

# MIKC\* MADS Domain Heterodimers Are Required for Pollen Maturation and Tube Growth in *Arabidopsis*<sup>1[W][OA]</sup>

Benjamin J. Adamczyk and Donna E. Fernandez\*

Department of Botany, University of Wisconsin, Madison, Wisconsin 53706–1381

MADS box genes encode transcription factors that play important regulatory roles at various stages in plant development. Transcripts encoding the MIKC\*-type (for MADS DNA-binding domain, Intervening domain, Keratin-like domain, and C-terminal domain) factors, a divergent clade, are enriched in mature pollen. Previous studies have shown that these proteins bind DNA as heterodimers, which form between S- and P-class MIKC\* proteins. In this study, *Arabidopsis* (*Arabidopsis thaliana*) pollen with little or no MIKC\* activity was produced by combining strong loss-of-function alleles of the S-class proteins AGAMOUS-LIKE66 (AGL66) and AGL104. Double mutant plants produce pollen but have severely reduced fertility due to reduced pollen viability, delayed germination, and aberrant pollen tube growth. Microarray analysis of the mutant pollen revealed that the loss of MIKC\* regulation has a major impact on pollen gene expression. Pollen competition assays involving various combinations of AGL65, AGL66, AGL104, and AGL94 mutant alleles provided genetic evidence that at least three heterodimers (AGL30-AGL104, AGL65-AGL104, and AGL30-AGL66) form and function in at least a partially redundant fashion in pollen. Analyses of transcript abundance in wild-type and mutant pollen indicated that AGL65-containing complexes are likely to be more abundant than the others and that accumulation of AGL30 and AGL94 transcripts increases in response to reductions in MIKC\* activity. These results were combined to create a model to describe MIKC\* heterodimer contributions in pollen.

The male gametophyte in angiosperms is highly specialized. During anther development, a microspore mother cell divides meiotically, giving rise to four haploid microspores, and each microspore then divides mitotically to create a vegetative cell and a generative cell. In *Arabidopsis* (*Arabidopsis thaliana*), the generative cell will divide once again prior to pollen maturity to create two sperm cells, which are enclosed within the larger vegetative cell (Boavida et al., 2005). Mature pollen has the unique characteristic of being able to respond to specific environmental cues with carefully regulated polar growth. Upon landing on a papilla cell, a pollen grain will adhere, rehydrate, and expand asymmetrically to form a pollen tube. The pollen tube must then navigate through the female reproductive tissues and into an unfertilized ovule. This activity is coordinated with the movement of the sperm cells, which travel down the pollen

tube to reach the egg and central cells of the female gametophyte (Wilhelmi and Preuss, 1997; Edlund et al., 2004). Much of the regulatory machinery that controls this carefully timed sequence of events has not been fully characterized.

The regulation of gene expression is likely to contribute to these processes. Recently, several groups have used microarray analyses to characterize the mRNA expression profiles of *Arabidopsis* pollen. These studies have revealed that the pollen transcriptome is distinct from that of sporophytic tissues (Pina et al., 2005) and changes as pollen develop to maturity. Transcript complexity has been found to decrease at the tricellular stage of development, relative to the unicellular and bicellular stages, and only 32% of the annotated genes in the *Arabidopsis* genome are expressed in mature pollen (Honys and Twell, 2004). This figure includes genes expressed in the sperm cells and genes expressed in the vegetative cells, which have distinct transcriptomes as well as distinct cell identities and functions (Borges et al., 2008). The presence of transcripts that encode transcription factors and the observation that actinomycin D, a transcription inhibitor, has an adverse effect on pollen germination and tube growth (Wang et al., 2008) suggest that transcription and transcriptional regulation are likely to play important roles during this developmental transition. Transcription factors that are preferentially expressed at this stage of the life cycle are particularly interesting in this regard. Among the transcripts that are specifically enriched during the late stages of pollen development are those encoding

<sup>1</sup> This work was supported by the University of Wisconsin-Madison Graduate School, the United States Department of Agriculture National Research Initiative Competitive Grants Program (grant no. 2001–35304–10887), and the National Science Foundation (grant no. IOS-0718598).

\* Corresponding author; e-mail dfernand@wisc.edu.

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[www.plantphysiol.org/cgi/doi/10.1104/pp.109.135806](http://www.plantphysiol.org/cgi/doi/10.1104/pp.109.135806)

members of the MIKC\* (for MADS DNA-binding domain, Intervening domain, Keratin-like domain, and C-terminal domain) clade of MADS domain regulatory factors.

The MADS box gene family in *Arabidopsis* is large and diverse and consists of at least 109 members (Kofuji et al., 2003; Parenicová et al., 2003; Nam et al., 2004). The family is divided into two large clades: type I and type II. There are 45 type II genes, which are also referred to as MIKC factors for the four domains they contain (see above; Alvarez-Buylla et al., 2000). The type II group can be further subdivided into 39 MIKC<sup>C</sup> and six MIKC\* (also called M- $\delta$ ) genes based on the inferred evolutionary history of the family (Henschel et al., 2002; Parenicová et al., 2003). MIKC\* proteins tend to have longer I domains and less-conserved K domains than the MIKC<sup>C</sup> proteins. Sequences encoding MIKC<sup>C</sup> and MIKC\* factors have been identified in many plant taxa, including bryophytes and lycopods, as well as in gymnosperms and angiosperms. This suggests that the MIKC\* and MIKC<sup>C</sup> genes have evolved independently for at least 450 million years (Henschel et al., 2002; Nam et al., 2004; Theissen et al., 2004).

A relatively small subset of the MADS domain gene family is expressed in pollen. Five of the six MIKC\* factors are pollen enriched (i.e. their transcripts accumulate at higher levels in pollen than at any other stage of the life cycle). We previously reported that *AGAMOUS-LIKE67* (*AGL67*; *At1g77950*) is preferentially expressed in embryonic tissue and that *AGL30* (*At2g03060*), *AGL65* (*At1g18750*), *AGL66* (*At1g77980*), *AGL94* (*At1g69540*), and *AGL104* (*At1g22130*) transcripts could be detected in inflorescence tissues (Lehti-Shiu et al., 2005). It has since been reported that the latter five genes are expressed specifically in pollen and that *AGL67* is not expressed in pollen (Kofuji et al., 2003; Verelst et al., 2007a, 2007b). In addition, transcripts of five other MADS box genes (*AGL18*, *AGL29*, *AGL49*, *AGL84*, and *At4g14530*) appear to be enriched in pollen relative to other stages of development (Kofuji et al., 2003; Honys and Twell, 2004; Pina et al., 2005). *AGL18* is the only MIKC<sup>C</sup>-type gene in this group, and it has been reported to act redundantly with *AGL15* to repress the transition to flowering (Adamczyk et al., 2007). The others encode type I factors of unknown function. While a role in pollen development has not been established for type I or MIKC<sup>C</sup> MADS box genes, the disruption of MIKC\* genes has been reported to have negative effects on pollen competitive ability (Verelst et al., 2007b).

