MYB108 Acts Together with MYB24 to Regulate Jasmonate-Mediated Stamen Maturation in Arabidopsis[1[OA]]

Ajin Mandaokar[2] and John Browse*

Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164–6340

In Arabidopsis (*Arabidopsis thaliana*), jasmonate is a key signal required for stamen and pollen maturation and thus for male fertility. Using transcriptional profiling, we have previously identified a set of 13 transcription factors that are proposed to be involved in controlling jasmonate responses in stamens. This finding suggests that a transcriptional cascade regulates the many developmental and biochemical pathways required to ensure fertility; however, the organization of this cascade is currently not understood. Here, we provide a genetic characterization of the role of MYB108 and map its relationship to MYB21 and MYB24, two other transcription factors involved in the jasmonate response in Arabidopsis stamens. Transcriptional profiling and analysis of plants expressing a MYB108:GUS fusion protein demonstrated that MYB108 expression is largely confined to sporophytic tissues of the stamen. Three allelic *myb108* mutants exhibited reduced male fertility that was associated with delayed anther dehiscence, reduced pollen viability, and decreased fecundity relative to wild type. These phenotypes were all found to be exacerbated in *myb108 myb24* double mutants, which also had shorter stamen filaments. Measurements of *MYB108* transcript levels in wild-type and mutant flowers showed that expression of this gene is strongly dependent on MYB21. Taken together, our results indicate that MYB108 and MYB24 have overlapping functions and act downstream of MYB21 in a transcriptional cascade that mediates stamen and pollen maturation in response to jasmonate.

Flower development in angiosperms is arguably the most complex and highly regulated developmental process in the plant kingdom. It entails individual programs for the development of male and female organs (stamens and carpels, respectively) and for the haploid gametophytes that these organs produce and nurture (Goldberg et al., 1993; Ma, 2005). The accessory floral organs (sepals, petals, and pedicel) have their own developmental programs (Coen and Meyerowitz, 1991; Yanofsky, 1995). Perhaps not surprisingly, most of the major plant hormones have been proposed to have roles in floral development, including auxin (Nagpal et al., 2005; Aloni et al., 2006), gibberellins (Weiss, 2000; Yu et al., 2004), cytokinin (Lindsay et al., 2006), abscisic acid (Wang et al., 1999), ethylene (Rieu et al., 2003), and jasmonate (Feys et al., 1994; McConn and Browse, 1996). Determining the mechanisms through which these hormones act is key to understanding flower development and plant fertility.

In self-pollinating plants, such as Arabidopsis (*Arabidopsis thaliana*), the development and maturation of floral organs requires a high level of coordination. Anther dehiscence and the release of mature pollen must occur when the stigmas (and ovules) are receptive. In addition, elongation of stamen filaments must occur shortly before anther dehiscence to correctly position the anther immediately above the stigmatic surface for efficient pollen transfer and subsequent fertilization (Stintzi and Browse, 2000; Ishiguro et al., 2001). Mutations that disrupt steps in pollen maturation, filament elongation, or anther dehiscence often reduce male fertility or make plants completely male-sterile. Forward and reverse genetic approaches have identified many genes required for full male fertility, and functional analysis of the proteins that these genes encode has provided information about the biochemical and developmental pathways involved in stamen and pollen development (Ma, 2005).

Jasmonic acid (JA) and its chemical derivatives (collectively known as jasmonates) are oxylipin signaling molecules that are key regulators of both stress responses and development in plants. In Arabidopsis, jasmonate signaling is required for fertility, and mutants that are deficient in jasmonate synthesis (McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000; Ishiguro et al., 2001; Park et al., 2002) or in jasmonate signaling (Feys et al., 1994; Chini et al., 2007; Thines et al., 2007) are male-sterile. These mutants show a characteristic set of developmental defects. Floral organs initially develop normally within the flower bud up to stage 12 of flower devel-

[1] This work was supported by the U.S. Department of Energy (grant no. DE–FG02–99ER20323) and by the Agricultural Research Center at Washington State University.

[2] Present address: DuPont Knowledge Centre, ICICI Knowledge Park, Turkapally, Shamirpeth Mandal, Hyderabad, India 500 078.

* Corresponding author; e-mail jab@wsu.edu.

The author responsible for the distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: John Browse (jab@wsu.edu).

[OA] Open access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.108.132597

opment (Smyth et al., 1990), but the anther filaments do not elongate sufficiently to place the anther locules above the stigma at the time of flower opening. The anther locules do not dehisce at the time of flower opening (although limited dehiscence does occur later) so that pollen is not released. Furthermore, although pollen on mutant plants develops to the trinucleate stage, it is predominantly inviable, with <4% of mutant pollen grains germinating (McConn and Browse, 1996; Stintzi and Browse, 2000). In jasmonate-synthesis mutants such as *fad3 fad7 fad8*, *aos*, and *opr3* (but not in the jasmonate-perception mutants), application of exogenous jasmonate is able to restore fertility (McConn and Browse, 1996; Stintzi and Browse, 2000; Park et al., 2002). Restoration is very stage specific. When flower clusters were treated with a single application of jasmonate, only flower buds that were in the middle of stage 12 at the time of application produced seed; buds at earlier or later stages of development remained sterile (Stintzi and Browse, 2000). These observations suggest that jasmonate triggers developmental programs required for stamen and pollen maturation.

In the flowers, as in other parts of the plant, the action of hormones such as jasmonate is mediated by transcription factors that initiate and coordinate gene expression programs. Genetic screens have identified many transcription factors whose expression is required for flower development and function (Yanofsky, 1995; Jack, 2004; Ma, 2005). However, for most processes, including jasmonate-regulated stamen and pollen maturation, it is likely that only a subset of the relevant transcription factors have been characterized, and as a result, we have no overall understanding of the transcriptional regulation that underlies the developmental and biochemical programs involved.

One problem that has limited genetic analysis of transcription factor action is the functional overlap that is often observed between two or more transcription factors (Meissner et al., 1999; Pontier et al., 2001; Zhang, 2003). Identifying (candidate) transcription factor partners usually requires some knowledge of which transcription factors are induced (and therefore putatively active) in response to a hormone or other signal. We took advantage of our ability to trigger stamen and pollen maturation in *opr3* plants with jasmonate to perform transcriptional profiling of gene expression in stamens in response to jasmonate treatment (Mandaokar et al., 2006). During the 22 h of the experiment, a total of 146 genes encoding known or putative transcription factors were induced or repressed in jasmonate-treated stamens compared with controls. A series of considerations and analyses refined this extended list to a set of 13 genes that we conclude are most likely to be key regulators of the stamen and pollen maturation processes triggered by jasmonate. These genes are strongly induced in *opr3* stamens following jasmonate treatment and are highly expressed in wild-type stamens relative to stamens from untreated (sterile) *opr3* flowers. Most of the 13 transcription factors identified appear to act specifically in flower development. For example, expression of the *MYB21* gene in stamens was induced nearly 400-fold by jasmonate and *myb21-1* null mutants showed similar symptoms of male sterility as described for *opr3* and several other jasmonate mutants (Mandaokar et al., 2006). Interestingly, null mutations in the *MYB24* gene, which is closely related to *MYB21*, do not lead to any detectable reduction in fertility or other difference from wild type, although in the *myb21-1* background *myb24* mutations did exacerbate the effects on flower development and fertility (Mandaokar et al., 2006). Mutant analyses of two additional genes from our list have independently identified *MYB32* and *IAA19* as having roles in stamen or pollen development, because mutations in either of these genes results in reduced male fertility (Preston et al., 2004; Tatematsu et al., 2004).

Among the transcription factors identified in our profiling experiments, MYB108 (At3g06490) was an additional promising candidate. *MYB108* transcript was increased more than 60-fold (relative to the zero-time control) at 22 h after jasmonate application. Treatment of flowers with 12-oxo-phytodienoic acid (OPDA), a precursor of jasmonate that does not restore fertility to *opr3* plants, did not result in a significant increase of *MYB108* transcript. Finally, transcript levels of *MYB108* in wild-type stamens were 20-fold higher than those in *opr3* stamens that were either untreated or treated with OPDA (Mandaokar et al., 2006). Here, we report that *MYB108* is a JA-inducible transcription factor gene with an important role in stamen development and male fertility in Arabidopsis. Reverse-genetic analysis demonstrates that MYB108 is required for correct timing of anther dehiscence. In combination with MYB24, it regulates three aspects of male fertility: filament elongation, anther dehiscence, and pollen viability. Results from additional genetic analysis indicate that *MYB108* expression is regulated by the upstream transcription factor, MYB21. We propose that *MYB108* and *MYB24* are two critical components of the JA-mediated transcriptional cascade that regulates final stages of stamen development and male fertility.

