Arabidopsis Nucleolin Affects Plant Development and Patterning

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Nucleolin is a major nucleolar protein implicated in many aspects of ribosomal biogenesis, including early events such as processing of the large 35S preribosomal RNA. We found that the Arabidopsis (Arabidopsis thaliana) par1 (parl) mutant, originally identified by its aberrant leaf venation, corresponds to the Arabidopsis nucleolin gene. parl mutants display parallel leaf venation, aberrant localization of the provascular marker Athβ-β-glucuronidase, the auxin-sensitive reporter DR5: β-glucuronidase, and auxin-dependent growth defects. PARL1 is highly similar to the yeast (Saccharomyces cerevisiae) nucleolin NUCLEAR SIGNAL RECOGNITION 1 (NSR1) multifunctional protein; the Arabidopsis PARL1 gene can rescue growth defects of yeast nsr1 null mutants. This suggests that PARL1 protein may have roles similar to those of the yeast nucleolin in nuclear signal recognition, ribosomal processing, and ribosomal subunit accumulation. Based on the range of auxin-related defects in parl mutants, we propose that auxin-dependent organ growth and patterning is highly sensitive to the efficiency of nucleolin-dependent ribosomal processing.

In eukaryotes, protein translation is accomplished by ribosomes that are synthesized in a subnuclear structure, called the nucleolus. Ribosomal biogenesis begins with the synthesis and processing of a large preribosomal RNA (pre-rRNA) and ends with ribosomal subunit assembly by the loading of ribosomal proteins onto mature rRNA and their subsequent transport into the cytoplasm (for review, see Woolford and Warner, 1991; Venema and Tollersve, 1999; Fromont-Racine et al., 2003; Nazar, 2004). The abundant nucleolar protein nucleolin is involved in many aspects of ribosomal biogenesis, including chromatin decondensation of ribosomal DNA, rDNA transcription, rRNA processing and maturation, ribosomal assembly, and nucleocytoplasmic transport of ribosomes (for review, see Tuteja and Tuteja, 1998; Ginisty et al., 1999). Nucleolin is found in organisms ranging from yeast (Saccharomyces cerevisiae) to plants to mammals. In yeast, nucleolin was first identified for its ability to bind nuclear localization signals (NLSs) and dubbed NUCLEAR SIGNAL RECOGNITION1 (NSR1; Lee et al., 1991). NSR1 has been suggested to bind to ribosomal proteins and pre-rRNA to facilitate their interaction (Xue and Mélese, 1994). Mutations in yeast NSR1 result in slow growth, accumulation of unprocessed 35S pre-rRNA, and subsequent reduction in 40S ribosomal subunits (Kondo and Inouye, 1992; Lee et al., 1992).

Mutations in nucleolins of plants have not been reported previously, to our knowledge. However, nucleolin has been shown to be light regulated and to have DNA helicase and ATPase activity in pea (Pisum sativum; Tong et al., 1997; Reichler et al., 2001; Nasirudin et al., 2005), to be cell-cycle regulated in alfalfa (Medicago sativa; Bögre et al., 1996), and to be part of a complex responsible for rRNA binding and in vitro prer-RNA processing in Arabidopsis (Arabidopsis thaliana; Sáez-Vasquez et al., 2004). While the phenotypic consequences of mutations in nucleolin proteins have not been described, those of ribosomal proteins have been reported in Arabidopsis. The pointed first leaf1 (pfl1)/ribosomal protein S18 (rps18), pfl2/rps13, and Arabidopsis minute-like1 (aml1)/rps5 mutants display developmental defects including growth retardation, narrow leaves with reductions in the palisade mesophyll layer, reduced fertility, and cotyledon vascular pattern defects (Van Lijsebettens et al., 1994; Ito et al., 2000; Weijers et al., 2001). Similar defects were exhibited in the recently described short valve1 (svo1)/ribosomal protein L24 (rpl24), though this mutant additionally displayed variable apical-basal gynoecium patterning defects that were not observed in pfl1/rps18, pfl2/rps13, and aml/rps5 mutants. The defects of svo1/rpl24 mutants were proposed to be auxin mediated through perturbations in the translation reinitiation of AUXIN RESPONSE FACTOR (ARF) transcripts, such as ETTIN (ETT)/ARF3 and MONOPTEROS (MP)/ARF5 (Nishimura et al., 2005). Interestingly, mutations in the auxin response genes ETT/ARF3 and MP/ARF5 also have vein pattern...
defects (Sessions and Zambryski, 1995; Przemeck et al., 1996; Sessions et al., 1997; Hardtke and Berleth, 1998; Nemhauser et al., 2000). Promotion of vein patterning and differentiation is known to involve the plant hormone auxin. Auxin’s role in providing positional information for vein formation is supported by the expression pattern of the DR5 auxin-responsive reporter gene and PINFORMED1, the effects of exogenously applied polar auxin transport (PAT) inhibitors (Mattsson et al., 1999, 2003; Sieburth, 1999; Scarpella et al., 2006), and a plethora of mutants with aberrant vascular anatomy and auxin physiology (for review, see Scarpella and Meijer, 2004). However, several vascular pattern genes have been identified that appear not to be involved directly in auxin signaling, but instead in the basic machinery that facilitates or regulates membrane traffic, cell cycle, and other cellular processes. Mutations in COTYLEDON VASCULAR PATTERN1 (CVP1), CVP2, and VARICOSE have normal auxin sensitivity, but display aberrant vein patterns (Carland et al., 1999, 2002; Deyholos et al., 2003; Carland and Nelson, 2004) associated in the first two cases with defects in sterol and phosphoinositol metabolism, and may have defects in endomembrane traffic required for PAT. Vena mutation phenotypes are also associated with defects in other signals, including xylem, brassinosteroids, cytokinin, and small peptides (Casson et al., 2002; Fukuda, 2004; Motose et al., 2004). It is currently unknown whether these diverse factors have direct or indirect roles in vein patterning, but it is remarkable that of the many plant morphological features, venation pattern is especially sensitive to perturbations in basic cellular machinery.