The *Arabidopsis* MIKC\* genes can be further subdivided into two groups, which represent monophyletic lineages. Proteins homologous to *AGL66*, *AGL67*, and *AGL104* in *Arabidopsis* have been designated as S-class proteins, while those homologous to *AGL30*, *AGL65*, and *AGL94* have been designated as P-class proteins. Analysis of rice (*Oryza sativa*), tobacco (*Nicotiana tabacum*), and *Arabidopsis* sequences has revealed that proteins in the same class (either S or P)

in different species are more closely related than two proteins from different classes within the same species (Nam et al., 2004). The S and P class distinction may have a functional significance. MADS domain proteins must form dimers in order to bind to their DNA targets (Riechmann et al., 1996), and previous studies have indicated that MIKC\* dimers include one P-class and one S-class protein (Parenicová et al., 2003; de Folter et al., 2005; Verelst et al., 2007a). Based on in vitro DNA-binding assays, it does not appear that MIKC\* proteins can homodimerize or that heterodimers of only P-class proteins or only S-class proteins can form (Verelst et al., 2007a). Five heterodimers have been postulated to form in pollen: *AGL65-AGL66*, *AGL65-AGL104*, *AGL30-AGL66*, *AGL30-AGL104*, and *AGL66-AGL94*. The first four heterodimer interactions have also been observed in planta using bimolecular fluorescence complementation in transiently transformed tobacco cells (Verelst et al., 2007a). Heterodimers have been shown to preferentially bind in vitro to MEF2-type CArG boxes of the form CTA(A/T)<sub>4</sub>TAG or N9-type sequences of the form CTA(TTT/AAA)TAG (Verelst et al., 2007a). Approximately 23% of the genes that encode tricellular- and mature pollen-specific transcripts contain a MEF2-type CArG motif within the 3-kb region upstream of the translational start site (Verelst et al., 2007a).

MIKC\* heterodimers may be major regulators of pollen maturation programs. Previous work has shown that 83.4% of transcripts that are enriched in mature pollen are down-regulated when the levels of MIKC\* heterodimers are reduced (Verelst et al., 2007b). Given the breadth of these transcriptional changes, it is surprising that the biological effects of the loss-of-function mutations that have been examined to date are not more pronounced. In vitro pollen germination rates are reduced in some mutant backgrounds (Verelst et al., 2007a), and some mutant combinations cause a decrease in the ability of pollen to compete for fertilization (Verelst et al., 2007b). However, the mutant plants are fully fertile, and in vivo changes in pollen germination and tube growth were not detected. The combinations of mutant alleles used previously may not have caused a sufficient disruption of MIKC\* proteins. We have combined strong loss-of-function alleles of the two pollen-expressed S-class genes *AGL66* and *AGL104* to generate plants that should lack all of the predicted heterodimers. The severely reduced fertility that the double mutant plants exhibit provides experimental support for the heterodimer model and establishes the essential nature of the contribution of the MIKC\* factors to plant reproduction. These plants showed reduced pollen viability, delayed pollen germination, and aberrant pollen tube growth, coupled with extensive changes in gene expression. Pollen competition assays and analyses of transcript abundance were used to establish the relative abundance and function of individual complexes and revealed that the system is marked by both a high degree of redundancy and a capacity for self-regulation.

## RESULTS

### Loss of MIKC\* Activity Affects Pollen Performance

To investigate the contributions of MIKC\* factors in vivo, T-DNA alleles were isolated for four of the five pollen-expressed MIKC\* genes. All of the MIKC\* loci, with the exception of *AGL30*, are located on chromosome I. A mutant allele could not be obtained for *AGL30*. One mutant allele each was isolated for *AGL65*, *AGL66*, and *AGL94*, and two alleles were isolated for *AGL104* (*agl104-1* and *agl104-2*). Reverse transcription (RT)-PCR using primers flanking the T-DNA insertion sites revealed that full-length transcripts accumulate at reduced levels in *agl104-1* pollen, while full-length transcripts could not be detected in the *agl104-2*, *agl65*, *agl66*, and *agl94* backgrounds. Transcript could be detected, however, using primers upstream of each T-DNA insertion. Additional information about the mutant alleles is provided in Supplemental Figures S1 and S2.

Each single mutant allele should result in the reduction or loss of two or more of the five predicted functional heterodimers: AGL66-AGL30, AGL66-AGL65, AGL66-AGL94, AGL104-AGL30, and AGL104-AGL65 (Verelst et al., 2007a, 2007b). To quantify the effect on pollen function, pollen competition assays were performed by placing pollen from a heterozygous plant onto a wild-type stigma and allowing pollen tubes to compete for unfertilized ovules (Fig. 1A). Progeny were then genotyped to determine the transmission rates. Single mutant pollen carrying *agl65*, *agl66*, *agl94*, *agl104-1*, or *agl104-2* alleles were indistinguishable from the wild type in terms of success as a pollen parent (Supplemental Fig. S3). Thus, loss or reduction of any two of the predicted heterodimers, or any set of heterodimers that include a common S protein, does not affect pollen function. This suggests that AGL66 and AGL104, in addition to being similar structurally, may act in a redundant fashion in MIKC\* regulatory complexes.

The simultaneous removal of AGL66 and AGL104 should eliminate all of the predicted MIKC\* complexes in pollen. To test this, competition assays were performed with pollen from a plant that was heterozygous for *agl104-2* and homozygous for *agl66* and pollen from a plant that was heterozygous for *agl66* and homozygous for *agl104-2*. In both cases, transmission of the double mutant combination was significantly lower than the expected rate of 50% (Fig. 1B). Transmission was reduced to 12% in one case ( $n = 50$ ) and not detected in the other ( $n = 29$ ). Thus, the loss of all MIKC\* complexes has a severe effect on pollen performance, but at least some of the double mutant pollen appear to be capable of successful fertilization.

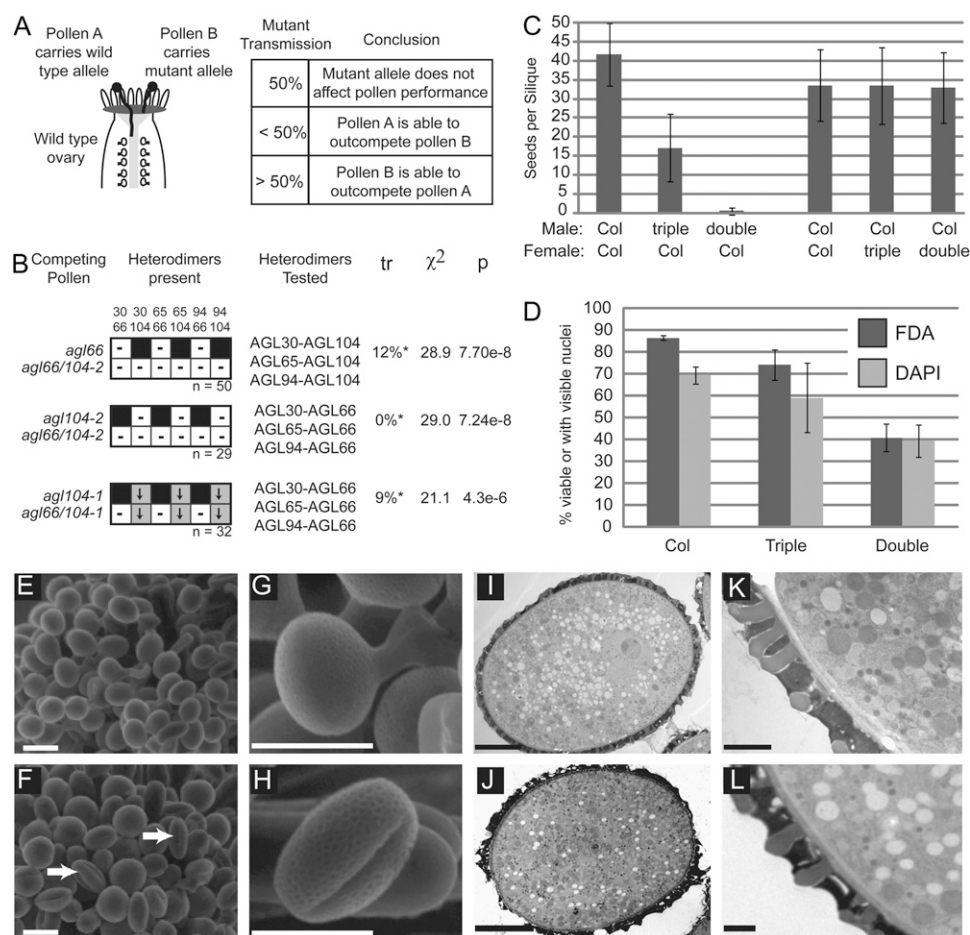
### *agl66 agl104* Gametophytes Are Compromised in Vivo

