Characterization of Arabidopsis Glutamine Phosphoribosyl Pyrophosphate Amidotransferase-Deficient Mutants[1]

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Using a transgene-based screening, we previously isolated several Arabidopsis mutants defective in protein import into chloroplasts. Positional cloning of one of the loci, *CIA1*, revealed that *CIA1* encodes Gln phosphoribosyl pyrophosphate amidotransferase 2 (ATase2), one of the three ATase isozymes responsible for the first committed step of de novo purine biosynthesis. The *cia1* mutant had normal green cotyledons but small and albino/pale-green mosaic leaves. Adding AMP, but not cytokinin or NADH, to plant liquid cultures partially complemented the mutant phenotypes. Both ATase1 and ATase2 were localized to chloroplasts. Overexpression of ATase1 fully complemented the ATase2-deficient phenotypes. A T-DNA insertion knockout mutant of the *ATase1* gene was also obtained. The mutant was indistinguishable from the wild type. A double mutant of *cia1*/*ATase1-knockout* had the same phenotype as *cia1*, suggesting at least partial gene redundancy between ATase1 and ATase2. Characterizations of the *cia1* mutant revealed that mutant leaves had slightly smaller cell size but only half the cell number of wild-type leaves. This phenotype confirms the role of de novo purine biosynthesis in cell division. Chloroplasts isolated from the *cia1* mutant imported proteins at an efficiency less than 50% that of wild-type chloroplasts. Adding ATP and GTP to isolated mutant chloroplasts could not restore the import efficiency. We conclude that de novo purine biosynthesis is not only important for cell division, but also for chloroplast biogenesis.

De novo biosynthesis of the purine ring is essential for plant growth and development. The major products, AMP and GMP, are the building blocks for DNA and RNA. AMP, when converted into ATP, is the major energy source for multiple cellular processes. Several important coenzymes, e.g. NAD and FAD, are also derived from the same pathway. In nodules of N-fixing tropical legumes, such as soybean (*Glycine max*) and cowpea (*Vigna unguiculata*), the pathway also plays a dominant role in primary nitrogen metabolism. The activity of enzymes in the purine biosynthesis pathway is enhanced considerably in nodules compared to other tissues. Therefore, most studies of purine biosynthesis in plants have used these legume nodules as materials and focused on the function of purine biosynthesis in nitrogen assimilation (for review, see Smith and Atkins, 2002). Studies on the role of purine biosynthesis in normal plant physiology or in non-N-fixing plants have been relatively few.

The location of purine biosynthesis within plant cells is still in dispute. The plant enzymes in the pathway are similar to those in *Escherichia coli*, except that each plant enzyme has an N-terminal extension that is presumed to function as an organelle-targeting signal (Smith and Atkins, 2002). Fractionation studies of nodules indicate that the pathway is located within plastids (Boland and Schubert, 1983; Shelp et al., 1983). However, recent reports indicate that the pathway is present in both mitochondria and plastids (Atkins et al., 1997) or in the cytosol (Sugiura and Takeda, 2000).

The enzyme Gln phosporibosyl pyrophosphate (PRPP) amidotransferase (EC 2.4.2.14) catalyzes the first committed step of de novo purine biosynthesis, the conversion of 5-phosphoribosyl-(α) 1-pyrophosphate to 5-phosphoribosyl-(β) 1-amine. It is subjected to feed-back regulation and forms the rate-limiting step in de novo purine biosynthesis (Zalkin and Nygaard, 1996). Despite its importance, studies on its function in vivo on plant growth and development have been lacking. Furthermore, in Arabidopsis, the enzyme is encoded by a family of three genes. The functional relationship of the three isozymes is also not known. In this article, the isozymes encoded by these three genes will be called ATase1 (At2g16570), ATase2 (At4g34740), and ATase3 (At4g38880), respectively. Partial cDNAs encoding ATase1 and ATase2 have been isolated from a cDNA library of young floral buds (Ito et al., 1994). ATase1 is expressed in roots and flowers, and ATase2 is expressed in roots, leaves, and flowers

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(Ito et al., 1994). ATase3 has been revealed by Arabidopsis genome sequencing and is expressed at very low levels in silique, cauline leaves, and roots (R. Boldt, unpublished data).

Most proteins in chloroplasts are encoded by the nuclear genome and synthesized in the cytosol as higher M_r precursors with N-terminal extensions called transit peptides. Import of these proteins into chloroplasts requires transit peptides and a translocon complex in the chloroplast envelope (for review, see Schleiff and Soll, 2000). Translocon components located in the outer membrane are called Toc (translocon at the outer-envelope-membrane of chloroplasts) proteins, and those in the inner membrane are called Tic (translocon at the inner-envelope-membrane of chloroplasts) proteins (Schnell et al., 1997). In addition, import also requires ATP and GTP. The exact function of these two purine nucleotides in chloroplast protein import is still not clear. However, it has been shown that ATP hydrolysis is essential for precursor protein binding and subsequent translocation across the envelope (Olsen et al., 1989; Theg et al., 1989). Nonhydrolyzable GTP analog can completely block precursor binding to chloroplasts (Kessler et al., 1994; Young et al., 1999). Furthermore, two of the three core components of the outer membrane translocon responsible for chloroplast protein import are GTPases (Kessler et al., 1994), further highlighting the importance of GTP in the import process.

We have designed a transgene-based screening strategy to isolate Arabidopsis mutants defective in chloroplast protein import (Sun et al., 2001). We have named these mutants *cia* (chloroplast import apparatus). Positional cloning of the *CIA1* locus revealed that it encodes ATase2. Characterization of the *cia1* mutant revealed the importance of de novo purine biosynthesis in both cell division and chloroplast biogenesis.

