Sex-Biased Lethality or Transmission of Defective Transcription Machinery in Arabidopsis

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ABSTRACT

Unlike animals, whose gametes are direct products of meiosis, plant meiotic products undergo additional rounds of mitosis, developing into multicellular haploid gametophytes that produce egg or sperm cells. The complex development of gametophytes requires extensive expression of the genome, with DNA-dependent RNA polymerases I, II, and III being the key enzymes for nuclear gene expression. We show that loss-of-function mutations in genes encoding key subunits of RNA polymerases I, II, or III are not transmitted maternally due to the failure of female megaspores to complete the three rounds of mitosis required for the development of mature gametophytes. However, male microspores bearing defective polymerase alleles develop into mature gametophytes (pollen) that germinate, grow pollen tubes, fertilize wild-type female gametophytes, and transmit the mutant genes to the next generation at moderate frequency. These results indicate that female gametophytes are autonomous with regard to gene expression, relying on transcription machinery encoded by their haploid nuclei. By contrast, male gametophytes make extensive use of transcription machinery that is synthesized by the diploid parent plant (sporophyte) and persists in mature pollen. As a result, the expected stringent selection against nonfunctional essential genes in the haploid state occurs in the female lineage but is relaxed in the male lineage.

IN flowering plants, three rounds of postmeiotic mitosis and development give rise to an eight-nucleate female gametophyte, one cell of which is the egg cell (SCHNEITZ *et al.* 1995; GROSSNIKLAUS and SCHNEITZ 1998; DREWS and YADEGARI 2002). Pollen, the male gametophyte, consists of three haploid cells, two of which are sperm cells. The three pollen cells are clonally related and are all descended from a single haploid meiotic product of a pollen mother cell (MCCORMICK 1993, 2004). The male gametophyte can survive independent of the sporophyte (the parent plant) and upon landing on a receptive flower, the pollen germinates and develops a pollen tube that elongates through the transmitting tract of the pistil, the female floral organ, to reach the ovary. Within the ovary, the pollen tube grows toward chemical signals emanating from the two synergid cells of the female gametophyte (HIGASHIYAMA 2002; HIGASHIYAMA *et al.* 2001, 2003; JOHNSON and PREUSS 2002). Upon reaching a synergid cell, adjacent to the egg, the pollen tube ruptures, releasing the sperm. One sperm cell fuses with the egg to give rise to the diploid embryo. The second sperm cell fuses with the female gametophyte's central cell, giving rise to the endosperm. Proper development of both embryo and endosperm as a result of double fertilization is required for seed maturation (RUSSELL 1993; GROSSNIKLAUS and SCHNEITZ 1998; YADEGARI *et al.* 2000).

Large-scale analyses of cDNA libraries generated from mRNAs purified from maize and wheat female gametophytes have shown that thousands of genes are expressed in female gametophytes (SPRUNCK *et al.* 2005; YANG *et al.* 2006). Comparative microarray-based transcript profiling analyses using ovules of Arabidopsis wild-type plants and mutants lacking embryo sacs have similarly identified large numbers of female gametophyte-specific genes (YU *et al.* 2005; JOHNSTON *et al.* 2007; JONES-RHOADES *et al.* 2007; STEFFEN *et al.* 2007). Collectively, expression-profiling studies combined with analyses of female gametophytic mutants (PAGNUSSAT *et al.* 2005) provide evidence for extensive transcriptional regulatory networks that are critical for the proper development of female gametophytes.

In Arabidopsis, ~62% of all genes in the genome are expressed during at least one stage of male gametophyte development, with ~10% of these transcripts being pollen specific (HONYS and TWELL 2003, 2004). Moreover, labeled UTP is incorporated into RNA in pollen and the transcription inhibitor, actinomycin D inhibits

pollen tube growth (Mascarenhas 1989, 1993; Honys and Twell 2004). These observations indicate that male gametophytes are actively engaged in the transcription of their haploid genomes.

The enzymes central to nuclear gene expression are DNA-dependent RNA polymerases I, II, and III (Pol I, Pol II, and Pol III), each of which is composed of between 12 and 17 subunits. Pol I is responsible for transcribing the 45S preribosomal RNAs (rRNAs) that are then processed into the 18S, 5.8S, and 25–28S (the latter size depends on the species) rRNAs that form the catalytic core of ribosomes. Pol II transcribes messenger RNAs (mRNAs) as well as RNAs that do not encode proteins, such as micro RNAs and small nuclear RNAs that guide mRNA and rRNA processing events. Pol III is primarily responsible for transcribing transfer RNAs (tRNAs) and repetitive 5S rRNA genes (Kassavetis *et al.* 1994; Paule and White 2000).

For purposes of gene and subunit nomenclature, Arabidopsis Pol I is denoted as nuclear RNA polymerase A (NRPA), Pol II is denoted as NRPB, and Pol III is denoted as NRPC. Their second-largest subunits, denoted as NRPA2, NRPB2, and NRPC2, respectively, are homologs of the β-subunits of eubacterial RNA polymerase. Together with the largest subunits, the β-like second-largest subunits help form the active sites of the enzymes and are essential for RNA synthesis. In *Arabidopsis thaliana*, the Pol I, Pol II, and Pol III second-largest subunits are encoded by single-copy genes located on chromosomes 1, 4, and 5, respectively (Larkin and Guilfoyle 1993; Onodera *et al.* 2005); see also phylogenetic analyses by Craig S. Pikaard and Jonathan Eisen discussed in Arabidopsis Genome Initiative (2000).

Contrary to our expectation that loss-of-function mutations in *NRPA2*, *NRPB2*, or *NRPC2* genes would be unrecoverable due to lethality in both the haploid male and female gametophytes, transgenic lines hemizygous for T-DNA disruptions of each gene can be identified and maintained. Detailed analysis of these lines revealed that the mutant RNA polymerase alleles are not transmitted through the female lineage due to the failure of mutant female gametophytes to complete their development. By contrast, the mutant alleles are transmitted to subsequent generations through the male gametophyte at moderate efficiency compared to wild type. Our data indicate that pollen can develop to maturity, grow pollen tubes, and carry out fertilization in the absence of functional RNA polymerase genes, apparently by utilizing transcription machinery synthesized premeiotically in pollen mother cells. By contrast, female gametophyte development is autonomous and requires transcription machinery generated *de novo* in the haploid state.

MATERIALS AND METHODS

Plant strains and growth conditions: *Arabidopsis thaliana* wild-type and T-DNA insertion mutants (ecotype Columbia in both cases) were grown at 22° with a 16-hr photoperiod. Gene locus identifiers for *NRPA2*, *NRPB2*, and *NRPC2* are At1g29940, At4g21710, and At5g45140, respectively. The T-DNA insertion alleles we named *nrpa2-1*, *nrpa2-2*, *nrpb2-1*, and *nrpb2-2* are carried within Torrey Mesa Research Institute (San Diego) transgenic lines: GARLIC_726_H01, GARLIC_918_C10, GARLIC_859_B04, and GARLIC_110_G08, respectively [GARLIC is the former name of the Syngenta Biotechnology's SAIL collection of T-DNA lines, available from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University]. The parental line for GARLIC_110_G08 was homozygous for the *qrt1-2* allele of the *QUARTET* gene (ecotype Columbia) (Preuss *et al.* 1994); other GARLIC lines are wild type at the *QRT* locus. The T-DNA allele *nrpc2-1* is present in Salk line 007865 (Alonso *et al.* 2003) obtained from the ABRC. Seeds of plants bearing the *nrpc2-2* (GABI_131_B09) allele were obtained from GABI-Kat (Rosso *et al.* 2003). The transgenic Arabidopsis line (SAIL _100_H07) carrying a *LAT52::GUS* reporter gene(s) inserted in an intergenic region was obtained from ABRC.

