pur4 Mutations Are Lethal to the Male, But Not the Female, Gametophyte and Affect Sporophyte Development in Arabidopsis[C][W]

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Purine metabolism is crucial in living cells and involves three complex pathways in plants: the de novo synthesis, the salvage, and the degradation pathways. The relative importance of each pathway in plant development and reproduction, however, is still unclear. We identified two T-DNA insertions in the Arabidopsis (*Arabidopsis thaliana*) *PUR4* gene (At1g74260) that encodes formylglycinamidine ribonucleotide synthase (EC 6.3.5.3), the fourth enzyme in the de novo purine biosynthesis pathway. The mutated alleles were never transmitted through the pollen of heterozygous plants but could be inherited through the female gametophyte, indicating that de novo purine synthesis is specifically necessary for pollen development. Because the *pur4* mutations were lethal to the male gametophyte, homozygous *pur4* plants could not be obtained. However, the reproductive phenotype of hetererozygous plants carrying the *pur4-2* mutated allele was more severe than that carrying the *pur4-1* mutated allele, and *pur4-2*/+ plants showed slightly delayed early development. We showed that the *pur4-2* allele produces an antisense transcript and that the amount of *PUR4* mRNA is reduced in these plants. Transient expression of a translational fusion with the green fluorescent protein in Arabidopsis plantlets showed that the formylglycinamidine ribonucleotide synthase protein is dually targeted to chloroplast and mitochondria, suggesting that at least some steps of the de novo purine biosynthesis pathway can take place in both organelles in Arabidopsis, a dual location previously thought to be a peculiarity of ureide-forming tropical legumes.

Purine nucleotides are major metabolites in living cells, at the crossroads of energy metabolism and the synthesis of essential cofactors and nucleic acids. In plants, purine molecules are also precursors for cytokinin metabolism and for the production of secondary metabolites such as ureides and alkaloids. Purine metabolism is complex, comprising a de novo synthesis pathway, which produces IMP, the direct precursor for AMP and GMP, from simple molecules; a salvage pathway allowing the recycling of purine rings at lower energetic cost; and a degradation pathway, which plays a central role in nitrogen metabolism (for review, see Moffatt and Ashihara, 2002; Stasolla et al., 2003; Zrenner et al., 2006).

De novo purine synthesis is thought to be crucial in dividing cells, because of the need for nucleotides in genome replication. However, in plants, high activity of salvage enzymes was reported in the cell proliferation phase of somatic embryogenesis (Stasolla et al., 2003). Therefore, it is not very clear how and when de novo purine synthesis is really crucial for plant cells and cannot be replaced by efficient recycling of preformed purines by the salvage pathway.

The genes encoding the enzymes that catalyze the first 10 steps of the purine de novo synthesis pathway have been identified in the Arabidopsis (*Arabidopsis thaliana*) genome (Boldt and Zrenner, 2003; van der Graaff et al., 2004). Most of these were cloned by functional complementation of *Escherichia coli* mutants before the completion of the Arabidopsis genome sequence (Senecoff and Meagher, 1993; Schnorr et al., 1994; Senecoff et al., 1996). The enzymes involved in this pathway in Arabidopsis are encoded by single copy genes, except for those catalyzing the first and eighth steps (amidophosphoribosyltransferase [ATase] and adenylosuccinate lyase), for which three and two genes, respectively, have been identified.

In plants, seven of the steps involve monofunctional enzymes, as in prokaryotes, whereas other eukaryotes have additional bifunctional enzymes (Smith and

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www.plantphysiol.org/cgi/doi/10.1104/pp.108.120014

Atkins, 2002). In addition, all of the plant enzymes carry N-terminal extensions, thought to direct them to organelles (Smith and Atkins, 2002). Analysis of the protein sequences using programs that predict protein subcellular localization suggests that de novo purine synthesis takes place in the plastids of plant cells (Smith and Atkins, 2002). Indeed, the ATase1 and ATase2 enzymes were localized to plastid stroma by immunolocalization of tagged proteins in electron microscopy and in fractionation experiments (Hung et al., 2004). In the N-fixing nodules of cowpea (*Vigna unguiculata*), however, activities for the de novo purine synthesis pathway were detected in both mitochondria and plastids (Atkins et al., 1997), suggesting that, at least in this species, the corresponding enzymes may be directed to both organelles. At present, therefore, the presence of the de novo purine synthesis pathway in mitochondria is considered a peculiarity of ureide-forming tropical legumes (Smith and Atkins, 2002).

In Arabidopsis, insertional and ethyl methanesulfonate mutations for ATase2 and an insertional mutation for ATase1 have been described, and in tobacco (*Nicotiana tabacum*), transgenic plants with reduced ATase were generated by expression of an antisense construct (Hung et al., 2004; van der Graaff et al., 2004). While the Arabidopsis *atase1* mutant has a wild-type phenotype (Hung et al., 2004), tobacco and Arabidopsis plants impaired in ATase activity have similar defects, including delayed growth and chlorotic leaves (Hung et al., 2004; van der Graaff et al., 2004). To our knowledge, no other mutations affecting the de novo purine synthesis pathway in plants have been reported.

Here, we report the characterization of two insertional mutations in the single copy gene (*PUR4*) encoding formylglycinamidine ribonucleotide synthase (FGAMS; EC 6.3.5.3), the fourth enzyme of the de novo purine biosynthesis pathway in Arabidopsis. This study provides strong evidence that de novo purine synthesis is crucial for male, but not female, gametophyte development. In addition, due to the suppression of *PUR4* expression by one of the mutations, the effect of impaired de novo purine synthesis could also be observed in the sporophyte. Furthermore, we show that Arabidopsis FGAMS is dually targeted to mitochondria and plastids, indicating that the de novo purine synthesis pathway, or at least a part of it, can be dually located in a nonlegume plant.

RESULTS

In a study aimed at identifying mutations that affect genes encoding proteins targeted to mitochondria or plastids and impaired in sexual reproduction (Berthomé et al., 2003), we identified two mutations in the *PUR4* gene (At1g74260). *pur4-1*, in the Columbia (Col0) genotype, was obtained from the Joe Ecker T-DNA insertional mutant collection (Salk Institute; SALK 050980); *pur4-2* was previously identified in the Wassilewskija (Ws) genotype by a screen designed to isolate gametophytic lethal mutations (Bonhomme et al., 1998).

We determined the complete sequence of the *PUR4* mRNA by sequencing overlapping amplification products from the cDNA and performing 3′ and 5′ RACE experiments. We detected a previously unreported intron in the 5′ untranslated region (UTR) of the gene and determined the 5′ and 3′ ends of the mRNA. The resulting sequence is aligned with the genomic sequence in Supplemental Figure S1. The *PUR4* gene structure is presented in Figure 1, showing the positions of the T-DNA insertions in the two mutant alleles. We analyzed both mutations by DNA hybridization analyses and sequencing (Supplemental Fig. S2). The *pur4-1* T-DNA insertion is complex, but its 5′ flanking sequence was confirmed by sequencing. The *pur4-2* T-DNA insertion is simple and was confirmed by sequencing the 5′ and 3′ flanking regions. In both cases, DNA hybridization showed that no other copy of the T-DNA was present in the plants carrying the mutated alleles, since the *nptII* probe on *Eco*RI digestions (only one site in each T-DNA) gave only one hybridization signal for both insertions.

