ATP represents the universal energy currency of all living cells. Due to both size and charge, adenylates do not cross biomembranes freely, making the involvement of highly specific transport proteins necessary. In eukaryotic cells mitochondria export ATP previously generated via oxidative phosphorylation at the matrix site in strict counter exchange to cytosolic ADP. The corresponding ADP/ATP carriers (AAC) function as dimers, comprising two identical subunits, each exhibiting six predicted transmembrane domains (Klingenberg, 1989). AAC proteins belong to the best characterized solute transporters and are the subject of numerous publications (Fiore et al., 1998).

We identified the plastidic ATP/ADP transporter as a second type of eukaryotic-adenylate carrier protein (Kampfenkel et al., 1995). Plastidic ATP/ADP transporters exist in all higher and lower plants analyzed so far (Linka et al., 2003), exhibit 11 to 12 predicted transmembrane domains (Winkler and Neuhaus, 1999), and do not show substantial structural similarities to the functional AAC homologs in mitochondria (Winkler and Neuhaus, 1999). In Arabidopsis (Arabidopsis thaliana), two plastidic ATP/ADP transporters are present, and both exhibit very similar biochemical transport properties when heterologously expressed in Escherichia coli (Mühlmann et al., 1998; Tjaden et al., 1998b).

The main function of plastidic ATP/ADP transporters is the supply of storage plastids with ATP (Schünemann et al., 1993; Kang and Rawsthorne, 1994; Neuhaus and Emes, 2000). In potato (Solania tuberosum) tubers, the plastidic ATP/ADP transporter exerts significant control on starch accumulation (Tjaden et al., 1998a), leading to a high-flux control coefficient within this metabolic pathway (Geigenberger et al., 2001). In contrast, a recently made metabolite flux analysis on developing rapeseed (Brassica napus) embryos indicated that ATP import into corresponding plastids is not required to achieve high rates of lipid biosynthesis (Schwender et al., 2004).

All orthologs of the plastidic ATP/ADP transporter, e.g. the two isoforms from Arabidopsis, a potato ortholog, or an ortholog from the primitive red alga Galderia sulfuralia, exhibit similar transport properties in respect to substrate specificity and substrate affinity (Mühlmann et al., 1998; Tjaden et al., 1998a, 1998b; Linka et al., 2003). Therefore, the observation of similar transport properties and the contradictory information on the involvement of plastidic ATP/ADP transporters in plastidic storage product synthesis (Tjaden et al., 2004)
expression analysis of AtNTT1

RESULTS

Expression Analysis of AtNTT1 and AtNTT2

Arabidopsis possesses two isoforms of the plastidic ATP/ADP transporter with similar biochemical transport properties. To reveal whether the presence of two independent transporter genes correlates with an organ- or development-specific expression pattern, we analyzed both the relative mRNA accumulation by northern-blot analysis and the promoter activity in transgenic plants carrying corresponding promoter-β-glucuronidase (GUS) fusions.

For reliable northern-blot analysis of isoform-specific mRNA accumulation it is required to use gene-specific probes. We generated probes specific for either AtNTT1- or AtNTT2 mRNA by using corresponding 3’-untranslated cDNA fragments (Fig. 1A). Although there is some minor cross hybridization, the probes used exhibited a sufficiently high specificity (Fig. 1A).

AtNTT1 mRNA accumulated strongest in root and stem tissue, and less in source leaves (Fig. 1B). In flowers and siliques the level of AtNTT1 mRNA was below or close, respectively, to the detection level (Fig. 1B). In contrast, AtNTT2 mRNA accumulated to similar amounts in roots, leaves, stem, and flower tissue (Fig. 1B). Similar to AtNTT1, the AtNTT2 mRNA was much less present in siliques (Fig. 1B).

To gain first evidence on the expression pattern of both plastidic ATP/ADP-transporter genes during early germination, we monitored the relative mRNA abundance within the first 6 d of development. Within this time span Arabidopsis develops a primary root and gains photosynthetic competence as revealed by accumulation of both chlorophyll and chlorophyll a/b-binding protein (CAB) mRNA (Fig. 1C). The level of AtNTT1 mRNA within this period of development remained close to the detection level without any substantial changes (Fig. 1C). This is different from the expression of AtNTT2, as latter mRNA strongly
accumulated in days 1 and 2, and declined from day 3 to a level still above the \textit{AtNTT1} mRNA (Fig. 1C).

To reveal whether plastidic ATP/ADP-transporter gene expression responds on altered sugar availabilities, we floated source leaf discs in either water (control) or 100 mM Glc or Suc (Fig. 1D). The incubation of leaf discs for 24 h in water did not alter the levels of \textit{AtNTT1} or \textit{AtNTT2} mRNA (Fig. 1D). In contrast, the presence of Glc or Suc strongly increased the accumulation of \textit{AtNTT1} mRNA but did not influence \textit{AtNTT2} mRNA concentration (Fig. 1D).

