An *Arabidopsis* Minute-like phenotype caused by a semi-dominant mutation in a *RIBOSOMAL PROTEIN S5* gene

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SUMMARY

Mutations in ribosomal protein (*RP*) genes in *Drosophila* lead to strong developmental phenotypes, expressed in the semi-dominant *Minute* syndrome. In plants, however, mutations in *RP* genes have so far only been reported to result in recessive developmental phenotypes. We present the analysis of an *Arabidopsis* promoter-trap line, in which a T-DNA insertion in an *RPS5* gene (*AtRPS5A*) causes semi-dominant developmental phenotypes. Most cell-division processes are delayed or disturbed in the heterozygous mutant, and development is completely arrested at an early embryonic stage in the homozygous mutant. By analogy with *Drosophila* *rp* mutants, we have named this mutant *Arabidopsis Minute-like 1* (*aml1*). As with other *Arabidopsis* RPs, *RPS5* is represented by a small gene family, but in contrast to other described plant RPs, this family comprises only two members. The *AtRPS5A* gene (mutated in *aml1*) is strongly expressed in dividing cells, whereas expression of the second *RPS5* gene, *AtRPS5B*, is lower than that of *AtRPS5A*, and is correlated with cell differentiation rather than cell division. From expression analyses we conclude that *AtRPS5A* is the most abundantly expressed *RPS5* gene in *Arabidopsis*. The *Minute*-like defects in the *aml1* mutant provide the first evidence that ribosome insufficiency leads to similar consequences in both plants and insects, and emphasize the general importance of efficient protein translation for cell proliferation in higher eukaryotes.

Key words: *Arabidopsis*, Semi-dominant mutation, Cell division, Ribosomal protein, *Minute* syndrome, Haplo-insufficiency

INTRODUCTION

The carriers of protein translation machinery in eukaryotic cells, the ribosomes, have a complex structure consisting of two subunits – the 60S (Large) and 40S (Small) subunit – that are each built from a considerable number of ribosomal proteins (RPs) and ribosomal RNAs (Moore, 1998). From one of the most well-studied models for ribosome structure and constitution, the rat, we now know that eukaryotic ribosomes consist of 82 different, highly conserved proteins (Wool et al., 1995).

The importance of the protein translation machinery in a given process can be studied by analysing the effect of a single *rp* mutation, as the absence of a single RP prevents assembly of the corresponding ribosomal subunit (Volarević et al., 2000). Notably, apart from expected defects such as growth arrest or delay, mutations in *RP* genes often result in specific defects in the development of an organism (Wool, 1996). In some cases, *RP* gene mutations affect DNA replication, RNA processing and DNA repair (Wool, 1996), suggesting roles for RPs that are additional to protein translation.

One of the most prominent examples of specific developmental defects due to *RP* gene mutations is the *Minute* syndrome in *Drosophila*. The *Minute* class of mutants was described, as early as in 1919 (Lambertsson, 1998), and today over 50 different *Minute* loci have been mapped. The *Minute* syndrome is characterised by semi-dominant phenotypes, such as delay in larval development, smaller body size and several pleiotropic morphological aberrations (e.g., short thoracic bristles), and recessive embryo lethality. Kongsuwan and co-workers (Kongsuwan et al., 1985) first showed that a *Minute* phenotype was caused by a deletion of a *RP* gene, and now, at least 11 *Minute* loci have been assigned to *RP* genes (Lambertsson, 1998), demonstrating that ribosome function is essential during specific stages of fly development and that individual RPs can easily become rate-limiting for various processes.

Only few mutations in *RP* genes have been described in the plant *Arabidopsis thaliana*. Two such mutations were identified during screens for either aberrant seedling phenotypes (van Lijsbetetens et al., 1994) or altered sensitivity to genotoxic stress (Revenkova et al., 1999). A third *rp* mutant, identified through sequence analyses of *Ds* transposon insertion sites, also appeared to show specific defects in seedling development (Ito et al., 2000). Strikingly, all three of the above *rp* phenotypes were found to be recessive. Semi-dominant phenotypes, as described for the *Drosophila Minute* mutants, have not been reported in plants. This might be a consequence of the fact that *RP* genes are single copy in *Drosophila* (Kay and Jacobs-Lorena, 1987), while in *Arabidopsis* each *RP* is represented by a small gene family.
(Cooke et al., 1997; Revenkova et al., 1999). Alternatively, plants may have evolved mechanisms to avoid the deleterious effect of ribosome insufficiency.

We report the identification and further characterisation of a semi-dominant mutation in an Arabidopsis RPS5 gene. We named the mutant Arabidopsis Minute-like 1 (aml1), because the semi-dominant growth retardation and floral and vascular defects and the recessive embryo lethality are analogous to defects observed in the Drosophila Minute mutants. Our findings show a striking similarity between the effect of ribosome insufficiency in flies and in plants and show that ribosomes can be limiting for plant growth and development.

MATERIALS AND METHODS

Plant material, plant growth and transformation

Arabidopsis thaliana ecotype C24 or Columbia seeds were surface sterilised by incubation for 1 minute in 70% ethanol, 15 minutes in 2% hypochlorite/0.01% Tween-20 and four rinses with sterile water. Seeds were imbibed for 2 to 4 days at 4°C in the dark before germination in regeneration rooms (21°C, 16 hour light/8 hour dark, 4000 lux) for seed set. Ploidy levels were analysed by counting the number of plastids per guard cell in epidermal strips.

