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Silencing of ribosomal protein L3 genes in *N. tabacum* reveals coordinate expression and significant alterations in plant growth, development and ribosome biogenesis

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Summary

The expression of ribosomal protein genes is coordinately regulated in bacteria, yeast, and vertebrates, so that equimolar amounts of ribosomal proteins accumulate for assembly into ribosomes. To understand how expression of ribosomal protein genes is regulated in plants, we altered expression of the large subunit ribosomal protein L3 (*RPL3*) genes in *Nicotiana tabacum* using post-transcriptional gene silencing (PTGS). L3 is encoded by two genes, *RPL3A* and *RPL3B*, with 80.2% amino acid sequence identity in tobacco. Two types of 'hairpin' RNA (hpRNA) vectors carrying the *RPL3A* or *RPL3B* sequences in both sense and antisense orientation were generated in order to alter the expression level of both *RPL3* genes. Tobacco plants transformed with a vector containing a 5′-terminal fragment of *RPL3A* gene displayed decreased *RPL3A* mRNA levels and a marked increase in the abundance of *RPL3B* mRNA. These results indicated that expression of the *RPL3* genes is coordinately regulated in tobacco. The transgenic plants that contained higher levels of *RPL3B* mRNA exhibited leaf overgrowth and mottling. Epidermal cells of these plants were increased in number and decreased in size. The precursor rRNA (pre-rRNA) and the mature rRNAs accumulated in these plants, suggesting that ribosome biogenesis is upregulated. Tobacco plants transformed with an hpRNA vector harboring the full-length *RPL3B* cDNA exhibited efficient silencing of both *RPL3A* and *RPL3B* genes, reduced L3 levels, and an abnormal phenotype characterized by a delay in development, stunting, and inhibition of lateral root growth. L3 deficiency led to a reduction in cell number and an increase in cell size, suggesting that L3 positively regulates cell division. Decreasing *RPL3* gene expression resulted in a decrease in accumulation of the pre-rRNA, establishing a prominent role for L3 in ribosome biogenesis in plants.

Keywords: ribosomal protein L3, post-transcriptional silencing, autoregulation, ribosome biosynthesis, regulation of cell size and number, rRNA processing.

Introduction

Extensive studies in yeast and vertebrates established that synthesis of functional ribosomes requires the coordinated assembly of 70–80 different ribosomal proteins with four species of rRNA to yield mature 40S and 60S ribosomal subunits (reviewed by Nomura, 1999). Ribosome biogenesis requires accumulation of the rRNAs and ribosomal proteins in precise stoichiometric quantities. The transcriptional and post-transcriptional mechanisms that regulate ribosome production differ among eukaryotes. Translational regulation is largely responsible for the stoichiometric accumulation of ribosomal proteins in mammalian cells (Amaldi and Pierandrei-Amaldi, 1990; Meyuhas and Hornstein, 2000), as well as in bacteria (Nomura, 1999). In contrast, ribosomal protein synthesis is tightly controlled at the level of transcription in yeast (Planta, 1997; Warner, 1999). Ribosomal proteins accumulate to high levels during growth in rich medium when cells divide rapidly and remain at low levels in minimal medium, indicating that their production is coupled to growth rate in yeast (Warner, 1999). Besides regulating transcription, yeast maintains equal levels of ribosomal proteins by targeting excess ribosomal proteins that fail to assemble into ribosomes

for rapid degradation (Maicas *et al.*, 1988). For example, when *RPL3* mRNA was overproduced in yeast, it accumulated in the cells and was efficiently translated (Maicas *et al.*, 1988); however, the excess L3 was rapidly degraded (Pearson *et al.*, 1982). Several ribosomal proteins have been shown to have roles outside the ribosome and regulate their own synthesis. For example, free L30 (Dabeva and Warner, 1993; Vilardell and Warner, 1997) and S14 (Fewell and Woolford, 1999) act as feedback inhibitors of the splicing of their own mRNA. Very little is known about the mechanisms that control ribosomal protein gene expression in plants. Evidence that ribosomal protein gene expression may be controlled at the post-transcriptional level was reported for L13 in *Brassica napus* and *Arabidopsis* (Saez-Vasquez *et al.*, 2000), maize P2a (Fennoy *et al.*, 1998) and maize S6 (Sanchez-de-Jimenez *et al.*, 1997).

Mutations in ribosomal proteins, rRNA, or other factors involved in ribosome synthesis have drastic effects on growth, development, and cellular differentiation. Mutations in individual ribosomal genes in *Drosophila melanogaster* caused the *Minute* phenotype, characterized by delayed development, reduced size, and other morphological characteristics such as thin bristles, reduced fertility, and reduced body size (Saeboe-Larssen *et al.*, 1998). The *Minute* phenotype is attributed to a cell-autonomous reduction in the rate of cell division (Lambertsson, 1998). Disruption of ribosomal protein genes in plants has also been shown to interfere with normal plant growth and development. A T-DNA insertion in S18A, one of the three S18 genes in *Arabidopsis*, caused the pointed first leaf (*pfl1*) phenotype characterized by pointed first leaves, reduced FW, and growth retardation (Van Lijsebettens *et al.*, 1994). A similar phenotype, named *pfl2*, was displayed by *Arabidopsis* plants with a Dissociation (Ds) insertion in the *S13A* gene (Ito *et al.*, 2000), also a member of a three-gene family with multiple expressed members. Inactivation of the *S5A* gene, one of the two differentially expressed members of the S5 family in *Arabidopsis*, by T-DNA insertion resulted in delayed or disturbed cell division and growth retardation (Weijers *et al.*, 2001). A T-DNA insertion in *Arabidopsis S27A* gene resulted in increased sensitivity to a genotoxic stress treatment, suggesting a role for S27 in DNA repair mechanisms (Revenkova *et al.*, 1999).

Studies of ribosomal protein L3 (RPL3) in the yeast *Saccharomyces cerevisiae* have revealed important functions for this protein in translation, ribosome biogenesis, and virus resistance. L3 participates in the formation of the peptidyltransferase center (reviewed by Noller, 1993, 1997) and is one of the first ribosomal proteins that associate with the rRNA during ribosome assembly (Herold and Nierhaus, 1987; Kruiswijk *et al.*, 1978). Mutations in the *RPL3* gene were initially identified by conferring resistance to the peptidyltransferase inhibitors, trichodermin, and anisomycin (Fried and Warner, 1981; Jimenez *et al.*, 1975). *S. cerevisiae* carrying another *RPL3* allele, maintenance-of-killer (*mak*)*8-1*, had decreased levels of free 60S ribosomal subunits, accumulated half-mer polysomes (Ohtake and Wickner, 1995; Wickner *et al.*, 1982) and did not maintain the killer virus because of increased programmed −1 frameshifting efficiency (Peltz *et al.*, 1999).

To elucidate the molecular mechanisms that regulate ribosomal protein gene expression and ribosome assembly in plants, we characterized the *RPL3* genes in *Nicotiana tabacum* using post-transcriptional silencing. We present the first evidence that expression of the two different *RPL3* genes is coordinately regulated in plants. Modifications in *RPL3* mRNA levels had a major effect on plant growth and development and revealed a significant role for L3 in regulating ribosome biogenesis.

Results

Analysis of L3 levels during various developmental stages in wild-type (Wt) N. tabacum

Ribosomal protein L3 is encoded by a single gene in yeast (Fried and Warner, 1981) and by two different genes in higher eukaryotes, including *Arabidopsis* (Barakat *et al.*, 2001; Kim *et al.*, 1990) and rice (Nishi *et al.*, 1993). Full-length cDNAs encoding two different *RPL3* genes, *RPL3A* (Accession number AY395738) and *RPL3B* (Accession number AY395739) were isolated from an *N. tabacum* leaf cDNA library. The two cDNAs shared 48% identity in the 5′ untranslated regions (5′ UTRs), while the open-reading frames (ORFs) were 80.2% identical. Both genes contained a 1170-bp ORF, encoding a 369 amino acid protein with a predicted molecular mass of 40.6 kDa and 94% sequence identity. Alignment of tobacco L3 protein sequences with other eukaryotic L3 proteins showed that they are highly similar to the *Arabidopsis* (86% average identity) and rice (89% average identity) L3 proteins and the single L3 in *S. cerevisiae* (65% identity).

Steady-state levels of the *RPL3A* and *RPL3B* mRNAs were analyzed in *N. tabacum* by quantitative real-time PCR and RNase protection analysis. For the real-time PCR analysis, first-strand cDNA was amplified with *RPL3A* and *B*-specific primers shown in Figure 1(a). One pair of PCR primers annealed to regions within the divergent 5′ UTR and the 5′ end of the *RPL3* genes in a region that showed 80% identity and amplified a 234-nucleotide (nt) fragment of the *RPL3A* gene between positions −29 and +205 and a 282-nt fragment of the *RPL3B* gene between positions −30 and +252 (PCR product 1). Another pair of primers annealed internally, near the 3′ end of the *RPL3* genes that showed 71% identity and amplified the sequences between positions +742 and +808 in *RPL3A* and the sequences between positions +714 and +779 in *RPL3B* (PCR product 2). Control