Genetic Analyses of the *pur4-1* and *pur4-2* Mutations

The selfed progeny of hemizygous plants from both T-DNA lines was screened for the T-DNA insertion, either on kanamycin-containing medium or by direct PCR genotyping. Plants homozygous for the *pur4* mutations were never recovered for either allele. The transmission efficiency of both mutations in selfed plants is shown in Figure 2 (see Supplemental Tables S1 and S2 for detailed results). The transmission rate of both mutant alleles was dramatically lower than expected for a Mendelian trait. Two types of mutations result in lower transmission rates in selfed progeny and the absence of homozygous mutants: (1) embryo-lethal mutations, which are transmitted to two-thirds of the viable progeny because of the loss of homozygous mutants; and (2) gametophyte-lethal mutations, which are transmitted to only half of the progeny because of the loss of either the male or the female gametes carrying the mutation. If the mutation is lethal for both gametes, it is not transmitted to offspring and is completely lost.

We found that the *pur4* mutations were transmitted even less efficiently than expected for a gametophyte-lethal mutation and that the *pur4-2* allele was transmitted less efficiently than the *pur4-1* allele. These results suggest that at least one of the two gametophytes is unable to transmit the mutated *pur4* alleles. In order to determine the cause of this transmission deficiency more precisely, we made reciprocal crosses with wild-type plants (Table I). When the *pur4*/+ plants were used as pollen donors, none of the resulting progeny carried either of the *pur4* mutations, showing that the male gametophyte could not transmit the *pur4* mutations. We conclude that the *pur4* mutations are male gametophyte lethal. Following

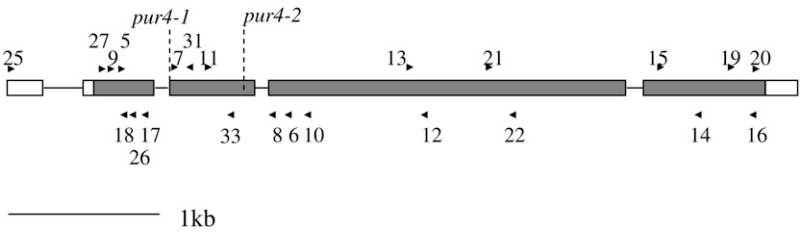

Figure 1. Schematic representation of the *PUR4* gene. Boxes and lines represent exons and introns, respectively, determined from the sequence of the mRNA obtained during this study. The protein coding sequence is shown in the gray boxes. Vertical dashed lines indicate the positions of the T-DNA insertions in the two mutant alleles. Numbered arrowheads show the positions of the primers used in this study (Supplemental Figs. S1 and S2; Supplemental Table S3).

crosses using the *pur4*/+ plants as the female parent, the two mutations were recovered, although often in less than half of the progeny (Table I). Therefore, we suspect that the *pur4* mutations also have an effect on the female gametophyte.

Overall, the transmission of the *pur4* mutant alleles was analyzed in progeny from 36 *pur4-1*/+ and 71 *pur4-2*/+ individual plants.

Phenotypic Analysis of the *pur4-1*/+ and *pur4-2*/+ Plants

Although all were heterozygous, the kanamycin-resistant plantlets carrying the *pur4-2* mutation showed delayed germination and early development, regardless of whether they resulted from selfing or outcrossing or were produced by *pur4-2*/+ plants themselves issued from selfing or backcrossing. In addition, the germination efficiency of seeds from *pur4-2*/+ plants was extremely variable (25%–100%) and often quite poor, whether produced by selfing or outcrossing or from plants themselves originating from selfing or backcrossing (data not shown). Sometimes, a few *pur4-2*/+ plantlets appeared to be chlorotic (Fig. 3A). The proportion (0%–2%) of these pale plants in (selfed or backcrossed) progeny was variable between individual progenies. When transferred to the greenhouse, pale plants grew slowly but finally reached the reproductive stage and set seeds at roughly the same time as wild-type siblings (Fig. 3B). All of the plants carrying the *pur4-1* mutation were indistinguishable from wild-type plants (Fig. 3A).

We observed that *pur4-2*/+ plants, issued either from selfing or backcrossing, had smaller anthers and produced less pollen than *pur4-1*/+ or wild-type plants (Fig. 3C). We Alexander stained and examined pollen in mature anthers of *pur4-1*/+ and *pur4-2*/+ plants issued from backcrosses (Fig. 3C) and scored viable (red-stained) or aborted (green-stained) pollen grains (Fig. 3D). In both wild-type controls, no aborted pollen was observed. In *pur4-1*/+ plants, however, half of the pollen was aborted, and because the mutation is not transmitted through pollen, we inferred that the dead pollen carries the *pur4-1* mutation. In *pur4-2*/+ anthers, more than half of the pollen had aborted. We concluded that some of the pollen grains carrying the wild-type *PUR4* allele also died in the *pur4-2*/+ background.

We carried out further cytological observations on anthers from *pur4-1*/+ and *pur4-2*/+ plants issued from backcrosses in order to define the precise stage at which pollen development is impaired (Fig. 4). In both heterozygous genotypes, pollen development was indistinguishable from that of the wild type until the vacuolated microspore stage (buds with a 1-mm pistil; data not shown). In buds with a 1.25-mm pistil, corresponding to pollen mitosis I (PMI) or soon after, we observed poorly stained cytoplasm and a remaining large vacuole in a large proportion (roughly half) of pollen grains in both heterozygous genotypes (Fig. 4A). In *pur4-1*/+ and *pur4-2*/+ buds with a 1.5-mm pistil, corresponding to pollen mitosis II (PMII) or soon after, about half of the pollen grains had almost completely collapsed. In addition, in *pur4-2*/+ anthers, some of the remaining normally shaped pollen grains also showed poorly stained cytoplasm (arrows in Fig. 4B). Again, the proportion of these poorly

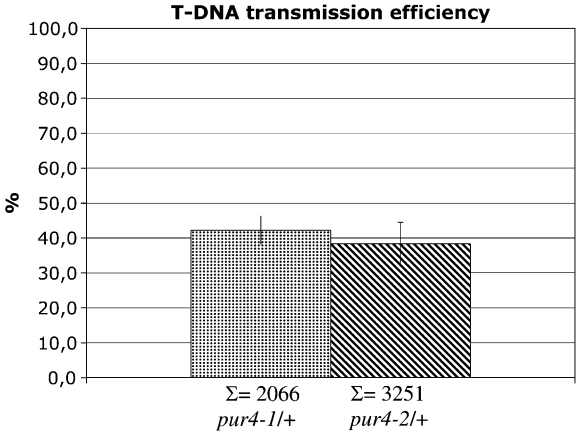

Figure 2. T-DNA transmission efficiency for *pur4-1*/+ and *pur4-2*/+ plants. Transmission of the T-DNA responsible for the mutations in the selfed progeny of heterozygous plants. The transmission of the T-DNA was screened by genotyping PCR for *pur4-1*/+ progeny and by selection on kanamycin-containing medium for *pur4-2*/+ progeny. The numbers under the bars indicate the total number of seedlings scored for each mutation. For each mutation, selfed progeny from several heterozygous individuals were analyzed separately and considered for the SD calculations. Details of the results for each progeny are given in Supplemental Tables S1 and S2.

