Running head: A role for RPL27a in female gametogenesis

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Ribosomal Protein RPL27a Promotes Female Gametophyte Development in a Dose-dependent Manner

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Summary: The dose of a ribosomal protein affects the level of plant fertility.
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ABSTRACT

Ribosomal protein mutations in *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 extra-ribosomal functions of ribosomal proteins. Here, we report that ovule development is sensitive to the level of ribosomal protein 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 *RPL27aC* and *RPL27aB* act redundantly and reveal a function for RPL27a in coordinating complex interactions between sporophyte and gametophyte during ovule development.
INTRODUCTION

Eukaryotic cytoplasmic ribosomes are comprised of two subunits, a large 60S and a small 40S subunit. The 60S subunit includes 25-26S, 5.8S and 5S 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; Tsukaya et al., 2012; McCann and Baserga, 2013; Terzian and Box, 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 extra-ribosomal function required for specific developmental processes.

In Arabidopsis, cytoplasmic ribosomal proteins are encoded by two to five genes (Barakat et al., 2001; Giavalisco et al., 2005; Carroll et al., 2007). Mutations in single ribosomal protein genes are sometimes gametophyte or embryo lethal (Weijers et al., 2001; Tzafrir 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, 2011; Stirnberg et al., 2012). Female fertility is also reduced in several ribosomal protein mutants. Mutations in the ribosomal proteins SHORT VALVE1 (STV1)/RPL24B, SUPPRESSOR OF ACAULIS (SAC52)/RPL10A, ARABIDOPSIS MINUTE-LIKE1 (AML1)/RPS5B and RPL27aC reduce female fertility (Weijers et al., 2001; Nishimura et al., 2005; Imai et al., 2008; Szakonyi and Byrne, 2011). 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 WALKERI (SWAI), SLOW
WALKER3 (SWA3/AtRH36) and NUCLEOLAR FACTOR1 (NOF1) encode nucleolar-localized proteins required for processing 18S pre-rRNA (Shi et al., 2005; Harscoet 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, ribosome maturation or in export of pre-ribosomes from the nucleus to the cytoplasm also reduce female fertility (Li et al., 2009; Chantha et al., 2010; Li 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, 2011). The \textit{RPL27aC} and \textit{RPL27aB} encoded proteins are highly conserved, differing in only 2 of 146 amino acids and in most tissues \textit{RPL27aC} expression is approximately twice that of \textit{RPL27aB} (Rhee et al., 2003; Laubinger et al., 2008). To compare the function of \textit{RPL27aC} and \textit{RPL27aB} we examined the phenotypes of T-DNA insertion mutations in both genes. As reported previously, two alleles of \textit{RPL27aC}, \textit{rpl27ac-2} and \textit{rpl27ac-3}, had T-DNA insertions in the promoter region and the 5' leader sequence of \textit{RPL27aC}, respectively, and transcript levels of both alleles were reduced relative to wild type (Szakonyi and Byrne, 2011) (Fig. 1A). Plants homozygous for either \textit{rpl27ac-2} or \textit{rpl27ac-3} had rosette leaves that were pointed and serrated compared to wild type and were similar to the semi-dominant heterozygous \textit{rpl27ac-1d/+} (Fig. 1, B, D and E; Fig. 3) (Szakonyi and Byrne, 2011).

Two alleles of \textit{RPL27aB}, \textit{rpl27ab-1} and \textit{rpl27ab-2}, had T-DNA insertions in the 5' leader sequence and in the coding region of \textit{RPL27aB}, respectively (Fig. 1A). Transcript levels of \textit{RPL27aB} in \textit{rpl27ab-1} were slightly reduced relative to wild type indicating this is a weak allele (Supplemental Fig. S1). The insertion in \textit{rpl27ab-2} is within the coding sequence and is predicted to be a null allele. Leaf shape in \textit{rpl27ab-1} and \textit{rpl27ab-2} was changed to a more pointed shape but this phenotype was less severe than the \textit{rpl27ac} mutants, indicating that \textit{RPL27aB} has a less significant role in leaf development than \textit{RPL27aC} (Fig. 1, F and G). This result is consistent with the lower level of expression of \textit{RPL27aB} compared with \textit{RPL27aC}. To test for redundancy between \textit{RPL27aC} and \textit{RPL27aB} we crossed homozygous mutants in each gene and analysed the F1 trans-heterozygotes \textit{rpl27ac-2/+ rpl27ab-1/+} and \textit{rpl27ac-2/+ rpl27ab-2/+} for phenotypes. Both trans-heterozygotes had pointed and serrated leaves (Fig. 1, H and I). Thus there is functional overlap between \textit{RPL27aC} and \textit{RPL27aB} in leaf development.