RESULTS

Identification of *myb108* T-DNA Insertion Mutants

Phylogenic analysis of the R_2R_3-MYB proteins in Arabidopsis places MYB108 in subgroup 20 of this transcription factor family (Stracke et al., 2001). Subgroup 20 has five other members, MYB78, MYB112, MYB2, MYB62, and MYB116 (Fig. 1A). The MYB21 and MYB24 transcription factors that have demonstrated roles in jasmonate regulation of stamen and pollen maturation (Mandaokar et al., 2006) are the two members in subgroup 19 and thus closely related to the subgroup 20 proteins.

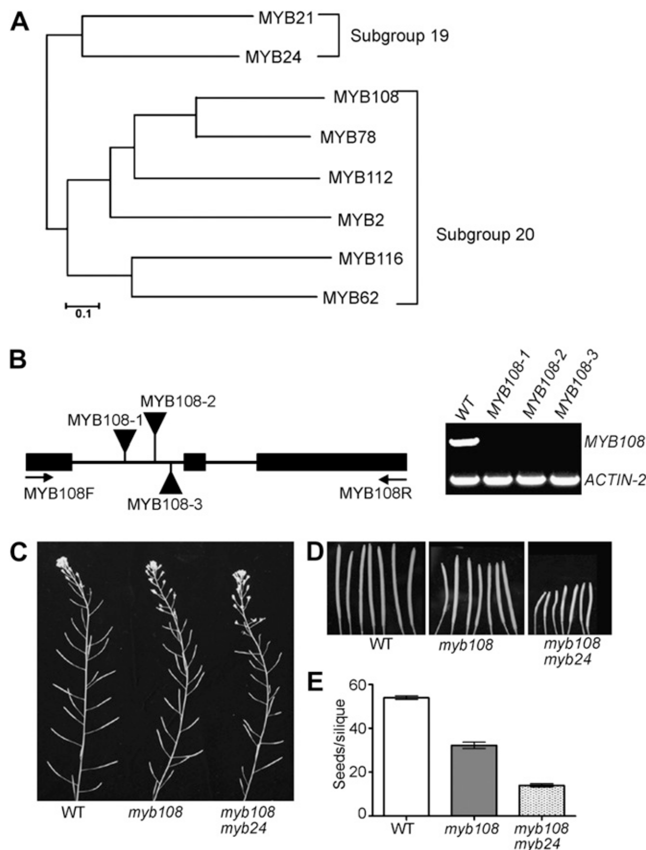


Figure 1. Phenotypic characterization of *myb108* and *myb108 myb24* mutants. A, Phylogenetic relationship of transcription factors in subgroups 19 and 20 of the R_2R_3-MYB family. Deduced amino acid sequences of eight MYB proteins were aligned and a rectangular cladogram constructed using Vector NTI software. Branch lengths are proportional to the degree of divergence, with the scale bar representing 10% change. B, Insertion mutants in *myb108*. The *myb108-1* (SALK_056061), *myb108-2* (SALK_024059), and *myb108-3* (CSHL_GT6213) alleles all contain insertions in the first intron of the gene (left). Locations of the MYB108F and MYB108R primers are shown. The right segment shows the results of RT-PCR with the *MYB108* primers (primers to *ACTIN2* as a control) on RNA from flower buds of wild type (WT) and the *myb108* mutants. C, Delayed flower senescence and reduced size of siliques on mutant plants, relative to WT, are symptoms of reduced fertility. D, Representative siliques from WT, *myb108*, and *myb108 myb24* plants. E, Seed numbers counted from mature siliques of WT, *myb108*, and *myb108 myb24*. The data are averages (± SEM) for 80 randomly selected siliques.

To examine the possible role of MYB108 in stamen development and fertility, we used a reverse genetic approach to identify homozygous T-DNA and transposon insertion mutants. We identified three insertion lines listed for *MYB108* in The Arabidopsis Information Resource database (www.arabidopsis.org): SALK_056061, SALK_024059, and CSHL_GT6213 (Martienssen, 1998; Alonso et al., 2003). These three lines were obtained from the Arabidopsis Stock Center at The Ohio State University and characterized as described under "Materials and Methods." Seed was collected from one homozygous plant of each line and designated *myb108-1* (SALK_056061), *myb108-2* (SALK_024059), and *myb108-3* (CSHL_GT6213). *myb108-1* and *myb108-2* are in the Columbia (Col-0) background, while *myb108-3* is derived from the Landsberg *erecta* (L*er*) ecotype. RNA prepared from developing flower buds of mutant plants was examined by reverse transcription (RT)-PCR using the MYB108F and MYB108R primers together with primers to the *ACTIN2* gene as a control. We could not detect the presence of any full-length transcript for *MYB108* in any of the three mutant lines (Fig. 1B), even though a strong PCR band was observed using RNA from wild-type flowers and *ACTIN2* transcript was detected in all the RNA samples.

The three T-DNA insertions are all in the first intron of the *MYB108* gene (Fig. 1B) and are predicted to preclude translation of both the R_2 and R_3 domains. Thus, it is highly unlikely that any truncated mRNA transcribed from the mutant gene would retain any biological function. T-DNA inserts in intronic sequences are sometimes spliced out of the pre-mRNA with the intron, thus allowing for some gene expression. However, our inability to detect *MYB108* transcript in the three mutant alleles indicates that each of the mutations is null.

When plants of all three mutant lines were grown side-by-side with wild-type controls, we could not detect any phenotype during vegetative growth. However, careful observation of newly opened flowers revealed a consistent phenotype for all three *myb108* alleles. Although the stamen filaments of *myb108* flowers had elongated to place the anthers level with or slightly above the stigmatic surface at the time of flower opening, anther dehiscence did not occur but instead was delayed by approximately 12 h relative to the corresponding Col-0 or L*er* wild type. This defect in anther dehiscence was associated with delayed senescence of stamens, petals, and sepals of flowers and reduced seed set for all three *myb108* alleles. Delayed senescence is often observed in male-sterile lines. The phenotype and seed set for *myb108-1* are shown in Figure 1, C to E. The delayed senescence of flower parts is reflected by the larger number of open flowers on *myb108* compared with wild type (Fig. 1C), while the smaller siliques on *myb108* (Fig. 1, C and D) indicate reduced seed set. Seed counts from 80 randomly selected siliques confirmed the reduced seed set with *myb108* siliques containing 32.2 ± 1.4 (mean ± SEM) seeds compared with 54.0 ± 0.8 for wild-type controls (Fig. 1E).

Expression Pattern of the *MYB108* Gene

Our previous results (Mandaokar et al., 2006) and data on *MYB108* expression from the Arabidopsis Gene Expression Atlas (Schmid et al., 2005) indicates that this gene is flower specific and is predominantly induced in stamens at stages 12 and 15 of flower development. To confirm and extend these results, we

first used quantitative, real-time PCR (qPCR) to reassess the induction of *MYB108* transcript in stamens following treatment of *opr3* flower buds with jasmonate or OPDA. In this experiment, *MYB108* was induced approximately 100-fold at 22 h after jasmonate treatment, but there was no response to OPDA (Fig. 2A). To determine if *MYB108* expression is dependent on activity of the SCF^{COI1} ubiquitination complex, we used RNA gel-blot analysis to measure *MYB108* transcripts in flowers of wild-type and *coi1*-mutant flowers that had been treated with 100 μM jasmonate or with a control solution. The results show high expression of *MYB108* in wild-type flowers with or without jasmonate treatment. By contrast, the *MYB108* transcript was not detected in *coi1* flowers even after jasmonate treatment (Fig. 2B). Taken together, these results indicate that *MBY108* is induced in stamens of wild-type flowers to levels comparable to those found in *opr3* stamens treated with jasmonate and that this induction is COI1 dependent.