Genotyping: To identify T-DNA disrupted alleles in segregating families, PCR was carried out using primers complementary to the T-DNA left border (5′-GCATCTGAATTTCA TAACCAATCTC-3′, 5′-CGTCCGCAATGTGTTATTAAG-3′, or 5′-CCCATTTGGACGTGAATGTAGACAC-3′) and primers specific for *NRPA2* (5′-AGAGAGGTAGAGAAACTCACG-3′ or 5′-ATAAACAGTTAGGCAAGCGAA-3′), *NRPB2* (5′-CGATTTGAG CTTCTACCGTTT-3′ or 5′-CCTAGAACATACCATGCGAAA-3′) or *NRPC2* (5′-CTCGCACAATGAAGGATGTTT-3′ or 5′-TAATTC TTGCCGCAAATTGAC-3′). Wild-type alleles of *NRPA2*, *NRPB2*, and *NRPC2* were identified using the gene-specific primers above in combination with 5′-GATGAGTTGGATAACACGA AC-3′ or 5′-AGCACCCTTTAAGCTACAAAG-3′ for *NRPA2*; 5′-CCATCAGACTCTGTCATCATA-3′ or 5′-ACGAAGGGTAA GCATGCAGTT-3′ for *NRPB2*; and 5′-AGCTACTCCAGGGGA GATTAT-3′ or 5′-GGCAAGTACTATAGCCCCCTG-3′ for *NRPC2*.

The unique genomic DNA/T-DNA junction sequences at both ends of the single T-DNA loci in *nrpa2-1*, *nrpa2-2*, *nrpb2-1*, *nrpb2-2*, *nrpc2-1*, and *nrpc2-2* alleles were amplified by PCR and verified by sequencing.

Production of transgenic plants: Genomic sequences for *NRPA2* (positions −1433 to +7346 relative to the translation start site), *NRPB2* (positions −338 to +6514), or *NRPC2* (positions −1947 to +10295) were amplified by PCR. Amplified gene sequences included promoter regions and all introns and exons. Resulting PCR products were captured in pENTR/D-TOPO and recombined into the Gateway recombination (Invitrogen)-compatible expression vector pEarley-Gate 302 (Earley *et al.* 2006). Resulting *NRPA2*, *NRPB2*, or *NRPC2* full-length transgenes were introduced into hemizygous plants bearing a corresponding mutant allele (+/ *nrpa2-1*, +/*nrpb2-1*, or +/*nrpc2-1*). Progeny of transgenic plants that were homozygous for the *nrpa2-1*, *nrpb2-1*, or *nrpc2-1* mutations and were rescued by the full-length transgenes were identified by PCR genotyping.

Confocal laser scanning microscopy: Examination of specimens was carried out using a Zeiss LSM confocal microscope system equipped with a Helium/Neon laser. Images were processed using Adobe Photoshop 7.0 software. Floral stages were defined according to Bowman (1994). Developmental stages of female gametophytes were defined according to Christensen *et al.* (1997).

Cytological and histochemical analysis of pollen: *In vitro* pollen germination was carried out as described by Hashida *et al.* (2007). Pollen were stained with 1 μg/ml DAPI in 20 mM Tris-HCl pH 7.65, 0.5 mM EDTA, 1.2 mM spermidine, 7 mM 2-mercaptoethanol, 0.4 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml FDA in 0.5 M sucrose, or Alexander solution (ftp://

ftp.arabidopsis.org/home/tair/Protocols/EMBOmanual/ch1. pdf). Pollen and self-pollinated pistils were incubated at 37° for 12 hr in GUS staining solution (50 mM sodium phosphate pH 7.2, 0.2% Triton X-100, 2 mM potassium ferrocyanide, 2 mM potassium ferricyanide, and 1 mg/ml X-Gluc).

RESULTS

Sex-biased defects in the transmission of mutant alleles encoding RNA polymerase I, II, and III second-largest subunits: We used a PCR-based strategy to verify the existence of T-DNA-disrupted alleles for the catalytic second-largest subunits of RNA polymerase I (alleles *nrpa2-1* and *nrpa2-2*), RNA polymerase II (alleles *nrpb2-1* and *nrpb2-2*), or RNA polymerase III (alleles *nrpc2-1* and *nrpc2-2*) (Figure 1A). We then genotyped the progeny resulting from self-fertilization of plants bearing these alleles. In all cases, individuals that carried a mutant RNA polymerase allele also carried a corresponding wild-type allele (Figure 1B and data not shown), indicating that these plants were hemizygous for the mutations. No plants homozygous for the Pol I (*nrpa2-1*, *nrpa2-2*), Pol II (*nrpb2-1*, *nrpb2-2*), or Pol III (*nrpc2-1* or *nrpc2-2*) mutant alleles were recovered, indicating that the alleles are all severe loss-of-function mutations in essential genes, consistent with the essential roles of Pol I, Pol II, and Pol III in nuclear gene expression.

Hemizygotes should outnumber homozygous wild-type siblings 67%:33% (2:1) among the progeny of a hemizygous parent bearing one copy of a defective essential gene, assuming that the homozygous mutant is inviable. However, as shown in Table 1, PCR-based genotyping revealed that only 8–38% of the progeny were hemizygous for Pol I (*nrpa2-1* or *nrpa2-2*), Pol II (*nrpb2-1* or *nrpb2-2*), or Pol III (*nrpc2-1* or *nrpc2-2*) mutant alleles (Table 1). Instead, the majority of the progeny possessed only wild-type alleles, indicating a defect in the transmission of the mutant RNA polymerase alleles.

To test for sex-biased defects in the transmission of the mutant alleles through the male or female gametophytes, Pol I hemizygotes (+/*nrpa2-1* or +/*nrpa2-2*), Pol II hemizygotes (+/*nrpb2-1* or +/*nrpb2-2*, *qrt1-2*; the latter is a Pol II mutant hemizygote in a homozygous quartet mutant background), or Pol III hemizygotes (+/*nrpc2-1* or +/*nrpc2-2*) were reciprocally crossed with wild-type (+/+) plants by hand-pollinating emasculated flowers. Resulting progeny were then genotyped by PCR. None of the mutant polymerase alleles were found to be transmitted to the progeny via the maternal parent (Figure 1, C–E; Table 2); instead all progeny of hemizygous (+/−) female plants crossed with wild-type (+/+) males were homozygous wild type (+/+). By contrast, the *nrpa2-1*, *nrpa2-2*, *nrpb2-1*, *nrpb2-2*, *nrpc2-1*, and *nrpc2-2* alleles were all pollen transmissible, such that 13–38% of the progeny inherited a mutant allele from the hemizygous paternal parent when crossed with a wild-type female (Table 2). Note, however, that equal numbers of hemizygous (+/−) and homozygous (+/+) progeny are expected from a (+/+) × (+/−) cross if the wild-type and mutant alleles are transmitted with equal efficiency; the male-transmitted Pol I, II, and III mutant alleles were not inherited at such high levels.

