

Ribosomal Protein RPL27a Promotes Female Gametophyte Development in a Dose-Dependent Manner¹[C][W][OPEN]

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Ribosomal protein mutations in *Arabidopsis* (*Arabidopsis thaliana*) result in a range of specific developmental phenotypes. Why ribosomal protein mutants have specific phenotypes is not fully known, but such defects potentially result from ribosome insufficiency, ribosome heterogeneity, or extraribosomal functions of ribosomal proteins. Here, we report that ovule development is sensitive to the level of Ribosomal Protein L27a (RPL27a) and is disrupted by mutations in the two paralogs *RPL27aC* and *RPL27aB*. Mutations in *RPL27aC* result in high levels of female sterility, whereas mutations in *RPL27aB* have a significant but lesser effect on fertility. Progressive reduction in RPL27a function results in increasing sterility, indicating a dose-dependent relationship between RPL27a and female fertility. RPL27a levels in both the sporophyte and gametophyte affect female gametogenesis, with different developmental outcomes determined by the dose of RPL27a. These results demonstrate that *RPL27aC* and *RPL27aB* act redundantly and reveal a function for RPL27a in coordinating complex interactions between sporophyte and gametophyte during ovule development.

Eukaryotic cytoplasmic ribosomes are comprised of two subunits, a large 60S and a small 40S subunit. The 60S subunit includes 25S or 28S, 5.8S, and 5S ribosomal RNA (rRNA) and approximately 47 ribosomal proteins, whereas the 40S subunit includes an 18S rRNA and approximately 33 ribosomal proteins. In plants and animals, reduced ribosomal protein function results in specific developmental phenotypes (Byrne, 2009; Warner and McIntosh, 2009; McCann and Baserga, 2013; Terzian and Box, 2013; Tsukaya et al., 2013). Currently, it is not known how ribosomal proteins modulate development. Potentially specific developmental phenotypes in ribosomal protein mutants are an outcome of ribosome haploinsufficiency and reduced global protein synthesis or reduced translation of specific proteins. Alternatively, ribosomal proteins, in addition to their role in translation, may have extraribosomal function required for specific developmental processes.

In *Arabidopsis* (*Arabidopsis thaliana*), cytoplasmic ribosomal proteins are encoded by two to five genes (Barakat et al., 2001; Giavalisco et al., 2005; Carroll et al., 2008). Mutations in single ribosomal protein genes are sometimes gametophyte or embryo lethal (Weijers et al., 2001; Tzafirir et al., 2004). However, many ribosomal protein mutants are viable. These mutants typically display a subtle change in leaf shape and may also have distinct developmental defects affecting embryo morphogenesis, inflorescence development, the transition to flowering, and plant stature (Van Lijsebettens et al., 1994; Ito et al., 2000; Pinon et al., 2008; Yao et al., 2008; Byrne, 2009; Fujikura et al., 2009; Falcone Ferreyra et al., 2010; Rosado et al., 2010; Horiguchi et al., 2011; Szakonyi and Byrne, 2011a, 2011b; Stirnberg et al., 2012). Female fertility is also reduced in several ribosomal protein mutants. Mutations in the ribosomal protein genes *SHORT VALVE1* (*STV1*)/*RPL24B*, *SUPPRESSOR OF ACAULIS52* (*SAC52*)/*RPL10A*, *ARABIDOPSIS MINUTE-LIKE1* (*AML1*)/*RPS5B*, and the Ribosomal Protein L27a gene *RPL27aC* reduce female fertility (Weijers et al., 2001; Nishimura et al., 2005; Imai et al., 2008; Szakonyi and Byrne, 2011b). *aml1* and *sac52-t1* are partially and fully gametophyte lethal, respectively. Although lower fertility in *stv1* and *rpl27ac* is associated with defective ovules, the nature of the fertility defect in these mutants has not been fully explored.

Female gametophyte development is also disrupted by mutations in a number of genes predicted to be involved in ribosome biogenesis. *SLOW WALKER1* (*SWA1*), *SWA3*/*Arabidopsis thaliana* RNA HELICASE36 (*AtRH36*), and *NUCLEOLAR FACTOR1* (*NOF1*) encode nucleolar-localized proteins required for processing 18S

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pre-rRNA (Shi et al., 2005; Harscoët et al., 2010; Huang et al., 2010; Liu et al., 2010). Mutations in other genes encoding proteins predicted to be involved in pre-rRNA processing and ribosome maturation or in export of preribosomes from the nucleus to the cytoplasm also reduce female fertility (Li et al., 2009, 2010; Chantha et al., 2010; Wang et al., 2012; Missbach et al., 2013). These mutants share similar phenotypes, where female gametophyte development is delayed and there is a failure in progression through gametophyte mitotic cell divisions. Transmission of these ribosome biogenesis mutants through the female is often reduced. This ostensibly reflects a requirement for active ribosome synthesis and sufficient ribosome levels to support morphogenesis of the gametophyte.

Here, we show that mutations in a number of different ribosomal protein genes lead to reduced seed set and an increase in the number of defective ovules in siliques. This is particularly apparent in mutants affecting ribosomal protein RPL27a. We show the two RPL27a genes, *RPL27aC* and *RPL27aB*, act redundantly and that ovule development is sensitive to the dose of RPL27a. *rpl27ac* and *rpl27ab* mutations are together female and male gametophyte lethal. Single *rpl27ac* mutants also result in some female gametophyte lethality. In the homozygous *rpl27ac-2* mutant, the mature embryo sac is frequently expelled from the ovule, suggesting RPL27a is necessary for maintaining a viable gametophyte. However, in the heterozygous *rpl27ac-2/+*, gametogenesis frequently fails early in development. This occurs independent of the genotype of the gametophyte, indicating somatic sporophyte cells in the mutant affect gametophyte development. Together, our data demonstrate that appropriate levels of RPL27a in the sporophyte and gametophyte are required for female gametophyte development and plant fertility.

RESULTS

Insertion Mutations in the Paralogs *RPL27aC* and *RPL27aB* Result in Altered Leaf Shape

Arabidopsis has three *RPL27a* genes. *RPL27aC* and *RPL27aB* are transcriptionally active, whereas *RPL27aA* is not expressed and is likely a pseudogene (Barakat et al., 2001; Szakonyi and Byrne, 2011b). The *RPL27aC*- and *RPL27aB*-encoded proteins are highly conserved, differing in only two of 146 amino acids, and in most tissues, *RPL27aC* expression is approximately twice that of *RPL27aB* (Rhee et al., 2003; Laubinger et al., 2008). To compare the function of *RPL27aC* and *RPL27aB*, we examined the phenotypes of transfer DNA (T-DNA) insertion mutations in both genes. As reported previously, two alleles of *RPL27aC*, *rpl27ac-2* and *rpl27ac-3*, had T-DNA insertions in the promoter region and the 5' leader sequence of *RPL27aC*, respectively, and transcript levels of both alleles were reduced relative to the wild type (Fig. 1A; Szakonyi and Byrne, 2011b). Plants homozygous for either *rpl27ac-2* or *rpl27ac-3* had

rosette leaves that were pointed and serrated compared with the wild type and were similar to the semidominant heterozygous *rpl27ac-1d/+* mutant (Fig. 1, B, D, and E; Szakonyi and Byrne, 2011b).