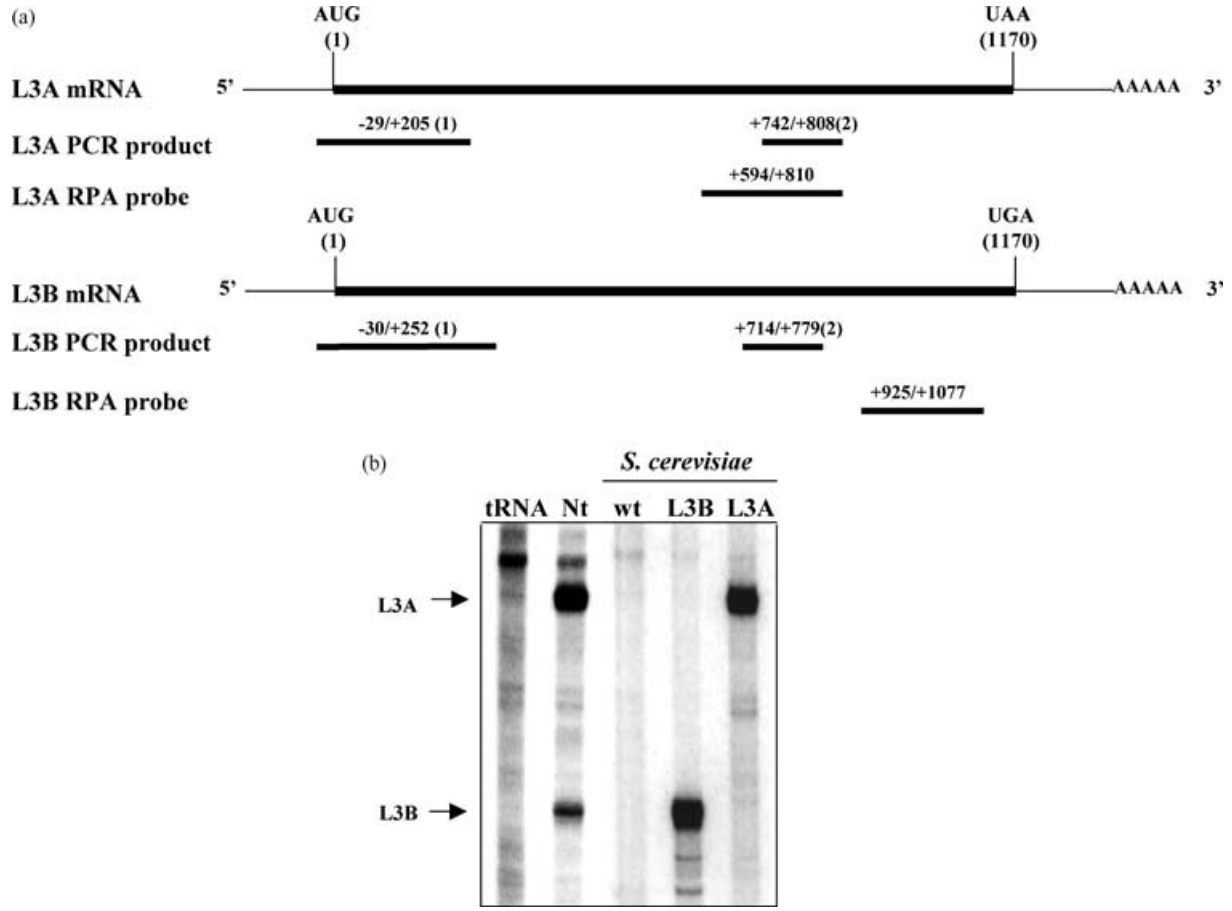

Figure 1. Primers and probes used for quantitative real-time PCR and RNase protection analysis.
(a) Schematic representation of *RPL3* mRNAs showing the position of the primers used in quantitative real-time PCR analysis. Primer set (1) annealed to sequences at positions −29 to +205 of *RPL3A* mRNA and −30 to +252 of *RPL3B* mRNA (PCR product 1). Primer set (2) annealed to sequences at positions +742 to +808 of *RPL3A* and at positions +714 to +779 of *RPL3B* mRNA (PCR product 2). RNase protection probes (RPA probe) were complementary to sequences at positions +594 to +810 of *RPL3A* mRNA and +925 to +1077 of *RPL3B* mRNA. The numbering was relative to the translation initiation site in both *RPL3* transcripts.
(b) RNase protection analysis of total RNA from Wt *N. tabacum* (Nt), untransformed yeast (Wt), and yeast harboring *RPL3A* or *RPL3B* cDNAs using L3A and L3B RPA probes shown in (a). tRNA represents RNase protection analysis using the yeast tRNA as a control.

amplifications demonstrated that that *RPL3A* and *RPL3B* primer pairs amplified only their cognate mRNA and no other non-specific amplification products were observed (data not shown).

To differentiate between the two mRNAs, RNase protection probes of different sizes complementary to divergent sequences near the 3′ end of each gene were synthesized. The RNase protection probes annealed and protected a 215-nt region between positions +594 and +810 of the *RPL3A* mRNA (L3A RPA probe) and a 152-nt region between positions +925 and +1077 of the *RPL3B* mRNA (L3B RPA probe). Probe specificity was verified after transforming yeast with *RPL3A* and *RPL3B* cDNAs and demonstrating that L3 riboprobes reacted only with their cognate mRNAs. As shown in Figure 1(b), *RPL3A* and *RPL3B* probes hybridized to their cognate mRNAs when both probes were used in the same reaction. Furthermore, we did not observe any RNase-protected fragments when total RNA from untransformed yeast (Wt) or when tRNA was used (tRNA).

RPL3 mRNA levels were quantified during different stages of growth in Wt tobacco by real-time PCR analysis. Tobacco tubulin A1 mRNA represented the internal control. As shown in Figure 2(a), in seedlings (S) and in young leaves of plants at the 4-leaf stage (4Y), both *RPL3A* and *RPL3B* mRNA levels were higher than those in young (Y) or mature (M) leaves of plants at later stages of development. An interesting observation was that *RPL3A* mRNA was present at approximately twofold higher levels than *RPL3B* mRNA throughout the plant development (Figure 2a). RNase protection analysis shown in Figure 2(b) verified the results of the real-time PCR analysis and demonstrated that *RPL3* mRNAs accumulated during active growth stages and decreased as the leaves matured. RNase protection analysis also confirmed that *RPL3A* and *RPL3B* mRNAs

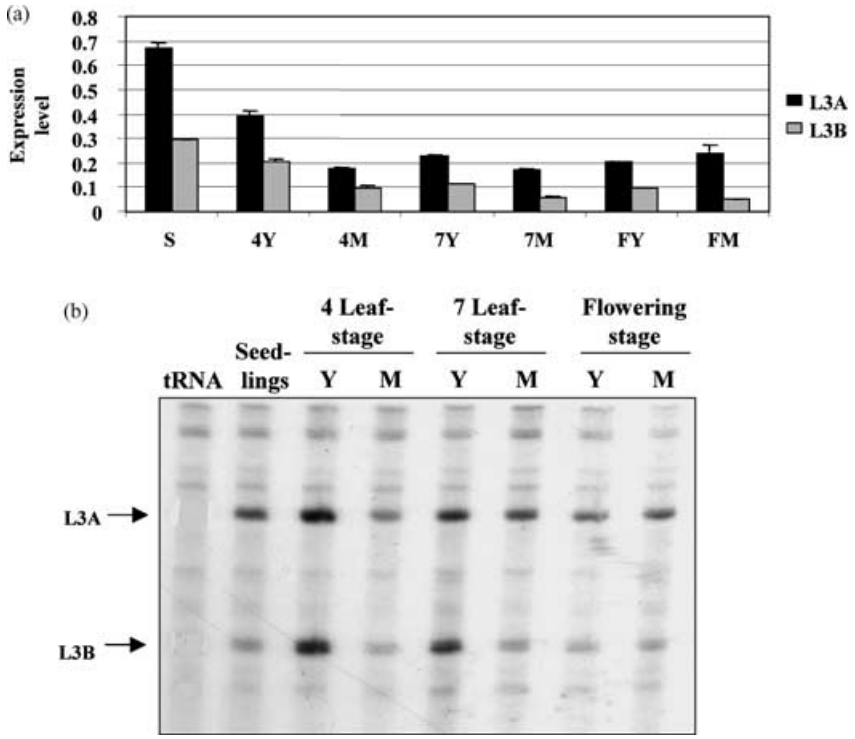

Figure 2. Analysis of *RPL3* mRNA levels in *N. tabacum* during various stages of development.
(a) Quantification of the *RPL3A* and *RPL3B* mRNA levels in 3-week-old tobacco seedlings (S), young or mature plants at 4-leaf stage (4Y and 4M), 7-leaf stage (7Y and 7M) and in flowering plants (FY and FM) by real-time PCR using primer set 2 (PCR product 2) shown in Figure 1(a).
(b) RNase protection analysis of total RNA extracted from 3-week-old seedlings (seedlings), young (Y), and mature (M) leaves of plants at 4-leaf stage, 7-leaf stage, and flowering stage. tRNA represents RNase protection analysis using the yeast tRNA as a control.

were expressed in tobacco leaves at an approximately 2 : 1 ratio.

Post-transcriptional silencing of RPL3 *genes reveals coordinate regulation of expression*

To investigate the functional role of the two different *RPL3* genes in *N. tabacum*, we sought to alter their expression by post-transcriptional gene silencing (PTGS) using vectors encoding self-complementary 'hairpin' RNAs (hpRNAs). The first 328-nt fragment of the *RPL3A* ORF (ΔL3A) was cloned in sense and antisense orientation separated by an intron from *A. thaliana omega-6 fatty acid desaturase* (*Fad*)*2* gene in NT698 (Figure 3a). The presence of a functional intron between the sense and antisense RNAs in the hpRNA-type vectors was previously shown to increase the efficiency of gene silencing in plants (Smith *et al.*, 2000; Wesley *et al.*, 2001).

The steady-state levels of *RPL3A* and *RPL3B* mRNAs were determined in four different transgenic lines using quantitative real-time PCR analysis. In each experiment, plants that were at the same developmental stage were used and the real-time PCR experiments were performed at least three times using two or three replicates per sample with comparable results. The *RPL3A* and *RPL3B* mRNA levels were normalized against the tobacco tubulin A1 mRNA, whose levels did not change and expressed relative to Wt plants. As shown in Figure 3(b), real-time PCR analysis of total RNA from plants at the vegetative stage using the 5′ (PCR product 1) primers revealed a decrease in the accumulation of *RPL3A* mRNA in lines 698-7 and 698-16 and a dramatic increase in the *RPL3B* mRNA levels in all transgenic lines compared with Wt plants. In lines 698-14 and 698-15 both *RPL3A* and *RPL3B* levels were increased relative to Wt plants.

These surprising results compelled us to analyze the *RPL3* mRNAs levels in the NT698 lines during a different stage in development, the reproductive growth. As shown in Figure 3(c), real-time PCR analysis of total RNA from plants at the reproductive stage using the 5′ primers revealed a greater decrease in the accumulation of *RPL3A* mRNA in lines 698-7, 698-14, and 698-16 (1.75–3.57-fold reduction compared with the Wt) than observed during the vegetative growth. The *RPL3B* mRNA levels were higher in all NT698 lines compared to Wt plants during the reproductive stage (Figure 3c).

To determine whether the increase in the *RPL3B* mRNA levels was because of accumulation of the full-length *RPL3B* mRNA, we performed additional real-time analysis using primers that annealed near the 3′ end of the *RPL3B* mRNA (Figure 1, PCR product 2). The results showed that all NT698 lines accumulated significantly higher levels of full-length *RPL3B* mRNA than the Wt plants (Figure 3d). Analysis of *RPL3A* mRNA levels using the 3′ primers showed that *RPL3A* mRNA levels decreased in transgenic lines during the reproductive growth (Figure 3d), as observed with the 5′-specific primers (Figure 3c).