RESULTS

The *cia1* Mutant Phenotypes

The *cia1* mutant has normal green cotyledons but small and albino/pale-green mosaic leaves (Fig. 1, A and B). To determine the nature of the mutation, *cia1* was back-crossed to the unmutagenized parental line. F_1 progeny were wild type in appearance. F_2 progeny segregated at a wild type to mutant ratio of 3:1 (333:114), indicating that *cia1* was a single-gene recessive mutation. Another mutant was discovered in an unrelated screen and had an identical phenotype to *cia1*. Complementation crosses indicated that these

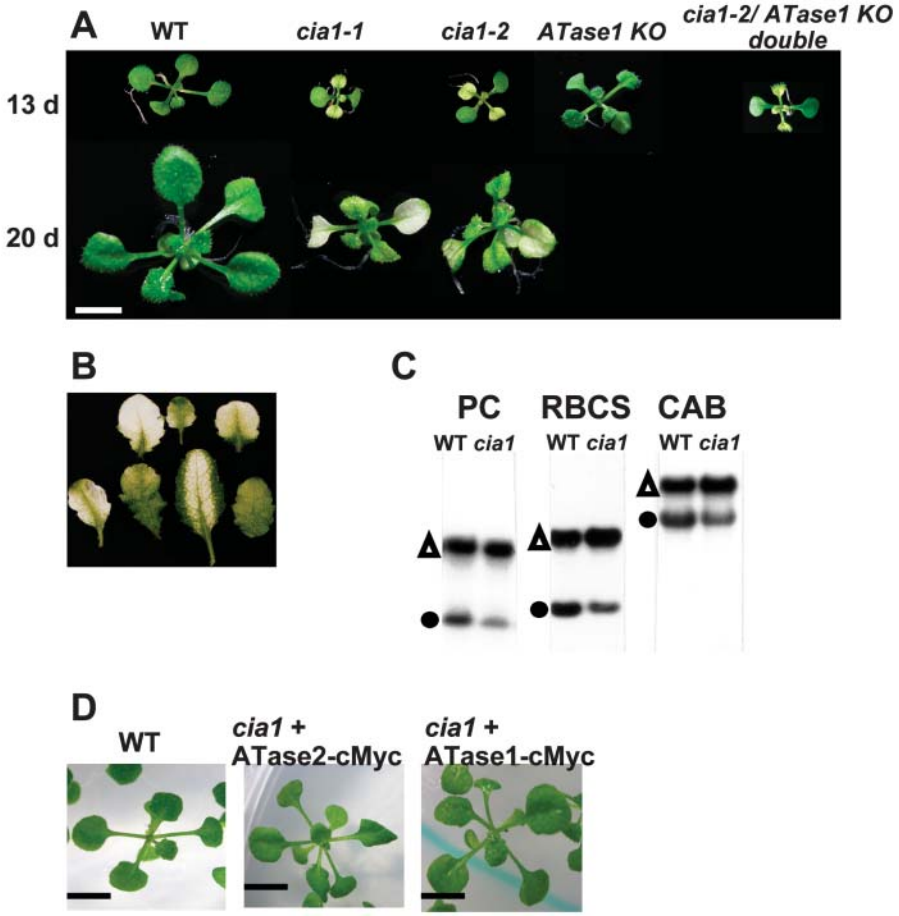

Figure 1. Phenotypes of various mutants. A, Morphology of wild-type (WT), *cia1-1* and *cia1-2*, *ATase1 knockout* (*ATase1 KO*), and *cia1-2/ATase1 KO* double mutant plants at 13 d and 20 d. Bar = 0.5 cm. B, Various mosaic patterns of the *cia1-2* mutant leaves. C, Import of precursor proteins into isolated wild-type and *cia1* chloroplasts. RBCS, small subunit of Rubisco; CAB, chlorophyll *a*/*b*-binding protein of PSII; PC, plastocyanin. White triangle, Precursor form of each protein bound on the chloroplast surface. Black circle, Imported mature form of each protein. D, Complementation of *cia1* by cDNA encoding ATase2-cMyc or ATase1-cMyc driven by the CaMV 35S promoter. Plants were grown on Murashige and Skoog agar plates for 18 d. Bar = 0.5 cm.