After approximately 2 weeks, seedlings were transferred to soil and grown in growth chambers (21°C, 60% relative humidity, 16 hour light/8 hour dark, 4000 lux) for seed set. Ploidy levels were analysed by counting the number of plastids per guard cell in epidermal strips. The presence of the 826 T-DNA was selected by germination on hygromycin-containing medium (Duchefa, 20 mg/l). Arabidopsis plants were transformed with Agrobacterium tumefaciens using the ‘floral-dip’ method (Clough and Bent, 1998) with minor modifications. Transgenic plants were selected on medium containing 30 mg/l phosphinothricin in the case of pAIRPS5A::GUS and pAIRPS5B::GUS or 75 mg/l kanamycin and 20 mg/l hygromycin in the case of the complementation construct.

Microscopy and histology

For whole-mount embryo analysis, silicues were slit open longitudinally on both sides of the septum with a hypodermic needle, fixed in a mixture of ethanol and acetic acid (3:1) for 1 hour and mounted in a drop of clearing solution (chloral hydrate: water: glycerol, 8:3:1). Embryos were viewed with a Zeiss Axiosplan II microscope equipped with differential interference contrast (DIC) optics. For cytology, silicues, roots or flower buds were fixed overnight at 4°C in 4% paraformaldehyde in a 0.1 M sodium cacodylate-buffer (pH 7.2), rinsed twice in the same buffer and dehydrated through a graded ethanol series before embedding in Technovit 7100 (Heraeus Kulzer, Germany). Tissues were fixed in a mixture of ethanol and acetic acid (3:1) for 1 hour at –20°C. Tissues were then washed twice for 5 minutes in washing buffer (0.1 M phosphate, pH 7.0, 10 mM EDTA, 2 mM K2Fe(CN)6) under vacuum.

Subsequently, staining buffer (0.1 M phosphate, pH 7.0, 10 mM EDTA, 1 mM K2Fe(CN)6, 1 mM K4Fe(CN)6:3H2O, 1 mg/ml X-Gluc) was infiltrated with a brief vacuum treatment and specimens were incubated for 5 minutes (pAIRPS5A::GUS seedlings) to overnight (aml1 mutant or pAIRPS5B::GUS seedlings) at 37°C. GUS-stained tissues were fixed in a 3:1 mixture of ethanol and acetic acid and cleared and mounted in a drop of clearing solution. All microscopic images were recorded using a Sony DKC-5000 digital camera, and compiled using Adobe Photoshop 5.5.

Measurements and morphometrical analyses

For cotyledon- and root measurements, seeds of the hemizygous aml1 mutant were germinated in a near-vertical position on MA medium without antibiotic selection. Four or 7 days after germination, seedlings were stained for GUS activity. Root and cotyledon size of GUS-positive and GUS-negative seedlings was measured from digital recordings using Scion Image software and statistical analysis (Student’s t-test) was performed in Microsoft Excel. To quantify vascular patterning in the mutant, the same seedlings were observed under dark-field illumination.

Molecular cloning

Molecular cloning was performed following standard procedures (Sambrook et al., 1989). Inverse PCR (I-PCR) was performed on Ncol-digested chromosomal DNA from 826 hemizygous plants using PCR primers in the GUS gene as described by Offringa and van der Lee (Offringa and van der Lee, 1995). EST clones homologous to the I-PCR fragment sequence (Accession Numbers H35978 and N37914) were obtained from the Arabidopsis Biological Research Centre (Columbus, OH). For promoter isolation, 1.7 kb fragments containing the promoter region of the AtRPS5A or AtRPS5B gene were amplified by PCR and fused to the eGFP::GUS::intron reporter gene (Quaedvlieg et al., 1998) in pGPTV-BAR (Becker et al., 1992) to yield AtRPS5A::GUS and AtRPS5B::GUS. Primers for PCR were based on genome sequence deposited in the GenBank database. For complementation of aml1, we amplified a 4.2 kb region spanning the entire AtRPS5A gene, including 2 kb promoter sequence from C24 genomic DNA using the following primers: RPS5AcompF GGCAGATCTGATACGTGTCCTTC and RPS5AcompR AGCCAGATGATCTACGTCAGTCTG. The entire fragment was ligated into pMOG800 (MOGEN International N.V., Leiden, The Netherlands). To detect complementation, we used primers 1 (GCTCACAACCTCCTCCTAGTGACGG), II (GAGGTGTGTAGTAGGTCGTTGTTGATCGTGG) and III (TGCCTAATGATGACCGCATCAG), and NPTII (TTGTCAGAGCCGACCTGTC) and NPTII6 (CACCATGATACCGCAGACG) in PCR reactions on DNA isolated from primary transformants with the MOG-RPS5 construct. Binary plasmids were transformed into A. tumefaciens strain LBA1115 by electroporation (den Dulk-Ras and Hooykaas, 1993). Sequence analysis was performed at Eurogentec (Belgium). DNA and protein sequences were analysed using the Vector NTI 5.5 software package (Informax, Bethesda, MD).