Here, we report the characterization and cloning of the parallel1 (parl1) mutant. The corresponding gene encodes a multifunctional protein that is homologous to the yeast nucleolin NSR1. Arabidopsis PARL1 complements the yeast nsr1 mutant and accumulates in a subnuclear domain that appears to be the nucleolus. The parl1 nucleolin deficiency causes morphological and developmental defects, including alterations in foliar organ shape and vein pattern, misexpression of developmental markers, and reduced apical dominance and fertility. In embryos, PARL1 is expressed ubiquitously in seedlings in areas of high cell division and proliferation, and later only in vascular cells of all organs. Since most parl1 effects appear to be auxin related, we suggest that auxin-dependent organ growth and patterning is particularly sensitive to nucleolin deficiency, possibly because auxin regulation depends on protein turnover and ribosome biogenesis in areas of growth.

RESULTS
The parl1 Mutant Has Morphological and Vein Patterning Defects

A single recessive allele of the parl1 mutant was identified in a chemical mutagenesis screen for vein patterning defects in the first pair of juvenile leaves of Arabidopsis (Clay and Nelson, 2005). The parl1 leaf phenotype was characterized by veins that were aligned with the proximal/distal axis of narrow, pointed parl1 leaves, a reduction in higher order tertiary and quaternary veins, and secondary veins that anastomose in the petiole and occasionally end freely at the leaf margins (compare Fig. 1, A and B). The vein patterns in the parl1 mutants resembled the normal, parallel vein pattern found in monocot species such as rice (Oryza sativa). While adaxial/abaxial arrangement of xylem and phloem appeared unperturbed, vein order was discernible by vein position only and not by vein size, as revealed in transverse sections of parl leaves (Fig. 1, C and D). In addition, the palisade mesophyll layer of the leaf was reduced in the parl1 mutant (Fig. 1C). Venation pattern was also affected in other organs of parl1 plants, including coryledons (compare Fig. 1, E and F), adult leaves, sepals, and petals. Seedlings with the parl1 mutation had narrower and slightly smaller coryledons and leaves, delayed leaf initiation, and reduced root length (1.6 ± 0.5 mm; 5.5 ± 0.8 mm in wild type; Fig. 1, G and H). Additionally, plant stature (19.3 ± 4.4 cm; 31.1 ± 2.9 cm in wild type), rosette size (3.7 ± 0.5 cm; 5.6 ± 0.6 cm in wild type), apical dominance (3.8 ± 0.9 lateral branches; 2.2 ± 0.5 in wild type), and fertility were reduced in mature parl1 mutants compared to wild-type plants (Fig. 1I). These defects suggest Arabidopsis PARL1 is involved in plant growth and patterning.

PARL1 Encodes a Nucleolin Family Protein

To identify the gene affected by the parl1 mutation, a map-based cloning approach was carried out. Genetic analysis of F2 progeny indicated parl1 segregated as a single recessive locus. Linkage to the bottom of chromosome one was initially established by recombination frequency of mapping lines at simple sequence length polymorphic marker nga111. Further mapping by chromosome walking localized the parl1 mutation to a region flanked by simple sequence length polymorphic markers F27K7 and F27J15 that contained 12 genes. DNA probes designed to be specific to this region were used to screen a transformation artificially competent (TAC) library (Liu et al., 1995). Two TACs, K1A14 and K139, completely complemented all defects of the parl1 mutation when transformed, while the other TACs in this region did not (Fig. 2A). This delimited the number of genes in the region to five, which were subsequently sequenced. In the parl1 mutant the only sequence change found in any of these genes was the insertion of two adenine bases into a canonical splice acceptor site at the end of exon 6 of the At1g48920 gene (Fig. 2B). This mutation disrupted the splice acceptor site, resulting in two RNA species containing part or all of the sixth intron in parl1-1 as detected by reverse transcription (RT)-PCR (data not shown) and by an increased size of the PARL1 transcript.
in parl1-1 versus that of the wild type by RNA-blot analysis (Fig. 2C). Both RNA species encode an immediate stop codon four amino acids later, resulting in a predicted truncated 196 amino acid protein. This insertional mutation was designated parl1-1. A second T-DNA allele was obtained, designated parl1-2, and the T-DNA insertion in exon 9 was confirmed by PCR. The parl1-2 allele was a complete null at the transcript level by RNA-blot analysis (Fig. 2C). Since both alleles have identical phenotypic defects, they are likely to be null mutations. To verify that the mutation in gene At1g48920 was responsible for the parl1-1 phenotypic defects, a construct containing the full-length cDNA of this gene was transformed into parl1-1. This construct completely complemented all phenotypic defects of the mutant, including all vein patterning defects of parl1-1 (Fig. 2D). We conclude PARL1 encodes At1g48920, which has high sequence homology to a nucleolin protein family member.

The PARL1 Protein Has Homology to Nucleolin Proteins in Plants and Yeast

While the National Center for Biotechnology Information protein BLAST searches using the PARL1 protein indicated PARL1 was most similar to a plant protein that we named PARL1 LIKE (PARLL1), similarity was also found to proteins annotated as nucleolin from a variety of organisms including humans, mice, chicken, worms, and yeast with 38%, 31%, 34%, 36%, and 34% overall protein identity, respectively. The PARL1 protein contains a putative bipartite NLS (amino acids 21–24 and 38–41) and three major regions involved in ribosomal biogenesis. An acidic/Ser-rich region (45% Ser, 34% aspartic or glutamic acid in yeast NSR1, and 24% and 32% in PARL1) resides in the amino-terminal part of the protein (amino acids 60–263), followed by two RNA recognition motifs (RRMs; amino acids 298–370 and 402–477, respectively) and a carboxyl-terminal Gly- and Arg-rich (GAR) domain (62% Arg or Gly in NSR1 and 69% in PARL1, amino acids 481–549; Fig. 2B). The acidic/Ser-rich region induces nucleolar chromatin decondensation through interaction with histone H1 (Jordon, 1987; Erard et al., 1988) and binds nontranscribed spacer regions in DNA that separate rRNA gene repeats to organize rDNA chromatin for transcription by RNA polymerase I (Olson and Thompson, 1983; Bouche et al., 1984; Eghbazi et al., 1988; Ghisolfi-Nieto et al., 1996). The RRM (also called RBD) domain consists of approximately 80 amino acid residues containing two highly conserved regions, called RNP motifs, that interact specifically with RNA, particularly with the external transcribed spacer region of primary rRNA transcripts (Ghisolfi et al., 1992; Burd and Dreyfuss, 1994; Johansson et al., 2004). A phylogenetic tree of the plant and yeast proteins was constructed using the neighbor-joining method and then analyzed using 1,000 replicates of bootstrap analysis. The most closely related protein to PARL1 supported in this tree was the paralogous PARLL1 protein from Arabidopsis

