Gibberellin Acts through Jasmonate to Control the Expression of MYB21, MYB24, and MYB57 to Promote Stamen Filament Growth in Arabidopsis

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Abstract

Precise coordination between stamen and pistil development is essential to make a fertile flower. Mutations impairing stamen filament elongation, pollen maturation, or anther dehiscence will cause male sterility. Deficiency in plant hormone gibberellin (GA) causes male sterility due to accumulation of DELLAs proteins, and GA triggers DELLAs degradation to promote stamen development. Deficiency in plant hormone jasmonate (JA) also causes male sterility. However, little is known about the relationship between GA and JA in controlling stamen development. Here, we show that MYB21, MYB24, and MYB57 are GA-dependent stamen-enriched genes. Loss-of-function of two DELLAs RGA and RGL2 restores the expression of these three MYB genes together with restoration of stamen filament growth in GA-deficient plants. Genetic analysis showed that the myb21-t1 myb24-t1 myb57-t1 triple mutant confers a short stamen phenotype leading to male sterility. Further genetic and molecular studies demonstrate that GA suppresses DELLAs to mobilize the expression of the key JA biosynthesis gene DAD1, and this is consistent with the observation that the JA content in the young flower buds of the GA-deficient quadruple mutant ga1-3 gai-t6 rga-t2 rgl1-1 is much lower than that in the WT. We conclude that GA promotes JA biosynthesis to control the expression of MYB21, MYB24, and MYB57. Therefore, we have established a hierarchical relationship between GA and JA in that modulation of JA pathway by GA is one of the prerequisites for GA to regulate the normal stamen development in Arabidopsis.

Introduction

Arabidopsis flowers are organized into four concentric whorls of distinct organs (sepals, petals, stamens and pistils). Stamens, the male reproductive organs of flowering plants, form the third whorl. Processes of stamen filament elongation and anthesis are precisely controlled so that they coincide with the pistil development to determine the fertility [1]. Mutations that impair stamen development such as filament elongation, pollen maturation, or anther dehiscence will result in male sterility [2,3]. Many genes have been found to control stamen development [4,5]. Stamen development is also subjected to hormonal control. For example, mutations affecting biosynthesis of two plant hormones gibberellin (GA) (e.g. ga1-3 mutation) and jasmonate (JA) (e.g. opr3 mutation) both confer male sterile phenotype due to failure of stamen filament elongation and of completion of anthesis and anther dehiscence [6,7].

A severe Arabidopsis GA-deficient mutant, ga1-3 exhibits retarded growth at both vegetative and reproductive stages [7]. The development of floral organs, especially petals and stamens, is impaired in the ga1-3 mutant. Detailed anatomical analysis showed that the male sterile phenotype of ga1-3 is due to the arrestment of stamen filament cell elongation and failure of completion of anthesis [8]. Application of exogenous GA can restore all the floral defects of ga1-3 [7]. Further studies revealed that the arrested floral development in ga1-3 is mediated by DELLA proteins [8,9]. DELLA family of putative transcription regulators [10,11] and have been revealed to function as negative regulators of GA response in diverse plant species including Arabidopsis, barley, rice and wheat etc [12–17]. There are five DELLA in Arabidopsis, namely GAI, RGA, RGL1, RGL2 and RGL3 [18,19]. Genetic studies have shown that RGA, RGL2 and RGL1 act synergistically in repressing petal and stamen development and GA triggers the degradation of these DELLA to promote floral development [8,9,20–22]. Severe JA deficient mutant opr3 and JA-signaling mutant coi1 also displayed retarded filament elongation, delayed anther dehiscence, and reduced pollen viability. As a consequence,
Gibberellin and jasmonate are plant hormones that mediate diverse plant developmental processes and responses to the environment. Deficiency in either gibberellin or jasmonate causes male sterility, in part due to the short stamen filament conferred. In this report, we sought to study the interaction between gibberellin and jasmonate during stamen filament development. We focused on three MYB genes, namely MYB21, MYB24, and MYB57, which have been proven to be essential for stamen filament development in Arabidopsis. These three MYB genes are regulated by both gibberellin and jasmonate. We performed various molecular analyses and found that GA activates the expression of DAD1 and LOXI, two genes essential for jasmonate biosynthesis. The hypothesis of GA regulating JA biosynthesis is proved by the fact that the JA content in the young flower buds of the ga1-3 gai-t6 rga-t2 rgl1-1 quadruple mutant is much lower than that in the WT. This evidence demonstrates that GA promotes the production of jasmonate and high level of jasmonate will induce the expression of MYB21, MYB24, and MYB57 to promote stamen filament development. This is most likely the first molecular and genetic evidence to show how gibberellin and jasmonate interact to control stamen filament development.

Author Summary

Gibberellin and jasmonate are plant hormones that mediate diverse plant developmental processes and responses to the environment. Deficiency in either gibberellin or jasmonate causes male sterility, in part due to the short stamen filament conferred. In this report, we sought to study the interaction between gibberellin and jasmonate during stamen filament development. We focused on three MYB genes, namely MYB21, MYB24, and MYB57, which have been proven to be essential for stamen filament development in Arabidopsis. These three MYB genes are regulated by both gibberellin and jasmonate. We performed various molecular analyses and found that GA activates the expression of DAD1 and LOXI, two genes essential for jasmonate biosynthesis. The hypothesis of GA regulating JA biosynthesis is proved by the fact that the JA content in the young flower buds of the ga1-3 gai-t6 rga-t2 rgl1-1 quadruple mutant is much lower than that in the WT. The evidence demonstrates that GA promotes the production of jasmonate and high level of jasmonate will induce the expression of MYB21, MYB24, and MYB57 to promote stamen filament development. This is most likely the first molecular and genetic evidence to show how gibberellin and jasmonate interact to control stamen filament development.

Identification of DELLA-Repressed Stamen-Enriched Genes

The ga1-3 mutant is retarded in floral development, suggesting that the transcriptome for floral development in the ga1-3 mutant must be kept at a repressive state. Conversely, the fact that the ga1-3 gai-t6 nga-t2 vgl1-1 vgl2-1 mutant (penta mutant) confers GA independent flowering suggests that the transcriptome responsible for floral development must have been constitutively activated in the penta mutant. We compared the expression profiles between ga1-3 and ga1-3 gai-t6 nga-t2 vgl1-1 vgl2-1 and identified 360 DELLA-repressed and 273 DELLA-activated genes essential for floral development [30]. To identify DELLA-repressed stamen-enriched genes, we examined expression of 43 DELLA-repressed genes in the sepal, petal, stamen and pistil via semi-quantitative RT-PCR. These 43 genes were chosen based on two criteria: 1) they are homologous to transcription factors known to regulate GA-response (e.g. MYB gene family) and 2) genes whose expression showed drastic changes between the ga1-3 and penta mutant [30]. Only genes whose expression are either enriched in the stamen or highly expressed in the stamen and also in some other floral organs but not ubiquitously highly expressed in all four floral organs were classified as the stamen-enriched genes. A total of 34 genes, including two APG-like genes (At1g73880, At1g73900) and three genes (IRX1, IRX3, IRX5) encoding the cellulose synthase subunits which are known to be enriched in the stamen, were identified as DELLA-repressed stamen-enriched genes (Figure 1; Table 1).

