Pollen Lethality: A Phenomenon in Arabidopsis RNA Interference Plants[C]

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To study gene function, generation of loss-of-function mutants by RNA interference (RNAi) approaches and T-DNA insertional mutagenesis are widely used in plant science and have proven to be very successful in determining gene functions. *AGAMOUS-LIKE18* (*AGL18*) is a MADS-box gene that is expressed during Arabidopsis (*Arabidopsis thaliana*) pollen development (Alvarez-Buylla et al., 2000; Pina et al., 2005). To reveal a function for *AGL18,* different transgenic *AGL18* RNAi populations were produced and a T-DNA insertion line from the Salk collection (Alonso et al., 2003) was analyzed in parallel. Surprisingly, a pollen lethality phenotype found in many RNAi lines was not detectable in the *AGL18* T-DNA knockout mutant. To investigate these contradicting results, we made a series of transgenic lines using different vectors, promoters, and reporter genes. Here, we present data proving that the abnormal pollen development observed in RNAi *AGL18* plants is not caused by loss of *AGL18* function but is a common feature occurring in transgenic RNAi populations.

Three different promoters, depicted in Figure 1A, were fused to the *AGL18* RNAi cassette, namely, the cauliflower mosaic virus 35S promoter, the *AGL18* promoter (Lehti-Shiu et al., 2005) and the pollen-specific *DEFH125* promoter (Lauri et al., 2006). These RNAi constructs were transformed into Arabidopsis wild-type plants using *Agrobacterium* strain GV3101. Among the 300 T1 plants examined from each transgenic population, we found that more than 20% of the plants failed to produce normal, wild type-like pollen. Instead, they developed anthers in which approximately 20% to 50% of the pollen was aborted by the time the anthers dehisced (Fig. 2; Table I). Except for the pollen defects (Fig. 2), these plants revealed a normal vegetative and reproductive development, producing wild type-like siliques under our growth conditions (data not shown). To determine whether the phenotype observed in these RNAi lines correlates with a reduced *AGL18* expression, we performed semiquantitative reverse transcription-PCRs with six transgenic T1 plants from one *AGL18*::*AGL18* RNAi population, all producing 50% aborted pollen (Fig. 1A). As shown in Figure 1D, *AGL18* transcription varied among the different individuals, and wild type-like as well as reduced *AGL18* expression levels were observed. No *AGL18* transcript was detectable in the *agl18-3* T-DNA mutant (Fig. 1, B and C), strongly suggesting that it represents a loss-of-function mutant. However, in contrast to the RNAi lines, *agl18-3* anthers produce wild type-like pollen, similar to two other recently analyzed *agl18* knockout lines (Lehti-Shiu et al., 2005). Taken together, these data indicate that the pollen lethality phenotype observed in *AGL18* RNAi plants is likely not related to loss of *AGL18* function.

To test if pollen development is generally affected by the RNAi mechanism, two genes were selected for which no function during pollen development has been reported. The heterologous fluorescent reporter *GFP* and *ROXY1*, a glutaredoxin involved in Arabidopsis petal development (Xing et al., 2005), were cloned into an RNAi cassette and expressed under the control of the pollen-specific *DEFH125* promoter and the cauliflower mosaic virus 35S promoter, respectively. Strikingly, more than 20% of both transgenic T1 plants displayed the pollen lethality phenotype, with 20% to 50% of the pollen being aborted (Table I). As these data were generated with the binary vector pGSA1252, a transgenic *DEFH125*::*DEFH125* RNAi population was produced using another binary vector, a derivative of pGPTV (Lauri et al., 2006). Again, 22.5% of the transgenic plants showed the pollen lethality phenotype (Table I). Thus, these data demonstrate that all different RNAi T1 populations tested here comprise a large proportion, about 20% to 30%, of T1 plants that produce 20% to 50% nonviable pollen (Table I), irrespective of the gene and binary vector type used for RNAi vector construction.