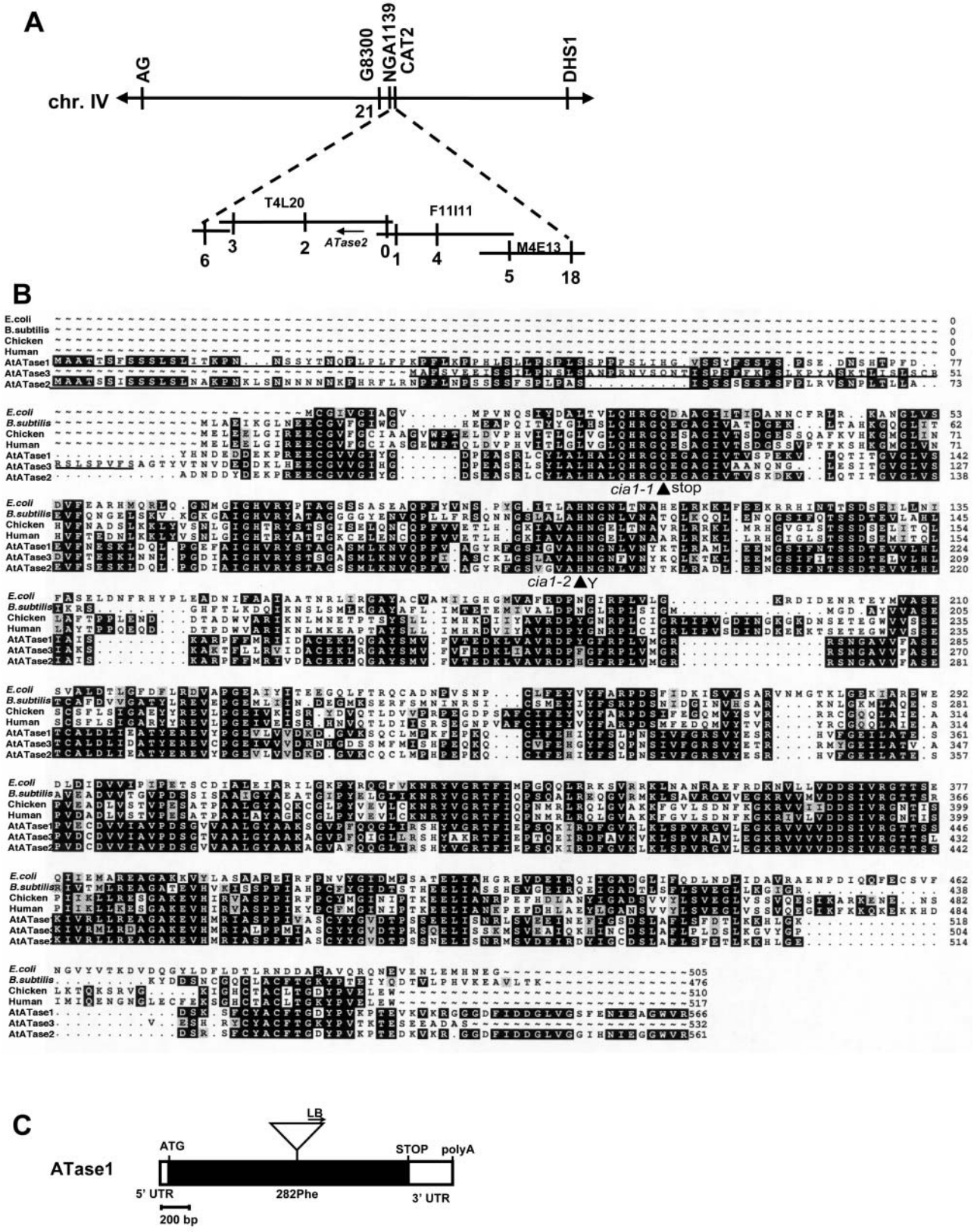

Figure 2. Molecular cloning of *CIA1* and positions of mutations or T-DNA insertion in *cia1-1*, *cia1-2*, and the *ATase1 knockout* line. A, Summary of positional cloning of *CIA1*. Vertical lines indicate positions of PCR-based markers. Values beneath the lines indicate number of recombinants. The direction of transcription of *ATase2* ORF is indicated (arrow). B, Comparison of the three Arabidopsis ATases (AtATase) with ATases from other species. Arrowheads indicate the positions of mutations in *cia1-1* and *cia1-2*. Predicted transit peptides in the three Arabidopsis ATases are underlined. C, Schematic representation of T-DNA insertion in the *ATase1* gene in the Salk_008888 line. The T-DNA inserts in the coding sequence of Phe residue 282. The *ATase1* gene has no intron. LB, T-DNA left border.

two mutants are allelic. The original *cia1* was named *cia1-1* and the new mutant *cia1-2*. Because a backcrossed line of *cia1-2* was obtained first, all experiments described in this article were performed with the *cia1-2* allele. The *cia1* mutant was much smaller than the wild type but was still viable and fertile even when directly germinated on soil. The leaves showed an irregular mosaic of green sectors among white portions. The mosaic pattern varied considerably, depending on growth, nutrient, and light conditions (Fig. 1B; data not shown).

Chloroplasts were isolated from *cia1* and wild-type plants and used for in vitro protein import experiments. Protein import efficiency of the mutant chloroplasts was about 50% that of wild-type chloroplasts (Fig. 1C). Import of precursors to the stroma-located small subunit of Rubisco (RBCS) and thylakoid-located chlorophyll *a*/*b*-binding protein (CAB) of PSII and plastocyanin (PC) were all reduced in the mutant, indicating a general defect in protein import into chloroplasts.

CIA1 Encodes ATase2

The *cia1* mutation was mapped to the lower arm of chromosome VI between the cleaved amplified polymorphic markers (CAPS) AG and DHS1 (Konieczny and Ausubel, 1993; Fig. 2A). Additional CAPS markers were identified using sequences available at the time of cloning. Data from three recombinant plants delimited the *CIA1* locus to a region contained by half of bacterial artificial chromosome T4L20 and a small portion of bacterial artificial chromosome F11I11. All the predicted open reading frames (ORFs) were sequenced from the wild type and the *cia1-2* mutant. Only one C-to-T mutation was found in the ORF predicted to encode ATase2. The missense mutation converts the conserved His at residue 187 to Tyr (Fig. 2B). Sequencing of the ATase2-encoding region in *cia1-1* revealed a C-to-T change that converts the Gln at residue 114 to a stop codon. Expression of the ATase2 cDNA alone (data not shown), or ATase2 cDNA fused in front of five tandem repeats of cMyc tag (Fig. 1D), under the direction of cauliflower mosaic virus 35S promoter (CaMV 35S), both fully complemented the *cia1-2* mutant (Fig. 1D). We therefore concluded that *CIA1* encodes ATase2. Because *cia1-1* and *cia1-2* were indistinguishable phenotypically, it is most likely that both alleles are null.

ATase converts PRPP to phosphoribosylamin, which is the first committed step in de novo purine biosynthesis (Fig. 3A). Nucleotide measurements revealed that the *cia1* mutant indeed had reduced levels of adenine and guanine nucleotides (Fig. 3B). The residual purine nucleotides in the mutant, and the fact that the mutant was still viable, are most likely due to the presence of the two other isozymes.

In non-N-fixing plants, de novo purine biosynthesis leads to three major groups of products: ATP/GTP,

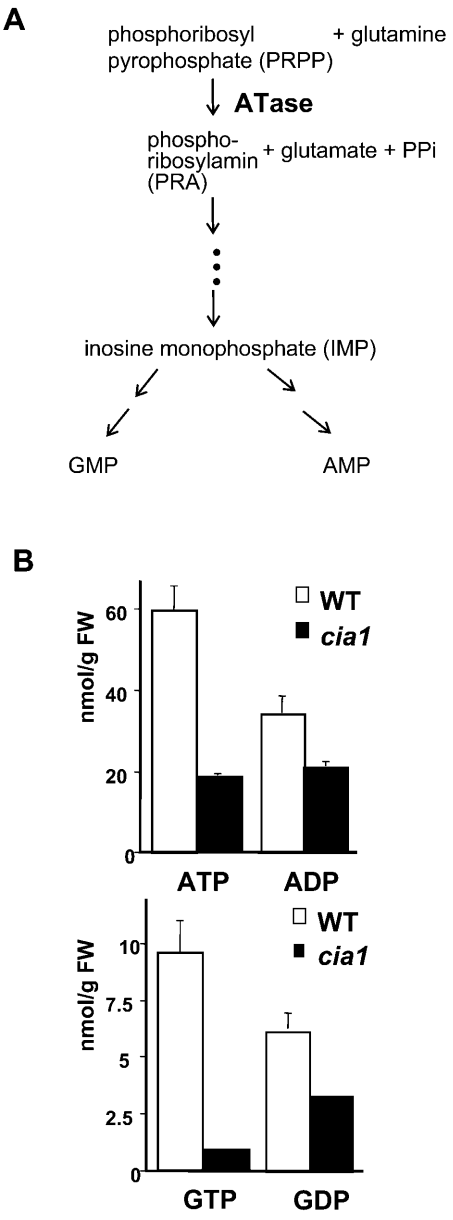

Figure 3. ATase and de novo purine biosynthesis. A, The reaction carried out by ATase. B, Purine nucleotide levels in wild type (WT) and *cia1*. Nucleotides were extracted by TCA and measured by HPLC, as described in "Materials and Methods." FW, fresh weight. Except for GDP measurement in the wild type, for which six different plants were measured, seven different wild-type plants and nine different *cia1* mutant plants were individually measured. Data points represent the average ± SE. For all four forms of nucleotides, wild type had significantly higher amounts of nucleotides than *cia1* ($P < 0.05$).

