Pollen Germinates Precociously in the Anthers of *raring-to-go*, an Arabidopsis Gametophytic Mutant[1]

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Pollen hydration is usually tightly regulated and occurs in vivo only when desiccated pollen grains acquire water from the female, thus enabling pollen tube growth. Pollen tubes are easily visualized by staining with decolorized aniline blue, a stain specific for callose. We identified a mutant, *raring-to-go*, in which pollen grains stained for callose before anther dehiscence. When *raring-to-go* plants are transferred to high humidity, pollen tubes dramatically elongate within the anther. As early as the bicellular stage, affected pollen grains in *raring-to-go* plants acquire or retain water within the anther, and precociously germinate. Thus, the requirement for contact with the female is circumvented. We used pollen tetrad analysis to show that *raring-to-go* is a gametophytic mutation, to our knowledge the first gametophytic mutation in Arabidopsis that affects early events in the pollination pathway. To aid in identifying *raring-to-go* alleles, we devised a new technique for screening pollen in bulk with decolorized aniline blue. We screened a new M_1 mutagenized population and identified several additional mutants with a *raring-to-go*-like phenotype, demonstrating the usefulness of this technique. Further, we isolated other mutants (*gift-wrapped pollen*, *polka dot pollen*, and *emotionally fragile pollen*) with unexpected patterns of callose staining. We suggest that *raring-to-go* and these other mutants may help dissect components of the pathway that regulates pollen hydration and pollen tube growth.

In most higher plants, the pollination pathway is initiated when a partially desiccated pollen grain contacts the stigma, acquires water from the stigma, and hydrates. In many plants, pollen hydration is tightly controlled (Heslop-Harrison, 1979). In species exhibiting sporophytic self-incompatibility, the control of water uptake provides a mechanism for preventing self-pollinations (Herrero and Hormaza, 1996). Despite this normally tight regulation of hydration, it is true nonetheless that pollen will also hydrate and grow a pollen tube if placed in a simple medium containing Suc, boric acid, and calcium (Taylor and Hepler, 1997).

In Arabidopsis, a self-compatible plant, once a pollen grain contacts a stigma papillar cell, the pollen coat, composed of lipids and proteins (Piffanelli et al., 1998; Dickinson and Elleman, 2000), softens into a gelatinous mixture and flows onto the papillar surface. This bridge between the pollen grain and papillar cell, sometimes termed a foot, establishes the route of water flow into the desiccated pollen grain (Elleman et al., 1992). Pollen hydration is complete when the oblong shape of the desiccated pollen grain becomes round. As diagrammed in Figure 1, the pollen grain hydrates and Ca^{2+} flows into the pollen grain; this influx triggers activation. Activation is characterized by cytoplasmic reorganization within

the pollen grain (Heslop-Harrison and Heslop-Harrison, 1992a, 1992b). This reorganization results in the formation of a cytoplasmic gradient of Ca^{2+} beneath the site of germination; this gradient is critical for polar tip growth (Heslop-Harrison and Heslop-Harrison, 1992a; Franklin-Tong, 1999). Associated with Ca^{2+} influx, the pollen grain deposits callose, a β-1,3-glucan, at one of the three pores, where the pollen tube will emerge. The pollen tube wall is an extension of the intine, or inner pollen wall, and is composed largely of callose (Schlupmann et al., 1993). The pollen tube extends by tip growth through the pistil to deliver the sperm to the embryo sac.

Here, we describe an unusual mutant in Arabidopsis, *raring-to-go* (*rtg*). Some of the pollen in *raring-to-go* plants can prematurely hydrate, germinate, and form pollen tubes within the anther. These pollen grains in the *raring-to-go* mutant thus appear capable of bypassing the need to contact the stigma in order to acquire water, hydrate, and germinate. Although not previously reported in Arabidopsis, pollen germination in the anther is not unknown in the plant kingdom. In fact, a wide diversity of plant species have a breeding system known as cleistogamy, wherein the flowers do not open or open only after fertilization (for review, see Lord, 1981). In a subset of cleistogamous species, all pollen germinates inside the anther. Fertilization mechanisms in such cleistogamous species are diverse. In *Lamium amplexicale*, pollen germinates in the anther and the tubes emerge through the anther stomium and grow to the wet stigma (Lord, 1979). In *Viola odorata*, pollen germinates in unopened anthers and grows through the anther wall to

reach the stigma (Mayers and Lord, 1984). An extreme case is seen in several genera of the Malpighiaceae: Here the anthers do not open but pollen tubes can grow through the anther filament tissue and enter the female tissue at the receptacle, completely bypassing the stigma (Anderson, 1980). It is remarkable that most studies on such cleistogamous species have remained descriptive and pollen tube growth within the anther is not genetically understood.
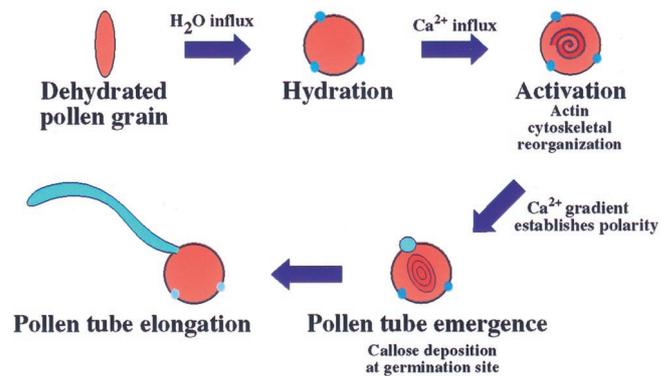
In Arabidopsis, other mutants are known to affect pollen hydration and germination. For example, pollen grains of some *eceriferum* mutants (Preuss et al., 1993; Hülskamp et al., 1995) fail to hydrate on the stigma; these mutants have defects in the biosynthesis of lipids deposited on the pollen coat. Plants with the *grp17-1* mutation lack an oleosin-domain protein in the pollen coat, resulting in delayed pollen hydration on the stigma (Mayfield and Preuss, 2000). In the *fiddlehead1* mutant, the leaf epidermis is capable of supporting pollen germination, due to lipid modifications that alter the permeability of the epidermal cuticle (Lolle et al., 1997; Pruitt et al., 2000). The *eceriferum*, *grp17-1*, and *fiddlehead1* genes act sporophytically. In contrast, we used tetrad analysis to show that the *rtg* gene acts gametophytically.