To perform a second independent approach to study regulation of gene expression, we generated transgenic plants harboring either an \textit{AtNTT1-promoter::GUS-} or \textit{AtNTT2-promoter::GUS} gene, respectively. During the first 6 d of development the \textit{AtNTT1} promoter is hardly active (Fig. 2A). At day 2 \textit{AtNTT1} promoter activity is slightly detectable in the center of the primary root (Fig. 2A), and at day 6 cells comprising the vascular structures in photosynthesizing cotyledons exhibited \textit{AtNTT1} promoter activities (Fig. 2A). In contrast to this, \textit{AtNTT2} promoter activity is very high at day 1, especially in the root tip and developing cotyledons (Fig. 2A). At day 3, highest \textit{AtNTT2} promoter activity is detectable in the root hair zone and at the basis of cotyledons. At day 6, we still observed

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**Figure 2.** Histochemical localization of \textit{GUS} expression under the control of either the \textit{AtNTT1} or \textit{AtNTT2} promoter in Arabidopsis seedlings and mature tissues. 

A, \textit{Promoter-GUS} activity in developing seedlings. Seeds were sown on Murashige and Skoog agar plates and harvested after 1, 2, 3, and 6 d after imbibition and analyzed for \textit{GUS} activity according to standard protocols. B, \textit{Promoter-GUS} activity in source leaves. Rosette leaves were harvested from plants, grown under short-day conditions, and \textit{GUS} stained. C, \textit{GUS} expression in flowers at different developmental stages.
strong AtNTT2 promoter activity in the root and in rapidly developing secondary leaves (Fig. 2A), still representing strong sinks.

In source leaves, both promoters are barely active. AtNTT1 promoter-GUS activity is detectable only in the vascular bundles located at the edge of the leaf (Fig. 2B), whereas AtNTT2 promoter activity was hardly detectable in the leaf. This result does not necessarily contradict the northern-blot analysis (Fig. 1B), as latter reflect the sum of AtNTT mRNA in total leaf tissue. In both flower tissue and developing siliques the AtNTT1 promoter activity is below the detection level (Fig. 2C). Petal crown leaves showed slight AtNTT2 promoter activity, which was, however similar to AtNTT1 promoter activity, nearly absent in developing siliques (Fig. 2C).

**Generation of Arabidopsis Mutants Exhibiting Reduced or Abolished Plastidic ATP/ADP-Transporter Gene Expression**

In the SALK library we identified a putative AtNTT1 knockout line exhibiting the T-DNA insertion in exon 1 (Fig. 3A). Corresponding heterozygous plants have been selfed to obtain homozygous mutants. By use of the gene-specific primers NTT1/1 and NTT1/2, we were able to amplify a PCR product of the expected size (about 2.4 kb) on wild-type DNA but not on DNA from AtNTT1::T-DNA plants (Fig. 3B).

The PCR product amplified on wild-type DNA has been sequenced to confirm the correct nucleotide sequence (data not shown). Using the primers NTT1/2 and left borderer (LB) we amplified a PCR product on DNA obtained from mutant plants but not from wildtype plants (Fig. 3B). The PCR product has been sequenced to confirm the insertion site (data not shown). To check that the T-DNA insertion into the AtNTT1 gene correlates with absence of the corresponding mRNA, we performed a reverse transcription (RT)-PCR analysis (Fig. 3C). As expected, we were able to demonstrate the presence AtNTT1 mRNA in wild-type leaf tissue but not in leaves from AtNTT1::T-DNA plants (Fig. 3C). Similarly, a northern-blot analysis demonstrated the presence of AtNTT1 mRNA in wild-type leaves but not in AtNTT1::T-DNA leaves (Fig. 3D, left section). Remarkably, the absence of AtNTT1 mRNA (Fig. 3, C and D) is not compensated by an increase of AtNTT2 mRNA (Fig. 3D, right section).

A putative AtNTT2 knockout line was available in the GARLIC library carrying the T-DNA insertion in exon 2 (Fig. 4A). Heterozygous plants have been grown and selfed to obtain a homozygous knockout line. The combination of the gene-specific primers NTT2/2 and NTT2/4 allowed amplification of a PCR product of the expected size (about 2.4 kb) on genomic wild-type DNA but not on DNA from homozygous AtNTT2::T-DNA plants (Fig. 4B). The use of the gene-specific primer NTT2/2 and the LB primer allowed amplification of a fragment of about 1.3 kb on genomic DNA from AtNTT2::T-DNA plants but not in wild-type DNA (Fig. 4B). The PCR products have been sequenced to demonstrate that the correct DNA fragments have been amplified and to confirm the position of the T-DNA insertion (data not shown). To prove that the T-DNA insertion in the AtNTT2 gene correlates with the absence of the corresponding mRNA, we performed RT-PCR and northern-blot analysis. The gene-specific primers NTT2/2 and NTT2/4 allowed to amplify a PCR product of the expected size on cDNA prepared from wild-type leaf tissue but not on cDNA prepared from AtNTT2::T-DNA plants (Fig. 4C). Similar to this, the northern-blot analysis revealed the absence of AtNTT2 mRNA in the homozygous knockout plants but showed the presence of this mRNA in wild-type leaf tissue (Fig. 4D, left section).