Although the *agl66 agl104-2* double mutant combination can be transmitted through pollen at rates as

high as 12% on wild-type females, recovering double homozygous plants following self-pollination of plants that were homozygous for *agl66* and heterozygous for *agl104-2* proved to be difficult. We were successful only when a limited quantity of pollen was placed onto the stigma of an emasculated parent, thereby minimizing pollen competition. Several homozygous double mutant plants were recovered, and each produced less than one seed per silique on average when allowed to set seed without intervention (Supplemental Fig. S4). Aside from the severely reduced fertility, homozygous plants were indistinguishable from wild-type plants. To further quantify the fertility defect, wild-type plants were emasculated and hand-pollinated with saturating amounts of wild-type and *agl66 agl104-2* pollen. Seed yield was reduced by 98% in the double mutant relative to the wild type. For comparison, the same test was performed using pollen from the previously described *agl65 agl66 agl104-1* mutant (Verelst et al., 2007b), which carries a weak allele of *agl104-1*, and a 59% reduction in seed yield was seen (Fig. 1C). However, when wild-type pollen was crossed onto females with these three genotypes, seed set was not affected. This indicates that the reduced fertility of the mutant plants is primarily due to a pollen defect and that the double mutant pollen is more strongly affected than the triple mutant. It also shows that even without competing pollen, double mutant pollen is severely compromised and has a low chance of successful fertilization.

To determine the basis of the defects in the *agl66 agl104-2* pollen, various assays were performed. A fluorescein diacetate (FDA) assay was used to examine pollen viability (Fig. 1D). Double and triple mutant pollen viability was reduced by 48% and 12% relative to the wild type, respectively. Pollen were also exposed to 4',6-diamidino-2-phenylindole (DAPI) in order to visualize nuclei in the mutant pollen. Approximately 40% of the double mutant pollen had at least one visible nucleus, while one or more nuclei were visible in approximately 70% of wild-type pollen (Fig. 1D). To examine pollen hydration on the stigma surface, scanning electron microscopy was used (Fig. 1, E–H). Pollen from wild-type individuals were able to adhere and rehydrate when in contact with a papilla cell, but double mutant pollen frequently failed to rehydrate.

These observations suggest that defects in pollen development occur prior to or during the final stage of maturation in *agl66 agl104-2* pollen. To determine whether there was a consistent cellular basis for the defect, stage 11 anthers (Smyth et al., 1990) were collected from wild-type and double mutant plants and pollen ultrastructure was examined by transmission electron microscopy. At this stage of development, pollen are primarily tricellular (Durberry et al., 2005) and are near maturity. Many of the pollen showed signs of structural damage, which may have occurred either before or during processing. However, at least some double mutant pollen were observed that

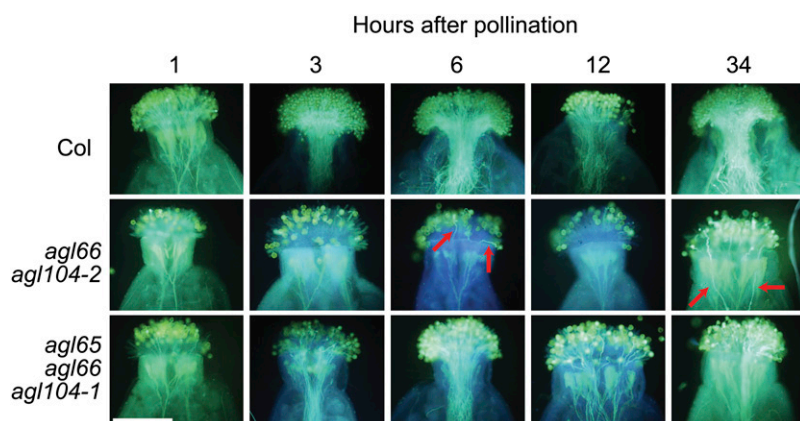


**Figure 1.** Analysis of *agl66 agl104-2* pollen. **A**, A summary diagram describing how pollen competition assays were carried out. **B**, Competition assays show that *agl66 agl104-2* pollen is significantly compromised relative to single mutant pollen. Shaded boxes illustrate which heterodimers (listed above the boxes) are present (black boxes), absent (white boxes), or reduced by the *agl104-1* weak allele (gray boxes). Transmission of the lower mutant genotype (tr),  $\chi^2$  statistic ( $\chi^2$ ), and *P* value (p) are also shown for each cross. Outcomes that differ significantly from the expected value of 50% ( $P < 0.01$ ) are indicated with an asterisk. **C**, Fertility is significantly reduced when double or triple mutant pollen is placed onto a wild-type female, but fertility is not affected by the genotype of the female.  $n \geq 13$  siliques for each sample. Col, Ecotype Columbia. **D**, Double and triple mutant pollen had reduced viability relative to the wild type as assessed by a FDA viability assay. Fewer double and triple mutant pollen had visible nuclei after pollen were exposed to DAPI. **E** to **H**, Both wild-type pollen (**E** and **F**) and double mutant pollen (**G** and **H**) adhere to wild-type stigmas, but some mutant pollen in contact with papilla cells fail to fully rehydrate (arrows). Bars = 20  $\mu$ m. **I** to **L**, Transmission electron microscopy images of mature wild-type pollen (**I** and **K**) and double mutant pollen (**J** and **L**). No ultrastructural changes are visible in the cytoplasm or cell wall of the double mutant pollen. Bars = 5  $\mu$ m in **I** and **J** and 1  $\mu$ m in **K** and **L**.

were indistinguishable from wild-type pollen (Fig. 1, I–L). These may represent the subset of double mutant mature pollen that are viable and capable of rehydration on the stigma.

