The R2R3-type *LoMYB20s* gene regulates programmed cell death and secondary wall biosynthesis, and affects anther development and dehiscence in Lily

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Abstract

Background Lilies are the widely cultivated cut flowers worldwide, while lily anthers carry a large amount of colored pollen dispersed easily to stain petals that makes serious problems for commerical sales. Improving pollen pollution in lily is one of the major goals of lily breeding.

Results In this study, we identified a putative R2R3 MYB transcription factor LoMYB20s from oriental lily (Lilium spp. 'Siberia'). LoMYB20s mainly expressed in anther wall during the late stages of lily anther development. Suppression of LoMYB20s by virus-induced gene silencing (VIGS) in lily led to a failure of the anthers to dehisce. Induction of LoMYB20s in DEX::LoMYB20s transgenic Arabidopsis caused the rosette leaves turning yellow and the inflorescences becoming procumbent and infertile. And the downstream genes of LoMYB20s were involved in multiple metabolic processes including jasmonate (JA) biosynthetic, gibberellin (GA)-deactivating, programmed cell death (PCD), and secondary wall biosynthesis (SWB). These results suggested that LoMYB20s participated in anther development and dehiscence possibly through regulating the PCD and SWB processes in a JA/GA-associated manner.

Conclusions Our results demonstrated the indispensable role of LoMYB20s in lily anther development and dehiscence, and provide a possibility of using LoMYB20s silencing to produce anther-indehicent lilies.

Background

During the late stage of anther development, tapetal degeneration is coordinated with microspore maturation and pollen wall formation, endothecium expansion and subsequent deposition of ligno-cellulosic thickening, and degeneration of the septum and stomium [1, 2]. Finally, endothecium dehydration induces anther dehiscence and pollen release as the flower opens [3, 4]. Pectin-degrading polygalacturonases were found to play important roles in anther wall degeneration and anther dehiscence [5, 6]. And programmed cell death (PCD) has also been variously contributed to the degeneration process [7, 8]. Endothecium secondary wall thickening (SWT) is thought to provide the structure for producing the mechanical force for the anther wall to roll outward under dehydration and the degeneration facilitate the process [4, 9]. Numerous loss-of-function mutants of SWT supported the its importance in anther dehiscence [10, 11, 12, 13, 14].

Multiple types of transcriptional factors (TFs) and enzymes have been found to participate in this late stage of anther development; however, overall, the process remains largely uncharacterized [15, 16, 17]. R2R3-MYBs comprise one of the largest families of plant TFs and are important regulators playing diverse roles in anther development [18, 19]. In *Arabidopsis*, *MYB21*, *MYB24*, and *MYB57* are critical components in the JA-mediated transcriptional cascade for stamen development and regulate overall filament elongation and anther dehiscence [20, 21]. In addition, the anther-specific gene *MYB26* governs SWT in the endothecium and functions upstream of the central TF genes involved in secondary wall (SW)

formation, including *NAC SWT promoting factor1* (*NST1*) and *NST2* [10, 11]; the *GAMYB*-like genes *MYB33*/*MYB65* facilitate tapetal and pollen development [22]; and *MYB80* is required for tapetal cell death, callose dissolution, exine formation, and pollen development in *Arabidopsis* [23, 24] and are functionally conserved in crops such as cotton, lily, and *Brassica* [25, 26, 27].

Lilies are amongst the most important and widely cultivated cut flowers worldwide; however, lily anthers carry a large amount of highly colored pollen. This easily becomes dispersed when the flower opens, leading to unsightly staining of the flowers themselves and of items that come into contact with it, such as clothing. Research into lily anther development and dehiscence will not only reveal the fundamental mechanisms involved in these processes, but will have practical implications in providing approaches for the control or prevention of pollen release in the breeding of new hybrid cultivars. In the present study, we report the functional characterization of a single R2R3-type *MYB* gene *LoMYB20s* from lily, which is specifically expressed in the late phase of anther development. Investigation of the effect of *LoMYB20s* silencing on lily anther development showed an inhibition on anther dehiscence, and it might be resulted from the obstruction of PCD-related anther tissue degeneration and the disturbance of natural SWB, possibly via a JA/GA (jasmonate/gibberellin) co-mediated regulatory pathway.

Results

LoMYB20s in lily belongs to the R2R3-type *MYB* gene family

As is well known, many *MYB*-family genes participate in the anther development process, in a variety of roles [20, 21]. In order to obtain *MYB* genes with roles in anther dehiscence in lily, we designed a degenerate primer based on the highly conserved R3 DNA-binding domain of the *MYB* family, and finally obtained a full-length sequence of a *MYB* gene from mature anthers by the RACE amplification method, which was deposited in Genbank with the designation *LoMYB20s* and the accession number KT759161. The full-length nucleotide sequence of *LoMYB20s* was 807 bp, encoding a deduced protein sequence of 190 amino acids. LoMYB20s has two MYB DNA-binding domains (Fig. 1a), and is a R2R3-type MYB protein [19]. A BLAST homology search of the sequence of LoMYB20s against the NCBI protein database revealed ten similar sequences (Fig. 1a), but no study of these MYBs has so far been reported. In *Arabidopsis*, the R2R3-type MYB factors that are encoded by 125 *MYB* genes have been categorized into 22 subgroups [19]. LoMYB20s is most closely related to MYB57, MYB21 and MYB24 from subgroup 20 of *Arabidopsis*, and then to MYBs from subgroup 19 (Fig. 1b).

There is only one copy of *LoMYB20s* in the lily genome

Southern hybridization was used to identify whether there were multiple copies or close homologs of *LoMYB20s* in the lily genome. The full-length sequence of *LoMYB20s* was used as a probe, and endonucleases *Bam*HI, *Eco*RI, *Hin*dIII, and *Xba*I were used for genomic DNA digestion. The results showed that two bands were visible in the *Eco*RI-digested product, and that only one band was visible in the other three endonuclease-digested products (Fig. 2). Given that there is a single *Eco*RI cleavage site in the

LoMYB20s sequence, the results suggested that *LoMYB20s* is a single-copy gene, with no close homologs in the lily genome.

LoMYB20s is mainly expressed in the late stages of lily anther development

Levels of expression of *LoMYB20s* were examined in a range of tissues of the lily plant, including stem, leaf, petal, ovary, stigma, filament, anther, anther wall, and pollen. *LoMYB20s* was found to be strongly expressed in petal, stigma, filament, anther, and anther wall, but not in leaf, stem, pistil, or pollen (Fig. 3a). Expression levels of the gene were also determined at different anther developmental stages. The various stages of anther development were easily distinguishable by anther color, which progressed from white to green, then to yellow, and finally to purple [28]. A low level of expression of *LoMYB20s* was detected at the white and green stages, which increased at the yellow and purple stages, and then declined following anther dehiscence (Fig. 3b). These results suggested that *LoMYB20s* probably functioned during the late phase of anther development.