We devised a new technique for screening pollen in bulk with histochemical stains, thereby greatly improving the efficiency of finding pollen mutants. To identify additional mutants with precocious pollen tubes, we screened an M_1 population by staining with the callose-specific stain decolorized aniline blue. Using this technique, we were able to identify several other *rtg*-like mutants, as well as novel mutants, including two (*gift-wrapped pollen*: *gwp1*, and *gwp2*) that appear to have pollen tubes within the pollen grain. These mutants should be useful tools for dissecting the roles that gametophyte-encoded proteins play during pollen hydration and pollen tube growth.

RESULTS

Identification of *raring-to-go*

While screening an M_2 population for male gametophytic mutations (Chen and McCormick, 1996), we noticed protrusions from some of the pollen released from anthers of one family. To further characterize this phenotype, pollen from the affected plants were stained with a variety of histochemical stains. Because the protrusions stained with decolorized aniline blue, a stain specific for callose, it seemed probable that these protrusions were pollen tubes. Further examination revealed some elongated pollen tubes within the anthers of these plants. The staining was similar to wild-type germinated pollen stained with aniline blue. Because of the premature pollen germination, we named this mutant *raring-to-go*. The flowers of *raring-to-go* plants appear otherwise normal and open normally; the anthers dehisce and shed
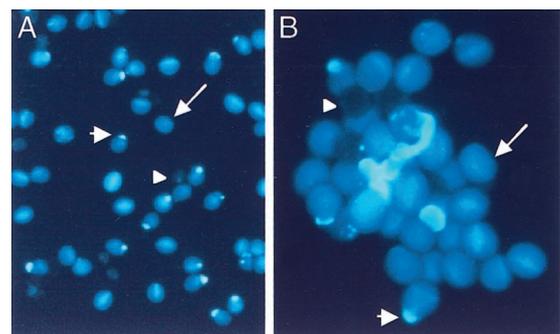

Figure 1. Schematic illustrating key features of the pollination pathway: pollen hydration, activation, and pollen tube extension.

pollen normally, and the affected plants exhibit full seed set.

Self and backcross progeny were generated. Figure 2 shows two fields of mature pollen from *raring-to-go* plants, and illustrates the range of phenotypes: There are pollen grains with pollen tubes, aborted pollen grains, and normal pollen grains. We classified the pollen phenotypes of 40 plants (230 flowers) with the *raring-to-go* phenotype. Overall, 26% of the pollen grains were aborted, 20% had pollen tubes, and 54% appeared normal. Counts from only 10 of the 230 flowers scored deviated significantly. To illustrate the extremes, one flower had 53% aborted grains, 7% with pollen tubes and 40% normal, whereas a flower on a different plant had 31% aborted grains, 32% with pollen tubes and 38% normal. In each of these 10 cases, the same plant had other flowers that fell within the mean.

raring-to-go Is Gametophytically Expressed

The ratio of 50% normal and 50% affected pollen grains was reminiscent of the ratios obtained with a previously characterized gametophytic mutant, *sidecar pollen* (Chen and McCormick, 1996); *sidecar pollen*

Figure 2. Pollen phenotypes found in *rtg*/+ plants. A, Field of pollen. B, Higher-magnification of another field. Note the two grains with pollen tubes, three non-stained (aborted) grains, and normal pollen. Representative pollen grains of the three phenotypes show wild-type (long arrow), *rtg* (short arrow), and aborted (arrowhead). Stained with aniline blue.
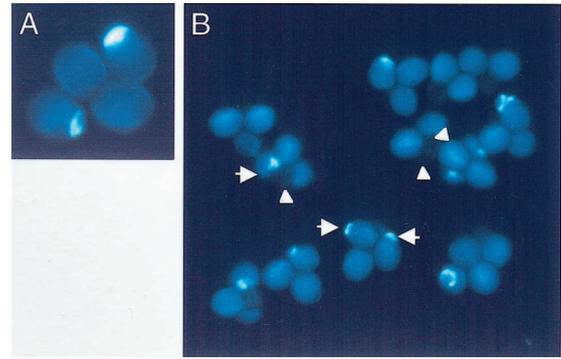
heterozygotes have approximately 50% normal and 50% affected (aborted or sidecar) pollen grains. By analogy, if the pollen with tubes and the aborted pollen grains can be grouped, then *rtg* might be a gametophytically expressed mutation, where half the pollen grains in a heterozygote (*rtg*/+) are not normal.

When *raring-to-go* plants were crossed as female to wild-type plants, about 50% of the F$_1$ progeny showed the phenotype. Expression of the phenotype in the F$_1$ suggests either that *rtg* is a dominant, sporophytic mutation or a gametophytic mutation. If *rtg* is a dominant sporophytic mutation, it might suggest that the anthers of the plant produce a signal that is normally produced by the stigma cells. Gametophytic expression would imply that 50% of the pollen grains initiate a pollination response (i.e. hydrate, germinate, and grow pollen tubes) in the absence of a signal from the stigma.