Pollen tube growth was examined *in vivo* by clearing fertilized flowers and staining with aniline blue. *agl66 agl104-2*, *agl65 agl66 agl104-1*, and wild-type pollen were each placed onto wild-type stigmas and given various lengths of time, up to 34 h, to germinate and elongate before being processed (Fig. 2). By 1 h after pollination, both wild-type and triple mutant pollen had produced a large number of pollen tubes. A few pollen tubes could be seen 3 h after pollination

with double mutant pollen; however, very few pollen had germinated and the tubes rarely extended beyond the papillae cells. At 12 h after pollination, a few elongated double mutant pollen tubes were seen in the transmitting tract of some gynoecia, although most did not have any visible pollen tubes. In pollinations with wild-type and triple mutant pollen, on the other hand, the transmitting tract was packed with pollen tubes after only 3 h. We conclude that limited and delayed pollen tube growth in the double mutant is a likely basis for the reduced fertility of the double mutant and the low rate of allele transmission in the presence of competing pollen.



**Figure 2.** Aniline blue staining of pollen tubes in vivo. Pollen were placed onto wild-type stigmas and stained to visualize pollen tube growth defects in MIKC\* mutant backgrounds. Pollen tube growth was inhibited in *agl66 agl104-2* pollen, while the growth of *agl65 agl66 agl104-1* pollen tubes was not consistently distinguishable from that of the wild type. Red arrows indicate *agl66 agl104-2* pollen tubes. Bars = 0.25 mm for all images. Col, Ecotype Columbia.

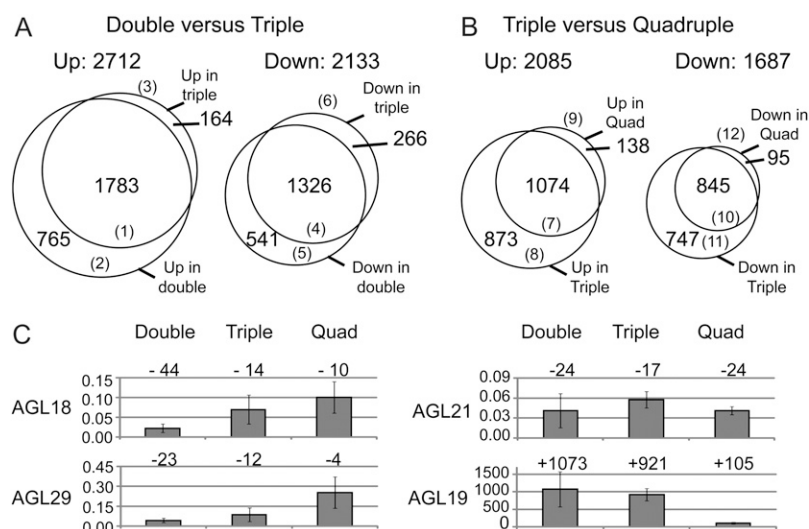
### Analysis of Target Genes

The *agl66 agl104-2* double mutant provided us with a useful tool to identify genes that are indirect or direct targets of MIKC\* regulatory activity. RNA was extracted from wild-type and mutant pollen, and cDNA was hybridized to Nimblegen whole-genome microarray chips. We also included *agl65 agl66 agl104-1* pollen in the analysis so our data could be directly compared with the Affymetrix chip results of Verelst et al. (2007b) and *agl65 agl66 agl94 agl104-1* pollen in order to examine pollen in which only AGL30-AGL104 complexes should be present. Purity of the pollen samples was verified by confirming that the expression of selected petal-, sepal-, stem-, and leaf-specific genes had low signal values (Supplemental Data File S1). Sixty-two percent of the genes were eliminated from the data set because of low expression levels or high variability between biological replicates. Of the remaining 11,612 genes, 2,712 (23.4%) were significantly up-regulated in the *agl65 agl66 agl104-1* or *agl66 agl104-2* mutant, while 2,133 (18.4%) were significantly down-regulated.

There was significant overlap between the double, triple, and quadruple mutant data sets, and a unique

set of regulated genes also appeared in each (Fig. 3, A and B). A higher number of genes were significantly affected in the double mutant than in the triple mutant. However, of the 1,783 genes significantly up-regulated in both backgrounds, 54% had a stronger fold change in the triple mutant than in the double mutant. Of the 1,326 genes that were down-regulated, 68% were more strongly affected in the triple mutant than in the double mutant. While the basis for this is unknown, one possibility is that compromised but viable pollen represent a larger portion of the population in the triple mutant samples. In the double mutant, severely compromised pollen die and do not contribute to the RNA pool. In comparing the triple and quadruple mutant data sets, with the exception of AGL94 itself, very few genes showed a strong fold change between the triple and quadruple mutant samples. A summary of differentially expressed genes is provided in Supplemental Data File S1.

Among the target genes discovered by microarray analysis were 11 non-MIKC\* MADS box genes. Probes for two of them, AGL52 and AGL97, are not available on the ATH1 Affymetrix array, and their expression in pollen has not been previously reported.



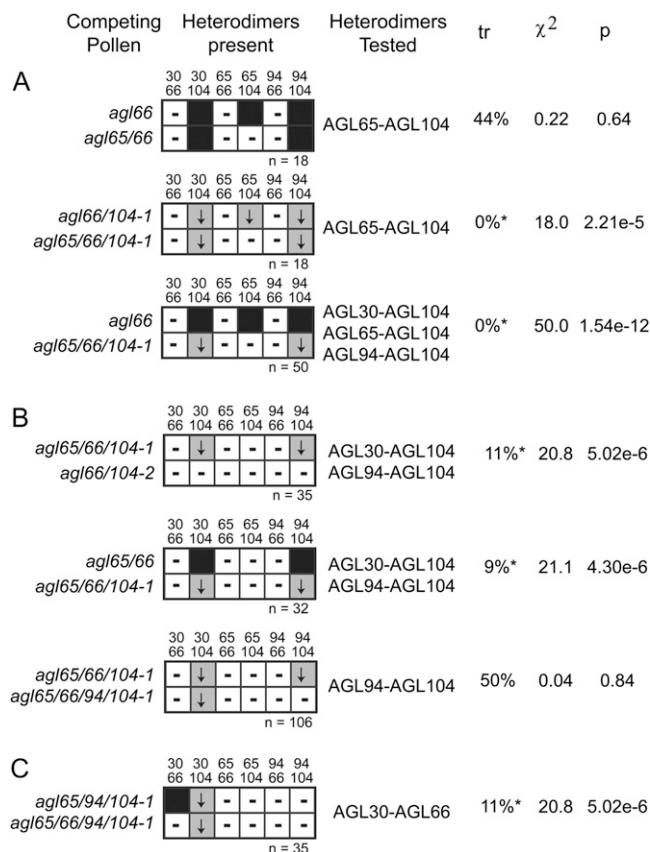
**Figure 3.** Analysis of MIKC\* target genes. A, Diagrams show the number of differentially expressed genes in *agl65 agl66 agl104-1* and *agl66 agl104-2* mutant pollen relative to the wild type, according to microarray analysis. While many of the same genes show changes in both mutants, a larger number of genes are affected in the double mutant, and targets are both up- and down-regulated. Numbers in parentheses correspond to categories in Supplemental Data File S1. B, Comparison of target genes in the triple mutant and *agl65 agl66 agl94 agl104-1* pollen. C, Differential expression of selected non-MIKC\* MADS box genes was confirmed by quantitative RT-PCR. The y axis values and numbers above the bars indicate fold change relative to the wild type.