Although both genes, AtNTT1 and AtNTT2, reside on chromosome 1, we crossed homozygous AtNTT1 and AtNTT2::T-DNA lines to receive null mutants (AtNTT1::T-DNA), lacking both functional plastidic ATP/ADP-transporter genes. Due to the relatively wide distance of both genes on chromosome 1, it appeared likely to receive null mutants due to crossover during meiosis. We screened about 100 indepen-
dent plants and identified 5 plants lacking intact genes from both transporters. That these plants represent homozygous null mutants has been demonstrated by PCR on genomic DNA (Fig. 5, A and B). The primer combinations NTT1/1 and NTT1/2, and NTT2/2 and NTT2/4, respectively, allowed amplification of expected PCR products on genomic DNA from wild-type but not from AtNTT1-2::T-DNA plants (Fig. 5A). The use of the primer combinations NTT1/2 and LB, and NTT2/2 and LB, in contrast, allowed amplification of expected DNA fragments on DNA from homozygous AtNTT1-2::T-DNA plants but not on DNA isolated from wild-type plants (Fig. 5B).

To prove that putative effects connected with the absence of functional AtNTT1 or AtNTT2 genes, or which are present in the double-knockout line are really due to reduced levels of corresponding gene products, we created further transgenic plant lines exhibiting strongly reduced levels of both mRNA species due to an RNAi effect. For this, we cloned a 418-bp fragment from AtNTT1 (corresponding to base positions 1,006–1,424 in the AtNTT1 cDNA; Kampfenkel et al., 1995) in sense and antisense orientation into the Hannibal vector (Fig. 5C). This cDNA fragment of AtNTT1 exhibits 92% sequence identity to the corresponding cDNA domain in AtNTT2 (see Möhlmann et al., 1998) leading to the expectation that the final RNAi construct might reduce the levels of both mRNA species simultaneously. After transformation of Arabidopsis plants, we received various independent transgenic plants with strongly reduced levels of AtNTT1 and AtNTT2 mRNA (Fig. 5D). Especially in lines 9, 10, and 14, both mRNA species were below the detection level (Fig. 5D). The absence of highly specific antisera detecting plastidic ATP/ADP transport proteins so far prevents a quantification of the final NTT protein levels in RNAi lines. However, as all RNAi lines generally exhibited physiological characteristics similar to null mutants (see below), we assume that they contained very strongly reduced transport activities, usually named knock down mutants.

Analysis of Germination and Growth Pattern of Wild-Type and Transgenic Arabidopsis Plants

After 1 d of germination, Arabidopsis shows a primary root of about 3 to 4 mm length, exhibiting a root hair zone of about 1 mm following the root tip (Fig. 6A). Mutant plants lacking the functional transporter

![Figure 4. Molecular characterization of homozygous AtNTT2 knockout mutants. A, Analysis of the AtNTT2-T-DNA-insertion line Garlic_288_E08.b1a.Lb13FA (designated AtNTT2::T-DNA). The insertion in AtNTT2::T-DNA is localized in the second exon. The primers used for PCR analysis are marked as arrows. Primer NTT2/4 was chosen from the AtNTT2-promoter region, primer NTT2/2 from the 3′-untranslated region, and GARLIC_LB-primer from the left border of the T-DNA. B, PCR analysis on genomic DNA of wild-type (WT) and homozygous AtNTT2::T-DNA mutants. C, RT-PCR analysis of the expression of the AtNTT2-genes in wild-type and in AtNTT2::T-DNA mutant plants. cDNA was isolated from rosette leaves. Actin PCR revealed correct PCR conditions. D, Northern-blot analysis of wild-type and AtNTT2::T-DNA mutant plants. Total RNA was extracted from rosette leaves. EtBr staining revealed equal RNA loading. Blots were hybridized with AtNTT1 or AtNTT2 specific probes, respectively.](https://www.plantphysiol.org/doi/10.1104/pp.136.1.3528)