DELLAs Repress the Expression of MYB21, MYB24, and MYB57

Three MYB genes, namely MYB21, MYB24 and MYB57, were among the identified DELLA-repressed stamen-enriched genes (Figure 1; Table 1). Based on the phylogenetic tree, MYB24 and MYB21 are classified into the subgroup 19 of R2R3-MYB family [36]. MYB57 shares high similarity with this subfamily and is a close member to this subfamily [37]. Overall, MYB21 shares 61.6% and 51.0% identity with MYB24 and MYB57, respectively (Figure 1; Table 1). The expression of these three MYB genes in the young flower buds were reduced to a very low level in ga1-3 but restored to the wild type (WT) level in the ga1-3 gai-t6 nga-t2 vgl1-1 vgl2-1 penta mutant (Figure 2A). In order to find out which DELLA (RGL1, RGL2, RGA and GAI) is more effective in repressing the expression of MYB21, MYB24 and MYB57, transcript levels of each individual MYB gene were studied in four quadruple mutants in which only one of the four DELLA genes remains intact. All three MYB genes were almost undetectable in the Q1 (ga1-3 gai-t6 nga-t2 vgl2-1, wild type for RGA) and barely detectable in the Q3 (ga1-3 gai-t6 nga-t1 nga-t2, wild type for col1) mutants. Application of exogenous JA onto the ga1-3 gai-t6 nga-t1 vgl2-1 quadruple mutant flower buds could restore the expression of MYB21, MYB24 and MYB57 whereas application of exogenous GA onto opr3 mutant flower buds failed to increase the expression of these three MYB genes. Most importantly, we showed that GA upregulates JA-biosynthetic genes DAD1 and LOXI and the JA content in the young flower buds of the GA-deficient quadruple mutant ga1-3 gai-t6 nga-t2 vgl1-1 is much lower than that in the WT and penta mutant. Therefore, we conclude that GA upregulates the DAD1 and LOXI expression to promote JA production to promote the expression of the three MYB genes necessary for stamen filament development.
Figure 1. Identification of DELLA-Repessed Stamen-Enriched Genes. At least three independent samples were used for RT-PCR analysis for each individual gene and a representative gel picture for each gene was shown here. Total 34 genes were identified as DELLA-repressed stamen-enriched genes (summarized in Table 1) based on their relative more abundant expression in the stamen than in one or more of the rest of the floral organs. Primer pairs corresponding to these genes were listed in Table S1. Se: sepal; Pe: petal; St: stamen; Pi: pistil.

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Table 1. RT-PCR examination of DELLA-down genes in different floral organs.

| Gene ID   | Gene description      | DELLA-Down |
|-----------|-----------------------|------------|
| At1g09610 | Hypothetical protein  | confirmed  |
| At1g17950 | MYB52                 | confirmed  |
| At1g52690 | Late embryogenesis abundant protein | confirmed |
| At1g70690 | Unknown               | confirmed  |
| At1g75880 | APG-like              | confirmed  |
| At1g75900 | APG-like              | confirmed  |
| At1g76240 | Hypothetical protein  | confirmed  |
| At1g78440 | Gibberellin 2-oxidase | confirmed  |
| At2g17950 | Homeodomain transcription factor | confirmed |
| At2g34870 | Berberrine bridge enzyme | confirmed |
| At2g34870 | Berberrine bridge enzyme | confirmed |
| At2g38080 | Unknown               | confirmed  |
| At3g01530 | Putative diphenol oxidase | confirmed |
| At3g11480 | Extensin-like         | confirmed  |
| At3g28000 | MYB21                 | confirmed  |
| At3g54770 | RNA binding protein   | confirmed  |
| At3g62020 | Germin-like protein   | confirmed  |
| At4g12730 | Putative pollen surface protein | confirmed |
| At4g12960 | Unknown               | confirmed  |
| At4g18780 | Cellulose synthase (IRX1) | confirmed |
| At4g34990 | MYB32                 | confirmed  |
| At5g12870 | MYB46                 | confirmed  |
| At5g17420 | Cellulose synthase (IRX3) | confirmed |
| At5g40350 | MYB24                 | confirmed  |
| At5g40300 | MYB57                 | confirmed  |
| At5g46300 | Terpene synthase      | confirmed  |
| At5g59120 | Subtilisin-like serine protease | confirmed |

Se: sepal; Pe: petal; St: stamen; Pi: pistil. Expression levels in different floral organs were based on the semi-quantitative RT-PCR results. "−" not detected, "−" faintly detected, "++" detected, "+++" strongly detected. DELLA-D: down-regulated by DELLA proteins.
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wild type for RGL2 mutants but were detected at high levels in the Q2 (ga1-3 rga-t2 rgl1-1 rgl2-1), wild type for GAI, and Q4 (ga1-3 rga-t2 rgl2-1), wild type for RGL1) mutants (Figure 2B), suggesting that RGA and RGL2, but not GAI nor RGL1, were the more effective DELLA’s in repressing the expression of these three MYB genes. Interestingly, we showed previously that while Q1 and Q5 mutants, as the ga1-3 mutant, were retarded in floral development both Q2 and Q4 mutants produced normal fertile flowers (Figure 2C) [8]. Therefore, it seems there is a nice correlation between normal floral development and the expression of MYB21, MYB24 and MYB57, suggesting that these three MYB’s are probably necessary for normal floral development.

MYB21, MYB24, and MYB57 Function Redundantly in Controlling Stamen Filament Elongation

Expression analysis showed that MYB21 and MYB24 [32,34] as well as MYB57 are flower-specific genes (Figure 2D). To determine if the spatial and temporal expression patterns of MYB21 and MYB24 correlate with their proposed role during stamen filament elongation, we examined MYB21 expression via in situ hybridization and generated pMYB24::GUS transgenic for examining MYB24 expression. Our in situ hybridization result showed that, starting from floral stage 12 [1,38], MYB21 is expressed in the anther vascular tissue and in cells at the junction between anther and stamen filament (Figure S2A,B) where rapid filament elongation is hypothesized to occur starting from the floral stage 13 after a successful pollination [1]. MYB21 expression is also detected in the nectaries and ovules (Figure S2A,B). Similarly, staining the young inflorescence of the pMYB24::GUS plants revealed that strong GUS activity was detected in the vascular tissue of stamen filament and sepal whereas only weak GUS activity was detectable in the petals starting from floral stage 12 (Figure S3A–E). GUS activity was also detected in the upper part of the pistils (Figure S3A–E).