Two alleles of *RPL27aB*, *rpl27ab-1* and *rpl27ab-2*, had T-DNA insertions in the 5' leader sequence and in the coding region of *RPL27aB*, respectively (Fig. 1A). Transcript levels of *RPL27aB* in *rpl27ab-1* were slightly reduced relative to the wild type, indicating this is a weak allele (Supplemental Fig. S1). The insertion in

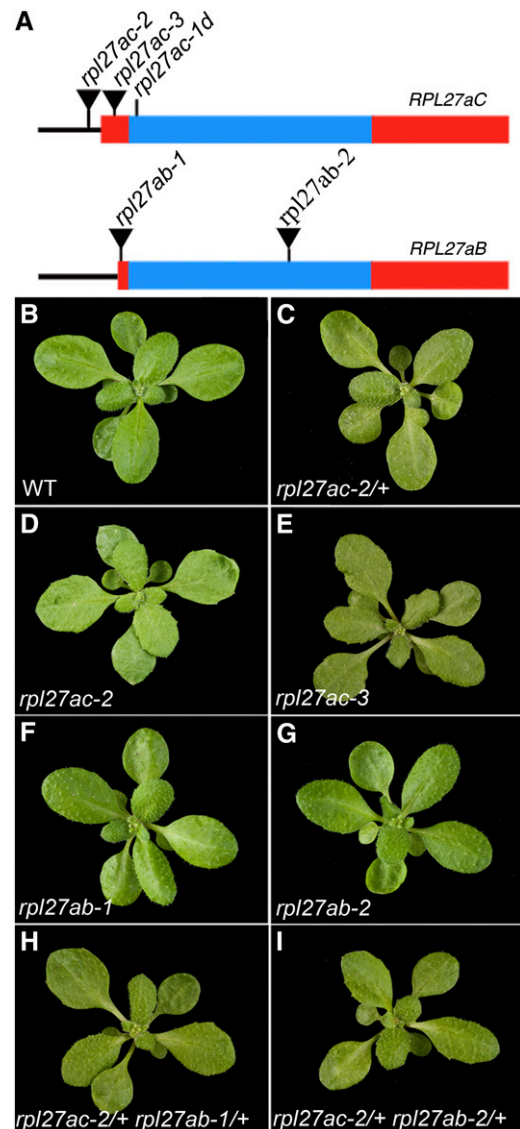


Figure 1. Leaf phenotypes of *rpl27ac* and *rpl27ab* mutants. A, Diagrammatic representation of *RPL27aC* and *RPL27aB* genes, showing exons, 5' and 3' leader sequences, sites of T-DNA insertions, and the location of the point mutation in *rpl27ac-1d*. B to I, Rosettes of the wild type (WT; B), *rpl27ac-2/+* (C), *rpl27ac-2* (D), *rpl27ac-3* (E), *rpl27ab-1* (F), *rpl27ab-2* (G), *rpl27ac-2/+ rpl27ab-1/+* (H), and *rpl27ac-2/+ rpl27ab-2/+* (I). [See online article for color version of this figure.]

rpl27ab-2 is within the coding sequence and is predicted to be a null allele. Leaf shape in *rpl27ab-1* and *rpl27ab-2* was changed to a more pointed shape, but this phenotype was less severe than the *rpl27ac* mutants, indicating that *RPL27aB* has a less significant role in leaf development than *RPL27aC* (Fig. 1, F and G). This result is consistent with the lower level of expression of *RPL27aB* compared with *RPL27aC*. To test for redundancy between *RPL27aC* and *RPL27aB*, we crossed homozygous mutants in each gene and analyzed the F1 transheterozygotes *rpl27ac-2/+ rpl27ab-1/+* and *rpl27ac-2/+ rpl27ab-2/+* for phenotypes. Both transheterozygotes had pointed and serrated leaves (Fig. 1, H and I). Thus, there is functional overlap between *RPL27aC* and *RPL27aB* in leaf development.

Insertion Mutations in *RPL27aC* and *RPL27aB* Reduce Fertility

rpl27ac-2 and *rpl27ac-3* had a change in leaf shape and were slightly slower growing than the wild type. Both mutants also had shorter siliques than the wild type. Examination of siliques in the mutants revealed a reduced number of seed and the presence of aborted or unfertilized ovules (Fig. 2A). In wild-type siliques, only 3.8% of ovules were defective and 96.2% of ovules were fertile and set seed. As reported previously, *rpl27ac-2* and *rpl27ac-3* siliques had 46.4% and 61.7% defective ovules, respectively (Fig. 2B; Szakonyi and Byrne, 2011b). The heterozygous *rpl27ac-3/+* mutant was indistinguishable from the wild type (Fig. 2B). By contrast, the heterozygous *rpl27ac-2/+* had wild type-shaped leaves (Fig. 1C) and was indistinguishable from the wild type, except siliques were found to have a high number of defective ovules (Fig. 2B). Heterozygous plants generated using a homozygous mutant as either the female or male parent did not lead to significant differences in the frequency of defective ovules. Heterozygous plants generated from *rpl27ac-2* crossed with wild-type pollen had 62.8% defective ovules ($n = 565$), and heterozygous plants generated from the wild type crossed with *rpl27ac-2* pollen had 60.6% defective ovules ($n = 676$). Surprisingly, the frequency of defective ovules was higher in the *rpl27ac-2/+* heterozygote than in the *rpl27ac-2* homozygous. Possibly, expression levels of *RPL27aC* in the ovule regulate expression of one or both *RPL27a* paralogs, and this regulation is disrupted to different extents in heterozygotes and homozygotes. A single copy of the transgene *RPL27aC:RPL27aC*, which carried the genomic promoter and coding region of *RPL27aC*, restored fertility in the *rpl27ac-2* mutant, confirming that the ovule defect is due to a reduction in *RPL27a* (Fig. 2B). Although there was a dramatic ovule defect in the heterozygous *rpl27ac-2/+*, reciprocal crosses between *rpl27ac-2/+* and the wild type demonstrated that the mutant allele was efficiently transmitted maternally (transmission efficiency = 54.1%, $n = 74$) and paternally (transmission efficiency = 56%,