cytokinin, and NAD/NADP and coenzymes (Smith and Atkins, 2002). We next investigated whether the mutant phenotypes could be complemented biochemically by exogenous addition of the three groups of products. Addition of AMP to plant liquid cultures restored the chlorophyll content and partially restored the size of mutant plants (Fig. 4, A and B). AMP added to agar plate media had no effect (data

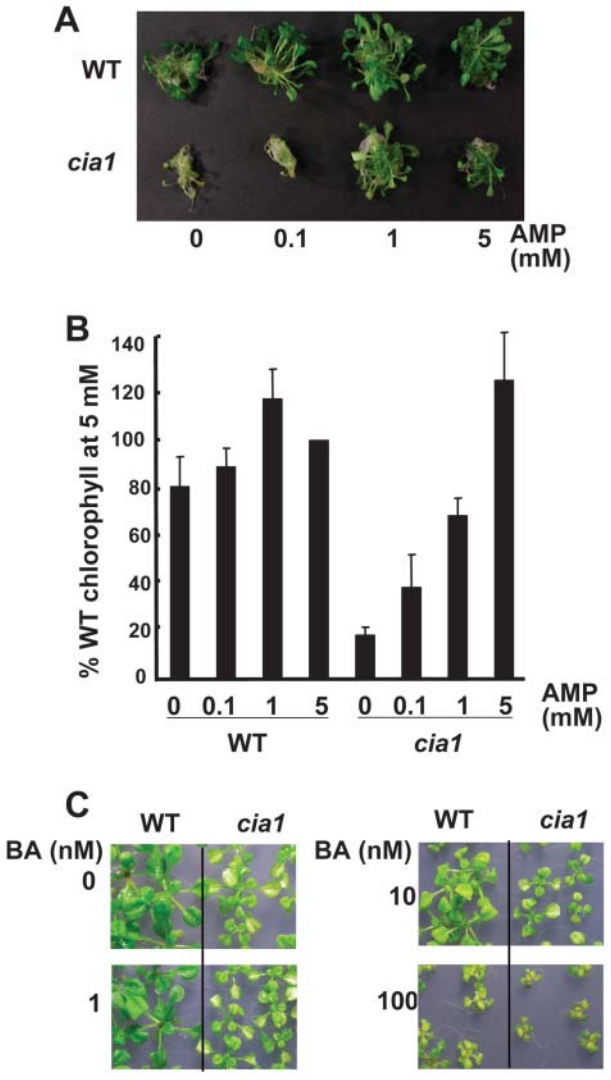

Figure 4. Complementation analysis of *cia1* by AMP and cytokinin. A, Morphology of wild-type (WT) and *cia1* mutant seedlings after growth in Murashige and Skoog liquid medium supplemented with various concentrations of AMP for 2 weeks. B, Chlorophyll content of the plants shown in A. Data points represent the average ± SD of three independent experiments except for wild type at 0.1 mM, which are from two independent experiments. At 0, 0.1, and 1 mM AMP conditions, wild type had significantly higher amounts of chlorophyll than *cia1* under the same AMP concentration ($P < 0.05$). At 5 mM ATP, the chlorophyll content of the wild type was not significantly higher than *cia1*. C, Morphology of wild-type and *cia1* mutant seedlings after growth on Murashige and Skoog agar plates supplemented with various concentrations of BA for 17 d.

not shown), suggesting that roots were unable to absorb AMP directly. The rescuing effect of AMP in liquid cultures may be due to the fact that AMP could be hydrolyzed to adenosine and adenine by phosphatases and nucleosidases, and possibly salvaged to adenine nucleotides by adenosine kinase and adenine phosphoribosyltransferase. IMP, GMP, FAD, and NADH had no effect on rescuing the mutant phenotypes even when added to plant liquid cultures (data not shown). Arabidopsis seedlings may not be able to absorb these compounds exogenously, or a deficiency in these compounds was not the major reason for the phenotypes of *cia1*. Low concentrations (1 and 10 nM) of synthetic cytokinin benzylaminopurine (BA) could not rescue the mutant phenotypes (Fig. 4C). At a higher concentration (100 nM), BA inhibited plant growth and made the mutant artificially resemble the wild type.

ATase2 Was Localized to the Stroma of Chloroplasts

We next investigated the localization of ATase2 in Arabidopsis leaf cells. The *cia1* mutant was transformed with the full-length ATase2 cDNA fused in front of five tandem repeats of cMyc tag, and the fusion construct was driven by the CaMV 35S promoter. The transformants were restored to wild-type appearance (Fig. 1D), indicating the fusion protein was functioning properly. Using the ATase2-cMyc-complemented transgenic plants, we first investigated the localization of ATase2 by fractionation. The protein detected in whole-cell extract was about 10 kD smaller than the in vitro-translated ATase2-cMyc fusion protein (Fig. 5, compare lanes 1 and 2), suggesting that

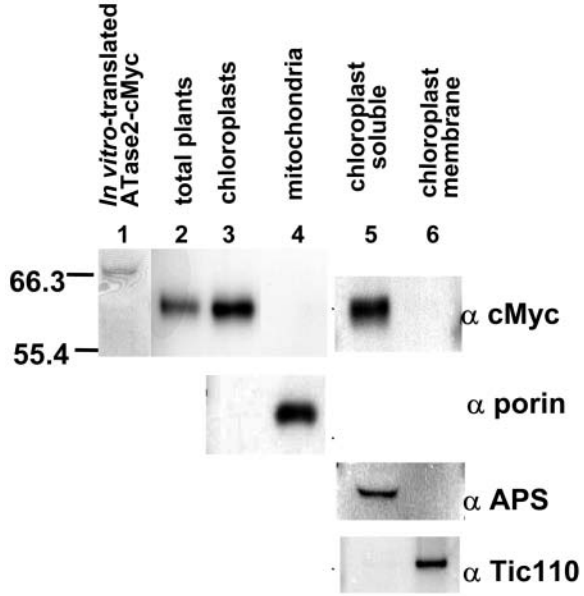

Figure 5. Localization of ATase 2-cMyc by fractionation. Chloroplasts and mitochondria (lanes 3 and 4, 6 μg of proteins in each lane) were isolated from the same batch of ATase2-cMyc-complemented *cia1* transformants. A portion of the chloroplasts was lysed and further separated into soluble and membrane fractions (lanes 5 and 6, 5 μg of proteins in each lane). Samples were analyzed by SDS-PAGE and immunoblots probed with antibodies against various proteins as indicated on the right. Lane 1, in vitro translated protein derived from the ATase2-cMyc cDNA and analyzed by SDS-PAGE and immunoblots probed with anti-cMyc monoclonal antibodies together with the chloroplast and mitochondrial fractions. Molecular masses for marker proteins ran on the anti-cMyc blot are indicated on the left. APS, Small subunit of ADP-Glc pyrophosphorylase.