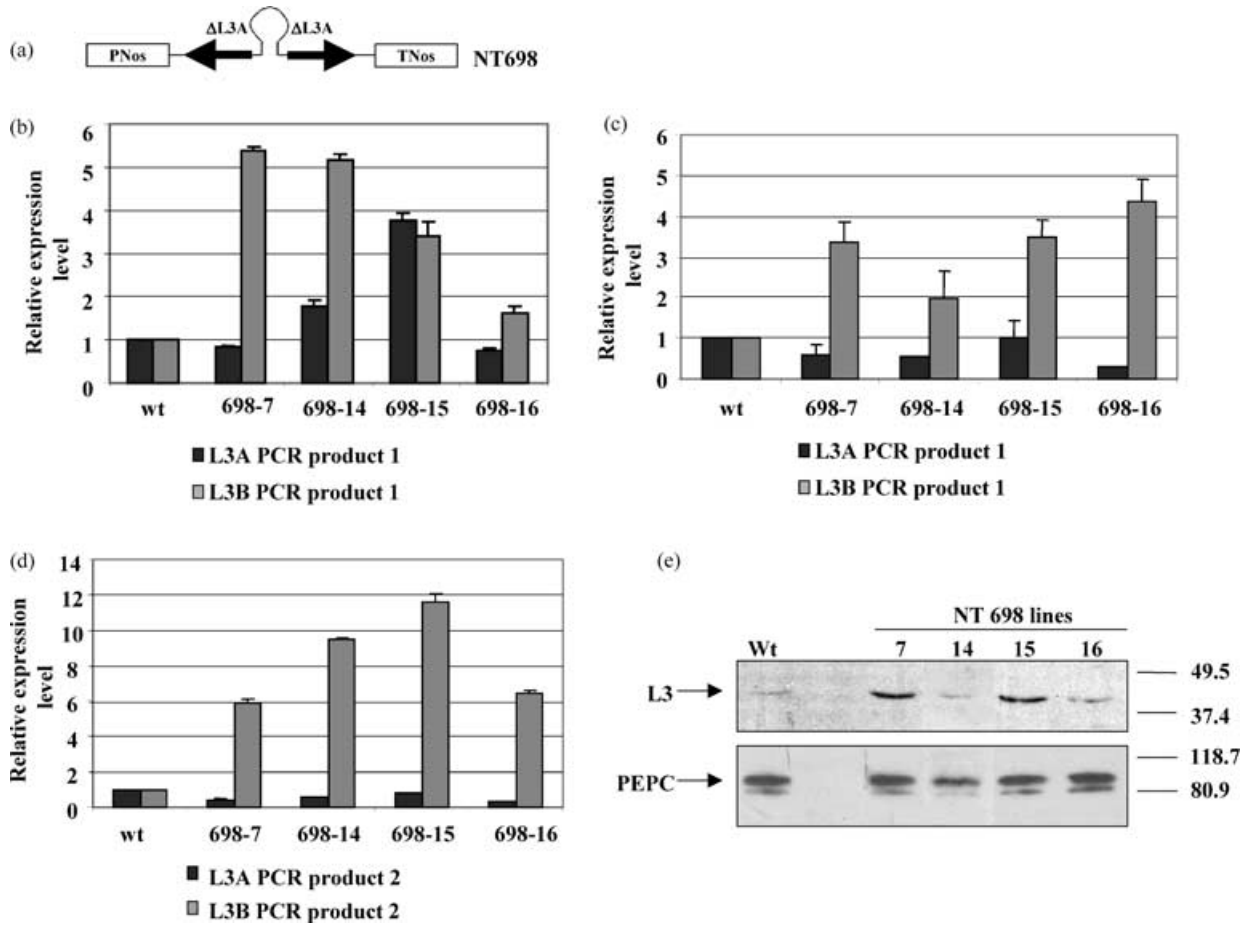

Figure 3. Steady-state levels of *RPL3A* and *RPL3B* mRNAs in transgenic lines containing NT698.
(a) Schematic representation of the transformation vector NT698. PNos represents the nopaline synthase (Nos) promoter, TNos is the Nos terminator, ΔL3A represents the 328-nt 5′-terminal fragment of the tobacco *RPL3A* gene.
(b) Real-time PCR analysis of the *RPL3A* and *RPL3B* mRNA levels in Wt and transgenic lines containing NT698 during vegetative growth using the primer set (1) shown in Figure 1(a).
(c) Real-time PCR analysis of the *RPL3A* and *RPL3B* mRNA levels in Wt and transgenic lines during reproductive growth using the primer set (1).
(d) Real-time PCR analysis of the *RPL3A* and *RPL3B* mRNA levels in Wt and transgenic lines during reproductive growth using the primer set (2). The *RPL3A* or *RPL3B* mRNA levels were normalized against tubulin A1 and expressed relative to Wt levels. The results represent the mean and the SD of three replicates for each plant.
(e) Immunoblot analysis of transgenic lines containing NT698 during vegetative growth. Total protein (10 μg) from Wt and transgenic tobacco lines (698-7, 698-14, 698-15, and 698-16) was analyzed by immunoblot analysis using polyclonal antiserum against tobacco L3B (1 : 2000). The blot was stripped and reprobed with polyclonal antibodies against maize PEP carboxylase (PEPC), 1 : 10 000. The positions of L3 and PEPC and the molecular weights of the markers are indicated (in kDa).

L3 protein levels were quantified in Wt and transgenic lines during vegetative growth using polyclonal antibodies raised against tobacco L3B (Figure 3e). A 44-kDa protein was detected, which most likely represents L3 encoded by both *RPL3A* and *L3B* genes, as the polyclonal antibodies used did not distinguish between the two different forms of L3. As observed in Figure 3(e), L3 protein levels were higher in 698-7 and 698-15 during vegetative growth compared to the Wt plants. Line 698-7 had the highest level of the *RPL3B* mRNA during vegetative growth (Figure 3b), while line 698-15 had higher levels of both *RPL3A* and *RPL3B* mRNAs during vegetative growth (Figure 3b).

To confirm the results of the real-time PCR analysis, we performed RNase protection analysis of total RNA extracted from transgenic lines and Wt plants at the vegetative stage (Figure 4a). The L3A and L3B RPA probes shown in Figure 1(a) were used and the signals were quantified using the PhosphorImager and normalized for equal loading (Figure 4b). A decrease relative to the Wt was detected in *RPL3A* mRNA levels in all lines with the exception of line 698-15, which, as previously observed by real-time PCR analysis (Figure 3b), accumulated higher levels of the *RPL3A* mRNA. As shown in Figure 4(b), the *RPL3B* mRNA levels were higher in all transgenic lines relative to Wt plants. These results provide evidence that expression of

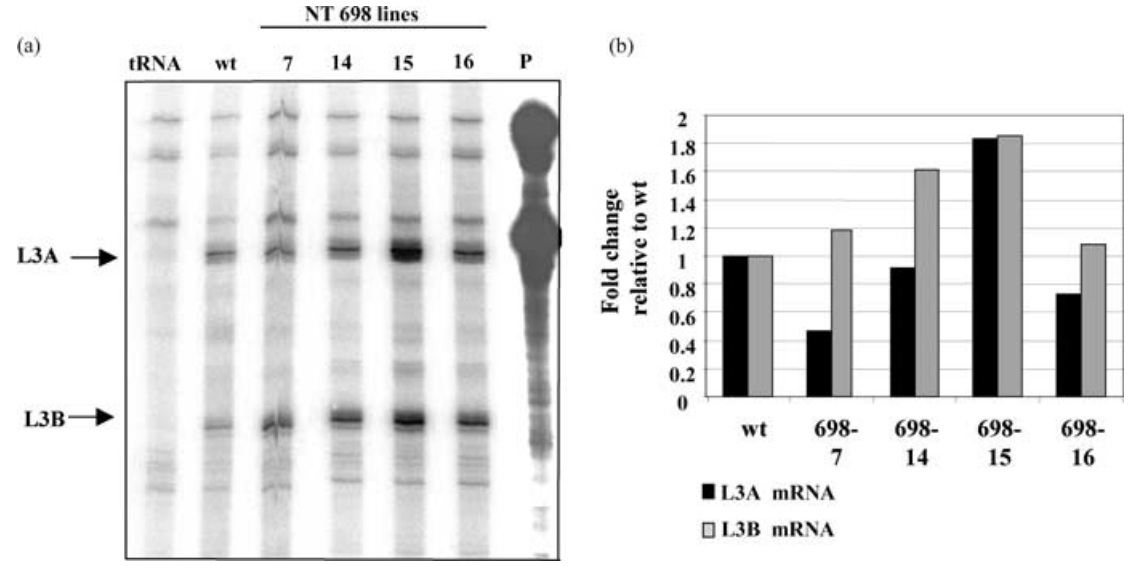

Figure 4. RNase protection analysis of transgenic plants containing NT698.
(a) Total RNA (15 μg) extracted from leaves of transgenic and Wt plants during vegetative growth was analyzed by RNase protection. Lane tRNA represents the negative control, containing 15 μg of yeast tRNA. The positions of the protected fragments corresponding to *RPL3A* and *RPL3B* are indicated with the arrows. Lane P corresponds to equal counts of the *RPL3A* and *RPL3B* probes before the RNase digestion.
(b) RNase protected fragments of *RPL3A* and *RPL3B* were quantified using the PhosphorImager and were plotted relative to the levels in Wt control plants.

the *RPL3A* and *RPL3B* genes is coordinately regulated in tobacco.

Silencing of RPL3B *mRNA leads to accumulation of truncated* RPL3B *RNAs*

Previous results indicated that a decrease in *RPL3A* mRNA level led to overaccumulation of the *RPL3B* mRNA. To determine if *RPL3B* mRNA can be silenced, we constructed another hpRNA vector, NT482, which contained the full-length *RPL3B* cDNA in both sense and antisense orientation, separated by a short (15 nt) DNA segment (Figure 5a). The *RPL3A* and *RPL3B* mRNA levels were examined by real-time PCR analysis in five independent transgenic tobacco lines containing NT482 and were normalized against tubulin A1 mRNA. As shown in Figure 5(b), real-time PCR analysis using both the 5′ and 3′ primers (PCR products 1 and 2) showed a decrease in the *RPL3A* mRNA accumulation in all lines containing NT482, compared to Wt tobacco plants. The extent of the decrease in *RPL3A* mRNA levels in the NT482 lines was greater in average than the decrease observed in the NT698 lines (e.g. 2–14.28-fold decrease as opposed to 1.75–3.57-fold). Analysis of the *RPL3B* mRNA levels in the NT482 lines using the 5′ primers (PCR product 1) showed a comparable decrease in the accumulation of *RPL3B* mRNA (Figure 5c). In contrast, *RPL3B* mRNA levels were 1.8–5.9-fold higher relative to Wt plants when the 3′ primers (PCR product 2) were used (Figure 5c). These results suggested that RNA fragments that contained only the 3′ part of the *RPL3B* mRNA, but not the 5′ part, accumulated in the transgenic lines.

