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Tetrad Analysis Possible in *Arabidopsis* with Mutation of the *QUARTET* (*QRT*) Genes

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Two *Arabidopsis thaliana* genes, *QRT1* and *QRT2*, are required for pollen separation during normal development. In *qrt* mutants, the outer walls of the four meiotic products of the pollen mother cell are fused, and pollen grains are released in tetrads. Pollen is viable and fertile, and the cytoplasmic pollen contents are discrete. Pollination with a single tetrad usually yields four seeds, and genetic analysis confirmed that marker loci segregate in a 2:2 ratio within these tetrads. These mutations allow tetrad analysis to be performed in *Arabidopsis* and define steps in pollen cell wall development.

In most higher fungi and in many algae and bryophytes, all four products of each meiosis are viable and are either encased in a single structure or remain adherent so that they can be recovered as a group (a tetrad). This feature provides the means to analyze allele segregation through individual meioses and is tremendously advantageous for genetic studies. Only with tetrad analysis is it possible to map centromeres genetically with high precision, to measure chromosomal gene conversion frequencies, and to detect every genetic exchange between chromatids (*1*). In addition, tetrad analysis expedites the construction of multiply marked strains, simplifies the creation of genetic maps, provides a simple method for distinguishing between nuclear and cytoplasmic forms of inheritance, and allows rapid identification of strains with lethal mutations or chromosomal rearrangements, such as reciprocal translocations, inversions, deletions, or duplications (*2*). Tetrad analysis is not possible in most higher eukaryotes; only one of the four products of female meioses typically survives, and although all four products of male meioses usually reach maturity, they are rarely packaged together. This report describes two *A. thaliana* mutations (*qrt1* and *qrt2*) that result in the release of viable pollen tetrads, permitting tetrad analysis to be performed in this flowering plant.

A visual screen of mutagenized plants of the Landsberg ecotype identified two mutations that dramatically affect pollen development (*3*). Unlike wild-type plants, which release single pollen grains, these *quartet* (*qrt*) mutants produce pollen that is arranged in a tetrahedron of four grains (Fig. 1, A and B). Examination of the fusion junction (Fig. 1C) indicated that portions of the outermost layer (exine) of the pollen wall are aberrant, extending between the pollen grains within each tetrad.

Although the *qrt* mutations result in fusion of the outer pollen wall, the cytoplasmic contents of each pollen grain remain separated. Sections of mature pollen grains showed no evidence of fusion of the cytoplasm or of the inner pollen wall (intine), and mechanical separation of the members of a tetrad typically did not affect pollen viability (*4*). When a single *qrt1* pollen tetrad was placed on the stigma surface such that only one pollen grain was in contact with a stigma cell (Fig. 1, D and E), only the

Table 1. Penetrance of the *qrt* phenotypes. Pollen from open flowers was dispersed on a glass slide, and the number of pollen tetrahedrons (tetrads), clusters of three (triads), two (dyads), or single (monads) pollen grains was determined. Percentages (in parentheses) represent the number of pollen grains in each class per total number of pollen grains.

Genotype	Number of tetrads	Number of triads	Number of dyads	Number of monads
+/+	0 (0)	0 (0)	38 (12.2)	547 (87.8)
qrt1/qrt1	1007 (98.7)	3 (0.22)	13 (0.64)	18 (0.44)
qrt2/qrt2	512 (97.5)	6 (0.86)	6 (0.57)	22 (1.05)
qrt1/+	0 (0)	0 (0)	27 (6.8)	738 (93.2)
qrt2/+	0 (0)	4 (1.3)	52 (11.6)	792 (88.0)

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pollen grain in direct contact with the stigma cell became hydrated. Thus, the pollen grains function individually.

Most (98%) of the pollen released from the *qrt* mutants was in a tetrad form, and no pollen tetrads were produced by the wild-type strain (Table 1). Each of the *qrt* mutants was backcrossed to wild type, and the heterozygous progeny did not produce pollen tetrads, indicating that the tetrad phenotype is recessive (Table 1). The frequency of dyads in *qrt1*/+ heterozygotes was not significantly higher than that observed in wild-type strains (Table 1), indicating that the pollen fusion phenotype is controlled by the parental genome, and not the genome of the haploid gametophyte.