**Insertion Mutations in \textit{RPL27aC} and \textit{RPL27aB} Reduce Fertility**

\textit{rpl27ac-2} and \textit{rpl27ac-3} had a change in leaf shape and were slightly slower growing than wild type. Both mutants also had shorter siliques than 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, \textit{rpl27ac-2} and \textit{rpl27ac-3} siliques had 46.4\% and 61.7\% defective ovules, respectively (Szakonyi and Byrne, 2011) (Fig. 2B). The
heterozygous rpl27ac-3/+ mutant was indistinguishable from wild type (Fig. 2B). By contrast, the heterozygous rpl27ac-2/+ had wild-type shaped leaves (Fig. 1C) and was indistinguishable from 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 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 regulates 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 wild type demonstrated that the mutant allele was efficiently transmitted maternally (transmission efficiency=54.1%, n=74) and paternally (transmission efficiency=56%, 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 wild type and the number of defective ovules in siliques was not significantly different to wild type (Fig. 2C). Homozygous rpl27ab-1 and rpl27ab-2 mutants were not noticeably different from 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 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 trans-
heterozygotes rpl27ac-2/+ rpl27ab-2/+ and rpl27ac-3/+ rpl27ab-2/+ relative to each single mutant. Ovule defects were significantly increased in both trans-heterozygotes 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 trans-heterozygotes 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 trans-heterozygotes, 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 the 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, STVI 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, 2011). To determine whether or not 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 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 glycine to aspartic acid. 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, 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 wild type these nine ribosomal protein mutants had variable effects on fertility. pgyl, pgyl2, rps23b, rpl39c and pfl2 did not reduce fertility, whereas rpl5a, rpl5b, rpl35a, pfl1 and stv1 had significantly more defective ovules than 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, 2011). 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-1dl/+ 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 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. Since 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. Semi-quantitative RT-PCR analysis demonstrated wild-type and mutant transcript levels were similar in \textit{rpl27ac-1d/+} (Supplemental Fig. S4A). By contrast, in wild-type plants that were also homozygous for \textit{RPL27aC:rpl27ac-1d} the level of mutant transcript was higher than that of wild type alone (Supplemental Fig. S4A). To obtain a more accurate estimate of wild-type and \textit{rpl27ac-1d} transcript levels in the different genetic backgrounds, RT-PCR products were cloned and individual clones were genotyped. Consistent with the semi-quantitative data, the alleles represented in the cloned RT-PCR products showed the level of mutant transcript was three times higher than that of wild type in plants carrying the \textit{RPL27aC:rpl27ac-1d} transgene (Supplemental Fig. S4B). Although the mutant allele was expressed at higher levels from the transgene it can be concluded that changes in the ratio of \textit{RPL27aC} to \textit{rpl27ac-1d} results in a gradual change in phenotype severity with changes in leaf shape paralleling changes in the frequency of defective ovules.