To test whether *rtg* was a gametophytic mutation, tetrad analysis was performed using *quartet1* (*qrt1*), a sporophytic recessive mutation that keeps all the products of a single meiosis together throughout pollen development (Preuss et al., 1994). If a mutation is sporophytically expressed but has low expressivity, we might expect the numbers of normal and affected pollen resulting from each meiosis to vary. However, if a mutation is gametophytic, the ratio of normal: affected pollen resulting from each meiosis should be 2:2. Table I shows the distribution of pollen phenotypes in the double mutant and, for comparison, the distribution of pollen phenotypes in the *scp; qrt1* double mutant (Chen and McCormick, 1996). Because most quartets in the *rtg*/+; *qrt1/qrt1* plants have two normal pollen grains to two affected pollen grains (i.e. either aborted or with a pollen tube; Fig. 3), we conclude that *rtg* is a gametophytically expressed mutation.

raring-to-go Pollen Grains Are Affected Beginning at the Bicellular Stage

The gametophytic expression of *rtg* suggests that the occurrence of both aborted pollen and pollen with pollen tubes is due to the same mutation.Therefore, it was of interest to determine how early pollen germination occurred and when aborted pollen grains were first observed. We sorted flower buds by

Figure 3. *rtg* is gametophytic. A, Single quartet from *rtg*/+; *qrt1/qrt1* plant illustrates two affected:two normal pollen grains, a distribution expected for a gametophytic mutation. B, Field of mature pollen from an *rtg*/+; *qrt1/qrt1* plant, *rtg* (short arrow) and aborted pollen (arrowhead) are marked in three quartets. Note the types of combinations possible: two *rtg*:two normal (center quartet), one *rtg*, one aborted: two normal (left quartet), and two aborted:two normal (right quartet). Stained with aniline blue.

size (Piffanelli et al., 1998) to examine different developmental stages. For each stage, we used sequential staining, first with 4',6-diamindino-2-phenylindole (DAPI) and then with aniline blue to determine if callose was present. This was necessary because when nuclei were near the aperture or in the growing pollen tube (Fig. 4A), they generally could not be seen after aniline blue staining. And with rare exceptions (Fig. 4A, inset) pollen tube protrusions were generally not visible when stained only with DAPI. Except for the aborted grains, all pollen (>400 scored) showed three nuclei (vegetative and two sperm) when stained only with DAPI (Fig. 4B).

If a pollen grain showed some callose deposition, we scored it as affected. The tetrad stage (not shown) and the unicellular stage (Fig. 4C) were equivalent to wild type. Pollen abortion is first evident at the bicellular stage (Fig. 4D), as is the first indication of callose deposition, in some of the grains (Fig. 4E). Furthermore, aborting pollen grains frequently had callose staining (Fig. 4F and data not shown). Callose staining is more extensive at the tricellular stage (Fig. 4, A and B) when protrusions from the pollen surface are obvious (Figs. 2 and 4F).
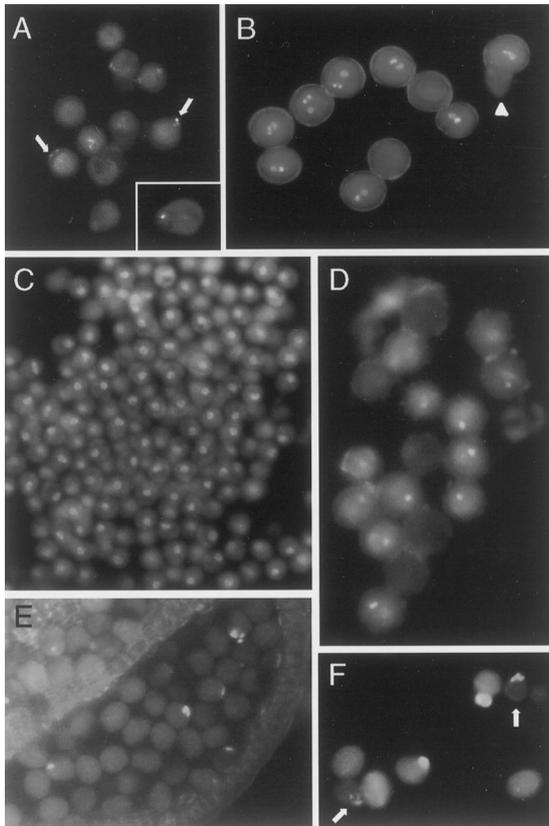
raring-to-go Pollen Does Not Completely Desiccate and Forms Wide Pollen Tubes

The process of staining with aniline blue unavoidably results in pollen hydration. However, Figure 5A shows that three different pollen phenotypes can be distinguished in pollen from dehiscent anthers of *rtg*/+ plants, even without aniline blue staining. There were oblong-shaped pollen grains identical to wild-type dehydrated pollen (Fig. 5B), there were some grains that were somewhat rounder in shape and that had bulges, and there were also aborted grains, some with bulges. We suggest that these phe-

Table I. *Tetrad analysis of rtg*

o, Normal pollen grain. x, Mutant pollen grain.

Plant	Tetrads Scored	Tetrad				
		(oooo)	(ooox)	(ooxx)	(oxxx)	(xxxx)
+/+; *qrt1/qrt1*[a]	434	98[b]	1.8	0	0.2	0
scp/+; qrt1/qrt1[a]	662	0	0	96.5	3.5	0
rtg/+; qrt1/qrt1	464	0	2.6	87.3	9.9	0.2

[a] Chen and McCormick (1996). [b] Percentage of tetrads with a given phenotype.

Figure 4. Pollen development in *rtg*/+ plants. A, Tricellular stage, stained with DAPI. Arrows mark nuclei near emerging pollen tube. B, Tricellular stage, stained with DAPI. The two grains whose nuclei are out of the plane of focus have pollen tube protrusions. Arrowhead marks aborted grain. C, Unicellular stage, stained with DAPI. D, Bicellular stage, stained with DAPI. Note that aborted cells lack nuclear staining. E, Bicellular stage, stained with aniline blue. F, Tricellular stage, stained with aniline blue. Arrows mark two aborting grains that lack cytoplasmic fluorescence but have fluorescent protrusions.

