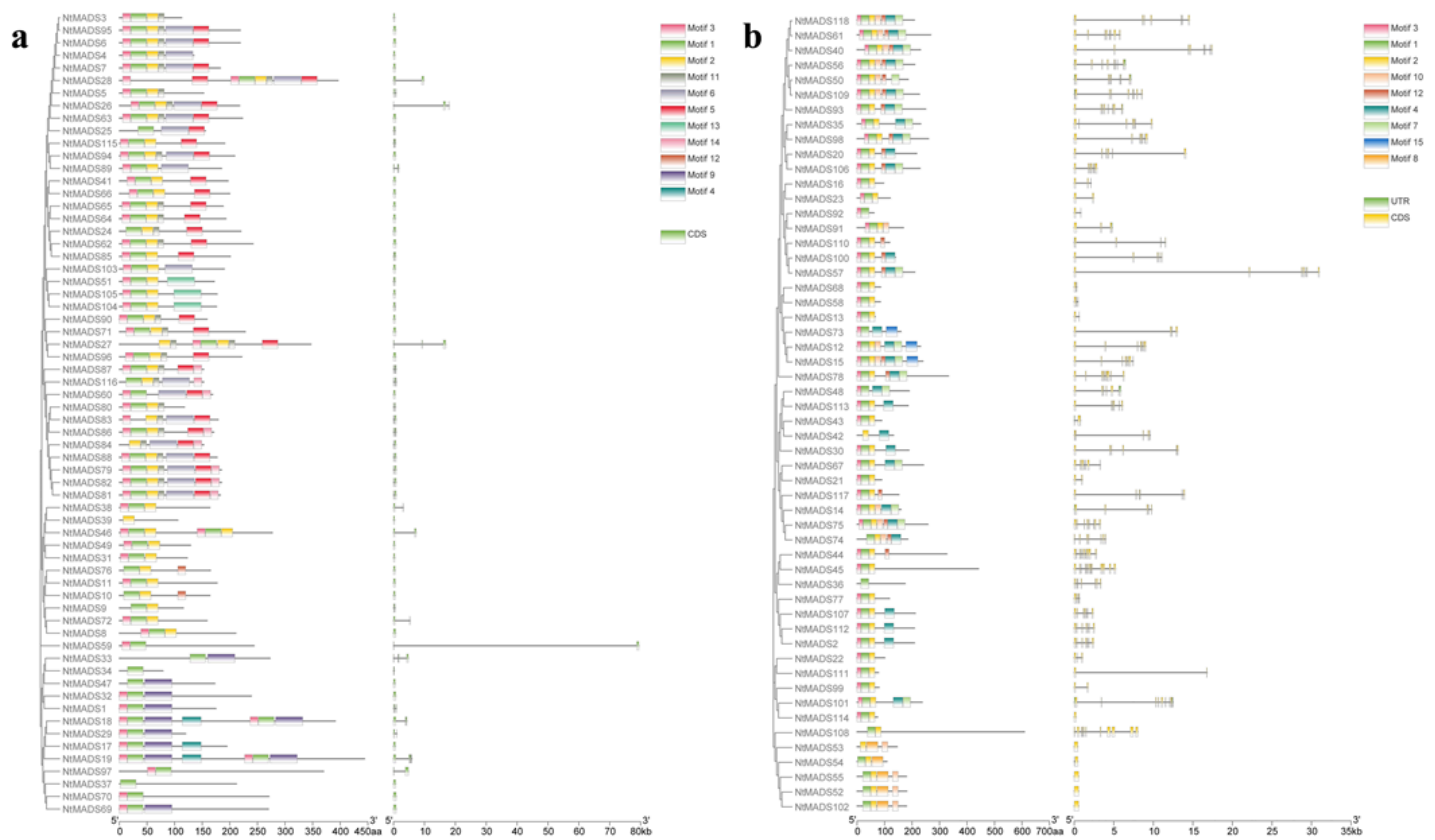


Figure 3

Gene structure and conserved protein motif analysis of tobacco Type I **(a)** and Type II **(b)** NtMADS-box genes