LoMYB20s silencing inhibits anther development and dehiscence

To examine the possible role of *LoMYB20s* in anther dehiscence, we silenced *LoMYB20s* in lily using virus-induced gene silencing (VIGS) [28]. Total RNA was extracted from anthers of lily plants infected with *Agrobacterium tumefaciens* carrying pTRV-*LoMYB20s* or the pTRV empty vector and analyzed by semi-quantitative RT-PCR (Fig. 4a). *LoMYB20s* was successfully silenced in the anthers of six lines of lilies (6/100), and representative *LoMYB20s*-silenced flowers of lily are shown in Fig. 4b. We observed that, in the *LoMYB20s*-silenced lilies, the flower petals were well developed and opened normally, and the filaments and stigmas appeared to be normally elongated; however, the anthers became slender, and indehiscent (Fig. 4b). Conversely, in the control lines, the anther walls could roll outwards and the anthers were able to dehisce normally. These results indicated that silencing of *LoMYB20s* affected anther development, and demonstrated that *LoMYB20s* plays important roles in anther dehiscence.

Dexamethasone (DEX) induction of *LoMYB20s* led to senescence and infertility in *Arabidopsis*

To further investigate the function of *LoMYB20s* in anther development, we constructed the pTA7001G-*LoMYB20s* vector, in which *LoMYB20s* is overexpressed only under DEX induction, and used this vector to transform *Arabidopsis thaliana*. Under the control treatment (no DEX treatment), DEX::*LoMYB20s* transgenic lines (*LoMYB20s*-expressing plants), wild-type (WT) plants, and pTA7001G transgenic plants (control plants) all grew, flowered, and produced siliques normally; the only noticeable effect was that the inflorescence stems of the *LoMYB20s*-expressing plants were a little short compared to those of the control plants (Additional file 1: Figure S1). Under DEX treatment, the control plants grew, flowered, and bore seed normally, whereas the *LoMYB20s*-expressing plants displayed serious leaf yellowing within a few days (Fig. 5a) and their inflorescences were poorly developed, or developed to be procumbent and with fewer siliques (Fig. 5b). These results indicated that overexpression of *LoMYB20s* caused early senescence of the transgenic plants, and severely influenced inflorescence development in *Arabidopsis*.

MYB homologs were induced in *LoMYB20s* transgenic *Arabidopsis*

In an attempt to explain the phenotypes of *LoMYB20s*-silenced lily anthers and *LoMYB20s*-expressing *Arabidopsis*, the expression levels were detected of potentially related genes in *Arabidopsis*, using RT-PCR (Additional file 1: Figure S2); this revealed that several different types of genes were obviously induced.

In *LoMYB20s*-expressing *Arabidopsis*, the expression of *LoMYB20s* and its closest *MYB* homologues was initially examined (Fig. 6a, Additional file 1: Figure S2a). As anticipated, the expression of *LoMYB20s* was only detected following DEX treatment, and not in the controls, indicating that the phenotype of the transgenic plants sprayed with DEX was consequent upon *LoMYB20s* expression. Then, the expression of the closest homologues of *LoMYB20s* in *Arabidopsis*, such as *MYB21*, *MYB24*, and *MYB57*, was determined. This revealed that both *MYB24* and *MYB57* were induced, though expression of *MYB21* was not detected. Next, the expression of various downstream genes of these homologous *MYBs* [20] was determined: the phenylalanine ammonialyase genes *PAL1* and *PAL2*, the terpene synthase genes *TPS11* and *TPS21*, the alternative oxidase gene *AOX1a*, and the auxin-related genes *SAUR63*, *IAA2*, *IAA3*, *IAA7*, *IAA19*, *ARF6*, and *ARF8*. Of these, only the expression of *PAL2* and *TPS21* showed clear changes.

Expression of PCD- and SWB-related genes was increased in *LoMYB20s* transgenic *Arabidopsis*

Given that the rosette leaves of the transgenic *Arabidopsis* seedlings showed premature yellowing, RT-PCR was first used to search for alterations in the expression of PCD-related genes (Additional file 1: Figure S2b). Several genes showed altered expression in the DEX-induced plants. Their expression was then precisely quantitated using qRT-PCR, which revealed dramatically higher levels of expression, relative to controls, in the *LoMYB20s*-expressing plants (Fig. 6b). These genes included, amongst others, three pathogen-elicited PCD-associated genes, *PR-1*, *PR-2*, and *PR-5* [29], a KDEL-tailed serine protease (CysP) gene, *CEP2* [23], a senescence-associated CysP gene, *SAG12* [31], a vascular xylem autophagy modulator gene, *MC9* [32, 33], and a senescence-associated TF gene, *WRKY53* [34].

As indicated previously, in *LoMYB20s*-silenced VIGS lilies, the anther wall was unable to roll outwards and the anthers dehisced abnormally, which suggested that SWB might be disrupted. Accordingly, we examined the expression in transgenic *Arabidopsis* of a number of genes involved in SWB (Additional file 1: Figure S2c). Among these, three negative regulator genes, *MYB7*, *MYB32*, and *KANT7*/*IRX11* [35, 36] , and two genes encoding proteins that interact with the *KANT7* gene product, *BLH6* and *OFP4* [37], were obviously induced (Fig. 6c). In addition, two effector genes involved in xylan biosynthesis, *FRA8*/*IRX7* [38] and *IRX15L*/*DUF579* [39], and an endo-PG gene, *QRT2* [5], were also sharply induced in *LoMYB20s*-expressing plants (Figure 6c).

Genes of JA and GA metabolism were altered in transgenic *Arabidopsis*

Among genes downstream of *LoMYB20s* that have been mentioned above, some have been reported to be JA- and/or GA-regulating genes, such as *MYB24*, *32*, *57*, *WRKY53*, *SAG12*, and *QRT2*. Therefore, the expression of genes involved in the metabolism and signaling of JA and GA was examined, including JA

biosynthetic and metabolic genes, *DAD1*, *LOX1-6*, *AOS*, *AOC*, *OPR3*, *ACX1*, *JMT*, and *JAR1*, the JA early signal transduction genes, *COI1*, *MYC2*, *MYC3*, and *MYC4*, GA-biosynthetic genes *GA20ox1-5* and *GA3ox1-4*, GA-deactivating genes *GA2ox1-8*, and DELLA genes (Additional file 1: Figure S2d). Two JA biosynthetic genes, *LOX1* and *JMT* [40], and three genes of GA deactivation, *GA2ox4*, *GA2ox6*, and *GA2ox8* [41], were induced in *LoMYB20s*-expressing plants compared to the control plants after DEX treatment (Fig. 6d). These results suggest that *LoMYB20s* could be involved in JA/GA-associated stamen developmental regulation.