notypes correspond to the three phenotypes we observed in pollen rehydrated in aniline blue.
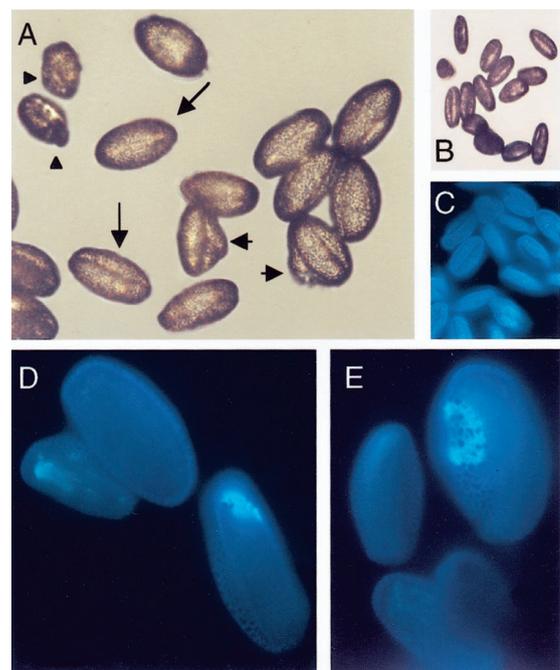
To further test whether there were differences in pollen hydration within the *rtg*/+ anthers, we adapted an in vitro inflorescence culture (Lardon et al., 1993) to deliver 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS), a water-soluble, pH-sensitive tracer that fluoresces upon excitation with UV light. HPTS has been used to monitor plasmodesmatal connections in developing meristems (Gisel et al., 1999). Inflorescences from *rtg*/+ plants and from Columbia-O (Col) wild-type plants were cut from the plant and the cut end placed in a solution of HPTS, at 0.125 μg μL^{-1}. Gisel et al. (1999) used HPTS at 2.5 mg mL^{-1}; we found that level toxic to the inflorescence cultures, the inflorescences stopped growing, and the flowers did not open. We tested serial dilutions in order to identify a concentration of HPTS that would allow anther dehiscence. Pollen grains from dehiscing anthers were gently transferred to slides 24 h after treatment and the pollen examined with a fluores-

cence microscope. The anthers and other parts of the inflorescence were brightly fluorescent (not shown), indicating efficient transport of the HPTS, via the vascular system and plasmodesmata (Gisel et al., 1999). Pollen within the locule is not connected to the HPTS source via the vascular system or plasmodesmata, and Figure 5C shows that Col wild-type pollen had negligible fluorescence after HPTS treatment. In Figure 5, D and E, representative pollen grains from HPTS-treated *rtg*/+ anthers are shown. In contrast to wild type, both fluorescent and nonfluorescent pollen grains are always seen when *rtg*/+ anthers are treated with HPTS. The percentage of HPTS staining pollen grains was equal to or less than the percentage of pollen grains with protrusions.

We suggest that the *rtg* pollen grains are able to acquire the HPTS from the moisture present within the anther locule, although the wild-type-appearing and the aborted grains in the same anther cannot. Although we did not directly quantitate water content in individual pollen grains, together these results suggest that at dehiscence, water content is higher in the nonaborted *rtg* pollen grains than it is in normal, oblong-shaped pollen, whether the normal pollen is from the same *rtg*/+ plant or from wild-type plants.

Pollen Tube Formation in *raring-to-go*

We used fluorescence microscopy to image pollen tube formation in affected *rtg* pollen grains. One

Figure 5. Pollen dimorphism within the anther of *rtg*/+ plants. A, Pollen released from dehiscent *rtg*/+ anther. Representative pollen grains of the three phenotypes show wild-type (long arrow), *rtg* (short arrow), and aborted (arrowhead). B, Pollen released from dehiscent wild-type anther. C, Pollen from wild-type inflorescence after 24 h incubation in HPTS. D and E, Pollen from *rtg*/+ inflorescence after 24 h incubation in HPTS.
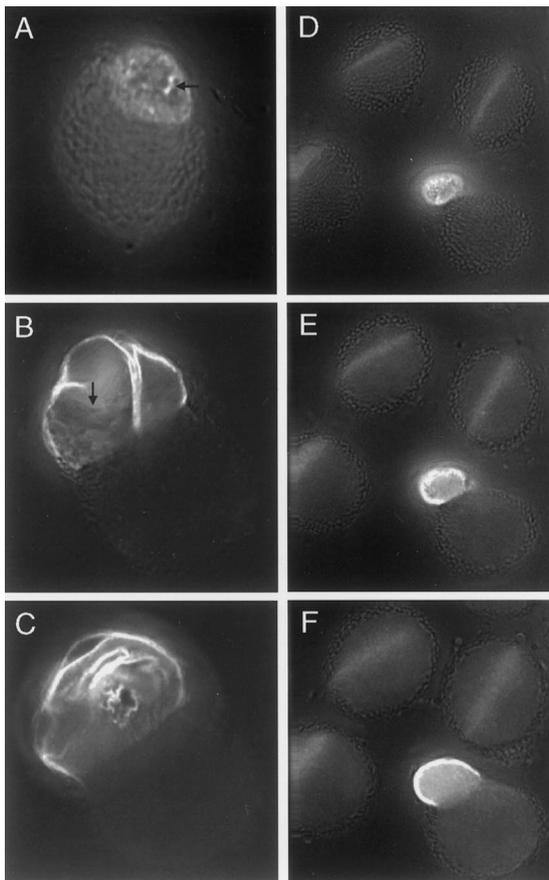
notable feature of *rtg* pollen tubes is that they are much wider (Figs. 2, 3, and 6) than wild-type pollen tubes, which are typically one-fifth the diameter of the grain. Sometimes the wide tube does not lengthen and a more normal width pollen tube arises out of the side of the wide *rtg* tube (not shown).

Within any flower of a *rtg*/+ plant, several stages of pollen tube extrusion are usually evident upon aniline blue staining. Pollen grains with only limited callose staining (that we interpret as an early stage) show an annulus ring below the intine surface, consistent with previous observations of callose staining during pollen germination (Heslop-Harrison and Heslop-Harrison, 1992b). The annulus ring is proposed to define the diameter of the pollen tube, and already at this early stage, the annulus ring diameter is enlarged (Fig. 6A) as compared with wild type (not shown). A few localized foci of brightly staining callose at the aperture represent ruptures in the exine where the growing pollen tube will emerge (Fig. 6A). In other pollen grains, one or more finger-like projections are apparent (not shown), and we interpret these as extensions emerging from the foci of callose