RT-PCR

Total RNA was extracted from 2-week-old seedlings according to Chang et al. (Chang et al., 1993). For RT-PCR, 10 μg of total RNA was treated with 50 U of DNaseI (Roche Biochemicals, The Netherlands) in DNaseI assay buffer (40 mM Tris-HCl, pH 7.5, 6 mM MgCl2, 1 μM RNaseguard (Roche Diagnostics, The Netherlands) during 1 hour at 37°C. The RNA was then purified by chloroform extraction and precipitated (0.5 volume 7.5 M NH4OAc, two volumes ethanol) at –80°C for 1 hour. The RNA pellet was resuspended in 9 μl RNAse free water and 1 μl oligo d(T)15 (0.5 μg/μl) (Roche Diagnostics, The Netherlands). After 5 minutes at 70°C, the RT mix (1x buffer concentration (supplied with enzyme), 0.5 mM dNTPs, 5 U MmuLV-RT (Roche Diagnostics, The Netherlands), 1 U RNaseguard) was added and kept at 42°C for 1 hour. The cDNA was then purified by phenol/chloroform and chloroform extractions and precipitated with 0.4 volume 7.5 M NH4OAc and two volumes ethanol. One-tenth of the total cDNA pool was used in all reactions. Fragments were amplified from these cDNA pools (or 10 ng C24 chromosomal DNA as a control for the primer pairs) using Taq polymerase (Roche Diagnostics, The Netherlands) according to the manufacturer’s procedures. Primer pairs were as follows: for AtRPS5A, 826g21F – CTTCAATTCCGGCGAGCCAAAACG and 826constR and – 826g21F – CGGCTAAAGATCCCTACCTTCC and 826constR. PCR conditions were as follows: 4 minutes at 95°C, 30 cycles of 15 seconds at 94°C, 30 seconds at 60°C and 1 minute at 72°C, followed by 5 minutes at 72°C.
Whole-mount in situ hybridisation
In-situ hybridisation on 5-day-old seedlings was performed as described (J. Friml, PhD thesis, University of Cologne, 2000) with slight modifications. For auxin-induction, seedlings were treated with 50 μM 1-NAA during 16 hours in liquid M-A medium (Masson and Paszkowski, 1992) in the dark. To detect RPS5A mRNA specifically, a 150 bp fragment of the AtRPS5A cDNA was PCR-amplified from the cloned cDNA (RPS5A.F CCGATCCGTTTTCGCTGTC and RPS5A.R GGAATTCGATGCTGACCGTACG) and cloned into pBluescript SK+ and KS+ vectors. Digoxigenin-labelled sense- or antisense RNA probes were synthesised using T7 RNA-polymerase (Promega, Leiden, The Netherlands) according the manufacturers procedures. For hybridisation we used 100 ng probe per ml hybridisation volume.

RESULTS

A promoter trap line that marks cell division
In an effort to identify new auxin-responsive genes, we screened a collection of transgenic Arabidopsis plant lines harbouring a promoter-trap T-DNA construct with the GUS gene as a reporter for promoter activity (Goddijn et al., 1993). Line 553-826 (hereafter referred to as line 826) was selected because seedlings showed auxin-responsive GUS activity in the root pericycle (R. O. and P. H., unpublished). In untreated seedlings, GUS activity was found in the apical meristems of both shoot and root (Fig. 1A-C), in lateral root primordia (Fig. 1D), young leaf primordia (Fig. 1B) and in the vascular tissue of cotyledons (Fig. 1E). This basal expression pattern was not enhanced by auxin (data not shown) and as auxin-induced expression was confined to a cell layer in which cell division is activated by auxin, we concluded that the GUS gene in the 826 line is regulated by a cell division-related, rather than by an auxin-specific pathway. This conclusion is supported by the fact that GUS activity was detectable in the most distal cells that represent the division zone of the root tip, but not in the mitotically inactive quiescent center (Fig. 1C) (Dolan et al., 1993).

The 826 T-DNA insertion is linked to a semi-dominant delay in embryo development, recessive embryo lethality and reduced gamete viability
Line 826 was tetraploid and contained four T-DNA loci. The line was backcrossed three times with the C24 wild type. A diploid plant, hemizygous for a single T-DNA insert (hereafter referred to as the 826 T-DNA), but which displayed the same GUS pattern as the original line, was selected. When seeds from this diploid 826 line were selected for the hygromycin resistance marker on the T-DNA, we observed a ratio of 0.62:1 resistant versus sensitive seedlings (n=1821). Because this ratio strongly deviated from the expected 3:1 ratio, we analysed embryo development to determine whether selective seed abortion contributed to the low number of seeds containing the 826 T-DNA. Embryo development clearly differed between wild-type and line 826 embryos. Most embryos in a wild-type siliques contained globular stage embryos, early embryogen stage embryos, and two-cell stage embryos. Later, torpedo stage embryos, heart stage embryos and early globular stage embryos were found (Fig. 2A). Late siliques contained nearly mature embryos, bent-cotyledon stage embryos and arrested globular stage embryos (Fig. 2B,C). Cells of these arrested embryos were swollen (Fig. 2Aii) and showed subcellular structures typical for mature embryos (Fig. 2D). Morphology of the endosperm appeared normal even in ovules that contained the most developmentally retarded class of embryos (data not shown), indicating that endosperm development is not severely affected in line 826. To test if the arrested embryos in line 826 represent the individuals homozygous for the 826 T-DNA, progeny from 31 hygromycin-resistant plants were analysed for segregation of the T-DNA. All 31 plants segregated for the 826 T-DNA. Based on this, we concluded that the development of hygromycin progeny did not progress to the seedling stage, but arrested prematurely during seed development. To determine linkage between presence of the 826 T-DNA and the mutant embryo phenotypes, GUS activity was analysed in embryos. Irrespective of the developmental stage, GUS activity was never detected in the most developmentally advanced embryos, whereas all GUS-positive embryos were retarded in their growth (Fig. 2E). Moreover, GUS activity in the most developmentally retarded class was always higher than in the intermediate class of embryos (compare Fig. 2Eii with 2Eiii), confirming the developmental separation of homo- and hemizygotes for the 826 T-DNA, and strongly suggesting that the T-DNA insertion in line 826 causes semi-dominant delay and recessive arrest of embryogenesis.