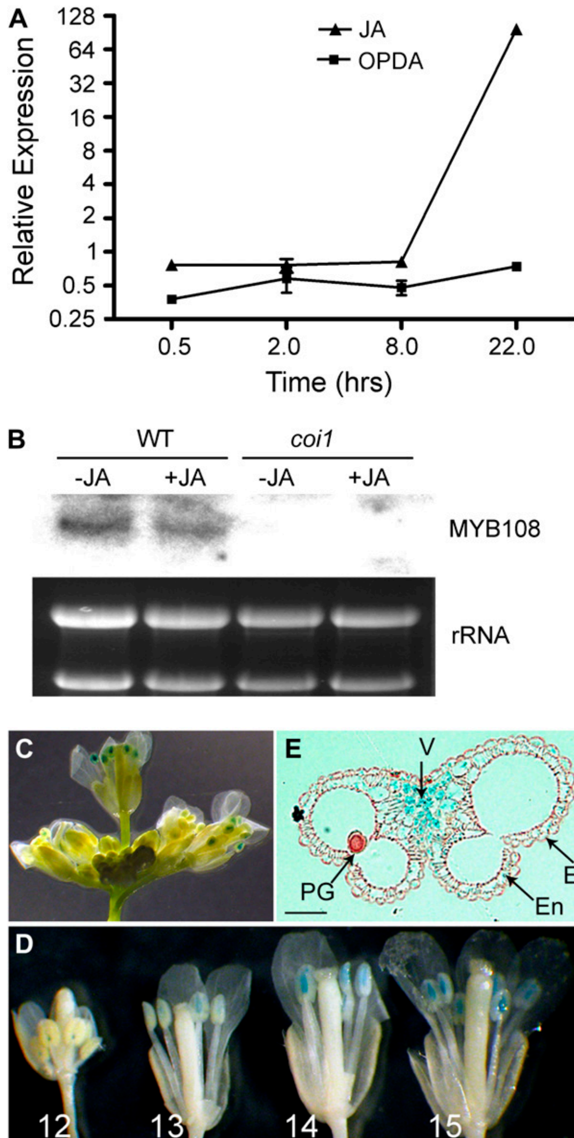


Figure 2. Expression of MYB108 is jasmonate and COI1 dependent and restricted to anthers in maturing flowers. A, *MYB108* transcript levels in *opr3* stamens following treatment with jasmonate (JA) or OPDA. qRT-PCR was carried out using total RNA as template and *ACTIN2* as a normalizing control. Data are means ± SEM of three replicates. B, RNA gel-blot analysis of *MYB108* in wild-type (WT) and *coi1* flower buds treated with a control solution (−JA) or a solution containing 100 μM jasmonate (+JA) and sampled 24 h later. Staining of rRNA bands with ethidium bromide (bottom) indicates equal loading of RNA samples. C to E, GUS expression in the MYB108 gene-trap line. C, Overview of the apical bud cluster. D, Flowers from stages 12 to 15. E, Cross section of an anther from stage 13. Abbreviations: E, epidermis; En, endodermis; PG, pollen grain; V, vascular bundle. Bar = 50 μm.

The *myb108-3* allele (derived from CSHL_GT6213) was generated in the L*er* genotype by transformation with a gene-trap construct based on the maize *Dissociation* (*Ds*) transposon (Martienssen, 1998). The construct is designed to generate in-frame protein-GUS fusions when the transposon inserts into an intron. Thus, heterozygous *myb108-3* plants are phenotypically wild type and allow analysis of the tissue specificity of *MYB108* expression. We performed histochemical staining for GUS activity at various stages of development from germination to maturity. GUS staining was observed very specifically in the stamens of flowers, particularly in the anthers (Fig. 2C). The intensity of staining increased progressively during flower maturation and opening (Fig. 2D), with maximum expression at stage 15 of flower development. Cross sections of anthers showed GUS staining throughout the maternal tissues with strongest expression in the vascular and connective tissue where the anther attaches to the filament (Fig. 2E). No GUS staining was observed in any other flower or vegetative organs, except for limited staining in the axial region of seedling leaves (data not shown). The results from these GUS reporter plants are consistent with data on the expression of *MYB108* transcript, described above, and indicate that MYB108 is likely specifically involved in regulation of late stages in stamen development.

myb78 and *myb112* Mutations Do Not Enhance the *myb108* Phenotype

The reduced fertility of *myb108* mutants, the strong induction of *MYB108* expression in response to jasmonate treatment of *opr3* stamens, and the anther-specific expression of the *myb108-3:GUS* reporter all indicate that the MYB108 transcription factor contributes to the regulation of stamen maturation and male fertility in response to jasmonate signaling. However, the reduction in fertility observed in *myb108* mutants is much less severe than in *myb21*, *opr3*, and other jasmonate mutants (Mandaokar et al., 2006). We therefore considered other MYB transcription factors that might have overlapping function(s) with MYB108.

All the proteins in subgroup 20 of the R_2R_3-MYB family contain a diagnostic WXPRL sequence in addi-

tion to the R_2 and R_3 domains and show higher sequence similarity to each other than to MYB proteins in other subgroups of the R_2R_3-MYB family (Stracke et al., 2001). The MYB2 protein has been shown to be a transcriptional activator of the dehydration-responsive gene *rd22* (Abe et al., 2003) and to act in abscisic acid signaling. It is therefore unlikely to have a function that overlaps with the function of MYB108. MYB116 and MYB62 are the subgroup 20 proteins most distantly related to MYB108 with sequence identities of 47% and 43%, respectively, and the corresponding genes are not strongly expressed in stamens of developing flower buds (data not shown). We considered these unlikely candidates for a shared role with MYB108 and did not investigate them further. MYB78 (At5g49620) is the closest homolog of MYB108 (Fig. 1A) with 66% sequence identity, although data from the Arabidopsis Gene Expression Atlas (Schmid et al., 2005) indicate that this gene is expressed at only very low levels throughout plant development. On the other hand, MYB112 (At1g48000) shows 62% amino acid identity to MYB108 and is expressed in stamens and other organs of stage 15 flowers.

We obtained T-DNA insertion mutants of *MYB78* (SALK_085369) and *MYB112* (SAIL_562_F10) and identified homozygous lines by PCR genotyping using the same strategy described above for *myb108* (Fig. 3, A and B). RT-PCR analysis using RNA from wild type, *myb78*, and *myb112* showed that each mutant lacked a full-length transcript of the corresponding gene (Fig. 3, A and B). Both the *myb78* and *myb112* knockout mutants were normal in growth, flower development, and seed set. We crossed each mutant with *myb108* plants and derived homozygous *myb108 myb78* and *myb108 myb112* double mutant lines. When these mutants were grown together with *myb108*, we did not detect differences in anther dehiscence or seed set between the double mutants and the parental *myb108* plants. These results indicate that it is unlikely that MYB78 or MYB112 has a function similar to MYB108.

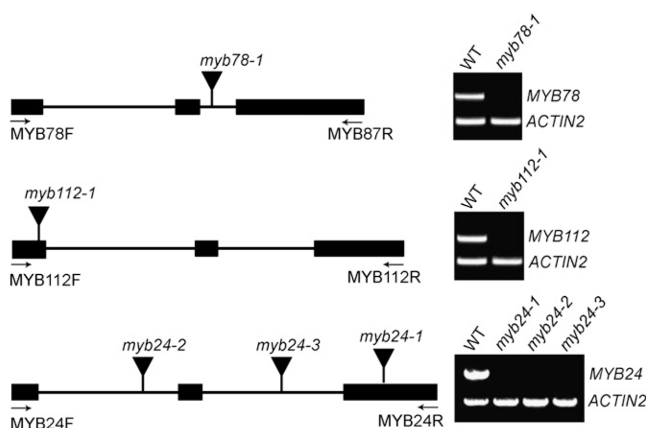


Figure 3. Insertion mutants in *myb78*, *myb112*, and *myb24*. For each gene, the left segment shows the structure of the gene locus, indicating the T-DNA insertion sites for each allele and the location of gene-specific primers used for molecular characterization of the mutants. The right segment shows the results of RT-PCR with the gene-specific primers (primers to *ACTIN2* as a control) on RNA from flower buds of wild-type (WT) and mutant plants.

Mutations in *myb24* Enhance Sterility of *myb108* Plants

We have not investigated the MYB transcription factors from subgroup 20 of the R_2R_3 family any further, because the low expression of these genes in stamens of wild-type flowers and especially the lack of significant induction by jasmonate in our transcriptional profiling experiment suggest that they are not involved in regulation of jasmonate responses. Instead, we next considered the possibility that the regulatory function of MYB24 overlaps with that of MYB108. The genes encoding these two transcription factors are very highly induced by jasmonate treatment of *opr3* flowers (Fig. 2A; Mandaokar et al., 2006). Furthermore, the *myb24-1* null mutant is fully fertile and indistinguishable from wild type in growth and development (Mandaokar et al., 2006), indicating that MYB24 function may be fulfilled by one or more other transcription factors in *myb24* mutants.