Figure 6. Deconvolution microscope images of selected pollen grains from *rtg*/+ plants. A, Optical section of *rtg* pollen grain 1. Arrow marks rupture in exine where callose stains brightly. B and C, Two different optical sections of *rtg* pollen grain 2. Arrow marks exine cap. D through F, Optical sections of three wild-type appearing grains and a typical *rtg* grain. Stained with aniline blue.
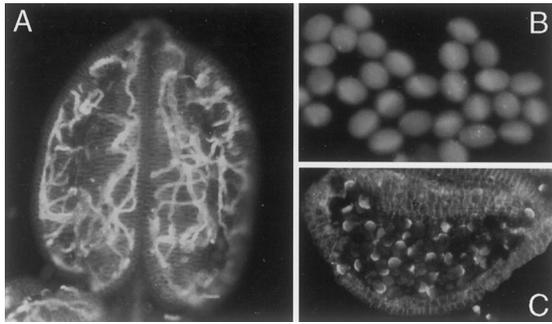
seen at the earlier stage. These finger-like projections seem to pop off the thin exine overlying the aperture. In pollen grains with slightly more developed tubes, these finger-like projections widen and coalesce and appear to twist out of the pollen grain, as is shown in two optical sections of one such pollen grain (Fig. 6, B and C). Figure 6, D through F, illustrates optical sections of three wild-type-appearing pollen grains, each with negligible callose staining, and one *rtg* pollen grain with a small tube. We cannot ascertain whether all tubes go through the stages illustrated in Figure 6, A through C, because many tubes (such as the one shown in Fig. 6, D–F), are already developed when the anther is dissected and the pollen is stained with aniline blue.

High Humidity Affects the *raring-to-go* Phenotype

Genetic mapping requires large populations of plants and precise phenotypic scoring. To our knowledge, pollen tubes in Arabidopsis anthers have not been previously reported, and the phenotype of the *rtg* mutant in the initial F$_1$ and F$_2$ generations was easy to distinguish from wild type. However, because hydration is the first step toward germination, it seemed possible that wild-type plants raised in high humidity might be able to form pollen tubes. Therefore, it was important to assess whether wild-type plants could ever form pollen tubes in the anther. First, pollen from *rtg*/+ plants was compared with wild-type pollen from Col plants, both grown in standard growth conditions under continuous daylight. The measured relative humidity was 70%. Under these conditions, dehiscent pollen from wild-type Col plants had negligible callose staining, whereas the *rtg*/+ plants had prominent callose staining (see Fig. 2). Sibling plants, one *rtg*/+ and one +/+, as well as a wild-type Col plant, were then placed in separate humidity chambers in which the measured relative humidity was >85%. After 2.5 d, pollen was collected from each plant and stained with aniline blue. Figure 7A shows that the *rtg*/+ plant had long pollen tubes trapped inside the anther. This dramatic phenotype suggests that humidity can certainly enhance the growth of pollen tubes in *rtg*/+ anthers, and the width of the pollen tubes under these conditions is more like those of wild-type tubes. However, the pollen from the +/+ sibling plant (not shown) and wild-type Col plant (Fig. 7B) still had negligible callose staining. The wild-type Col plant was further maintained at >85% relative humidity. Even after 12 d there was no pollen tube outgrowth or elongation (Fig. 7C), although all the pollen showed some marginal callose deposition within the grain, frequently in a half-moon shape. We suggest that this pattern of callose deposition is due to a stress response.

Figure 7. Effects of 85% relative humidity on dehiscent pollen. A, Pollen within anther from an *rtg/+* plant after 2.5 d. B, Pollen within anther from a wild-type plant after 2.5 d. C, Pollen within anther of a wild-type plant after 12 d. Stained with aniline blue.

Transmission of *raring-to-go*

We have shown that *rtg* pollen has a greater content of water upon dehiscence than does wild-type-appearing pollen within the same anther (Fig. 5, C–E), and that under high humidity *rtg* pollen has the capacity to grow long pollen tubes (Fig. 7A). However, under normal growth conditions *rtg* does not appear to transmit through the male. When F_1 plants with the *rtg* phenotype (*rtg/+*) were allowed to self-pollinate, the F_2 plants segregated 1:1 for *rtg*: wild-type, rather than in the expected 3:1 ratio. This F_2 ratio suggests either that *rtg* homozygotes are inviable, or that transmission through one of the parents is impaired. Reciprocal crosses showed that female transmission was normal, but crosses using *rtg/+* as the male donor yielded only wild-type progeny. Thus, the name *raring-to-go* is somewhat of a misnomer and does not reflect success at fertilization. Perhaps the *rtg* pollen grains that contact the stigma are delayed in elongating their pollen tubes and therefore fail when competing with wild-type pollen.
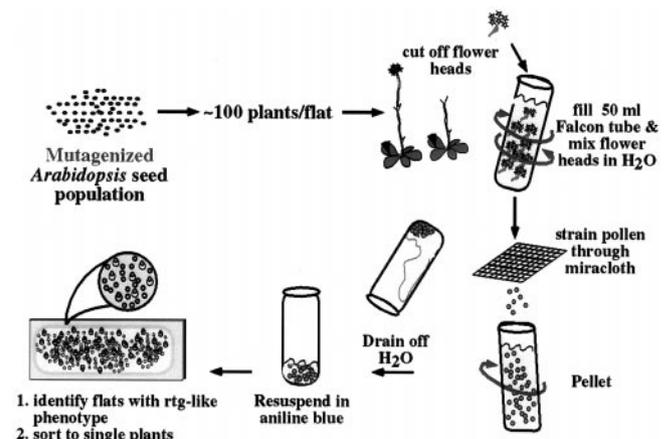
New Screening Technique Yields *rtg*-Like Mutants and Other Mutants with Unexpected Patterns of Callose Staining