To investigate their roles in GA-mediated floral organ development, we identified T-DNA insertion mutant lines corresponding to these three MYB genes from the Salk Institute Genomic Arabidopsis Laboratory (SIGnAL) database. Mutant alleles were confirmed (data not shown) and designated as myb21-t1 (SALK_042711) for MYB21, myb24-t1 (SALK_017221) for MYB24, and myb57-t1 (SALK_065776) for MYB57 (Figure 3A). myb24-t1 and myb57-t1 are both likely null alleles since MYB24 and MYB57 transcripts were undetectable in myb24-t1 and myb57-t1
multiflora mutant flowers, respectively (Figure 3B). On the other hand, MYB21 transcripts were still detectable in myb21-t1 although its level was greatly reduced in the mutant, suggesting that myb21-t1 is likely a leaky allele (Figure 3B). After two rounds of backcross, we found that myb24-t1 and myb57-t1 mutant plants were phenotypically indistinguishable from the WT control plant (Figure 3C; Table 2). However, in myb21-t1 the early developed flowers bore short stamens (Figure S4) with greatly reduced fertility and only the late developed flowers yielded proper seed settings (Figure 3C; Table 2). A close look at the matured early flowers in myb21-t1 showed that the stamens did produce pollen (Figure 3D, panel d). Cross-pollinating the pollen onto the myb21-t1 stigma yielded seeds that were homozygous for myb21-t1 and onto the WT stigma yielded myb21-t1 heterozygous seeds (data not shown), demonstrating that the short stamen is responsible for the partial sterile phenotype. Although myb21-t1 is likely a leaky allele, the short stamen phenotype conferred by the myb21-t1 mutation is identical to a MYB21 null allele we obtained later from Gabi-Kat stock (stock number N311167, data not shown). The WT-like phenotype displayed by myb24-t1 and myb57-t1 and mild floral phenotype displayed by myb21-t1 suggest that these MYB genes might function redundantly during stamen development. To prove this hypothesis, crosses were made among homozygous myb24-t1, myb21-t1 and myb57-t1 plants. Three

Figure 2. MYB21, MYB24I and MYB57 Are RGA- and RGL2-Repressible Floral Specific Genes. (A) RT-PCR analysis shows that the expression of the three MYB genes in the young flower buds are greatly reduced in ga1-3 but restored to the WT level in ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1 (penta). (B–C) RT-PCR analysis shows that the repressed expression of the three MYB genes in ga1-3 was restored in ga1-3 gai-t6 rga-t2 rgl2-1 (Q2) and ga1-3 rga-t2 rgl1-1 rgl2-1 (Q4) two quadruple mutants but not in ga1-3 gai-t6 rgl1-1 rgl2-1 (Q1) and ga1-3 gai-t6 rga-t2 rgl1-1 (Q3) two quadruple mutants (B). This restoration of MYB expression nicely correlates with the recovery of fertility in Q2 and Q4 (C). Total RNA used in RT-PCR analysis was extracted from the young flower buds. (D) RT-PCR analysis shows that the three MYB genes are floral specific genes. IF, inflorescence; CL, cauline leaves; RL, rosette leaves; IT, internodes; RT, roots; SL, siliques. (E) Amino acid alignment of MYB21, MYB24 and MYB57 proteins. The conserved R2 and R3 domains and the NYWSV/ME/DDLWP/S motif are highlighted in red, blue and green, respectively.

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Regulating the Stamen Filament Development. (A) Schematic diagram shows the respective T-DNA insertions in the three MYB genes. Black box: exon; black line: intron; triangle: T-DNA insertion site. (B) RT-PCR analysis of RNA for RT-PCR and northern analysis was extracted from the young flower buds. (C) Comparison of main shoots bearing siliques among different mutant lines as indicated. Genotypes for different mutant lines as indicated. (D) Comparison of the stamen flower buds. (C) Comparison of main shoots bearing siliques among different mutant lines as indicated. Genotypes for flowers a-h in (D) corresponds to that showed in (C). doi:10.1371/journal.pgen.1000440.g003

double mutants (myb21-t1 myb24-t1, myb21-t1 myb57-t1, and myb24-t1 myb57-t1) and one triple mutant (myb21-t1 myb24-t1 myb57-t1) were generated and used in our phenotypic analysis. The flower development of myb24-t1 myb57-t1 double mutant at all stages was indistinguishable from the WT control (Figure 3D; Table 2) [1]. Stamens in mature flowers of the myb21-t1 myb24-t1 double mutant were shorter than that of the myb21-t1 single mutant and shorter stamens were also observed in majority of the late developed mature flowers and some of the later flowers (Figure 3D; Figure S4) and its seed settings displayed an intermediate phenotype between myb21-t1 single and myb21-t1 myb24-t1 double mutants (Figure 3C and 3D; Table 2). Interestingly, cross-pollination showed that the short stamens in both myb24-t1 myb21-t1 and myb21-t1 myb57-t1 double mutant plants produced viable pollens (data not shown), suggesting that the short stamen (Figure S4) is responsible for the reduced fertility in these mutants.

The myb21-t1 myb24-t1 myb57-t1 triple mutant, as the myb21-t1 myb24-t1 double mutant, had short stamens but was even more severely sterile than myb21-t1 myb24-t1 (Figure 3C and 3D; Figure S4; Table 2). For myb21-t1 myb24-t1 double and myb21-t1 myb24-t1 myb57-t1 triple mutant plants, we occasionally observed that while, in the same inflorescence, most of the flowers did not set or set very few seeds, some were able to develop normal siliques filled up with seeds (Figure 3C and 3D). Cross pollination showed the pollens produced by myb21-t1 myb24-t1 myb57-t1 triple mutant plants were partial viable (data not shown), suggesting the short stamens in the triple mutants are the main cause of the sterility. It is possible that environmental factors (e.g. temperature) may influence male fertility in these mutants, an observation also reported for MYB23 and MYB65 [24]. Therefore, MYB21, MYB24 and MYB57 function redundantly to control the stamen filament development in the late developed flowers.

Since MYB21 and MYB24 are also expressed in sepals and petals, we examined the sepal and petal development in the single, double and triple myb mutants. As shown in Figure 3D, sepal development appeared normal in all mutants whereas petal development varied in different mutants. Petals in the myb24-t1 and myb57-t1 two single mutants grew to a final length longer than the pistil, as that did the WT petals. Petals in the myb21-t1 single, myb24-t1 myb57-t1 and myb21-t1 myb57-t1 two double mutants grew to a final height parallel to the pistil (Figure 3D). Petals in the myb21-t1 myb24-t1 double mutant grew just out of the sepals but ended at a lower level than the stigma (Figure 3D). The growth of petals in the myb21-t1 myb24-t1 myb57-t1 triple mutant was arrested and the petals never grew out of the sepals (Figure 3D).

myb21-t1 myb24-t1 is Epistatic to gai-t6 rga-t2 rgl1-t1 rgl2-t1 in Controlling Stamen Filament Elongation MYB21 and MYB24 were repressed in ga1-3 but their expressions were restored to the WT level in the ga1-3 gai-t6 rga-t2 rgl1-t1 rgl2-t1 penta mutant, suggesting that GA regulates MYB21 and MYB24 through inactivating DELLA proteins. Application of