\textbf{Ovule and Female Gametophyte Defects in \textit{rpl27ac-2}}

To understand the role of \textit{RPL27a} in ovule development, we examined the morphology of developing and mature \textit{rpl27ac-2} ovules. In wild type, the megaspore mother cell (MMC) is a single large sub-epidermal 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. Post-meiosis the ovule has a single functional megaspore and three degenerate megaspores (Fig. 4B). The functional megaspore undergoes three rounds of mitosis leading to an FG5 stage embryo sac with eight nuclei (Fig. 4C). Subsequently, in the FG6 stage of development, the two polar nuclei fuse to form a central cell nucleus (Fig. 4D). In \textit{rpl27ac-2}, the frequency of an abnormal cell arrangement was 18\% of ovule primordia, somewhat higher than that of wild type (Fig. 4, E and I). Despite this defect, subsequent early stages of female gametophyte development in \textit{rpl27ac-2} were not readily distinguished from wild type. 12.3\% of post-meiosis \textit{rpl27ac-2} ovules had a large cell in the position of a degenerate megaspore (Fig. 4, F and J). Since these cells were not apparent in subsequent stages of gametogenesis they may represent delayed degeneration of non-functional megaspores. Defects in \textit{rpl27ac-2} ovules became more obvious in the final stages of ovule development. At FG5, when wild type had completed mitotic divisions, 31.4\% of \textit{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 wild type had a
large 7-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 background (Ler), we also examined rpl27ac-2 ovule development in the Columbia 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 ET2634 and antipodal cell marker GT3733 was initially detected in the majority of wild-type and mutant ovules (Fig. 5, A, D, F, I). In slightly older pistils, the frequency of ovules expressing synergid and antipodal markers decreased in rpl27ac-2 (Fig. 5, C, E, H, 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, the central cell marker 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 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 and instead is due to physical displacement of the mature gametophyte from the ovule.

Female Gametophyte Defects in rpl27ac/+

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 pre-meiosis ovule primordia was similar in rpl27ac-2/+ and rpl27ac-2 mutants. As with rpl27ac-2 siliques, pre-meiosis ovules with an abnormal arrangement of cells occurred at a slightly higher frequency in rpl27ac-2/+ compared with wild type (Fig. 6A). At later stages a significant proportion of ovules in rpl27ac-2/+ siliques were found to have only a single
nucleus. At maturity all ovules in rpl27ac-2/+ heterozygotes had normal integuments. 33% of ovules had a normal FG6-stage embryo sac, whereas 77% of ovules had gametophytes with only a single nucleus (Fig. 6B). The post-meiosis cell specific markers pFM2:GUS and pAt1g21670:nls-vYFP, which are expressed in the functional megaspore and in the developing gametophyte in wild type (Olmedo-Monfil et al., 2010; Tucker et al., 2012) (Fig. 6, C and E) 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). Since transmission of rpl27ac-2 is not affected in the rpl27ac-2/+ mutant, arrested gametogenesis occurs irrespective of the contribution of the genotype 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., 2012). 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 extra-ribosome 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, 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 masks or limits the contribution of these ribosomal proteins to ovule development. By contrast, stv1-2, which is likely a null allele, and rpl27ac mutants had dramatic semi-dominant effects on fertility. Two other semi-dominant 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 effects 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 indicate 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 extra-ribosome 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 (ETT)/ARF3, ARF5* and *ARF7* via uORFs 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 signalling.

The ovule defects resulting from mutation in RPL27a highlights 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 the 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-Saez 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 \textit{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 \textit{rpl27ac-2/+} mutants, it appears that the functional megaspore fails to undergo mitotic division. By comparison, ribosome biogenesis mutants \textit{swa1, swa2, swa3/atrh36} and \textit{nof1} that are female gametophyte lethal initiate megagametogenesis but do not complete mitotic cell divisions (Shi et al., 2005; Li et al., 2009; Harscoet et al., 2010; Huang et al., 2010; Liu et al., 2010). In \textit{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 \textit{swa2} and \textit{swa3} are also asynchronous and arrest with two to eight nuclei, whereas defective embryo sacs in \textit{nof1} have 4 nuclei. In a number of ways \textit{rpl27ac-2/+} is phenotypically similar to plants that are heterozygous for a novel semi-dominant allele of \textit{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 \textit{ago5-4/+} mutants are partially sterile, and like \textit{rpl27ac-2/+}, female gametophyte development aborts after meiosis and prior to mitotic divisions. This defect is in part mediated by \textit{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 \textit{Drosophila}, ribosomal protein mutants are the classic \textit{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; Farrar et al., 2008; Gazda et al., 2008; Bolze et al., 2013). Different models have been
proposed to explain ribosomal protein mutant phenotypes including ribosome heterogeneity, extra-ribosome 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 in development is to be established the data we present demonstrating 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 thaliana* (Arabidopsis) mutants rpl27ac-1d, pgyl-1 and pgy2-1 have been described previously (Pinon et al., 2008; Szakonyi and Byrne, 2011). rpl27ac-2 (SALK_142534), rpl27ac-3 (GABI_066_H03), rpl27ab-1 (SAIL_540_E01), rpl27ab-2 (GABI_104A06), pfl-1, pfl2 and stvl-2 were obtained from The European Arabidopsis Stock Centre (NASC) 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, 2011; Kleinboelting et al., 2012). rpl5a-2 (Salk_089798), which has been published as ae6-2 and oli5-2 (Yao et al., 2008; Fujikura et al., 2009), was also obtained from NASC. rpl5b-3 (GT16460) was obtained from Cold Spring Harbor Laboratory (Sundaresan et al., 1995). rps23b-1, and rpl35a-1, 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 wild type and rpl27ac-1d/+ , and *RPL27aC:*rpl27ac-1d in wild type were previously reported (Szakonyi and Byrne, 2011). rpl27ac-1d carrying *RPL27aC:*rpl27ac-1d was generated by crossing with *RPL27aC:*rpl27ac-1d in wild type to the heterozygous mutant. ET2634, GT3733, pMEA:GUS, pFM2:GUS and AT1g21670:nls-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 Columbia (Col) background were backcrossed four to five times to Landsberg erecta (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:nls-vYFP (Col). rpl27ac-2 in the Col background was used for crosses to pFM2:GUS and to AT1g21670:nls-vYFP. Plants were grown either in soil or on Murashige and Skoog media at 22°C with a day length of 16 hours.