Figure 1. The plant phenotypes of the Arabidopsis parl1 mutant. A and B, Cleared leaves of wild type and the parl1 mutant, respectively, viewed under dark-field illumination. C and D, Transverse 2 μm plastic sections of parl1 and wild-type juvenile leaves, respectively. Arrows in C point to some of the spaces in the palisade mesophyll layer of the parl1 mutant leaf. Insets of C and D depict midvein cross sections of parl1 and wild-type juvenile leaves, respectively, at 63× magnification. E and F, Cleared cotyledons of wild type and the parl1 mutant, respectively, viewed under dark-field illumination. G, Top view of wild-type (left) and parl1 mutant (right) 2-week-old seedlings. H, Wild-type (left four) and parl1 mutant (right four) 1-week-old seedling roots. I, Mature Col wild-type (left) plants next to shorter and more bushy parl1-1 (middle), and parl1-2 plants displaying reduced fertility.
PARL1 Can Substitute for the Yeast NSR1 Protein

Given the homology between PARL1 and the well-characterized NSR1 nucleolin protein of yeast, we tested whether the Arabidopsis PARL1 protein could functionally rescue growth defects characteristic of null nsr1 yeast mutants. With this aim, a characterized yeast nsr1 knockout line was obtained, sporulated, and dissected for tetrad analysis. As expected, haploid spores of the nsr1 heterozygotes segregated in a one to one ratio of slow growing nsr1 knockout to NSR1 wild-type haploid spore colonies (Fig. 4A). Homozygous nsr1 knockout haploids were transformed with either a Gal-inducible protein expression vector harboring the Arabidopsis PARL1 cDNA or the vector alone. Transformants were grown on Gal-inducing media lacking uracil and as expected, nsr1 containing the vector alone, but not untransformed nsr1, grew slowly on this media (Fig. 4B, compare plate regions 4 and 5 with 1). Wild-type yeast transformed with the vector alone grew well on this media (Fig. 4B, plate regions 6 and 7), as did nsr1 mutants transformed with the inducible Arabidopsis PARL1 construct (Fig. 4B, plate regions 2, 3, and 8). This demonstrates that the Arabidopsis nucleolin rescues growth defects of the yeast mutant nsr1. Galactose induction liquid growth assays confirmed our results. We found nsr1 to have a doubling time of 3 h, which was 1.5 times slower than wild-type yeast, in agreement with previously reported doubling times (Kondo and Inouye, 1992). When nsr1 mutant yeast containing the inducible Arabidopsis PARL1 construct was monitored for growth in the inducing media, the doubling time was found to be the same as wild-type yeast. The rescue of yeast nsr1 mutants by PARL1 suggests a functional role for the Arabidopsis nucleolin in the process responsible for the growth defects characteristic of nsr1 mutants, which has been attributed to defects in 35S pre-rRNA processing.

Unprocessed 35S Pre-rRNA Is Increased in Arabidopsis parl1 Mutant Seedlings

Inhibition of endonucleolytic cleavage in the 5′ external transcribed spacer at site A0 has been found in nsr1 mutants of yeast. This step is one of the earliest corresponding to At3g18610. Orthologous proteins to PARL1 protein were also found in rice (two proteins), pea, alfalfa, and Nicotiana tabacum (Fig. 3). All of these proteins were homologous to nucleolin from yeast (NSR1 in yeast and GAR2 in Schizosaccharomyces pombe in Fig. 3). We conclude Arabidopsis PARL1 is a nucleolin family member homologous to the yeast NSR1 protein.
processing events of the pre-rRNA (Kondo and Inouye, 1992; Lee et al., 1992) and this cleavage is conserved in all eukaryotes (Venema and Tollervey, 1999; Fromont-Racine et al., 2003). In Arabidopsis, site A0 is located 1275 in the 35S pre-rRNA and it is the primary cleavage site (Saéz-Vasquez et al., 2004; Shi et al., 2005; Fig. 4C). To investigate whether Arabidopsis parl1 mutant plants had defects in pre-rRNA processing at site A0 we isolated total RNA from Columbia (Col) and parl1-2 seedlings and performed RT-PCR with U1 and U2 primers to detect unspliced 35S pre-rRNA at site A0. The amount of unprocessed 35S pre-rRNA was much higher in the parl1-2 mutant seedlings than in Col wild-type seedlings (Fig. 4D). These results show that PARL1 of Arabidopsis, like its yeast homolog NSR1, participates in 35S pre-rRNA processing.

PARL1 Is Expressed Early in Embryonic Development and in Vascular Cells

To investigate the expression pattern of nucleolin in Arabidopsis, a PARL1 promoter GUS fusion construct, P

PARL1::GUS, was characterized to evaluate the PARL1 expression pattern. PARL1 was expressed early in development in the funiculus of the seed and ubiquitously throughout the globular stage embryo (Fig. 5A). Strong, ubiquitous GUS expression was also observed later in both the globular and heart stages of embryonic development (Figs. 5, B and C) coincident with in situ hybridization results (data not shown). Expression of the P

PARL1::GUS construct was enriched in vascular cells throughout the seedling and was vascular specific in cotyledons (Fig. 5, D–F). In young leaf primordia P

PARL1::GUS expression was initially strong and ubiquitous (Fig. 5, D and F), whereas later expression was restricted to the leaf tip and developing vascular cells (Fig. 5, G and H). Further analysis of the P

PARL1::GUS construct in other tissues revealed expression of the PARL1 gene in regions associated with cell division (root and leaf tips) as well as in vascular cells of roots and flowers (Fig. 5, D, F, and I). Taken together the expression analyses demonstrate the PARL1 gene is expressed early and ubiquitously in embryonic development, in regions associated with cell division, and in vascular cells of all organs late in development.