Table I. *Results of reciprocal crosses between pur4-1/+ and pur4-2/+ plants and wild-type plants*

Maternal Parent of the Cross	Paternal Parent of the Cross	*pur4*/+ Progeny[a]	Wild-Type Progeny[a]	χ^2 to the 1:1 Segregation
pur4-1 no. 1	Col0	33	65	10.45[b]
pur4-1 no. 11	Col0	33	30	0.14
Col0	*pur4-1* no. 2	0	223	–
pur4-2 no. 8	Ws	66	91	3.98[c]
pur4-2 no. 6	Ws	9	14	1.09
Ws	*pur4-2* no. 8	0	61	–

[a]The progeny genotype was scored by PCR genotyping for *pur4-1*/+ and by growth on kanamycin-containing medium for *pur4-2*/+. [b]This result is different from the tested hypothesis with an α risk of $P < 0.01$. [c]This result is different from the tested hypothesis with an α risk of $P < 0.05$ (1 degree of freedom).

stained pollen grains was variable among *pur4-2*/+ plants. Next, we stained mature pollen with 4′,6-diamino-phenylindole (DAPI) and found that some of the nonaborted pollen grains in *pur4-2*/+ anthers had abnormal nuclei numbers, suggesting impaired pollen mitoses (Fig. 4C).

Finally, we scored the number of fully developed seeds and the number of empty spaces (holes) in mature siliques of plants issued from backcrosses. The results are shown in Fig. 5. Both heterozygous mutants showed lower seed set than their wild-type siblings. Again, the phenotype was more severe for the *pur4-2* allele.

The *pur4-2* Mutation Leads to the Suppression of *PUR4* mRNA

We analyzed the expression of the *PUR4* gene in *pur4-2*/+ plants issued from selfing compared with wild-type plants by performing quantitative reverse transcription (RT)-PCR analyses on total RNA from different organs (data not shown). In all organs, the *PUR4* mRNA was accumulated at less than 20% of the wild-type level in *pur4-2*/+ plants. We repeated quantitative RT-PCR analysis on plantlets issued from the backcross. Results of the expression level of PUR4 in several individual plants are shown in Figure 6A. We observed that *pur-4*/+ plantlets accumulated between 15% and 30% of the amount of *PUR4* mRNA found in their wild-type siblings. Seedlings carrying the *pur4-1* mutation accumulated approximately 50% of the wild-type level of *PUR4* mRNA (data not shown).

The low level of *PUR4* mRNA in *pur4-2*/+ plants suggests that the accumulation of the *PUR4* transcript is affected by a suppression mechanism in these plants. A possible explanation could be that an aberrant RNA is produced from the mutated allele, leading to a decrease of the *PUR4* transcript. To test this hypothesis, we performed RT-PCR experiments with primers allowing the detection of sense and antisense transcripts in plants issued from the backcross. The results are shown in Figure 6, B and C. The sense transcript originating from the *PUR4* allele was detected in both *pur4-2*/+ and wild-type plants (Fig. 6B). Oppositely, we detected an antisense transcript only in *pur4-2*/+ plants (Fig. 6C). A partial sequence of the *pur4-2*-specific antisense RNA was obtained by sequencing several RT-PCR products. Alignment of the resulting sequences with the *PUR4* gene and the pGKB5 T-DNA sequences is shown in Supplemental Figure S1. We conclude that the *pur4-2* allele produces an antisense chimeric transcript, overlapping the *PUR4* and T-DNA sequences, which may interfere with *PUR4* mRNA accumulation.

The N-Terminal Portion of the PUR4 Protein Directs GFP to Mitochondria and Chloroplasts

The enzymes for de novo purine synthesis are predicted to be targeted to plastids, and the Arabidopsis ATase, which catalyzes the first step of the pathway, was shown to be present in chloroplasts (Hung et al., 2004). However, in tropical legumes, this pathway was localized to both mitochondria and plastids (Atkins et al., 1997; Smith and Atkins, 2002). Therefore, we decided to investigate the subcellular localization of the PUR4 protein in Arabidopsis. We constructed a translational fusion between the first 94 amino acid residues of the PUR4 protein and the GFP under the control of the 35S promoter using Gateway technology. The resulting chimeric gene possesses the entire 5′ UTR of the *PUR4* gene. Transient expression experiments were performed in *Nicotiana benthamiana* leaves (data not shown) and Arabidopsis plantlets via *Agrobacterium* infiltration, using the PUR4-GFP resulting vector and a vector expressing a potato (*Solanum tuberosum*) formate dehydrogenase (FDH)-DsRed2 fusion known to be targeted to mitochondria (Ambard-Bretteville et al., 2003). Figure 7 shows an example of the typically observed patterns in Arabidopsis adaxial cotyledon cells. Both GFP and DsRed2 signals exhibited very similar distribution patterns in cells and could be superposed in an overlay (Fig. 7, A, B, and D). Both signals colocalized in optical sections, indicating that both proteins are targeted to the same organelles. Optical sections also revealed that the GFP signal in PUR4-GFP-expressing cells was also visible in the chloroplasts, identified by the autofluorescence of the