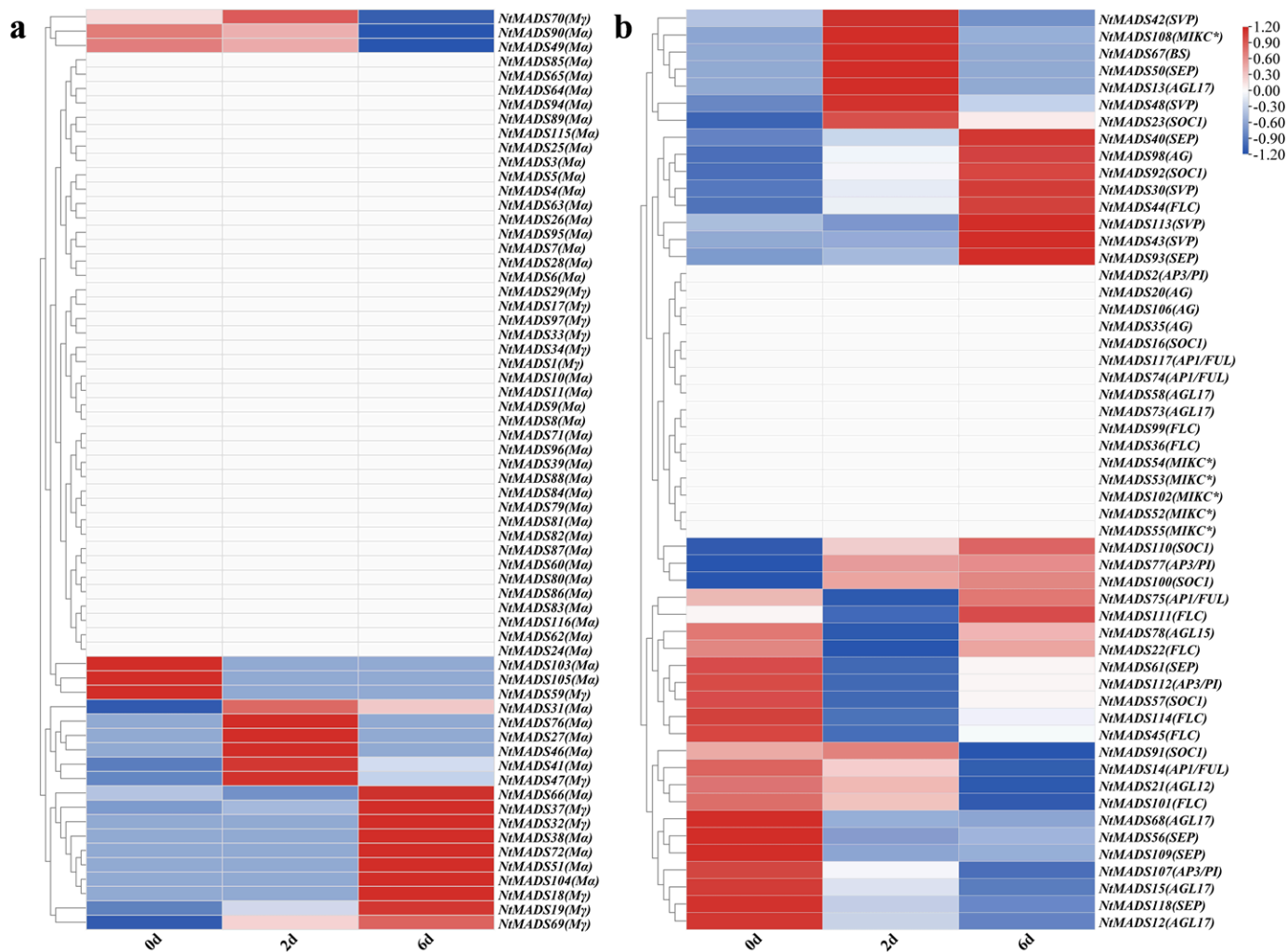


Figure 5

Expression of Type I **(a)** and Type II **(b)** MADS-box genes in axillary buds of tobacco at 0, 2, and 6 days after topping