**Table 2. Seed settings in different mutants grown under LD condition.**

| Col-0/mutant | Number of Siliques | Number of Siliques With Seeds | Percentage of Siliques With Seeds |
|--------------|--------------------|-------------------------------|----------------------------------|
| Col-0        | 21.1±5.1           | 20.7±5.0                      | 98.1±2.7                        |
| myb57        | 22.3±5.7           | 20.3±6.6                      | 89.4±1.27                       |
| myb24        | 28.1±3.8           | 27.3±3.8                      | 97.3±2.9                        |
| myb21        | 26.3±4.8           | 17.1±5.4                      | 64.2±10.7                       |
| myb24myb57   | 26.3±6.9           | 22.6±6.8                      | 85.1±6.3                        |
| myb21myb57   | 30.6±6.6           | 9.4±6.5                       | 29.5±17.3                       |
| myb21myb24   | 30.8±9.5           | 5.6±4.3                       | 16.9±11.8                       |
| myb21myb24myb57 | 33.4±7.5     | 1.6±1.5                       | 4.1±3.6                         |

*Siliques formed in primary inflorescence of plants were scored at 45 days. doi:10.1371/journal.pgen.1000440.t002
exogenous GA could not rescue the stamen development in myb21 myb24 mutant (data not shown), suggesting that MYB21 and MYB24 are needed in GA-mediated stamen development. To further confirm this hypothesis, we crossed myb21 myb24 with ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1 to generate two hexa mutants (hexa1: ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1; hexa2: ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1 myb24-t1) and one hepta mutant (ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1 myb21-t1 myb24-t1). The two hexa mutants overall appeared similar to each other and had wildtype-like stamens and were largely fertile (Figure 4A; Figure S5). This observation demonstrated that myb21-t1 myb24-t1 double mutants are epistatic to DELLA mutations. SEM analysis showed that the short stamen phenotype in the hepta mutant was due to reduced cell length (Figure 4B and 4C), rather than to a reduction in cell number (Figure 4D). Therefore, MYB21 and MYB24 act downstream of DELLAAs in GA signaling pathway to control the stamen filament development.

GA Application Fails to Induce the Expression of MYB21, MYB24 and MYB57 in GA-Deficient Mutant

We showed in the above that the expression of MYB21, MYB24 and MYB57 was repressed in the ga1-3 gai-t6 rga-t2 rgl1-1 quadruple mutant (wild type for RGL2) but restored to normal in the ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1 penta mutant (Figure 2A and 2B). Mandaokar et al reported that the expression of MYB21 and MYB24 was downregulated in opr3 mutant and application of exogenous JA could restore their expression [32]. These results suggest that there might be a crosstalk between the GA and JA pathways in regulating the expression of MYB21, MYB24 and MYB57 during stamen development. Genetically, there are three possible ways of interaction between GA and JA. Firstly, GA might act through the JA pathway to regulate the expression of these MYB genes. In this case it is expected that JA application onto ga1-3 gai-t6 rga-t2 rgl1-1 would induce the expression of MYB21, MYB24 and MYB57 whilst GA application onto opr3 would have no effect on their expression. Conversely, JA may act upstream of the GA pathway to regulate the expression of these three MYB genes. In this case, GA application onto opr3 would induce whilst JA application onto ga1-3 gai-t6 rga-t2 rgl1-1 would have no effect on the expression of MYB21, MYB24 and MYB57. The third possibility is that GA and JA may not act in a hierarchical manner but rather via parallel pathways to regulate the expression of the three MYB genes. If this is the case, GA application onto opr3 and JA application onto ga1-3 gai-t6 rga-t2 rgl1-1 would probably both induce the expression of the three MYB genes. To find out which is the likely case, we first examined the effect of GA application on JA-deficient mutant opr3 and found that GA application failed to rescue the opr3 mutant phenotype and failed to induce the expression of MYB21, MYB24 and MYB57 in opr3 even at 96 hrs after GA treatment (Figure 3A). Failure in induction of expression of MYB21, MYB24 and MYB57 in GA-treated opr3 mutants could be due to inactivation of GA signaling in JA-deficient background. GA3ox1 and GA20ox2 are two key genes that contribute to the biosynthesis of bioactive GA and these two genes are under negative feedback regulation by GA signaling pathway (GA-down) [30]. On the other hand, GA2ox1 is a GA-up gene responsible for GA catabolism [30]. Examination of the GA3ox1 and GA20ox2 and GA2ox1 expression in GA-treated opr3 mutants showed expected GA-response (Figure 3B). Meanwhile, expression of GA3ox1 and GA2ox1 appeared normal in opr3 (Figure 6A). These results suggest that JA-deficiency specifically blocks the GA-signaling leading to the induction of MYB21, MYB24 and MYB57 expression but not the negative feedback pathway for GA-biosynthesis.

JA Application Restores the Expression of MYB21, MYB24 and MYB57 in GA-Deficient Mutant

We then studied the effect of JA application on ga1-3 gai-t6 rga-t2 rgl1-1 (GA-deficient) by examining the expression of MYB21, MYB24 and MYB57 in the young flower buds at 18, 48, 72 and 96 hrs post-treatment. As expected, LOX2, a JA-response gene, was strongly upregulated by JA application at 18 hrs post treatment (Figure 5C). Interestingly, we observed that JA-treatment induced high expression of MYB21 and MYB24 and weak expression of MYB57 in the ga1-3 gai-t6 rga-t2 rgl1-1

Figure 4. myb21-t1 myb24-t1 is Epistatic to ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1 in Controlling Stamen Filament Elongation. (A) Comparison of the stamen phenotype among ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1 (penta), ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1 myb21-t1 myb24-t1 (hepta), ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1 myb21-t1 myb24-t1 (hexa1) and ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1 myb21-t1 myb24-t1 (hexa2). (B) SEM of stamen filament epidermal cells in the penta, hepta, hexa1 and hexa2 mutants. Segments shown were all from the middle part of the filament. Some individual cells were outlined with black lines for easy visualization. (C) Comparison of stamen and pistil lengths among different genotypes. Filament and pistil lengths were measured from SEM pictures (n = 30). (D) Average number of epidermal cells per stamen filament in penta, hepta, hexa1 and hexa2. n: number of stamens used in counting. doi:10.1371/journal.pgen.1000440.g004
quadruple mutant at 18 hrs post treatment (Figure 5D). However, examination of $\text{GA}_3\text{ox1}$ and $\text{GA}_{20}\text{ox2}$ (two GA-down genes) and $\text{GA}_{20}\text{ox1}$ (GA-up gene) showed that JA treatment did not obviously change the expression patterns of these three GA-response genes in the $\text{ga1-3 gai-t6 rga-t2 rgl1-1}$ quadruple mutant (Figure 5C). These data suggested that JA signaling might mediate a specific branch of GA signaling to regulate the expression of the three MYB genes.