![Figure 5. Molecular characterization of the double-knockout mutant (designated AtNTT1-2::T-DNA) and RNAi mutant. A, Amplification of AtNTT1- and AtNTT2-specific PCR products. B, Identification of the T-DNA in the AtNTT1 and AtNTT2 gene in the double-knockout mutant. C, Structure of the RNAi construct. A 418-bp fragment from AtNTT1cDNA was cloned in sense and antisense orientation into the pHANNIBALL vector. D, Northern-blot analysis of wild-type plants and different RNAi-lines. Five independent RNAi-lines were tested. Total RNA was extracted from rosette leaves. EtBr staining shows equal RNA loading. Blots were hybridized with AtNTT1 or AtNTT2 specific probes, respectively.](https://www.plantphysiol.org/doi/10.1104/pp.136.1.3528)
gene *AtNTT1* showed a similar size and shape of the primary root as wild-type roots (Fig. 6A). In contrast, mutant plants lacking a functional *AtNTT2* gene and RNAi line 10 exhibited less developed primary roots (Fig. 6A). RNAi lines 9 and 14 showed similarly reduced roots (data not shown). After 1 d of germination, *AtNTT2::T-DNA* plants and RNAi lines showed a substantially reduced number of rooted seedlings when compared to wild-type or *AtNTT2::T-DNA* plants (Fig. 6B).

To reveal whether plastidic ATP/ADP transporters are important for development of photosynthetically competent chloroplasts we analyzed chlorophyll accumulation within the first days of development. For this we germinated wild-type and mutant seeds for 5 d in a growth chamber under short-day conditions and analyzed the resulting chlorophyll content. Wild-type and *AtNTT1::T-DNA* seedlings showed similar levels of chlorophyll (Fig. 7, A and B). In contrast to this, knockout plants lacking a functional *AtNTT2* gene; RNAi lines 10, 9, 14; and the null mutant showed a reduced seedling size (Fig. 7A; data not shown) and a strongly reduced average chlorophyll content (Fig. 7B; data not shown). Wild-type plants contained about 0.33 μg chlorophyll/plant, whereas chlorophyll in *AtNTT2::T-DNA* plants amounted to only 0.20 μg/plant (Fig. 7B). Both plants from RNAi line 10 and null mutants contained less than one-sixth of the chlorophyll present in wild-type seedlings (Fig. 7B).

To reveal whether the reduced chlorophyll level observed in some mutant lines is due to an impaired chlorophyll biosynthesis per se or might also correlate with alterations of the whole thylakoid system we examined the chloroplast ultrastructure by transmission electron microscopy. The ultrastructure of chloroplasts in 5-d-old wild-type plants exhibits a well-organized intraorganell membrane system, comprising grana and stroma thylakoids (Fig. 7C). In contrast, low-chlorophyll-containing chloroplasts from RNAi line 10 exhibited less thylakoids; especially the number of grana stacks appeared to be strongly reduced in this mutant (Fig. 7C).
To complete our picture on the effects of altered NTT gene expression on chloroplast development, we additionally analyzed the accumulation of nuclear-encoded chloroplast protein during initiation of deetiolation. For this, we germinated wild-type, RNAi, and null mutant seedlings for 6 d in the dark and illuminated etiolated seedlings for 8 or 24 h (at 100 μmol quanta m⁻² s⁻¹). Subsequently, the change in the chlorophyll content was monitored, and the accumulation of chlorophyll-binding protein CP24, as indicator for altered plastidic protein import/maturity capacity, was examined by western-blot analysis.

After 6 d of dark incubation, the chlorophyll levels in wild-type and all mutant seedlings were similarly low and amounted to less than 0.01 mg/plant (Fig. 8A). After 8 and 24 h of illumination the chlorophyll level in wild-type leaves increased already to about 0.045 mg/g fresh weight (FW) and 0.160 mg/g FW, respectively (Fig. 8A). In contrast, RNAi lines 10, 9, 14, and the null mutant showed much less chlorophyll accumulation, amounting to only about 0.050 mg/g FW after 24 h of illumination (Fig. 8A; data not shown). This reveals that also during the sudden induction of deetiolation, mutant plants showed a reduced capacity for chlorophyll synthesis.

In none of the Arabidopsis lines analyzed was CP24 detectable after 6 d of dark germination (Fig. 8B). However, appreciable levels of CP24 were present in wild-type seedlings after already 8 h of light induction (Fig. 8B). CP24 levels in corresponding RNAi tissue (lines 10, 9, and 14) and null mutants were significantly lower than in wild-type tissue (Fig. 8B, and data not shown). After 24 h of light incubation, CP24 levels were high in wild-type tissue and still significantly lower in RNAi- (lines 10, 9, and 14) and null mutants (Fig. 8B; data not shown).