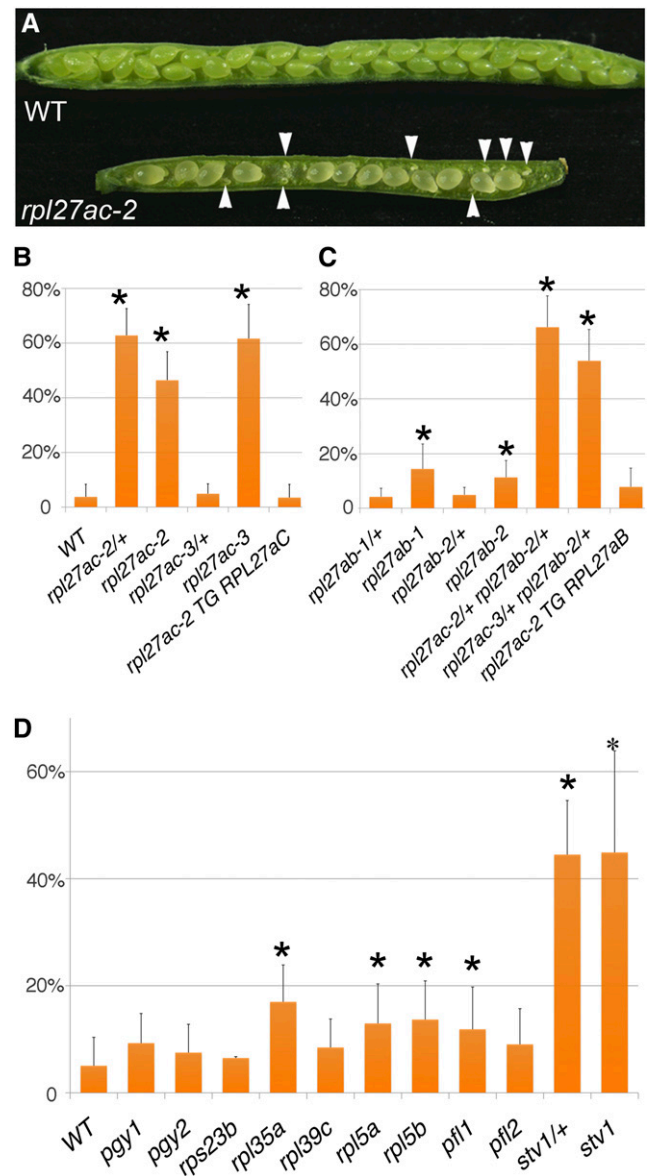


Figure 2. Fertility defects in *rpl27a* and ribosomal protein mutants. A, Wild-type (WT; top) and *rpl27ac-2* (bottom) open siliques, with defective ovules in the mutant marked (arrowheads). B, Percentage of defective ovules in siliques of the wild type ($n = 581$), *rpl27ac-2/+* ($n = 565$), *rpl27ac-2* ($n = 521$), *rpl27ac-3/+* ($n = 587$), *rpl27ac-3* ($n = 579$), and *rpl27ac-2* carrying the transgene *RPL27aC:RPL27aC* (*rpl27ac-2 TG RPL27aC*; $n = 824$). C, Percentage of defective ovules in siliques of *rpl27ab-1/+* ($n = 572$), *rpl27ab-1* ($n = 549$), *rpl27ab-2/+* ($n = 626$), *rpl27ab-2* ($n = 548$), *rpl27ac-2/+ rpl27ab-2/+* ($n = 612$), *rpl27ac-3/+ rpl27ab-2/+* ($n = 594$), and *rpl27ac-2* carrying the transgene *RPL27aB:RPL27aB* (*rpl27ac-2 TG RPL27aB*; $n = 932$). D, Percentage of defective ovules in siliques of the wild type ($n = 690$), *pgy1* ($n = 603$), *pgy2* ($n = 598$), *rps23b* ($n = 630$), *rpl35a* ($n = 604$), *rpl39c* ($n = 601$), *rpl5a* ($n = 532$), *rpl5b* ($n = 562$), *pil1* ($n = 556$), *pil2* ($n = 585$), *stv1/+* ($n = 668$), and *stv1* ($n = 560$). Error bars are the SD (Student's *t* test, * $P < 0.05$). [See online article for color version of this figure.]

$n = 75$). This indicates ovule development depends on appropriate levels of RPL27a in the sporophyte.

Mutations in *RPL27aB* also affected ovule development. Heterozygous *rpl27ab-1/+* and *rpl27ab-2/+* plants were indistinguishable from the wild type, and the number of defective ovules in siliques was not significantly different from the wild type (Fig. 2C). Homozygous *rpl27ab-1* and *rpl27ab-2* mutants were not noticeably different from the wild type, except siliques showed 14.4% and 11.3% defective ovules, respectively (Fig. 2C). The frequency of defective ovules in *rpl27ab* mutants was higher than the wild type but less than that in *rpl27ac* mutants.

Given that *RPL27aC* and *RPL27aB* acted redundantly in leaf development, we examined whether these genes are redundant in ovule development by testing whether *RPL27aB* can complement the *rpl27ac-2* ovule phenotype. A single copy of the transgene *RPL27aB:RPL27aB* carrying the promoter and coding region of *RPL27aB* completely restored fertility in *rpl27ac-2* (Fig. 2C). We also determined whether these two ribosomal protein genes act redundantly in ovule development by comparing fertility in the transheterozygotes *rpl27ac-2/+ rpl27ab-2/+* and *rpl27ac-3/+ rpl27ab-2/+* relative to each single mutant. Ovule defects were significantly increased in both transheterozygotes compared with either single heterozygous or homozygous mutants (Fig. 2, B and C). *rpl27ac-2/+ rpl27ab-2/+* and *rpl27ac-3/+ rpl27ab-2/+* had 66% and 54% defective ovules, respectively. Like *rpl27ac-2/+*, these defective ovules may have been due to sporophytic effects but may also have included ovules that were gametophyte lethal. A screen of F2 progeny from the two transheterozygotes failed to recover plants that were double homozygous *rpl27ac rpl27ab*. Mutants that were homozygous for *rpl27ac* and heterozygous for *rpl27ab* or homozygous for *rpl27ab* and heterozygous for *rpl27ac* were also not recovered. This indicated that the two mutant alleles were not transmitted together through either the female or the male and were therefore gametophyte lethal. In the transheterozygotes, 25% of the defective ovules were therefore likely to be the result of gametophytic lethality. The remaining defective ovules are predicted to be due to reduced RPL27a in the sporophyte. Transcriptional fusions of the RPL27a gene promoters to a nuclear-localized venus yellow fluorescent protein reporter (*nls-vYFP*) showed both genes were expressed in all cells of the ovule (Supplemental Fig. S2). The two *RPL27a* genes therefore have overlapping expression and both contribute to ovule development.

Mutations Disrupting Different Ribosomal Proteins Reduce Fertility

Although mutants in the ribosomal protein genes *RPL27aC*, *RPS5B*, *STV1*, and *SAC52* have ovule defects, this phenotype has not been reported for mutations in other ribosomal protein genes (Weijers et al., 2001; Nishimura et al., 2005; Imai et al., 2008; Szakonyi