Discussion

Regarding its homology to MYB proteins in *Arabidopsis*, LoMYB20s is most closely related to MYB21, MYB24, and MYB57 (Fig. 1b). MYB21, MYB24, and MYB57 are closely related to each other and they have been demonstrated to have a role in the JA regulation of pollen maturation, filament elongation, and anther dehiscence [21]. In contrast to the shorter filaments and petals displayed in the delayed dehiscence phenotypes exhibited by *myb21, myb21myb24*, or *myb21myb24myb57* mutants [20, 42], only slender and indehiscent anthers were observed in *LoMYB20s*-silenced lily flowers (Fig. 4b). In transgenic *LoMYB20s*-expressing *Arabidopsis* plants, *MYB21* expression was only slightly influenced, compared to *MYB24* and *MYB57*, which were strongly induced (Additional file 1: Figure S2, Fig. 6a). MYB21, MYB24, and MYB57 are the key TFs in JA-regulated anther development, but the mechanism by which mutations in *MYB21*, *MYB24*, or *MYB57* cause the phenotypes mentioned above has not been elucidated. Several genes have been reported to be downstreams of *MYB21* or *MYB24*, such as *PAL*, *AOX1a*, and *TPS11* and *TPS21* [42, 43]. Among these genes, only *PAL2* and *TPS21* were significantly induced in *LoMYB20s*-expressing *Arabidopsis* plants after DEX induction (Additional file 1: Figure S2a, Fig. 6a). A number of auxin response and JA biosynthesis genes have been shown to display obvious changes in *myb21myb24* double mutants, including *SAUR63*, *IAA2*, *IAA3*, *IAA7*, *IAA19*, *DAD1*, and *LOX2* [42], but none of them showed significant change in *LoMYB20s*-expressing plants in the present work (Additional file 1: Figure S2a). It would appear that *LoMYB* differentially affected the expression of these *MYBs* and their downstream genes and that, perhaps as a consequence of this, *LoMYB20s* regulated anther dehiscence more specifically than its homologs.

Given that *LoMYB20s*-expressing *Arabidopsis* plants exhibit a phenotype of early senescence after DEX induction (Fig. 5a), it is reasonable to suspect that *LoMYB20s* participates in the regulation of PCD in lily anthers, where it is expressed. In the *Arabidopsis MYB* family, several *MYB*s have been reported to be associated with PCD, including *MYB33*, *MYB65* [22], *MYB80* [23], *MYB101* [44], and *MYB30* [45]. None of these genes showed changes in expression in *LoMYB20s*-expressing plants after DEX induction (Additional file 1: Figure S2b). But one of the downstream targets of *MYB30*, *PR-1* [46] showed strongly altered expression in both *LoMYB20s*-expressing and control plants, compared to WT plants (Fig. 6b). Amongst other four *PR* genes, *PR-2* and *PR-5* were strongly expressed only in *LoMYB20s*-expressing plants (Fig. 6b). *PR-1*, *PR-2*, and *PR-5* are signature genes in plant defense responses to pathogenic infection [47], which also play important roles in various different abiotic stress responses [48, 49, 50, 51]. The expression of *PR* genes is activated by the SA sensor protein NPR1, which stimulates transcriptional

activity in response to pathogenic infection [52]. In addition, cold stimulation could also cause the induction of *PR* genes, which is mediated by a plasma membrane-tethered NACTF, NTL6, independently of NPR1-mediated SA signaling [53]. In the present work, the expression levels of *NPR1* and *NTL6* were unchanged in *LoMYB20s*-expressing plants (Additional file 1: Figure S2b), implying that *PR-1*, *PR-2*, and *PR-5* may play roles in anther development that are distinct from the known biotic and abiotic stress responses.

Furthermore, we found that the expression levels of three cell-death-related protease genes, *CEP2*, *SAG12*, and *MC9* were remarkably higher in *LoMYB20s*-expressing plants than in control plants (Additional file 1: Figure S2b, Fig. 6b). The *CEP2* homolog *CEP1* has been reported to be involved in the tapetal PCD process [24], and *SAG12* was identified from leaves in the late stage of senescence that were visibly yellow [31]. Here, a gene upstream of *SAG12*, the senescence-associated TF gene *WRKY53* [34], was also induced in *LoMYB20s*-expressing plants after DEX induction (Fig. 6b). Lastly, the cysteine-type peptidase gene *MC9* is involved in the modulation of autophagy, to confine cell death to the appropriate target cells during *Arabidopsis* vascular xylem differentiation [33]. Taken together, the results suggested that *LoMYB20s* might have a function in the regulation of the PCD process.

Specific cells such as those comprising conducting vessels and fibers deposit a SW rich in cellulose, hemicellulose, and lignin, with lesser amounts of pectin [54]. In the anther endothecium, SW deposition is required for the generation of the tensile force necessary for stomium rupture to release pollen grains [11]. The indehiscence of lily anthers in *LoMYB20s*-silenced flowers (Fig. 4b), and the procumbent inflorescence stems of *LoMYB20s*-expressing *Arabidopsis* plants (Fig. 5b) suggested that either silencing or, conversely, ectopic expression of *LoMYB20s* might compromise SW deposition. Among many SWB regulator genes (Additional file 1: Figure S2c), the expression of *MYB7*, *MYB32*, and *KNAT7* was dramatically induced in *LoMYB20s*-expressing plants (Fig. 6c). These three genes are direct targets of the secondary master switches of the SWB regulatory network, *MYB46* and *MYB83* [36, 55]. But neither these two *MYBs* nor other genes directly downstream of them, such as *MYB43*, *MYB52*, *MYB54*, *MYB58,* and *MYB*63, and *IRX1*, *IRX3*, *IRX8*, *IRX9*, and *IRX14* were affected in *LoMYB*-expressing plants, suggesting that *LoMYB20s* probably promoted the expression of *MYB7*, *MYB32*, and *KNAT7* through other unknown pathways.

MYB7 and MYB32 are negative regulators in lignin biosynthesis [56], and KNAT7 and its interacting proteins OFP4 and BLH6 are also inhibitors of SWB [57, 58]. Although there are another two genes, FRA8 and IRX15L, involved in glucuronoxylan biosynthesis were also induced by *LoMYB* (Fig. 6c), they are not the main enzymes in xylan biosynthesis [56]. So based on the symptom of the decreased inflorescence stem strength (Fig. 5b) and the expression induction of the negative regulator genes in *LoMYB20s*–expressing plants (Fig. 6c), we are inclined to believe that *LoMYB20s* negatively regulated SWB in anther development. Previous studies also discovered some negative regulators of SWB which were involved in the regulation of anther dehiscence [59, 60, 61], but how these negative regulators coordinate with positive regulators acting on anther wall development is yet to be identified and explained.

Plant hormones interact with each other through signaling crosstalk in multiple stress responses and developmental processes. A previous study reported that GA suppressed DELLAs to promoted the expression of *DAD1* and *LOX1*, up-regulated JA production, and promoted the expression of *MYB21*, *MYB24*, and *MYB57* [20]. In the present work, we found that *LoMYB20s* could promote the expression of both JA biosynthetic and GA-deactivating genes (Fig. 6d). This result implied an antagonistic relation between JA and GA in *LoMYB20s* mediated regulatory network. Previous studies have observed the antagonistic interaction of JA and GA in the regulation of plant growth and defense, and the synergistic interaction of JA and GA in regulation of stamen development [20, 62]. The findings reported here suggested some possibility of this antagonistic JA–GA interaction in *LoMYB20s* regulated specific PCD- and SWB-related processes in anther development.

A biomechanical model describing anther opening incorporates the outer epidermal cell layer dehydration, and the endothecial layer uneven SWT in some model plant and crops [9]. In our previous work, we exhibited a reversible open and close process in mature anthers of lily controlled by the ambient humidity, and a *LoPIP2*-silencing induced anther indehiscent phenotype [28]. Here, we found the silencing of a *LoMYB20s* gene also induced lily anther indehiscent phenotype (Fig. 4), which might be related to natural PCD and SWB damage (Fig. 6). Accompanied with the development of genetic transformation system of lily [63, 64], these genes could be good candidates for developing anther-indehiscent lilies by molecular breeding in the future.