When heterozygous *qrt*/+ plants were self-pollinated, the tetrad phenotype was observed in the resulting progeny at a ratio of approximately 3 Qrt^+:1 Qrt^- (*qrt1*, 187:42; *qrt2*, 75:26), indicating that both *qrt1* and *qrt2* mutations result from alteration of individual Mendelian loci. Crosses of *qrt1* to *qrt2* plants yielded wild-type progeny, demonstrating that these isolates have mutations in different genes. *QRT1* was mapped to chromosome 5 [at position 84 ± 1.5 centimorgans (cM)], and *QRT2* was mapped to chromosome 3 (at 3 ± 2 cM) (*5*).

The tetrahedral arrangement of pollen grains in each *qrt* mutant suggested that the tetrads may be the products of a single meiosis. Gene segregation could be scored directly within tetrads with any visible gene product expressed in pollen after the completion of meiosis. We used a β-glucuronidase (GUS) transgene under the control of the postmeiotic LAT52 promoter (*6*) as a pollen marker. To examine gene segregation in the pollen tetrads, we crossed the *qrt1* isolate to a strain containing a LAT52-GUS construct, inserted at a single site in the genome (*7*). The resulting F_1 plants (*qrt1*/+, LAT52-GUS/+) were self-fertilized, and segregation of the LAT52-GUS transgene was examined in F_2 *qrt1*/*qrt1* plants with the chromogenic substrate X-gluc (*8*). The LAT52-GUS/+ plants produced two GUS-containing pollen grains in each tetrad (Fig. 1F), confirming that these tetrads resulted from single meiotic events.

Segregation of the GUS marker in a 2:2 ratio was observed in most *qrt1* tetrads (379/485) from a GUS/+ strain. Staining of three or four members of a tetrad was never detected, but some tetrads had one (15%) or zero (7%) stained pollen grains. In *qrt1* tetrads from GUS/GUS controls, staining of four pollen grains was most prevalent (59/126, Fig. 1G), but tetrad staining patterns of 3 GUS^+:1 GUS^- (28%), 2:2 (11%), 1:3 (3%), and 0:4 (11%) were also observed. Unstained pollen may be inviable or too immature to express the LAT52-GUS construct. Of tetrads with fewer than the expected number of stained pollen grains, many (55%) contained aborted pollen. Thus, *qrt* mutations make tetrad analysis possible for a marker or markers visibly scored in pollen. Aberrant development of pollen may be scored as deviations from expected genetic ratios.

Because it also would be useful to analyze gene segregation in whole plants, the capacity of the pollen tetrads to fertilize eggs was examined. Both the GUS-expression and vital-staining experiments (*4*) indicated that approximately half of the tetrads from the *qrt1* isolate contained four viable pollen grains. In addition, DNA staining with 4,6-diamino-2-phenylindole (*9*) showed that, like wild-type pollen grains, each member of the tetrad contained two sperm nuclei and one vegetative nucleus (*10*).

Each wild-type pollen grain forms a tube that delivers the sperm cells to a single female gametophyte, resulting in the production of a seed (Fig. 1I) (*10*). To test whether the *qrt1* tetrads form normal pollen tubes, we pollinated stigmas with single tetrads from a *qrt1* isolate. To avoid potential contamination from other pollen grains, we pollinated male-sterile *cer6-2* mutant strains (*11*). When tetrads were placed on the stigma surface so that all four pollen grains were likely to become hydrated, four pollen tubes emerged from a single tetrad cross (Fig. 1J) (*12*). Four tubes were