and Byrne, 2011b). To determine whether other ribosomal protein mutants affect fertility, the frequency of defective ovules in siliques of several ribosomal protein mutants was examined. Five of the ribosomal protein mutants were isolated as enhancers of the leaf shape mutant *asymmetric leaves1 (as1)*. All of these mutants had pointed, serrated leaves and, in an *as1* mutant background, had a piggyback phenotype with ectopic lamina outgrowths on the adaxial side of the leaf. These included the previously reported mutants *piggyback1-1 (pgy1-1; corresponding to rpl10ab)* and *pgy2-1 (corresponding to rpl9c; Pinon et al., 2008)* and three new *pgy* mutants, designated *rps23b-1*, *rpl35a-1*, and *rpl39c-1*, which were found to have mutations in ribosomal protein genes *RPS23B*, *RPL35A*, and *RPL39C*, respectively (Supplemental Fig. S3). *rps23b* had a point mutation that changed the second amino acid from Gly to Asp. *rpl35a* and *rpl39c* had point mutations in the 5' leader sequence and at the first exon-intron junction, respectively, and both mutations reduced the level of wild-type transcript (Supplemental Fig. S3). In addition, previously reported mutations *rpl5a-2*, *rpl5b-3*, *pointed first leaf1-1 (pfl1-1)*, *pfl2*, and *stv1-2* were included in this analysis (Van Lijsebettens et al., 1994; Ito et al., 2000; Nishimura et al., 2005; Yao et al., 2008; Horiguchi et al., 2011). Compared with the wild type, these nine ribosomal protein mutants had variable effects on fertility. *pgy1*, *pgy2*, *rps23b*, *rpl39c*, and *pfl2* did not reduce fertility, whereas *rpl5a*, *rpl5b*, *rpl35a*, *pfl1*, and *stv1* had significantly more defective ovules than the wild type (Fig. 2D). The number of defective ovules was most extreme in *stv1*. Homozygous *stv1* had a high frequency of defective ovules, as previously reported (Nishimura et al., 2005). As with *rpl27ac-2/+*, heterozygous *stv1/+* plants had siliques with a high proportion of defective ovules (Fig. 2D). Based on these results, it can be concluded that a number of different ribosomal proteins promote plant fertility.

The Dose of RPL27a Determines the Severity of Leaf and Ovule Defects

To examine the potential effect of RPL27a dose on development, we took advantage of the dominant-negative *rpl27ac-1d* allele, which is predicted to produce a protein that interferes with wild-type function (Szakonyi and Byrne, 2011b). Plants in which the copy number of *RPL27aC* varied relative to mutant *rpl27ac-1d* were generated by placing transgenes that carried either the wild-type (*RPL27aC:RPL27aC*) or mutant allele (*RPL27aC:rpl27ac-1d*) into wild-type or *rpl27ac-1d/+* genetic backgrounds (Fig. 3). In the heterozygous *rpl27ac-1d/+* and homozygous *rpl27ac-1d* mutants, there were 20.1% and 91.9% defective ovules, respectively (Fig. 3). *RPL27aC:RPL27aC* fully suppressed *rpl27ac-1d/+* leaf and ovule phenotypes. Furthermore, plants homozygous for *RPL27aC:RPL27aC* in a wild-type background were indistinguishable from the wild

type, indicating there are no phenotypic consequences from an increased copy number of *RPL27aC*. Wild-type plants hemizygous for *RPL27aC:rpl27ac-1d* had pointed, serrated leaves and 16.6% defective ovules. Wild-type plants homozygous for *RPL27aC:rpl27ac-1d* had more severe changes in leaf shape, and siliques had 38.1% defective ovules. *rpl27ac-1d/+* plants hemizygous for *RPL27aC:rpl27ac-1d* had the most severe leaf shape change and had siliques with 74.3% defective ovules. We did not recover *rpl27ac-1d/+* plants homozygous for *RPL27aC:rpl27ac-1d*. Because wild-type plants homozygous for *RPL27aC:rpl27ac-1d* had more severe phenotypes than *rpl27ac-1d/+*, we speculated that the mutant transgene was more potent than the wild-type gene, either due to higher levels of transcription or due to differences in levels or stability of the proteins. To test whether this was due to differences in transcript levels, we took advantage of an *HpaII* polymorphism that distinguished transcripts from the two alleles. Semiquantitative reverse transcription (RT)-PCR analysis demonstrated that wild-type and mutant transcript levels were similar in *rpl27ac-1d/+* (Supplemental Fig. S4A). By contrast, in wild-type plants that were also homozygous for *RPL27aC:rpl27ac-1d*, the level of mutant transcript was higher than that of the wild type alone (Supplemental Fig. S4A). To obtain a more accurate estimate of wild-type and *rpl27ac-1d* transcript levels in the different genetic backgrounds, RT-PCR products were cloned

and individual clones were genotyped. Consistent with the semiquantitative data, the alleles represented in the cloned RT-PCR products showed the level of mutant transcript was 3 times higher than that of the wild type in plants carrying the *RPL27aC:rpl27ac-1d* transgene (Supplemental Fig. S4B). Although the mutant allele was expressed at higher levels than the transgene, it can be concluded that changes in the ratio of *RPL27aC* to *rpl27ac-1d* results in a gradual change in phenotype severity, with changes in leaf shape paralleling changes in the frequency of defective ovules.

Ovule and Female Gametophyte Defects in *rpl27ac-2*

To understand the role of RPL27a in ovule development, we examined the morphology of developing and mature *rpl27ac-2* ovules. In the wild type, the megaspore mother cell is a single large subepidermal cell at the distal end of ovule primordium in 97.6% of ovules (Fig. 4A), and only 2.4% of primordia had alterations in this pattern, most often with more than one large subepidermal cell. Postmeiosis, the ovule has a single functional megaspore and three degenerate megaspores (Fig. 4B). The functional megaspore undergoes three rounds of mitosis, leading to female gametophyte stage 5 (FG5) embryo sac with eight nuclei (Fig. 4C). Subsequently, in the FG6 stage of