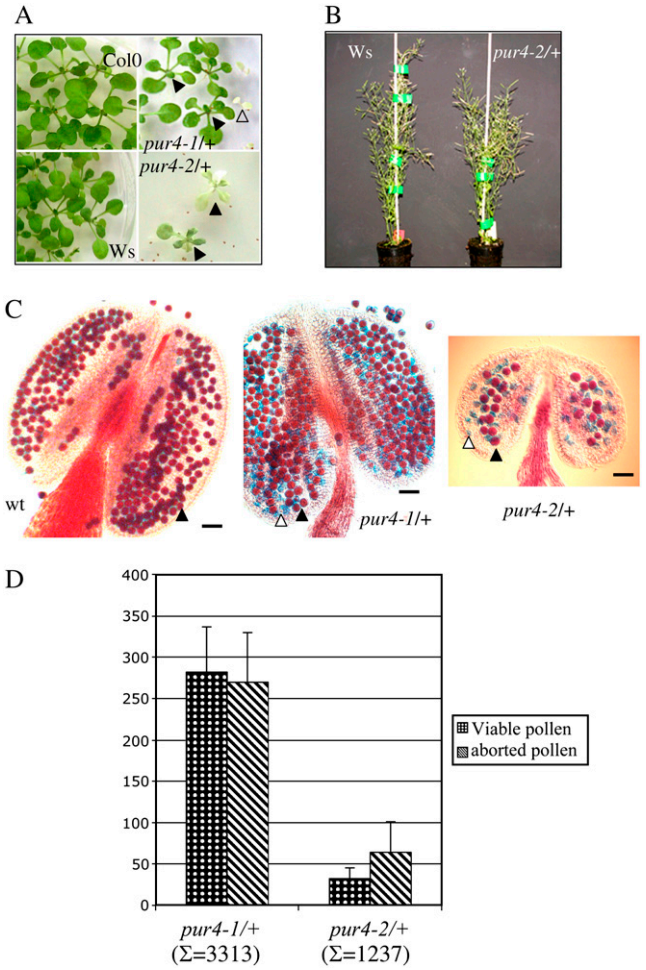

Figure 3. A, In vitro segregation of kanamycin-resistant and kanamycin-sensitive seedlings for the *pur4*/+ hemizygous mutants on medium supplemented with 100 mg L^{-1} kanamycin. Wild-type controls were germinated on nonselective medium. Photographs were taken at 15 d after sowing. The clear arrowhead indicates a typical kanamycin-sensitive seedling, and the black arrowheads point at kanamycin-resistant plantlets. Note the phenotype of *pur4-2*/+ kanamycin-resistant seedlings. B, Typical fully grown plants. The *pur4-2*/+ plant was from a pale seedling as seen in A. C, Alexander staining of anthers to detect viable and aborted pollen. wt, Wild type. Both wild-type accessions gave the same results; here, Col0 is shown. Note that anthers from *pur4-2*/+ plants are smaller than wild-type and *pur4-1*/+ anthers. The black arrowheads indicate normal, viable pollen grains, and the white arrowheads indicate aborted pollen grains. Bars = 40 μm. D, Pollen viability in the heterozygous plants carrying the *pur4-1* and *pur4-2* mutations. Pollen viability was determined by counting normal and aborted pollen after Alexander staining. The total number of counted pollen grains is given. Analyzed anthers were from six different *pur4-1*/+ plants and 13 different *pur4-2*/+ plants. In control experiments carried out on wild-type plants of either the Ws or Col0 genotype, no aborted pollen was observed. [See online article for color version of this figure.]

chlorophyll (Fig. 7, B–D). These results strongly suggest that the Arabidopsis FGAMS is localized to both organelles, as shown previously for other enzymes of the de novo purine biosynthesis pathway of legumes (Smith and Atkins, 2002).

DISCUSSION

Arabidopsis FGAMS Is Targeted to Mitochondria and Chloroplasts

Our results strongly suggest that the N-terminal portion of the Arabidopsis PUR4 protein, most probably containing targeting sequences, directed GFP to mitochondria as well as chloroplasts. Until now, the de novo purine synthesis pathway was thought to occur in plastids, with the exception of the nodules of ureide-forming legumes, in which the pathway was detected in both mitochondria and plastids (Atkins et al., 1997; Smith and Atkins, 2002). Convincing evidence for the plastidial localization of the pathway was the unambiguous localization of ATase in chloroplast, and not mitochondria, by fractionation and immunogold-labeling electron microscopy (Hung et al., 2004). In addition, several enzymes of the de novo purine synthesis pathway, encoded by the *PURD*, *PUR4/PURL*, *PURH*, *PURA*, and *PURB1* genes, were found in the plastid proteome (Kleffmann et al., 2004; Peltier et al., 2006). However, enzymes encoded by *PUR4/PURL* and *PURM*, which catalyze subsequent reactions in the pathway, were identified in the mitochondrial proteome (Ito et al., 2006). Noticeably, FGAMS was found in both organelle proteomes. This is in good agreement with our observations of the dual localization of the PUR4-GFP fusion protein. Indeed, it was recently pointed out that dual targeting of proteins to mitochondria and plastids is more prevalent than expected in the first place (Millar et al., 2006). Dual targeting to mitochondria and plastids was reported for enzymes involved in tRNA function, such as tRNA synthetases (Duchene et al., 2005) and tRNA nucleotidyl transferase (von Braun et al., 2007), and for other enzymes formerly thought to be solely addressed to plastids, such as Gln synthetase (Taira et al., 2004). Among the mechanisms found to induce the dual targeting of single gene products are the generation of translation products with different N-terminal extensions using alternative start codons (von Braun et al., 2007) and the use of ambiguous addressing sequences (Karniely and Pines, 2005). The *PUR4* gene carries three in-frame start codons that could be alternatively used to encode products with slightly different lengths of addressing sequences. Within the first 30 amino acids of the coding sequence, several Arg residues can be found that could also play a role in dual targeting (Pujol et al., 2007). Additional studies, involving modified UTR and coding sequences, would be necessary to identify the elements determining the dual targeting of FGAMS. Nevertheless, our results, together with organelle proteome analyses, strongly suggest that at least part of the de novo purine synthesis pathway may also occur in mitochondria in a nonlegume species. The cell and organ type(s) in which mitochondria are involved in this pathway still remains to be investigated.

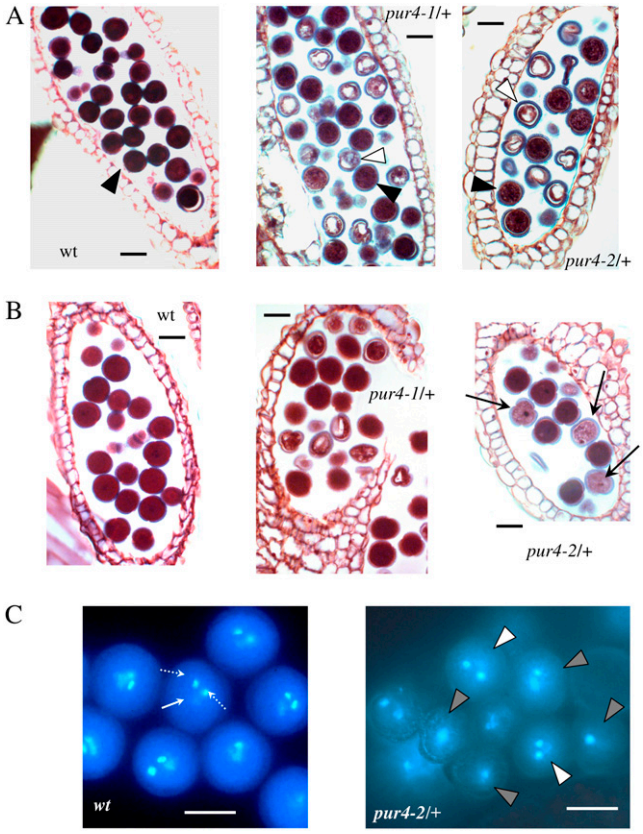

Figure 4. Cytological analysis of pollen development in *pur4-1*/+ and *pur4-2*/+ plants. A and B, Toluidine blue staining of anther sections. Anthers were embedded in resin, sectioned, and stained as described in "Materials and Methods." Both wild-type (wt) accessions gave the same results; therefore, only one is shown here. In A, anthers are from buds with a 1.25-mm pistil (i.e. at, or soon after, PMI); in B, anthers are from buds with a 1.5-mm pistil (i.e. at, or soon after, PMII). The black arrowheads indicate normal-looking pollen grains, the white arrowheads indicate aborted pollen grains, and the arrows indicate pollen grains that did not abort after PMI but have abnormally clear cytoplasm. C, DAPI staining of pollen grains from fresh mature wild-type and *pur4-2*/+ anthers (buds with a 1.75-mm pistil). Anthers were dissected from freshly harvested flower buds and stained as described in "Materials and Methods." The white arrowheads indicate normal trinucleate pollen grains, with one vegetative nucleus (plain white arrow) and two reproductive nuclei (dashed white arrows). The gray arrowheads indicate abnormal pollen grains. Bars = 20 μm. [See online article for color version of this figure.]