Besides the direct import of ATP via a plastidic ATP/ADP transporter, plastids may regenerate endogenous ATP via glycolytic enzyme activities. To raise evidence on an up-regulation of plastidic glycolytic activity during deetiolation in mutants, we quantified the level of mRNAs encoding enzymes and transporters involved. For this we germinated wild-type or RNAi plants for 6 d in the dark and illuminated subsequently etiolated seedlings for 8 h before cDNA was prepared. Gene-specific primers were chosen to amplify about 500-bp fragments coding for either plastidic phosphoglycerate kinases 1 and 2; pyruvate kinases 1, 2, and 3; or plastidic triose P/P, Glc 6-P/P, phosphoenolpyruvate/P transporters 1 and 2, and xylose 5-P/P transporter. We observed increased mRNA levels of plastidic PGK1, PKI, and PK3 in RNAi plants compared to wild-type plants (Fig. 9), whereas the mRNA levels of the PGK2, PK2, and all plastidic phosphate transporters have not been changed substantially in mutant tissues (Fig. 9).

Wild-type and AtNTT1-::T-DNA plants grown for 50 d under short-day conditions exhibited an average rosette size of about 12 cm (Fig. 10A; data not shown). AtNTT2-::T-DNA plants, RNAi, or null mutants showed, however, a strongly reduced average size of the leaf rosette, approaching only 6 and 3 cm on average (Fig. 10A; data not shown). Interestingly, under long-day conditions (16 h light/d), the growth difference between wild-type plants and null mutants is nearly abolished (Fig. 10B).

The observation that RNAi and null mutants exhibited severely impaired growth tempted us to study physiological and morphological changes in these mutants in more detail. As the impaired growth is due to processes connected to a reduced plastidic ATP supply under conditions of long-night phases we first focused on changes in starch levels at the end of the day and night phase. However, we did not observe specific changes in transitory starch metabolism in AtNTT1-; AtNTT2-::T-DNA; RNAi lines 10, 9, and 14; or in null mutants when compared to wild-type leaves (plants were grown under short-day conditions). All plant lines exhibited starch contents equivalent to about 30 μmol C6/mg chlorophyll at the end of the day and about 7.5 μmol C6/mg chlorophyll at the end of the night.

**Seed Quality Produced by Arabidopsis Plants with Reduced Plastidic ATP/ADP-Transporter Activity**

To analyze the effect of reduced plastidic ATP/ADP-transporter activity on seed quality, we grew...
Arabidopsis wild-type and mutant plants under long-day conditions, a light period required for induction of flowering. Fully developed seeds from wild type, AtNTT1- and AtNTT2::T-DNA-, RNAi, and AtNTT1-2::T-DNA mutants were collected from opened siliques, and the seed weight, the lipid content, and the protein levels were quantified (Fig. 11).

Wild-type and AtNTT1::T-DNA seeds exhibited an average weight of 23 μg/seed (Fig. 11A). AtNTT2::T-DNA, RNAi, and AtNTT1-2::T-DNA seeds exhibited reduced average weights leading to 19, 20, and 18.5 μg/seed, respectively (Fig. 11A). Lipids represent the main storage product in Arabidopsis seeds. Both wild-type and AtNTT1::T-DNA seeds accumulated similar levels of storage lipids amounting to about 7.2 to 7.5 μg lipid/seed (Fig. 11B). In contrast, AtNTT2::T-DNA and RNAi seeds exhibited only 5.8 and 6.0 μg lipid/seed, respectively, and seeds from AtNTT1-2::T-DNA plants still showed only 4.5 μg lipid/seed (Fig. 11B). The protein in wild-type and AtNTT1::T-DNA seeds has been estimated to be about 4.8 μg/seed (Fig. 11C). AtNTT2::T-DNA, RNAi, and null mutants showed reduced protein levels approaching 3.3, 3.9, and 3.8 μg/seed, respectively (Fig. 11C).

**DISCUSSION**

**Regulation of NTT Isoform Expression**

ATP represents a uniquely important cellular-energy source and is required in most cell compart-
chloroplastic ATP/ADP transporter prevents ATP export into the cytosol but allows ATP import during the night phase; and (2) the growth differences of null mutants grown under either short- or long-day conditions strikingly demonstrate that nocturnal ATP uptake into the chloroplasts is required for proper plant development (Fig. 10, A and B). This ATP is, however, in Arabidopsis not required for degradation of transitory starch (see "Results"; Nittyla et al., 2004) but for other, still unidentified processes.