GA Suppresses DELLA to Upregulate the JA-Biosynthesis Gene $\text{LOX1}$ and $\text{DAD1}$

Considering the fact that JA application was able to induce the expression of MYBs in the $\text{ga1-3 gai-t6 rga-t2 rgl1-1}$ mutant it is reasonable to argue that JA biosynthesis, instead of JA signaling pathway, is likely affected in the $\text{ga1-3 gai-t6 rga-t2 rgl1-1}$ mutant. To test this hypothesis, we examined the expression of known or putative JA biosynthesis genes including $\text{DAD1}$ (Defective in anther dehiscence 1), $\text{LOX1}$ (Lipoxygenase 1), $\text{LOX2}$ (Lipoxygenase 2), $\text{AOS}$ (Allene oxide synthase), $\text{AOC1}$ (Allene oxide cyclase 1), $\text{AOC2}$ (Allene oxide cyclase 2), $\text{AOC3}$, $\text{AOC4}$, $\text{LOX2}$ and $\text{OPR3}$ (OPDA reductase 3) in La-er WT, Ws WT, $\text{gai-3 gai-t6 rga-t2 rgl1-1}$ quadruple and penta mutants (Figure 6A), suggesting that JA-deficiency does not affect GA biosynthesis. Expression of these genes in GA-related mutants was more complicated. We found that the expression levels of $\text{AOS}$, $\text{AOC1}$, $\text{AOC3}$, $\text{AOC4}$, $\text{LOX2}$ and $\text{OPR3}$ did not show significant differences in all GA-related mutants when compared to the La-er WT control (Figure 6A), suggesting these genes are probably regulated in a GA-independent fashion. The expression of $\text{AOC2}$ was obviously induced in $\text{gai-3}$ and then reduced to the WT level in the quadruple and penta mutants (Figure 6A), suggesting $\text{AOC2}$ is a GA-down gene. In contrast, the expression of $\text{LOX1}$ was significantly reduced in $\text{gai-3}$ but was restored both in the $\text{gai-3 gai-t6 rga-t2 rgl1-1}$ quadruple and penta mutants (Figure 6B), suggesting that although $\text{LOX1}$ is a GA-up gene and its expression is not repressed by RGL2. Interestingly, $\text{DAD1}$ expression was found to be downregulated to approximately 20% of the WT level in both $\text{gai-3}$ and the $\text{gai-3 gai-t6 rga-t2 rgl1-1}$ quadruple mutant whereas was restored to approximately 60% of the WT level in the penta mutant (approximately three folds increase in penta versus $\text{opr3}$ mutants. We found that only $\text{MYB21}$, $\text{MYB24}$ and $\text{MYB57}$ showed reduced expression in the $\text{opr3}$ mutant whereas all the other genes, including GA-biosynthesis genes, expressed similarly in the $\text{opr3}$ mutant and Ws WT control (Figure 6A and 6B).

Figure 5. JA-Deficiency Specifically Blocks GA-Signaling Leading to the Induction of Expression of $\text{MYB21}$, $\text{MYB24}$, and $\text{MYB57}$. (A–B) Semi-quantitative analysis of $\text{MYB21}$, $\text{MYB24}$, $\text{MYB57}$ (A), $\text{GA}_{20}\text{ox1}$, $\text{GA}_{30}\text{ox1}$ and $\text{GA}_{20}\text{ox2}$ (B) expression in the $\text{opr3}$ mutant flowers at 18, 48, 72 and 96 hrs after GA treatment. Data were averaged from 2–4 batches of independently treated samples and ACTII was used as the normalization control. The graph was drawn based on Log$_{10}$ scale of the ratio of the expression levels of GA treated versus untreated samples. (C–D) Semi-quantitative analysis of $\text{LOX2}$ (in red line), $\text{GA}_{20}\text{ox1}$, $\text{GA}_{30}\text{ox1}$ and $\text{GA}_{20}\text{ox2}$ (C), $\text{MYB21}$, $\text{MYB24}$ and $\text{MYB57}$ (D) expression in the $\text{gai-3 gai-t6 rga-t2 rgl1-1}$ (Q3) mutant flowers at 18, 48, 72 and 96 hrs after JA treatment. Data were averaged from 2–4 batches of independently treated samples and ACTII was used as the normalization control. The graph was drawn based on Log$_{10}$ scale of the ratio of the expression levels of JA treated versus untreated samples. doi:10.1371/journal.pgen.1000440.g005
Q3) (Figure 6A and 6C), indicating that GA may regulate DAD1 expression via suppression of RGL2.

JA Levels Are Greatly Reduced in the Young Flower Buds of the ga1-3 gai-t6 rga-t2 rgl1-1 Quadruple Mutant

One expected consequence of downregulation of DAD1 expression by RGL2 is the reduction of JA levels in the ga1-3 gai-t6 rga-t2 rgl1-1 quadruple mutant (Q3). To test this hypothesis, we measured the JA contents in the young flower buds in WT, opr3, the Q3 quadruple mutant and the ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1 penta mutant (penta). The data obtained clearly showed that the JA content was greatly reduced in the young flower buds of the quadruple Q3 mutant whereas was restored in the penta mutant when compared to that in the WT and opr3 mutant (Figure 6D; Table S2).

GA Application Induces DAD1 Expression Prior to the Induction of MYB21, MYB24, and MYB57

DAD1 is a stamen specific gene encoding chloroplastic phospholipase A1 protein that catalyzes the first step of JA biosynthesis. Mutation in DAD1 resulted in a typical JA-deficient phenotype in stamen development [40], a phenotype similar to that of myb21-t1 myb24-t1 double mutant. As mentioned earlier, JA likely acts downstream of GA to regulate the expression of MYB21, MYB24 and MYB57. To study whether there is a correlation between GA-regulated DAD1 expression and MYB21, MYB24 and MYB57 expression, we treated the ga1-3 gai-t6 rga-t2 rgl1-1 quadruple mutant with GA. We first confirmed the GA-responsiveness in the quadruple mutant plants by examining the expression of known GA-response genes GA3ox1, GA20ox2 and GA2ox1 (Figure 7A). Then we examined the expression of DAD1
and the three MYB genes MYB21, MYB24 and MYB57. Surprisingly, compared to the induction of MYB21 and MYB24 expression by JA treatment which is detectable at 18 hrs post treatment (Figure 5D), GA induction of the expression of these two MYB genes in ga1-3 gai-t6 rga-t2 rgl1-1 happens much later and only became detectable at 72 hrs (Figure 7B). More interestingly, GA induction of the expression of MYB21 and MYB24 in the ga1-3 gai-t6 rga-t2 rgl1-1 quadruple mutant (Figure 7B). Our data suggest that GA might first induce the expression of DAD1 to promoter JA production then via JA signaling to regulate the expression of MYB21 and MYB24.

Expression of MYB21, MYB24, and MYB57 Is Necessary But Insufficient for Normal Stamen Filament Elongation in ga1-3 gai-t6 rga-t2 rgl1-1

As shown in the above, MYB21, MYB24 and MYB57 act downstream of DELLAs in controlling stamen filament elongation. Expression of MYB21, MYB24 and MYB57 was repressed and floral development was arrested in the ga1-3 gai-t6 rga-t2 rgl1-1 Q3 quadruple mutant (Figure 2B and 2C). Regarding the fact that JA content is reduced in the young flower buds of Q3 we questioned whether restoration of expression of these MYBs by exogenous application of JA could rescue the stamen development to the ga1-3 gai-t6 rga-t2 rgl1-1 Q3 plants. We analyzed the flowers of JA-treated ga1-3 gai-t6 rga-t2 rgl1-1 plants and found that repeated JA application was unable to rescue the stamen development (Figure 8) though JA could restore the expression of the three MYB genes (Figure 5D), indicating that expression of MYB21, MYB24 and MYB57 alone was insufficient for normal stamen development in the ga1-3 gai-t6 rga-t2 rgl1-1 mutant. Furthermore, we found that exogenous application of GA to the ga1-3 gai-t6 rga-t2 rgl1-1 plants was able to induce the expression of MYB21, MYB24 and MYB57 (Figure 7B) and recover normal floral development (Figure 8). Taken together, our results demonstrate that besides these JA-inducible MYBs, other important GA-regulated JA-independent factors are needed for normal stamen filament development in ga1-3 gai-t6 rga-t2 rgl1-1.