**Molecular Biology**

*RPL27aC* and *RPL27aB* promoters were amplified with the primers 5’-ATGCGAATTTCGTCACGTAAGGAAGAATCGTGTC-3’ and 5’-ATGCGGATCTTTTGCGCAGATCTGCTA -3’ (*RPL27aC*) and 5’-ATGCGAATTCCCCCGGTGAAGCTTGAAAATA-3’, and 5’-ATGCGGATCTTTTAGTATCAGATCTAGGGTTTTGAA-3’ (*RPL27aB*). Each PCR product was cloned upstream of the reporter gene 3XNLS-vYFP. The promoter and reporter gene were cloned into the binary vector pBARMAP and transformed into Arabidopsis (Adamski et al., 2009; Tucker et al., 2012). *rps23b-1, rpl35a-1* and *rpl39c-1* were cloned using Ler x Col mapping populations. To confirm gene cloning by complementation 2399 bp, 1607 bp and 1137 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-day-old plants and used for RT-PCR amplification with the primers 5’-ATGACAACCAGATTCAAGAAGAAC-3’ and 5’-CGCTCTTTCCAAAATCCAAA-3’. PCR products were digested with *HpaII* and separated on a 4% agarose gel. *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), 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 β-glucuronidase (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.

**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 wild type and *RPL27aC:rpl27ac-1d.*

**Supplemental Figure S5.** *rpl27ac-2* ovules phenotypes in Columbia background.

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FIGURE LEGENDS

Figure 1. Leaf phenotypes of \textit{rpl27ac} and \textit{rpl27ab} mutants. A, Diagrammatic representation of \textit{RPL27aC} and \textit{RPL27aB} genes, showing exons (blue), 5’ and 3’ leader sequences (red), sites of T-DNA insertions and the location of the point mutation in \textit{rpl27ac-1d}. B-I, Rosettes of wild type (B), \textit{rpl27ac-2/+} (C), \textit{rpl27ac-2} (D), \textit{rpl27ac-3} (E), \textit{rpl27ab-1} (F), \textit{rpl27ab-2} (G), \textit{rpl27ac-2/+ rpl27ab-1/+} (H), \textit{rpl27ac-2/+ rpl27ab-2/+} (I).