The strong accumulation of AtNTT1 mRNA in leaf discs incubated on high Glc or Suc concentrations (Fig. 1D) indicates that this gene belongs to a large group of sugar up-regulated genes (Koch, 1996) required to reprogram chloroplasts into starch-accumulating ATP-importing storage plastids. In this respect AtNTT1, beside the plastidic Glc6P/Pi transporter (Quick et al., 1995), a second plastid envelope transporter protein up-regulated upon sugar application. The specific need for ATP supply into heterotrophic plastids is further indicated by the demonstration of AtNTT2 promoter activity in rapidly developing root tips and cotyledons and in petals (Fig. 2, A and C). This is indicative of a high ATP import into corresponding plastids, which is substantiated by the demonstration of ATP uptake and the presence of several ATP-dependent anabolic reactions in isolated chromoplasts, root plastids, and premature chloroplasts (Robinson and Wiskich, 1977; Kleining and Liedvogel, 1980; Kleppinger-Sparace et al., 1992).

Why Does Reduced ATP Supply into Developing Plastids Impair Leaf and Root Development?

Both the northern-blot analysis and the promoter-GUS analysis indicate that especially AtNTT2 expression is high in root tips and cotyledons of developing seedlings (Fig. 1C). This observation tempted us to study the effect of altered plastidic ATP/ADP-transporter activity on both root formation and establishment of photosynthetic competence (Figs. 6, A and B, 7, A–C, and 8). The deletion of a functional AtNTT1 gene in Arabidopsis (Fig. 3, A–C) does not result in an impaired root formation of young seedlings (Fig. 6, A and B), nor did it appear that chlorophyll accumulation or seedling development was affected (Fig. 7, A and B). This observation nicely correlates with the relatively low expression of AtNTT1 in corresponding tissues (Figs. 1C and 2A). In strong contrast, the absence of a functional AtNTT2 gene (Fig. 4, A–C) or the reduction of both mRNA species (AtNTT1 and AtNTT2) in RNAi mutants (Fig. 5D) led to a strongly decreased formation of primary roots in young seedlings (Fig. 6, A and B) and a retarded chlorophyll accumulation (Fig. 7B) corresponding to a reduced growth rate (Figs. 7A and 10A).

The impaired root development is most likely due to an inhibited rate of fatty-acid synthesis. In plants this process takes place exclusively in plastids, and in case of root plastids the process has been characterized to be strictly dependent upon ATP import rather than on internal ATP regeneration via glycolytic reactions (Kleppinger-Sparace et al., 1992). The localization of AtNTT2 expression in the root tip, representing the meristematic zone of cell division and elongation, is therefore fully consistent with a high demand for fatty-acid synthesis in this tissue. In addition, heterotrophic plastids are known as a cellular site for energy-consuming amino acid biosynthesis (Neuhaus and Emes, 2000). Therefore, reduced rates of amino acid synthesis in mutant lines might also contribute to an impaired root development.

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**Figure 11.** Seed quality analysis of wild-type and transgenic plants. Plants were grown for 35 d on soil under short-day conditions. Subsequently the light phase was prolonged to induce flowering (16 h light) until the life cycle was completed. Seeds from wild-type, AtNTT1::T-DNA, AtNTT2::T-DNA, AtNTT1-2::T-DNA, and RNAi were collected, and seed weight (A), lipid content (B), and protein content (C) in dry seeds were quantified. Data represent the mean of three independent experiments.
In the case of developing leaf tissue several processes are negatively affected by reduced plastidic ATP/ADP-transporter activity. First, the accumulation of chlorophyll is delayed in AtNTT2::T-DNA-, AtNTT1-2::T-DNA, and RNAi seedlings (Figs. 7A and 8A). Second, the generation of functional thylakoid structures is impaired in plants with strongly reduced plastidic ATP import capabilities (Fig. 7C); and third, the accumulation of nuclear-encoded proteins in developing-mutant chloroplasts is reduced (Fig. 8B). Both chlorophyll synthesis and protein import are dependent upon the presence of ATP at the stromal site (Soll and Tien, 1998; Buchanan et al., 2000), and the inhibition of both processes in mutant plastids strikingly show that alternative routes for ATP regeneration do not compensate for insufficient import capacity. Interestingly, we did not observe an accumulation of CP24 preprotein in mutant tissues exhibiting impaired accumulation of the mature CP24 protein (Fig. 8B). This observation might indicate that a so far unknown signaling exists between the developing chloroplast and the nucleus regulating the expression of genes encoding for chloroplastic proteins. It should be mentioned here that we were not able to compare altered NTT mRNA accumulation in transgenic mutant lines with alterations of corresponding transport protein levels. For such analysis we will attempt to generate isoform-specific antisera in the near future.

Reduced ATP Supply into Developing Seed Plastids Limits Lipid Accumulation

We showed in the past that starch accumulation in potato tubers is strongly affected by altering the plastidic ATP/ADP-transporter activity (Tjaden et al., 1998a) leading to a high metabolic-flux control coefficient (Geigenberger et al., 2001). Therefore, we analyzed whether reduced plastidic ATP import capacity governs the end-product accumulation in Arabidopsis embryos to a similar extent as observed in potato. This analysis was further encouraged, since experiments on isolated rapeseed seed-embryo plastids showed that the highest rates of fatty-acid synthesis depend upon the supply with exogenous ATP (Eastmond and Rawsthorne, 1998; Rawsthorne, 2002), whereas a recently developed mathematical carbon-flux model indicated that net ATP import is not required for maximal fatty-acid synthesis in rapeseed embryos (Schwender et al., 2004).