Four insertion alleles of *myb24* were identified in our previous work, but only *myb24-1* (isolated through the Arabidopsis Knockout Facility at the University of Wisconsin) was characterized in detail. We therefore prepared RNA samples from flower buds of *myb24-1*, *myb24-2* (homozygous derivative from SALK_030452), and *myb24-3* (homozygous derivative from SAIL_284_F01) and used them as templates for RT-PCR using primers MYB24F and MYB24R designed to the 5′ and 3′ ends, respectively, of the *MYB24* coding sequence. As shown in Figure 3C, these primers detected full-length *MYB24* transcript in RNA from wild-type controls but not in any of the three *myb24* alleles. We conclude that each of these mutant lines contains a null mutation in the *MYB24* gene.

A cross between *myb108-1* and *myb24-1* produced F_1 plants that were allowed to produce selfed F_2 seed. A total of 62 F_2 plants were analyzed and four of these showed greatly reduced seed set compared with *myb108* and wild type. The 62 plants were genotyped by PCR (using primer combinations that distinguish wild-type and mutant alleles of the two genes) and only the four plants with very low seed set were homozygous *myb108 myb24* double mutants. The 58:4 segregation in this experiment is a good fit to the 15:1 ratio ($\chi^2 = 2.7$; $P > 0.1$) expected for two recessive loci. We also made double mutants between *myb108-2* and *myb24-2* and between *myb108-2* and *myb24-3*. In both these cases, the double mutant segregants in the F2 population had greatly reduced seed set relative to the *myb108* parental allele. The consistency of this result indicates that the reduced fertility of the *myb108 myb24* mutants is the result of mutations in only these two genes and is not affected by any other mutations that might potentially be present in the individual *myb108*

or *myb24* lines. The detailed characterization reported below uses results from the *myb108-2 myb24-2* mutant, and consistent results were obtained from the two additional lines.

The cumulative effect of the *myb108* and *myb24* mutations was evident in the phenotype of flowering shoots and sample siliques from *myb108 myb24* plants (Fig. 1, C and D). Seed counts from 80 randomly selected siliques of *myb108 myb24* indicated that seed production was only 13.1 ± 0.6 (mean ± SEM) seeds per silique compared with 32.2 ± 1.4 for the *myb108* single mutant (Fig. 1E). When *myb108 myb24* flowers were pollinated with wild-type pollen, seed set was normal. However, when pollen from double-mutant plants was used to pollinate emasculated, wild-type flowers, seed set was consistently very low (Table I). These results indicate the *myb108 myb24* plants are female-fertile but male-sterile and are consistent with the notion that the mutations substantially block jasmonate responses in stamens of double-mutant plants.

We treated *myb108*, *myb108 myb24*, and *opr3* flower buds with jasmonate by dipping the apical bud clusters in a solution of 100 μM methyl jasmonate. Following this treatment, *opr3* plants produced three to six elongated siliques filled with seed, as expected for this mutant (Mandaokar et al., 2006). By contrast, jasmonate treatment did not enhance seed set on *myb108* or *myb108 myb24* plants. This lack of response is consistent with the mutations in *MYB108* and *MYB24* blocking jasmonate actions downstream of the initial responses mediated by SCF^{COI1} and the JAZ proteins (Chini et al., 2007; Thines et al., 2007).

Delayed Anther Dehiscence in *myb108 myb24* Double Mutants

Because we had observed delayed anther dehiscence in the *myb108* mutant, we speculated that the *myb24* mutations might further delay dehiscence and set out to investigate this process in detail. As in other jasmonate mutants (Feys et al., 1994; McConn and Browse, 1996; Sanders et al., 2000; Ishiguro et al., 2001), the timing of flower development and anthesis in *myb108* and *myb108 myb24* mutants is indistinguishable from wild type. Morphological changes are limited to the stamens and to delayed dehiscence of the petals and sepals, which is a result of reduced fertilization (Butenko et al., 2003). We grew wild-type, *myb108*, and *myb108 myb24* plants together and measured the timing of anther dehiscence in the flowers.

Table I. *Reciprocal crosses between myb108 myb24 and wild type show reduced seed set from myb108 myb24 pollen*

Data are means ± SEM for nine siliques of each cross.

Genotypes	No. of Seeds/Silique	
	Wild-Type Female	Wild-Type Male
myb108-2 myb24-2	13.0 ± 2.4	56 ± 2.0
myb108-2 myb24-3	12.1 ± 2.4	55 ± 2.0

The most mature unopened flower bud in each flower cluster was identified (flower 0), and the set of six open flowers below it was treated as a developmental series (Fig. 4A). The unopened flower buds contained no dehisced anthers, but the earliest open flowers on wild-type plants consistently had six dehisced anthers (Fig. 4B). By contrast, in *myb108*, anther dehiscence did not occur in the first and second open flowers. A more pronounced delay was observed in the *myb108 myb24* double mutants, with no anther dehiscence occurring until flower 4 and with full dehiscence in flower 5, which corresponds to a stage when pistil elongation has already begun.

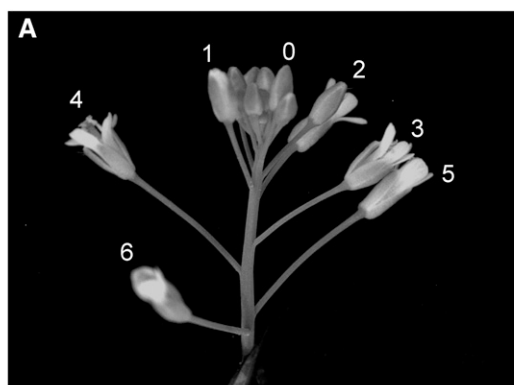
Reduced Filament Elongation of *myb108 myb24* Stamens

A second important aspect of the *myb108 myb24* phenotype was discovered through measuring stamen lengths. In flowers 1 through 4 in the series, stamen length for *myb108 myb24* was slightly but significantly less than for wild type, with *myb108* indistinguishable from wild type (Fig. 5A). Because stamen length is particularly important to position anthers for releasing the pollen onto the stigmatic surface, we also measured carpel lengths and calculated the stamen:carpel length ratio. The results in Figure 5B show that in wild type, this is greater than one in all the open flowers, while the ratio for *myb108 myb24* is consistently less than one.

The combined effects of delayed anther dehiscence and reduced stamen elongation mean that pollen in *myb108 myb24* flowers is inefficiently transferred to the stigmatic surface and is often deposited on the side of the style (Fig. 5C). Microscopic examination of *myb108 myb24* flowers typically revealed 20 to 50 (occasionally up to 100) pollen grains on the stigmatic surface compared with several hundred pollen grains on the stigmas of wild-type flowers. Taken together, these results show that stamens on the double mutant are inefficient at transferring pollen to the stigma.

Pollen from *myb108 myb24* Mutants Has Reduced Viability and Germination

The poor seed set achieved using *myb108 myb24* as a pollen donor (Table I) implies that *myb108 myb24* pollen is defective. To investigate this more closely, we examined pollen viability using double staining with fluorescein diacetate and propidium iodide. Fluoroscein diacetate is taken up by living cells and converted to fluoroscein, which emits blue-green light under UV irradiation (Heslop-Harrison and Heslop-Harrison, 1970). Propidium iodide is excluded from living cells but labels dead cells with red-orange fluorescence under UV irradiation (Regan and Moffatt, 1990). The results in Figure 6 show the greatly reduced viability of *myb108 myb24* pollen relative to pollen from either wild-type or *myb108* flowers. Counts of fluoroscein-stained pollen grains from 10 sample microscope fields in each of three separate experiments



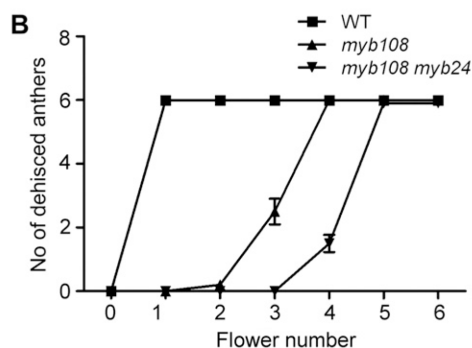


Figure 4. Delayed anther dehiscence in *myb108* and *myb108 myb24* mutants. A, Flower cluster showing the developmental series of flowers used to investigate the time course of anther dehiscence. B, The number of dehiscent anthers observed for wild type (WT), *myb108*, and *myb108 myb24* flowers at each of the stages shown in A. Data are means ± SEM for 10 flower clusters of each line.

indicated that viability of *myb108 myb24* pollen by the criterion of this vital stain was consistently 20% to 25% while wild-type pollen was ≥90% viable.