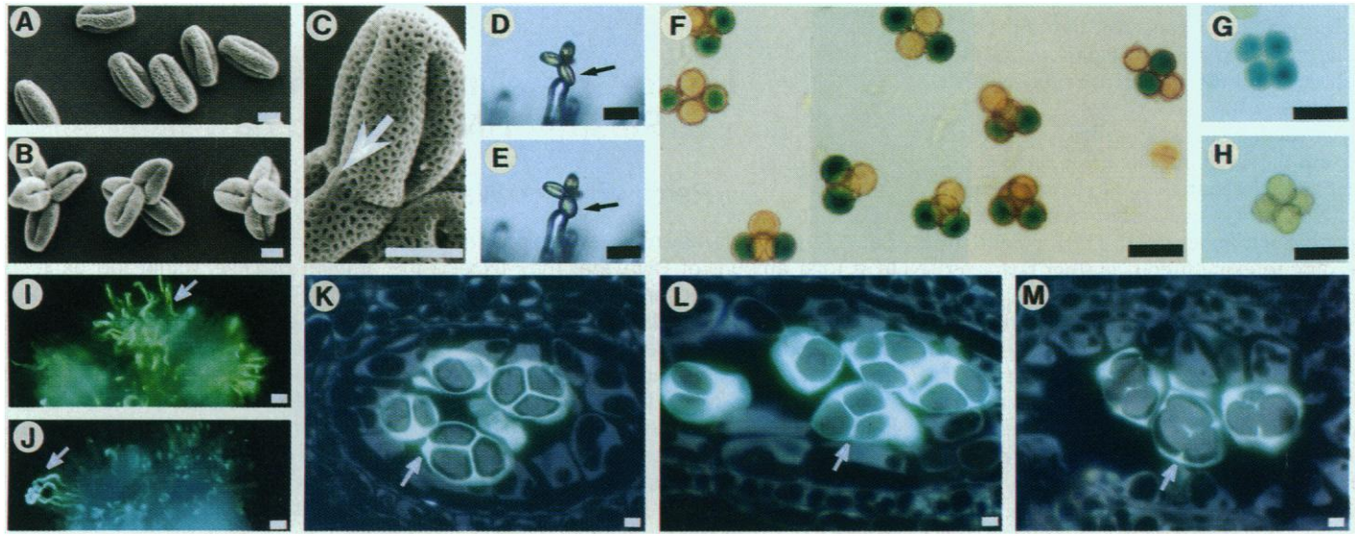

Fig. 1. (**A**) Scanning electron micrograph depicting wild-type pollen and (**B**) pollen from the *qrt1* mutant. (**C**) Exine walls fuse the pollen grains within a *qrt1* tetrad (arrow); similar results were observed for *qrt2* pollen. (**D**) A *qrt1* pollen tetrad immediately after it was placed on a stigma cell. (**E**) As in (D), 20 min later. The pollen grain in contact with the stigma (arrow) has expanded, whereas the other members of the tetrad remain desiccated. The *qrt2* tetrads behaved similarly. (**F**) Segregation (2:2) of X-gluc staining (indicating GUS activity) in pollen tetrads from a *qrt1*/*qrt1*, LAT52-GUS/+ plant. (**G**) GUS/GUS homozygotes show four GUS^+ pollen grains within the tetrad, whereas isogenic plants that do not inherit the transgene (**H**) produce unstained pollen. (**I**) Self-fertilization of *Arabidopsis* plants typically results in germination of >100 pollen grains, each of which forms a pollen tube that emits a blue-green fluorescence when stained as described (*12*). Arrow indicates a single pollen tube. (**J**) Four pollen tubes emerge from a *qrt1* pollen tetrad (arrow) placed on a stigma of a male-sterile *cer6-2* strain (*11*). (**K**) Section of a wild-type anther showing pollen grains immediately after meiosis. Arrow indicates a tetrad of developing microspores separated by the temporary callose wall (bright staining) (*15*). (**L**) The *qrt1* mutants at the same stage as (K). No defects in callose deposition are apparent (arrow). (**M**) The *qrt2* mutants deposit only patches of callose between the developing pollen grains (arrow). Size bars indicate 10 μm (A and B and K to M) and 100 μm (D to J).

produced in half of the pollinations scored (9/19), and most other pollinations (6/19) produced three pollen tubes. Of 23 crosses allowed to mature, nine resulted in the development of three or four seeds, and 68% of these seeds germinated. Pollination with *qrt2* tetrads resulted in similar seed yields. These results with *Arabidopsis* are promising because, in some plant species, pollination with one or a few pollen grains rarely produces seeds (*13*). Thus, tetrad analysis in *Arabidopsis* can make use of phenotypes both of the pollen and the progeny. Moreover, tetrad analysis in plants does not require the microdissection typical of many microbial systems.

What is the nature of the biochemical defect that leads to tetrad formation? Most plants produce pollen monads, but a few species (such as water lilies, cattails, heath, and evening primrose) which are not widely used for genetic analysis release pollen in a tetrad form (*14*). The potential usefulness of these pollen tetrads was recognized early, but efforts to carry out tetrad analysis were hindered by technical difficulties (*13*). The capacity to form pollen tetrads has arisen many times through evolution and is viewed as an example of evolutionary convergence (*14*). In plants that form pollen monads, pollen mother cells secrete callose (a β-1 → 3 glucan) as the daughter cells undergo cytokinesis, so that a callose wall separates each meiotic product (Fig. 1K) (*15*). After the developing pollen grains have initiated synthesis of the exine, the callose wall disappears (*16*). In those species that produce permanent pollen tetrads, the callose wall often is absent or prematurely dissolves (*14*).