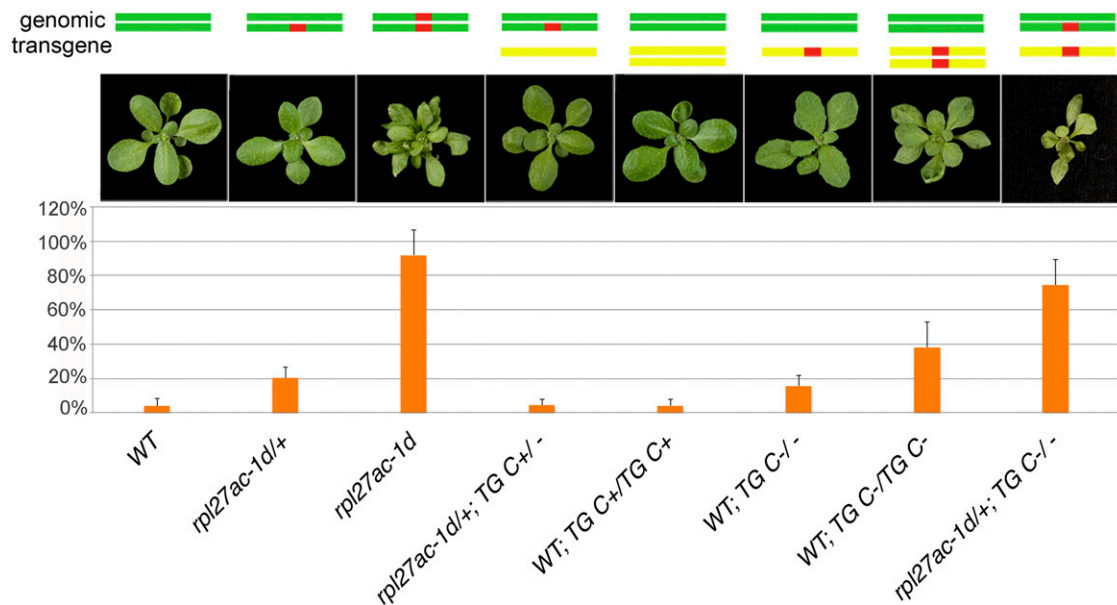


Figure 3. Leaf and fertility defects reciprocally correlate with relative levels of functional RPL27a. Diagrams at the top represent the genomic wild-type (WT) *RPL27aC* and *rpl27ac-1d* alleles (top) and *RPL27aC:RPL27aC* and *RPL27aC:rpl27ac-1d* transgenes (bottom). Plant rosettes and the percentage of defective ovules for each genotype are shown below the diagrams. Genotypes are, from left to right, the wild type ($n = 581$), *rpl27ac-1d/+* ($n = 578$), *rpl27ac-1d* ($n = 401$), *rpl27ac-1d/+* hemizygous for the transgene *RPL27aC:RPL27aC* (TG C+; $n = 2,109$), wild-type homozygous for the transgene *RPL27aC:RPL27aC* ($n = 1,836$), wild-type hemizygous for the transgene *RPL27aC:rpl27ac-1d* (TG C-; $n = 1,239$), wild-type homozygous for the transgene *RPL27aC:rpl27ac-1d* ($n = 1,801$), and *rpl27ac-1d/+* hemizygous for the transgene *RPL27aC:rpl27ac-1d* ($n = 1,366$). Error bars are the sd. [See online article for color version of this figure.]

development, the two polar nuclei fuse to form a central cell nucleus (Fig. 4D). In *rpl27ac-2*, the frequency of an abnormal cell arrangement was 18% of ovule primordia, somewhat higher than that of the wild type (Fig. 4, E and I). Despite this defect, subsequent early stages of female gametophyte development in *rpl27ac-2* were not readily distinguished from the wild type. After meiosis, 12.3% of *rpl27ac-2* ovules had a large cell in the position of a degenerate megaspore (Fig. 4, F and J). Because these cells were not apparent in subsequent stages of gametogenesis, they may represent delayed degeneration of nonfunctional megaspores. Defects in *rpl27ac-2* ovules became more obvious in the final stages of ovule development. At FG5, when the wild type had completed mitotic divisions, 31.4% of *rpl27ac-2* ovules had no embryo sac (Fig. 4G). Another 48% of ovules had short integuments and/or an embryo sac in an abnormal position in the ovule (Fig. 4K). At FG6, when the wild type had a large seven-cell embryo sac, 50.1% of *rpl27ac-2* ovules did not have an embryo sac (Fig. 4H) and 22.8% of ovules had a small and displaced embryo sac (Fig. 4L). To rule out the potential influence of the *Landsberg erecta* (*Ler*) background, we also examined *rpl27ac-2* ovule development in the Columbia (*Col-0*) background and found no significant differences compared with *rpl27ac-2* in *Ler* (Supplemental Fig. S5). The increase in frequency of ovules without an embryo sac in

late stages of development indicates that sufficient levels of RPL27a are required to maintain a viable female gametophyte.

Female gametophyte synergid-, antipodal-, and central cell-specific markers (Huanca-Mamani et al., 2005; Groß-Hardt et al., 2007; Olmedo-Monfil et al., 2010; Tucker et al., 2012) were used to confirm the *rpl27ac-2* ovule phenotype. In maturing ovules, GUS expression from the synergid cell marker enhancer trap ET2634 and antipodal cell marker gene trap GT3733 was initially detected in the majority of wild-type and mutant ovules (Fig. 5, A, D, F, and I). In slightly older pistils, the frequency of ovules expressing synergid and antipodal markers decreased in *rpl27ac-2* (Fig. 5, C, E, H, and J). The pattern of both markers was abnormal in some *rpl27ac-2* ovules, with expression displaced distally toward the micropyle (Fig. 5, B and G). Likewise, GUS expression from the central cell promoter of *MEDEA* (*pMEA:GUS*) occasionally showed abnormal expression in *rpl27ac-2* and was reduced to a small region toward the micropyle of the ovule (Fig. 5, K–M). The frequency of ovules expressing the central cell marker *pMEA:GUS* was also reduced in *rpl27ac-2* compared with the wild type. The expression pattern of these gametophyte cell markers suggests that the primary defect in *rpl27ac-2* is not due to a lack of cellular differentiation, delay in development, or arrest of development

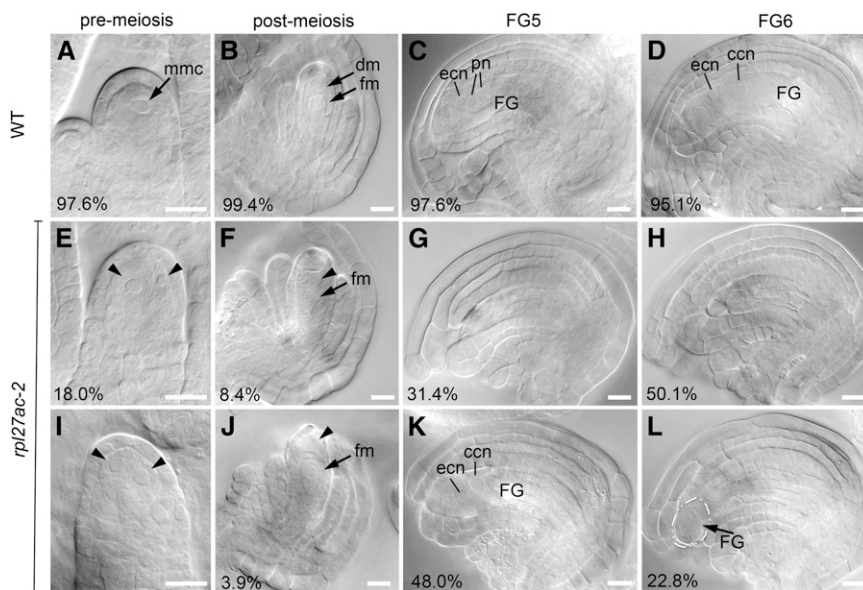


Figure 4. Ovule phenotypes in *rpl27ac-2*. A to D, Wild-type (WT) ovules. Percentage of normal ovules is indicated. A, Pre-meiosis ovule ($n = 371$). B, Postmeiosis ovule ($n = 313$). FG5 stage ovule with two integuments surrounding an eight-cell embryo sac or functional gametophyte ($n = 460$; C) and FG6 stage ovule with polar nuclei fused to form a seven-cell gametophyte ($n = 425$; D). E to L, Abnormal ovules from *rpl27ac-2*. Percentage of abnormal ovules is indicated. E and I, Pre-meiosis ovules with enlarged cells (arrowheads; $n = 323$). F to J, Postmeiosis ovules with functional megaspore and enlarged cell (arrowhead; $n = 310$). FG5 stage ovules with normal integuments and no embryo sac (G) and with short integuments and protruding embryo sac ($n = 523$; K). FG6 stage ovules with no embryo sac (H) and with a small embryo sac (outlined; $n = 587$; L). FG, Functional gametophyte; ccn, central cell nucleus; dm, degenerate megaspore; ecn, egg cell nucleus; fm, functional megaspore; mmc, megaspore mother cell; pn, polar nucleus. Bars = 10 μm .