Recessive embryo lethality has been observed in several other Arabidopsis mutants (Patton et al., 1991; Castle et al., 1993; Yadegari et al., 1994; Uwer et al., 1998; Albert et al., 1999). Still, the 0.62:1 ratio observed in the hemizygous line 826 deviates strongly from the 2:1 segregation ratio expected in the case of embryo lethality. To determine if transmission of the 826 T-DNA to the next generation was affected by the viability of mutant gametes, we performed reciprocal crosses between the diploid, hemizygous 826 line and C24 wild-type plants, and scored for segregation of the T-DNA locus in the resulting progeny. When using 826 plants as a male parent, we found 15% of the progeny to be hygromycin resistant (n=41). In the case of full viability of the male gamete, one would expect this to be 50%. Thus, we conclude that the viability of pollen that carries the 826 T-DNA is decreased by approximately 70%. Using 826 plants as a female parent, we found 26% of the progeny to be hygromycin resistant (n=312). The viability of mutant female gametes is thus reduced by 50%. From these data the segregation ratio expected for a self-pollination was calculated to be 0.53:1 (resistant versus sensitive), which closely approaches the observed 0.62:1 ratio. The GUS gene in line 826 is expressed in pollen (not shown), implying that the decreased pollen viability is a result of the presence of the 826 T-DNA.

Semi-dominant defects during post-embryonic development in line 826
We noticed that the hygromycin-resistant progeny of the diploid, hemizygous 826 line showed a variety of semi-dominant phenotypes. Seedlings carrying the T-DNA, although variable in size (Fig. 1A), were generally smaller than wild-type seedlings. We observed an average 20% reduction in cotyledon length, 4 days after germination, and a 40%
reduction of root length, one week after germination (Table 1). Hypocotyl length in 826 seedlings did not differ significantly from the wild type. It was shown that hypocotyl growth in seedlings is achieved by cell-elongation, and not by cell division (Gendreau et al., 1997). We therefore suspect that only cell division related processes are disturbed in 826 seedlings. We did however, observe a significant difference in lateral root number (data not shown).

Cotyledons of the 826 line appeared rounded in shape (Figs 1A, 3A). To analyse the nature of this shape change, we studied venation patterns in mutant and wild-type seedlings in more detail. Vascular development in wild-type cotyledons is highly reproducible, resulting in a recognisable pattern (for a detailed description see Berleth et al. (Berleth et al., 2000) and references therein). Nonetheless, slight differences in developmental stages among individual seedlings cause stochastic variation of vascular pattern complexity (Fig. 3B). In 826 hemizygous seedlings, the vascular strands were often shorter and poorly interconnected (Fig. 3A), frequently leading to aberrant, simplified patterns. The lack of interconnections between xylem strands correlated with the absence of protoxylem strands (data not shown), indicating a defect in cell division rather than vascular tissue differentiation. In general, vascular patterning was less complex in the 826 hemizygotes (Fig. 3B). The decrease in cotyledon size is correlated with a simplification of the vascular pattern. However, this does not answer the question of whether cotyledons are smaller due to incomplete vascular tissue development, or whether vascular tissue formation is compromised because cotyledons are smaller. In any case, the defect in vascular pattern correlates with the observed expression of the GUS gene in the vascular tissue in cotyledons (Fig. 1E). Similar expression was also seen with the promoter of the Arabidopsis CDC2A gene, which marks cells competent for cell division (Hemerly et al., 1993). No obvious defects were observed in other leaf types such as rosette leaves, bracts, sepals, petals and siliques valves.

Plants hemizygous for the 826 T-DNA developed normal
The receptacle of a young flower.

strand (compare top with bottom arrow) in a cross-section through from the floral meristem, as indicated by the absence of one vascular bud from an 826 hemizygous plant. (D) The floral defect originates four to five stamens, as clearly shown in a cross-section of a flower-axis. (C) Hemizygous 826 flowers carry only x

represented on the shows the percentage of seedlings falling into the categories

Fig. 3. Vascular and floral defects in hemizygous 826 plants. (A) Abnormal venation pattern in a cotyledon of a plant hemizygous for the 826 T-DNA. Note that the central vein is short and a secondary vein is interrupted. (B) Vascular pattern complexity in the most advanced cotyledon of wild-type (GUS negative, white bars) and hemizygous 826 (GUS positive, black bars) seedlings. The y-axis shows the percentage of seedlings falling into the categories represented on the x-axis. (C) Hemizygous 826 flowers carry only four to five stamens, as clearly shown in a cross-section of a flower bud from an 826 hemizygous plant. (D) The floral defect originates from the floral meristem, as indicated by the absence of one vascular strand (compare top with bottom arrow) in a cross-section through the receptacle of a young flower.

Table 1. Organ size of Arabidopsis wild-type and hemizygous 826 seedlings

<table>
<thead>
<tr>
<th></th>
<th>+/+ (GUS negative)</th>
<th>826/GUS positive</th>
<th>Reduction (%)</th>
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<tr>
<td></td>
<td>Length (mm)±s.e.m.</td>
<td>n</td>
<td>Length (mm)±s.e.m.</td>
</tr>
<tr>
<td>Root length*</td>
<td>4 dag</td>
<td>3.46±0.16</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>7 dag</td>
<td>3.75±0.49</td>
<td>113</td>
</tr>
<tr>
<td>Cotyledon length†</td>
<td>4 dag</td>
<td>1.19±0.02</td>
<td>90</td>
</tr>
<tr>
<td>Hypocotyl length‡</td>
<td>4 dag</td>
<td>2.22±0.05</td>
<td>45</td>
</tr>
</tbody>
</table>

GUS negative (+/+ and GUS positive (+/826) seedlings were measured after staining.