FGAMS Is Crucial for Pollen Development

We characterized two insertional mutations in the single copy gene encoding the enzyme that catalyzes the fourth step of de novo purine synthesis in Arabidopsis. From our results, it appears that the *pur4-1* allele is most likely a null mutation. First, the insertion is in the second protein-encoding exon; second, it appears to be associated with a partial deletion of this exon, together with a rearrangement of a small part of the gene (Supplemental Fig. S2). We showed that the *pur4-2* allele produces an antisense RNA that overlaps the T-DNA and the *PUR4* gene and is spliced at abnormal positions (Fig. 6; Supplemental Fig. S1). Abnormal splicing of the *pur4-2* transcript is consistent with its antisense direction. Due to the presence of the antisense RNA from the *pur4-2* allele, it is likely that the decrease in *PUR4* mRNA observed in *pur4-2*/+ heterozygous plants (Fig. 6) is caused by a suppression mechanism.

We observed no pollen transmission of either *pur4* mutation in the progeny of more than 100 heterozygous plants issued either from selfing or backcrossing. These results show unambiguously that *pur4* null alleles are male gametophyte lethal. In addition, staining of mature pollen showed that at least half of the pollen from *pur4-1*/+ and *pur4-2*/+ plants aborted (Fig. 3, C and D). These results show that FGAMS activity is essential for proper pollen development. FGAMS, therefore, is the second enzyme involved in a metabolic pathway that is necessary for male gametophyte development. Recently, Ser palmitoyltransferase, an enzyme involved in the de novo synthesis of sphingolipids, was shown to be essential for pollen development (Teng et al., 2008). Other mutations in genes encoding proteins involved in nutritional or metabolic pathways and that affect male gametophyte development are the genes encoding a plasma membrane H^+-ATPase (*AHA3*; Robertson et al., 2004) and a Glc-6-P translocator (*GPT1*; Niewiadomski et al., 2005; for review, see Twell et al., 2006).

Our results tend to indicate that de novo purine biosynthesis is necessary for the formation of normal, viable pollen grains. The only other step of de novo purine biosynthesis for which mutants have been described is the first step of the pathway, catalyzed by ATase. Arabidopsis plants carrying mutations in the two genes encoding ATase were reported to have delayed growth but no pollen abnormalities (Hung et al., 2004; van der Graaff et al., 2004). Furthermore, the occurrence of homozygous mutants clearly shows that pollen carrying the two *atase* mutations is viable. Similarly, tobacco plants with impaired ATase activity due to silencing were not reported to display fertility

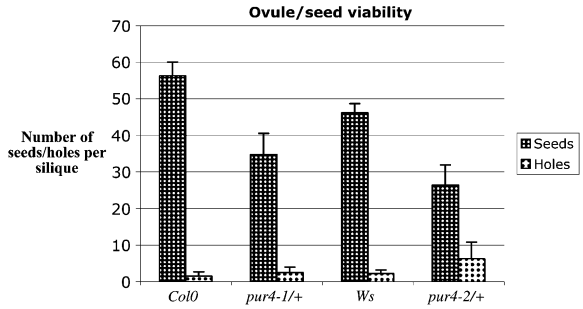

Figure 5. Ovule and/or seed viability analysis. Ovule and/or seed viability was examined by detecting empty spaces (holes) in mature, indehiscent siliques after selfing. The total number of siliques (seeds) observed was as follows: 60 (2,117) for *pur4-1*/+; 56 (1,482) for *pur4-2*/+; 10 (562) for Col0; and 10 (462) for Ws.

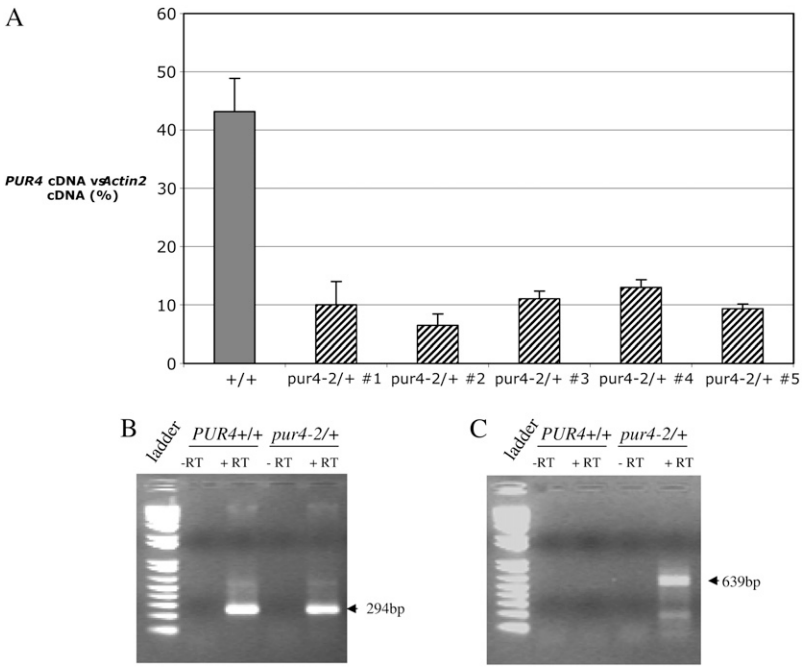

Figure 6. Expression analysis of *PUR4* in *pur4-2*/+ plants. A, Quantitative RT-PCR analyses of *PUR4* mRNA in wild-type and *pur4-2*/+ siblings from a backcross. PCR amplifications were achieved on cDNA samples from five individual *pur4-2*/+ plants and a wild-type sibling. Results are expressed as percentage of *ACTIN2* cDNA. Means and SD from two technical repetitions are shown. B and C, RT-PCR amplification of mRNAs from the *PUR4* and *pur4-2* alleles in the wild type and *pur4-2*/+ siblings from a backcross. PCR with primer sets 20/28 (B) and 28/33 (C) was performed on cDNA samples (+RT) from seven individual *pur4-2*/+ plants (only one is shown) and a wild-type sibling. Controls in which reverse transcriptase was omitted are shown (−RT). The sizes of the amplification products are shown on the right.