Overexpression of MYB21 Restored Stamen Filament Elongation and Fertility to opr3 Flowers

To test our hypothesis that GA acts through JA to control expression of the MYB genes to promote filament elongation, we fused MYB21 gene with the CaMYB33S promoter (pCAMBIA1301 vector) and this construct was used to generate transgenic plants in the opr3 mutant background. Semi-quantitative RT-PCR showed that MYB21 was overexpressed in the transgenic plants in the opr3 background (Figure 9A). We found that overexpression of MYB21 could restore the stamen filament growth (Figure 9B) and restore

![Figure 8. MYB21, MYB24, and MYB57 Are Necessary but Insufficient to Complete the Normal Stamen Filament Development.](https://doi.org/10.1371/journal.pgen.1000440.g008)

**Figure 8.** MYB21, MYB24, and MYB57 Are Necessary but Insufficient to Complete the Normal Stamen Filament Development. Pictures are shown to compare the stamen phenotype in JA or GA repeatedly treated ga1-3 gai-t6 rga-t2 rgl1-1 (Q3) and opr3 plants with respective untreated controls.
doi:10.1371/journal.pgen.1000440.g008

![Figure 9. Overexpression of MYB21 Rescues the Stamen Filament Growth and Fertility to the opr3 Mutant.](https://doi.org/10.1371/journal.pgen.1000440.g009)

**Figure 9.** Overexpression of MYB21 Rescues the Stamen Filament Growth and Fertility to the opr3 Mutant. (A) RT-PCR analysis of MYB21 and OPR3 gene expression in WT, opr3 and opr3 MYB21OE-1. Total RNA was extracted from the young flower buds. ACTIN was used as the normalization control. (B) Comparison of the flowers at stage 14 in different genotypes. The flower in opr3 MYB21OE-1 shows elongated filament than that in opr3. (C and D) Comparison of seed set in different genotypes as shown (C) and of plant growth of WT (Col-0) (50 days old), opr3 (50 days old) and opr3 MYB21OE-1. The third plant from left was an opr3 MYB21OE-1 plant with primary shoot (50 days old) whereas the last plant was a 60-day-old opr3 MYB21OE-1 with axillary shoots after its primary influence has been removed earlier. White arrows highlight siliques with seed set, red arrows highlight sterile siliques.
doi:10.1371/journal.pgen.1000440.g009
the fertility (Figure 9C and 9D) to the opr3 mutant partially. Together with the fact that loss of function of four DELLAs (GA1, RGA, RGL1 and RGL2) could not restore the fertility and filament elongation to the coil mutant (Figure S6), we have now provided strong evidence to show that GAs act through JA to control expression of the MYB57 to promote stamen filament elongation.

**Discussions**

In this report, we first identified 34 DELLa-repressed stamen-enriched genes by RT-PCR analysis of candidate genes selected from microarray data [30]. We then selected MYB21, MYB24 and MYB57 for detailed genetic analysis because GAMYBs are the best characterized transcription factors involved in GA-response. We showed that *Arabidopsis* MYB21, MYB24 and MYB57 are highly expressed in the stamen. The stamen-enriched expression pattern is consistent with the observation that the myb21-t1 myb24-t1 myb57-t1 triple mutant is impaired in the stamen development, especially in the stamen filament elongation. During the course of studying these three MYBs, Mandaokar et al. reported that MYB21 and MYB24 are also JA-inducible [32] which immediately attracted our attention to study the hierarchical relationship between GA and JA in regulating the expression of these three MYBs.

We first tested the responses of GA- and JA-deficient mutants (i.e. ga1-3 gai-t6 rga-t2 rgl1-1 Q3) quadruple mutant and opr3 mutant, respectively) to GA and JA treatments and found that GA-treatment induced the expression of MYB21, MYB24 and MYB57 in the GA-deficient plants whereas GA-treatment failed to do so in the JA-deficient plant. This result suggests that JA likely acts downstream of GA pathway to control the expression of these three MYBs. It is possible that JA acts downstream by modulating the stability or activity of DELLAs proteins to induce the expression of the three MYBs. If this is the case, we would expect that JA-treatment would lead to RGL2 degradation or would change the expression patterns of GA-response genes in ga1-3 gai-t6 rga-t2 rgl1-1. However, we found that neither the RGL2 protein level (data not shown) nor the expression patterns of three GA-response genes GA2ox1, GA3ox1 and GA20ox1 were obviously altered in the JA-treated ga1-3 gai-t6 rga-t2 rgl1-1 plants at 18 hrs post treatment although the three MYBs are highly expressed at this time point, suggesting that destabilization or inactivation of DELLAs proteins is unlikely the cause for JA-induced expression of MYBs in ga1-3 gai-t6 rga-t2 rgl1-1. Alternatively, it is possible that GA suppresses DELLAs to promote JA production or modulate JA-signaling to induce the expression of the three MYBs. The fact that JA application can restore the expression of the three MYBs in the GA-deficient background strongly suggests that, at least in part, JA biosynthesis is impaired in the ga1-3 gai-t6 rga-t2 rgl1-1 mutant.

JA biosynthesis is accomplished by a sequential biochemical reactions mediated by JA-biosynthesis genes including DADI, LOX1, 2, AOS, AOC1, 2, 3, 4 and OPR3 and is regulated by OPDA compartmentalization and a JA-mediated positive feedback loop [41]. Biotic and abiotic stresses also induce JA formation [42–44]. In our experiment, we found that JA biosynthesis gene DADI was greatly down-regulated in both ga1-3 single and ga1-3 gai-t6 rga-t2 rgl1-1 quadruple mutants but partially restored to a relatively high level in the penta mutant, suggesting that GA is required for the expression of DADI to control the production of JA via repression of DELLAs proteins. In flowers of *dadi* null mutant, the JA levels were only 22% of that of WT [40], demonstrating that limited initial substrate generation by DADI reaction acts as a control point for JA biosynthesis in flowers. Therefore, it is highly possible that reduced expression of DADI in ga1-3 gai-t6 rga-t2 rgl1-1 or ga1-3 mutant may result in relative low JA production. This hypothesis is strongly supported by the observation that JA content was greatly reduced in the young flower buds of the GA-deficient quadruple mutant ga1-3 gai-t6 rga-t2 rgl1-1 (Q3 mutant). Furthermore, the fact that the induction of DADI expression happens prior to the expression of MYB57 by GA in the ga1-3 gai-t6 rga-t2 rgl1-1 mutant strongly support our hypothesis that GA may regulate the MYB57 expression via mobilization of the biosynthesis of JA. A recent report showed that DADI expression is directly controlled by AGAMOUS (AG) [45]. Interestingly, Yu et al. reported that AG expression was down regulated in the GA-deficient mutant ga1-3 and exogenous GA application promoted the AG expression [20]. It will be interesting to study if there is a relationship among DELLAs, AG and DADI in the future. High level of JA would induce the expression of the three MYB genes essential for stamen development. In addition to DADI, we also observed that expression of LOX1 was down-regulated in ga1-3 mutant and restored to the WT level in the penta mutant. On the other hand, another JA biosynthesis gene AOC2 was up-regulated in ga1-3 mutant. These observations suggested that GA may be one of the endogenous signal involved in the regulation of JA biosynthesis genes.