The reciprocal crossing data summarized in Tables 1 and 2 indicate a lack of transmission of the mutant polymerase second-largest subunit alleles through female gametophytes and a partial defect in their transmission through the male gametophyte. Similar allele transmission behavior was observed for the RNA polymerase subunit mutant *nrpb12a* (supplemental Table S1). The homolog of *NRPB12a* in yeast is a single-copy gene whose encoded protein is incorporated into all three nuclear polymerases (Pol I, II, and III). As was the case for the second-largest subunit mutants, homozygous *nrpb12a* mutants were not recoverable. Moreover, *nrpb12a* mutant alleles were transmitted via pollen but not through the female gametophytes. Collectively, our results indicate that male-specific transmissibility of defective RNA polymerase alleles is a general characteristic of RNA polymerase subunit genes and not a peculiarity of second-largest subunit genes.

Defective RNA polymerase alleles cause female gametophyte developmental arrest: Lack of maternal transmission of the Pol I (*nrpa2-1* or *nrpa2-2*), Pol II (*nrpb2-1* or *nrpb2-2*), or Pol III (*nrpc2-1* or *nrpc2-2*) alleles prompted an examination of siliques (seed pods) of self-pollinated hemizygous +/*nrpa2-1*, +/*nrpa2-2*, +/*nrpb2-1*, +/*nrpc2-1*, +/*nrpc2-2*, or +/*nrpb2-2*, *qrt1-2* plants. Siliques of these plants contain small unfertilized ovules interspersed with an equal number of normal seeds; as an example, a silique from a +/*nrpa2-1* plant is shown in Figure 2A. Whereas wild-type plants produce 51–58 seeds per silique, siliques of Pol I (*nrpa2-1* or *nrpa2-2*), Pol II (*nrpb2-1* or *nrpb2-2*), or Pol III (*nrpc2-1* or *nrpc2-2*) mutant hemizygotes contain only 25–27 mature seeds (Figure 2B).

Defects in seed set caused by the polymerase mutations were rescued by transforming Pol I, Pol II, or Pol III hemizygotes with full-length *NRPA2*, *NRPB2*, or *NRPC2* genomic clone transgenes expressed from their endogenous promoters (Figure 2B). Southern blot and segregation analyses showed that the transgenes in each case were integrated in multiple copies at a single locus (data not shown) such that the plants tested in Figure 2B were hemizygous for the polymerase mutant alleles as well as being hemizygous for the rescuing transgene loci. As a result, seed set is rescued by the transgenes to a level intermediate between the mutant and wild-type phenotypes. This is due to the independent segregation of the transgenes and polymerase alleles such that only half of the gametophytes bearing a mutant polymerase allele inherit a rescuing transgene. Collectively, our data indicate that functional RNA polymerases are essential for one or more critical aspects of female gametophyte development, fertilization, or seed development.
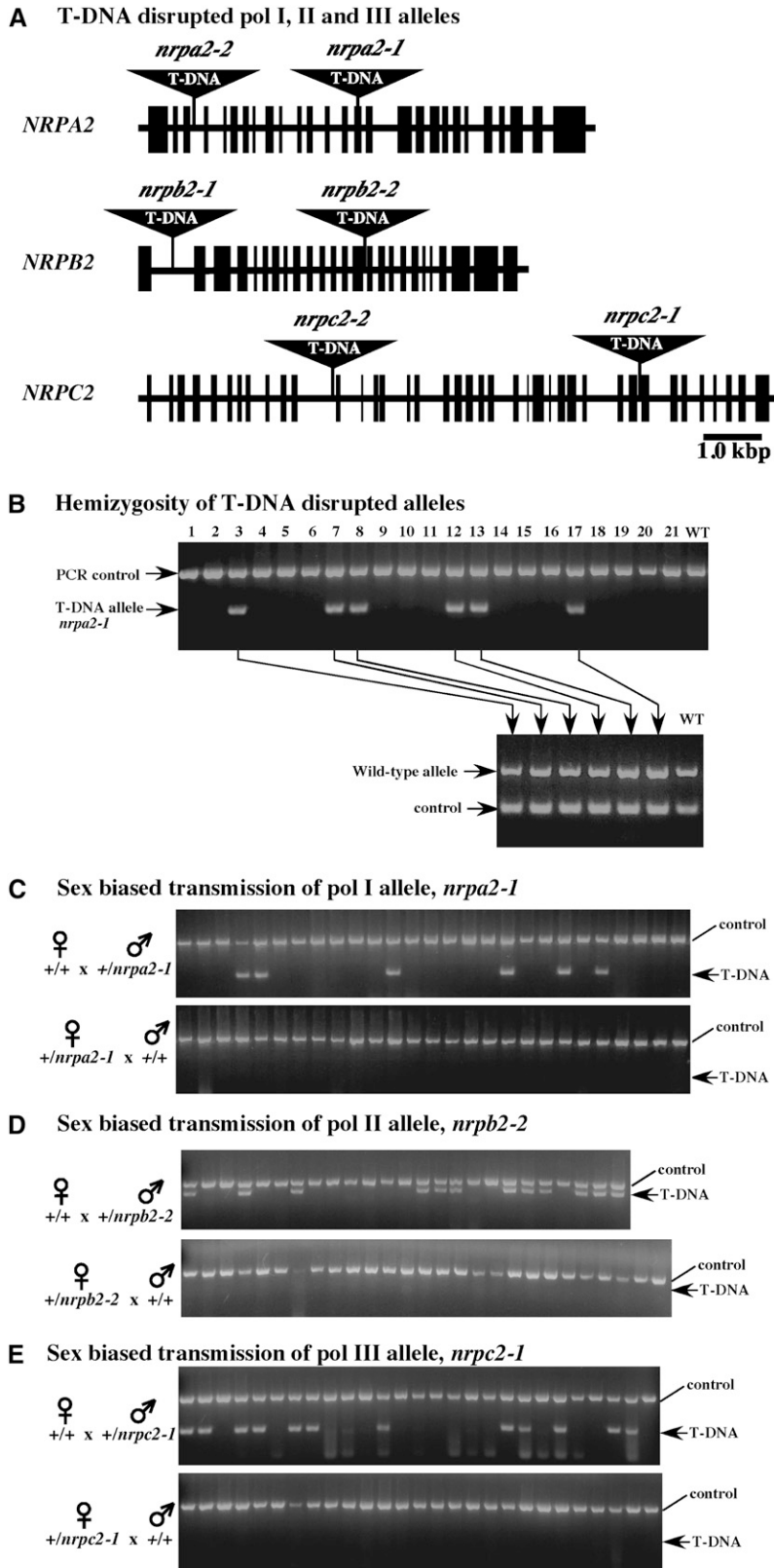
FIGURE 1.—Sex-biased transmission of disrupted alleles for second-largest subunits of RNA polymerases I, II, and III (*NRPA2*, *NRPB2*, and *NRPC2*, respectively). (A) Structures of the *NRPA2*, *NRPB2*, and *NRPC2* genes showing the positions of *nrpa2-1*, *nrpa2-2*, *nrpb2-1*, *nrpb2-2*, *nrpc2-1*, and *nrpc2-2* T-DNA insertions. Solid boxes represent exons. (B) PCR-based genotyping of progeny of a self-fertilized +/*nrpa2-1* hemizygote. Disrupted alleles were detected using a T-DNA-specific primer in conjunction with a gene-specific primer. Wild-type alleles were detected using primers that flank the T-DNA insertion site. (C–E) PCR-based detection of T-DNA disrupted alleles in progeny generated from reciprocal crosses between wild-type (+/+) and +/*nrpa2-1*, +/*nrpb2-2*, and +/*nrpc2-1* hemizygotes.