Figure 2. Fertility defects in \textit{rpl27a} and ribosomal protein mutants. A, Wild-type (top) and \textit{rpl27ac-2} (bottom) open siliques with defective ovules in the mutant marked (arrowheads). B, Percentage of defective ovules in siliques of wild type (n=581); \textit{rpl27ac-2/+} (n=565); \textit{rpl27ac-2} (n=521); \textit{rpl27ac-3/+} (n=587); \textit{rpl27ac-3} (n=579) and \textit{rpl27ac-2} carrying the transgene \textit{RPL27aC:RPL27aC} (\textit{rpl27ac-2 TG RPL27aC}) (n=824). C, Percentage of defective ovules in siliques of \textit{rpl27ab-1/+} (n=572), \textit{rpl27ab-1} (n=549); \textit{rpl27ab-2/+} (n=626), \textit{rpl27ab-2} (n=548); \textit{rpl27ac-2/+ rpl27ab-2/+} (n=612); \textit{rpl27ac-3/+ rpl27ab-2/+} (n=594) and \textit{rpl27ac-2} carrying the transgene \textit{RPL27aB:RPL27aB} (\textit{rpl27ac-2 TG RPL27aB}) (n=932). D, Percentage of defective ovules in siliques of wild type (n=690); \textit{pgy1} (n=603); \textit{pgy2} (n=598); \textit{rps23b} (n=630); \textit{rps35a} (n=604); \textit{rps39c} (n=601); \textit{rpl5a} (n=532); \textit{rpl5b} (n=562); \textit{pfl1} (n=556); \textit{pfl2} (n=585); \textit{stv1/+} (n=668) and \textit{stv1} (n=560). Error bars are the standard deviation. * Student’s t-test P < 0.05.

Figure 3. Leaf and fertility defects reciprocally correlate with relative levels of functional \textit{RPL27a}. Diagrams at the top represent genomic wild-type \textit{RPL27aC} allele (green), genomic \textit{rpl27ac-1d} allele (green and red), \textit{RPL27aC:RPL27aC} transgene (yellow) and \textit{RPL27aC:rpl27ac-1d} transgene (yellow and red). Plant rosettes and the percentage of defective ovules for each genotype are shown below the diagrams. Genotypes are, from left to right, wild type (n=581); \textit{rpl27ac-1d/+} (n=578); \textit{rpl27ac-1d} (n=401); \textit{rpl27ac-1d/+ hemizygous for the transgene RPL27aC:RPL27aC} (TG C+) (n=2109); wild type homozygous for the transgene \textit{RPL27aC:RPL27aC} (TG C+) (n=1836); wild type hemizygous for the transgene \textit{RPL27aC:rpl27ac-1d} (TG C-) (n=1239); wild type homozygous for the transgene \textit{RPL27aC:rpl27ac-1d} (n=1801) and \textit{rpl27ac-1d/+} hemizygous for the transgene \textit{RPL27aC:rpl27ac-1d} (n=1366).
**Figure 4.** Ovule phenotypes in rpl27ac-2. A-D, Wild-type ovules. Percentage of normal ovules is indicated. Pre-meiosis ovule (n=371) (A). Post-meiosis ovule (n=313) (B). FG5 stage ovule with two integuments surrounding an eight-cell embryo sac or functional gametophyte (FG) (n=460) (C) and FG6 stage ovule with polar nuclei fused to form a seven-cell gametophyte (n=425) (D). E-L, Abnormal ovules from rpl27ac-2. Percentage of abnormal ovules is indicated. Pre-meiosis ovules with enlarged cells (arrowheads) (n=323) (E, I). Post-meiosis ovules with functional megaspore and enlarged cell (arrowhead) (n=310) (F-J). 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). ccn, central cell nucleus; dm, degenerate megaspore; ecn, egg cell nucleus; fm, functional megaspore; mmc, megaspore mother cell; pn, polar nucleus. Scale bars = 10 µm.

**Figure 5.** Maturing female gametophytes are expelled from the ovule in rpl27ac-2. A-C, Synergid marker ET2634 expression pattern in wild-type 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-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]. Scale bars = 10 µm.

**Figure 6.** Ovule phenotypes in rpl27ac-2/+ . A, Abnormal ovule from rpl27ac-2/+ plant at the pre-meiosis stage with several enlarged cells (arrowheads) (n=349). The percentage of abnormal ovules is indicated. B, Ovule 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-F, Post-meiosis 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. Scale bars = 10 µm.
Figure 1. Leaf phenotypes of rpl27ac and rpl27ab mutants. A, Diagrammatic representation of RPL27ac and RPL27ab genes, showing exons (blue), 5' and 3' leader sequences (red), sites of T-DNA insertions and the location of the point mutation in rpl27ac-1d. B-I, Rosettes of wild type (B), rpl27ac-2/+ (C), rpl27ac-2 (D), rpl27ac-3 (E), rpl27ab-1 (F), rpl27ab-2 (G), rpl27ac-2/+ rpl27ab-1/+ (H), rpl27ac-2/+ rpl27ab-2/+ (I).
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