To further test pollen viability and function, we measured pollen germination on artificial media (Pickert, 1988). Pollen from the mutants was transferred to the agar media either from newly dehiscent anthers (flowers 4 and 5 in Fig. 4) or by cutting open anthers from full open flowers (flowers 2 and 3). In either case, germination of *myb108 myb24* was considerably lower than germination of wild-type or *myb108* pollen (Fig. 6B). For wild type, counts from 10 samples (>1,000 pollen grains in total) showed an average of 80% germination. By contrast, <10% germinated pollen grains were observed in 20 samples (>1,000 sample grains) from *myb108 myb24* stamens.

MYB108 Expression Is Dependent on MYB21

Available evidence indicates that MYB21 is a key regulator of the transcriptional cascade that brings about jasmonate-dependent maturation of stamens and pollen in Arabidopsis, and *myb21* mutant plants have severely reduced fertility (Mandaokar et al., 2006). Following jasmonate treatment of *opr3* stamens, the *MYB21* gene is induced earlier than either *MYB24* or *MYB108*, suggesting that the MYB21 transcription factor may control the expression of one or both of these genes.

As part of an investigation of MYB21 function, we conducted transcriptional profiling of genes expressed in stamens of *myb21* mutant plants after treatment with 100 μM jasmonate. Comparison of gene expression in *myb21* with expression in wild-type, *opr3* plants treated with jasmonate, and *opr3* plants untreated or treated with the inactive precursor, OPDA, will allow us to evaluate the extent to which MYB21 controls the jasmonate response in stamens. Using the data from Affymetrix arrays as a digital northern (Toufighi et al.,

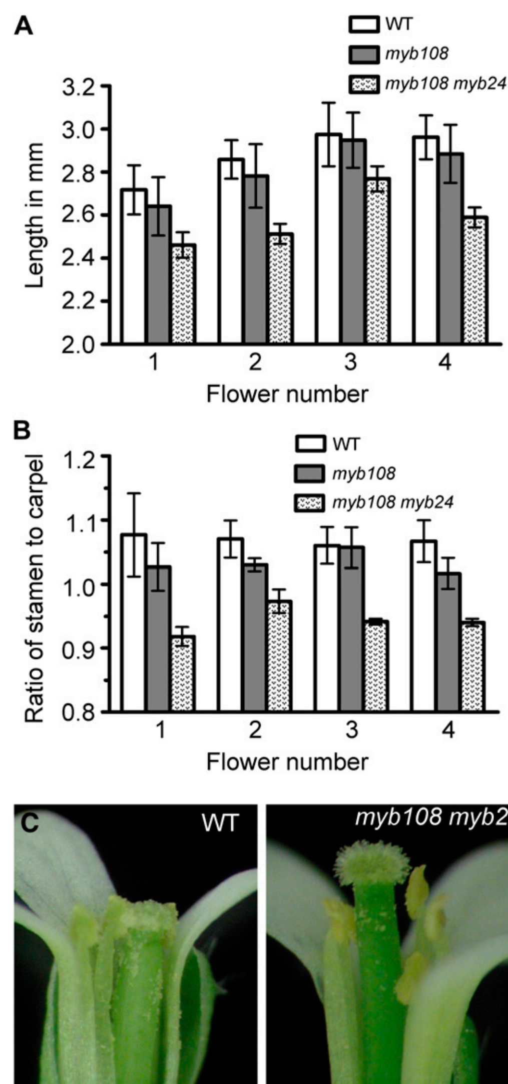


Figure 5. Stamen height is reduced in the *myb108 myb24* mutant. A, Average length of filament plus anther measured for 12 long stamens from wild-type (WT), *myb108*, and *myb108 myb24* plants at stages 1 to 4 shown in Figure 4A. Data are means ± SEM. B, The ratio of stamen length to carpel length in WT, *myb108*, and *myb108 myb24* flowers. C, Deposition of pollen on the carpels of WT and *myb108 myb24* flowers.

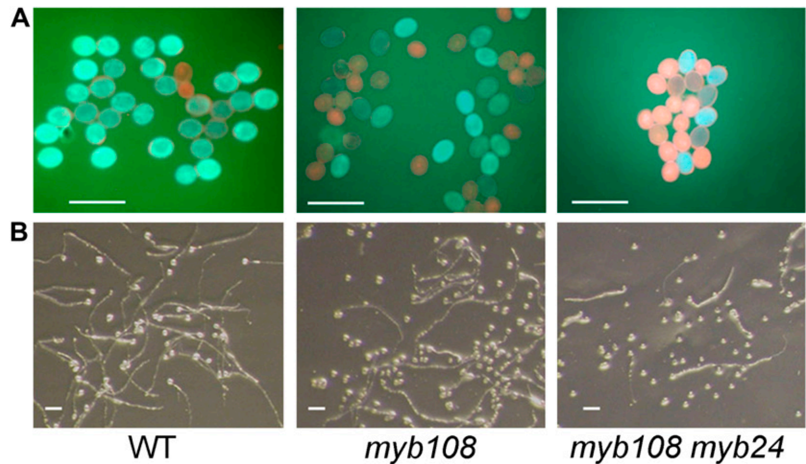


Figure 6. Reduced pollen viability and germination in *myb108* and *myb108 myb24* mutants. A, Pollen from wild-type (WT) and mutant lines was stained with fluorescein diacetate and propidium iodide. This protocol stains viable pollen blue-green and inviable pollen red-brown. B, Pollen harvested from mature open flowers was incubated on germination medium for 10 h.

2005), it is clear that both *MYB108* and *MYB24* are highly expressed in wild-type stamens, approximately 700 expression units when the arrays are normalized to a median of 125 (Mandaokar et al., 2006; Fig. 7A). However, in stamens from *myb21*, *MYB108* expression is only 112, indicating that expression of this gene is very dependent on the MYB21 transcription factor. Expression of *MYB24* is also decreased in the *myb21* mutant but to a much smaller extent, indicating that *MYB24* expression is substantially independent of MYB21. Comparisons with untreated *opr3* stamens indicates that *MYB24* is induced 140-fold in the *myb21* mutant background, while *MYB108* is induced <4-fold.

We carried out a second investigation of *MYB108* expression using qPCR to measure *MBY108* transcript levels in stamens of wild-type and mutant plants (Fig. 7B). The results confirm that *MYB108* expression is greatly reduced in the *myb21* mutant relative to wild type. *MYB108* expression is decreased only to a small extent by the *myb24* mutation, but, in the *myb21 myb24* double mutant and in the *opr3*, mutant *MYB108* transcripts are lower than in *myb21* and only 10% to 15% of those in wild type. Taken together, these results indicate that *MYB24* induction by jasmonate is largely independent of MYB21 but that MYB21 and MYB24 both contribute to induction of the *MYB108* gene.

DISCUSSION

The role of jasmonate in plant reproduction has been established through the identification and characterization of mutants deficient in jasmonate synthesis or perception (Feys et al., 1994; McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000; Ishiguro et al., 2001; Park et al., 2002; von Mallek et al., 2002). Our previous transcript profiling experiments showed that jasmonate initiates a transcriptional cascade in Arabidopsis stamens that is required for stamen maturation and male fertility (Mandaokar et al., 2003, 2006). Using reverse genetics, we have previously established a role for two early transcription factors MYB21 and MYB24 in this cascade. Mutation of *MYB21* severely reduces fertility in Arabidopsis in that anther filaments are short, anthers do not dehiscence, and pollen viability is also affected to a large extent. *myb24* mutants are phenotypically wild

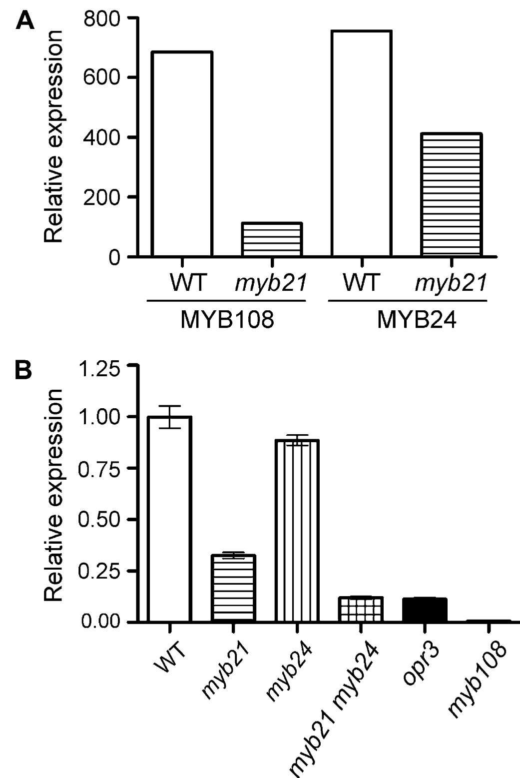


Figure 7. Regulation of *MYB108* and *MYB24* expression by MYB21. A, Expression of *MYB24* and *MYB108* transcripts in stamens of wild-type (WT) and *myb21* plants. Data are derived from transcriptional analysis of RNA samples using Affymetrix ATH1 microarrays. B, Expression of *MYB108* transcript in flowers from WT and mutant plants. qPCR was used to measure *MYB108* transcript in RNA prepared from apical flower clusters. qPCR of *ACTIN2* transcript was used as a calibrator, and *MYB108* expression plotted relative to WT = 1. Data are means ± SEM for three replicates.

type, but this mutation exacerbates the phenotype of *myb21* (Mandaokar et al., 2006). Here, we show that another MYB transcription factor, MYB108, plays an overlapping role with MYB24 to regulate late stages of stamen development and male fertility.