To further investigate the defects in ovule development and female transmission of mutant alleles (Figure 1, C–E; Table 2), ovaries of flowers at floral stage 13 (BOWMAN 1994), a stage just prior to flower opening, were examined by confocal laser scanning microscopy (CLSM). Female gametophytes develop relatively synchronously (CHRISTENSEN *et al.* 1997) such that gametophytes that have undergone all three rounds of mitosis

TABLE 1

Genotypes of progeny of Pol I, II, and III hemizygotes

Parental genotype	% homozygous wt (+/+)	% hemizygous (+/−)	% homozygous mutant
+/nrpa2-1	76 (62/82)	24 (20/82)	0 (0/82)
+/nrpa2-2	63 (32/51)	37 (19/51)	0 (0/51)
+/nrpb2-1	86 (18/21)	14 (3/21)	0 (0/21)
qrt1-2, +/nrpb2-2	80 (67/84)	20 (17/84)	0 (0/84)
+/nrpc2-1	62 (39/63)	38 (24/63)	0 (0/63)
+/nrpc2-2	92 (45/49)	8 (4/49)	0 (0/49)

Mutant alleles *nrpa2-1, nrpa2-2, nrpb2-1, nrpb2-2, nrpc2-1,* and *nrpc2-2* are underrepresented among the progeny of self-fertilized hemizygotes. Numbers in parentheses represent the number of individuals displaying a given genotype and the total number of individuals examined. wt, wild type.

(female gametophyte stages FG5–FG7; see Figure 3A) are observed at floral stage 13 in wild-type pistils (Figure 3B and Table 3). By contrast, in floral stage 13 pistils of hemizygous plants segregating mutant alleles for Pol I (+/nrpa2-1 or +/nrpa2-2), Pol II (+/nrpb2-1 or +/nrpb2-2, qrt1-2), or Pol III (+/nrpc2-1 or +/nrpc2-2), ~50% of the female gametophytes arrest after only one or two rounds of mitosis (2–4 nuclei), at developmental stages FG2–FG4 (Table 3, Figure 3, C–G, and supplemental Figure S1). The other ~50% of the gametophytes in these ovaries display normal development, as in wild-type plants, consistent with the 1:1 segregation of wild-type and mutant alleles within the siliques of plants hemizygous for the mutations.

Detailed examination of ovules within +/nrpa2-1 plants indicated that female gametophytes lacking functional Pol I arrest most frequently at the two-nucleus stage (FG2 and FG3; Figure 3, C and D and Table 3) and were not observed to progress beyond the four-nucleus stage. Similar results were observed for hemizygous plants bearing the *nrpa2-2* Pol I mutant allele (Table 3 and supplemental Figure S1, A and B).

As shown in Table 3, Figure 3, E–G, and supplemental Figure S1, most of the *nrpb2-1, nrpb2-2,* and *nrpc2-1* female gametophytes arrested after the second mitotic division (FG4), at the four-nucleus stage, whereas the majority of *nrpc2-2* female gametophytes displayed developmental arrest at the two-nucleus stage (FG2 and FG3). The difference in the severity of the *nrpc2-1* and *nrpc2-2* alleles is presumably due to the relative locations of the T-DNA insertions, with the T-DNA in the stronger *nrpc2-2* allele occurring in an earlier intron (see Figure 1).

Collectively, the microscopic analyses suggest that female gametophytes carrying defective alleles for RNA polymerases I, II, or III arrest early in development, at or prior to the four-nucleus stage, FG4.

Certation explains reduced male transmissibility of defective polymerase alleles: As shown in Table 2 and Figure 1, C–E, *nrpa2-1, nrpa2-2, nrpb2-1, nrpb2-2, nrpc2-1,* and *nrpc2-2* alleles are all transmitted via the male gametophyte. However, homozygous wild-type individuals outnumber hemizygous individuals among the progeny of self-fertilized hemizygotes or among the progeny of wild-type females outcrossed with a hemizygous male (Tables 1 and 2). These data indicate that male gametophytes bearing wild-type RNA polymerase alleles are either more viable or more successful at fertilization than are male gametophytes bearing mutant polymerase alleles.

TABLE 2

Male-specific transmission of Pol I, II, and III mutant alleles

Parental genotype		Genotypes of progeny	
Female parent	Male parent	% homozygous wt (+/+)	% hemizygous (+/−)
+/nrpa2-1	+/+	100 (55/55)	0 (0/55)
+/nrpa2-2	+/+	100 (46/46)	0 (0/46)
+/nrpb2-1	+/+	100 (52/52)	0 (0/52)
qrt1-2, +/nrpb2-2	+/+	100 (42/42)	0 (0/42)
+/nrpc2-1	+/+	100 (56/56)	0 (0/56)
+/nrpc2-2	+/+	100 (47/47)	0 (0/47)
+/+	+/nrpa2-1	75 (42/56)	25 (14/56)
+/+	+/nrpa2-2	62 (24/39)	38 (15/39)
+/+	+/nrpb2-1	79 (38/48)	21 (10/48)
+/+	qrt1-2, +/nrpb2-2	70 (19/27)	30 (8/27)
+/+	+/nrpc2-1	67 (36/54)	33 (18/54)
+/+	+/nrpc2-2	87 (45/52)	13 (7/52)

Paternally biased transmission of *nrpa2-1, nrpa2-2, nrpb2-1, nrpb2-2, nrpc2-1,* and *nrpc2-2* alleles. Wild-type (+/+) plants were reciprocally crossed with +/nrpa2-1, +/nrpa2-2, +/nrpb2-1 (in *qrt1-2* mutant background); +/nrpb2-2, +/nrpc2-1, and +/nrpc2-2 and resulting progeny were genotyped. Numbers in parentheses are the number of progeny displaying the specified genotype out of the total number of progeny examined.
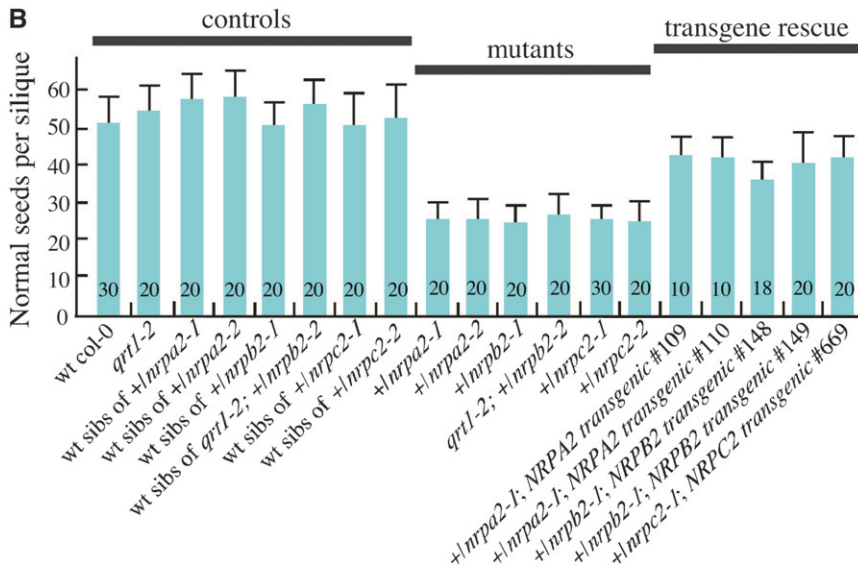
A