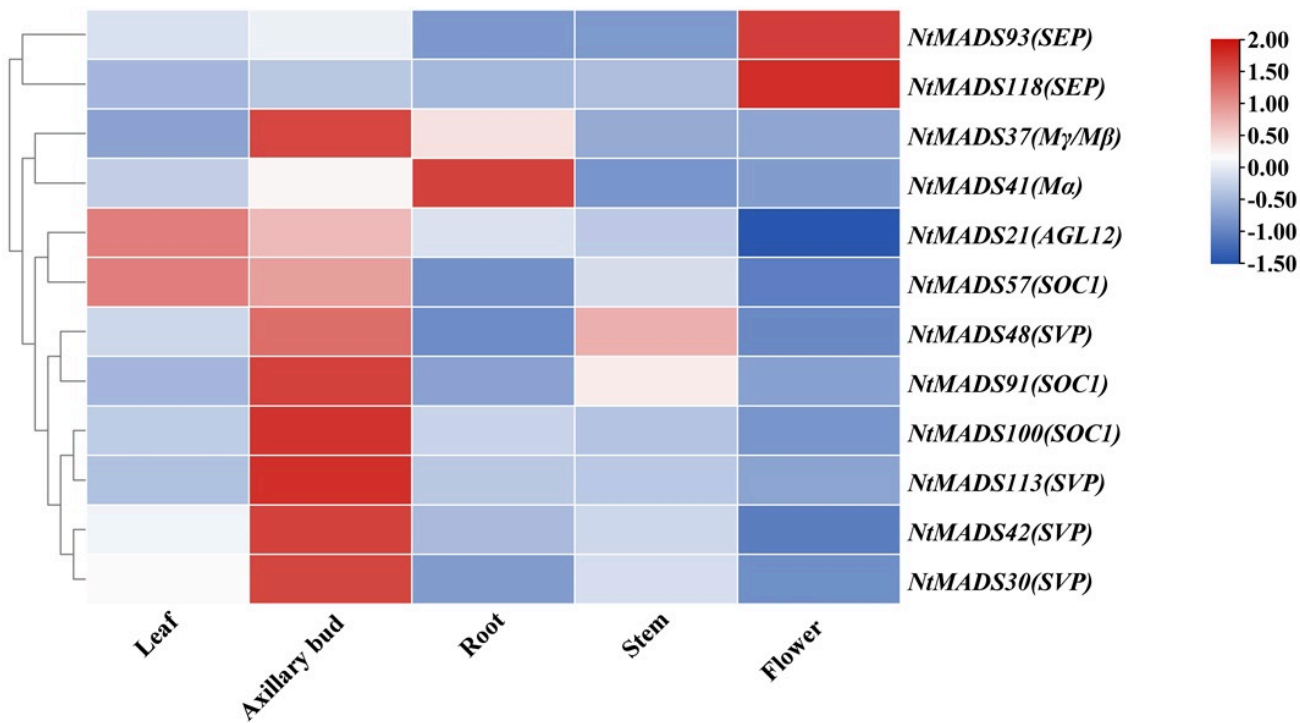


Figure 6

Expression patterns of NtMADS-box genes in different tissues of tobacco

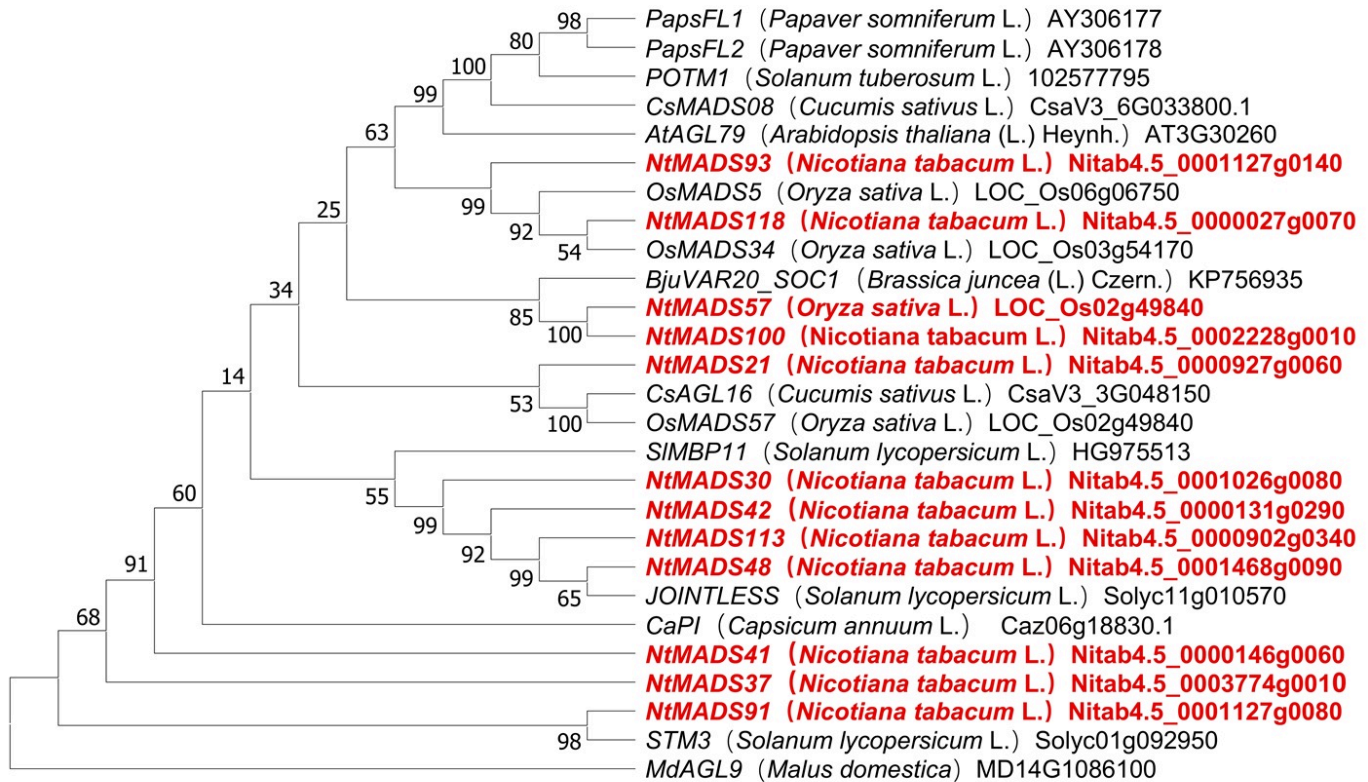


Figure 7

Phylogenetic tree of 12 NtMADS-box genes and reference MADS-box genes

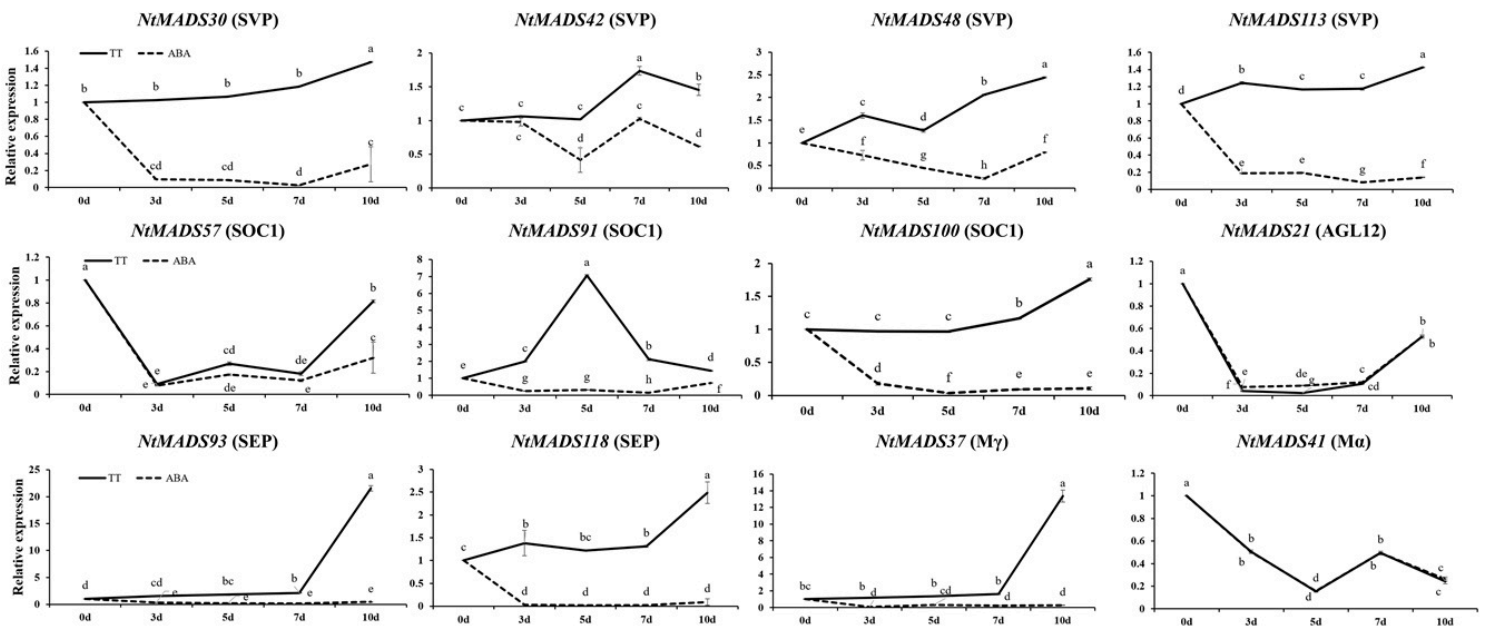


Figure 8

Expression patterns of NtMADS-box genes following ABA treatment. TT, topping treatment; ABA, topping followed by exogenous ABA treatment. Different lowercase letters indicate significant differences among means at $P < 0.05$. Data are expressed as mean $\pm$ SE ($n=3$)