Conclusion

The roles of the lily flower-specific gene *LoMYB20s* were investigated. Silencing of *LoMYB20s* in cut flowers of lily led to a failure of anthers swelling and dehiscence. Overexpression of *LoMYB20s* in *Arabidopsis* promoted the expression of genes related to SWB and PCD processes and involved in JA and GA metabolism. The results suggest a possible role of *LoMYB20s* in the regulation of PCD and SWB processes in lily anther development and open opportunities for the development of anther-indehiscent lilies.

Methods

Plant materials

Oriental hybrid lily cultivar ‘Siberia’ is cultured worldwide and is one of the most popular bouquets of corm varieties on the Chinese floral market [28]. The flowers of ‘Siberia’ were obtained from Beijing Shengsitong Eco-Technology Co., Ltd. (China). The flower stems were placed in water immediately after harvesting and delivered instantly to the laboratory. The flower stems were then re-cut underwater to a length of ~80 cm and placed in deionized water. Lily anther development was divided into four stages according to the distinctive colors visible at the anther surface: white, green, yellow, and purple [28].

Cloning of *LoMYB20s* genes

Total RNA was extracted from yellow anthers of lily using Trizol reagent (Invitrogen, USA). cDNAs for cloning the 5' and 3' ends of *MYB* were prepared from 1 μg of total RNA from lily anthers, using the SMART^{TM} RACE cDNA Amplification Kit (Clontech, USA). A degenerate primer, 5'-KCWARRTGGGGRAAYAGGTGGTC-3', was used, together with “Universal Primer A Mix” provided in the kit, to amplify potential *MYB* 3' sequences in lily. Then the *MYB* gene-specific primer, 5'-GGACCCTCCAAACATTCTATATCTATCT-3', was designed as a reverse primer based on the obtained 3' sequence and used with “Universal Primer A Mix” to amplify the *MYB* 5' sequence. Finally, the complete sequence of the *MYB* gene was obtained using specific primers designed according to the 5' and 3' ends of *MYB*. Multiple sequence alignment was carried outusing the EBI ClustalW server (*http://www*.ebi.ac.uk/clustalw/) and visualized using BioEdit (Ibis Biosciences, Carlsbad, USA). Phylogenetic and molecular evolutionary analysis was undertaken using MEGA version 5 [65].

Southern blot analysis of *LoMYB20s*

Southern blot analysis was carried out using the DIG Application Manual for Filter Hybridization (Roche, Switzerland). Genomic DNA was extracted from lily anthers using the CTAB method and digested with *Bam*HI, *Eco*RI, *Hin*dIII, and *Xba*I. Ten μg of each digested product was separated on an agarose gel (0.8%) and then transferred onto a positively charged nylon membrane (Roche) by capillary transfer using 20×SSC buffer; the DNA was then UV-cross linked to the filter at a strength of 70,000 μj m^{-2}. The filter was then prehybridized and hybridized against the DIG-labeled full sequence of the *MYB* gene probe. The primers for the probe were: *LoMYB20s*FU, 5'-GGACCCTCCAAACATTCTATATCTATCT-3' and *LoMYB20s*FL, 5'-CAGCATGAAAAGCAATAAAGTTCAATTAC-3'. Hybridization was performed at 42^{o}C and the probe was washed at 60^{o}C. DNA blots were visualized following film development (FUJI Photo Film, Japan) for 1 h.

Reverse transcriptase polymerase chain reaction (RT-PCR) analysis

To determine the expression levels of the *LoMYB20s* gene in various different organs of lily plants, samples of leaf, stem, petal, ovary, stigma, anther, anther wall, and pollen were obtained from cut flowers (leaves and stems attached) that were about to open. In determining the expression pattern of the *LoMYB20s* gene in lily anthers at different developmental stages, the stages of anther development were distinguished by color [28]. To determine the expression levels of *LoMYB20s* and candidate related genes in transgenic *Arabidopsis*, the above-ground parts of *Arabidopsis* plants were collected on the 5th day following DEX treatment. Total RNA from multiple organs of lily flower was extracted using a General Plant Total RNA Extraction Kit RP3301 (BioTeke, China). Total RNA from lily anthers at different developmental stages or from *Arabidopsis* plants was extracted using a TRIpure Total RNA Extraction Reagent RP1001 (BioTeke, China). Reverse transcription was carried out using 1 μg of RNA sample and ImProm-II^{TM} Reverse Transcriptase (Promega, USA). Semi-quantitative RT-PCR analysis was carried out using lily *TIP* (KJ543466), *L-actin* (DQ019459.1), or *Arabidopsis ACTIN7* (At5g09810) as the internal control. Primer pairs used for RT-PCR detection are listed in Additional file 1: Table S1.

Virus-induced gene silencing (VIGS) of *LoMYB20s* in lily anthers

Silencing of *LoMYB20s* was performed using VIGS, with pTRV vectors (pTRV1 and pTRV2) [66]. A 426-bp fragment was amplified from the 5' region of *LoMYB20s* using the primers 5'-gaggtaccTACCATGGACAAGAGAGTGAT-3' and 5'-ctggatccgGCTCGCTTGGCTCGTG -3'. The fragments were inserted into the *Kpn*I and *Bam*HI restriction sites of pTRV2 to form the pTRV2-*LoMYB20s* construct. pTRV2-*LoMYB20s*, pTRV1, and pTRV2 were transformed individually into *Agrobacterium tumefaciens* GV3101. For infiltration, *Agrobacterium* suspensions containing a 1:1 (v/v) ratio of either (i) pTRV1 and pTRV2-*LoMYB20s* or (ii) pTRV1 and pTRV2 (control) were prepared. Lily plants were infected when the flower buds were ~4 cm in length and contained white anthers at an early stage of development [28]. Approximately ~15 days after infiltration, the flowers were photographed, and the anthers were collected for characterization.

Arabidopsis transformation and morphological observations

To transform the *LoMYB20s* gene into *Arabidopsis*, we first amplified the *LoMYB20s* ORF, using as primers: forward, 5'-aaactcgagACTCGAGATGGACAAGAGAGTGATCCCT-3'; reverse, 5'-aaaactagtCGTCTCCATTGAAAGACTGCATAG-3'. The resultant product was ligated into the pTA7001G vector using the *Xho*I and the *Spe*I sites. The constructed vectors were then introduced into *Agrobacterium tumefaciens* strain GV3101. Transformations with *Arabidopsis* were performed using the flower-dip method, as described by Clough and Bent [67].

For further analysis, homozygous lines were selected on 1/2 MS medium with 25 mg L^{-1} hygromycin B (Hyg). Homozygous plant lines carrying pTA7001G or pTA7001G-*MYB*, along with WT plants, were placed on 1/2 MS medium for 10 days, and then transplanted to 8-cm square pots filled with a 1:1 mixture of peat and vermiculite under greenhouse conditions (23±1^{o}C, 16/8 h (day/night), and 80-100 lux m^{-2} s^{-1} illumination) for 15 days. Plants to be treated with DEX were sprayed daily with 30 μM DEX from the 26th day for a further 15 days, while non-treated lines were sprayed with 1‰ aqueous alcohol as negative controls (DEX powder was dissolved in ethanol to give a stock solution of 30 mM; this was kept at -20^{o}C and diluted with ddH_2O to give a working concentration of 30 μM). The above-ground parts of each line were sampled for gene expression examination at the 30th day, 4 h after being sprayed with DEX. The morphologies of the whole plants were observed and photographed on the 30th and 45th days.