To confirm these results, we carried out RNase protection analysis using total leaf RNA from lines containing NT482. The results shown in Figure 5(d) confirmed that *RPL3A* mRNA level decreased in all transgenic lines. RNase-protected fragments corresponding to the full-length *RPL3B* probe (152 nt), as well as fragments shorter than the full-length probe (indicated with arrowheads in Figure 5d), accumulated in all transgenic lines containing NT482, but not in Wt plants. Northern blot analysis of transgenic lines containing NT482 confirmed the RNase protection results and indicated increased abundance of RNA fragments that migrated close to the full-length *RPL3* mRNA (data not shown).

Analysis of L3 protein levels in transgenic lines containing NT482 indicated that L3 levels were reduced in lines 5, 11, and 18, consistent with the reduction in *RPL3A* and *RPL3B* mRNA levels in these lines (Figure 5e). A similar reduction was not detected in lines 9 and 12 possibly because of variation in L3 expression during development. These results demonstrate that tobacco plants transformed with the NT482 vector exhibit efficient silencing of both *RPL3A* and *RPL3B* genes. Silencing of the *RPL3B* gene involved accumulation of distal fragments corresponding to the 3′ part of the *RPL3B* mRNA.

Accumulation of siRNAs in transgenic plants during silencing of RPL3 *genes*

Post-transcriptional gene silencing is associated with the accumulation of low-molecular-weight RNAs that are similar in sequence to the co-suppressed gene (Hammond *et al.*, 2000; Zamore *et al.*, 2000). To confirm that the *RPL3A*

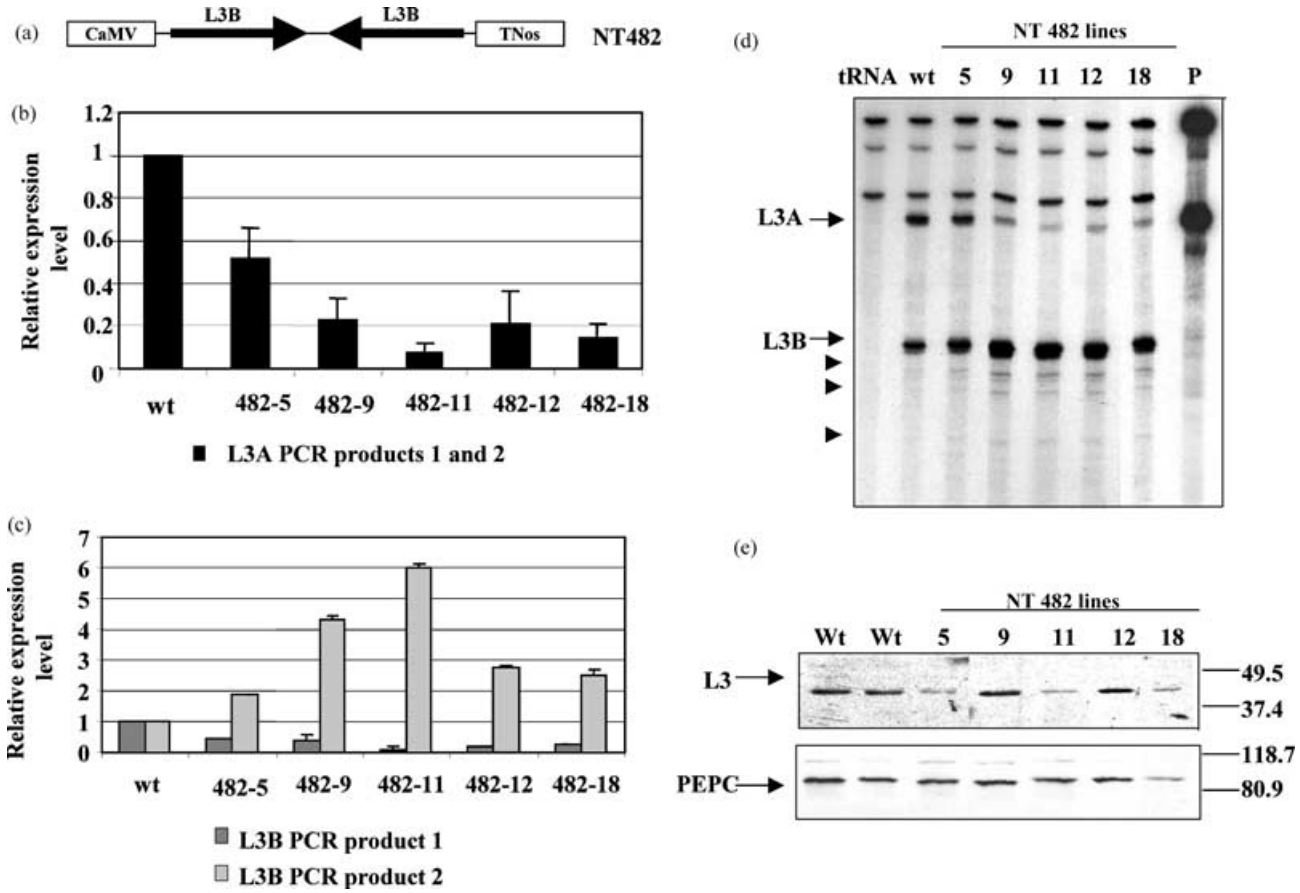

Figure 5. Seady-state levels of *RPL3A* and *RPL3B* mRNAs in transgenic lines, containing NT482.
(a) Schematic representation of the plant transformation vector NT482. CaMV represents the 35S promoter from the cauliflower mosaic virus, TNos is the Nos terminator, and L3B represents the entire coding region of the tobacco *RPL3B* gene.
(b) Real-time PCR analysis of the *RPL3A* mRNA levels in Wt and in transgenic tobacco lines containing NT482 during vegetative growth using primer sets (1) and (2) shown in Figure 1(a).
(c) Real-time PCR analysis of *RPL3B* mRNA levels in Wt and transgenic lines containing NT482 using primer sets (1) and (2). The *RPL3A* or *RPL3B* mRNA levels were normalized against tubulin A1 and expressed relative to Wt levels. The results represent the mean and SD of three replicates per plant.
(d) RNase protection analysis of lines containing NT482. Total RNA (15 μg) extracted from leaves was analyzed by RNase protection analysis and separated on a denaturing polyacrylamide gel. Lane tRNA represents RNase protection analysis using 15 μg of yeast tRNA. Lane P represents equal counts of the *RPL3A* and *RPL3B* probes before the RNase digestion. The arrows correspond to the positions of the protected fragments and the shorter RNA species.
(e) Immunoblot analysis of transgenic lines containing NT482. Total protein (10 μg) from Wt and transgenic tobacco lines NT482-5, NT482-9, NT482-11, NT482-12, and NT482-18 was analyzed by immunoblot analysis using polyclonal antiserum against tobacco L3 (1 : 2000). The blot was stripped and re-probed with polyclonal antibodies against maize PEPC, 1 : 10 000. The positions of L3 and PEPC and the molecular weights of the markers are indicated (in kDa).

and *RPL3B* mRNAs are silenced in NT482 lines as a consequence of PTGS, we attempted to detect such small interfering RNAs (siRNAs) using an *in vitro* transcribed RNA probe that corresponds to the sense (S) strand of the *RPL3B* gene. Previous Northern blot analysis indicated that this probe hybridizes to both *RPL3A* and *RPL3B* genes. As shown in Figure 6, siRNAs of approximately 25 nt in length were present in four out of the five NT482 lines analyzed. Lines 482-11 and 482-18, which had the lowest levels of *RPL3* mRNA and protein (Figure 5b,e) had higher levels of siRNAs. As a positive control for the hybridization, we analyzed synthetic oligonucleotides corresponding to the sense (S) and the antisense (A) strands of the *RPL3B* cDNA on the same gel. The sense *RPL3B* probe hybridized to the antisense strand oligonucleotide (Figure 6, lane A) and not to the sense strand oligonucleotide (lane S) or to the total Wt tobacco RNA. These results demonstrate that *RPL3*-specific siRNAs accumulate in transgenic lines containing NT482, indicating that the decrease in the level of *RPL3A* and *RPL3B* mRNAs in these lines is a consequence of PTGS. In contrast, we did not detect siRNAs using the same probe in the NT698 lines after numerous attempts.

Transgenic plants with altered levels of L3 mRNAs exhibit characteristic phenotypes

The reduction in *RPL3A* mRNA level and the increase in the *RPL3B* mRNA level in NT698 lines resulted in dramatic

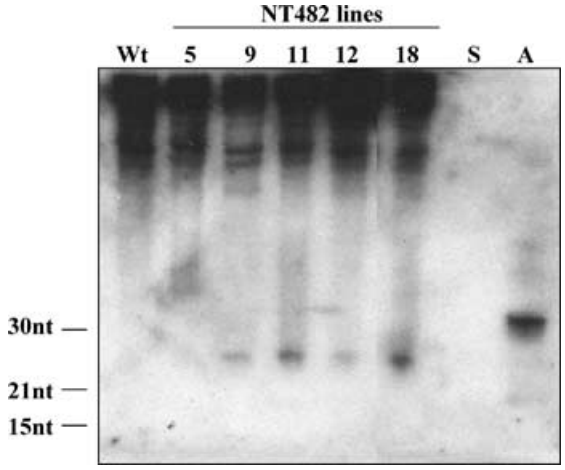

Figure 6. L3-specific small antisense RNAs accumulate in silenced NT482 tobacco.
Total leaf RNA (30 μg) from NT482 plants (R2 generation) was separated on a 15% polyacrylamide gel, blotted onto nitrocellulose membrane and hybridized with an *in vitro* transcribed ^{32}P-labeled RNA probe corresponding to the sense strand of the *RPL3B* gene. Lane Wt represents wild-type tobacco. Lanes S and A represent oligonucleotides homologous to the sense and antisense strands of the *RPL3B* cDNA, respectively. Low-molecular-weight size markers were produced by *in vitro* transcription of pBS-SK plasmid linearized with *Xho*I, *Kpn*I, or *Apa*I to generate 15-, 21-, and 30-nt RNA fragments.

phenotypic changes. Plants containing NT698 were stunted and had a shorter and thicker main shoot compared with Wt plants (Figure 7b). Their leaves increased in size during vegetative growth to an average area that is 1.5–2-fold larger than Wt leaves (Figure 7f). Furthermore, they showed mottling on their upper leaf surface (Figure 7d) compared to Wt plants (Figure 7e). However, during flowering plants containing NT698 showed a general inhibition in organ growth (Figure 7c). Their leaves were narrower and smaller in size than the leaves of Wt plants. The flowers were smaller and fewer than those in controls. They did not open fully and were shed before seeds were formed (Figure 7c). Line NT698-15, which showed a reduction in the *RPL3A* mRNA level only during the reproductive growth and an increase in the *RPL3B* mRNA level throughout development, exhibited leaf overgrowth, but not any of the growth inhibition characteristics observed in the other lines containing NT698 (Figure 7a). However, as shown in Figure 7(f), it had larger leaves during the vegetative growth stage.