Genetic studies have shown that MYB21, MYB24 and MYB57 are indispensable for stamen development. The stamen phenotype of myb21-t1 myb24-t1 myb57-t1 triple mutant is similar to that of JA-deficient mutants including opr3 and dadi mutants. Overexpression of MYB21 restored the stamen filament elongation and fertility to the opr3 flowers, strongly suggesting that JA-mediated stamen filament growth is mainly through the MYB pathway. Both ga1-3 single and ga1-3 gai-t6 rga-t2 rgl1-1 quadruple mutants showed a more severe flower phenotype than myb21-t1 myb24-t1 myb57-t1 triple mutant. The fact that expression of these MYB5 in ga1-3 gai-t6 rga-t2 rgl1-1 plants was not enough to rescue the mutant flower phenotype indicates that these MYB5 are necessary but not sufficient for GA-mediated floral development. These data also indicate that modulation of JA pathway may be only one of the branches of GA function in regulating stamen development.

Active cross-talk between different hormone signaling pathways have been revealed in many developmental processes [46]. For example, it was reported that auxin was necessary for GA-mediated *Arabidopsis* root growth by promoting GA-dependent degradation of DELLA proteins [47]. In contrast, ethylene inhibits *Arabidopsis* root growth by delaying the GA-induced destabilization of DELLA [48]. Recently, the complexity of interactions between ethylene and GA signal transduction pathways were analyzed by using combinations of different ethylene and GA related mutants [49]. Hormone-hormone interaction also plays an important role in controlling flowering. For example, it was found that stress induced hormone ethylene control floral transition via DELLA-dependent regulation of floral meristem-identity genes LEAF and SUPPRESSOR OF OVEREXPRESS of CONSTANS I (SOC1) [50]. We have here established a linear relationship between GA and JA in that GA modulates the expression of DADI that in a likely scenario to promote JA biosynthesis and in return JA induces the expression of MYB21, MYB24 and MYB57 to control the normal stamen development in *Arabidopsis*.

**Materials and Methods**

**Plant Materials**

Plants were grown as described previously [19]. Mutant lines (Le-er background) ga1-3, Q3 (ga1-3 gai-t6 rga-t2 rgl1-1) and penta (ga1-3 gai-t6 rga-t2 rgl1-1 rgl2-1) were described previously [8]. Mutant lines (Col-0 background) myb21-t1 (SALK_042711), myb24-t1 (SALK_017221) and myb57-t1 (SALK_065776) were...
obtained from Arabidopsis Biological Resource Centre at the Ohio State University [51] and verified using primer pairs listed in Table 3. These lines were backcrossed twice to purify the genetic background and were then used for all experiments described in this paper. Double mutants were generated from crosses between the relevant single mutants. Triple mutant myb21-11 myb24-11 myb57-11 was obtained from cross between myb21-11 myb24-11 and myb57-11. Hexa1 (ga1-3 ga6-16 nga-t2-1 nga1-1 nga2-1 myb21-t1), hexa2 (ga1-3 ga6-16 nga-t2-1 nga1-1 nga2-1 myb24-t1) and hepta (ga1-3 ga6-16 nga-t2-1 nga1-1 nga2-1 myb21-11 myb24-t1) mutants were in Ler background via cross-pollination of myb21-11 myb24-11 to the ga1-3 ga6-16 nga-t2-1 nga1-1 nga2-1 penta mutant four times. SEM of the penta, hexa1, and hepta mutants was performed as described previously [9]. The opr3 mutants is in theWs background [6].

Hormone Treatment

Both the ga1-3 ga6-16 nga-t2-1 nga1-1-1 (Q3) and opr3 mutants plants (~27 days old) were sprayed with mock (0.1% ethanol v/v), GA3 (10^{-3} M) [Sigma] or MeJA (0.015% v/v) [Sigma]. After treatment, young inflorescences were collected at different time point (18 hrs, 48 hrs, 72 hrs and 96 hrs) for total RNA extraction. For observing rescue of stamen development, mutant plants were repeatedly treated (once a week) with GA or JA.

RT-PCR and Northern Analysis

Different organs (sepal, petal, stamen, pistil and peduncle) of stage 11–12 flowers were dissected under microscope and pooled for RNA extraction. Flowers younger than stage 11 were pooled as young flower buds for RNA extraction. Total RNA was extracted from the young flower buds of respective genotypes treated with or without GA and JA using Tri Reagent (Molecular Research Center, Cincinnati, OH). The residue genomic DNA in the total RNA was removed via treatment with RNase-free DNase I [Invitrogen, USA]. The total RNA further purified with the RNA was purified with the RNA was purified with the Sep-pak C18 cartridge. The cartridge was washed with 300 μL of methanol, 30% methanol 300 μL of ddH2O was added to the combined extracts and this solution was applied onto the separation standard. Samples were analyzed by a Thermo TSQ Quantum Ultra LC-MS-MS system. 10 μL of sample was injected onto a Hypersil Gold column (150×2.1, 3 μm). The mobile phase comprised solvent A (0.1% formic acid) and solvent B (methanol) used in a gradient mode [time/concentration of A/concentration of B(min/%/%) for 0/90/10; 1/90/10; 10/10/90; 15/90/10; 16/90/10, 28/90/0]. The machine was run with a spray voltage 4800 v, atomization flow 30 mL/min, auxiliary flow: 2 mL/min, capillary temperature temperature 380°C, lens compensation voltage 77 v, molecular ions m/z 135 [JA], collision energy 15 eV and signal collection interval 15–19 min.