Quantitative RT-PCR (qRT-PCR) analysis of *LoMYB20s*-regulated genes

The expression of *LoMYB20s*-related genes was initially examined in *LoMYB20s*-expressing and WT *Arabidopsis* plants by semi-quantitative RT-PCR. Genes showing obvious changes in expression between *LoMYB20s*-expressing plants and control plants were selected for qRT-PCR analysis. qRT-PCR was performed using 2×Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific, USA) and the Mx3005P QPCR System (Agilent, USA), and relative mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method [68]. Three biological replicates were performed for qRT-PCR. The *ACTIN7* gene was used as a reference for normalization. All primers used for RT-PCR and qRT-PCR are listed in Additional file 1: Table S1.

Abbreviations

DEX: dexamethasone; GA: gibberellin; JA: jasmonate; PCD: programmed cell death; PR: pathogenesis-related; SWB: secondary wall biosynthesis; SWT: secondary wall thickening; VIGS: virus-induced gene silencing.

Declarations

Acknowledgements

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Authors' contributions

BH, JH, and ZT designed the study. ZT performed the majority of the experiments. QL and AJK assisted with experiments. ZT, JH, and BH analysed the data and wrote the paper. JG read and provide helpful discussions. All authors have read and approved the manuscript.

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Availability of data and materials

The data sets supporting the results of this article are included within the article and its additional file.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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Additional File Information

Additional file 1: **Figure S1.** Growth phenotypes of transgenic *Arabidopsis* lines of DEX::*LoMYB20s* plants. **Figure S2.** The semi-quantitative PCR detection of related genes. **Table S1.** Primer sequences used in this study.

Figures

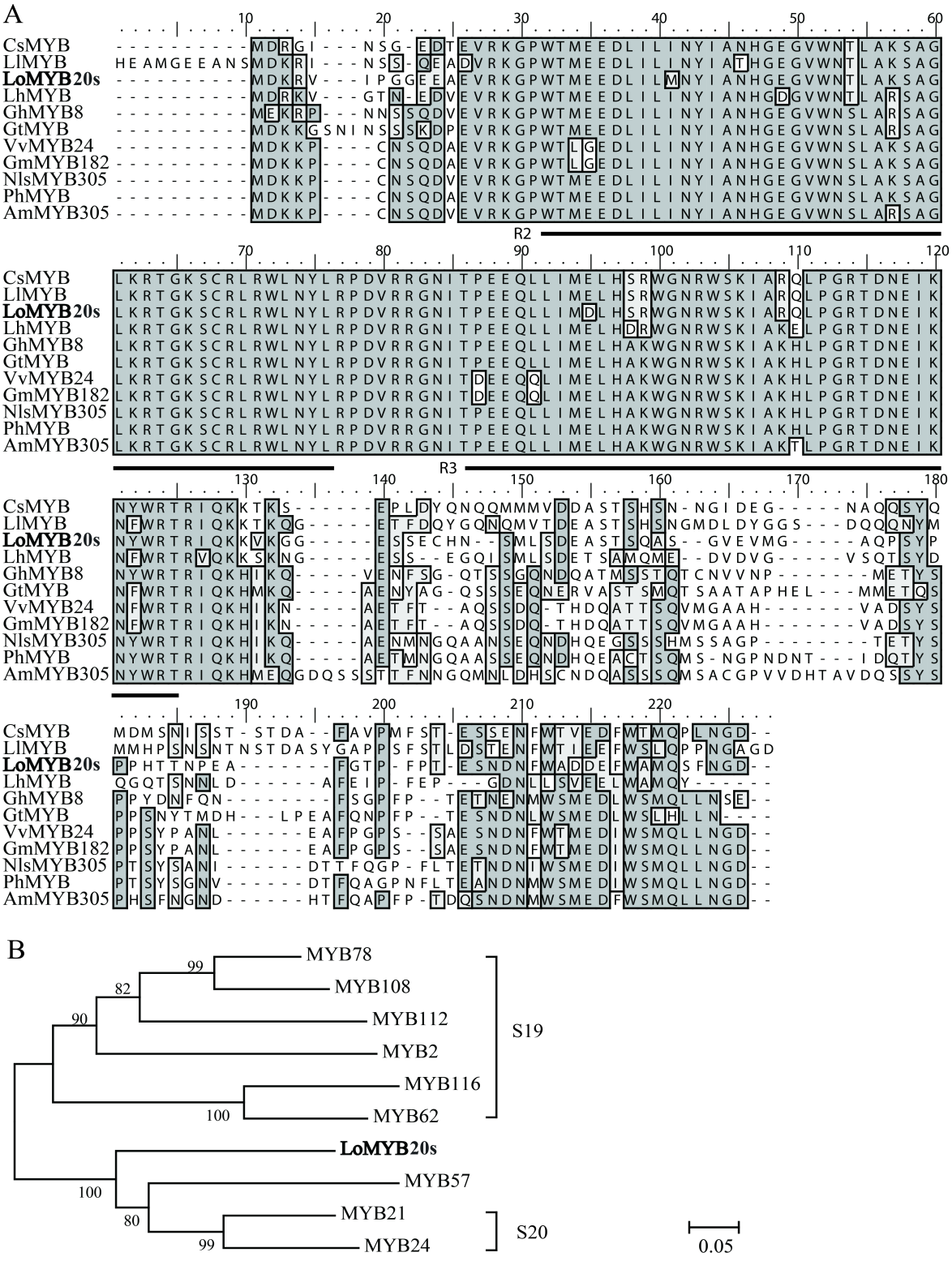

Figure 1

Comparison of the deduced amino acid sequence of LoMYB20s with those of MYBs from other species. a Amino acid alignment of LoMYB20s and its most closely related MYBs from other plant species. Identical and similar amino acids are colored dark gray and light gray, respectively. Black lines under the

sequences indicate the R2 and R3 domains of the MYB family, respectively. b Phylogenetic relationship between LoMYB20s and its most closely related MYB subgroup members in Arabidopsis. Branch lengths are proportional to accumulated amino acid substitutions. Bootstrap values indicate the divergence of each branch.