+/nrpa2-1   normal
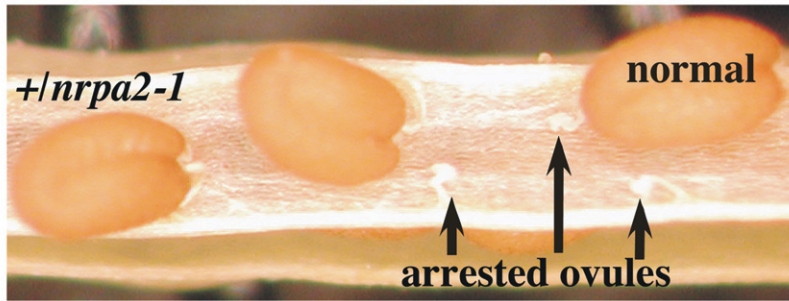
arrested ovules

FIGURE 2.—Failed seed development in siliques of *nrpa2-1, nrpa2-2, nrpb2-1, nrpb2-2, nrpc2-1,* and *nrpc2-2* hemizygotes. (A) A silique of a hemizygous +/*nrpa2-1* plant. Normal seeds and undeveloped (arrested) ovules occur in a silique of a hemizygous plant. (B) Average amounts of normal seeds per silique from wild-type and hemizygous plants. Numbers of siliques examined are indicated.

To investigate the influence of defective RNA polymerase alleles on pollen development and viability using tetrad analysis, we generated lines that carry a Pol I (*nrpa2-1*), Pol II, (*nrpb2-1*), or Pol III (*nrpc2-1*) mutant allele in the *quartet* (*qrt*) mutant background. The quartet mutation causes the four pollen that develop from the four meiotic products (microspores) to remain associated with one another, rather than dissociating into individual pollen grains. Thus, pollen tetrads of plants hemizygous for the polymerase mutants include two pollen-bearing mutant polymerase alleles and two bearing wild-type polymerase alleles.

Pollen tetrads were examined by DAPI (4′,6-diamidino-2-phenylindole), FDA (fluorescein diacetate), or Alexander staining (Figure 4, A–H). DAPI staining of chromatin in pollen of *quartet* (*qrt1-2*) mutant plants; Pol I hemizygote *quartet* (+/*nrpa2-1; qrt1-2*), Pol II hemizygote *quartet* (+/*nrpb2-1; qrt1-2* as well as +/*nrpb2-2; qrt1-2*), or Pol III hemizygote *quartet* (+/*nrpc2-1; qrt1-2*) plants revealed the normal pattern of one diffuse vegetative cell nucleus and two compact sperm cell nuclei in each of the four attached pollen (Figure 4, B and F, and data not shown). FDA and Alexander staining detected no differences in viability among the individual pollen in tetrads of wild-type or mutant plants (Figure 4, C, D, G, and H, and data not shown).

Two of the pollen in each tetrad of a polymerase mutant hemizygote carry defective RNA polymerase alleles and lack wild-type alleles. In the case of the *nrpb2-2* hemizygotes, the mutant alleles are tagged by a *LAT52::GUS* reporter gene that is present within the T-DNA inserted into the Pol II *NRPB2* gene (Figure 4, I and J). The *LAT52* promoter is specifically expressed in mature pollen and pollen tubes, thereby allowing the pollen bearing the mutant *nrpb2-2* alleles to be visualized by GUS staining. Equal numbers of GUS-positive (blue) and GUS-negative pollen are present in *nrpb2-2/+* pollen quartets, indicating that wild-type and mutant pollen develop in equal abundance and that the *nrpb2-2* mutant allele segregates normally (Figure 4, I and J).

It is noteworthy that mRNA-encoded proteins, such as the GUS enzyme, are synthesized by RNA polymerase II and require the distinctive 5′7-methylguanosine caps and poly A tails of Pol II transcripts to be translated. Pol I and Pol III transcripts lack these features and are not translated. Despite the disruption of the gene encoding the essential Pol II second-largest subunit (NRPB2), the GUS enzyme is clearly expressed from the *LAT52* promoter in *nrpb2* mutant pollen (Figure 4, I and J). Expression of the GUS gene cannot be attributed to stored GUS mRNA transcribed premeiotically; if so, it would be
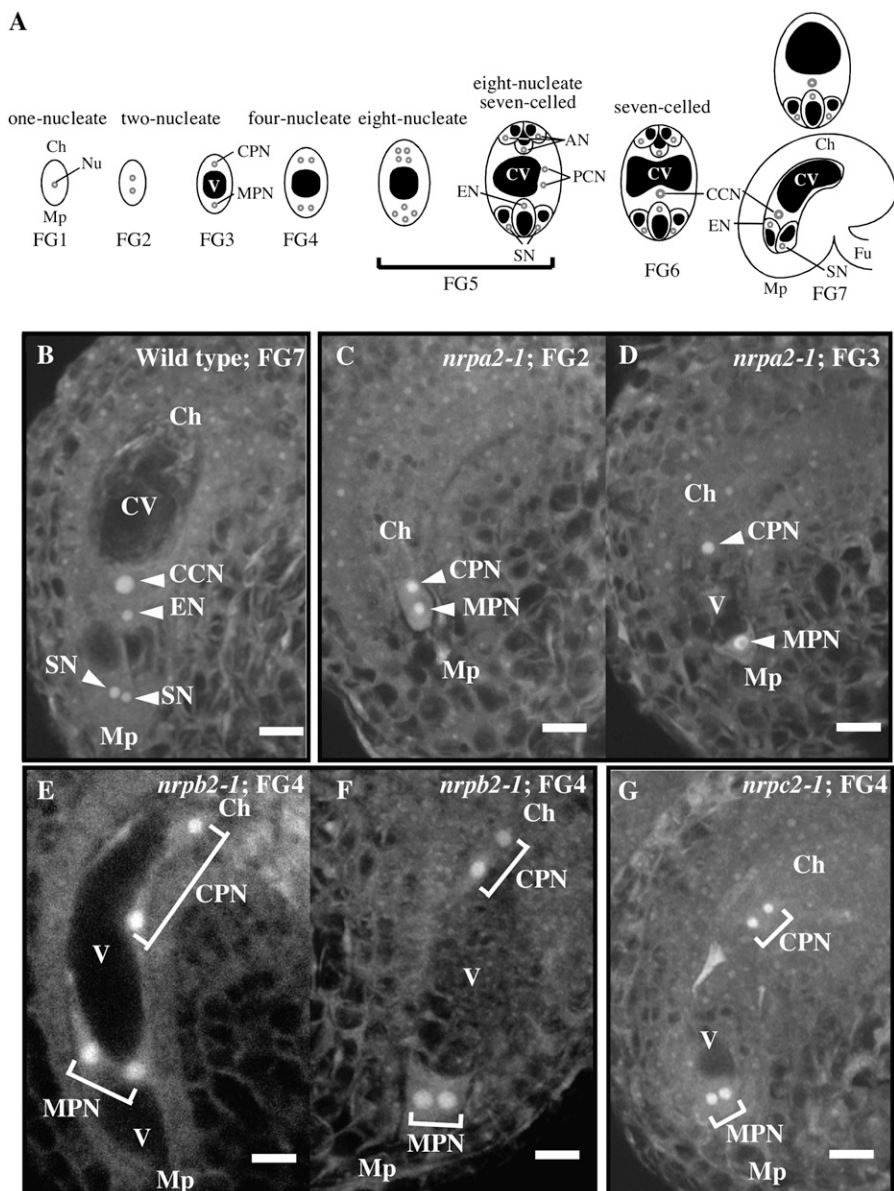
FIGURE 3.—Developmental arrest of mutant female gametophytes in flowers just prior to anthesis was visualized by confocal fluorescence microscopy. (A) Stages of female gametophyte development (FG1–FG7), according to CHRISTENSEN et al. (1997). Mp, micropylar pole; Ch, chalazal pole; Nu, nucleus; V, vacuole; CPN, chalazal pole nucleus; MPN, micropylar nucleus; AN, antipodal cell nucleus; CV, central cell vacuole; EN, egg cell nucleus; PCN, polar cell nucleus; CCN, central cell nucleus; SN, synergid cell nucleus; Fu, funiculus. (B) A wild-type female gametophyte, at floral stage 13, that is fully developed (FG7). The nuclei and vacuoles for the 2N central cell, the egg cell, and two synergid cells are apparent. (C and D) nrpa2-1 female gametophytes arrested at the two-nucleate stage (FG2 and FG3). (E and F) nrpb2-1 female gametophytes arrested at the four-nucleate stage. (G) A nrpc2-1 female gametophyte arrested at the four-nucleate stage. Scale bars, 10 μm.