Four others, *AGL18*, *AGL19*, *AGL21*, and *AGL29*, were examined by quantitative PCR (Fig. 3C). *AGL18*, *AGL21*, and *AGL29* were strongly down-regulated in the double mutant, and *AGL18* and *AGL29* were similarly affected, but to a lesser degree, in triple and quadruple mutant backgrounds. *AGL19*, which shows little or no expression in wild-type pollen, is significantly up-regulated in the double mutant and may be affected to a lesser degree in the triple and quadruple mutant backgrounds. These data show that a broader MADS domain regulatory network exists in pollen and that at least some of its components are more strongly affected in the double mutant than in the triple mutant.

#### AGL65-AGL104, AGL30-AGL104, and AGL30-AGL66 Heterodimers Regulate Pollen Activity

The disruption of all MIKC\* activity has a severe effect on pollen performance, but no single MIKC\* protein appears to play an essential role. To probe the degree of redundancy and to determine whether particular heterodimers are functional in vivo, we compared the pollen performance of various mutant combinations through competition assays. Although there is no direct evidence that AGL94-AGL104 complexes form, this sixth heterodimer was also considered in our analysis because AGL104 and AGL66 are closely related and AGL94 has been shown to interact with AGL66. The weak *agl104-1* allele was used to determine whether quantitative differences in MIKC\* levels in pollen are also important. To minimize the effect of linkage and maximize the sensitivity, the competition assays were set up such that only one allele was segregating and there were only two possible genotypes in the progeny. According to this strategy, any outcome that is statistically different from 50% is significant.

In the first set of experiments (Fig. 4A), the effect of removing AGL65-AGL104 complexes was examined. For this, competitions were carried out between *agl66* and *agl65 agl66* pollen as well as between *agl66 agl104-1* and *agl65 agl66 agl104-1* pollen. In each case, the competing pollen differ with regard to the presence of AGL65-AGL104, but the inclusion of the weak *agl104-1* allele in the second experiment also reduces the levels of AGL30-AGL104 and AGL94-AGL104 heterodimers. We found that loss of AGL65-AGL104 has a significant effect on pollen performance, but only when the other AGL104-containing complexes are reduced. This was further confirmed by a competition with *agl66* pollen and *agl65 agl66 agl104-1* pollen (Fig. 4A). In this case, the transmission of the triple mutant combination was also severely compromised relative to the single mutant. These results suggest that the AGL104-containing complexes act in a partially redundant fashion, but the experiments also demonstrate that AGL65-AGL104 is functional and likely to regulate downstream targets that are important for pollen activity.



**Figure 4.** Pollen competition assays in higher order MIKC\* mutants. Competitions were carried out in various mutant backgrounds to eliminate functional redundancy between MIKC\* heterodimers and examine the contribution of specific heterodimers. Shaded boxes illustrate which heterodimers (listed above the boxes) are present (black boxes), absent (white boxes), or reduced by the *agl104-1* weak allele (gray boxes). Transmission of the lower mutant genotype (tr),  $\chi^2$  statistic ( $\chi^2$ ), and P value (p) are also shown for each cross. Outcomes that are significantly different from 50% ( $P < 0.01$ ) are indicated with an asterisk. The crosses that demonstrate AGL65-AGL104 (A), AGL30-AGL104 (B), and AGL30-AGL66 (C) activity contribute positively to pollen competitive ability.

Next, the roles of AGL30-AGL104 and AGL94-AGL104 were examined by comparing *agl66 agl104-2* double mutant pollen with *agl65 agl66 agl104-1* triple mutant pollen, which contain only these two heterodimers, at lower levels than in the wild type. In this case, the *agl104-2* T-DNA was transmitted at a frequency of 11%, suggesting that one or both of these two heterodimers control genes that confer a competitive advantage (Fig. 4B). An additional assay comparing *agl65 agl66 agl104-1* and *agl65 agl66* pollen showed that simply reducing the levels of these heterodimers is sufficient to reduce competitive ability. In this case, the *agl104-1* allele had a 9% transmission frequency, suggesting that these heterodimers do not need to be completely eliminated in order to see an impact on pollen function.

Although physical interactions between AGL94 and AGL104 have not been detected previously (Verelst et al., 2007b), genetic tests might reveal the existence of this heterodimer through its contributions to pollen function. To test this, *agl65 agl66 agl104-1* and *agl65 agl66 agl94 agl104-1* pollen were used in a competition experiment (Fig. 4B). While both pollen contain AGL30-AGL104 at reduced levels, the AGL94-AGL104 heterodimer should be more strongly disrupted in the quadruple mutant. The quadruple mutant pollen was not at a disadvantage relative to triple mutant pollen, suggesting that the elimination of AGL94-AGL104 (if it exists) did not contribute to the transmission defects in two previous competition assays. We conclude that AGL30-AGL104 is sufficient to produce largely functional pollen.

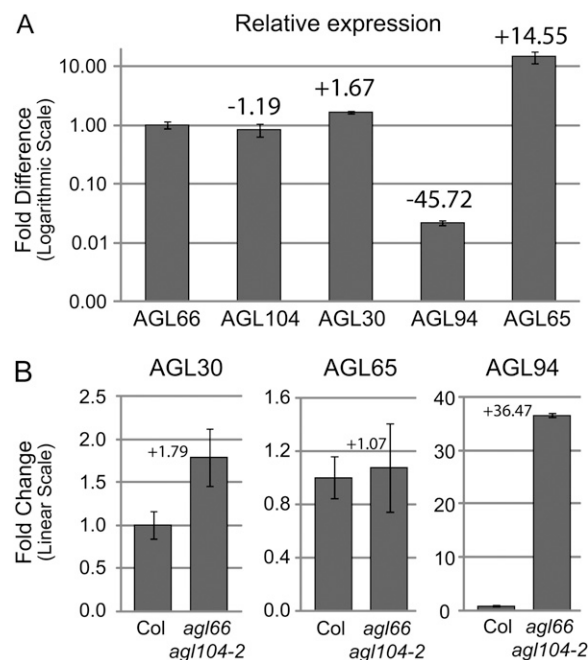
A previous competition experiment had shown that *agl65 agl66 agl104-1* pollen is compromised relative to *agl65 agl104-1* pollen (Verelst et al., 2007b). Because the triple mutant is deficient in AGL30-AGL66 and AGL94-AGL104, this result suggested that AGL30-AGL66 is also a functional heterodimer. To confirm this, a competition was performed between *agl65 agl94 agl104-1* and *agl65 agl66 agl94 agl104-1* pollen (Fig. 4C). Although both pollen contain low levels of AGL30-AGL104, the impact of losing AGL30-AGL66 is clear and severe. The *agl66* mutant allele transmission was 11%, suggesting that AGL30-AGL66 has a major regulatory role in pollen.

Taken together, these pollen competition experiments provide evidence for *in vivo* function for at least three MIKC\* heterodimers. They also show that MIKC\* heterodimers contribute quantitatively and in a partially redundant fashion in Arabidopsis pollen.