defects (van der Graaff et al., 2004). The discrepancy between our mutant phenotype and that of ATase mutants seems to indicate that not all steps of the pathway are crucial to male gametophyte development. We can hypothesize that the amount of FGAM, the product of the fourth step of the pathway, is limiting in pollen development as early as PMI, while 5-phosphoribosylamine, the product of the first step of the pathway, is in large amounts in the pollen during development and tube growth. We propose that limiting amounts of pathway substrates and products result in specific steps of the pathway becoming preferentially significant for functions in different organs, explaining the differences in *atase* and *pur4* mutant phenotypes.

pur4 Mutations Only Partially Affect Female Gametophyte Development

Interestingly, both *pur4* mutations are successfully transmitted through the ovules, ensuring that the mutant alleles were not directly lost after the first generation. However, for both mutants, the efficiency of transmission through the female gametophyte was sometimes lower than for the wild-type allele (Table I), and heterozygous *pur4*/+ plants produce fewer seeds than their wild-type counterparts, regardless of whether they are issued from selfing or backcrossing (Fig. 5). These results suggest that the *pur4* mutations also affect the viability of the female gametophyte, but with lower penetrance than for the male gametophyte. Therefore, we infer that FGAM synthesis is less limiting during female than male gametophyte development. It is unlikely that embryo sac development requires fewer purines than pollen development; for example, it involves three successive divisions, whereas male gametophyte development involves only two divisions. Consequently, we propose that the female gametophyte benefits from purine inputs from surrounding cells, conferring tolerance to purine synthesis deficiency. Purines could be supplied into the developing embryo sac from the three other meiosis products, ensuring recycling of their contents, perhaps through the purine salvage pathway, to the advantage of the cells entering gametophytic development, before their collapse. Alternatively, sporophytic cells, which tightly surround the female gametophyte throughout development, could be a direct source of purines. Another possibility, which cannot be excluded at the moment, is that FGAM accumulates in the megaspore, reducing the need for FGAM synthesis during embryo sac development.

PUR4 Is Probably Important for the Plant Sporophyte…

Gametophyte-lethal mutations can be of two types, as pointed out by Feldmann et al. (1997) in the first report of these mutations. The first type includes mutations that affect functions specifically necessary for the gametophytic phase of the life cycle. For example, this is the case of a pectin methylesterase encoded by the *VGD1* gene and necessary for pollen tube growth in the style (Jiang et al., 2005). The second type includes mutations that affect functions necessary throughout the whole life cycle and that therefore have a deleterious effect in haploid cells but, due to the presence of the wild-type allele, the sporophytic heterozygous tissues are healthy. Because diploid plants homozygous for a gametophyte-lethal mutation cannot be obtained, it is not always simple to determine

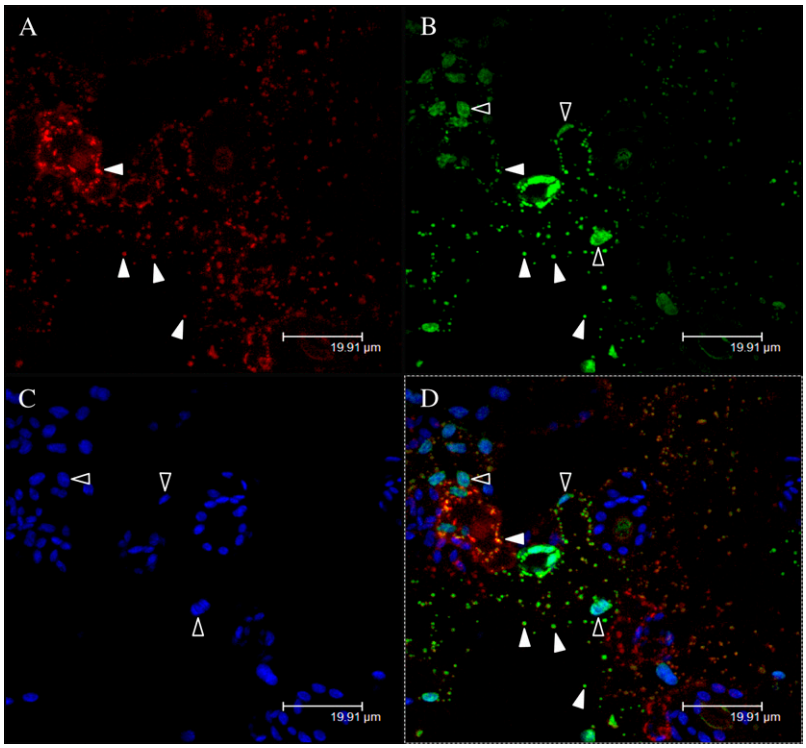

Figure 7. Cellular localization of the PUR4-GFP fusion in Arabidopsis adaxial cotyledon cells. Confocal images are shown for DsRed2 (A) and GFP (B) fluorescence and chlorophyll autofluorescence (C) in Arabidopsis adaxial cotyledon cells transiently expressing both PUR4-GFP and FDH-DsRed2 fusion proteins. D shows the overlay. White arrowheads show fluorescence signals specifically obtained in mitochondria, and clear arrowheads indicate fluorescence signals acquired in chloroplasts.

which of these types is a gametophytic mutation and to explore its effects on the sporophyte. One possible way to avoid this obstacle is to mimic a mutation at the homozygous state in the diploid either by suppressing the expression of both alleles, using RNA interference or silencing, or using dominant negative mutations. For example, dominant negative mutants were used to explore the role of rhoGTPases, which are necessary for normal pollen tube growth, in the development of Arabidopsis plants (Li et al., 2001).

We observed that plants carrying the *pur4-2* mutation accumulated lower levels of *PUR4* mRNA than wild-type or *pur4-1*/+ plants (Fig. 6A; data not shown). The detection of an antisense RNA transcript originating from the *pur4-2* allele provides a possible explanation for this observation (Fig. 6B). The sequence analysis of this antisense RNA shows that it results from a transcriptional fusion of the *PUR4* gene and the T-DNA and displays aberrant splicing (Supplemental Fig. S1), indicating that its transcription starts in the T-DNA sequence. Its presence, therefore, could lead to the partial degradation of *PUR4* mRNA, resulting in lower expression of the gene in *pur4-2*/+ plants.