present in all four pollen of the tetrad. Moreover, the LAT52 promoter has previously been shown to be expressed only postmeiotically, making it a useful male-gametophyte-specific marker (EADY et al. 1994; TWELL et al. 1990). We conclude that Pol II transcription takes place in nrpb2-2 mutant pollen despite the lack of a functional NRPB2 allele.

Examination of pollen germination and pollen tube growth in vitro revealed no differences among pollen tubes that grew from pollen quartets consisting of two pollen-bearing defective RNA polymerase alleles and two pollen-bearing wild-type alleles, at least up to a pollen tube length of 100–150 μm (Figure 4, K and L, and data not shown). Self-pollinated pistils of qrt1-2; +/nrpb2-2 plants stained for GUS also reveal pollen tube growth from pollen bearing the disrupted allele in vivo (Figure 4, M and N). Most of the GUS-stained tubes

from nrpb2-2 pollen are observed at the stigma and upper portions of the ovary (Figure 4, M and N; Figure 5C). However, in rare cases, tubes from nrpb2-2 pollen are observed in the distal portion of the ovary (Figure 4N, images at top right and bottom). Collectively, these observations suggest that in pollen that do not encode endogenous functional RNA polymerase II, Pol II-dependent GUS activity is sustained during pollen development and early pollen tube growth.

To test the hypothesis that pollen bearing Pol I (nrpa2-1 or nrpa2-2), Pol II (nrpb2-1 or nrpb2-2), or Pol III (nrpc2-1 or nrpc2-2) mutant alleles are at a competitive disadvantage compared to wild-type pollen, we determined the distribution of seeds bearing mutant alleles within the siliques of self-pollinated hemizygous plants. Due to the previously demonstrated lethality of the 50% of female gametophytes that inherit a

TABLE 3

Female gametophyte development in polymerase mutants

Plant genotype	Pistil identification no.	No. of female gametophytes at specified developmental stages							Total
		FG1	FG2	FG3	FG4	FG5	FG6	FG7	
wt col-0	1					7	2	8	17
	2					1	2	11	14
	3					3	1	12	16
	4						1	14	15
qrt1-2	1						1	12	13
	2					5	3	9	17
	3							13	13
+/nrpa2-1	1		4	4	1		2	4	15
	2		2	4	2		1	3	12
	3		4	4	2	7	2	4	23
	4		1	6			1	9	17
+/nrpa2-2	1		3	6	1	2	1	7	20
	2		2	7	1	4	1	1	16
	3		1	4	1	7	3	1	17
	4		1	6	4	2	1	7	21
+/nrpb2-1	1			2	8		1	4	15
	2			1	4		2	2	9
	3			3	3	1	1	7	15
	4				9		2	8	19
	5			1	10		2	9	22
	6			4	10			8	22
qrt1-2, +/nrpb2-2	1			2	6	2	1	4	15
	2			5	8	3	2	3	21
	3			1	3			8	12
+/nrpc2-1	1			4	12	2	1	7	26
	2			2	7	2	1	5	17
	3			2	3	3	1	7	16
	4			4	4	1	2	6	17
+/nrpc2-2	1		3	10	1	3	1	6	24
	2		2	10			1	8	21
	3		3	6	1		3	6	19
	4		1	8			1	9	19
	5		2	7	1		1	8	19

Developmentally arrested *nrpa2-1*, *nrpa2-2*, *nrpb2-1*, *nrpb2-2*, *nrpc2-1*, and *nrpc2-2* female gametophytes. Pistils from flowers just prior to anthesis (flower opening) were fixed, and female gametophytes within these pistils were classified according to their developmental stage (FG1–FG7). wt, wild type.

mutant polymerase allele (depicted as ovules with an "X" through them in Figure 5A), only the 50% of female gametophytes that bear wild-type alleles are available to be fertilized. Therefore, any mutant alleles detected in the seeds are inherited via the male gametophytes (refer to Figure 1, C–E, and Table 2). Seeds were collected from the top one-third of the silique, which is nearest to the stigma where the pollen germinates to initiate the growth of pollen tubes, or from the middle or bottom one-third of the silique. Following germination of the seeds, resulting plants were genotyped (Figure 5B). This test revealed that mutant alleles were found most frequently among seeds that developed within the top one-third of the siliques; 35–50% of these seeds develop as hemizygotes (note that a frequency of 50% is ex-

pected if there is no difference in the fitness of wild-type and mutant pollen). The frequency of hemizygous seeds within the middle portions of the siliques were significantly reduced (11–21%) in comparison with the top one-third, except for the *nrpa2-2* allele that was detected in 16 of the 23 sibs examined. In the bottom one-third of the siliques, where fertilization of the ovules would require the growth of the longest pollen tubes, hemizygotes represented only a small proportion of the seeds (0–11%).