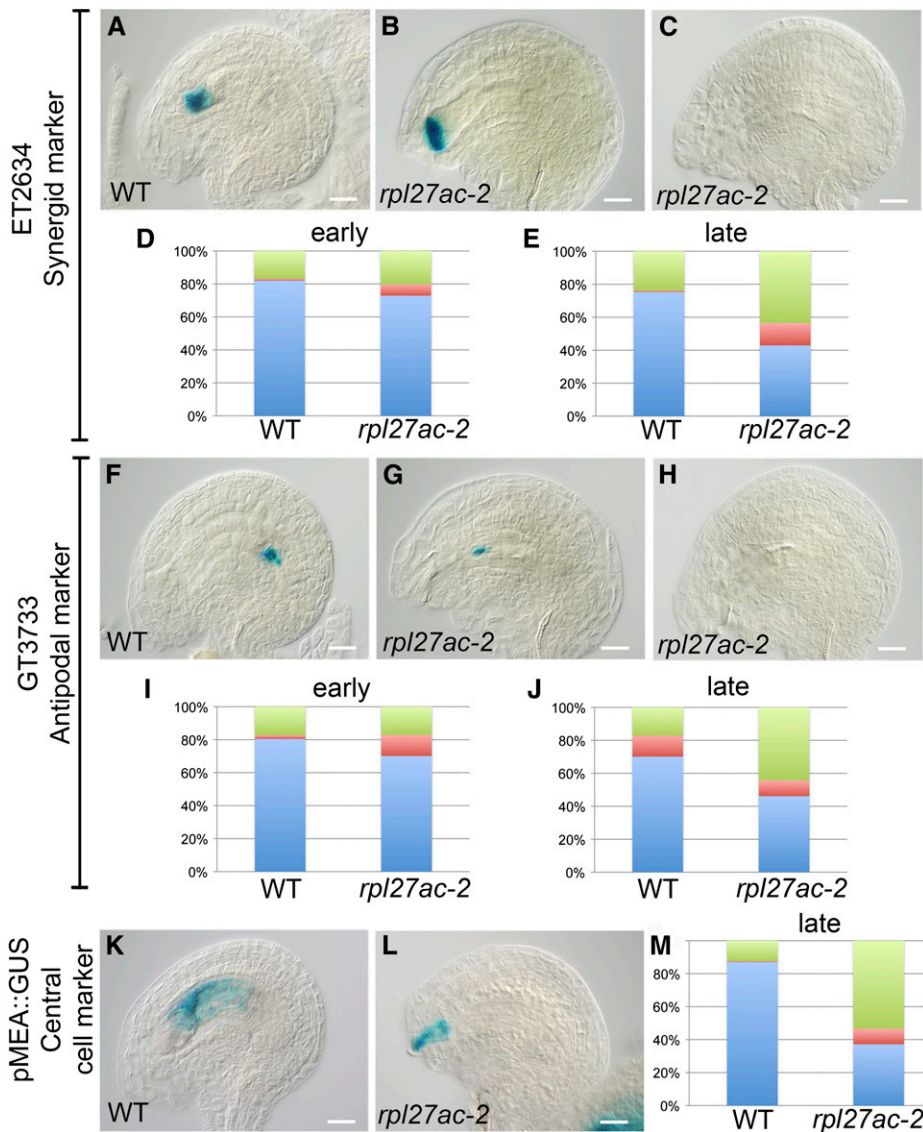


Figure 5. Maturing female gametophytes are expelled from the ovule in *rpl27ac-2*. A to C, Synergid marker ET2634 expression pattern in wild-type (WT) ovule (A) and abnormal *rpl27ac-2* ovules (B and C). D and E, Percentage of wild-type synergid marker expression pattern (blue), abnormal expression pattern (red), and no expression (green) in ovules at early-stage (wild type, $n = 176$; *rpl27ac-2*, $n = 175$; D) and late-stage (wild type, $n = 823$; *rpl27ac-2*, $n = 850$; E) development following cell specification. F to H, Antipodal marker GT3733 expression pattern in wild-type ovule (F) and abnormal *rpl27ac-2* ovules (G and H). I and J, Percentage of wild-type antipodal marker expression pattern (blue), abnormal expression pattern (red), and no expression (green) in ovules at early-stage (wild type, $n = 170$; *rpl27ac-2*, $n = 158$; I) and late-stage (wild type, $n = 854$; *rpl27ac-2*, $n = 892$; J) development following cell specification. K and L, Central cell marker *pMEA::GUS* expression pattern in wild-type ovule (K) and abnormal *rpl27ac-2* ovule (L). M, Percentage of wild-type *pMEA::GUS* expression pattern (blue), abnormal expression pattern (red), and no expression (green) in ovules at late-stage development (wild type, $n = 192$; *rpl27ac-2*, $n = 520$). Bars = 10 μm .

and, instead, is due to physical displacement of the mature gametophyte from the ovule.

Female Gametophyte Defects in *rpl27ac-2/+*

rpl27ac-2/+ heterozygote plants had no obvious phenotypes except for short siliques with a high frequency of defective ovules. To determine whether defective ovules in the heterozygote *rpl27ac-2/+* and homozygote *rpl27ac-2* were the same, we examined the morphology of developing and mature ovules in *rpl27ac-2/+* plants. The morphology of young premeiosis ovule primordia was similar in *rpl27ac-2/+* and *rpl27ac-2* mutants. As with *rpl27ac-2* siliques, premeiosis ovules with an abnormal arrangement of cells occurred at a slightly higher frequency in *rpl27ac-2/+* compared with the wild type (Fig. 6A). At later stages, a significant proportion of ovules in

rpl27ac-2/+ siliques was found to have only a single nucleus. At maturity, all ovules in *rpl27ac-2/+* heterozygotes had normal integuments. Thirty-three percent of ovules had a normal FG6 stage embryo sac, whereas 77% of ovules had gametophytes with only a single nucleus (Fig. 6B). The postmeiosis cell-specific markers *pFM2::GUS* and *pAt1g21670::nls-vYFP*, which are expressed in the functional megaspore and in the developing gametophyte in the wild type (Fig. 6, C and E; Olmedo-Monfil et al., 2010; Tucker et al., 2012), were used to determine the identity of the single cell in *rpl27ac-2/+*-defective ovules. These markers were expressed in the single cell of *rpl27ac-2/+* ovules, indicating that gametophyte development had undergone meiosis but had not proceeded to mitotic divisions (Fig. 6, D and F). Because transmission of *rpl27ac-2* is not affected in the *rpl27ac-2/+* mutant, arrested gametogenesis occurs irrespective of the contribution of the genotype

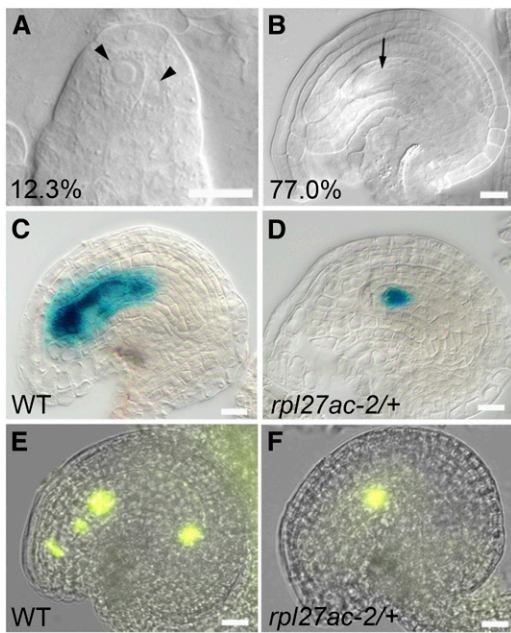


Figure 6. Ovule phenotypes in *rpl27ac-2/+*. A, Abnormal ovules from *rpl27ac-2/+* plant at the premeiosis stage with several enlarged cells (arrowheads; $n = 349$). The percentage of abnormal ovules is indicated. B, Ovules from *rpl27ac-2/+* plant at the FG6 stage with normal integuments and an embryo sac with a single nucleus (arrow; $n = 370$). The percentage of abnormal ovules is indicated. C to F, Postmeiosis cell-specific markers *pFM2:GUS* (C and D) and *pAt21670:nls-vYFP* (E and F) in wild-type (C and E) and abnormal *rpl27ac-2/+* (D and F) ovules. Bars = 10 μm . [See online article for color version of this figure.]

of the gametophyte and depends on reduced levels of RPL27a in the sporophyte.