Next, we examined whether not only RNAi constructs, but also transgenes in general, such as antisense constructs (*35S*::*AGL18* antisense), reporter gene constructs like *GUS* (*ROXY1*::*GUS*; *AGL17*::*GUS*) and *GFP* (*35S*::*GFP*), as well as overexpression constructs (*35S*::*DEFH125*) or even empty vectors (pBarA; pGSA1252; pGSA1285), also disturb normal pollen development. We observed that in all different T1 populations, more than 10% of the transgenic plants showed the pollen lethality phenotype (Table I). To conclude, occurrence of the pollen lethality phenotype

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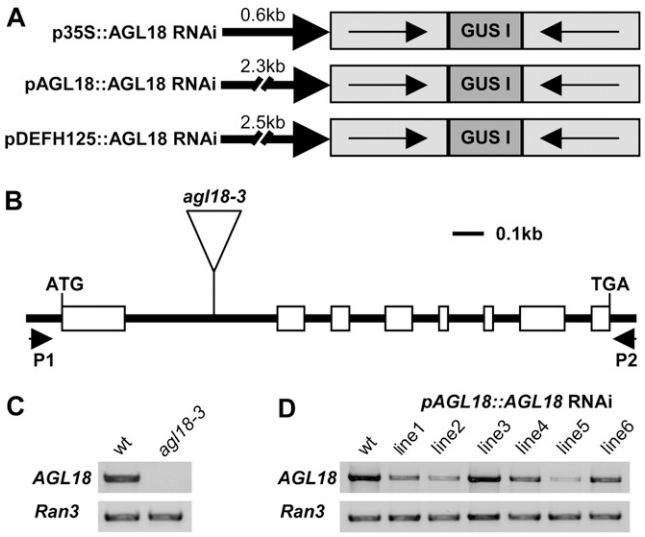

Figure 1. Gene structure and expression analysis. A, *AGL18* RNAi constructs (derivatives of the binary RNAi vector pGSA1252; http://chromdb.org/rnai). *AGL18* cDNA (without MADS box, gray boxes) in sense (→) and antisense (←) orientation, separated by a 370-bp GUS intron (GUS I, green box), was cloned into the RNAi cassette driven by three different promoters (bold arrows, promoter length indicated above). B, *AGL18* gene structure and T-DNA insertion site of the *agl18-3* (NASC ID, N875156) allele. P1 (5′-TAATCTCTTCTCTCTA-TATCTCTTCTC-3′) and P2 (5′-AGATGAAATAAAGCAAAAGAACAGC-CAG-3′) are primers used for reverse transcription-PCR in C and D. C and D, Comparison of *AGL18* transcript level between wild-type Columbia plants, *agl18-3* T-DNA, and six different RNAi T1 mutants, harboring the *AGL18*::*AGL18* RNAi construct. Total RNA was isolated from inflorescences of 6-week-old plants grown under long-day conditions (16 h light/8 h dark, 22°C). cDNA was produced from 4 μg of total RNA, treated with RNase-free DNase I (Roche) and using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions. PCRs were performed with 31 cycles. Five microliters from 25-μL reactions were loaded on ethidium bromide-stained 1.0% (w/v) agarose gels. Images were scanned with the phosphor imager Typhoon 8600 (Amersham Biosciences). As a control, *Ran3* (*At5g55190*) was amplified using the primers 5′-ACCAGCAAACCGTGGATTACCCT-AGC-3′ and 5′-ATTCCACAAAGTGAAGATTAGCGTCC-3′.

seems to be a general, common phenomenon among transgenic plant populations. However, the number of plants with this phenotype was significantly increased in all investigated transgenic RNAi populations.

Then, we determined at which stage pollen development is affected in the RNAi lines. To make pollen abortion analysis easier, three selected *AGL18*::*AGL18* RNAi lines exhibiting 50% pollen lethality were crossed into a *qrt1-2* mutant background, where the four meiosis products of the pollen mother cells do not separate but stick together as tetrads (Francis et al., 2006). In the three F2 populations, plants showed identical pollen lethality phenotypes and pollen development was further analyzed. Until the end of meiosis, no obvious morphological differences among haploid uninucleate microspores were detectable (data not shown). Onset of abnormal pollen development was determined to occur at a late uninucleate microspore stage. Normally, big vacuoles are formed, moving the nuclei to the microspore wall (Fig. 3, B and C). However, microspores lacking formation of a big vacuole degenerate their nuclei and cytoplasm and get aborted (Fig. 3, C–H). Occasionally, pollen quartets containing four wild type-like pollen grains were formed (Fig. 3J). Also, other deviations from a 2:2 ratio of aborted pollen to nonaborted pollen were observed in the quartets (data not shown), inferring that pollen defects in these RNAi plants might result from sporophytic effects (Johnson-Brousseau and McCormick, 2004).