*Root length was measured in seedlings either 4 or 7 days after germination (dag).

†Cotyledon length was measured in the proximal-distal axis of both cotyledons of each seedling.

‡The means were significantly different when tested with Student’s t-test (P<0.05).

n, number of measurements; s.e.m., standard error of the mean.
recessive embryo lethality, decreased gamete viability and several semi-dominant defects, such as delay in development and reduced body size (Lambertsson, 1998). The similarity between the Drosophila Minute mutants and our mutant line prompted us to rename our 826 line *aml1* for *Arabidopsis* Minute-like 1.

**RPS5 is represented by a small gene family in *Arabidopsis***

A search of the TIGR *Arabidopsis* gene index (http://www.tigr.org/tdb/agi) with the *AtRPS5* gene sequence revealed the presence of a second class of *RPS5* ESTs in *Arabidopsis*. The initially identified gene will be referred to as *AtRPS5A*, and the second copy will be referred to as *AtRPS5B*. We sequenced full-length cDNAs of both *RPS5* genes and found that they were 78% identical at the nucleotide level and contained an open reading frame of 621 bp, encoding a protein of 207 amino acids with a predicted molecular mass of 22.9 kDa. The two predicted proteins show 94% identity. Alignment of a number of *RPS5* sequences from different organisms revealed many invariant amino acids, as well as a few plant-specific residues (Fig. 4A). Only two of the amino acid substitutions between the two family members are at residues that are otherwise conserved in eukaryotic *RPS5* proteins, and the two proteins are thus very likely to be functionally redundant.

Southern analysis and a survey of the completed sequence of the *Arabidopsis* genome confirmed the presence of two gene copies in *Arabidopsis*. In the *aml1* mutant, a third hybridising band was observed, which represents the T-DNA insertion in *AtRPS5A* (not shown). The *AtRPS5A* gene resides on P1 clone MEC18 (Accession Number, AP002040) and maps at 18 cM on chromosome III. The *AtRPS5B* gene is located on BAC F3G5 (Accession Number, AC005896) and maps at 69.0 cM on chromosome II. No other mutants with similar phenotypes to *aml1* were found to map near these two loci.

The gene structure of the two *RPS5* copies is identical. Both contain five exons and four introns (ranging from 79-385 bp), the first of which is located 4 bp upstream of the translational start codon and the other three within the coding region (Fig. 4B). Both the sequence (49% identity) and length of the introns (Fig. 4D) are conserved between the two gene copies. The transcripts share a number of characteristics with mammalian RP transcripts (Wool et al., 1995). Three pyrimidine stretches are found within the 5'-UTR of the mRNA (at −34, −46 and −53 bp relative to the ATG in *AtRPS5A*; −29, −35 and −59 in *AtRPS5B*). The translation initiation context is G(T)/C AUUG, the termination codon is UAA and both transcripts have an A/T-rich, poly-adenylated 3'-UTR (71% A/T in *AtRPS5A* and 68% A/T in *AtRPS5B*). This shows that not only the amino acid sequence, but also mRNA characteristics are strongly conserved between different
species. In both genes, a conserved motif is present directly upstream of the transcribed region that is referred to as the telo box. This box is found in many plant genes that are involved in protein synthesis and is thought to act in concert with the Tef1 box to direct expression of genes to foci of cell division in plants (Regad et al., 1995; Tremousaygue et al., 1999). The Tef1 box consensus sequence was found in the promoter sequence of the AtRPS5A gene. Only a weak similarity to the Tef1 box consensus sequence is present in the promoter of AtRPS5B.

**AtRPS5A and AtRPS5B are differentially expressed**

Despite the presence of two expressed RPS5 family members in Arabidopsis, a mutation in only one gene does result in semidominant Minute-like phenotypes. This suggests that AtRPS5A and AtRPS5B are differentially expressed and prompted us to compare the expression pattern of both genes.

RT-PCR analysis with primer sets specific for either of the genes revealed that AtRPS5A is expressed several-fold stronger than AtRPS5B in seedlings (Fig. 4E). The RT-PCR data are supported by the occurrence of 36 independent ESTs representing AtRPS5A and only 12 ESTs representing AtRPS5B in the Arabidopsis gene index (AtGI).

**AtRPS5A expression**

The strict correlation between the Minute-like phenotypes in the am11 mutant and the expression pattern of the RPS5::GUS fusion gene suggested that this fusion gene perfectly reproduces the expression pattern of the AtRPS5A gene. To confirm this, we performed whole-mount in situ hybridisation on wild-type seedlings using a probe specific for the AtRPS5A gene. The antisense probe detected a strong hybridisation signal in young leaf primordia (Fig. 5A,B) and the shoot apical meristem (Fig. 5B). Furthermore, we found hybridisation signal in the vasculature of cotyledons (Fig. 5C). In roots, we found strong signals in the distal tip (Fig. 5D,E). Upon closer inspection, the region that shows this signal appears to be the division zone of the root meristem.

In accordance with the cell-division-correlated expression, the QC cells do not show a detectable signal (Fig. 5E). Upon treatment for 16 hours with the synthetic auxin 1-NAA, we found hybridisation signals in lateral root primordia (Fig. 5F). As hybridisation using a sense control probe lacked the signals described above (Fig. 5G), these expression signals confirm the pattern of RPS5::GUS activity detected in the am11 mutant (Fig. 1; data not shown). We therefore conclude that the RPS5A::GUS gene expression genuinely reflects the expression of the AtRPS5A gene.