DISCUSSION

In plants and animals, mutations in cytoplasmic ribosomal protein genes result in delayed growth as well as specific developmental defects (Byrne, 2009; Bhavsar et al., 2010; Gilbert, 2011; Horiguchi et al., 2012; Tsukaya et al., 2013). In Arabidopsis, most ribosomal protein mutants have pointed rosette leaves, and this shared phenotype suggests defects are due to reduced ribosome function. However, phenotypic differences between ribosomal protein mutants have been reported, and this may be due to different patterns of gene expression or to extraribosomal function of some ribosomal proteins (Falcone Ferreyra et al., 2013).

Here, we show variable effects of mutations in different ribosomal protein genes on female fertility. Mutations in five ribosomal protein genes, *PGY1*, *PGY2*, *RPL39C*, *RPS23B*, and *PFL2*, did not reduce fertility. *pgy2-1* and *rpl39c-1* are likely weak alleles, whereas *pgy1-1* and *pfl2* are predicted null alleles. Mutations in five other ribosomal protein genes, *RPL5A*, *RPL5B*, *RPL27aB*, *RPL35A*, and *PFL1*, showed a moderate reduction in fertility. *rpl5b-3* and *rpl35a-1* are predicted to be weak alleles, and *rpl27ab-2*, *rpl5a-2*,

and *pfl1* are predicted null alleles. Potentially, residual expression from weak alleles and redundancy with other ribosomal protein gene family members mask or limit the contribution of these ribosomal proteins to ovule development. By contrast, *stv1-2*, which is likely a null allele, and *rpl27ac* mutants had dramatic semidominant effects on fertility. Two other semidominant ribosomal protein mutants induce gametophyte defects. *aml1* and *sac52-t1* mutants are homozygous embryo lethal, but heterozygotes display reduced transmission of the mutant allele through female and male gametes (Weijers et al., 2001; Imai et al., 2008; Falcone Ferreyra et al., 2013). The effect of multiple ribosomal protein mutants on fertility is consistent with an essential role for the ribosome in cell viability. However, the phenotype of *rpl27ac-2/+* mutants also indicates this ribosomal protein has a more specific role in development. RPL27a may be involved in ribosome regulation of translation of genes required for ovule development or may have an extraribosomal function that serves to regulate expression of genes required for ovule development. STV1, RPL4, and RPL5 ribosomal proteins promote the translation of the AUXIN RESPONSE FACTOR (ARF) genes *ETTIN/ARF3*, *MONOPTEROS/ARF5*, and *ARF7* via upstream open reading frames in the 5' leader sequences of these target genes (Nishimura et al., 2005; Rosado et al., 2012). Potentially, ribosomal proteins that have extreme effects on fertility and ovule development are disrupting the control of genes involved in auxin signaling.

The ovule defects resulting from mutation in RPL27a highlight general and specific roles for a ribosomal protein in development. The two *RPL27a* genes in Arabidopsis are functionally redundant. Both genes have a role in fertility, and we propose that both genes contribute to the total cellular pool of RPL27a. Gradual reduction in the level of RPL27a results in a progressive and stochastic reduction in the level of fertility. The consequences for female gametophyte development appear to depend on the level of ribosomal protein in the sporophyte and gametophyte. Mutations in the two *RPL27a* genes are not transmitted together through female or male gametes, suggesting minimum levels of RPL27a are necessary for gametophyte viability. Likewise, the ribosomal proteins RPL4, RPL5, RPL36a, and RPS6 are each encoded by two genes, and for each of these genes, mutations in both paralogs are not transmitted together through gametes (Yao et al., 2008; Fujikura et al., 2009; Creff et al., 2010; Rosado et al., 2010; Casanova-Sáez et al., 2014). This indicates that a minimal threshold of ribosomes is required for gametophyte viability and reflects an essential role for ribosomes in cell viability.

In the homozygous *rpl27ac-2* mutant, female gametophyte development proceeds through megagametogenesis, but there is a subsequent failure to maintain the mature embryo sac within the ovule. The large multinucleate gametophyte may require sufficient ribosome levels to carry out essential cellular and metabolic functions. However, this explanation is difficult

to reconcile with ovule defects in the *rpl27ac-2/+* mutant, where gametophyte development appears to have arrested following meiosis. Multiple factors appear to be involved in coordinating sporophytic tissues and the gametophyte during ovule development (Bencivenga et al., 2011). Potentially, the distinct heterozygote and homozygote phenotypes reflect the need for balanced levels of RPL27a in the sporophyte and gametophyte.

In *rpl27ac-2/+* mutants, it appears that the functional megaspore fails to undergo mitotic division. By comparison, ribosome biogenesis mutants *swa1*, *swa2*, *swa3/atrh36*, and *nof1* that are female gametophyte lethal initiate megagametogenesis but do not complete mitotic cell divisions (Shi et al., 2005; Li et al., 2009; Harscoët et al., 2010; Huang et al., 2010; Liu et al., 2010). In *swa1*, defective embryo sacs are asynchronous with development arrested at the functional megaspore stage or at two, four, or eight nuclei stages of development. Defective embryo sacs in *swa2* and *swa3* are also asynchronous and arrest with two to eight nuclei, whereas defective embryo sacs in *nof1* have four nuclei. In a number of ways, *rpl27ac-2/+* is phenotypically similar to plants that are heterozygous for a novel semidominant allele of *ARGONAUTE5* (*AGO5*; Tucker et al., 2012). *AGO5* is a putative effector of small RNA silencing pathways that is expressed in somatic cells during megasporogenesis. Heterozygous *ago5-4/+* mutants are partially sterile, and like *rpl27ac-2/+*, female gametophyte development aborts after meiosis and prior to mitotic divisions. This defect is, in part, mediated by *AGO5* expression in sporophyte tissues (Tucker et al., 2012). The similarity in phenotypes suggests a potential link between pathways targeted by *AGO5* and RPL27a function that remains to be explored.