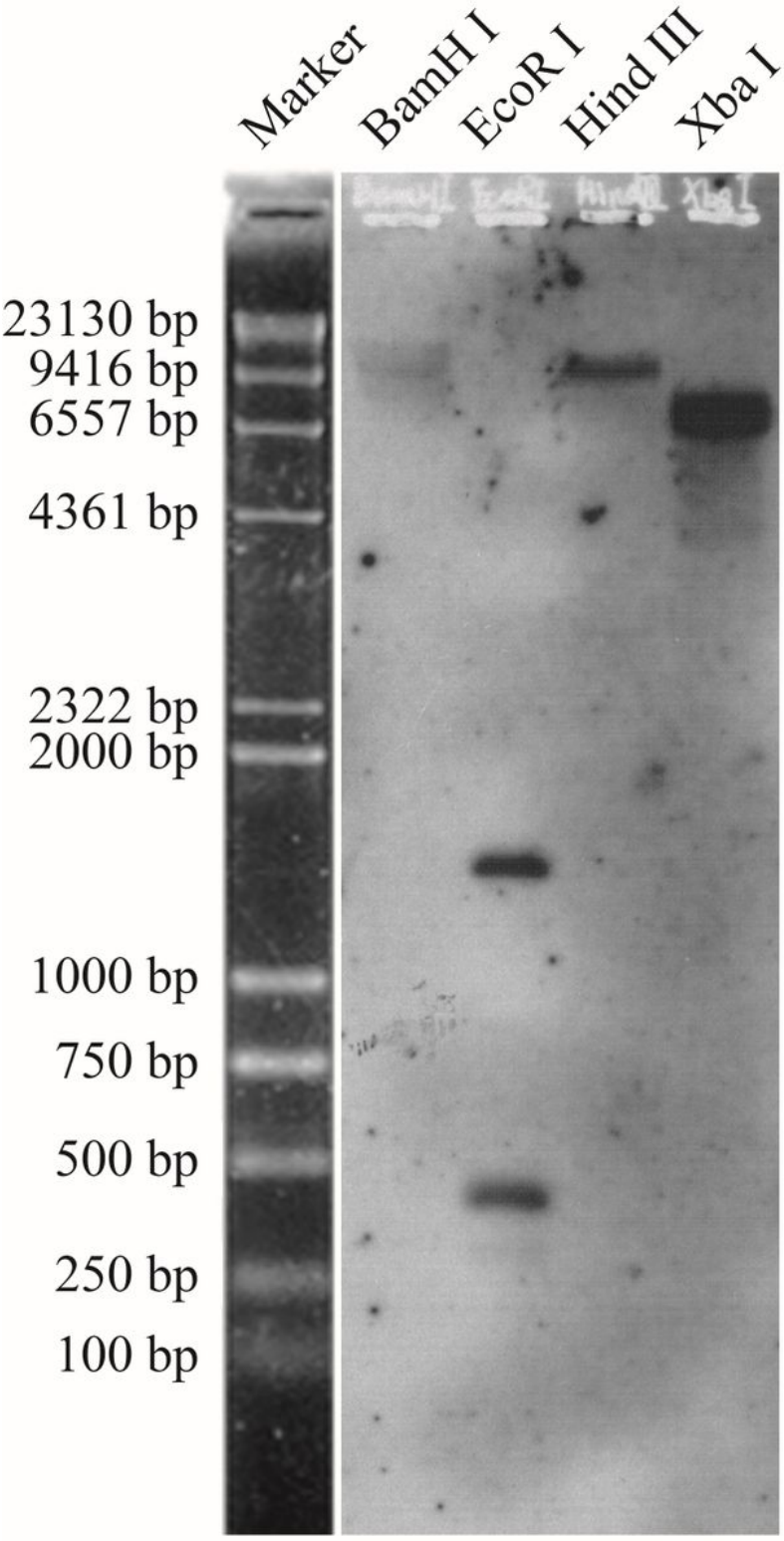

Figure 2

Southern blot of LoMYB20s in lily genome. Endonucleases BamHI, EcoRI, HindIII, and XbaI were used for genomic DNA digestion. 10 μg of each digested product was separated by electrophoresis and then transferred onto a positively charged nylon membrane. The membrane was hybridized with a DIG-labeled LoMYB20s probe.

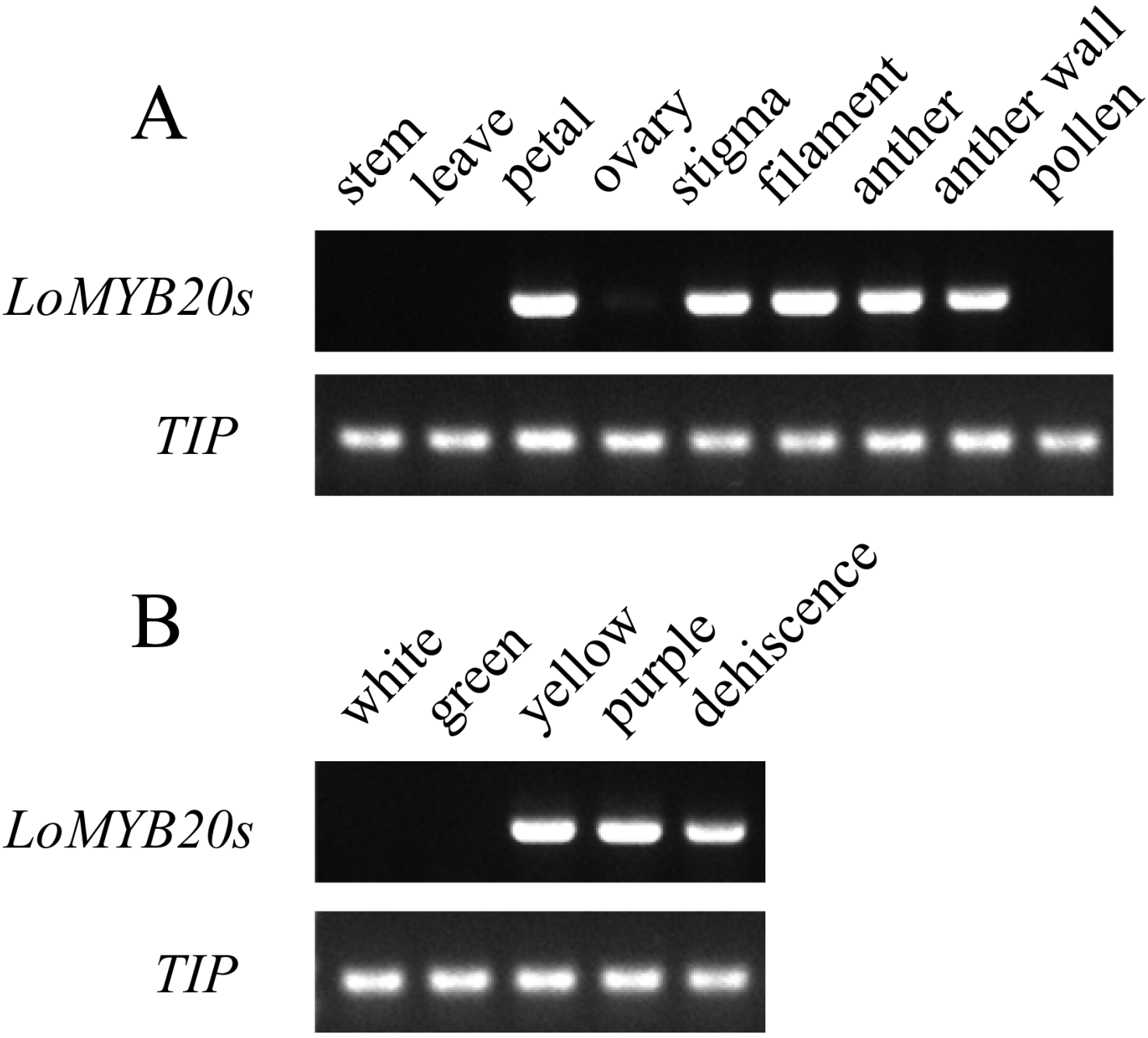

Figure 3

Expression analysis of LoMYB20s transcripts. a Expression levels of LoMYB20s in different organs of lily cut flower. b Expression levels of LoMYB20s at different stages of anther development. The stages of anther development were distinguished by anther color, which ranged from white (early developmental stage), through green (middle developmental stage), to yellow (late developmental stage), and finally to purple (fully mature stage), followed by dehiscence.