To address the spatiotemporal regulation of AtRPS5A gene transcription in the absence of the am11 mutation, we constructed a promoter::reporter gene fusion. GUS activity in pAtRPS5A::GUS seedlings could already be detected after 10 minutes of histochemical staining at the sites of cell division, i.e. in the shoot apical meristem, the youngest leaf primordia and the division zone of the root meristem (Fig. 6A,C). Strong expression was also found in lateral root primordia, where intense GUS staining was already visible during the first division of a single pericycle cell (data not shown). Weaker GUS activity was detected in non-dividing pericycle cells (Fig. 6B) that are considered to be competent for cell division (Hemerly et al., 1993; Doerner et al., 1996). Expression of GUS was also seen in all axillary meristems, floral meristems and flower primordia, and typically faded as organs matured. In general, the pattern of pAtRPS5A::GUS expression was identical to the AtRPS5A mRNA distribution pattern and to that of the RPS5A::GUS fusion in the am11 mutant (Figs 1, 5). Apparently, no cis-regulatory elements other than the promoter region are necessary to provide the complete AtRPS5A gene expression pattern.

**AtRPS5B expression**

As the gene structure of the two AtRPS5 genes in Arabidopsis is highly homologous, we reasoned that, as with AtRPS5A, the promoter activity of AtRPS5B would reflect its mRNA expression pattern. As predicted by the RT-PCR analysis, GUS reporter gene expression in pAtRPS5B::GUS lines was much weaker than in pAtRPS5A::GUS lines. The sites of pAtRPS5A::GUS and pAtRPS5B::GUS expression partially overlapped, but while AtRPS5A is expressed in dividing cells (Figs 1, 5A,C), AtRPS5B expression seems correlated with differentiation and elongation of cells in young growing tissues (Fig. 6D-F). Expression of AtRPS5B in the root was found in epidermal cells at the tip, several cells proximal from the initials (Fig. 6D), and was later observed in the trichoblast cell files and in root hairs (Fig. 6H), cell types where AtRPS5A expression is undetectable (Fig. 6G). In an in situ hybridisation experiment using a probe that recognised both mRNAs, we could reproduce this trichoblast-specific AtRPS5B expression (data not shown). Weaker expression of pAtRPS5B::GUS was found in developing lateral root primordia and in the shoot (Fig. 6F). Expression is absent from the pericycle layer (Fig. 6E), as opposed to that of pAtRPS5A::GUS (Fig. 6B). In contrast to AtRPS5A, expression of AtRPS5B was not observed in the SAM, but rather in the distal parts (and trichome cells) of young leaf primordia (Fig. 6F).

In the pAtRPS5A::GUS lines, GUS expression was found in the embryo from the zygote stage onward (Fig. 7A-E). GUS expression increased in strength until the octant stage and was strongly reduced after the late heart stage (Fig. 7B-E). Already during the first stages of embryo development, GUS expression was detected in the dividing endosperm (Fig. 7A-C). This activity decreased with the progression of endosperm development (Fig. 7C,D). pAtRPS5B::GUS lines showed only weak GUS expression at early stages of embryogenesis (Fig. 7G,H). During the transition to the heart stage, GUS activity increased and became preferentially localised to the inner cell layers, excluding the apical protoderm (Fig. 7I,J). During later stages, this pattern progressed, marking the inner cell types of the embryo axis and the cotyledons at late-torpedo stage (Fig. 7K). No GUS activity was found in the endosperm of pAtRPS5B::GUS lines until the globular stage of embryo development (Fig. 7G-J). The differential expression patterns of AtRPS5A and AtRPS5B in the embryo are schematically summarised in Fig. 7F.

GUS expression in both promoter::GUS lines was induced by treatment with IAA in the pericycle and in the newly formed lateral root primordia. Slight auxin-induction of both genes was confirmed in a micro-array experiment (B. van der Zaal, unpublished). The finding that lateral root initiation is marked by the expression of both gene copies explains why we did not observe a clear defect in lateral root formation in the heterozygous am11 mutant. GUS expression was not induced
by 1 or 10 \( \mu \text{M} \) zeatin, 10 \( \mu \text{M} \) NPA, or by mechanical wounding (data not shown).

**DISCUSSION**

A semi-dominant mutation in a plant ribosomal protein gene

Protein synthesis by the translational carriers, the ribosomes, is an essential feature of living cells. Mutations in genes encoding the structural components of ribosomes, the ribosomal proteins and ribosomal RNAs, have been described in various organisms. In addition to growth arrest or delay, these mutations often induce developmental aberrations (Wool, 1996). In *Drosophila*, such defects are expressed in the *Minute* syndrome, a semi-dominant phenotype affecting multiple developmental processes (Lambertsson, 1998).

Diverse mutant screens in *Arabidopsis thaliana* have identified mutations in several *RP* genes. The first to be identified was a recessive mutation in the *RPS18A* gene, which results in plants with pointed leaves (van Lijsebettens et al., 1995). The second *RP* gene to be identified by a mutation in *Arabidopsis* was the *AtRPS27A* gene. A recessive mutation in this gene caused plants to be more sensitive to a genotoxic stress treatment (Revenkova et al., 1999). Recently, Ito et al. (Ito et al., 2000) used a reverse genetics approach to identify a recessive mutation in the *AtRPS13A* gene. As with *AtRPS18A*, this mutation results in pointed leaves, and in addition, has impaired root growth and trichome branching.