Because *raring-to-go* was isolated from a nondirected screen, we could not ascertain the frequency of such a phenotype. In addition, we were interested in determining if mutational analysis could be used to dissect the pollination response pathway (Fig. 1). We devised a new technique for screening pollen in bulk (Fig. 8) to aid in identifying *raring-to-go* alleles or other genes with similar phenotypes. Because we were particularly interested in recovering *raring-to-go* alleles and *raring-to-go* is gametophytic, we chose to screen an M_1 population instead of an M_2 population. By screening an M_1 population, we were preferentially selecting for dominant sporophytic or gametophytic mutations. This strategy has been shown previously to be successful in identifying new gametophytic mutations (Chen and McCormick, 1996). From approximately 6,000 M_1 plants screened,
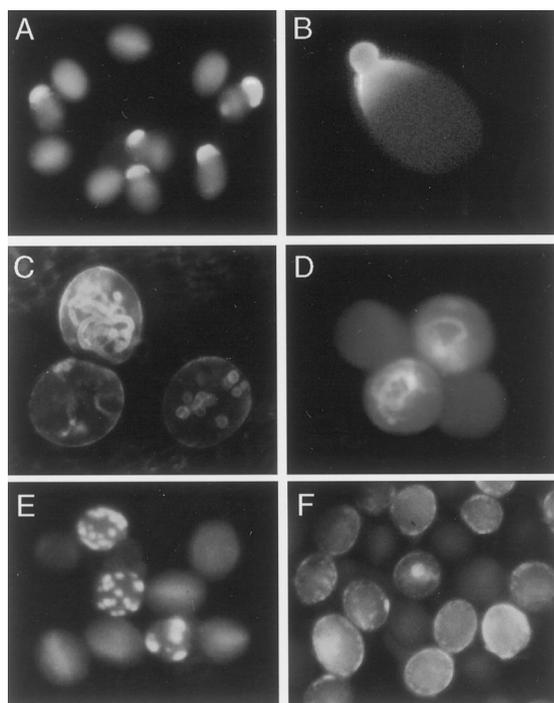
we identified 52 plants whose pollen stained with aniline blue while still in the anther. Because M_1 plants are chimeric for the mutation, each putative mutant was self-pollinated, and 50 M_2 seed were planted to test for transmission. We have further characterized 15 lines with distinct and transmitted phenotypes.

We have confirmed seven *rtg*-like mutants. Six of these look just like *raring-to-go* (one is shown in Fig. 9A), whereas the seventh, shown in Figure 9B, exhibits a subtle but consistent difference in the width of the pollen tube protrusions. Figure 9 also shows pollen from three other selected M_2 families, to illustrate the range of mutants we obtained from this screen. The mutants *gift-wrapped pollen1* (Fig. 9C) and *gift-wrapped pollen2* (not shown) were so named because some of the grains appear to have ribbons tied in bows within the pollen grain. The ribbons appear to be pollen tubes; optical sections (not shown) through such pollen grains show that the tubes are continuous, although in other pollen grains in these lines the structures staining with aniline blue are less extensive. When pollen from *gwp1/+* plants is stained with DAPI, all pollen grains show three stained nuclei, and under bright-field illumination there is no obvious pollen phenotype (not shown). However, like *rtg*, the *gwp1* mutant does not transmit through the male, as shown by reciprocal crosses, and because the F_2 population segregates 1:1 (not shown). As we showed for *rtg* (Fig. 3 and Table I), *gwp1* is also gametophytic (Fig. 9D). Of 220 quartets counted in the *gwp1/+*; *qrt1/qrt1* double mutant, 81% had two affected grains and 15% had one affected grain.

Figure 9E shows *polka dot pollen*, a mutant in which approximately 50% of the pollen shows brightly stained globules of callose. The screen yielded three isolates of *pdp*. In somatic cells, callose is formed in response to stress. The mutant *emotionally fragile pollen* (Fig. 9F) was so named because, in the absence of any apparent stress, 50% of the pollen shows a diffuse callose staining. Affected pollen in *efp* some-

Figure 8. Schematic of screen used to identify *rtg*-like mutants and other mutants affecting pollination.

Figure 9. Examples of new mutant lines with aberrant patterns of callose staining. A, *rtg* like. B, *rtg* like with subtle difference in tube width. C, *gift-wrapped pollen1* (*gwp1*). D, *gwp1/+; qrt1/qrt1*. E, *polka dot pollen* (*pdp*). F, *emotionally fragile pollen* (*efp*). Stained with aniline blue.

times shows more intense staining near the pollen wall. The screen yielded three isolates of *efp*.

Mapping

We used simple sequence length polymorphism (SSLP) and cleaved amplified polymorphic sequences (CAPS) markers with a Landsberg *erecta* (Ler)/Col F_2 population of 720 plants to localize *rtg* to the top arm of chromosome 3, between the CAPS markers cytosolic glyceraldehyde-3-phosphate-dehydrogenase and manganese superoxide dismutase. We have further mapped *rtg* to a region covered by two bacterial artificial chromosome (BAC) clones. We generated an SSLP from the sequence of one of these BAC clones, and have one recombinant in the population.

In the more recent mutant screen, we identified seven additional mutants whose phenotypes are similar to *rtg*. Several male gametophytic mutations have reduced (Chen and McCormick, 1996; Park et al., 1998) or no transmission through the male. Therefore, rather than performing allelism tests of the new isolates with the original isolate, these *rtg*-like mutants and other mutants identified in the screen have been outcrossed to Ler to generate mapping populations and thereby determine if any map to the same region of chromosome 3. So far, one of the *rtg*-like mutants maps to same region as *rtg* (no recombinants

in 38 plants when the SSLP tightly linked to *rtg* is assayed), suggesting that we have recovered an *rtg* allele. If so, sequence analysis of *rtg* alleles will help in identifying which candidate protein is encoded by *rtg*, because the annotation of the BAC clones reveals mostly hypothetical proteins of unknown function.

We have roughly mapped *gwp1*, *gwp2*, and *efp1*. The *gwp1* mutant is linked to *Erecta* on chromosome 2 (one recombinant in 56 plants). The *gwp2* and *efp1* mutants show no linkage to *Erecta*. Furthermore, *gwp2* and *efp1* are not alleles of *rtg* because they independently assort (approximately 45% recombination) from the SSLP marker that is tightly linked to *rtg* on chromosome 3.