In our search for additional transcription factors in the jasmonate signaling cascade, we identified *MYB108* as an important candidate gene, because this gene is very strongly induced between 8 and 22 h after jasmonate treatment of *opr3* stamens, it is not detectably expressed in untreated or OPDA-treated *opr3* plants (Mandaokar et al., 2006), and it is highly expressed in wild-type stamens. Reverse-genetic analysis using T-DNA insertion mutants showed that *MYB108* is required for correct timing of anther dehiscence (Fig. 4B), a critical part of anther development, and that loss of MYB108 results in reduced fertility.

Our examination of GUS staining in the *myb108-3* gene-trap mutant revealed that MYB108 expression is tightly constrained to the maternal tissue of the stamen and to a short period of time corresponding to stages 12 to 15 of flower development. No GUS staining of pollen was observed, nor of any other floral organs. The primary phenotype of *myb108* is delayed dehiscence and in Arabidopsis dehiscence is believed to be facilitated by water movement out of the anther via the vasculature (Bonner and Dickinson, 1990; Ishiguro et al., 2001). The expression of MYB108 in the connective tissue and vascular bundles of the anther is thus consistent with its regulating genes required for water movement and anther dehydration.

Expression data from the Arabidopsis Gene Expression Atlas (Schmid et al., 2005) indicates that *MYB108* transcript is modestly induced by salt treatment and during osmotic stress. Interestingly, a specific mutation at the *MYB108* locus, *botrytis susceptible1* (*bos1*), was isolated on the basis of its susceptibility to *Botrytis cinerea*, and in wild-type plants, *MYB108/BOS1* is induced in *Botrytis*-infected tissue (Mengiste et al., 2003). As well as being susceptible to some pathogens, *bos1* plants were shown to be impaired in tolerance of water deficit, salt, and oxidative stress. However, *bos1* plants do not have reduced fertility and do not show delayed anther dehiscence (T. Mengiste, personal communication). We are currently unable to fully explain the differences in reproductive phenotype between our three *myb108* null alleles and the *bos1* mutant. However, the *bos1* mutant is unusual in that homozygous and heterozygous mutant plants express very high levels of apparently full-length *MYB108* transcript in vegetative tissue (Mengiste et al., 2003). The T-DNA in *bos1* is inserted immediately 5′ to the ATG start codon of the open reading frame and there is also a 314-bp deletion that involves the 151-bp 5′ untranslated region and 163 bp of the promoter (Mengiste et al., 2003). A reasonable explanation for the full fertility of *bos1* plants would be the production of at least some full-length, functional MYB108 protein in *bos1* anthers.

Considering the modest reduction in fertility of the *myb108* null mutants, we considered the possibility that additional transcription factors might also be involved. To test this possibility, we made double mutants with the closely related homologs of *MYB108*. Null mutants for two closely related transcription factors, *myb78* and *myb112*, showed wild-type phenotypes. When we generated double mutants with *myb108*, both *myb108 myb78* and *myb108 myb112* failed to show any exacerbation of the *myb108* phenotype, suggesting that these genes are not involved in jasmonate-regulated anther development processes. Consistent with these results, our transcript profiling experiments showed no induction for either of these genes by jasmonate (Mandaokar et al., 2006). However, the generation of double mutants between *myb108* and *myb24* demonstrated the related contributions that the MYB108 and MYB24 transcription factors make in controlling the jasmonate response in stamens, because all the *myb108 myb24* lines showed greatly reduced fertility relative to either of the parental mutants.

All three aspects of jasmonate-regulated stamen function are more strongly affected in the *myb108 myb24* double mutants than in the *myb108* parental lines. Filament elongation is not significantly affected in *myb108*, but in the double mutant it is reduced sufficiently to compromise positioning of the anthers above the stigma. A substantial delay in anther dehiscence is the most obvious aspect of the *myb108* phenotype (Fig. 4B), and the delay is longer in *myb108 myb24* flowers. Pollen germination is reduced by approximately 35% in *myb108* relative to wild type, but by 90% in the double mutant (Fig. 6). These results indicate that MYB108 and MYB24 both function to activate jasmonate-responsive genes required for correct stamen and pollen maturation.

Because Arabidopsis flowers produce pollen in considerable excess (Jürgens et al., 2001), the severely deficient pollen and stamen functions in *myb108 myb24* still allow some seed set (Fig. 1, C–E). By contrast, mutants that are completely blocked in jasmonate synthesis or jasmonate signaling, such as *opr3*, *coi1*, *myb21*, and the *JAZ1Δ3A* transgenic plants, are more completely sterile (Feys et al., 1994; Stintzi and Browse, 2000; Mandaokar et al., 2006; Thines et al., 2007). We infer that one or more additional transcription factors may partially compensate for the loss of MYB108 and MYB24 function.

We have recently proposed that the active form of jasmonate is jasmonoyl-Ile, which acts by enhancing the affinity of SCF^{COI1} ubiquitin ligase for JAZ repressor proteins (Thines et al., 2007). This suggests a model in which ubiquitination and subsequent degradation of JAZ proteins leads to activation of primary transcription factors that then induce the expression of additional transcription factors and other components of the jasmonate-induced pathways. We propose that, in flower organs, the *MYB21* and *MYB24* genes are principal targets of this signaling. The results reported

here, together with our previous results (Mandaokar et al., 2006; Thines et al., 2007), indicate a complex relationship among the transcription factor genes and proteins that mediate jasmonate responses in stamens. The data in Figure 7 indicate that MYB21 is one of the transcription factors contributing to maximum expression of the *MYB24* gene. MYB21 or MYB24 is required for expression of *MYB108*, and both of these transcription factors are necessary for maximum accumulation of *MYB108* transcript. The timing of gene induction following jasmonate treatment of *opr3* stamens is consistent with these observed dependencies. Transcriptional profiling indicates that *MYB21* is induced first, followed by *MYB24* and finally *MYB108* (Mandaokar et al., 2006; Fig. 2A). Additional MYB transcription factors are induced by jasmonate and, at least in some cases, required for full male fertility (Preston et al., 2004; Tatematsu et al., 2004; Mandaokar et al., 2006). In plants and other organisms, the MYB transcription factors have often been shown to regulate gene transcription in concert with bHLH proteins, which include the MYC transcription factors. Our transcript profiling experiments identified early- and late-induced *bHLH* genes, including those encoding bHLH31 (At1g59640), bHLH152 (Atlg22380), and bHLH145 (At5g50010; Mandaokar et al., 2006). It is possible that regulation of the expression of these genes involves interactions analogous to those described here for the *MYB* genes. Indeed, the interactions may extend between the MYB and bHLH classes and to other transcription factors involved in the jasmonate response in stamens. One explanation for such a complex interrelationship is that it allows robust regulation of stamen and pollen maturation processes that are essential for reproductive fitness.