Plants containing NT482, which had reduced levels of *RPL3A* and *RPL3B* mRNAs, exhibited an evident delay in development, characterized by slower elongation of the main shoot, marked delay in flowering and seed formation, and inhibition of lateral growth. The overall height of the NT482 lines was reduced by 1/3 or more compared to Wt controls 3 weeks after germination (Figure 8a–c). All NT482 lines showed a 2–4-week delay in flowering compared with Wt tobacco plants, had smaller and fewer flower organs, and consequently, reduced seed yield. The degree of growth inhibition was variable among the plants containing NT482 and correlated with the reduction in total *RPL3* mRNA levels. The most severe phenotype was observed in line 482-11, which contained the lowest amount of total *RPL3* mRNA and protein (Figure 5). In addition to the delay in shoot elongation and flowering, line 482-11 also showed morphological anomalies in leaf shape and size during reproductive growth and reached only 80% of Wt plant height (Figure 8b).

These observations indicate that a decrease in the expression level of *RPL3* resulted in slower growth and development. Conversely, an increase in the *RPL3B* expression had a stimulatory effect on plant organ growth during the vegetative stage. These results also demonstrate that various organs of the plant are affected differently by the variation in the accumulation of *RPL3* mRNAs and argue for a functional specialization of L3A and L3B in tobacco.

Cell size and number are affected in transgenic plants with altered L3 levels

The characteristic phenotypic changes observed in the transgenic plants with altered levels of *RPL3* mRNAs may be a consequence of alteration in cell growth or division. To address this, we examined the epidermal layer of Wt and transgenic plants using light microscopy. Epidermal peels from the 6th to 8th fully expanded leaves of 30-day-old plants containing NT698, NT482, and Wt plants were stained with 1% Toluidine Blue and cell size and number were analyzed (Figure 9a). In plants containing NT698, cell size decreased to approximately 20–50% of Wt plants (Figure 9b) and cell number increased by 15–45% relative to Wt plants (Figure 9c). These results indicate that the leaf hypertrophy observed during vegetative growth of plants containing NT698 results from an increased number of cells per organ. Line 698-14, which contained high levels of *RPL3B* mRNA and the most severe alterations in phenotype (Figure 7) showed the greatest decrease in cell size and increase in cell number.

Analysis of epidermal cells in plants containing NT482 revealed that cell size is doubled in lines 482-5, 482-9, and 482-18 and almost tripled in line 482-11, relative to Wt plants (Figure 9d). Epidermal cell analysis showed that transgenic lines containing NT482 had 40% fewer cells per unit area than Wt plants (Figure 9e). Line 482-11, which had the lowest levels of *RPL3* mRNA and protein, had the greatest increase in cell size and decrease in cell number. These results demonstrate that a reduction in the overall *RPL3* mRNA level in lines containing NT482 resulted in a decrease in cell number and an increase in cell size, suggesting that L3 positively regulates cell division.

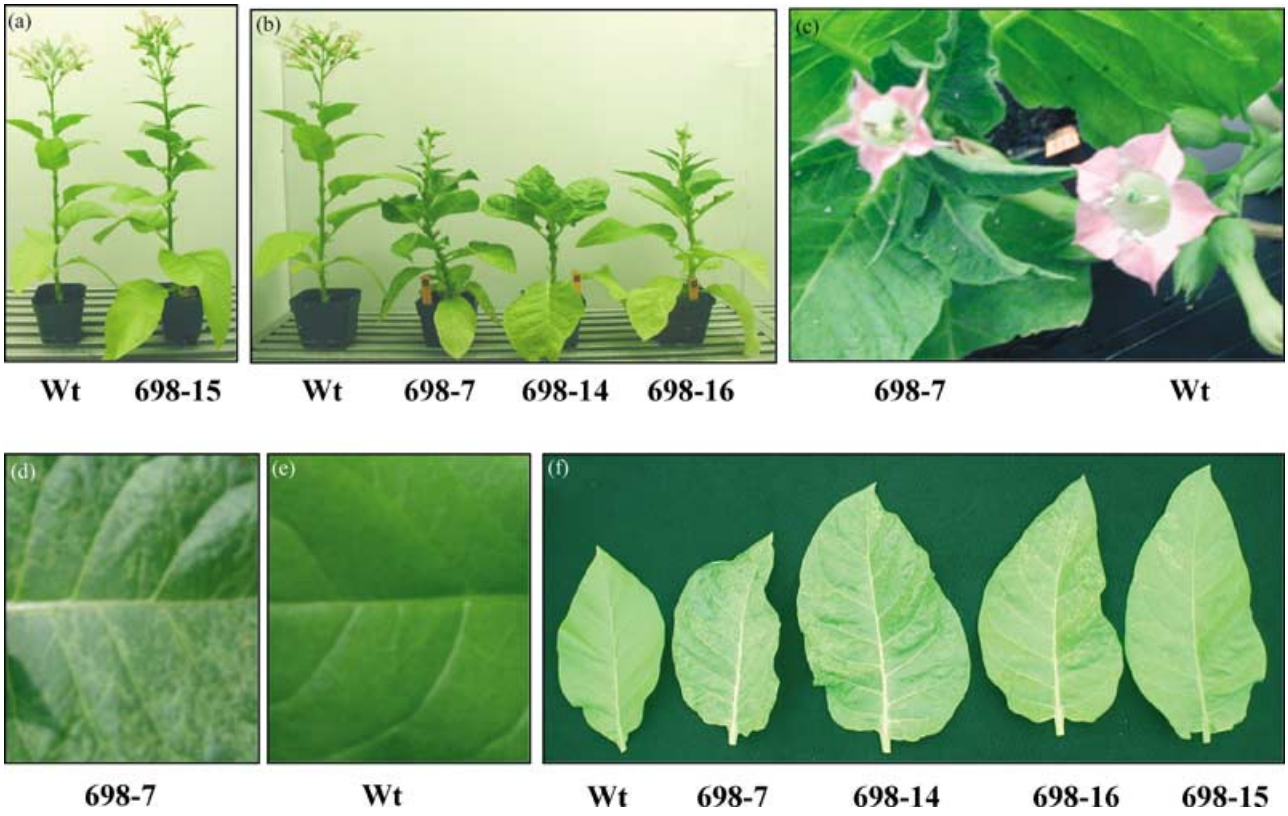

Figure 7. Effect of altered *RPL3* expression on growth and development of transgenic lines containing NT698.
(a) Comparison of a Wt (left) tobacco plant and a line 698-15 (right), which expresses high levels of *RPL3A* and *RPL3B* mRNA.
(b) Comparison between a Wt tobacco and three independent transgenic lines, 698-7, 698-14, and 698-16, expressing lower levels of *RPL3A* mRNA and higher levels of *RPL3B* mRNA. Note the stunted phenotype and the enlarged and mottled leaves.
(c) Comparison of a flower from line NT698-7 (left) and from a Wt plant (right).
(d) An example of the mottling observed on the upper leaf surface of 698 lines.
(e) Surface of Wt leaves.
(f) Comparison of the leaves of Wt and transgenic plants containing NT698 during vegetative growth.

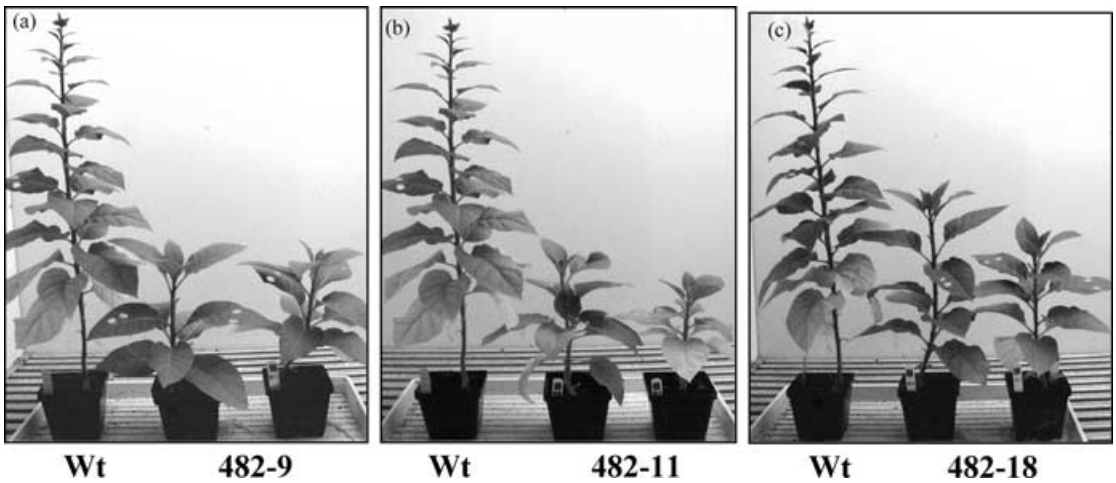

Figure 8. Effect of altered *RPL3* expression on growth and development of transgenic lines containing NT482.
The photographs were taken after 4 weeks of development and represent a comparison of a Wt plant and plants from transgenic lines: (a) 482-9, (b) 482-11, and (c) 482-18.