Arabidopsis Nucleolin Is Localized to the Nucleus and a Subnuclear Region

While nucleolin is thought to be the most abundant protein in the nucleolus and contains a bipartite NLS, multiple protein domains are needed for its nucleolar localization and accumulation (Schmidt-Zachmann and Nigg, 1993; Yan and Mélese, 1993). To reveal the subcellular localization of the PARL1 protein in wild-type plants, an amino-terminal GFP fusion to the PARL1 protein, P35S::GFP:PARL1, was constructed in the pEGAD vector (Cutler et al., 2000). The fluorescent protein product of the pEGAD vector alone localizes diffusely in the cytoplasm and nucleoplasm of Arabidopsis root cells (Cutler et al., 2000). The nuclei of cotyledon and leaf primordia cells expressing the P35S::GFP:PARL1 construct were clearly seen by differential interference contrast optics (Fig. 5, J and M) and 4′,6-diamino-phenylindole (DAPI) staining (Fig. 5, L and O), respectively. When the P35S::GFP:PARL1 construct was viewed in the same cells under UV illumination, nuclear fluorescence was observed in cotyledon (Fig. 5K) and leaf (Fig. 5N) cells. This nuclear fluorescence was most strongly localized to a subnuclear region, presumably the nucleolus (Fig. 5, K and N), and was clearly observed by confocal microscopy (Fig. 5, P and Q). These results are consistent with the localization of nucleolin reported in other systems (Bugler et al., 1982; Shaw and Jordan, 1995) and with a role for Arabidopsis nucleolin in ribosomal processing and biogenesis.

Early Steps in Vein Differentiation Are Defective in parl1 Mutants

Since Arabidopsis parl1 mutants have defects in vein patterning and PARL1 is expressed in vascular cells (this article, Figs. 1 and 5), we wanted to assess how early parl1 acts in leaf vein differentiation. With this aim, parl1 was crossed to the Athb8::GUS reporter line to generate a homozygous parl1/Athb8::GUS line and the reporter was observed in comparable stages to the
wild type/Athb8:GUS (due to the delayed leaf initiation and slow growth of parl1). Athb8:GUS is a reporter line that marks cells in the provascular stage of development before lignification is observed and is one of the earliest characterized reporters for vein development (Baima et al., 1995; Kang and Dengler, 2002; Scarpella et al., 2004, 2006). In wild-type plants, expression of this reporter was initially visible in both wild-type and the parl1/Athb8:GUS line leaf primordia as a single file of expression that extended from the petiole to the tip of leaf blade, marking cells that later differentiate into the midvein (Fig. 6A, inset). Next, as previously reported, two pairs of expression traces connecting to the central line in high-arching loops were observed in the Athb8:GUS line that mark cells destined to become secondary vein loops (Fig. 6A). While parl1 mutants resembled the wild type for early midvein expression, in subsequent stages multiple traces of Athb8:GUS expression extended from the petiole into the leaf primordia, and often ended freely (Fig. 6B). This expression pattern change continued with subsequent vein initiation in parl1 (Fig. 6D). In wild-type plants, the expression of Athb8:GUS marked the closed, interconnected vein pattern observed in normal leaves at later stages of development and expansion (Fig. 6C). However, parl1 at this same stage displayed an Athb8:GUS expression pattern that was unconnected and consisted of freely ending parallel veins (Fig. 6D). These alterations in Athb8:GUS expression suggest PARL1 acts early in vein patterning.

**Auxin Response Maxima Are Altered in parl1 Leaves**

The parl1 defects in leaf vein patterning suggest that responsiveness to the plant hormone auxin may be disrupted in parl1 mutants. To investigate the level and localization of auxin response in developing Arabidopsis parl1 leaves, we monitored GUS activity in a homozygous parl1-2 mutant seedlings. RT of RNA from 2-week-old wild-type (lanes 1, 2, 5, 6) and parl1-2 (lanes 3, 7) Arabidopsis seedlings using primers as directed in Figure 4C (lanes 1–3) and ubiquitin control primers (lanes 5–7).
of emerging leaf primordia, prior to midvein differentiation (Fig. 6E). Following this expression, a set of apical loops was observed extending from the initial zone of tip expression toward the base of the leaf. These loops were parallel to the leaf margin and presaged secondary vein formation (Fig. 6F). Next, this expression began to turn off in a basipetal fashion, except for expression in the tip. Concurrently, expression was observed in the middle and basal sites of the leaf that foretell higher order tertiary and quaternary vein formation in these regions (Fig. 6G). At later stages, tip expression persisted while apical loop expression ceased and differentiated secondary vein loops were visible. Expression was restricted to the most basal leaf positions, which presaged higher order vein formation in this region (Fig. 6H).

In par1 mutant leaves, DR5:GUS expression was initially correctly localized in the tip at comparable stages, suggesting the incipient midvein pattern was normal (Fig. 6I). This is consistent with the normal pattern of AtHB8:GUS expression at the incipient midvein, as described above, and the normal subsequent differentiation of the midvein. This apparently normal tip expression of DR5 persisted into the next

Figure 5. PARL1, expressed early in embryonic development, in regions associated with cell division and in vascular cells, is localized to the nucleus. A to C, PPARL1:GUS expression in the Arabidopsis globular (A and B) and heart stage (C) embryos, respectively. D to I, PPARL1:GUS expression in later stages. D to F, 4-d-old Arabidopsis seedlings and emerging leaves (F) viewed under bright field. E is a magnification of the region boxed in D to show vascular expression. G and H, Dark-field images of developing leaves of 5- (G) and 6-d-old (H) Arabidopsis seedlings. Yellow arrow denotes leaf tip expression in G, while white arrows point to vascular expression traces. I, Bright-field image showing floral expression. J to O, P35S:GFP:PARL1 localization in the cotyledon (J-L) and leaf primordia (M-O) of Arabidopsis seedlings viewed under differential interference contrast optics (J and M) and UV illumination with filters for GFP expression (K and N) and DAPI staining (L and O). Arrows in J to L denote the nucleus. P and Q, Confocal laser microscopy of 1-week-old seedling Arabidopsis roots expressing P35S:GFP:PARL. P and Q are 189 and 50 μM boxes, respectively.
stage, when subsequent veins were initiated. However, *parl1* mutants failed to show DR5:GUS expression in apical loops, as is observed in wild type (Fig. 6J) and subsequently failed to differentiate veins in apical loops. At the next stage and subsequent stages, the persistent tip expression typical of DR5:GUS in wild-type leaves was drastically mislocalized in *parl1* leaves of comparable stages (compare Fig. 6, K–M with Fig. 6, G and H). In the mutant, expression ceased at the tip, and ectopic DR5:GUS expression appeared in one or two foci offset from the tip. This mislocalization was observed at 9 d after germination (DAG) in 66% (*n* = 502) and at 10 DAG in 86% (*n* = 349) of *parl1*/DR5:GUS leaves and never (7 DAG, *n* = 178; 8 DAG, *n* = 274) in the wild type. The lack of apical loop expression and the mislocalization of tip expression in the *parl1*/DR5:GUS line suggest PARL1 acts prior to the acquisition of preprocambial identity in agreement with our *parl1*/Athb8:GUS expression results and that auxin responses are disrupted in Arabidopsis *parl1* mutants.