The metabolic function of the *PUR4* product implies that its depletion in sporophytic tissues would have severe consequences, at least in parts of the plant and/or at time points in development where large amounts of purine nucleotides are needed. However, the purine synthesis salvage pathway can provide cells with purine nucleotides and is activated in important phases of development, such as embryo formation and seed germination (Stasolla et al., 2003). In addition, a mutation in the gene for a salvage pathway enzyme, *APRT* (for *ADENOSINE PHOSPHORIBOSYLTRANSFERASE*) leads to male sterility (Gaillard et al., 1998), indicating that the sporophytic tissue salvage pathway is necessary for pollen development. The partial suppression of *PUR4* in *pur4-2*/+ plants provides an interesting tool for exploring how much FGAMS is required by the plant and during which developmental programs. *pur4-2*/+ plantlets germinated and developed more slowly than wild-type siblings at very early stages of development. Despite this slow growth, however, they reached the flowering stage at about the same time as wild-type siblings (Fig. 3). Also, when sown in vitro, a few of the *pur4-2*/+ seedlings appeared chlorotic at early stages, but they recovered normal greening after transfer to the greenhouse. Nevertheless, the overall vegetative phenotype of plants partially depleted of *PUR4* mRNA is not very different from that of wild-type plants. Moreover, the severity of the observed phenotype was always heterogeneous, even between siblings; this type of heterogeneity was previously observed for a phenotype resulting from antisense suppression (Golovkin and Reddy, 2003). It is likely that a complete null mutation would not be viable, and the amounts of *PUR4* mRNA still present in *pur4-2*/+ plants might be sufficient to ensure nearly normal activity. For example, the FGAMS enzyme could be very stable and accumulate in partially suppressed *pur4-2*/+ plants at almost normal levels during the slow growth of the plants. Alternatively, FGAM might not be very limiting during vegetative growth or the de novo pathway might be

efficiently compensated for by the salvage pathway. Interestingly, we observed lower germination efficiency of seeds from the *pur4-2/+* plants, in variable proportions but probably regardless of their genotype, as suggested by transmission of the mutated allele. This lower germination, therefore, might result from a maternal effect on the purine pool available for germination in the progeny.

…and Particularly for Sustaining Pollen Development

Interestingly, all *pur4-2/+* plants, originating from selfing or backcrosses, displayed a more severe pollen phenotype than *pur4-1/+* plants (Fig. 3, C and D). Indeed, *pur4-2/+* plants have smaller anthers and produce less pollen than wild-type and *pur4-1/+* plants. This suggests that although the vegetative phenotype of most of these plants was nearly normal, the amount of remaining *PUR4* mRNA in sporophytic tissues of reproductive organs is limiting for pollen development. Analysis of *PUR4* mRNA accumulation in anther tissues would be necessary to test this hypothesis. In addition, our cytological analysis suggests that, in *pur4-2/+* anthers, some wild-type pollen grains aborted at a later stage than *pur4* pollen (Figs. 3, C and D, and 4). DAPI staining of pollen in *pur4-2/+* plants suggested that PMII (and maybe PMI) was affected in some pollen of these plants (Fig. 4C). Considering our findings, it is likely that, although pollen development needs active de novo purine synthesis in the gametophyte, it is also dependent on the purine synthesis pathway in the surrounding sporophyte. Therefore, our results, together with those obtained regarding the *aprt* mutant (Gaillard et al., 1998), suggest that both the de novo and salvage purine synthesis pathways are necessary in sporophytic tissues to sustain male gametophyte development. We also observed that *pur4-2/+* plants produce fewer seeds, and their siliques contain more empty spaces, than wild-type and *pur4-1/+* plants (Fig. 5). However, we cannot conclude whether the *pur4-2* allele affects female gametophyte or embryo development (or both).

Altogether, our results demonstrate that de novo purine synthesis, and particularly the fourth step, catalyzed by FGAMS, is crucial for plant reproduction, particularly in male gametophyte development, but probably also in the sporophytic tissues sustaining pollen and embryo sac developments. Together with other reports, this suggests that both salvage and de novo synthesis pathways play an important role in the anther during pollen development. Further studies are needed to decipher their respective roles and to what extent reciprocal compensation occurs between the two pathways.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of Arabidopsis (*Arabidopsis thaliana*) ecotype Col0 carrying the *pur4-1* mutation (SALK 050980) and seeds of Arabidopsis ecotype Ws carrying the *pur4-2* mutation (FST 064G02) were obtained from the Salk Institute collection (Alonso et al., 2003) and the Versailles collection (Bechtold et al., 1993) of T-DNA insertional mutants, respectively. When the use of wild-type siblings as controls is not specified, wild-type Arabidopsis Col0 and Ws seeds were used as controls.

Seeds grown in vitro for genetic analyses and for kanamycin resistance assays were surface sterilized for 10 min with a solution containing 10% (v/v) commercial bleach (Minichlor; Hygiena) diluted in 95°C ethyl alcohol and 0.05% (v/v) Teepol 610 (Serva). Seeds were rinsed thoroughly with 95°C ethyl alcohol and dried overnight in a sterile hood. Seeds were sown on Arabidopsis medium (Estelle and Somerville, 1987) with or without kanamycin (100 mg L^{-1}) and germinated in a growth chamber (16-h-light/8-h-dark cycle, 21°C, 50% hygrometry) after a cold treatment for 48 h at 4°C. Plants used for phenotypic and cytological analyses were grown in the greenhouse at 20°C to 25°C under a 16-h-light/8-h-dark cycle. For RNA extraction from separated organs, plants were grown hydroponically in the greenhouse as described above. Arabidopsis plantlets of the Landsberg *erecta* genotype used for transient expression experiments were grown in vitro as described above.

Molecular Analyses

Primers used in this study are shown in Supplemental Table S3, and the positions of those targeted to the *PUR4* gene are shown in Figure 1.

Extraction of Nucleic Acids

For genotyping PCR, genomic DNA was quickly extracted according to Edwards et al. (1991). For DNA hybridization experiments, genomic DNA was extracted from plantlets or leaves as described by Bouchez et al. (1996). Total RNA was extracted from roots, leaves, stems, and flowers of 4-week-old plants using Trizol reagent (Invitrogen). One hundred micrograms of total RNA was treated with 30 units of RNase-free DNase I using the RNeasy plant mini kit cleaning procedure (Qiagen) and eluted with RNase-free water. The quality of RNA was verified by gel electrophoresis using either ethidium bromide staining or an Agilent RNA 6000 nano kit and Agilent 2100 bioanalyzer (Agilent Technologies).

PCR Genotyping

T-DNA insertions were confirmed using specific primers from either the right or left border of the T-DNA and gene-specific primers (primers 1/2 and 3/4). The *PUR4* wild-type allele was amplified using a pair of gene-specific primers (primers 5/6 and 7/8). PCR annealing temperatures and expected sizes of amplification products are presented in Supplemental Table S4 for all of the primer pairs used in this study. PCR amplifications were performed with 100 ng of genomic DNA as template in a reaction volume of 25 μL containing 1× *Taq* buffer, 1.5 mM $MgCl_2$, each deoxynucleotide at 0.2 mM, each primer at 0.5 μM, and 1 unit of *Taq* DNA polymerase (Fermentas). The PCR was run under the following conditions: 5 min of initial denaturation at 95°C, 30 cycles of 30 s at 95°C, 30 s at specific annealing temperature, and 1 min kb^{-1} (depending on the amplification product size) at 72°C, followed by 10 min at 72°C. Amplification products obtained with gene-specific primer/right border or left border primer combinations were cloned and sequenced. For each PCR product, four independent clones were sequenced.