**CalMV35S::MYB21 Transgenic Plants**

For MYB21 overexpression construct, the Arabidopsis MYB21 was cloned into an overexpression vector using a primerF: (5'-agctctagaATggagaaag agggagagagc-3') and a primerR: (5'-atctctacactatcactatcactaatga-3') through XbaI and SacI sites. The overexpression vector, which was derived from pCAMBIA1301, contains the CaMV35S promoter to drive the expression of MYB21. The plasmids was confirmed by sequencing and introduced into Agrobacterium tumefaciens and then introduced into heterozygous opr3/opr3 plant by flower dip method [19]. More than 20 transformed lines were obtained based on PCR analysis. Homozygous opr3 mutants were identified using Opr3-RP (5'-tctaaatgtagagagaccg-3') and Opr3-UP (5'-GGCA-GAGTATTATGCTCAACG-3').

| Mutant lines | Primer pairs used for T-DNA insertion verification | Primer pairs used to amplify fragment spanning T-DNA insertion |
|--------------|-----------------------------------------------|---------------------------------------------------------------|
| myb21-11     | LBA1: TGGTCACAGCTAGGGGCACGCA              | 4334F: ATCGTGCCATTTTCCTCCAT                                      |
|              | 5355R: TTGATAATGACGTGAGTGAGAGA         | 5577R: CGCCGCCGGAAATGTTACATAGT                                  |
| myb24-11     | LBA1: TGGTCACAGCTAGGGGCACGCA              | 4566F: TGCCGATTCTACCAACAC                                       |
|              | 4975R: CTACATCTACGTCGAGCAAATAA          | 4975R: CTACATCTACGTCGAGCAAATAA                                   |
| myb57-11     | LBA1: TGGTCACAGCTAGGGGCACGCA              | 3411F: CATGGTAGAAGTGTCTTGGAACT                                    |
|              | 3411F: CATGGTAGAAGTGTCTTGGAACT            | 4511R: TAAACATAAACAACGCTCCTCCCT                                  |

Table 3. Primer pairs used for genotyping MYB mutants.
**Table 4. Gene specific primer pairs used in RT-PCR analysis.**

| Genes       | Primers                                    | Genes       | Primers                                    |
|-------------|--------------------------------------------|-------------|--------------------------------------------|
| AtMYB21     | 5' TCTAGGAGGACCAACTCCTACC 3'               | LOX1        | 5' GTGTCGGCTATTTATTGGTG 3'                 |
|             | 5' AAGTCGAGTCGCTTGTACATC 3'               |             | 5' GCTCTGACGCCCTTCCTGTG 3'                |
| AtMYB24     | 5' ATGGATGGTGTGTCAGAATGTC 3'              |             | 5' GCAGCTCGACGCGCTTCATCT 3'               |
|             | 5' AGAAGGAGTAAGCTTCTGCTGCTGCTGCT 3'       |             |                                           |
| AtMYB57     | 5' GTGGTACGCACTTGAACGGG 3'                |             |                                           |
|             | 5' TACGCAAGAAGGAGAAAGCT 3'               |             |                                           |
| GA2ox1      | 5' AATCATCGCTGCACTACAGTG 3'              | AOC2        | 5' CAGGAGACATCGATGACTGACTC 3'             |
|             | 5' AAGCGGGAACCATGAGGGA 3'                |             |                                           |
| GA3ox1      | 5' TCTAGGAGGAGGAAGGTCTTCTGCTGCTGCTGCT 3' |             |                                           |
| GA20ox2     | 5' AAGTGTCTATGACGTTGCTGCTGCTGCTGCTGCTGCT 3' |             |                                           |
| DAD1        | 5' GGCGGGGCAAGGAAGGAAAGCTCT 3'           | OPR3        | 5' CAGGGAACCCACAGGAAAGCTCTC 3'            |
|             | 5' GTTTCGAGGCTCTGCTGCTGCTGCTGCTGCTGCT 3' |             |                                           |
| LOX1        | 5' TTCTAGGAGGAGGAAGGTCTTCTGCTGCTGCTGCT 3' |             |                                           |
|             | 5' AAGGTGGTTACGCTGCTGCTGCTGCTGCTGCTGCT 3' |             |                                           |

**pMYB24::GUS Transgenic Plants**

To make the pMYB24::GUS construct, a 3098 bp (68 bp upstream of MYB24 start codon ATG) genomic DNA fragment was PCR amplified from Col-0 genomic DNA using primers 18F (PstI, 5' TCTAGGAGGACCAACTCCTACC 3') and 2934R (5' GTGTCGGCTATTTATTGGTG 3'). PCR amplifications of promoter regions were performed using PhiTaq DNA polymerase (Stratagene). The amplified DNA fragment was digested with PstI and then cloned into PstI/Ncol-cleaved pCAMBIA 1301 vector and their sequences were confirmed by sequencing. The pMYB24::GUS fusion construct was then introduced into Arabidopsis thaliana ecotype Col-0 plants using flower dip method [19]. More than three independent lines were examined at various stages of floral development in this study.

**In Situ Hybridization**

Whole inflowers which included unopened flower buds were fixed and in situ hybridization was carried out as described before [8]. Antisense and sense probes of MYB21 (+294 to +801 nt, nt stands for nucleotides, the A of the start codon ATG = 1) for in situ hybridization were DIG-labeled by in vitro transcription.

**Supporting Information**

**Figure S1** Phylogenetic Tree Showing the Relationship among MYB21, MYB24, and MYB57 and Other MYBs. Found at: doi:10.1371/journal.pgen.1000440.s001 (9.39 MB TIF)

**Figure S2** MYB21 Expression Patterns. (A) Cross section of an anther showing that MYB21 is expressed in the vascular tissue. VT, vascular tissue. (B) Transverse section of a stamen showing that MYB21 is expressed in the region linking stamen filament and the anther where fast cell elongation occurs. Found at: doi:10.1371/journal.pgen.1000440.s002 (6.07 MB TIF)

**Figure S3** MYB24 Expression Patterns. (A) GUS staining of a young inflorescence from a pMYB24::GUS plant. (B-E) GUS staining of flowers at various stages after floral stage 11. GUS activity is clearly detectable after the floral stage 12 (C). Flowers were sequentially taken from the same inflorescence. Found at: doi:10.1371/journal.pgen.1000440.s003 (10.33 MB TIF)

**Figure S4** Analysis of Stamen and Pistil Length in Different MYB Mutants. (A) Ratio of length of stamen to its respective pistil in flowers at the floral stage 12. (B) Number of flowers used in the analysis in (A). Found at: doi:10.1371/journal.pgen.1000440.s004 (13.51 MB TIF)

**Figure S5** myb21-t1 myb24-t1 Is Epistatic To ga1-3 gai-t6 rga-t2 rgl2-1. Pictures showing whole inflowers from hepta (myb21-t1 myb24-t1 ga1-3 gai-t6 rga-t2 rgl2-1), hexa1 (myb21-t1 ga1-3 gai-t6 rga-t2 rgl2-1) and hexa2 (myb24-t1 ga1-3 gai-t6 rga-t2 rgl2-1) mutants. Found at: doi:10.1371/journal.pgen.1000440.s005 (5.91 MB TIF)

**Figure S6** coi1 Mutation Is Epistatic To ga1-3 gai-t6 rga-t2 rgl2-1 (penta) in Stamen Filament Elongation. Flowers from different genotypes at the floral stage 14 were compared. (A) Latex WT; (B) penta mutant; (C) coi1 mutant; (D) coi1 penta mutant. Found at: doi:10.1371/journal.pgen.1000440.s006 (6.83 MB TIF)

**Table S1** List of Primers Used in Identifying Stamen-Enriched Genes. Found at: doi:10.1371/journal.pgen.1000440.s007 (0.06 MB DOC)

**Table S2** JA Contents in Young Flower Buds. Found at: doi:10.1371/journal.pgen.1000440.s008 (0.03 MB DOC)

**Author Contributions**

Conceived and designed the experiments: DX JP. Performed the experiments: DX SS LX HMS ZC. Analyzed the data: DX SS LX ZC. Contributed reagents/materials/analysis tools: LX. Wrote the paper: DX JP.
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