Finally, we tested if the pollen lethality phenotype is heritable. Six T1 plants from different transgenic populations (two from *AGL18*::*AGL18* RNAi; two from *35S*::*GFP*; two from pGSA1252) were selfed over five generations and the pollen lethality phenotype was still detectable in the progeny. Given the observation that no plant could be scored producing more than 50% lethal pollen, this suggests that the aborted pollen contains the transgene and the lethal pollen phenotype might be transmitted through the female. To test this idea, pollen from the investigated transgenic plants showing 50% pollen lethality was used to pollinate wild-type plants. Of the 334 F1 plants analyzed, none showed the pollen lethality phenotype. However, when transgenic plants were fertilized with wild-type pollen, pollen abortion was still detectable in about 50% of the F_1 progeny (146/300 analyzed plants). To conclude, these data indicate that the observed pollen lethality phenotype in the different transgenic plants is inheritable and transmitted through the female.

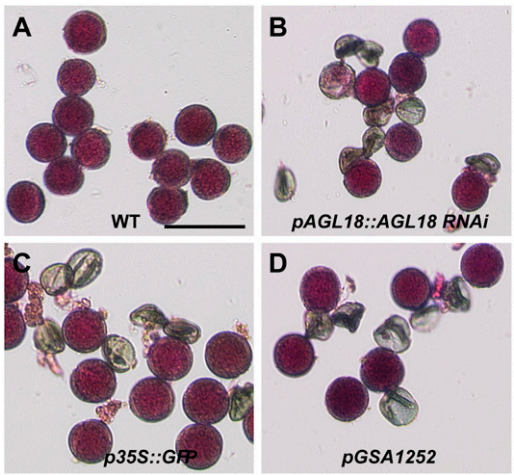

Figure 2. Lethal pollen phenotypes in representative transgenic plants harboring RNAi, reporter gene constructs, and empty vectors, respectively. Pollen was isolated from anthers after dehiscence and examined by Alexander staining (Alexander, 1969). Five hundred pollen grains were counted from each T1 plant. A, Wild-type (Columbia) pollen grains. B, Pollen grains from an *AGL18*::*AGL18* RNAi plant. C, Pollen grains from a *35S*::*GFP* plant. D, Pollen grains from a transgenic plant harboring the empty vector pGSA1252. Bar = 50 μm. [See online article for color version of this figure.]

Table I. *Percentage of plants showing the pollen lethality phenotype in transgenic Arabidopsis populations*

Pollen lethality phenotype is defined as the production of 20% to 50% aborted pollen per anther. Percentage of plants exhibiting this phenotype in each transgenic population was determined by three independent scorings, each comprising 100 transgenic T1 plants. Information on vectors is available upon request. Values are depicted as means ± SD.

Genotype	Percentage of Plants with Pollen Lethality Phenotype
Wild type	0.0 ± 0.0
AGL18::*AGL18* RNAi	28.3 ± 2.5
DEFH125::*AGL18* RNAi	25.3 ± 2.2
DEFH125::*GFP* RNAi	23.8 ± 2.0
DEFH125::*DEFH125* RNAi	22.5 ± 2.0
35S::*ROXY1* RNAi	22.1 ± 2.0
35S::*AGL18* RNAi	21.6 ± 1.9
35S::*GFP*	14.6 ± 1.7
ROXY1::*GUS*	14.2 ± 1.5
AGL17::*GUS*	13.5 ± 1.5
pBarA	13.3 ± 1.5
35S::*AGL18* antisense	11.7 ± 1.3
35S::*DEFH125*	11.4 ± 1.3
pGSA1252	11.1 ± 1.0

DISCUSSION

Our data demonstrate that at least 10% of transgenic Arabidopsis plants produce 20% to 50% nonviable pollen in anthers and this phenomenon is independent of the construct types used for transformation. However, the number of plants with this pollen phenotype was 2 to 3 times higher when the RNAi constructs were used for generation of transgenic plants (Table I). This pollen lethality phenotype will be easily overlooked if the focus is not on investigating pollen development, as transgenic plants do not display other obvious deviations from wild-type development.