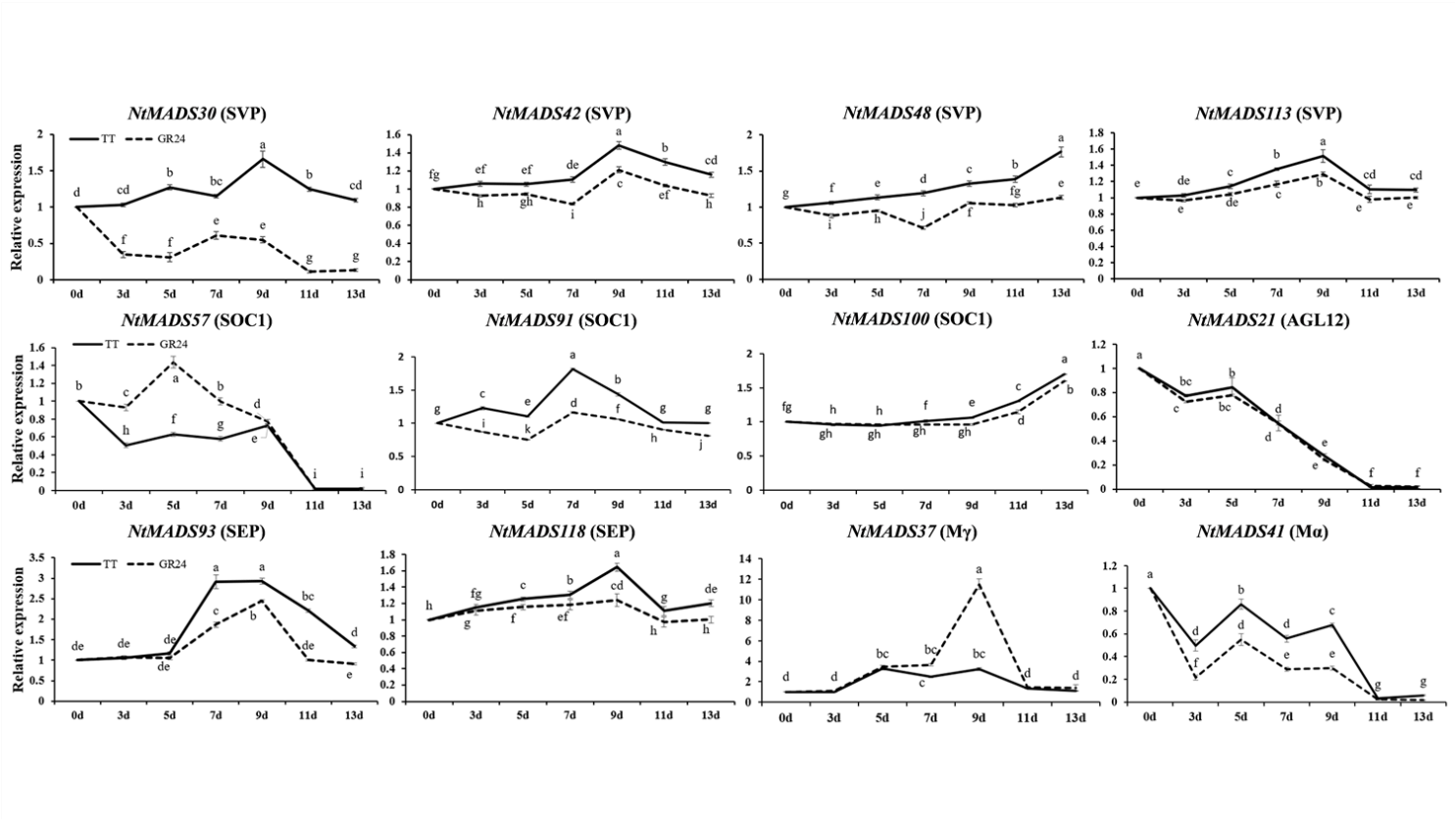


Figure 9

Expression patterns of NtMADS-box genes following GR24 treatment. TT, topping treatment; GR24, topping followed by exogenous GR24 treatment. Different lowercase letters indicate significant differences among means at $P < 0.05$. Data are expressed as mean $\pm$ SE ($n=3$)

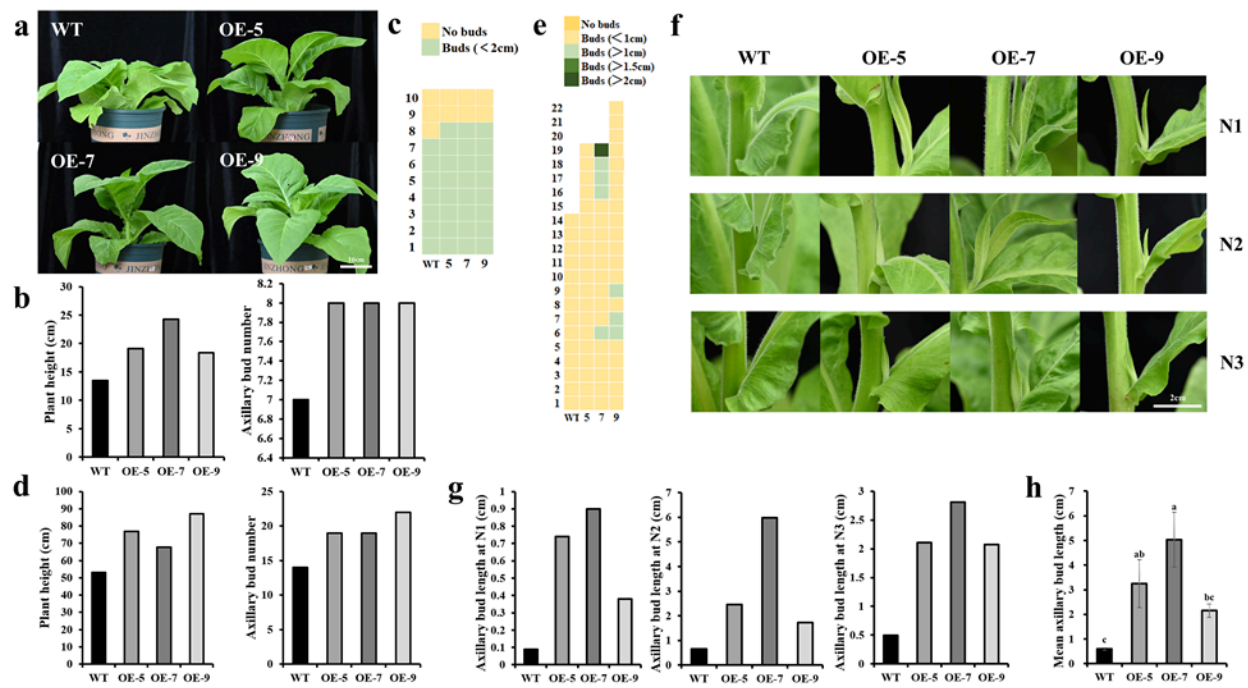


Figure 10

Overexpression of *NtMADS91* promotes the development of tobacco axillary buds. **a** Phenotypes of plants with different genotype at 30 days after transplanting. **b, c** Statistics of plant height, axillary bud number, and axillary bud length at each node at 30 days after transplanting. **d, e** Statistics of plant height, axillary bud number, and axillary bud length at each node at 60 days after transplanting. **f, g** Phenotype and length of axillary buds at the first to third nodes (N1-3), numbered downward from the apical bud, at 75 days after transplanting. **h** Mean axillary bud length calculated using the N1-3 nodes as biological replicates. WT, wild type; OE-5, OE-7 and OE-9 represent *NtMADS91* overexpression lines

Supplementary Files

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