### Analysis of Relative Transcript Abundance

Given that functional redundancy exists between MIKC\* heterodimers, their relative abundance may be an important factor that determines the strength of their contributions in pollen. Quantitative RT-PCR was used to measure mRNA transcript levels for the five pollen-expressed MIKC\* genes in mature wild-type pollen (Fig. 5A). Less than a 20% difference in transcript accumulation was detected for the S-class proteins AGL66 and AGL104. Thus, if translation and turnover rates are equal, the abundance of AGL66- and AGL104-containing heterodimers should be similar. In contrast, transcript levels differ by greater than 2 orders of magnitude between the different P-class factors. AGL65 transcripts are more abundant than AGL30 transcripts, and both are more abundant than AGL66 and AGL104. AGL94, however, accumulates at much lower levels than other MIKC\* transcripts in pollen.

The effect of loss of MIKC\* activity on P-class transcript accumulation was also examined using quantitative RT-PCR (Fig. 5B). Changes in S-class transcript accumulation could not be examined be-



**Figure 5.** Quantitative RT-PCR analysis of MIKC\* transcript accumulation. A, Expression of MIKC\* genes in wild-type mature pollen. Numbers above the bars indicate fold differences in the transcript levels of various MIKC\* genes relative to AGL66 transcript levels. Note the logarithmic scale on the y axis. B, Expression of P-class MIKC\* genes in *agl66 agl104-2* mutant pollen. Numbers above the bars indicate fold change relative to wild type. The y axis is a linear scale. Col, Ecotype Columbia.

cause the mutant alleles changed the nature of the transcripts. In the pollen of *agl66 agl104-2* double mutants, AGL65 transcript accumulation was essentially unchanged relative to the wild type. AGL30 transcripts increased to almost twice the level found in the wild type. AGL94 transcripts became much more abundant, increasing 36-fold, but still below the AGL30 and AGL65 transcript levels in wild-type pollen. A similar up-regulation of AGL94 was also seen in *agl65 agl66 agl104-1* triple mutant pollen (data not shown).

## DISCUSSION

### MIKC\* Activity Is Required for Pollen Germination and Tube Growth

MIKC\* proteins have been proposed as major regulators of pollen maturation (Verelst et al., 2007b); however, definitive evidence that they play an essential role in plant reproduction has not been provided previously. Previous studies were limited by the lack of strong loss-of-function alleles for AGL30 and AGL104. In the most severe mutant combination reported previously (*agl65 agl66 agl104-1* triple mutant), the AGL30-AGL104 heterodimer is still present, although at reduced levels. Pollen competition assays show that pollen performance is impaired in the triple

mutant background, but the homozygous plants do not show an obvious fertility defect.

We have created plants that carry the stronger *agl104-2* allele along with the *agl66* allele, and severe defects in pollen function are apparent. This mutant combination should result in disruption of all of the predicted MIKC\* heterodimers, including AGL30-AGL104. Homozygous double mutant plants produce few seeds, and aniline blue staining showed that, even on wild-type females, pollen germination is delayed and limited and few pollen tubes extend into the transmitting tract. The mutant pollen that are capable of growing tubes may correspond to those that show few cellular alterations relative to the wild type. This suggests that the mutations are not fully penetrant, which is perhaps fortunate, because we would have failed to recover homozygous double mutant plants otherwise. This may be due to remaining, but not readily detected, full-length or truncated transcripts in the mutant backgrounds. Although the defects appear to be exclusively on the male side, it is interesting that the mutant pollen appears to function somewhat better in mixed pollinations, as in a competition assay, than when pollen from a homozygous plant is used. The double mutant combination is transmitted to up to 12% of the progeny when both single mutant and double mutant pollen are present (Fig. 1B), and we were able to recover homozygous double mutant plants after pollinations with a limited pollen load. However, it is extremely difficult to obtain seeds when a homozygous mutant self-pollinates or is used to pollinate a wild-type female plant. This suggests that the wild-type pollen may condition the transmitting tract in a way that facilitates the growth of mutant pollen. This may involve the release of signaling molecules from the pollen or female tissues or both.

Verelst et al. (2007b) have suggested that MIKC\* activity regulates the process of pollen maturation, based on their observation that transcripts enriched in mature pollen grains were more likely to be down-regulated in the triple mutant relative to transcripts that are enriched in immature tricellular pollen. Since their data, as well as our own, were derived from mature pollen RNA, it is possible that transcripts enriched in earlier stages of development are also down-regulated in immature mutant pollen. The phenotypes that were seen in the double mutant are consistent with their hypothesis, although MIKC\* proteins could also be acting during other stages of pollen development. Pollen viability is reduced in the double mutant, and although cell death could occur in earlier stages of development, the dead mutant pollen is comparable in size and shape to fully developed wild-type pollen. Pollen tube defects could be a consequence of developmental changes that occur prior to germination. However, it is also possible that MIKC\* proteins have a role within the growing pollen tube, as recent microarray data have shown that *AGL65*, *AGL66*, *AGL30*, and *AGL104* transcripts are enriched in pollen tubes (Wang et al., 2008). These four tran-

scripts are not enriched in isolated sperm cells (Borges et al., 2008), however, indicating that they are specific to the vegetative cell of the pollen tube.

### MIKC\* Heterodimers Form a Redundant and Dynamic Regulatory Network

Pollen performance was only inhibited in mutant backgrounds in which both *AGL66* and *AGL104* were disrupted, indicating that only one S-class protein is required for normal pollen activity. *AGL66* and *AGL104* represent a duplicated pair, which originated following a large-scale duplication of a portion of chromosome 1 (Blanc et al., 2000). They subsequently diverged in sequence, although the two proteins still share greater than 70% identity. Within the 60 codons that encode the MADS domain, there are 22 synonymous base pair changes and one nonsynonymous base pair change between *AGL66* and *AGL104*. The preference for synonymous changes indicates that there may be some advantage associated with the maintenance of two S-class proteins. The interaction of *AGL94* with *AGL66*, but not *AGL104*, in yeast two-hybrid assays (Verelst et al., 2007a) suggests that *AGL66* and *AGL104* may not be completely functionally redundant. It is possible that subfunctionalization has occurred and *AGL66* and *AGL104* now have unique as well as shared protein-protein interactions. It is interesting in this regard that rice also contains two S-class MIKC\* proteins. *OsMADS40* (Os08g38590) and *OsMADS108* (Os06g11970) are putative homologs of *AGL66* and *AGL104*, and they are both enriched in rice pollen according to massively parallel signature sequencing data (<http://mpss.udel.edu/rice/>). The presence of multiple S-class proteins may be ancient or evolutionarily favored, although it is not known whether the rice MIKC\* protein interactions resemble those in *Arabidopsis*.