**Auxin Response and PAT Inhibition in *parl1* Leaves**

Auxin transport and auxin response are coordinated in many plant pattern formation events, including leaf vein patterning (Reinhardt et al., 2003; Scarpella et al., 2006). As auxin response was disrupted in *parl1* leaves, we wanted to investigate the role of auxin transport in *parl1*/DR5:GUS expressing leaves. In wild-type leaves, the inhibition of PAT by naphthalphthalamic acid (NPA) and similar inhibitors leads to restriction of DR5:GUS expression to the leaf margins, possibly reflecting the accumulation of auxin in these regions (Fig. 6, N and P; Mattsson et al., 2003). Subsequent venation is restricted to the leaf margins. In agreement with this previous report, 1 μM NPA treatment of wild-type plants restricted DR5:GUS expression to the leaf margin (Fig. 6, N and P; Mattsson et al., 2003). In contrast, this marginal expression was absent in 1 μM NPA-treated leaves of the *parl1* mutant (compare Fig. 6, N and P with Fig. 6, O and Q). Thus the expression pattern of DR5:GUS was similar in both NPA-treated and -untreated *parl1* leaves.

**The Inhibition of Auxin Transport Enhances Defects in *parl1* Leaf Shape and Vein Pattern**

Inhibition of PAT has been shown to disrupt not only vein patterning, but also leaf development simultaneously (Mattsson et al., 1999; Sieburth, 1999). To investigate the effect of auxin transport inhibition on *parl1* development and patterning, wild-type and mutant plants were grown on plates containing varying concentrations of the PAT inhibitor NPA. At low concentrations of NPA (0.5 μM) the wild-type leaf vein pattern and morphology were radically altered. There was an increase in marginal venation and the number

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**Figure 6.** Disruptions in Athb8:GUS and DR5:GUS expression in *parl1* leaves. A and C, Athb8:GUS expression in days 4 (A, inset), 5 (A), and 7 (C) of wild-type seedlings. One trace of expression extends from existing vasculature into each leaf primordia of wild type in A. B and D, Athb8:GUS expression in *parl1* seedlings (days 7 and 9). Multiple traces are observed extending into *parl1* leaves in B and D, as well as unconnected vein loops (D). E to M, Auxin response localization viewed by DR5:GUS in developing leaves. E to H, DR5:GUS localization in wild-type leaves at days 4, 5, 7, and 8. I to M, DR5:GUS localization in *parl1* leaves days 6, 7, 9, and 10. L and M, Two images of the auxin response tip mislocalization in *parl1* are shown for day 10. N to Q, Auxin response localization viewed by DR5:GUS localization in leaves of plants grown on media containing the PAT inhibitor NPA. N and P, DR5:GUS expression in wild-type leaves days 8 and 10 treated with 1 μM NPA. O and Q, DR5:GUS expression in *parl1* mutant leaves days 9 and 11 treated with 1 μM NPA. All size bars correspond to 100 μM. Stages between wild-type and *parl1* mutants are comparable, as the age differences are due to the 2-d delay in leaf initiation and slow growth of *parl*. 

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of traces in the petiole. This change was accompanied by a reduction in higher order veins (compare Fig. 7, A and B). As previously reported (Mattsson et al., 1999; Sieburth, 1999), striking changes in leaf morphology were also observed at low NPA concentrations, as leaves became epinastic, darker in color, reduced in size, and the petiole was shortened (compare Fig. 7, E and F). This effect was more severe in wild-type plants at higher concentrations of NPA (Fig. 7, G and H).

In parl1 mutant leaves, leaf shape was converted to a paddle shape appearance at low concentrations of NPA (compare Fig. 7, I and J). The vein pattern in parl1 leaves was transformed by NPA treatment into a mass of parallel vein traces penetrating the leaf from the petiole (compare Fig. 7, M and N). Arching secondary veins were found only occasionally at the tip of NPA-treated parl1 leaves (Fig. 7O). These results suggest NPA treatment of parl1 plants led to the enhancement of parl1 leaf shape and vein pattern defects.

DISCUSSION

Ribosomes are responsible for protein translation in living eukaryotic cells, and their biogenesis is a complex process involving the coordination of rDNA, rRNA, RNA polymerase I, and a plethora of nonribosomal/ribosomal proteins. Nucleolin is a nonribosomal multifunctional protein involved in many steps of ribosomal biogenesis. We report here the identification and cloning of a mutation in the PARL1 gene, which encodes nucleolin of Arabidopsis. The parl1 mutants display several aberrations in venation pattern of all foliar organs, including the parallel alignment of veins along the proximal/distal leaf axis, the parallel exit of multiple veins from the petiole, and a reduction in higher order venation. These defects in leaves are associated with the mislocalization of activity from the provascular Athb8:GUS and auxin-responsive DR5:GUS reporters. We also show Arabidopsis parl1 mutants have an accumulation of unprocessed 35S prerRNA that is consistent with our results demonstrating PARL1 is able to rescue mutants in the yeast nucleolin gene NSR1. These data suggest that auxin-dependent growth and patterning processes, including vein patterning, are particularly sensitive to perturbations in ribosomal processing. From these results we suggest that PARL1 is a multifunctional Arabidopsis nucleolin that has a role in ribosomal RNA processing and auxin-dependent growth and patterning.