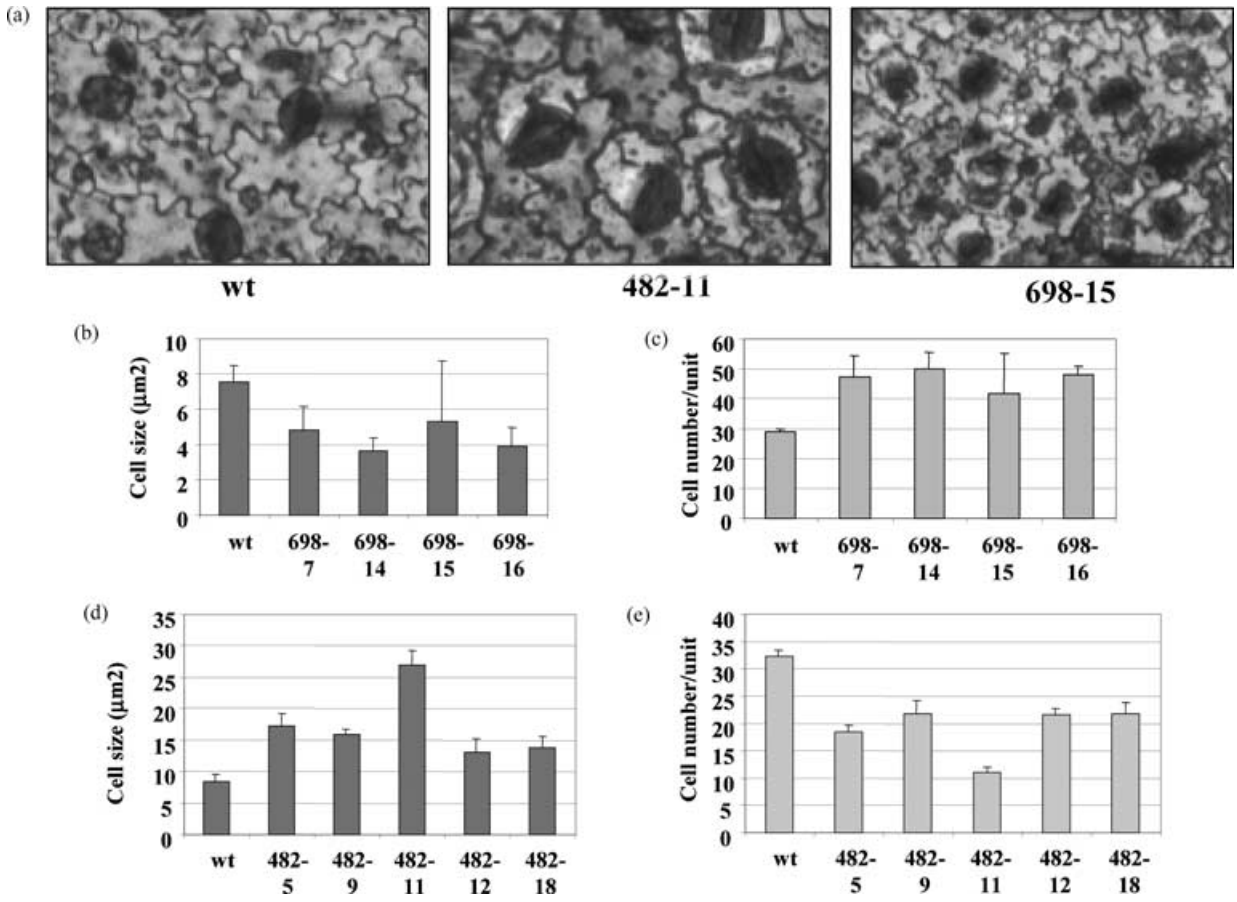

Figure 9. Alteration of *RPL3* expression affects cell size and number in *N. tabacum*.
(a) Comparison of epidermal cells from the abaxial distal portion of mature leaves from transgenic (482-11 and 698-15) and Wt plants at the same stage of development. Hand peels of epidermis were stained with Toluidine Blue 1% and viewed using the light microscope.
(b) Comparison of cell size between the Wt and transgenic plants containing NT698. The values represent the average size of 30 epidermal cells per plant line ($n = 30$). The vertical lines represent the SD. The SD for line 698-15 was larger than the other lines because of the high variability of the cell size and number in this line during the vegetative and the reproductive growth stage. Cell size was measured using ADOBE PHOTOSHOP. The average cell size and the SD were calculated using Microsoft Excel.
(c) Comparison of the number of cells per area in control and transgenic plants containing NT698. The plotted values represent the average of three separate cell counts per area (200 μm^2) per plant. The vertical lines represent the SD.
(d) Comparison of cell size between the Wt and transgenic plants containing NT482. The values represent the average area of at least 30 epidermal cells per plant line ($n = 30$).
(e) Comparison of the number of cells per area in control and transgenic plants containing NT482. The plotted values represent the average of three separate cell counts per area (200 μm^2) per plant. The cell size and number of the transgenic plants were significantly different from those of Wt plants ($P < 0.01$).

Precursor rRNA (pre-rRNA) overaccumulates in the plants with altered RPL3 *mRNA levels*

As L3 is one of the first proteins that associate with the rRNA during ribosome assembly in yeast and *Escherichia coli* (Herold and Nierhaus, 1987; Kruiswijk *et al.*, 1978), we examined the level of pre-rRNA in transgenic tobacco plants with altered levels of *RPL3* mRNAs using real-time PCR analysis. The 5140-bp tobacco intergenic spacer (IGS), which is located between regions coding for the 18S and 28S rRNA, has been divided into seven regions based on its sequence structure (Borisjuk *et al.*, 1997). The IGS sequence is processed during maturation of the rRNAs (Brown and Shaw, 1998). For the real-time PCR amplification, specific primers were designed for the region VII of the tobacco IGS, located upstream of the mature 18S rRNA sequence. These primers were specific for the extragenic spacer region and detected only the 35S rRNA and not the mature 28S or the 18S rRNAs (data not shown). Random oligonucleotides were used to prime cDNA synthesis using the total RNA preparations that were used to examine the levels of *RPL3* transcripts in NT482 and NT698 lines (Figures 3 and 5). As controls, total RNA from Wt plants at the same developmental stage was used. As shown in Figure 10(a), pre-rRNA accumulated at levels slightly lower than those in Wt plants (Wt-1) in transgenic lines containing 482, which had lower levels of both *RPL3* mRNAs. In contrast, the level of the pre-rRNA in NT698 lines was

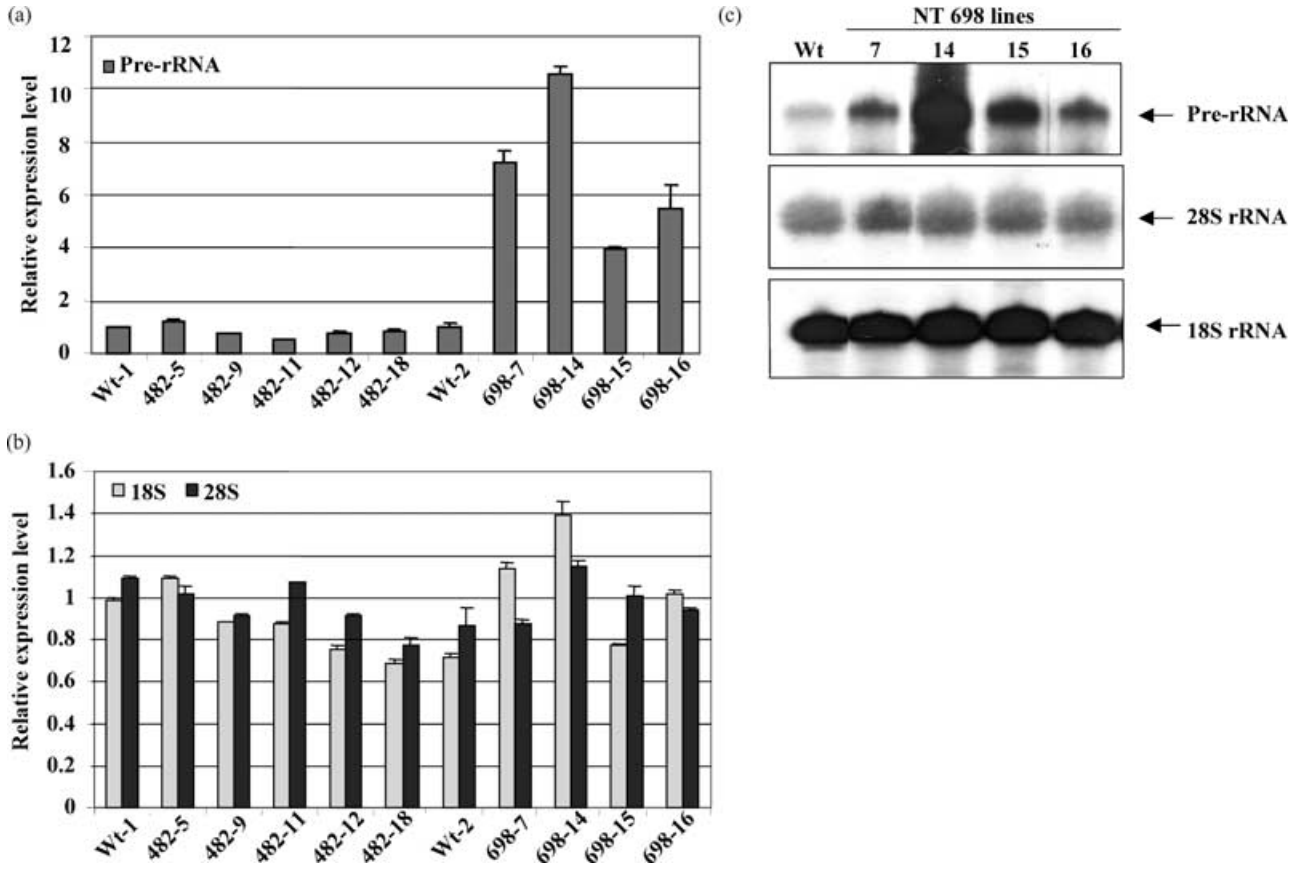

Figure 10. Steady-state levels of precursor and mature rRNAs in tobacco containing NT482 and NT698.
(a) Real-time PCR analysis of pre-rRNA levels in NT482 and NT698 lines. Random-primed cDNA synthesized from 5 μg of total RNA was amplified by real-time PCR using primers specific for the region VII of the *N. tabacum* IGS. The pre-rRNA levels were normalized against 28S rRNA and expressed relative to Wt levels. The results represent the mean and SD of three replicates per plant.
(b) Mature rRNA levels in NT482 and NT698 lines. Random-primed cDNA was amplified by real-time PCR using primers specific for *N. tabacum* 18S and 28S rRNA. Results are the mean of three replicates.
(c) Northern blot analysis of total RNA (10 μg) from Wt and NT698 lines using oligonucleotides specific for the *S. cerevisiae* 5′-external transcribed spacer (pre-rRNA) and for the mature rRNA sequences (28S rRNA and 18S rRNA). The blot was probed with the pre-RNA probe first and stripped and re-probed with the 28S, followed by the 18S rRNA probes.