Why so many transgenic lines show this pollen phenotype, particularly in RNAi transgenic populations, is still a puzzle. One possible explanation is the positional effect of the T-DNA integration. T-DNA insertions can cause chromosome rearrangements and deletions that can lead to pollen developmental defects (Nacry et al., 1998; Tax and Vernon, 2001; Oh et al., 2003). It has been estimated that about 3,500 genes are expressed in the anther (Scott et al., 2004), and random targeting of these genes by T-DNA insertions might cause, directly or indirectly, pollen developmental defects. Alternatively, off-target effects might be involved; these have been reported from animal studies and require as few as 11 bp of sequence identity to direct silencing of nontargeted transcripts (Jackson et al., 2003; Birmingham et al., 2006; Moffat et al., 2007). Using *GFP* and *AGL18* coding region sequences as well as binary vector sequences to blast the whole Arabidopsis genome or Arabidopsis Genome Initiative transcripts (www.arabidopsis.org) easily identifies a number of matching 16 to 22 nucleotides that may mediate, via an off-targeting mechanism, a higher frequency of lethal pollen formation in the RNAi populations.

Currently, RNAi and T-DNA mutant analysis are broadly and successfully applied approaches to study gene functions. However, pollen lethality is a by-product affecting about 10% of transgenic plants regardless of the construct type used, and the phenomenon is enhanced to more than 20% if RNAi technology is applied to analyze pollen development. Given our observations, it is highly recommendable to combine RNAi data with independent T-DNA mutant analysis and complementation studies. This seems to be particularly crucial for analysis of genes in species such as *Antirrhinum majus* and *Zea mays*, where raising larger T1 populations is hampered by laborious transformation techniques and where pollen sterility, obtained in only a few transgenic T1 plants, could lead to misinterpretation of observed pollen lethality phenotypes.

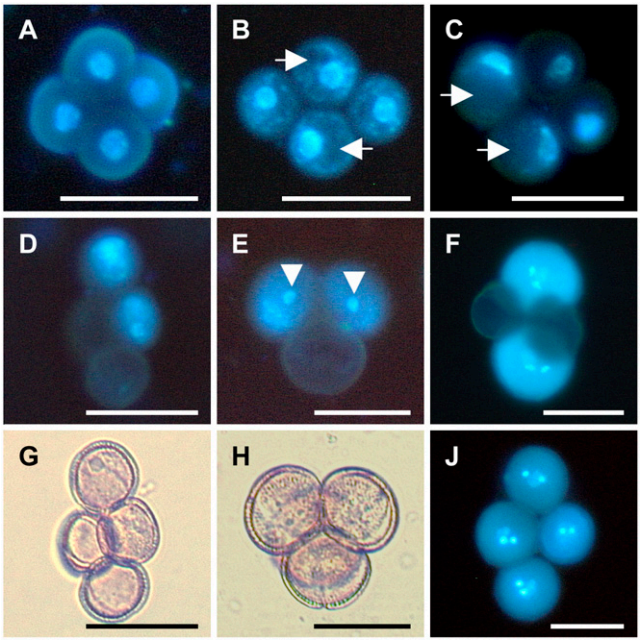

Figure 3. Pollen development analysis of T1 *AGL18*::*AGL18* RNAi plants forming 50% nonviable pollen in a *qrt1-2* background. Representative microspores, isolated from anthers at different uninucleate and binucleate stages and stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (1 μg/mL dissolved in PBS), are shown. A, Early uninucleate microspore stage. Due to the *qrt1-2* background, microspores still stick together, revealing that wild type-like nuclei are located in the center of the cells. B and C, At the late uninucleate microspore stage, formation of big vacuoles (arrows) in two microspores moves nuclei from the center to the cell wall. The two other microspores are lacking big vacuoles and likely degenerated later. D, Nuclei disappeared in two microspores; others just completed the first mitotic division. E, At the two-cell stage, two microspores proceeded further in development and generative cells are visible (arrowheads). F, Only wild type-like microspores underwent second mitosis and contain two sperm cells and one big vegetative cell; the two other pollen grains were aborted. G and H, Bright-field images of Figure 3, D and F (respectively). J, Four wild type-like pollen grains in the quartet at a mature stage. Bar = 25 μm. [See online article for color version of this figure.]

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