Figure 7. Auxin transport inhibition studies using varied NPA concentrations. Col wild-type (A–D) and parl1 (M–P) cleared leaves of 17-d-old plants grown on increasing NPA concentrations (0, 0.5, 1, and 100 μM, respectively) viewed under dark-field illumination. Top view displaying plant morphology of wild-type (E–H) and parl1 (I–L) 17-d-old plants grown on increasing NPA concentrations (0, 0.5, 1, and 100 μM, respectively). Two representative wild-type seedlings grown on 100 μM NPA are shown in H.
Ribosomal Biogenesis and PARL1

PARL1 is predominantly localized to the nucleus and in a subnuclear structure presumed to be the nucleolus, consistent with localization of nucleolin in other systems (Bugler et al., 1982; Shaw and Jordan, 1995). We also demonstrated that Arabidopsis PARL1 functionally complements its yeast homolog NSR1. Defects in yeast NSR1 include a decrease in 35S rRNA and an increase in 35S pre-rRNA that presumably result in a decrease in the ratio of 40S to 60S ribosomal subunits and a slow growth phenotype (Kondo and Inouye, 1992; Lee et al., 1992). Similarly, Arabidopsis parl1 mutants are characterized by a slight slow growth phenotype and by an increase in unprocessed 35S pre-rRNA in seedlings.

Whether a decrease in the ratio of 40S to 60S ribosomal subunits is directly responsible for the slow growth of yeast nsr1 and Arabidopsis parl1 mutants remains to be determined, but phenotypic similarities between parl1 and Arabidopsis ribosomal protein mutants suggest this may be the case. Developmental defects including growth retardation, narrow leaves with reductions in the palisade mesophyll layer, reduced fertility, and cotyledon vascular pattern defects are displayed in the ribosomal protein mutants pfl1/tps18, plf2/rps1, amn1/tps5, and str1/rpl24 (Van Lijsebettens et al., 1994; Ito et al., 2000; Weijers et al., 2001; Nishimura et al., 2005). Since the patterning and growth defects of str1/rpl24 and parl1 seem to be auxin mediated, we suggest these processes are highly sensitive to changes in ribosomal processes. While the ribosomal subunit localization of these mutants is unknown, it is likely that the loss-of-function mutations in these genes are localized to the 40S ribosomal subunit, which we predict is also defective in parl1. This may suggest PARL1 and ribosomal proteins affect ribosome biogenesis independently. On the other hand, PARL1 may interact directly with ribosomal proteins. Yeast NSR1 has been suggested to bind to ribosomal proteins and pre-rRNA to facilitate their interaction (Xue and Mélese, 1994). As PARL1 can substitute for NSR1 in yeast, this may also be the case for nucleolin in Arabidopsis.

It is unlikely that the increase in unprocessed 35S pre-rRNA in the parl1 mutant is due to direct RNA cleavage activity of PARL1. Nucleolin has a number of enzymatic activities including autodegradation (Olson et al., 1990; Chen et al., 1991; Fang and Yeh, 1993), DNA and RNA helicase activities (and thus DNA/RNA binding activity; Tuteja et al., 1995), and DNA-dependent ATPase activity (Tuteja et al., 1997), but not RNA-cleaving activity. The role of PARL1 may be to directly assemble this pre-rRNA processing complex on pre-rRNA via its RNA binding activity, as it does in vertebrates, rather than to cleave rRNA directly (Ginisty et al., 2000). In yeast the pre-rRNA complex also contains the DEAD BOX RNA HELICASE3 protein (Dbp3p; Krogan et al., 2004). Double mutants between a verified Salk T-DNA insertion line in the Arabidopsis homolog of Dbp3p, dbp3/rna helicase5 (rh5), and parl1-2 are indistinguishable from parl1-2 mutants (Supplemental Fig. S1), suggesting these genes act in a genetic pathway involved in ribosomal RNA processing in Arabidopsis, as in yeast (Krogan et al., 2004). If this complex proves to include RNA polymerase I, it would link the role of nucleolin in chromatin remodeling and rDNA transcription with the role of PARL1 in pre-rRNA processing.

Interestingly, the aforementioned Arabidopsis pre-rRNA processing complex dissociated into smaller complexes, which was obviated by isolating the complex from cauliflower (Brassica oleracea), a tissue rich in meristematic cells (Sáez-Vasquez et al., 2004). This suggests a connection between a pre-rRNA processing role for PARL1 and cell division in plants. Supporting this idea, alfalfa nucleolin is absent in stationary cells, but highly expressed before the onset of DNA replication at both the transcriptional and protein levels in logarithmically dividing cells (Bögé et al., 1996). Furthermore, red light treatment induces pea nucleolin transcription 1 h prior to rRNA synthesis and coincides with increased cell division rates in pea apical internodes as well as increased nuclear number/weight of pea plumules (Reichler et al., 2001). These data support a link between nucleolin and cell division in plants that may explain the growth defects and reduction in higher order venation characteristic of Arabidopsis parl1.

Alternatively, slow growth and lack of proliferation may be due to other defects in ribosome biogenesis. Preribosomal particles are assembled in specific regions of the nucleolus, moved to the nucleoplasm, and then shuttled through nuclear pores (utilizing ATP) to the cytoplasm where protein synthesis occurs (Milkeriet et al., 2001; Cheutin et al., 2002; Lei and Silver, 2002). In light of the fact that this process requires the utilization of ATP, it is interesting that pea nucleolin has demonstrated ATPase activity (Nasirudin et al., 2005). It is unknown if this activity is affected in yeast nsr1 and Arabidopsis parl1 mutants or if this could account for their slow growth defects. PARL1 may shuttle ribosomal components to/from the nucleus via an ATPase activity and ability to bind NLSs of ribosomal proteins. In fact, NSR1 was isolated from yeast based on its capability to bind NLSs (Lee et al., 1991). Whether or not defective shuttling of ribosomal components accounts for growth defects of yeast nsr1 and Arabidopsis parl1 mutants is unknown.