MATERIALS AND METHODS

Plant Material and Methyl Jasmonate Treatment

The T-DNA insertion lines in the Col-0 ecotype of Arabidopsis (*Arabidopsis thaliana*) Hegn., SALK_056061, SALK_024029, SALK_030452, SALK_01722, and SAIL_284_F10, were identified using the SIGnAL database (http://signal.salk.edu/cgi-bin/tdnaexpress) and obtained from the Arabidopsis Biological Resource Center at The Ohio State University. The gene trap line CSHL_6213 was obtained from Cold Spring Harbor Laboratory and is in the genetic background of the L*er* ecotype. Wild-type and T-DNA insertion mutant seeds were sown on soil and stratified at 4°C for 2 d. The plants were gown in a controlled growth chamber in a continuous light condition (150 μmol m^{-2} s^{-1}) at 22°C. The *opr3*, *myb108*, and *myb108 myb24* mutants were treated by spraying separately with 0.1% Tween 20 (control) and 0.03% methyl jasmonate (Bedoukian Research) in 0.1% Tween 20 on inflorescences. Development of siliques was observed 1 week after the treatment.

Isolation of Homozygous T-DNA Insertion Mutants

Screening of homozygous insertion mutants was done by genomic PCR using a combination of gene specific and T-DNA left border (LB) primers. Approximately 12 plants of each line were grown on soil. Using DNA from each plant and oligonucleotide primers designed to the 5′ and 3′ ends of the *MYB108* coding sequence (MYB108F and MYB108R, respectively) and primers matching sequences in the LB of the T-DNAs or in the transposon, we confirmed the site of insertion in each line by PCR and identified plants that were homozygous for each mutation. LB primer for SALK lines, 5′-TGGTTCACGTAGTGGGCCATCG-3′; for line GT6213, 5′-ACCCGACCG-GATCGTATCGGT-3′; and for SAIL lines is LB3, 5-TAGCATCTGAATTTCA-TAACCAATCTCGATACAC-3′. Gene-specific forward and reverse primers for *MYB108*: MYB108F 5′-AATGGAGAAGGTCGCTGGAACTCT-3′ and MYB108R 5′-AACCAGCTGAGGTTACTCTGCTCT-3′; *MYB78*: MYB78 F 5′-ATGGGTGACAAAGGAAGGAGCTTAA-3′ and MYB78R, 5′-TCAGA-AGCTTCCATTGTCGTGGAC-3′; *MYB112*: MYB112F 5′-AGAAGAAGTC-GAAGAAGTCGAGAA-3′ and MYB112R 5′-CTACTGTATGAGCCACTTG-TTGAGC-3′; and *MYB24*: MYB24F 5′AGAGAAAGTAGTGGTGGGTCT-GGA3′ and MYB24R 5′-GCCAAAGATCATCGACGCTCCAAT-3′.

Promoter-GUS Fusion and Histochemical Analysis

The gene trap line from CSHL was used to study the staining pattern of GUS in various tissues. These lines were developed by transposon mutagenesis in Arabidopsis using *Ds* from maize (*Zea mays*) that has been engineered to carry a *uidA* (GUS) reporter gene and an *NPTII* kanamycin resistance gene. In gene trap construct, the reporter gene is preceded by a triple splice acceptor and by a short intron so that insertion into chromosomal introns leads to reporter gene expression via alternate splicing in each reading frame (Martienssen, 1998). Following transposition, GUS will express if it is integrated in the gene. The promoter of the genes drives the expression of GUS. Gene trap line GT6213 is obtained from the Cold Spring Harbor Laboratory. The insertion is in the first intron of the *MYB108* gene. Because the reporter carries a triple splice acceptor, its insertion will result in a translational fusion with the first exon of *MYB108*. The *MYB108* promoter then regulates the expression of this translation fusion.

The staining of GUS was done according to standard protocol (Weigel and Glazebrook, 2002). Individual flowers from plants were removed and placed in the GUS substrate buffer (50 mM sodium phosphate buffer, pH 7.0, 0.1% Triton X-100, 3 mM potassium ferricyanide, 3 mM potassium ferrocyanide, and 1 mM 5-bromo-4-chloro-3-indolyl-β-glucuronic acid). After a 5-min vacuum infiltration, samples were placed in a 37°C incubator overnight. The next day, tissue was fixed in 50% ethanol, 5% acetic acid, and 3.7% formaldehyde for overnight and dehydrated through an ethanol series. Samples were processed through ethanol to CitriSolv and then into paraffin. The paraffin-embedded samples sat at 62°C for 3 d, after which blocks were cast. Longitudinal and transverse sections of anthers (15 μm) were deparaffinized in CitriSolv, dehydrated in oil under a coverslip, and observed under a microscope.

RT-PCR and qRT-PCR

Total RNA from flowers of wild type and all the mutants was isolated using Trizol and purified using an RNeasy kit (Qiagen) according to the manufacturer's instructions. Transcript presence in wild-type, *myb108*, *myb78*, *myb112*, and *myb24* plants was measured by one-step RT-PCR (Invitrogen) with 100 ng of RNA isolated from flowers using the same set of primers used for genomic PCR. The PCR conditions were 42°C for 50 min and 30 cycles of the following: 94°C for 20 s, 58°C for 30 s, and 72°C for 2 min (the final synthesis step was extended for 5 min). Amplification products were analyzed with agarose gel electrophoresis.

For qRT PCR, total RNA was isolated from inflorescence of wild-type, *opr3*, *myb21-1*, *myb24-1*, and *myb21-1 myb24-1* plants, and first-strand cDNA synthesis was performed on 2 μg of total RNA using SuperScriptIII cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. Reaction was performed in a 20-μL volume and diluted 2-fold after the completion of reaction. qRT-PCR was performed on a Stratagene MX3000p using the SYBR Green I dye method (Stratagene). Reaction mixture (20 μL) contained 2 μL of the first-strand cDNA, 2.0 μL 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM of each sequence-specific primers, 0.6 μL of ROX dye (diluted to 1:500), and 10 μL SYBR Green mix (Invitrogen) with *Taq* polymerase. The amplification protocol was 95°C (2 min) and 40 cycles of amplification cycle (95°C [15 s], 55°C [30 s], and 72°C [30 s]). All the experiments were carried out in triplicate and repeated twice on two biological samples. *MYB108* mRNA level was determined by normalizing with the *ACTIN2* cDNA of each sample. The relative transcript level of *MYB108* in *opr3*, *myb21-1*, *myb24-1*, and *myb21-1 myb24-1* line was compared using wild-type (Col-0) as a calibrator. The gene-specific primer sequences used for *MYB108* were forward 5′-AATGGAGAAGGTCGCTGGAACTCT-3′ and reverse 5′-CGTTGTCCGT-TCTTCCCGGTAAAT-3′; the primer sequences for *ACTIN2* were forward,

5′-GGTGATGGTGTGTCTCACACTG-3′ and reverse, 5′-GAGGTTTCCATCTCCTGCTCGTAG-3′. The quality control was carried out using both electrophoresis analyses on a 1.5% agarose gel and dissociation curve analysis performed immediately after the end of amplification.

RNA-Blot Analysis

Flowers of wild-type and *coi1* plants were treated with 100 μM methyl jasmonate in 0.1% Tween 20 solution. Control treatment contained only 0.1% Tween 20. Samples were collected 24 h after treatment. RNA extraction and RNA gel-blot analysis were performed as described previously (Mandaokar et al., 2003).

Pollen Viability and Germination Test

Double staining with fluorescein diacetate and propidium iodide was performed using the method of Regan and Moffatt (1990). A stock solution of 2 mg /mL fluorescein diacetate was made in acetone and added drop wise to 17% Suc (w/v) until the solution became cloudy. Propidium idodite was diluted to 1 mg/mL in water and diluted to 100 μL/mL with 17% Suc (w/v). Equal amounts of fluorescein diacetate and propidium iodide solutions were mixed together and were added to freshly isolated pollen on a glass slide. The pollen were covered with a coverslip and viewed under UV light under a microscope (Olympus IX70).

Pollen germination assay was done according to the methods of Pickert (1988). Pollen was isolated from mature flowers by gently releasing them from the anther locules onto a petri dish (5 cm) containing pollen germination medium. Pollen from *myb108* and *myb108 myb24* mutants was released by manually opening the anther locule. The medium consisted of tap water, 20% (w/v) Suc, 80 μg mL^{-1} boric acid, and 10 μg mL^{-1} myoinositol at pH 5.8 to 6.0. The plates were incubated in the dark for 5 to 10 h at room temperature, and pollen germination was observed in a microscope (Leica MZ8).

Measurements of Stamen Length

To measure the stamen and carpel length of wild type, *myb108*, and *myb108 myb24*, sepal and petals were removed from unopened flower buds and open flowers. The series of pictures of stamen and carpel were taken under the microscope at a fixed magnification. From these images, the length of stamens and carpel was measured. The actual length of stamen was calculated by dividing the magnification.

ACKNOWLEDGMENTS

We thank Craig Whitney for taking care of plants and Jim Wallis for helpful comments on this manuscript.