The extent of mutant pollen tube growth fits with the distribution of hemizygous seeds following fertilization. A nonmutant transgenic line in which a T-DNA bearing the *LAT52*::GUS reporter gene inserted into an intergenic region was used as a control for comparison to
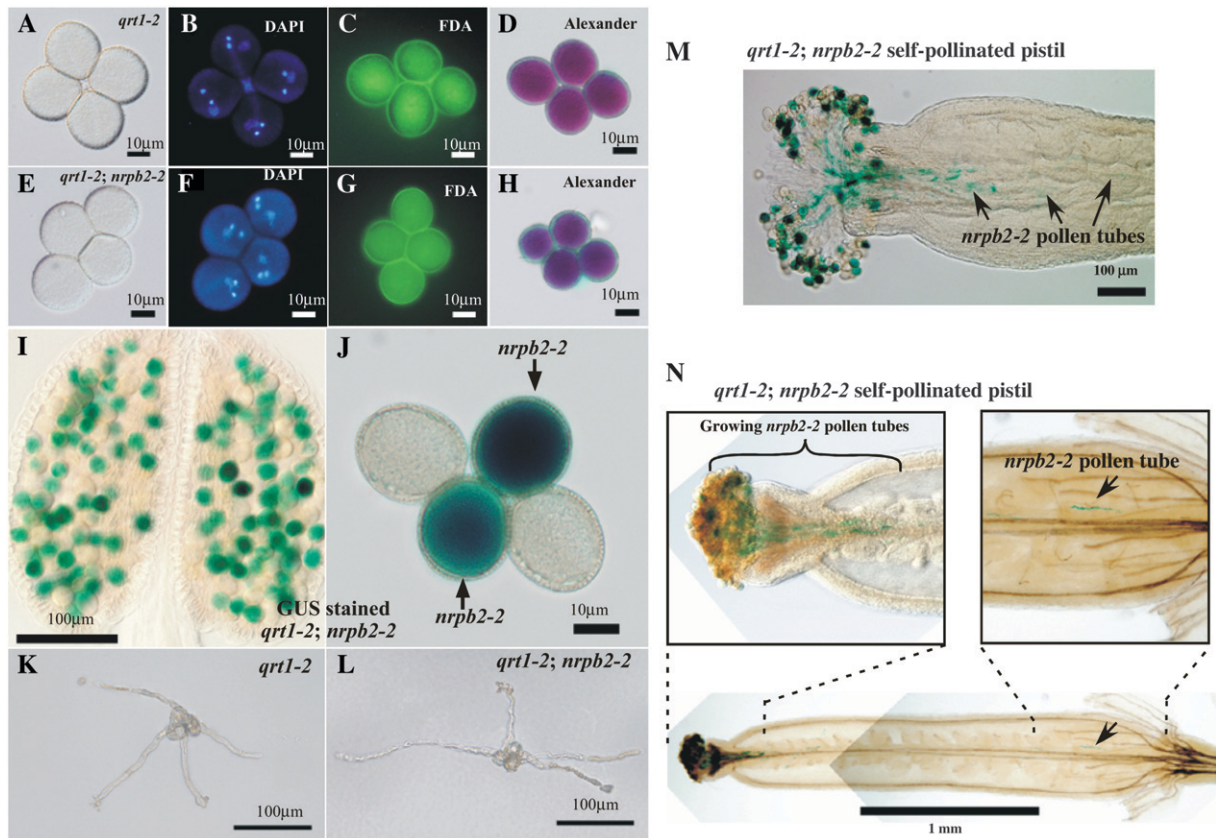
FIGURE 4.—Development and early tube elongation of pollen are unaffected by defects in RNA polymerases. (A–H) Cytological examination of mature pollen from *qrt1-2* (a–d) and *qrt1-2; +/nrpb2-2* (e–h). (a and e) Bright-field microscopy; (b and f) DAPI staining test; (c and g) FDA staining test; (d and h) Alexander staining test. (I and J) *LAT52::GUS* expression in pollen defective for the Pol II subunit (*nrpb2-2* pollen). (K and L) Germinating *qrt1-2* (k) and *qrt1-2; nrpb2-2* (l) pollen. Pollen was incubated for 18 hr at 22° in a germination medium and its images were captured. Note that four tubes of quartet pollen from wild-type (k, *qrt1-2*) and mutant (l, *qrt1-2; +/nrpb2-2*) plants grew equally in this assay, to a length of ∼100–150 mm. (M and N) Self-pollinated pistils from *qrt1-2; +/nrpb2-2* plants. *LAT52::GUS* was expressed during pollen tube growth in the absence of the functional allele of a catalytic subunit of Pol II. A considerable number of *nrpb2-2* pollen tubes (blue stained) was present in the top portions of the pistils. Note that a tube from *nrpb2-2* pollen grew into ∼2.0 mm in length (N).

nrpb2-2. Whereas GUS-stained *nrpb2-2* pollen tubes are rarely observed deeper than the top one-third of the pistil, GUS-stained control pollen tubes are easily detected throughout the top and middle one-thirds of the pistils and can be observed all the way to the base of the pistil (Figure 5C). Taken together, our results suggest that pollen germination, early pollen tube elongation, and fertilization are not severely affected by the lack of functional alleles for the RNA polymerase I, II, or III subunits. However, sustained pollen tube growth presumably requires *de novo* synthesis of essential RNA polymerase genes such that mutant pollen are at a competitive disadvantage compared to wild-type pollen, the phenomenon known as certation (HERIBERT-NILSSON 1920).

DISCUSSION

Genetic analyses have identified a large number of female gametophytic mutants in Arabidopsis, a signifi-