Nucleolins and Auxin Responses

Why does the parl1 nucleolin deficiency cause a reduced auxin responsiveness? Based on current views of the connection between venation pattern and PAT (Rolland-Lagan and Prusinkiewicz, 2005; Scarpella et al., 2006), the venation pattern defects in parl1 should correspond to a lack of marginal auxin accumulation during leaf growth, and multiple leaf tip sources, causing a predominance of tip to base vein orientation. This is consistent with patterns of auxin
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exciting challenge for further research.

exhibited by ribosomal protein mutants provides an

of auxin-dependent growth and patterning phenotypes

from STV1's influence on ETTIN/ARF3 translation and cotyledon vein patterning defects resulting

do exactly this. Mutants in STV1 not only have ribosomal

for translation reinitiation, has recently been shown to

Interestingly, the ribosomal protein STV1 required

for translation reinitiation. Root length measurements taken from the hypocotyl/root junction to the root tip were measured on plants (100 of each genotype) grown for 4 d vertically on agar media. For phenotypic quantification, 4-week-old plants (30 of each genotype) were measured for plant height from the rosette base to the stem tip, for rosette width across the center in the widest part of the rosette, and for the number of lateral branches the number of lateral branches from the main stem was counted.

Mutant Isolation

The parl1-1 allele was isolated from a leaf vein pattern screen of Col-0 as described by Clark and Nelson (2005). The parl1-2 and dbp3/rh5 insertion alleles were identified from the Salk Insertion Sequence Database (Alonso et al., 2003). The parl1-2 allele corresponds to Salk 002764, and genotyping was carried out using LBB1 and the following gene-specific primers: PARSF, 5'-TGG CCT ACC ATG GAA TTC A-3' and PARSR, 5'-AGT TGC TGT CAC CAA GAA G-3' (LBB1 + PARSF gives the parl1-2 insertion-specific product).

The dbp3/rh5 allele corresponds to Salk 002509 and was verified to be 29 bp downstream (still in exon 7) of The Arabidopsis Information Resource annotation by genotyping using LBB1 and the following gene-specific primers: DRP3F, 5'-TGG AGA CCT ACA GCT TCC-3' and DRIP3R, 5'-TGG GTG TAT GCT GGC ACC T-3' (LBB1 + DRIP3R gives the dbp3/rh5 insertion-specific product). The cvp2-2 allele was isolated in a leaf vein pattern screen of activation tagging lines generated by D. Weigel (Weigel et al., 2000) and is a recessive mutation resulting from insertion of the T-DNA into the 1,988 bp of the CVP2 gene.

Histology and GUS Staining

Organs were fixed in 3:1 ethanol to acetic acid, dehydrated in an ethanol series, cleared in Histoclear (National Diagnostics), mounted in 2:1 Permoun (Fisher Scientific) to xylene, and viewed under dark-field optics. For GUS analysis, tissues were stained at the same hour daily and incubated overnight at 37°C in GUS buffer described by Donnelly et al. (1999), except 5 mM instead of 3 mM K3F3(CN)6 was used. For parl1 mutants, samples at comparable stages refer to the 2 d delay in leaf initiation of parl1 compared to wild-type plants. Tissues were fixed in acetone, transferred into ethanol overnight, and mounted as described above. Histological analysis was performed using plastic and paraffin sectioning. For this specimens were fixed, dehydrated, embedded, sectioned, and stained as described by Clark and Nelson (2005). For DAPI staining, whole seedlings were stained for 5 min by submersion in 1 μg/mL DAPI, washed twice in water in an eppendorf tube, and then mounted in 75% glycerol. All specimens were examined with a Zeiss Axio phot microscope, except for confocal microscopy, which was performed on a Bio-RAD 1024 Laser Confocal Unit mounted on a Zeiss Axiovert 10 inverted microscope.

Genetic Mapping and Plant Transformation Vector Construction

Plants homozygous for the parl1-1 mutation were outcrossed to wild-type Landsberg erecta plants to generate F2 recombinant inbred mapping lines. DNA from 20 mutant plants was used with simple sequence length polymorphic markers (Bell and Ecker, 1993; Ponce et al., 1998) to establish chromosomal linkage of the parl1-1 mutation, and DNA from 823 plants was used with Cremer’s insertion/deletion and single-nucleotide polymorphism markers (Jander et al., 2002) to finely map the parl1-1 mutation to a region spanned by two BACs. Mapping primers are available upon request. TAC filters were probed with 1-kb genomic probes spaced approximately 20 to 40 kb apart based on BAC sequences of F271/15 and F27/7 using a formamide-based buffer, according to manufacturer’s instructions provided for ZetaProbe GT membranes (Bio-Rad). Positive TAC clones were ordered based on their hybridization signals.

The PARL1 cDNA (stock no. C105460) GenBank accession number BT005793 generated by Yamada et al. (2003) was PCR amplified and subcloned into XbaI/BamHI sites of the pJL119 (KAN) binary vector for complementation and into HindIII/BamHI sites of the pE5AG binary vector (Cutler et al., 2000).

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (Arabidopsis thaliana) ecotype Col-0 seeds were grown under continuous white light (300 μE m⁻² s⁻¹) either on 0.75% agar media consisting of Murashige and Skoog basal salts (Sigma-Aldrich), Haughn and Somerville (1986) nutrient solution, 0.5 g/L MES, and 10g/L Suc, or on soil (2:1 mix of Fafard Super Fine Germination Mix;vermiculite; Conrad Fafard). The auxin transport inhibition experiments used the agar media described above supplemented with NPA. Exposure of imibed seeds to light is referred to as 0 DAG in this study. Root length measurements taken from the hypocotyl/root junction to the root tip were measured on plants (100 of each genotype) grown for 4 d vertically on agar media. For phenotypic quantification, 4-week-old plants (30 of each genotype) were measured for plant height from the rosette base to the stem tip, for rosette width across the center in the widest part of the rosette, and for the number of lateral branches the number of lateral branches from the main stem was counted.

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in frame to generate the amino-terminal GFP fusion P\textsubscript{dsr1}GFP-PARL1. The PARL1 promoter-GUS fusion construct (P\textsubscript{dsr1}:GUS) was generated by amplification of upstream sequence (2,361 bp) and genomic sequence from the ATG through the second intron (474 bp) from TAC K1A14 and subcloned in frame with the GUS gene into Sall/BamHI sites of the pHBlu1.1 binary vector (CLONTECH). Constructs were introduced into wild type and/or parl1-1 by the Agrobacterium tumefaciens-mediated floral dip method of Clough and Bent (1998). Transformants were selected on agar media containing 15 \mu M glucose or 50 \mu M kanamycin.