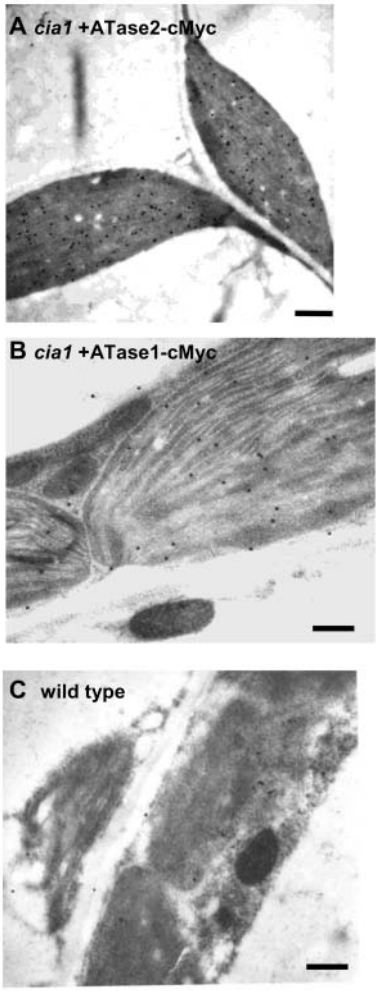

Figure 6. Localization of ATase2-cMyc and ATase1-cMyc by immunogold-labeling electron microscopy. Leaf sections from ATase2-cMyc- (A) and ATase1-cMyc-complemented (B) *cia1* transformants and the nontransformant control plant (C; wild type) were hybridized with anti-cMyc antibodies and gold-conjugated secondary antibodies. Bars = 0.5 μm.

cleavage of an N-terminal targeting signal had occurred within cells. When mitochondria and chloroplasts were isolated from the transgenic plants, ATase2-cMyc was detected only in chloroplasts, not in mitochondria (Fig. 5, lanes 3 and 4). Control hybridization with anti-mitochondrial porin antibodies showed that the chloroplast fraction was not contaminated by mitochondria. ATase2-cMyc fusion protein was further localized to the soluble fraction of chloroplasts (Fig. 5, lane 5), indicating the protein was localized in the stroma of chloroplasts. Control hybridization with antibodies against the stroma-located small subunit of ADP-Glc pyrophosphorylase and the inner envelope membrane-located Tic110 showed that the chloroplast membrane and soluble fractions were not contaminated with each other.

We further confirmed the fractionation data using electron microscopy and immunogold labeling (Fig. 6). Leaf tissue sections were hybridized with anti-cMyc antibodies and then with gold particle-conjugated secondary antibodies. Gold particles were only detected in chloroplasts of the ATase2-cMyc transgenic plants, not in other organelles, cytosol, or extracellular space (Fig. 6A). No specific hybridization was detected in nontransformed control plants (Fig. 6C).

Expression of the Three ATase Genes in Cotyledons

It has been shown that ATase2 is expressed in roots, leaves, and flowers (Ito et al., 1994). Since the cotyledons of *cia1* were wild type in appearance, we suspected that ATase2 was not expressed in cotyledons and thus no phenotype was observed there. To examine this possibility, we performed further expression analysis focusing on the cotyledons. Total RNA was isolated from cotyledons and leaves of 5- or 10-d-old wild-type seedlings and analyzed by semiquantitative reverse transcription (RT)-PCR. As shown in Figure 7, the expression of ATase2 was still higher than ATase1 in cotyledons. Therefore, it is more likely that the reason for the *cia1* mutant having normal cotyledons is that cotyledons perform much less de novo purine biosynthesis. Early processes during seed germination are mainly determined by the purine salvage pathways (Moffat and Ashihara, 2002; Stasolla et al., 2003). Adenine and guanine bases and nucleosides from storage organs are recycled by the salvage pathways, and their contribution to the overall nucleotide pool seems to be critical for the early phase of germination. The de novo pathway of purine synthesis becomes

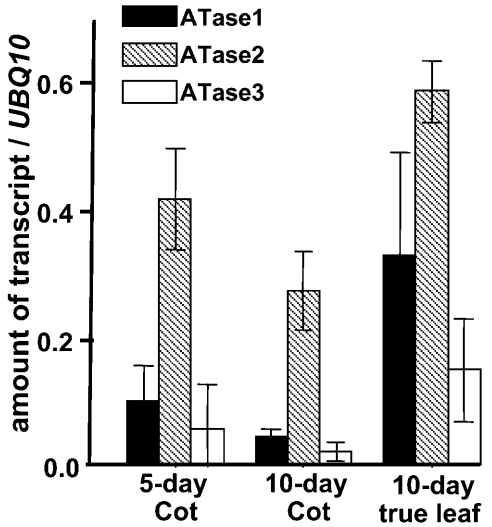

Figure 7. Expression of three ATase genes in cotyledons (Cot) from 5-and 10-d-old seedlings and the first true leaf from 10-d-old seedlings of wild-type plants. Total RNA was isolated and reverse transcribed into first-strand cDNA. Amounts of transcripts were analyzed by gene-specific primers and semiquantitative RT-PCR, and normalized to the amount of *UBQ10* transcript. Data points represent the average ± SE of three independent experiments. *ATase2* had a higher expression level than *ATase1* in 5- and 10-d-old cotyledons ($P < 0.05$).