Received November 14, 2008; accepted December 3, 2008; published December 17, 2008.

LITERATURE CITED

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) *Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell **15:** 63–78

Aloni R, Aloni E, Langhans M, Ullrich CI (2006) Role of auxin in regulating *Arabidopsis* flower development. Planta **233:** 315–328

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, et al (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science **301:** 653–657

Bonner LJ, Dickinson HG (1990) Anther dehiscence in lycopersicon-esculentum. 2. Water relations. New Phytol **115:** 367–375

Butenko MA, Patterson SE, Grini PE, Stenvik GE, Amundsen SS (2003) Inflorescence deficient in abscission controls floral organ abscission in *Arabidopsis* and identifies a novel family of putative ligands in plants. Plant Cell **15:** 2296–2307

Chini A, Fonseca S, Fernández G, Adie B, Chico JM, Lorenzo O, García-Casado G, López-Vidriero I, Lozano FM, Ponce MR, et al (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature **448:** 666–671

Coen HS, Meyerowitz EM (1991) The war of the whorls: genetic interactions controlling flower development. Nature **353:** 31–37

Feys BJF, Benedetti CE, Penfold CN, Turner JG (1994) *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell **6:** 751–759

Goldberg RB, Beals TP, Sanders PM (1993) Anther development: basic principles and practical applications. Plant Cell **5:** 1217–1229

Heslop-Harrison J, Heslop-Harrison Y (1970) Evaluation of pollen viability by enzymatically induced fluorescence; intracellular hydrolysis of fluorescein diacetate. Stain Technol **45:** 115–120

Ishiguro S, Kawai-Oda A, Ueda J, Nishida I, Okada K (2001) The DEFECTIVE IN ANTHER DEHISCIENCE gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in *Arabidopsis*. Plant Cell **13:** 2191–2209

Jack T (2004) Molecular and genetic mechanisms of floral control. Plant Cell **14:** 279–289

Jürgens A, Witt T, Gottsberger G (2001) Pollen grain numbers, ovule numbers and pollen-ovule ratios in Caryophylloideae: correlation with breeding system, pollination, life form, style number, and sexual system. Sex Plant Reprod **14:** 279–289

Lindsay DL, Sawhney VK, Bonham-Smith PC (2006) Cytokinin-induced changes in *CLAVATA1* and *WUSCHEL* expression temporally coincide with altered floral development in *Arabidopsis*. Plant Sci **170:** 1111–1117

Ma H (2005) Molecular genetic analyses of microprogenesis and microgametogenesis in flowering plants. Annu Rev Plant Biol **56:** 393–434

Mandaokar A, Kumar VD, Amway M, Browse J (2003) Microarray and differential display identify genes involved in jasmonate-dependent anther development. Plant Mol Biol **52:** 775–786

Mandaokar A, Thines B, Shin B, Lange BM, Choi G, Koo YJ, Yoo YJ, Choi YD, Choi G, Browse J (2006) Transcriptional regulators of stamen development in Arabidopsis identified by transcriptional profiling. Plant J **46:** 984–1008

Martienssen RA (1998) Functional genomics: probing plant gene function and expression with transposons. Proc Natl Acad Sci USA **95:** 2021–2026

McConn M, Browse J (1996) The critical requirement for linolenic acid is for pollen development, not photosynthesis, in an *Arabidopsis* mutant. Plant Cell **8:** 403–416

Meissner RC, Jin J, Cominelli E, Denekamp M, Fuertes A, Greco R, Kranz HD, Penfield S, Petroni K, Urzainqui A, et al (1999) Function search in a large transcription factor gene family in *Arabidopsis*: assessing the potential of reverse genetics to identify insertional mutations in R2R3 MYB genes. Plant Cell **11:** 1827–1840

Mengiste T, Chen X, Salmeron J, Dietrich R (2003) The BOTRYTIS SUSCEPTIBLE1 GENE encodes an R2R3MYB transcription factor protein that is required for biotic and abiotic stress responses in *Arabidopsis*. Plant Cell **15:** 2551–2565

Nagpal P, Ellis CM, Weber H, Ploense SE, Barkawi LS, Guilfoyle TJ, Hagen G, Alonso JM, Cohen JD, Farmer EE, et al (2005) Auxin response factors ARF6 and ARF8 promote jasmonic acid production and flower maturation. Development **132:** 4107–4118

Park JH, Halitschke R, Kim HB, Baldwin IT, Feldmann KA, Feyereisen R (2002) A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in Arabidopsis due to a block in jasmonic acid biosynthesis. Plant J **31:** 1–12

Pickert M (1988) In vitro germination and storage of trinucleate Arabidopsis-thaliana L. pollen grains. Arabidopsis Inf Serv **26:** 39–42

Pontier D, Miao ZH, Lam E (2001) Trans-dominant suppression of plant TGA factors reveals their negative and positive roles in plant defense responses. Plant J **27:** 529–538

Preston J, Wheeler J, Heazlewood J, Li SF, Parish RW (2004) AtMYB32 is required for normal pollen development in Arabidopsis thaliana. Plant J **40:** 979–995

Regan SM, Moffatt BA (1990) Cytochemical analysis of pollen development in wild-type *Arabidopsis* and a male-sterile mutant. Plant Cell **2:** 877–889

Rieu I, Wolters-Arts M, Derksen J, Mariani C, Weterings K (2003) Ethylene regulates the timing of anther dehiscence in tobacco. Planta **217:** 131–137

Sanders PM, Lee PY, Biesgen C, Boone JD, Beals TP, Weiler EW, Goldberg

RB (2000) The *Arabidopsis* DELAYED DEHISCENCE1 gene encodes an enzyme in the jasmonic acid synthesis pathway. Plant Cell **12:** 1041–1061

Schmid M, Davison TS, Henz SR, Pape UJ, Demar M, Vingron M, Schölkopf B, Weigel D, Lohmann JU (2005) A gene expression map of Arabidopsis thaliana development. Nat Genet **37:** 501–506

Smyth DR, Bowman JL, Meyerowitz EM (1990) Early flower development in *Arabidopsis*. Plant Cell **2:** 755–767

Stintzi A, Browse J (2000) The *Arabidopsis* male-sterile mutant, *opr3*, lakes the 12-oxophytodienoic acid reductase required for jasmonate synthesis. Proc Natl Acad Sci USA **97:** 10625–10630

Stracke R, Werber M, Weisshaar B (2001) The R2R3-MYB gene family in Arabidopsis thaliana. Curr Opin Plant Biol **4:** 447–456

Tatematsu K, Kumagai S, Muto H, Sato A, Watahiki MK, Harper RM, Liscum E, Yamamoto KT (2004) MASSUGU2 encodes Aux/IAA19, an auxin-regulated protein that functions together with the transcriptional activator NPH4/ARF7 to regulate differential growth responses of hypocotyl and formation of lateral roots in *Arabidopsis thaliana*. Plant Cell **16:** 379–393

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J (2007) JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. Nature **448:** 661–665

Toufighi K, Brady SM, Austin R, Ly E, Provart NJ (2005) The Botany Array Resource: e-Northerns, expression angling, and promoter analyses. Plant J **43:** 153–163

von Mallek B, van der Graaff E, Schneitz K, Keller B (2002) The Arabidopsis male-sterile mutant dde2-2 is defective in the ALLENE OXIDE SYNTHASE gene encoding one of the key enzymes of the jasmonic acid biosynthesis pathway. Planta **216:** 187–192

Wang M, Hoekstra S, van Bergen S, Lamers GE, Oppedijk BJ, van der Heijden MW, de Priester W, Schilperoort RA (1999) Apoptosis in developing anthers and the role of ABA in this process during androgenesis in Hordeum vulgare L. Plant Mol Biol **39:** 489–501

Weigel D, Glazebrook J (2002) Arabidopsis: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 171–280

Weiss D (2000) Regulation of flower pigmentation and growth: multiple signaling pathways control anthocyanin synthesis in expanding petals. Physiol Plant **110:** 152

Yanofsky MF (1995) Floral meristems to floral organs: genes controlling early events in *Arabidopsis* flower development. Annu Rev Plant Physiol Plant Mol Biol **46:** 167–188

Yu H, Ito T, Zhao Y, Peng J, Kumar P, Meyerowitz EM (2004) Floral homeotic genes are targets of gibberellin signaling in flower development. Proc Natl Acad Sci USA **101:** 7827–7832

Zhang JZ (2003) Overexpression analysis of plant transcription factors. Curr Opin Plant Biol **6:** 430–440