Considering the importance of protein translation in general developmental processes and the fact that disruption of *RP* genes in *Drosophila* often results in semi-dominant phenotypes, the question arises why mutations in the *Arabidopsis* *RP* genes discussed above do not result in a semi-dominant phenotype. A possible explanation comes from the observation that in *Drosophila*, RPs are mostly represented by a single gene (Kay and Jacobs-Lorena, 1987). It was proposed that the *Minute* syndrome is caused by haplo-insufficiency (Sæbøe-Larssen et al., 1998), i.e. if one functional gene copy per diploid genome is insufficient for complete gene function, this results in a loss-of-function phenotype. Evidence for this interpretation comes from the finding that a number of *Minute* mutants were shown to have a 50% decrease of mRNA levels in the heterozygote, and that there is a proportional relationship between the amount of *RP* mRNA and the severity of the *Minute* phenotype (Sæbøe-Larssen and Lambertsson, 1996; Lambertsson, 1998). Additional support for the haplo-insufficiency hypothesis comes from experiments in which partial *Minute* mutants were obtained in *Drosophila* when the *RPA1* or *Rp49* mRNA was over-expressed in antisense orientation (Qian et al., 1988; Patel and Jacobs-Lorena, 1992).

**Fig. 5.** Localisation of *AtRPS5A* mRNA by in situ hybridisation. Five-day-old wild-type seedlings were hybridised with an *AtRPS5A*-specific antisense (A-F) or sense (G) probe. Untreated seedlings (A-E,G) as well as NAA-treated seedlings (F) were used. Expression is detected in young leaf primordia (A). (B) A close-up of the apical shoot region of a seedling. Expression is confined to the leaf primordia (lp1 and lp2) and the shoot apical meristem (sam). A significant signal is detected in the vasculature of cotyledons (C), and the primary root tip (D). (E) A close-up of a root apical region. A strong signal is present in all dividing cells, but not in the quiescent center (qc). NAA-induced lateral root primordia stain strongly for the *AtRPS5A* signal (F). (G) The absence of a signal in a sense-probe control on an untreated seedling root.

**Fig. 6.** *AtRPS5A* and *AtRPS5B* are differentially expressed in seedlings. Four-day-old seedlings from *pAtRPS5A::GUS* (A-C) and *pAtRPS5B::GUS* (D-F) plants were stained for GUS activity during 10 minutes and 2 hours, respectively. The *AtRPS5A* promoter is strongly active in the division zone of the primary root tip (A) and the SAM and leaf primordia (C), and is weaker in the pericycle of the root (B). *pAtRPS5B* is active in the epidermal cells of the primary root tip (D), in a zone more distal from the tip than *pAtRPS5A*. Activity is almost absent in the root pericycle (E), but present in lateral root primordia (not shown). GUS activity is also found in the distal, differentiating cells of the shoot primordium, but absent from the SAM (F). *AtRPS5B* (H), but not *AtRPS5A* (G) is expressed in root hairs.
Fig. 7. The AtRPS5A and AtRPS5B genes are differentially expressed during embryogenesis. Siliques of 
pAtRPS5A::GUS (A-E) and pAtRPS5B::GUS (G-K) plants were stained for GUS activity for 2 and 8 hours, respectively. 
(A) In pAtRPS5A::GUS lines, GUS activity is already observed at the 2-cell stage in both embryo and endosperm. 
GUS activity remains strong through the octant (B) and globular stage (C), and decreases after the transition (D) and 
late torpedo stage (E). Activity is strongest in the peripheral cell layer (C-E). Expression of pAtRPS5B::GUS in embryos 
is weak until the globular stage (G,H), but increases during the late globular (I), heart (J) and late torpedo (K) stages. 
Note that expression is strongest in the inner cell layers (I-K), and is visible in the provascular tissue (K). 
(F) Schematic representation of the differential expression pattern of AtRPS5A (yellow) and AtRPS5B (blue). Places 
where both genes are expressed are marked with green. (i) 2-cell, (ii) octant, (iii) globular, (iv) transition, (v) heart and 
(vi) torpedo stage.

All three Arabidopsis RP genes for which mutants were previously described have at least two additional copies in the 
Arabidopsis genome (van Lijssebettens et al., 1994; Revenkova et al., 1999; Ito et al., 2000) and, at least for AtRPS18, there is 
a large overlap in the expression domain of the different gene copies (van Lijsebettens et al., 1994). Therefore, haplo-
sufficiency is not likely to occur in mutants of these genes.

We describe the first mutation in an Arabidopsis RP gene, AtRPS5A, which results in semi-dominant phenotypes. In 
contrast to what has been previously concluded, based on EST data analysis (Cooke et al., 1997), we show that AtRPS5A belongs to a gene family that comprises only two members in 
Arabidopsis. Detailed analysis of the spatiotemporal expression of both family members by mRNA in situ hybridisation and 
promitter::GUS fusions shows clearly that the two gene copies are differentially regulated. The processes affected in the amil 
mutant can only be seen in tissues/organs where AtRPS5A and AtRPS5B expression do not overlap. This closely resembles the situation in Drosophila Minute mutants, where for each RP only one functional gene is present in the genome.

Analogous to the situation in Drosophila, where mutations in the RPS5 gene result in a Minute phenotype (McKim et al., 
1996), our results indicate that the semi-dominant amil phenotypes are caused by haplo-insufficiency. In the complementation experiment we obtained a plant homozygous for the amil mutation, that harboured an extra wild-type gene copy. If the RPS5::GUS would have a dominant-negative effect, the complemented homozygous amil mutant (ratio wild-
type: mutant=1:2) would either be lethal, or at least have a more severe phenotype than the hemizygous mutant (ratio wild-
type: mutant=1:1). Instead, this plant looked phenotypically similar to the hemizygote (data not shown) and did not grow 
less vigorously, implying that the semi-dominance of the amil mutation is caused by haplo-insufficiency rather than by 
dominant-negative effects.