3.98–19.5-fold higher than that in the control plants. Accumulation of the pre-rRNA in NT698 lines correlated with the increase in the total *RPL3B* mRNA levels. Transgenic line 698-14, which had the greatest decrease in cell size and increase in cell number (Figure 9b,c), accumulated the highest levels of the pre-rRNA during the vegetative growth (Figure 10a).

To determine whether the increase in the pre-RNA level in the transgenic plants affected the steady-state levels of the mature rRNA, we quantified the levels of 28S and 18S rRNA using real-time PCR analysis. As shown in Figure 10(b), 18S and 28S rRNAs accumulated approximately at control levels in 482 lines. Among the NT698 lines, line 698-14 accumulated 1.6 times more 18S rRNA and 1.4 times more 28S rRNA than Wt plants (Figure 10b).

To confirm these results, we investigated the levels of the pre-rRNA and the mature rRNAs using Northern blot analysis. Oligonucleotides specific to the *S. cerevisiae* 5′ external transcribed spacer (ETS) of the 35S pre-rRNA and to sequences within the mature 28S and 18S rRNAs were used as probes. As shown in Figure 10(c), pre-rRNA accumulated to higher levels in all transgenic lines containing NT698, line 698-14 showing the highest accumulation. In contrast, no significant increase was detected by Northern blot analysis in the levels of the 28S and 18S rRNAs compared to the control plants. Because of the overall abundance of the mature 28S and the 18S rRNAs, small differences in their steady-state levels might not have been detectable by Northern blot analysis. These results demonstrate that alterations in the level of *RPL3* mRNAs interfere with the accumulation of pre-rRNA in transgenic plants.

Discussion

To determine how expression of the two different *RPL3* genes is regulated in *N. tabacum*, we have used hpRNA

constructs to alter their expression. Our results provide evidence that expression of *RPL3A* and *RPL3B* genes is coordinately regulated in tobacco. The NT698 construct, carrying a hpRNA containing the ΔL3A fragment decreased the accumulation of the *RPL3A* mRNA and increased the accumulation of the *RPL3B* mRNA. The observation that a reduction in *RPL3A* mRNA level led to an increase in *RPL3B* mRNA level suggested that L3 might have a negative regulatory effect on its own expression. A decrease in L3 protein caused by a reduction in the accumulation of *RPL3A* mRNA may have resulted in the increased accumulation of the *RPL3B* mRNA. A similar situation was described for the *S. cerevisiae* genes encoding ribosomal proteins, S14A and S14B. A deficit of S14 protein, caused by the deletion or inactivation of *S14A*, resulted in a 10-fold derepression of *S14B* in yeast (Li *et al.*, 1995). S14 repressed S14B expression by binding to a stem-loop structure at the 5′ end of its mRNA (Fewell and Woolford, 1999). Autogenous regulation of ribosomal protein mRNA accumulation at the post-transcriptional level was also described for the human homologs of S14 (Tasheva and Roufa, 1995), *Xenopus laevis*, and yeast homologs of L34 (previously known as L2; Bozzoni *et al.*, 1984; Presutti *et al.*, 1991) and the yeast L30 (previously known as L32; Dabeva and Warner, 1993; Vilardell and Warner, 1997). An alternative possibility is that if ΔL3 is translated in transgenic plants containing NT698, it may have exerted a dominant negative effect on *RPL3* gene expression. Expression of ΔL3 in yeast had a *trans*-dominant effect on programmed −1 ribosomal frameshifting and killer virus maintenance (Peltz *et al.*, 1999).

The NT482 construct, which contained the full-length *RPL3B* cDNA in sense and antisense orientation, led to a decrease in the accumulation mRNA from both *RPL3* genes. The decrease observed in the mRNA levels of *RPL3* genes was consistent with PTGS, as shown by the accumulation of siRNAs homologous to *RPL3* sequences. Silencing of the *RPL3B* mRNA did not increase the *RPL3A* mRNA levels, indicating that either the regulation operates only in one direction or the silencing mechanism triggered in plants containing NT482 resulted in a more efficient degradation of the endogenous L3 mRNAs than in plants containing NT698.

An interesting observation was the differential accumulation of the distal fragments of the *RPL3B* mRNA, during silencing in transgenic plants containing NT482. Accumulation of RNA products that were truncated at their 5′ end suggested an endonucleolytic cleavage of terminated transcripts, rather than premature termination of transcription and degradation of the message in a 5′ to 3′ direction. Further analysis of these transcripts indicated that they contained sequences corresponding to the 3′ UTR (+1165/+1247) (data not shown). These results argue against degradation of the *RPL3B* mRNA in a 3′ to 5′ direction. Accumulation of truncated RNAs were previously described as the initial products of L1 pre-mRNA turnover in *X. laevis* (Caffarelli *et al.*, 1987) and during post-transcriptional regulation of L2 mRNA in yeast (Presutti *et al.*, 1991), suggesting that a mechanism similar to PTGS might occur during turnover of ribosomal protein mRNAs in plants. In several examples of PTGS in plants, RNAs corresponding to the proximal or distal parts of the mature mRNA have been identified (Litiere *et al.*, 1999; Metzlaff *et al.*, 1997; Tanzer *et al.*, 1997; Van Eldik *et al.*, 1998). The *RPL3B* RNA fragments that accumulate in plants containing NT482 may be the result of a nucleolytic cleavage associated with PTGS and may be relatively stable because of either binding of L3 or formation of complex RNA secondary structures.

Immunoblot analysis revealed that L3 accumulated at lower levels in several transgenic lines containing NT482 and at higher levels in several transgenic lines containing NT698. However, there was variation in L3 levels among the transgenic lines analyzed, and we did not observe a correlation between *RPL3* mRNA and protein levels in every transgenic line. This may be because of variation in expression during different stages of plant development (Figure 2) or because of the existence of a post-transcriptional mechanism to regulate the balanced accumulation of L3 protein in rapport with the level of other ribosomal proteins and rRNAs. A lack of correlation between the *RPL3* mRNA and protein levels was previously observed in yeast. When present in excess, the mRNAs for ribosomal proteins L3 and L29 were translated less efficiently so that their synthesis did not exceed the synthesis of other ribosomal proteins (Pearson *et al.*, 1982). Furthermore, terminating the synthesis of L3 and L29 in yeast resulted in lack of accumulation of other large subunit ribosomal proteins, suggesting that synthesis of L3 and L29 is regulated post-transcriptionally as a function of the extent they are assembled (Nam and Fried, 1986).

Analysis of the L3 mRNA levels in Wt tobacco plants indicated that *RPL3* mRNA levels are high in young leaves during active growth and lower in mature leaves and during flowering. Tobacco lines containing NT482, which had reduced level of total *RPL3* mRNA, exhibited a developmental delay, a decrease in the cell number and a significant increase in the epidermal cell size compared to Wt plants. Conversely, the NT698 lines that overaccumulated *RPL3B* mRNA exhibited reduced plant size, leaf overgrowth, overproliferation of the leaf epidermal cells, a marked decrease in the cell size, and alterations in the cell shape. Previous studies have shown that in organs with determinate growth, such as leaves, control of cell proliferation is related to the control of cell size. Plants can adjust cell size in order to compensate for alterations in cell proliferation (reviewed by Tsukaya, 2003). Therefore, the observed variation in cell size in transgenic tobacco plants with altered level of *RPL3* mRNA may be a consequence of a

compensatory mechanism to maintain an optimal organ size.

The severity of the phenotypes correlated with the abundance of *RPL3* mRNA in transgenic plants. Line NT482-11 accumulated the lowest levels of *RPL3* mRNA and showed the greatest increase in the cell size and the decrease in the cell number. Lines NT698-7 and NT698-14, which showed a similar increase in the level of the *RPL3B* mRNA during vegetative growth (Figure 3b), exhibited a similar reduction in cell size and an increase in cell number (Figure 9). However, the magnitude of the effect on the final leaf size was greater in line NT698-14, which exhibited higher levels of *RPL3A* mRNA during the vegetative stage than in line NT698-7 (Figure 7), suggesting the existence of additional levels of leaf size control. Line NT698-14, which exhibited the largest leaf size among the NT698 lines, accumulated the highest levels of the precursor and mature rRNA species (Figure 10).

RPL3 is one of the five essential proteins for peptidyltransferase activity of the large ribosomal subunit (Schulze and Nierhaus, 1982). Recently, it has been shown that yeast ribosomes containing the mutant forms of L3 (*tcm1*, *mak8-1*) have decreased peptidyltransferase activity (Meskauskas *et al.*, 2003). The reduction in the peptidyltransferase activity in transgenic plants with altered *RPL3* mRNA level may be responsible for the reduction in growth. However, a reduction in peptidyltransferase activity does not lead to significant growth phenotypes in yeast (Meskauskas *et al.*, 2003). An alternative mechanism through which L3 might affect growth is by impairing ribosome biogenesis and the protein synthetic activity of the plant. Ribosome assembly in yeast occurs in the nucleolus, where an ordered assembly of ribosomal proteins begins on a 35S pre-rRNA, leading to the formation of a 90S pre-ribosomal particle (Venema and Tollervey, 1999). In plants, rRNA-processing pathway is similar to that of other higher eukaryotes, and several processing sites showing conservation with the yeast counterparts have been identified in maize and wheat (Brown and Shaw, 1998; Vincentz and Flavell, 1989).

To determine if altered levels of *RPL3* mRNA affect the abundance of the pre-rRNA in plants, we measured the steady-state levels of the 35S pre-RNA using primers specific for the IGS region of tobacco, which detect only the 35S pre-rRNA and not the mature 28S or the 18S rRNAs (Borisjuk *et al.*, 1997). Analysis of transgenic tobacco containing NT698 showed a 3.98–10.5-fold increase in the level of the pre-rRNA compared to Wt plants. This elevation may result from stimulation of the rDNA transcription, leading to a higher proliferation rate in these lines. Alternatively, the pre-rRNA accumulation in NT698 lines might be a direct consequence of impaired processing at cleavage sites within the pre-rRNA. Depletion or mutation of *L16* (Moritz *et al.*, 1990), *L1* (Deshmukh *et al.*, 1993), *L32* (Vilardell and Warner, 1997), or *L25* (van Beekvelt *et al.*, 2001) in yeast resulted in accumulation of the 35S pre-rRNA through a delay at the early cleavage sites and resulted in a decrease in the accumulation of the 60S subunit. In contrast, NT698 lines that contained high levels of the pre-rRNA, accumulated higher levels of the mature rRNAs, suggesting that ribosome assembly is upregulated in these plants. These results provide a link between ribosome biogenesis and control of cell division and suggest that ribosome biogenesis is associated with increased proliferative activity in plants (Mager, 1988; Tushinski and Warner, 1982).