No obvious defect in callose wall synthesis was observed in pollen from *qrt1* strains (Fig. 1L). In contrast, *qrt2* plants deposited only patches of callose between the developing microspores (Fig. 1M), potentially leading to exine fusion. Further characterization of the mechanism that gives rise to pollen tetrads in *qrt* strains may ultimately provide the means for performing tetrad analysis in plant species other than *Arabidopsis*. The large number of chromosomal markers (now nearly 1000) coupled with the potential for tetrad analysis serve to make *Arabidopsis* a powerful system for genetic studies, comparable with more classical microbial systems.

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3. Mutagenized seed stocks were obtained from Lehle Seeds (Tucson, AZ), and pollen from M2 individuals was examined in a dissecting microscope. In an initial screen of 800 plants, two independent mutations, *qrt1* and *qrt2*, were identified. Subsequent screening has yielded a total of four isolates with the *quartet* phenotype from 2800 M2 strains.
4. D. Preuss, S. Y. Rhee, R. W. Davis, data not shown. Pollen viability was determined by staining with fluorescein diacetate [J. Heslop-Harrison and Y. Heslop-Harrison, *Stain Technol.* **45**, 115 (1970); S. M. Regan and B. A. Moffatt, *Plant Cell* **2**, 877 (1990)]. Mechanical separation of tetrads was performed by several passages through a pipette.
5. Polymerase chain reaction mapping markers were as described by A. Konieczny and F. M. Ausubel [*Plant J.* **4**, 403 (1993)]. The *qrt* isolates (Landsberg ecotype) were crossed to a wild-type Columbia strain, and mutant F_2 segregants were scored. For *QRT1*, linkage to *DFR* (10 recombinants/48 total) and to *LFY3* (4 recombinants/50) was observed. *QRT2* is tightly linked to *GAPC* (1 recombinant/60). The indicated map positions correspond to those listed in AAtDB version 1.3 [J. M. Cherry, S. W. Cartinhour, H. M. Goodman, *Plant Mol. Biol. Rep.* **10**, 308 (1992)].
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7. For plant transformations, a binary Ti vector containing the LAT52-GUS transgene (*6*) was introduced into the *Agrobacterium* strain GV3101 that contained the helper plasmid pMP90 [C. Koncz and J. Schell, *Mol. Gen. Genet.* **204**, 383 (1986)]. Self-pollination of a GUS/+ heterozygote yielded approximately 1/4 GUS/GUS progeny, 1/2 GUS/+, and 1/4 +/+, (with a segregation ratio of 6:16:6), indicating that the transgene had integrated at a single chromosomal locus.
8. Pollen grains were incubated for 20 min in 90% (v/v) acetone, mounted and air-dried on a glass slide, and then incubated for >5 hours in 50 mM sodium phosphate buffer, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and X-gluc [5-bromo-4-chloro-3-indolyl glucuronide], (0.5 mg/ml) pH 7.0 [R. A. Jefferson, *Plant Mol. Biol. Rep.* **5**, 387 (1987)]. In negative controls, pollen from plants that did not inherit the GUS transgene remained unstained (Fig. 1H).
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15. Pollen grains were fixed in 2.5% glutaraldehyde as described (*11*), dehydrated in a graded series (30, 50, 70, 80, 95, and 100%) of ethanol, and infiltrated with L. R. White resin (Polysciences) as described by the manufacturer. The resin was polymerized by incubation at 60°C for 24 hours. Sections of 2 to 3 μm were cut with a glass knife and mounted on glass slides. Aniline blue staining was performed as described (*11*).
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Liquid-Crystalline Mesophases of Plasmid DNA in Bacteria

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Bacterial plasmids may often reach a copy number larger than 1000 per cell, corresponding to a total amount of DNA that may exceed the amount of DNA within the bacterial chromosome. This observation highlights the problem of cellular accommodation of large amounts of closed-circular nucleic acids, whose interwound conformation offers negligible DNA compaction. As determined by x-ray scattering experiments conducted on intact bacteria, supercoiled plasmids segregate within the cells into dense clusters characterized by a long-range order. In vitro studies performed at physiological DNA concentrations indicated that interwound DNA spontaneously forms liquid crystalline phases whose macroscopic structural properties are determined by the features of the molecular supercoiling. Because these features respond to cellular factors, DNA supercoiling may provide a sensitive regulatory link between cellular parameters and the packaging modes of interwound DNA in vivo.

The problem of DNA storage transcends the specific topic of plasmid packaging: Most extrachromosomal DNA, including many viruses, mitochondrial and chloroplast DNA, as well as substantial portions of the prokaryotic chromosomal DNA, adopts a nucleosome-free interwound conformation (*1*). In contrast to the solenoidal DNA organization encountered in nucleosomal complexes, such a conformation pre-

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