DISCUSSION

We have identified an unusual pollen mutant. Some of the pollen in *raring-to-go* prematurely germinates inside the anther at an immature developmental stage, but fails to complete fertilization after dehiscence and subsequent contact with the stigma. Even though pollen tube elongation in *raring-to-go* occurs more readily at high humidity, these pollen tubes do not appear to grow toward the female, either through the filament or through the open stomium. It is notable that high humidity (>88%) influences directional guidance of growing pollen tubes, at least in *Nicotiana alata* (Lush et al., 1998). However given our results, *rtg* is clearly different from some cleistogamous species, where all pollen grains germinate within the anther and fertilization is successful. Nonetheless, because it is defined genetically, *rtg* may provide insight into mechanisms involved in pollen germination in such cleistogamous species.

The importance of water early in pollination has been demonstrated in *cer* mutants (Preuss et al., 1993; Hülskamp et al., 1995), which are blocked in germination and all subsequent steps of the pollination pathway as a result of failure to hydrate. We have demonstrated that some pollen grains from *rtg/+* plants have a higher water content than wild-type pollen (Fig. 5D, E). The HPTS dye was transferred to the pollen grains in *rtg/+* anthers during the 24 h before dehiscence; thus, even if the *rtg* grains were already hydrated they still were able to acquire more water, while the wild-type and aborted pollen grains within the same anther could not. Pollen germination in *rtg/+* anthers can begin at the bicellular stage (Fig. 4), when pollen grains are still in a hydrated state. Therefore, we cannot tell if the *rtg* mutation allows premature water uptake or if the *rtg* mutation allows premature germination when the pollen is already somewhat hydrated during pollen maturation.

Pollen tube growth is dependent on an available water source and it is generally accepted that water acts as a solvent to dissolve nutrients needed to sustain pollen tube growth. Pollen tube growth in affected *rtg* pollen grains is dependent on the avail-

ability of water. Under high humidity, pollen tubes dramatically elongate in *rtg*/+ anthers (Fig. 7A). The loss of water during pollen dehydration and anther dehiscence under normal growth conditions (70% humidity) appears to limit continued pollen tube growth in *rtg*/+ plants.

Pollen viability in other plants has been shown to be affected by the hydration status during dispersal and by the conditions of rehydration (Crowe et al., 1989; Hoekstra et al., 1992; Heslop-Harrison, 1999). In lily (*Lilium longiflorum*), pollen is metabolically inactive upon anther dehiscence. Lily pollen removed from the anther 1 d before dehiscence can germinate, although germination frequency is much improved if the pollen is allowed to dry before germination (Lin and Dickinson, 1984). In Arabidopsis, as in most plants, pollen is dehydrated and metabolically inactive upon anther dehiscence, and thus can be viable for long periods (Stanley and Linskens, 1974). Once in contact with the stigma, metabolic activity is restored during hydration. Because some of the pollen grains in *rtg* plants have germinated and retain water at the time of dehiscence, these pollen grains remain metabolically active and lack a dormancy period before germination. In contrast, in *Cucurbita pepo* and in several species of Gramineae, pollen is dehisced in a hydrated state and remains metabolically active. In these species, pollen viability is short lived (Dumas et al., 1983; Nepi and Pacini, 1993). Perhaps the affected pollen in *rtg*/+ plants is similarly dessication intolerant, but this alone cannot explain the aborted pollen in *rtg*.

Pollen viability in *rtg* is likely compromised by a combination of factors. First, germination of pollen occurs in the anther of *rtg*/+ plants at an immature developmental stage. In 70% of the angiosperms bicellular pollen is released from the anthers and pollen mitosis II occurs after germination and pollen tube penetration into the female tissue. Arabidopsis normally releases mature tricellular pollen; thus, in Arabidopsis, precocious germination at the bicellular stage (Fig. 4, D and E) disrupts normal development and any crucial steps occurring near the end of pollen maturation may not be completed (Lin and Dickinson, 1984). Second, in *rtg*/+ plants, germinated pollen is held for some time in the anther prior to dehiscence. In the Arabidopsis mutant *ms35* (Dawson et al., 1999), pollen development appears to be normal, but the anthers do not dehisce. Only low seed set is obtained from crosses using pollen dissected from *ms35* anthers, suggesting that pollen retained in the anthers loses viability over time. In a similar manner, in the Arabidopsis mutant *delayed-dehiscence1*, some pollen degeneration is observed in mutant anthers (Sanders et al., 1999). In *rtg*/+ plants, affected pollen experiences a prolonged period in the anther post-germination and an increasingly limited water supply as the anthers dessicate, which likely slows or stalls pollen tube growth. Taken together, these factors likely account for abortion of some of the affected pollen grains.

Mature pollen can be easily germinated in vitro, and germination and the initial stages of pollen tube growth occur quite rapidly, sometimes within a matter of minutes. This makes it difficult to characterize discrete stages of germination and incipient pollen tube formation in detail. Affected *rtg* pollen grains have a range of pollen phenotypes during development, which we believe represent different stages of pollen germination, pollen tube formation, and elongation. We have illustrated early pollen tube formation (Fig. 6, A–F) in *rtg*. Affected *rtg* pollen grains from *rtg*/+ plants grown at 70% humidity have pollen tubes that are wider than those of wild type, but when the same plant is grown at high humidity, pollen tube width in affected pollen (Fig. 7A) is comparable to wild type. Because *rtg* exhibits a range of developmental stages that can be influenced by environmental factors, *rtg* pollen should be useful in studying a variety of early germination responses. For example, *rtg* should be useful in examining the dynamics of actin filament organization (Kost et al., 1998; Gibbon et al., 1999).

If *rtg* is a loss-of-function mutant, it would suggest that the RTG protein is necessary to inhibit pollen hydration or germination, until contact with a receptive stigma in some way overcomes the inhibition. A useful analogy for this scenario is provided by the *viviparous* (*vp*) mutants in maize (*Zea mays*), whose seeds precociously germinate on the ear. *vp* mutants either do not synthesize, or are insensitive to, the dormancy hormone abscisic acid (McCarty, 1995). To our knowledge there is no evidence that abscisic acid plays a role in preventing pollen germination, but it is certainly possible that some unknown inhibitor fills this role in pollen.