Table I. *The cia1 mutant has reduced numbers of leaf cells compared to wild type*

	24 d	35 d
Cell Size		
Wild type	127.6 ± 34.2 143.2 ± 35.1	135.4 ± 42.4
cia1	111.9 ± 22.9 111.6 ± 26.3	108.6 ± 42.4
Cell Number		
Wild type	92.3 ± 4.9 75.5 ± 0.6	91 ± 1.0
cia1	44.6 ± 2.8 34.7 ± 2.1	48.3 ± 0.6

The first true leaves from two 24-d-old plants and one 35-d-old plant were measured. For cell-size measurement, 418 and 204 cells from 24-d-old wild-type and mutant leaves, respectively, and 55 and 39 cells from 35-d-old wild-type and mutant leaves, respectively, were measured. Wild-type leaves had significantly larger cells and a higher number of cells ($P < 0.05$).

fully operative only at later stages of germination (Ashihara, 1983; Stasolla et al., 2003).

Functional Relationship of ATase1 and ATase2

In Arabidopsis, all enzymes in the de novo purine biosynthesis pathway are encoded by single genes, except those for ATase (Smith and Atkins, 2002), adenylosuccinate lyase, and IMP dehydrogenase (Boldt and Zrenner, 2003). We therefore investigated the functional relationship between the two major isoforms, ATase1 and ATase2. The *cia1* mutant was transformed with the full-length ATase1 cDNA fused in front of five tandem repeats of cMyc tag and the fusion construct was directed by the CaMV 35S promoter. The transformants were restored to wild-type appearance (Fig. 1D), indicating that ATase1 could functionally substitute ATase2 when directed by a constitutive promoter. Furthermore, ATase1 was most likely also localized in the same subcellular compartment as ATase2. The localization of ATase1 was confirmed by immunogold labeling (Fig. 6B) to be in the stroma of chloroplasts.

We then obtained a T-DNA insertion knockout mutant of the *ATase1* gene (Salk_008888) from the Arabidopsis Biological Resource Center (ABRC; Fig. 2C). The mutant was indistinguishable from the wild type (Fig. 1A). A double mutant of *cia1/ATase1 knockout* was indistinguishable from *cia1* (Fig. 1A), suggesting that ATase2 was the major isozyme for plant growth.

Further Characterizations of *cia1* Mutant Phenotypes

We further investigated whether the smaller size of mutant leaves was due to reduced cell numbers, cell size, or both. Scanning electron microscopy revealed no major difference in cell size or numbers between the wild type and the mutant in cotyledons (data not shown). By contrast, in mature true leaves, *cia1* mutant had slightly smaller cell size but only about half the cell number of wild-type plants (Table I). This result confirmed the role of ATase in cell division.

We further characterized the protein import defect of *cia1*. We first tested the effect of supplementing ATP and GTP during the in vitro import reaction. Under sufficient light, when the wild-type chloroplasts had sufficient ATP from photosynthesis, addition of extra ATP or GTP had little effect on the import efficiency of wild-type chloroplasts (Fig. 8A). The same treatment slightly increased the amount of proteins imported in the mutant chloroplasts, suggesting that mutant chloroplasts were indeed deficient in ATP and GTP. However, the amount of proteins imported was still much less than that of wild-type chloroplasts. We further checked the amount of translocon proteins present in the mutant plastids by immunoblots. When equal numbers of plastids were analyzed, *cia1* plastids had slightly lower amounts of outer membrane translocon proteins like Toc159 and Toc33 (Fig. 8B). However, it is unclear whether this small reduction in some

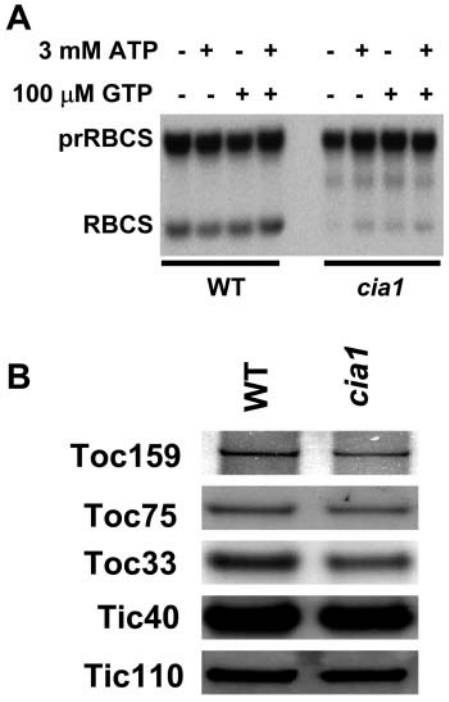

Figure 8. Analysis of the protein import defect of *cia1* chloroplasts. A, Effect of adding ATP and GTP to import reactions. Chloroplasts were isolated from 28-d-old wild-type (WT) and *cia1* mutant plants and used to perform in vitro protein import experiments supplemented with the indicated amounts of either ATP, GTP, or both. Positions of the precursor form of RBCS (prRBCS) bound on the chloroplast surface or mature RBCS imported are indicated. B, Amount of translocon proteins in wild-type and *cia1* mutant chloroplasts. Chloroplasts were isolated from 28-d-old wild-type and *cia1* mutant plants and analyzed by SDS-PAGE and immunoblots probed with antibodies against various translocon proteins as indicated on the left. Equal numbers of plastids were loaded in each lane.

translocon components could result in the import defect observed in *cia1* mutant chloroplasts.

DISCUSSION

Although de novo purine biosynthesis has been studied extensively in animals and legumes of higher plants, studies in other plant species have been relatively few. Arabidopsis offers the advantage of a fully sequenced genome and vast resources of genetic and genomic tools. Our characterization of the Arabidopsis ATase mutants offered insights into the roles of de novo purine biosynthesis in normal plant physiology in non-N-fixing plants. We showed that both ATase1 and ATase2 were located in chloroplasts of leaf cells. We confirmed the role of de novo purine biosynthesis in cell division and further showed that its deficiency has a detrimental effect in chloroplast biogenesis. Because the *cia1* mutant plants have very low levels of ATP and GTP (Fig. 3B), a decrease in energy charge may be altering growth and chloroplast protein import significantly. The exact mechanism linking de novo purine biosynthesis to chloroplast biogenesis requires further study.

In Arabidopsis, ATase is one of the few enzymes in the de novo purine biosynthesis pathway that is encoded by multiple genes. Therefore, it is interesting to study the role played by each isozyme. From the expression pattern and our mutant analysis, ATase2 is clearly the major isoform. However, although the *cia1/ATase1 knockout* double mutant has the phenotype of *cia1* and the expression level of ATase3 is very low, our preliminary data indicate that a *cia1* mutant transformed with an ATase1 antisense construct is lethal (data not shown), suggesting ATase3 or a yet unknown mechanism of purine biosynthesis is responsible for the survival of the *cia1/ATase1 knockout* double mutant.