While disruption of several other ribosomal protein genes in plants have been shown to interfere with growth and development, effects of enhanced expression of a single ribosomal protein on growth, development or ribosome biogenesis have not been reported. Our preliminary results indicate that mRNA levels of *RPS6* and *RPL2* are also increased in transgenic lines 698-14 and 698-15, which contain higher levels of the *RPL3B* mRNA and are decreased in lines 482-11 and 482-18, which contain lower levels of the *RPL3A* and *RPL3B* mRNAs (data not shown).

These results suggest that enhanced expression of L3 can lead to increased ribosome biogenesis involving more than 70 other ribosomal proteins and four rRNA species. While it is generally accepted that a stoichiometric balance of ribosomal proteins is important for ribosome assembly and function, studies with *Drosophila Minutes* have shown that each ribosomal protein has a threshold requirement and a level of expression that may differ between cell types (Cramton and Laski, 1994; Lambertsson, 1998). Thus, if expression of a particular ribosomal protein is close to the threshold level in a particular cell type, a reduction in its expression may produce phenotypes that are cell-type specific and different from those produced by a mutation in another ribosomal protein gene (Cramton and Laski, 1994; Lambertsson, 1998). For example, apoptosis was induced by suppressing *RPS3a* expression, while constitutive expression of *RPL7* mediated cell cycle arrest and apoptosis in animal cells (Naora and Naora, 1999). Furthermore, studies in yeast suggested that *RPL3* expression is uncoupled from the coordinated expression of other ribosomal protein genes. A tryptophan aspartic acid (WD)-repeat-containing protein, termed regulator-of-ribosome-biogenesis 1 protein (Rbp1p) that plays a role in the assembly of the 60S ribosomal subunit and ribosomal protein expression, binds to free L3 and regulates its expression, uncoupling it from the coordinated expression of other ribosomal protein genes (Iouk *et al.*, 2001). As L3 is among the first proteins to assemble with the rRNA, its synthesis may be more stringently regulated at the post-transcriptional and translational levels because controlling ribosome assembly at an early step will prevent unnecessary accumulation of ribosomal proteins or rRNAs. Further studies will address if increasing expression of other ribosomal protein genes will have similar effects on ribosome biogenesis and the

precise role of L3 in coordination of ribosome assembly in plants.

Experimental procedures

Plant transformation and vector construction

Ribosomal protein L3A (*RPL3A*) and *L3B* cDNAs were isolated from a tobacco cDNA library and subcloned in the pBS-SK vector. *RPL3B* cDNA was released as a *Bam*HI–*Xho*I fragment from the pBS-SK plasmid. NT482 was generated by triple ligation of two *Bam*HI–*Xho*I fragments into the *Bam*HI site of the pEL103 binary vector (gift from E. Lam, Rutgers University, New Brunswick). The 328-nt region of *L3A* cDNA (ΔL3A) was PCR-amplified using specific primers containing *Eco*RI–*Xho*I and *Bam*HI–*Xba*I sites and subcloned into the respective sites of the pKannibal (gift from P. Waterhouse, CSIRO Plant Industry Canberra, Australia). NT698 was generated by subcloning *ΔL3A* sequence in sense orientation as an *Eco*RI–*Xho*I fragment, and in reverse orientation as a *Bam*HI–*Xba*I fragment into pKannibal and then subcloning it as a *Not*I fragment into the *Not*I site of the pART27 binary transformation vector (gift from P. Waterhouse, CSIRO Plant Industry Canberra). *N. tabacum* cv. Samsun NN was transformed by *Agrobacterium*-mediated transformation.

Real-time PCR analysis

Total RNA was extracted from plant tissue using TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. cDNA was synthesized from 5 μg of total RNA in a 20-μl reaction, containing 1× first-strand buffer (Invitrogen), 40 U μl^{-1} RNA Guard RNase inhibitor (Promega, Madison, WI, USA), 0.5 μg poly d(T) oligonucleotide or 50 ng random primers (Promega), 40 mM dNTPs and Superscript II (Invitrogen) reverse transcriptase. Quantification of transcript levels by real-time PCR analysis was performed either using a Bio-Rad iCycler iQ Multi-Color Real-Time PCR Detection System or an ABI Prism 7000 Sequence Detection System using the manufacturers' protocols. The following protocol was used for the real-time PCR amplification using the Bio-Rad Cycler: step one – 3 min at 95°C for one cycle; step two – 1 min at 95°C, 30 sec at 60°C (or 62°C for tubulin), 30 sec at 72°C, repeated 40 times; step three – 1 min at 95°C, followed by melting curve analysis from 55 to 95°C. The following protocol was used for the ABI Prism 7000 Sequence Detection System: step one – 10 min at 95°C; step two, repeated 40 times – 15 sec at 95°C and 1 min at 60°C. Primers used for the quantitative real-time PCR are as follows: tobacco L3A forward: 5′-CCGTCTATTGCAGATAGGAG-3′ and L3A reverse: 5′-GTGGAGTTTTGATCCAGGT-3′; L3A795F: 5′-GGTCTGCGTAAGGTTGCTTGT-3′ and L3A861R: 5′-CACGGGCAACTGTGTAGGAA-3′; tobacco L3B forward: 5′-AGCTACGAGCTATTACAGG-3′ and L3B reverse: 5′-CATAGGAGGCGTTTCAATTAT-3′; L3B842F: 5′-AACCCGTCTCCCACGTAAGA-3′ and L3B907R: 5′-GGATGCCAAGCACCAATACA-3′; tobacco rRNA intergenic region IGS-VII, forward: 5′-TTGTTCGGAGTCGGTTT-3′ and reverse: 5′-GGCAGATAGGAGCCAAT-3′; tobacco tubulin A1, forward 5′-TAAACGCCGATCCTTTGAC-3′ and reverse, 5′-GTTATTGGCCGCATCT-3′; tobacco 28S rRNA, forward: 5′-CCTAGTACGAGAGGAACCGTTGA-3′ and reverse: 5′-GCGCCACTGGCTTTTCA-3′; tobacco 18S rRNA, forward: 5′-CGTCCCTGCCCTGCCCTTTGTACA-3′ and reverse: 5′-AACATTTCACCGGATCATTCAT-3′. Computations were performed using the Bio-Rad sequence detection software in combination with Microsoft Excel. Levels of unknowns were obtained by interpolating their starting quantity values on a standard curve obtained with dilutions of the *in vitro* transcribed plant cDNA. Transcript levels were normalized against tobacco tubulin A1 RNA as an internal control.

RNase protection analysis

To generate the RNase protection probes, a 266-nt fragment of the *RPL3A* cDNA, extending from nt 354 to 810 was amplified by PCR using specific *RPL3A* primers and cloned into the pBSII-SK vector (Promega) to generate NT634. *RPL3A* probe was obtained by *in vitro* transcription of *Nde*I-linearized NT634 using T3 phage polymerase (Promega). This probe protects a 216-nt fragment of the *RPL3A* mRNA from nt 594 to 810. A 223-nt fragment of *RPL3B*, extending from nt 365 to 1077, was amplified by PCR using specific *RPL3B* primers and cloned into pGEM-T vector (Promega) to generate NT584. *RPL3B* probe was obtained by *in vitro* transcription of *Eco*RV-linearized NT584 using SP6 phage polymerase (Promega). This probe protects a 152-nt-long fragment of the *RPL3B* mRNA, from nt 925 to 1077. RNase protection assay was carried out as previously described by Parikh *et al.* (2002). Total RNA (15 μg) was re-suspended in hybridization buffer (40 mM PIPES, pH 6.4, 1 mM EDTA, 0.4 M NaCl, 80% formamide), incubated with *RPL3A*- and *RPL3B*-specific probes at 70°C for 15 min and at 50°C for 10 h. RNase A and RNase T1 were added, followed by incubation at 37°C for 20 min. A 1 : 4 (v/v) solution of proteinase K and SDS 10% was added, followed by incubation at 37°C for 20 min. RNA was then precipitated and separated on a sequencing gel. The identity and specificity of the protected bands were verified using *S. cerevisiae* expressing tobacco *RPL3A* and *RPL3B* cDNAs. RNAs were quantified using a PhosporImager (Amersham Biosciences, Piscataway, NJ, USA) and IMAGEQUANT 5.2 system.

Immunoblot analysis

Total protein was extracted from leaf tissue collected from Wt and transgenic plants using extraction buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 2 mM DDT, and 1 mM phenylmethylsulfonyl fluoride). After centrifugation at 14 000 ***g*** for 10 min, and the supernatant was collected and transferred to a fresh Eppendorf tube. The concentration of protein in each sample was measured using the Bradford assay. Total protein (10 μg) was separated on a 12.5% SDS–PAGE, blotted onto nitrocellulose membranes and probed with polyclonal antibodies against tobacco L3 (1 : 2000). The blot was stripped and re-probed with antimaize PEP carboxylase polyclonal antibodies (1 : 10 000).

Small interferring RNA analysis

Total RNA was extracted from plant tissue using TRIZOL reagent (Invitrogen) following manufacturer's protocol, digested with RQ1 DNase (Promega), and re-suspended in diethyl pyrocarbonate-treated deionized water. For RNA blot analysis, 15 μg of total RNA was separated on a 15% polyacrylamide gel containing 7 M urea in TBE buffer (45 mM Tris-borate, pH 8.0, 1 mM EDTA), transferred to Hybond-N+ membranes using the TransBlot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA, USA) and UV cross-linked. ^{32}P-labeled, *in vitro* synthesized *RPL3B* transcripts were hydrolyzed to fragments of approximately 50 nt using 200 mM carbonate solution and used as probes for hybridization to the RNA blots. The blots were pre-hybridized for 1 h and then hybridized overnight using the indicated probe at 42°C in ExpressHyb Hybridization solution (BD Biosciences Clontech, Palo Alto,

CA, USA), washed at 42°C in 2× SSC, 0.2% SDS, and exposed to film (Eastman Kodak, Rochester, NY, USA).

Microscopy

Fully developed leaves (numbers 5–8) were collected from 30-day-old transgenic and control plants grown in the greenhouse. Fragments of approximately 0.5 cm^2 were peeled from the epidermal layer of the distal leaf area, stained with Toluidine Blue 1%, and visualized using a Nikon D-Eclipse C1 microscope (Micron-Optics, Atlanta, GA, USA). The recorded images were transferred to computer and analyzed using ADOBE PHOTOSHOP. The calculations and the Student's *t*-test were performed using Microsoft Excel. Analyses of at least three independent images per leaf per plant type were used to calculate the cell number and area.

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