If *rtg* is a loss-of-function mutation, and there is normally an inhibitor that prevents precocious pollen germination, then how do we explain the phenotypes of *efp*, *pdp*, and *gwp*? In each of these mutants, like in *rtg*, mature pollen stains for callose within the anther (Fig. 9) and thus each exhibits some aspects of precocious behavior. If *rtg* is a loss-of-function allele, then some of our new isolates might be alleles of *rtg*, albeit with quite variant phenotypes. However, we have shown that *gwp1*, *gwp2*, *efp1*, and *rtg* are not allelic.

In contrast, if *rtg* is a gain-of-function mutant, it might suggest that the downstream events that normally occur after contact with the stigma are constitutively activated. Perhaps *rtg* and the other new mutants may define discrete steps in the pollination pathway (Fig. 1). For example, some pollen in *emotionally fragile pollen* and *polka dot pollen* can clearly accumulate callose before contact with the female, but perhaps these mutants cannot properly localize callose at the germination pore, a necessary step for subsequent germination. In *gift-wrapped pollen* plants,

callose also accumulates and in some cases pollen tubes appear to form, but polar outgrowth doesn't occur. Perhaps *gwp* cannot establish or maintain the Ca^{2+} gradient necessary for the proper orientation of the pollen tube. As we saw with *rtg*, in each of these mutants the affected pollen grains exhibit a range of phenotypes. For example, in *gift-wrapped pollen* plants, the percentage of pollen grains containing pollen tube-like structures varies, although *gwp* plants always have pollen with diffuse, and sometimes abundant, callose deposits. In *emotionally fragile pollen* plants, callose staining is mainly diffuse, with occasional brighter fluorescence near the surface of some of the affected grains. In *polka dot pollen* plants, there is variation in the number and size of globules present in affected pollen grains. These variations suggest that *gwp*, *pdp*, and *efp* may also be influenced by environmental factors. Because we screened an M_1 population, the pollen phenotypes we identified are either the result of a pollen gametophytic mutation or a sporophytic dominant mutation. We showed that *rtg* and *gwp1* are gametophytic (Figs. 3 and 9D). Because approximately 50% of the pollen grains in the other new mutants are affected, it is likely that most will also be lesions in gametophytically acting genes.

Other screens using histochemical staining have been successful in identifying pollen mutants affected in pollen cell division and cell fate. For example, the *sidecar pollen* (Chen and McCormick, 1996) and *gemini pollen* mutants (Park et al., 1998) were identified in screens using DAPI staining. These screens were quite tedious because they required dissection and microscopic examination of individual plants. In contrast, we successfully incorporated a method of pooling pollen, limiting individual plant analysis to a small number of M_1 plants. Screens based on segregation distortion have also successfully identified gametophytic mutants (Bonhomme et al., 1998; Howden et al., 1998; Grini et al., 1999), but required subsequent histochemical analysis to identify the particular developmental stage affected. Our screen targeted mutants affected at a discrete stage because we used aniline blue staining as a marker for germination (Figs. 8 and 9). The screen was not exhaustive. Nonetheless, isolation of several *rtg*-like and novel pollen mutants effectively demonstrates the usefulness of the bulk pollen screening technique. We suggest that bulked pollen screening, used with other histochemical stains, might be useful in identifying other interesting and unexpected cellular phenotypes.

MATERIALS AND METHODS

Plant Materials and Mutant Isolation

The *rtg* mutant was identified in progeny from a previously generated Col ethyl methanesulfonate-mutagenized population (gift of Bob Fischer, University of California, Berkeley). Pollen was collected and prepared for staining as previously described (Chen and McCormick, 1996) or pollen was released from dehiscent anthers by squashing flowers in a histochemical stain (see below for stains used).

To screen for new mutants, 0.2 g (approximately 10,000 seeds) of Col were ethyl methanesulfonate mutagenized and sown on germination medium (Chen and McCormick, 1996). Seedlings were transplanted and each flat was scored for pollen phenotypes by staining bulk-collected pollen (Kulikauskas and McCormick, 1997). Arabidopsis plants have a terminal inflorescence and side branches. Because only the terminal inflorescences are cut off to pool the pollen, the side branch inflorescences were checked to identify the affected plant.

For humidity treatments, plants were placed in a chamber constructed from two magenta boxes, one inverted on top of the other, allowing air flow only at the point where the boxes join. The relative humidity inside these chambers was >85%.

Inflorescences from *rtg*/+ and wild-type Col plants were cultured using a system developed for *Brassica napus* flowers (Lardon et al., 1993). An opening was made in the lid of each well of a 24-well tissue culture plate (Corning) by inserting a hot needle. The peduncles of the inflorescences were inserted through the hole in the lid and incubated in HPTS (Molecular Probes, Eugene, OR) at 0.125 μg μL^{-1} in water. The lid of the culture plate was covered with aluminum foil to prevent photodegradation. Flowers that had already undergone anthesis were removed prior to culture.

Microscopy

DAPI (Molecular Probes) at 1 μg mL^{-1} was used to stain nuclei and decolorized 0.1% (w/v) aniline blue was used to stain callose (Regan and Moffatt, 1990). Immersion oil 518 N (Zeiss, Thornwood, NY) was used for observation of dry pollen and HPTS-treated pollen. An Axiophot compound microscope (Zeiss) was used for fluorescent and light microscopy. Images were photographed using Ektachrome 160T color slide film (Kodak, Rochester, NY). Some images were acquired with a deconvolution microscope (Applied Precision, Issaquah, WA).

Genetic Analysis

To perform pollen tetrad analysis, *rtg*/+ plants (Col) or *gwp1*/+ plants (Col) were crossed as females to homozygous Ler *quartet1* (Preuss et al., 1994). Those F_1 progeny whose pollen stained with aniline blue were selfed, and the F_2 progeny were scored to identify the *rtg*/+; *qrt1/qrt1* or *gwp1*/+; *qrt1/qrt1* double mutants.

Mapping

Heterozygous plants in Col background were crossed as females to wild-type Ler. F_1 progeny were scored and several F_1 plants exhibiting pollen staining with aniline blue were self-pollinated to obtain an F_2. Genomic DNA

was isolated from F_2 progeny using the method of Edwards et al. (1991). The mutations were mapped using PCR-based markers SSLP and CAPS as previously described (Chen and McCormick, 1996).

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