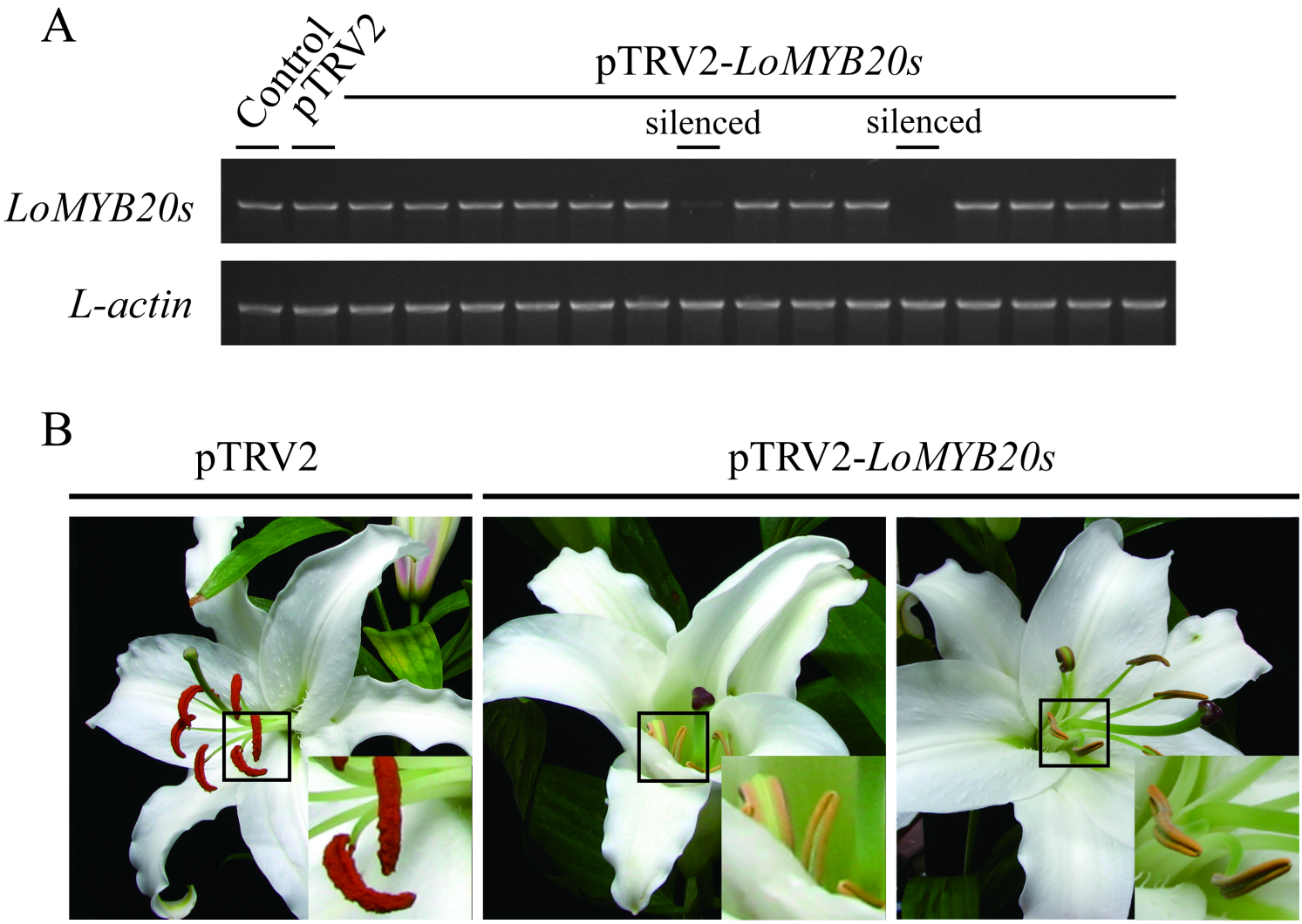

Figure 4

Silencing of LoMYB20s in lily anthers by VIGS. a RT-PCR analysis of LoMYB20s in lily anthers infected with Agrobacterium tumefaciens carrying pTRV2-LoMYB20s. Control, anthers from non-infiltrated flowers; pTRV2, anthers from flowers infiltrated with Agrobacterium containing the TRV empty vector (pTRV1 + pTRV2); pTRV2-LoMYB20s, anthers from flowers infiltrated with Agrobacterium containing TRV-LoMYB20s (pTRV1 + pTRV2-LoMYB). b Morphologies of flowers and anthers in which the expression of LoMYB20s had been silenced. The photographs were taken ~15 days after infiltration. The picture in the lower right corner of each photograph is a 2.5-times amplification of the region indicated by the black box.