The mosaic patterns of *cia1* mutant leaves are irregular and variable. We have observed that, under conditions that favor rapid growth, e.g. high light or supplementing Suc to growth media, more white sectors are produced. By contrast, under low light or on media without Suc, although *cia1* plants grow even smaller, *cia1* leaves can be evenly pale green without white sectors (data not shown). It is possible that there is a threshold level of purine nucleotides that needs to be met for normal chloroplast biogenesis. Under rapid growth conditions, purine nucleotides are channeled into cell division, the purine nucleotide level in the mutant cytosol drops below the threshold, and chloroplasts fail to develop normally. White sectors are then observed on mutant leaves. When the division and growth rate of leaf cells are slower, purine nucleotides have time to accumulate in the cytosol of mutant cells, eventually reaching the threshold level.

The isolation of *cia1-1* from our screen indicated that a deficiency in ATase2 would decrease chloroplast protein import efficiency since our screen is based on accumulation or transient retention of chloroplast precursor proteins in the cytosol (Sun et al., 2001). Import experiments using chloroplasts isolated from the mutant confirmed this defect. However, supplementation of additional ATP and GTP to the import reaction did not restore the import efficiency of mutant chloroplasts to the wild-type level. It is possible that the *cia1* mutation could have an effect on the transport of ATP into the stroma of chloroplasts, where ATP is used during protein translocation (Theg et al., 1989; Leheny and Theg, 1994), e.g. by affecting the amount of the ATP/ADP translocator on the chloroplast inner envelope membrane. Alternatively, a deficiency in purine nucleotides or other compounds derived from the same pathway may have caused irreversible damage to chloroplasts. A small, but consistent, reduction of several Toc translocon components was observed. It is not clear whether such a small reduction could cause the import defect observed in the in vitro import experiments. More studies are required to understand the relationship between de novo purine biosynthesis and reduced chloroplast protein import, defective chloroplast biogenesis, and the irregular leaf mosaic patterns.

MATERIALS AND METHODS

Molecular Cloning of *CIA1*

The *cia1-1* mutant was isolated by a transgene-based screening strategy as described by Sun et al. (2001). All molecular characterizations of the *cia1* mutant were performed with lines that had been back-crossed at least three times. The *cia1* mutant was crossed to the ecotype Landsberg *erecta* of Arabidopsis. DNA was isolated from 1,025 F_2 mutant seedlings for mapping of the *cia1* locus. CAPS and simple sequence length polymorphism markers, as shown in Figure 2A, were used for positional cloning. Full-length cDNA of *CIA1* was amplified from first-strand cDNA of total leaf mRNA by *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and subcloned into the *Sma*I site of a modified pCAMBIA 1390 containing a CaMV 35S promoter. This subclone was transformed into *Agrobacterium tumefaciens* and introduced into the *cia1* mutant by the floral dip method (Clough and Bent, 1998).

In Vitro Translation of Precursor Proteins, Chloroplast Isolation, and Protein Import

The plasmids containing cDNA clones for the precursors to RBCS, PC, and CAB of PSII were first transcribed in vitro and then translated with [^{35}S]Met (Perry et al., 1991) in wheat germ extracts (purchased from Promega, Madison, WI). ATase2-cMyc was transcribed and translated in the TNT-SP6-coupled system (Promega).

For chloroplast isolation, Arabidopsis plants were grown on 1× Murashige and Skoog agar medium with Gamborg's vitamins and 2% Suc at 22°C under a 16-h-light/8-h-dark cycle for 4 weeks. Chloroplasts were isolated using the protoplast procedure as described by Fitzpatrick and Keegstra (2001). For in vitro import experiments, chloroplasts were incubated with [^{35}S]Met-labeled precursor in import buffer supplemented with ATP, GTP, or both. Reactions were incubated at room temperature for 15 min and stopped by sedimenting the intact chloroplasts through a 40% Percoll cushion. Intact chloroplasts were washed once with import buffer and analyzed by SDS-PAGE on 4% to 12% NuPAGE gels (Invitrogen, Carlsbad, CA) with MES running buffer. Pictures of gels were taken from x-ray films generated by exposing the dried gels to Kodak (Rochester, NY) MS film with a BioMax TranScreen-LE intensifying screen.

Immunoblotting of Translocon Proteins

The number of chloroplasts was counted on a hemocytometer under a phase-contrast microscope. After electrophoresis, proteins were transferred to Immobilon-P PVDF membranes (Millipore, Bredford, MA). The membranes were blocked for 30 min with Tris-buffered saline plus Tween 20 (TBST; 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, and 0.05% Tween 20) containing 5% (w/v) nonfat dry milk. Antibodies against Toc75, Arabidopsis Toc33, Toc159, Tic110, and Tic40 were generated against *Escherichia coli* overexpressed and purified full-length pea Toc75 precursor, Arabidopsis Toc33 residues 1 to 256, Arabidopsis Toc159 residues 1,224 to 1,503, Arabidopsis Tic110 residues 431 to 1,016, and Arabidopsis Tic40 full-length precursor (Chou et al., 2003). Except for the Tic40 construct, all constructs were in the pET22b vector (Novagen, Madison, WI) with a C-terminal 6× His tag. Alkaline phosphatase-conjugated secondary antibodies were used for detection. Mouse monoclonal anti-cMyc antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Biochemical Complementation of *cia1*

Wild-type and mutant plants were grown in 1× Murashige and Skoog liquid media with Gamborg's vitamins, 2% Suc, and 0.05% MES under continuous light at 22°C with constant orbital shaking at 125 rpm. Various amounts of AMP or BA, as indicated in the figures, or 0.1, 1, or 5 mM of IMP, GMP, or NADP were added to the liquid media. Continual observation of phenotypes was made during 4 weeks. Seedling chlorophyll contents were measured as described by Lichtenthaler (1987).

Nucleotide Measurements

Wild-type and mutant plant leaf materials (500–600 mg) were frozen in liquid nitrogen and homogenized. Nucleotides were extracted by the trichloroacetic acid (TCA) method described by Hajirezaei et al. (2003). The nucleotide content in the TCA extracts was measured by HPLC using a Kontron HPLC system (Kontron, Eching, Germany) fitted with a Partisil-SAX anion-exchange column and detected by absorption at OD_{254}. Nucleotides were identified and quantified by comparison with respective nucleotide standards as described by Geigenberger et al. (1997).