As given in Figure 11, AtNTT1::T-DNA did not show altered seed weight, lipid, and protein content when compared to wild-type seeds, whereas AtNTT2::T-DNA seeds showed reduced weight, which correlates with reduced levels of lipids and storage protein (Fig. 11, A–C). Strongest reduction of the lipid content showed seeds generated from double-knockout mutants as these seeds contained only about 50% of the lipid content present in wild-type seeds (Fig. 11B). This result is surprising, because the expression level of NTT1 and NTT2 mRNA in developing siliques and seeds is remarkable low (Figs. 1B and 2C). Obviously, even low mRNA levels allow the maintenance of sufficient plastidic ATP import capacity.

It is important to note that the reduced seed oil phenotype is evident under long-day conditions, where the effects of gene knockout on whole-plant physiology, and hence maternal carbon supply to the embryo, were absent. These effects on storage product content are therefore likely to be specific to alterations to NNT gene expression in the seed. From this result we conclude that, similar to rapeseed and cauliflower (Brassica oleracea) inflorescence plastids (Möhmann et al., 1994; Eastmond and Rawsthorne, 1998), Arabidopsis embryo plastids need to import cytosolic ATP to achieve the highest rates of lipid synthesis. This conclusion is fully consistent with recent observations that the overall energy status of developing rapeseed seeds correlate with lipid synthesis (Vigeolas et al., 2003).

However, Arabidopsis embryo plastids obviously possess, in addition to ATP/ADP-transporter proteins, endogenous sources for ATP regeneration because the absence of both transporter activities does not correlate with a total loss of storage product (Fig. 11B). In general two other metabolic pathways might allow stromal regeneration of ATP: First, chlorophyll-containing embryo plastids might regenerate ATP by photo-phosphorylation. Secondly, stromal-located glycolytic sequences might regenerate ATP at the enzymic steps catalyzed by phosphoglycerate kinase (PGK), or pyruvate kinase (PK). We would like to exclude the first possibility, since in case of rapeseed the light transmission into the developing seed tissue is supposed to be too low (Eastmond et al., 1996). Moreover, rapeseed mutants showing strongly reduced chlorophyll levels in developing embryos did not contain less lipids than wild-type plants (Tsang et al., 2003). These independent observations point to stromal glycolysis as the alternative source for endogenous ATP resynthesis. This assumption is substantiated by the demonstration that Glc 6-phosphate is a very suitable carbon precursor for fatty-acid synthesis in rapeseed plastids (Kang and Rawsthorne, 1996). In addition, the observation that developing leaf plastids from RNAi plants exhibited increased accumulation of plastidial PGK- and PK mRNAs during deetiolation (Fig. 9) might indicate that heterotrophic Arabidopsis plastids use endogenous glycolysis for ATP resynthesis. The latter observation is moreover in full agreement with the demonstration of a DHAP-driven ATP production in etioplasts from dark-grown barley leaves (Batz et al., 1992). Obviously, some types of heterotrophic plastids use endogenous glycolysis for stromal ATP production, which contribute to energize anabolic reactions and compensate partly the lack of plastidic ATP import capacity in mutant plants (Figs. 6, 7, 8, 10, and 11). Moreover, the observation that mRNA coding for plastidic glycolytic enzymes specifically accumulated in deetiolating plastids from RNAi plants (Fig. 9) might indicate that the stromal ATP
(energy) status is sensed and governs expression of genes allowing regeneration of ATP by alternative sources.

CONCLUSION

Arabidopsis contains two isoforms of plastidic ATP/ADP transporter to allow an optimal spatially and developmentally regulated adaptation of gene expression. Surprisingly, Arabidopsis does not need plastidic ATP/ADP-transporter activity to pass through the complete developmental cycle. However, plastidic ATP/ADP-transporter activity is required for a controlled development of young tissues, especially shown for roots and cotyledons, and is required in mature chloroplasts at night. The absence of plastidic ATP import in developing embryo tissue correlates with a reduction of lipid accumulation, which however still occurs at appreciable levels. This observation points to an ATP regeneration by stromal-located glycolytic enzymes, which seems to participate on ATP provision.