Sequencing of the Transcripts

RT-PCR experiments were carried out to determine the full-length *PUR4* mRNA sequence. The absence of DNA contamination in total RNA was tested by PCR as described above using *PUR4* gene-specific primers 27 and 31. First-strand cDNA was synthesized as follows: 500 ng of total RNA was mixed with 1 μg of dT_{18} primer, and the volume was adjusted to 10.5 μL with RNase-free water. Samples were denatured at 65°C for 5 min. The following mix composed of 1× RT buffer, 0.625 mM dNTPs, 10 mM DTT, and 20 units of RNase inhibitor (Fermentas) was added to the samples to reach a final reaction volume of 19 μL. A total of 200 units (1 μL) of RevertAid H minus M-MuLVRT (Fermentas) was added (in RT-minus samples, it was replaced by 1 μL of RNase-free water), and samples were incubated for 1 h at 42°C. Reactions were terminated at 70°C during 15 min. One microliter (2 units) of RNase H (Fermentas) was finally added to the reaction, and samples were incubated for

20 min at 37°C. PCRs were run as described above using 2 μL of first-strand cDNA product. Four pairs of gene-specific primers (9/10, 11/12, 13/14, and 15/16), producing four overlapping amplification products, first-strand cDNA from wild-type plants, and AB gene high-fidelity *Taq* polymerase (Promega) were used for the sequencing of the *PUR4* mRNA. 5′ UTR and 3′ UTR PCR products were obtained using 5′ and 3′ RACE systems (Invitrogen) with 5′ and 3′ gene-specific primers (17/18 and 19/20).

To detect the antisense mRNA PCR product, a first PCR was run with 5 μL of first-strand cDNA obtained as above from *pur4-2*/+ plants using primers 28 and 33. The extension of the antisense mRNA into the T-DNA was demonstrated using primers 27 and 3 (targeting the pGKB5 left border). The sequences of both RT-PCR products were assembled to give the partial sequence given in Supplemental Figure S1.

For both RNAs, each PCR product was purified using the NucleoTrap kit (Macherey-Nagel) and cloned using the TOPO TA cloning kit (Invitrogen). For each PCR product, four independent clones were sequenced.

Expression Studies

Real-time RT-PCR was carried out using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). A specific primer set (21/22) was designed to amplify a 211-bp fragment on the *PUR4* cDNA. A standard curve was generated from duplicate series of five DNA template dilutions to test PCR efficiencies. PCR was conducted in duplicate in the presence of 1 ng of cDNA, 1.2 μL of each primer (2.5 μM), 5 μL of SYBR Green Mastermix, and distilled water to a final volume of 10 μL. PCR conditions were as described above, with 10 min at 95°C and 40 cycles at 95°C for 10 s and 60°C for 10 s. The results were standardized by comparing the data obtained for the *ACTIN2* gene (primer set 23/24). The quantification of gene expression was performed using the comparative cycle threshold number method.

Transient Expression Experiments

The subcellular localization of the PUR4 protein was predicted in silico using four different software programs: TargetP version 1.1 (Emanuelsson et al., 2000), Predotar version 1.03 (Small et al., 2004; www.inra.fr/Internet/Produits/Predotar/), MitoProtII (Claros and Vincens, 1996; www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter), and IPSORT (Bannai et al., 2002; www.HypothesisCreator.net/iPSORT/). The sequence coding for the putative targeting peptide was amplified using the primer pair 25/26. Using the Gateway cloning technology (Invitrogen), the 605-bp amplification product was shuttled to the pDONR207 entry vector by a BP recombination reaction, sequenced, and subsequently transferred to the pGreen0229 GFP binary vector (Lurin et al., 2004) by a LR recombination reaction. The resulting binary vector was electroporated into *Agrobacterium tumefaciens* C58C1 strain (Koncz et al., 1984) harboring the transformation helper pSoup (Hellens et al., 2000). The PUR4-GFP fusion protein was transiently expressed in Arabidopsis cotyledons via agroinfiltration as described by Marion et al. (2008). *Agrobacterium* carrying a binary vector that allows the expression of a FDH-DsRed2 fusion protein, kindly provided by C. Colas des Francs-Small, was coinfiltrated with that carrying the PUR4-GFP fusion. This vector is a modified version of the vector described by Ambard-Bretteville et al. (2003) in which the GFP coding sequence was substituted by the DsRed2 coding sequence (C. Colas Des Francs-Small, personal communication). Cotyledons were harvested at 72 h after infiltration and were mounted on a glass slide. Fluorescent proteins were visualized in adaxial cotyledon cells using a spectral Leica SP2 AOBS confocal microscope (Leica Microsystems) equipped with an argon laser and a HeNe laser. Different fluorochromes were detected using laser lines 488 nm (GFP) and 543 nm (DsRed2 and chlorophyll autofluorescence). The images were coded green (GFP), red (DsRed2), and blue (chlorophyll autofluorescence), giving yellow colocalization when green and red signals overlap and white colocalization when green and blue signals overlap in merged images. Microscopic observations were carried out using a Leica HC PL APO 633/1.20 Water Corr/0.17 Lbd.BL objective. Each image shown represents the projection of optical sections taken as a Z series. To determine the specificity of the signals, sequential scans were performed using in between line mode.

Cytological Analyses

All cytological analyses were performed on *pur4*/+ plants issued from backcrosses.

Pollen viability was evaluated after Alexander staining (Alexander, 1969). After staining, the number of viable and collapsed pollen grains was counted.

Toluidine blue O staining was carried out as described by Trump et al. (1961) on resin-embedded sections of flower buds corresponding to five individual plants for each genotype. Freshly harvested flower buds were treated using the Technovit 7100 kit. Sections (12 μm) were cut using a microtome equipped with a glass blade (Leica RM2165). Sections were mounted on a glass slide and stained.

DAPI staining of mature pollen was done according to Coleman and Goff (1985) on anthers of freshly harvested flower buds. Observations were made on flowers from 10 plants for each genotype.

Samples were visualized with a Leitz DIAPLAN microscope under UV light for DAPI staining and in bright-field conditions for toluidine blue O and Alexander staining. Photographs were captured with a Leica DFC 480 camera.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number EU091297.

Supplemental Data

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

Supplemental Figure S1. Sequence alignments.

Supplemental Figure S2. Analysis of T-DNA insertions in the *PUR4* gene.

Supplemental Table S1. *pur4-1* transmission in the selfed progeny of heterozygous *pur4-1*/+ plants.

Supplemental Table S2. Kanamycin-resistant segregation in the selfed progeny of heterozygous *pur4-2*/+ plants.

Supplemental Table S3. Primers used.

Supplemental Table S4. Annealing temperatures for PCR and amplification product sizes for primer combinations used in this work.

ACKNOWLEDGMENTS

We gratefully thank S. Bonhomme for a thorough critical reading of the manuscript and advice on gametophytic mutants. We thank M. Laloue and C. Mézard for stimulating discussions. We are grateful to S. Bonhomme, C. Horlow, and M. Grelon for generously providing information from their previous screen. The generous gift of the FDH-DsRed2 fusion by C. Colas des Francs-Small is gratefully acknowledged. We thank A. Martin-Canadell, who took care of the plants in the greenhouse. We are grateful to O. Grandjean for his valuable help in confocal microscopy. The constant support of P. Guerche, head of the laboratory, is gratefully appreciated.

Received March 28, 2008; accepted April 22, 2008; published April 25, 2008.

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