Because the system is highly redundant, loss of individual complexes will only have a measurable impact on pollen performance in genetic backgrounds in which most of the other heterodimers are disrupted. By disrupting functionally redundant heterodimers, three MIKC\* heterodimers (*AGL65*-*AGL104*, *AGL30*-*AGL104*, and *AGL30*-*AGL66*) were identified as likely contributors to the regulation of pollen development. If protein abundance reflects transcript abundance and the capacity for heterodimer formation is similar for all three P-class factors, we can expect complexes containing *AGL65* to be at least 10 times more abundant than *AGL30*-containing complexes and over 600 times more abundant than *AGL94*-containing complexes. Because *AGL104* and *AGL66* transcripts are not as abundant as either *AGL65* or *AGL30* transcripts, the availability of S-class binding partners may limit complex formation in the system. Because there is potentially a large excess of *AGL65* and a smaller excess of *AGL30* in the system, elimination of either one would affect the composition of complexes but might not have a large impact on the total number of MIKC\*

complexes in the cell. While AGL65 heterodimers likely regulate the most targets in wild-type pollen, AGL30 heterodimers may take over some of those regulatory roles when AGL65 heterodimers are absent, as in *agl65* mutants. The feedback regulation of AGL30 and AGL94 potentially adds to the capacity of the system for compensation. When MIKC\* activity is limited (*agl66 agl104-2* or *agl65 agl66 agl104-1* mutants), AGL30 and AGL94 transcripts, and presumably proteins, become more abundant.

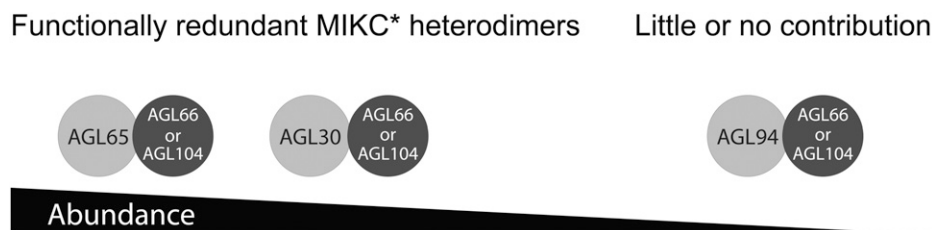
What role does AGL94 play in this regulatory system? If AGL94 heterodimers contribute, their low abundance may explain why AGL94 removal does not affect pollen performance in a measurable way when AGL30 heterodimers are present. Without a loss-of-function allele for AGL30, a definitive genetic test cannot be set up. Pollen competition assays showed that *agl66 agl94* double mutant pollen was comparable to either *agl66* or *agl94* single mutant pollen (data not shown) and *agl65 agl66 agl104-1* triple mutant pollen was comparable to *agl65 agl66 agl94 agl104-1* quadruple mutant pollen (Fig. 4B; Supplemental Fig. S5). Although these experiments do not provide evidence that AGL94 heterodimers form and function, neither do they preclude this. Heterodimers that include AGL94 may simply be functionally redundant with other MIKC\* heterodimers. The up-regulation of AGL94 in the double and triple mutants is not likely to have a negative impact, because quadruple mutant pollen performance was comparable to that of the triple mutant. The differences seen between quadruple and triple mutant regulatory targets could be due in part to the elevated expression of AGL94 in the triple mutant.

Based on the redundancy and relative expression levels of the MIKC\* genes, we propose a model for the relative contribution of MIKC\* heterodimers in pollen (Fig. 6). In this model, four of the five predicted heterodimers act in a partially redundant and quantitative manner to regulate pollen development. Any possible heterodimers that require AGL94 have little or no contribution in pollen. From this, we would predict that pollen from an *agl30 agl65* double mutant, if it could be obtained, would be comparable to *agl66 agl104-2* pollen. If this is not the case, it would provide

evidence that AGL94 complexes play important regulatory roles.

### MIKC\* Regulation Affects a Large Suite of Genes in Pollen

The dramatic phenotypic effects seen in the double mutant may be due to changes in a small number of pivotal genes or they may reflect the collective effect of changes in many genes. Of the 4,415 genes that show changes, only 654 (14.8%) contain at least one of the MEF2 or N9 CARG box target sequences that MIKC\* factors prefer (Verelst et al., 2007a; Supplemental Data File S1). Chromatin immunoprecipitation studies will be needed to determine how many of these potential binding sites are occupied by MIKC\* factors. Those that show binding may represent direct targets; however, genes that are indirect targets may be equally important for pollen viability and in vivo function. In order to identify potentially important direct and indirect target genes, one criterion that can be applied is the magnitude of the changes between the double and triple mutants, which show strong and weaker effects, respectively, on fertility. Of the 1,306 genes that were uniquely altered in the double mutant relative to the wild type (765 up-regulated, 541 down-regulated), only 251 of these genes showed a 1.5-fold or greater difference in expression in the double versus the triple mutant background. Of the 3,109 genes that changed in both the double and triple mutants relative to the wild type, a smaller set of 172 genes showed 1.5-fold or greater differences in expression in the two backgrounds (Supplemental Data File S1). The set of genes that emerged from this analysis included members of the LCR (low-molecular-weight Cys-rich) family of proteins. Of the 13 LCR proteins significantly affected in the double and triple mutant backgrounds, 12 were expressed at lower levels in the double mutant than in the triple mutant, and six fell into the more restricted categories described above. LCR proteins are related to members of the pollen coat protein family in *Brassica* (Vanoosthuyse et al., 2001), which has been shown to interact with S-locus proteins that mediate the self-incompatibility response (Takayama et al., 2001). The role of LCR proteins in Arabidopsis is not well under-



**Figure 6.** Model showing the relative contributions of different MIKC\* heterodimers in Arabidopsis pollen. AGL65-AGL104, AGL30-AGL104, and AGL30-AGL66 were each shown to contribute to pollen function. AGL94 may not play a major role in pollen when other P-class proteins are present. Redundancy exists between MIKC\* heterodimers, and differential levels of P-class transcript abundance likely affect the contribution of each heterodimer.

stood, and additional work will be needed to determine whether the down-regulation of LCR proteins in the double mutant contributes to the mutant phenotype.

The double mutant also shows differential regulation of 11 non-MIKC\*-type MADS box genes, suggesting that a broader MADS domain regulatory network is active in pollen. Within this set, quantitative RT-PCR showed that some MADS box genes, such as *AGL18*, *AGL19*, *AGL21*, and *AGL29*, may be more strongly affected in the double mutant (Fig. 3C). Mutant lines have been characterized for *AGL18* (Adamczyk et al., 2007), *AGL19* (Schonrock et al., 2006), and *AGL29* (Verelst et al., 2007b), but no pollen transmission defects have been reported. Thus, the reduced fertility of the double mutant cannot be attributed solely to the down-regulation of any of these individual factors. *AGL18* is the most highly expressed MADS box gene in pollen and is sharply down-regulated in double mutant pollen. *AGL18* transcript accumulation in 11-d-old *agl66 agl104-2* seedlings is similar to that in the wild type, however (data not shown), suggesting that MIKC\* factors only control the pollen portion of the expression pattern. This was also the case for the MADS box genes *MAF3* and *AGL19*, which show changes in mutant pollen but not in mutant seedlings (data not shown). Transcripts from the type I gene *AGL97* also accumulate at reduced levels in double mutant pollen. Of the eight non-MIKC\* proteins that were shown to interact with MIKC\* proteins in yeast two-hybrid assays (de Folter et al., 2005), *AGL97* is the only one that is expressed in pollen. Because *AGL97* was found to interact with both *AGL65* and *AGL104*, it may be a member of MIKC\* regulatory complexes.