**Total RNA Isolation and RNA Gel-Blot Analysis**

Total RNA was isolated from 2-week-old seedlings with TRIzol (Gibco BRL) according to the manufacturer’s instructions. Ten micrograms of total RNA was electrophoresed in 1.2% formaldehyde-agarose gel, transferred to a ZetaProbe GT blotting membrane, UV cross-linked and baked for 1.5 h at 80°C. Blots were stained with 0.02% methylene blue for 5 min, destained for 10 to 15 min in 20% ethanol, and the stained rRNA bands were visualized with a Gel Doc 2000 (Bio-Rad). The PARL1 cDNA was labeled with \textsuperscript{32}P and hybridized overnight at 42°C to the RNA blot. The blot was washed at 55°C in 0.5 × SSC/0.5% SDS for 10 min, then in 0.2 × SSC/0.25% SDS, and exposed to Eastman Kodak X-OMAT AR film overnight at ~70°C.

**RT-PCR**

Two micrograms of total RNA from 2-week-old seedlings was reverse transcribed with 200 units of Superscript II (Invitrogen). The resulting cDNA:RNA hybrids were treated with 10 units of DNaseI (Roche) at 37°C for 30 min, purified on a Qiaquick PCR column (Qiagen), and used as a template to amplify pre-35S (35 cycles) and UBC (35 cycles). PCR conditions were as follows: 94°C for 15 s, 52°C for 15 s, and 72°C for 30 s. PCR products (882 bp for pre-35S and 366 bp for UBC) were electrophoresed in 1.5% agarose gels and visualized with GelDoc 2000. Primer sequences for pre-35S, designated U1 and U2, were as reported by Shi et al. (2005). UBC primer sequences were as follows: UBCF, 5′-TCA AGA GCC TCT AAC AAG A-3′ and UBCR, 5′-CTT TGC TCA ACA ACA TCA CG-3′.

**Sequence Analysis**

Amino acid sequence alignments were performed using the ClustalX program. Phylogenetic analyses were performed with Phylogenetic Analysis Using Parsimony, version 4.0. The Tetrahymena thermophila sequence was used as an outgroup because of its similarity to PARL1 and the roughly equivalent distance between ciliates and both yeast (Saccharomyces cerevisiae) and plant sequences. Shime mold sequences were also used as outgroups with identical results. All heuristic and neighbor-joining searches were performed by tree bisection reconnection branch swapping with option MULPARS in effect and generated similar trees. All tree searches were subjected to bootstrap analysis with 1,000 replications.

**Yeast Strains and Experiments**

The nsr1 mutant (BY7473) was obtained as a heterozygous diploid knockout (Open Biosystems). The PARL1 cDNA was subcloned in frame into the XbaI/HindIII sites of the pEGH yeast expression vector containing the GAL1 inducible promoter and the URA3 gene (Mitchell et al., 1993). BY7473 (MATa/MATa; MATA/ MATA met15Δ/0/lys2Δ his3Δ1 leu2Δ1 ura3Δ1 nsr1Δ1:KAN) was sporulated and tetrads were dissected and selected for homozygous nsr1 mutants. The nsr1 mutant haploids were transformed with pEGH alone or pEGH containing the full-length Arabidopsis PARL1 cDNA. All transformed yeast were grown at 30°C on either inducing media (5.75 g/L yeast nitrogen base [Becton, Dickinson, and Company], 0.77g/L CSM-URA [CLONTECH], 10 mM potassium phosphate, 0.1% [w/v] dextrose, and 2% [w/v] Gal or noninducing media (5.75 g/L yeast nitrogen base [Becton, Dickinson, and Company], 0.77g/L CSM-URA [CLONTECH], 10 mM potassium phosphate, and 2% [w/v] dextrose).

For liquid growth complementation experiments, three samples each of both Aparl1 mutant haploid strains containing either pEGH alone or pEGH containing the full-length Arabidopsis PARL1 cDNA, as well as three samples of the wild-type haploid strain transformed with pEGH alone were transferred from noninducing plates and grown in 5 mL noninducing media overnight. As described by Rechler et al. (2001), equal amounts of each culture were then transferred to another 5 mL of noninducing media and grown to early log phase. Equal amounts of these cultures were then transferred to inducing media and grown to saturation while growth rate was monitored hourly by OD\textsubscript{600} readings. This experiment was performed in triplicate.

Sequence data can be found in the GenBank database (http://www.ncbi.nlm.nih.gov/) under the following accession numbers: PARL1 (At4g8920); PARL1 (At5g38610). GenBank accession numbers for the sequences analyzed in the phylogenetic analyses are as follows: ArabidopsisPARL1 (NP_175322), ArabidopsisPARL1 (NP_188491), OsativaOSNi40058K23.21 (XP_473925), OsativaOSNi40056006.24 (XP_480492), Psativirusnuclecold (AAA42086), M.sativumn1 (CAA61298), N.tabacumnucleolin (BAC02896), T.saccharomycesnucleolarphospho (AA96781), S.pombeGAR2 (NP_595331), and S.cerevisiae NSR1p (NP_011675).

**Supplemental Data**

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

**Supplemental Figure S1.** Double mutant analysis of parl1 ctxp2 and parl1 dbp3/nhs.

**Note Added in Proof**

Since the completion of this work, two relevant papers have appeared: Kojima et al. (2007) and Pontivanne et al. (2007) (Kojima H, Suzuki T, Kato T, Enamoto K, Sato S, Kato T, Tabata S, Sáez-Vasquez J, Echeverria M, Nakagawa T, et al [2007] Sugar-inducible expression of the nucleolin-1 gene of Arabidopsis thaliana and its role in ribosome synthesis, growth and development. Plant J 49: 1053–1063; Pontivanne F, Matia I, Douet J, Tourmente S, Medina FJ, Echeverria M, Sáez-Vasquez J [2007] Characterization of AnNLC-L1 reveals a central role of nucleolin in nucleolus organization and silencing of AnNLC-L2 gene in Arabidopsis. Mol Biol Cell 18: 369–379). These studies support our findings on the roles of nucleolins in Arabidopsis growth and development. They also show sugar-induced expression of nucleolin and ribosomal proteins and a role for nucleolin in the organization of the nucleolus and rDNA chromatin structure, respectively.

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