cant fraction of which correspond to mutant alleles of transcription factors (PAGNUSSAT *et al.* 2005). Our demonstration that mutations in RNA polymerases I, II, and III cause female gametophyte lethality are generally consistent with these findings and indicate that the female gametophyte is dependent on endogenous transcription machinery synthesized *de novo* during gametophyte development. In the absence of functional RNA polymerase subunits, female gametophytes can often progress to the two-nucleate stage, but typically arrest before, or shortly after, the second of the three mitotic divisions required for development of mature gametophytes. It is noteworthy that the SeedGenes Project database (http://www.seedgenes.org/index.html) (TZAFRIR *et al.* 2003, 2004) includes information for two T-DNA insertion alleles of *nrpb2*, named *emb 1989-1* and *emb 1989-2*. Embryos fail to develop in 90–94% of ovules bearing these mutant alleles, consistent with the female gametophytic lethal phenotype we describe in this article. However, 6–10% of *emb 1989-1* and *emb 1989-2* ovules are reported to arrest as preglobular embryos, indicating that
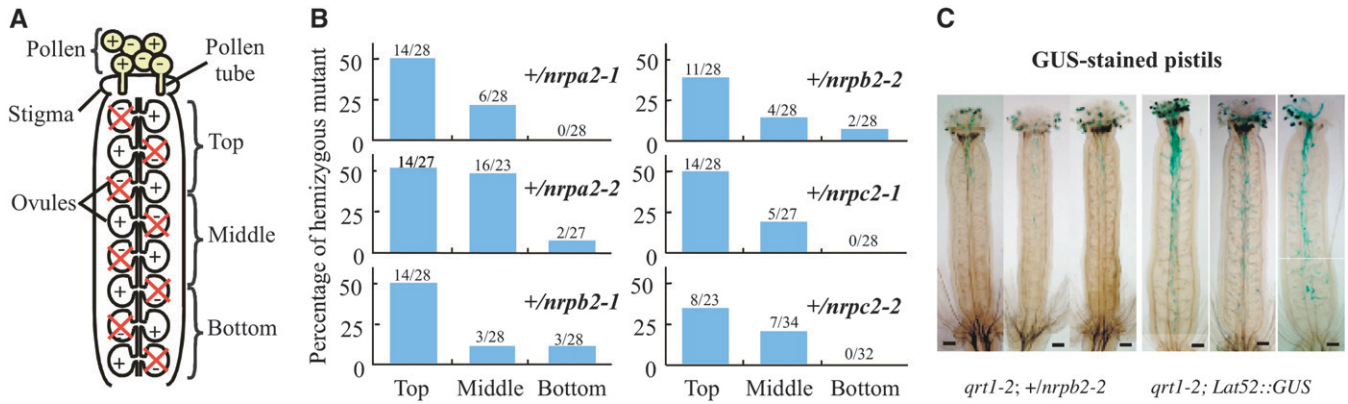
FIGURE 5.—Reduced paternal transmission of *nrpa2-1*, *nrpa2-2*, *nrpb2-1*, *nrpb2-2*, *nrpc2-1*, and *nrpc2-2* alleles relative to wild-type alleles in self-fertilized hemizygotes is due to decreased, competitive fertilization of ovules farthest from the stigma. (A) A diagram of the female floral organ (the pistil), whose surface (the stigma) is the site where a pollen grain germinates and initiates formation of a pollen tube. Half of the pollen of a hemizygote has wild-type (+) RNA polymerase alleles and half are mutant (−), but all develop and mature. Likewise, within the ovary of a hemizygote, half of the ovules are wild type and half are mutant with respect to the RNA polymerase alleles. However, the latter fail to develop (denoted with an "X") such that mutant alleles in fertilized ovules and seeds are derived from the male gametophyte. (B) Seeds collected from the top, middle, and bottom portions of siliques of the hemizygotes were germinated and resultant plants were genotyped. The numbers of plants of each genotype are indicated. Note that mutant alleles are more abundant in seeds developing nearest the stigma, at the top of the siliques, where the shortest pollen tubes would be needed to reach the ovules. (C) Self-pollinated pistils from *qrt1-2; +/ nrpb2-2* plants and transformants hemizygously carrying a *LAT52*::*GUS* reporter gene(s) inserted in an intergenic region (*qrt1-2; LAT52*::*GUS*). Pollen tubes from *qrt1-2; nrpb2-2* pollen (blue stained) were present in top portions of the pistils, while control pollen tubes (*qrt1-2; LAT52*::*GUS*) were observed all the way from the tops to the bottoms of the pistils.

the female gametophytes in these cases had completed development and had been fertilized, but produced embryos that were then unable to complete development. Cloning and sequencing of the region that defines the junction between the *NRPB2* gene and the T-DNA revealed that the T-DNA in *emb 1989-1* inserted 34 nucleotides upstream from the translation start site (Y. Onodera, data not shown). Because the protein coding region is not disrupted, it is possible that the *emb 1989-1* allele is partially functional, which may explain how development can sometimes proceed to stages beyond what we have observed for the *nrpb2-1* and *nrpb2-2* alleles. We currently lack analogous data concerning the precise location of the T-DNA in the *emb 1989-2* allele.

A recent study of developing and mature pollen showed that 61.9% of all Arabidopsis genes are expressed during at least one stage of male gametophyte development, with 9.7% of the transcripts being pollen specific (Honys and Twell 2004). A large number of transcription factors are expressed during pollen development, suggesting that orchestrated waves of transcription are essential for pollen maturation. Mature pollen is also known to contain proteins, ribosomes, mRNAs, rRNAs, and tRNAs that are synthesized postmeiotically during pollen maturation or pollen tube growth (Mascarenhas 1975, 1989). Therefore, we were surprised to find that functional alleles of RNA polymerases I, II, and III are not absolutely required in the haploid pollen genome to complete pollen development, germination, pollen tube growth, or fertilization. The simplest explanation is that transcription in pollen

bearing defective polymerase alleles is conducted using RNA polymerases, or stored mRNAs encoding RNA polymerase subunits, that are synthesized premeiotically in the hemizygous microspore mother cell and are then partitioned into the microspores following meiosis. The one functional allele is apparently sufficient for microspore mother cells to load microspores with enough polymerase to support subsequent pollen development and postgermination pollen functions, including pollen tube growth and fertilization.

Transcript profiling using DNA microarray technology has shown that mRNAs encoding the core subunits for nuclear RNA polymerases are present within unicellular microspores at similar or greater abundance than in sporophytic tissues (Honys and Twell 2004). However, in mature pollen, mRNAs encoding transcription factors, RNA processing proteins, and translation machineries are less abundant than in vegetative tissues of the plant (Honys and Twell 2003; Pina *et al.* 2005; Grennan 2007). This holds true for transcripts encoding the core subunits for nuclear RNA polymerases I, II, and III, which either are not detected in mature pollen or are present at very low levels (Honys and Twell 2003; Pina *et al.* 2005). The idea that maternally derived polymerase subunit mRNAs are stored for translation late in pollen development is not readily supported by these observations, but the possibility cannot be ruled out. An alternative hypothesis is that polymerase proteins derived from the microspore mother cell, or translated from mRNAs partitioned into the unicellular microspores, persist in mature pollen. Plants hemizygous for a

single-copy transgene expressing a polymerase subunit-GFP fusion protein would be useful for testing this hypothesis. If the transgene were capable of rescuing plants that were homozygous for null alleles of the corresponding endogenous genes, one would expect the GFP marker to segregate 2:2 among the pollen. If GFP were observed in all pollen, this would indicate maternal loading of the polymerase subunit. Regardless of whether stored mRNA or stored protein is responsible for allowing the transmission of mutant polymerase alleles through the pollen, there are enough of the stored molecules to complete pollen development, germination, and fertilization. These developmental events are thought to span a period of at least 90 hr (BOWMAN 1994). However, additional *de novo* synthesis of Pol I, II, and III is apparently needed for full pollen vigor and for growth of pollen tubes long enough to reach the ovules farthest from the stigma.

Given the reduced fitness of mutant pollen relative to wild-type pollen, deleterious mutant polymerase alleles are unlikely to become widespread among a population. However, some gene evolution phenomena would seem to be favored by allowing mutant alleles to persist in the population for some period of time. For instance, a characteristic of the RNA polymerase I transcription system is that it evolves rapidly, such that the transcription machinery of one species cannot transcribe the rRNA genes of an unrelated species (GRUMMT *et al.* 1982; MIESFELD and ARNHEIM 1984; DOELLING and PIKAARD 1996). Species specificity appears to be explained by the rapid evolution of rRNA gene sequences and the corresponding coevolution of the transcription machinery, such that changes in gene sequences can be tolerated as a result of compensatory changes in the proteins that bind these sequences (or vice versa). Because haploid selection against defective alleles is less stringent in the male gametophyte than in the female gametophyte, at least for subunits of RNA polymerases I, II, and III, it is tempting to speculate that the male lineage could be the conduit for transmitting mutations that might initially be deleterious but could be tolerated if a compensatory mutation in an interacting protein or DNA sequence were to occur. Transmitting mutations at moderate frequency via the pollen would presumably buy time for such compensatory mutations to occur. However, the null hypothesis is that the capacity to transmit mutations in essential housekeeping genes such as RNA polymerases via pollen has no evolutionary advantage and is merely an unintended consequence of pollen development.

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