Analysis of mutant pollen has shown that regulation by MIKC\* heterodimers is necessary for successful pollen development and function. Like some well-characterized MIKC<sup>C</sup> factors, MIKC\* proteins appear to act near the top of a regulatory hierarchy and their activity influences the expression of many downstream genes. This study has highlighted the importance of transcriptional regulation in pollen, and future work will be aimed at further characterizing additional players in the transcriptional network.

## MATERIALS AND METHODS

### Plant Material

Experiments were conducted using *Arabidopsis* (*Arabidopsis thaliana*) plants of the Columbia-0 ecotype. All mutant lines were obtained from the SALK T-DNA collection (Alonso et al., 2003). These included SALK\_009651 (*agl65*), SALK\_072108 (*agl66*), SALK\_016078 (*agl94*), SALK\_98698 (*agl104-1*), and SALK\_066443 (*agl104-2*). Each line was backcrossed to the wild type a minimum of two times before being used for experiments. Plants were grown at 22°C with 16-h days and 8-h nights. Light was maintained at approximately 125  $\mu\text{E m}^{-2} \text{s}^{-1}$  with a mixture of cool fluorescent and incandescent bulbs. Pollen was isolated by gently agitating open flowers in water and filtering the solution through a 70- $\mu\text{m}$  nylon mesh (Falcon no. 352350). Pollen was concentrated by centrifugation at 10,000g and stored at -80°C prior to RNA extraction.

### RT-PCR Analysis

RNA was extracted from hydrated mature pollen according to the phenol extraction method described previously (Lehti-Shiu et al., 2005). cDNA was synthesized from 1  $\mu\text{g}$  of RNA using MMLV reverse transcriptase (Promega) according to the manufacturer's instructions. For quantitative RT-PCR analysis, a dilution series and standard curve were created for each set of gene-specific primers (Supplemental Table S1). Ct (threshold cycle) values were plotted on the standard curves to determine the initial quantity in each reaction. Initial quantities were normalized to an actin control reaction for each cDNA template. Reactions were carried out on RNA samples representing three independent biological replicates for each genotype and two technical replicates for each biological replicate.

### Competition Experiments and Plant Genotyping

For each pollen competition assay, stage 12 wild-type flower buds (Smyth et al., 1990) were emasculated. After waiting 12 to 24 h to ensure that the stigma was receptive, a saturating amount of pollen from a donor plant was then placed onto the stigma. The competitions were configured such that the expected transmission was always 50% and differences in the recombination rate between loci would have no impact on the outcome. Donors were typically heterozygous at one locus and homozygous mutant or wild type at other loci, as specified. Progeny were genotyped by extracting DNA from seedling tissue as described previously (Adamczyk et al., 2007), followed by PCR amplification with allele-specific primers (Supplemental Table S1). Any seedling that did not carry a mutant allele that was homozygous in the pollen donor was assumed to result from self-pollination and was excluded from the analysis.

### Visualization of Pollen

Pollen viability was assessed using a 20  $\mu\text{M}$  FDA treatment (Heslop-Harrison and Heslop-Harrison, 1970). Pollen nuclei were visualized by staining with 1  $\mu\text{g mL}^{-1}$  DAPI. For each assay, three biological replicates were scored for each pollen genotype. Pollen tube growth was visualized by placing mutant or wild-type pollen onto the stigma of a wild-type flower emasculated approximately 1 d beforehand and then, after a given length of time, placing the gynoecium into a 1:3 acetic acid:ethanol fixative. Gynoecia were dehydrated, treated with 8 M NaOH, and stained with decolorized aniline blue as described by Mori et al. (2006). To visualize pollen hydration and adhesion to the stigma, open flowers were collected and placed directly on the stage of an environmental scanning electron microscope (FEI Quanta; FEI).

To examine pollen at the subcellular level, anthers from stage 12 flowers (Smyth et al., 1990) were dissected into a 0.1 M Suc solution, frozen using a Balzers HPM 010 high-pressure freezer, and stored in liquid nitrogen prior to freeze substitution in 2% OsO<sub>4</sub>. Substitution and subsequent embedding of samples in Epon resin (Ted Pella) were carried out as described previously by Otegui and Staehelin (2004). Eighty- to 100-nm sections were cut and stained with uranyl acetate (8% in 50% ethanol) and Reynolds lead citrate (Reynolds, 1963) before imaging with a CM120 transmitting electron microscope (Philips).

### Microarray Analysis

RNA was collected and extracted from mature pollen samples, as described above, from wild-type, *agl65 agl66 agl104-1*, *agl66 agl104-2*, and *agl65 agl66 agl94 agl104-1* homozygous plants. Three biological replicates were collected for each genotype, and the quality of each sample was assessed using the Agilent Bioanalyzer 2100. RNA was reverse transcribed, and amino-allyl labeling was carried out using the Ambion Amino Allyl MessageAmp kit (Ambion) according to the manufacturer's protocol. Labeled cDNA was hybridized to three Nimblegen ATH6 60mer expr X4 four-plex arrays (Nimblegen-Roche) and scanned with an Axon400B scanner (Axon Systems). Robust multichip average normalization was performed using NimbleScan version 2.3 software. CYBER-T (Baldi and Long, 2001) was used to determine the false discovery rate for each gene in the data set, and Microsoft Excel was used to organize and interpret the data. Genes with a posterior probability of differential expression of 0.80 or greater between two samples were considered significantly different, implying that there was at least an 80% chance that the given gene was not a false positive. This cutoff corresponded with a log-transformed *P* value of 0.013. An intensity threshold was established based on the signal intensity from highly expressed petal-, sepal-, stem-, and pedicel-specific genes that are known not to be expressed in pollen. These genes were

selected using the Biomarker tool in Genevestigator (Hruz et al., 2008). This threshold was used to eliminate genes from the data set that are not expressed in the four pollen samples or that show excessive variability between biological replicates. Details about this calculation are summarized in Supplemental Data File S1. All microarray data have been submitted to Array Express (accession no. E-MTAB-91; <http://www.ebi.ac.uk/arrayexpress>).

## Supplemental Data

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

**Supplemental Figure S1.** Description of MIKC\* mutant alleles.

**Supplemental Figure S2.** T-DNA junction sequence for *agl104-1* and *agl104-2* alleles.

**Supplemental Figure S3.** Single mutant pollen competition assays.

**Supplemental Figure S4.** The inflorescence of an *agl66 agl104-2* homozygous plant.

**Supplemental Figure S5.** Aniline blue staining of *agl65 agl66 agl94 agl104-1* pollen tubes in vivo.

**Supplemental Table S1.** Nucleotide sequences for the primers used in genotyping and RT-PCR.

**Supplemental Data File S1.** Excel file describing the genes given in Figure 3, data describing the purity of the samples used for microarray analysis, and lists of genes differentially expressed in the double and triple mutants.

## ACKNOWLEDGMENTS

The authors thank John Beirne, Connie Vo, Kelsey Weis, and Pamela Hall for help with plant genotyping, Dr. Ravi Palanivelu for advice on pollen imaging, and Dr. Peter Dowd for helpful comments on the manuscript. Seed stocks for the mutant lines were obtained from the Arabidopsis Biological Resource Center. Imaging was performed at the Plant Imaging Center, Department of Botany, University of Wisconsin-Madison.

Received January 21, 2009; accepted February 6, 2009; published February 11, 2009.

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