Construction of Transgenic Plants

DNA encoding five tandem copies of cMyc was amplified from plasmid Myc6mNotch1C and cloned into the *Eco*RV site of pSP72 (Promega), producing the plasmid pSP72-cMyc. The ATase2 full-length cDNA was amplified from first-strand cDNA of Arabidopsis leaf mRNA (Sun et al., 2001) with an N-terminal primer containing a *Bam*HI site at the end and a C-terminal primer containing an *Eco*RI site at the end and mutating the stop codon to Glu. The amplified fragment was subcloned into the *Bam*HI/*Eco*RI site of pSP72-cMyc. The ATase2-cMyc fragment was then excised with *Pvu*II and *Eco*RV and cloned into the *Sma*I site of pCAMBIA 1390 containing a 35S promoter.

DNA encoding five tandem copies of cMyc was amplified from plasmid Myc6mNotch1C and cloned into the *Eco*RV site of pBluescript SK (Stratagene), producing plasmid pBluescript-cMyc. ATase1 full-length cDNA was amplified from first-strand cDNA of Arabidopsis leaf mRNA with an N-terminal primer containing an *Xba*I site at the end and a C-terminal primer containing a *Bam*HI site at the end and mutating the stop codon to Gly. The amplified fragment was subcloned into the *Bam*HI/*Xba*I site of pBluescript-cMyc. The ATase1-cMyc fragment was then excised with *Hin*cII and *Xba*I, and cloned into the *Sma*I/*Xba*I site of pCAMBIA 1390 containing a 35S promoter. The ATase1-cMyc and ATase2-cMyc pCAMBIA 1390-35S constructs were introduced into Arabidopsis by Agrobacterium-mediated transformation.

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes.

Fractionation

Protoplasts were isolated from 4-week-old ATase2-cMyc transgenic plants and lysed by forcing them through two layers of nylon mesh (20 and 10 μm; Fitzpatrick and Keegstra, 2001). Chloroplasts and mitochondria were isolated from the lysate (Werhahn et al., 2001). In brief, chloroplasts were collected by centrifugation at 4,200*g* for 5 min and then purified on a 50% Percoll continuous gradient. For the mitochondrial fraction, supernatant from the 4,200*g* centrifugation was further centrifuged at 3,000*g* for 5 min and then at 17,000*g* for 20 min, and the pellet fraction was separated on a Percoll step gradient consisting of 1:1:1 ratio of 40%:23%:18% Percoll by centrifugation at 70,000*g* for 45 min. Mitochondria were retrieved from the 23%/40% Percoll interface. To get chloroplast subfractions, total chloroplasts were lysed hypotonically by resuspending in 25 mM HEPES/KOH, pH 8.0, and 4 mM $MgCl_2$, and separated into soluble and membrane fractions by centrifugation at 100,000*g* for 45 min.

Electron and Confocal Microscopy and Immunogold Labeling

Leaves from wild-type and transgenic plants were cut into small sections (about 2 mm^2) and then fixed in 0.1 M phosphate buffer (pH 7.0) with 4% (w/v) paraformaldehyde and 0.1% (v/v) glutaraldehyde overnight at 4°C. The leaf sections were washed with 0.1 M phosphate buffer (pH 7.0), dehydrated with a series of ethanol solutions, and infiltrated with London Resin Gold (Electron Microscopy Sciences, Fort Washington, PA). Polymerization was conducted with UV (360 nm) at −20°C for 24 h and then at room temperature for 48 h. Ultrathin sections were put on nickel grids. The grids were first blocked with TBST buffer containing 4% (w/v) bovine serum albumin at room temperature for 1 h, and then incubated with 1:25 diluted anti-cMyc rabbit polyclonal antibodies (Santa Cruz Biotechnology) for 1 h. The grids were then washed in TBS, incubated for 1 h with 20 nm gold-conjugated goat anti-rabbit IgG (dilution 1:25; British Biocell International, Cardiff, UK), and washed in TBS and then distilled water. Selected sections were further stained with uranyl acetate.

Cell numbers and size were examined by cutting the first true leaves of 24- or 35-d-old wild-type or mutant seedlings at the base of petioles. The lower side of leaves was examined directly with a Zeiss (Jena, Germany) LSM 510 laser scanning confocal microscope. Three rows of cells along the vein were measured and counted from the base of the petiole to two-thirds of the length of the excised leaves. Cell sizes were determined using the program Metamorph (Adobe, Mountain View, CA). Cell numbers were counted by assembling continuous images into one single picture of the leaves.

Quantification of ATase Transcripts

Amounts of ATase transcripts were analyzed by RT-PCR. RNA was isolated from cotyledons and the first true leaves of 5- and 10-d-old seedlings using Trizol (Invitrogen, Carlsbad, CA) and reverse transcribed into first-strand cDNA (Sun et al., 2001). Semiquantitative RT-PCR was performed as described (Halford, 1999). In brief, the detection linear range of the ATase primers and UBQ10 primers was first tested by amplifying with 16, 19, 22, and 25 cycles of PCR and then the optimal cycle numbers were selected. All cDNA samples were then amplified with the UBQ10 primers to determine their relative cDNA concentration. Samples with the same amount of first-strand cDNA were then amplified with the ATase and UBQ10 primers according to the optimized cycle numbers previously determined. PCR products were analyzed with agarose gels and stained with SYBR Green I (Sigma-Aldrich, St. Louis). Fluorescent signal intensities were quantified with a LAS1000 plus Luminescence Image Analyzer (Fuji Film, Tokyo). Sequences of the specific primers are as follows: UBQ10 (forward, 5′-ggatctcactcgcgaccg-3′; reverse, 5′-cttcttaagcataacagagacgag-3′), ATase1 (forward, 5′-gcttaagttgtctcctgttcgtgg-3′; reverse, 5′-gcttaagttgtctcctgttcgtgg-3′), ATase2 (forward, 5′-cctctaaatcagctctcaatttcg-3′; reverse, 5′-caactccttcgtttccgctcagag-3′), and ATase3 (forward, 5′-gtgtatgaaacacgccgcatgtac-3′; reverse, 5′-ctatcttcaaactcgtggtaccgc-3′).

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