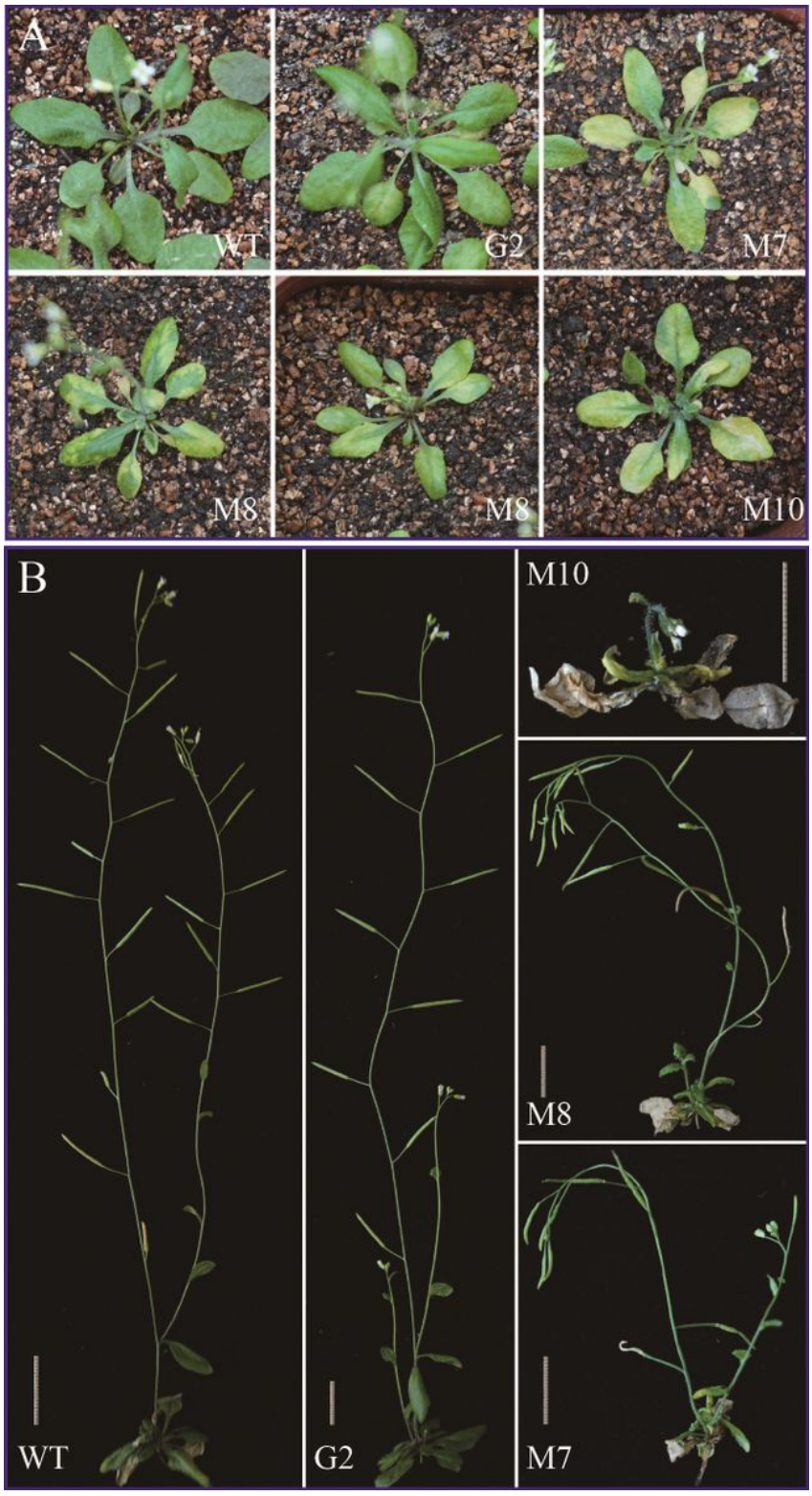

Figure 5

The altered phenotypes of transgenic Arabidopsis DEX::LoMYB20s plant lines. The plants of WT (wild-type), G2 (empty vector), and M7, M8, and M10 lines (DEX::LoMYB20s) were sprayed with 30 μM DEX (+DEX) or ddH2O (-DEX with 1‰ aqueous alcohol) once per day. a The prematurely senescent leaves of the DEX::LoMYB20s lines. The pictures were taken 5 d after the first DEX treatment. b The inhibited

development of the inflorescence stems of the DEX::LoMYB20s lines. The pictures were taken 20 d after the first DEX treatment.The bar in each picture represents 2 cm.

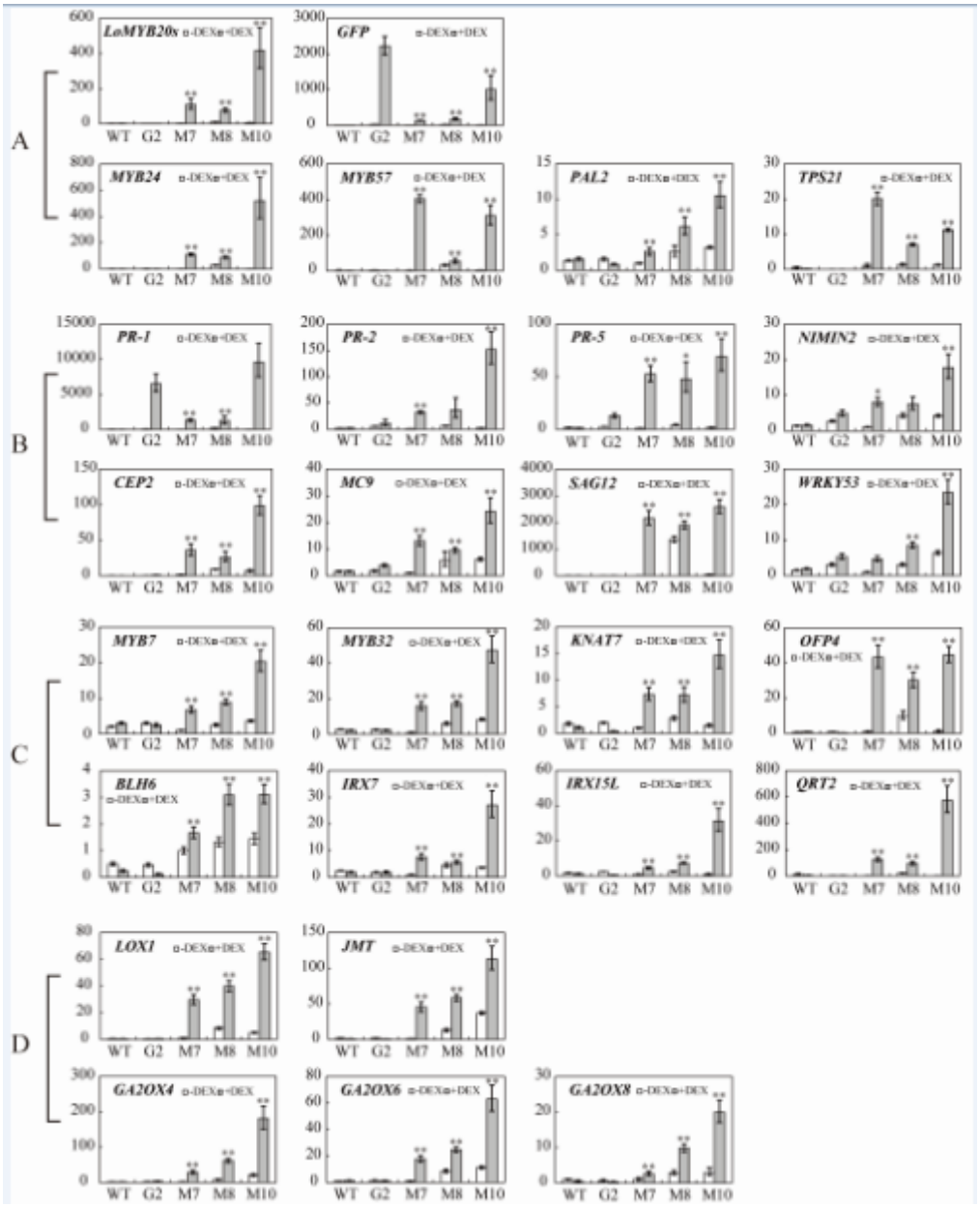

Figure 6

qRT-PCR analysis of the expression of LoMYB20s-regulated genes. WT: wild-type; G2: pTA7001G empty vector transgenic line; M7, M8, and M10: DEX::LoMYB20s transgenic lines. Plants were sprayed with 30 μM DEX (+DEX) or ddH2O (-DEX with 1‰ aqueous alcohol) once per day, and the samples were collected 4 h after DEX treatment on the fifth day. Asterisk above an error bar indicates a significant difference (Student's t-test, n=3) compared with the G2+DEX plants. * indicates a difference of $P < 0.05$, and ** indicates a difference of $P < 0.01$. a LoMYB20s and its Arabidopsis homologs and downstream genes; b plant PCD-related genes; c SWB regulator and effector genes; d JA and GA biosynthesis and signal genes.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1n.pdf