Comparisons can be made between loss of ribosomal protein function in plants and animals. In *Drosophila melanogaster*, ribosomal protein mutants are the classic *Minute* mutants that are slow growing and small in size but also have specific developmental defects, including eye- and wing-patterning defects, and reduced fertility (Lambertsson, 1998; Marygold et al., 2007). In zebrafish and mice, reduced ribosomal protein function results in defects affecting brain, skeleton, eye, and ear development (Amsterdam et al., 2004; Oliver et al., 2004; Uechi et al., 2006; McGowan et al., 2008; Kondrashov et al., 2011; Watkins-Chow et al., 2013). In humans, Diamond-Blackfan anemia and isolated congenital asplenia are associated with mutations in ribosomal proteins (Draptchinskaia et al., 1999; Willig et al., 1999; Gazda et al., 2006, 2008; Farrar et al., 2008; Bolze et al., 2013). Different models have been proposed to explain ribosomal protein mutant phenotypes, including ribosome heterogeneity, extraribosomal function, and targeting of specific transcripts for translational regulation (Byrne, 2009; Warner and McIntosh, 2009; Horiguchi et al., 2012; Xue and Barna, 2012). Although the mechanism of RPL27a function in development is to be established, the data

we present demonstrating that female fertility is determined in a dose-sensitive manner by the level of RPL27a further define specific developmental phenotypes of ribosomal proteins.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) mutants *rpl27ac-1d*, *pgy1-1*, and *pgy2-1* have been described previously (Pinon et al., 2008; Szakonyi and Byrne, 2011b). *rpl27ac-2* (SALK_142534), *rpl27ac-3* (GABI_066_H03), *rpl27ab-1* (SAIL_540_E01), *rpl27ab-2* (GABI_104A06), *pfl-1*, *pfl2*, and *stv1-2* were obtained from The European Arabidopsis Stock Centre and GABI-Kat (Van Lijsebettens et al., 1994; Ito et al., 2000; Scholl et al., 2000; Sessions et al., 2002; Alonso et al., 2003; Nishimura et al., 2005; Szakonyi and Byrne, 2011b; Kleinboelting et al., 2012). *rpl5a-2* (Salk_089798), which has been published (Yao et al., 2008; Fujikura et al., 2009), was also obtained from The European Arabidopsis Stock Centre. *rpl5b-3* (GT16460) was obtained from Cold Spring Harbor Laboratory (Sundaresan et al., 1995). *rps23b-1*, *rpl35a-1*, and *rpl39c-1* were isolated in the mutagenesis screen described previously (Byrne et al., 2002; Pinon et al., 2008). The mutations in these genes were identified by map-based cloning and confirmed by complementation of the *as1 pgy* phenotype with a transgene carrying the full-length genomic region of the respective gene. Lines carrying *RPL27aC:RPL27aC* in the wild type and *rpl27ac-1d/+* and *RPL27aC:rpl27ac-1d* in the wild type were previously reported (Szakonyi and Byrne, 2011b). *rpl27ac-1d* carrying *RPL27aC:rpl27ac-1d* was generated by crossing with *RPL27aC:rpl27ac-1d* in the wild type to the heterozygous mutant. ET2634, GT3733, *pMEA:GUS*, *pFM2:GUS*, and *AT1g21670:mls-vYFP* were as previously reported (Huanca-Mamani et al., 2005; Groß-Hardt et al., 2007; Olmedo-Monfil et al., 2010; Tucker et al., 2012). *rpl27ac-2*, *rpl27ac-3*, *rpl27ab-1*, *rpl27ab-2*, and *rpl5a-2* in the Col-0 background were backcrossed four to five times to *Ler*, and unless otherwise stated, these alleles in the *Ler* background were used in analyses. All mutants and marker lines were in the *Ler* background, except *pfl-1* (C24), *pfl2* (No-O), *pFM2:GUS*, and *AT1g21670:mls-vYFP* (Col-0). *rpl27ac-2* in the Col-0 background was used for crosses to *pFM2:GUS* and to *AT1g21670:mls-vYFP*. Plants were grown either in soil or on Murashige and Skoog media at 22°C with a day length of 16 h.

Molecular Biology

RPL27aC and *RPL27aB* promoters were amplified with the primers 5'-ATGCCGAATTCGTCACGTAAGGAAGAATCGTGTC-3' and 5'-ATGCCGATCCITTCGCCGATCTGCTACACT-3' (*RPL27aC*) and 5'-ATGCCGAATTCGCCGTTGAAGCTTGAATA-3' and 5'-ATGCCGATCCITTTAGTATCAGATCTAGG-GITTTGAA-3' (*RPL27aB*). Each PCR product was cloned upstream of the reporter gene *3XNLS-vYFP*. The promoter and reporter genes were cloned into the binary vector pBAMAP and transformed into *Arabidopsis* (Adamski et al., 2009; Tucker et al., 2012). *rps23b-1*, *rpl35a-1*, and *rpl39c-1* were cloned using *Ler* × Col-0 mapping populations. To confirm gene cloning by complementation, 2,399-, 1,607-, and 1,137-bp genomic regions encompassing *RPS23B*, *RPL35A*, and *RPL39C*, respectively, were cloned into pMDC123, and the resulting constructs were transformed into *Ler* (Clough and Bent, 1998; Curtis and Grossniklaus, 2003). For *RPL27aC* gene expression analysis, mRNA was isolated from 10-d-old plants and used for RT-PCR amplification with the primers 5'-ATGACAACCAGATTCAGAAGAAGAC-3' and 5'-CGCTCTTCCAAAATCCAAA-3'. PCR products were digested with *HpaII* and separated by agarose gel electrophoresis. *HpaII* cleaves the wild type but not the *rpl27ac-1d* mutant allele. Undigested PCR products were also cloned into pCR8 (Invitrogen), and the allele in individual clones was determined by *HpaII* digestion.

Microscopy

For whole-mount analysis of ovules, gynoecia were dissected from flowers and fixed overnight in ethanol:acetic acid (9:1, v/v) and then dehydrated with 80% and then 70% ethanol prior to clearing with chloral hydrate:glycerol:water solution (8:3:1, w/v/v). Cytochemical staining for GUS activity was performed on developing gynoecia as previously described (Byrne et al., 2002). Following staining, ovules were dissected from gynoecia and mounted in 10% glycerol. Cleared and GUS-stained tissues were analyzed on a Zeiss Axiophot microscope and imaged with an Olympus DP72 digital camera. For fluorescence

analysis, ovules were mounted in 10% glycerol and examined on a Zeiss Zeiss AxioImager microscope.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers RPL27aC (At1g70600), RPL27aB (At1g23290), PGY1 (At2g27530), PGY2 (At1g33140), RPS23B (At5g02960), RPL35A (At3g09500), RPL39C (At4g31985), RPL5A (At3g25520), RPL5B (At5g39740), PFL1 (At1g22780), PFL2 (At4g00100), and STV1 (At3g53020).

Supplemental Data

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

Supplemental Figure S1. *rpl27ab-1* has reduced transcript levels.

Supplemental Figure S2. Expression pattern of *RPL27aB* and *RPL27aC* in developing ovules.

Supplemental Figure S3. Mutations in *rps23b*, *rpl35a*, and *rpl39c*.

Supplemental Figure S4. Relative transcript levels of the wild type and *RPL27aC:rpl27ac-1d*.

Supplemental Figure S5. *rpl27ac-2* ovules' phenotypes in Col-0 background.

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