Differential expression of the RPS5 gene copies in Arabidopsis
To date the expression of only two plant gene families encoding RPs has been thoroughly analysed. RPL16 is 
represented by three gene copies in Arabidopsis, one of which shows strong expression in all proliferating tissues, while 
another is expressed in more specific cell types (Williams and Sussex, 1995). The AtRPS18 gene family comprises three 
members, two of which are strongly expressed in a similar pattern (van Lijsebettens et al., 1994). Interestingly, at least one 
copy of the AtRPS18 and AtRPL16 genes shares an almost identical gene expression pattern with AtRPS5A. For the 
AtRPS5 gene family, AtRPS5A can be considered as the major gene copy, which, like AtRPL16B and AtRPS18A (PFL1), is 
expressed in proliferating cells, i.e. in meristems, pericycle cells and embryos. A single transcription factor or a family of 
transcription factors with the same DNA-binding specificity could well regulate this common expression. For example, 
the co-ordinate expression of RP genes in yeast is thought to be mediated by RAP1 and ABF1, transcription factors 
that contain binding sites in the promoters of these genes (Mager and Planta, 1990). One candidate for such a motif in plants is the 
Tef1 box. This box was found in the promoter of AtRPS5A, while only a weak resemblance to the consensus sequence was 
found in AtRPS5B. The same motif is also present in the promoters of AtRPS18A (PFL1) (van Lijsebettens et al., 1994), 
AtRPL16A (Williams and Sussex, 1995) and AtRPL16B (D. W. and R. O., unpublished) and in a number of other promoters of 
genes that code for proteins that contribute to the translational apparatus (Regad et al., 1995). Regad et al. (Regad et al., 1995) 
have shown binding of proteins from nuclear extracts to the 
Tef1 box. However, to date no further studies have been published concerning the nature of such a Tef1 box-binding 
protein. Considering the exact stoichiometry of ribosome biosynthesis, it is very likely that specific plant transcription 
factors act coordinately to regulate the expression of at least one copy of each ribosomal protein gene family. Such 
transcription factors might act as master switches to increase-
or decrease the translational status of a cell. Considering the 
importance of this process in development, the identification and modulation of such master switches should provide tools 
for the modification of plant growth and stature. Similarly,
enhanced expression of the mitotic cyclin CYC1At in Arabidopsis has been shown to accelerate growth and increase biomass (Doerner et al., 1996).

The Arabidopsis Minute-like phenotype

The delay in development in Drosophila Minute mutants has been shown to be due to a cell-autonomous reduction of the rate of cell division (Morata and Ripoll, 1975). In our studies, amll phenotypes are associated with disturbed cell division rather than with reduced cell growth. One of the best examples is that the only part of the seedling that is not decreased in size in the amll mutant is the hypocotyl. Indeed, it has been shown that elongation growth of the hypocotyl is not dependent on cell division (Gendreau et al., 1997). By analogy, in mice it was shown that a functional knockout of the RPS6 gene affected cell division, but not cell expansion (Volarević et al., 2000).

In embryos that are homo- or heterozygous for the amll mutation cell division respectively stops or is delayed. Further, the semi-dominant post-embryonic amll phenotypes, combined with prominent expression of AtRPS5A in the root meristem, in cotyledon primordia and the vascular tissue of cotyledons and in floral organs, and the low levels or absence of AtRPS5B expression in these organs, indicate that the amll mutation affects cell division.

Expression of AtRPS5B is detected in the embryo, but it is significantly weaker than that of AtRPS5A. Apparently, the expression of AtRPS5B does not supply sufficient RPS5 protein to compensate for the presence of the mutant amll allele, even in the heterozygous state. Strikingly, the homozygous amll embryo still reaches the globular stage of development in the absence of proper RPS5 expression. It is possible that weak expression of AtRPS5B until the globular stage may partially rescue the loss of AtRPS5A expression. However, even the significantly stronger AtRPS5B expression during the globular stage is not able to rescue the amll embryo, making it more likely that the embryo develops until the globular stage because of maternal RPs that are deposited in the egg cell. This explanation is in accordance with the large number of Arabidopsis embryo mutants that arrest at the globular stage (Patton et al., 1991; Castle et al., 1993; Yadegari et al., 1994; Uwer et al., 1998; Albert et al., 1999), some of which are also disrupted in housekeeping genes. The analysis of RP accumulation in the gametophyte and embryo is needed to address this question. Preliminary data show that the AtRPS5B gene is strongly expressed in the egg cell before fertilization (D. W. and R. O., unpublished).

To our knowledge, this is the first report describing an Arabidopsis mutant with a semi-dominant delay in embryo development. In this respect, it will be interesting to determine the AtRPS5B mutant phenotype. Based on the differential expression pattern of the two AtRPS5 gene copies, we predict that a mutation in this AtRPS5B gene will lead to recessive phenotypes related to delayed cell growth and differentiation, including the differentiation of root hairs and trichomes.

Conclusion

The analysis of the Arabidopsis amll mutant reveals a striking similarity between plant and insect development in that both systems have an absolute need for a functional and efficient translational machinery. This again shows that basic cellular processes are extremely conserved in species that are evolutionarily distant, and that the research on plant cellular processes may provide essential information on the biology of other complex multicellular organisms. Our results show for the first time that expression of genes encoding components in the translational machinery can be rate limiting for plant development. The coordinate expression of such genes may be an interesting target to improve regeneration or plant stature in agronomically important crops.

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