MATERIALS AND METHODS

**AtNTT1 (AtNTT1::T-DNA) and AtNTT2 (AtNTT2:: T-DNA) Knockout Mutant Plants**

The heterozygous AtNTT1::T-DNA mutant plant (Salk_013530) was provided by the SALK library. In that mutant the T-DNA is located in the first exon of AtNTT1 (locus At1g80300) on bp position 777. The heterozygous AtNTT2::T-DNA mutant (GARLIC, 288_E06b.1b.Lrb3Fa) was provided by the Torrey Mesa Research Institute (San Diego). In that mutant the T-DNA is located in the second exon of AtNTT2 (locus At1g35500) on bp position 1015.

To confirm that we generated homozygous mutants after backcrossing, we used gene- and T-DNA-specific primers. For PCR on genomic DNA the following primers were used: NTT1/I (5'-TTTTCTCTTCTGTAATCGGCGAGGAGACTG-3'); NTT2/I (5'-CTTTTCTTCCCCCACAACAAAACAAATA-3'); SALK_LB (5'-ACTCACTCCATCTGGGGTATATT-3'); NTT2/I (5'-CTCCTCTCCTCTACCCAGGGC-3'); NTT2/2 (5'-CCAAACTCCCAACCATTTTACATC-3'); and GARLIC_LB (5'-TAGATGCTAACATTCTAATAACAAATCCCGATAAC-3').

**Generation of Double-Knockout Plants**

(AtNTT1-2::T-DNA)

To generate double-knockout mutants (designated AtNTT1-2::T-DNA) lacking both functional plastidic ATP/ADP-transporter genes, homozygous AtNTT1::T-DNA and homozygous AtNTT2::T-DNA mutant plants were crossed. Although both genes reside on chromosome 1, we were able to identify double-knockout mutants by use of the primers given above.

**Generation of RNAi Mutants**

Transgenic RNAi plants were generated to achieve strongly reduced mRNA-levels of both AtNTT1 and AtNTT2. For Arabidopsis (Arabidopsis thaliana) transformation the pART27 vector (Gleave, 1992) was used. For this we cloned a 418-bp fragment from AtNTT1 (corresponding to bp positions 1,006–1,424) in sense and antisense orientation into the pHANNIBALL vector (Torrey Mesa Research Institute). In that mutant the T-DNA is located in the second exon of AtNTT2 (locus At1g35500) on bp position 1015.

To confirm that we generated homozygous mutants after backcrossing, we used gene- and T-DNA-specific primers. For PCR on genomic DNA the following primers were used: NTT1/I (5'-TTTTCTCTTCTGTAATCGGCGAGGAGACTG-3'); NTT2/I (5'-CTTTTCTTCCCCCACAACAAAACAAATA-3'); SALK_LB (5'-ACTCACTCCATCTGGGGTATATT-3'); NTT2/I (5'-CTCCTCTCCTCTACCCAGGGC-3'); NTT2/2 (5'-CCAAACTCCCAACCATTTTACATC-3'); and GARLIC_LB (5'-TAGATGCTAACATTCTAATAACAAATCCCGATAAC-3').

**Generation of AtNTT1::Promoter-GUS and AtNTT2::Promoter-GUS Plants**

For the generation of promoter-GUS constructs the binary vector pGPTV (Becker et al., 1992) containing the β-glucuronidase (uidA) gene from Escherichia coli was used. For the generation of promoter-GUS fusion constructs a promoter region of about 1.4 kb of either the AtNTT1 or AtNTT2 gene was cloned upstream of the GUS gene. The promoter region of the AtNTT1 or AtNTT2 gene (including 21 bp of the coding region) was amplified by PCR from genomic DNA. Both promoters were sequenced to check that the correct products were amplified. For amplification of the AtNTT1 promoter the following primers were used: JR3-sense, 5'-TGGAGGATCTGTGCTCAGGA-3'; and JR2ant, 5'-AAGAGAGAAGCCCATTTCCATTCGTTCC-3'. For amplification of the AtNTT2 promoter the following primers were used: JR3-sense, 5'-TGGAGGATCTGTGCTCAGGA-3'; and JR2ant, 5'-AAGAGAGAAGCCCATTTCCATTCGTTCC-3'. For the generation of AtNTT1::Promoter-GUS constructs the binary vector pHANNIBALL was subcloned as Ncol fragments into pART27, and the final plasmid was subsequently transformed into Agrobacterium. Transformation of Arabidopsis was conducted according to the floral-dip protocol.
translocator-fp, (Ae5g54800) 5'-TTCCATGACGACGATCCAA-3'; Glc-6-phosphate translocator-rp, (Ae5g54800) 5'-ACCCAGGTTACAACCTCTTG-3'; xylulose-5-phosphate translocator-fp, (Ae5g17630) 5'-CCGTTGCTATCCGA-TTCA-3'; xylulose-5-phosphate translocator-rp, (Ae5g17630) 5'-GCCTGTGAA- GCTACGGTTAGA-3'; Actin-fp, 5'-TGTACGCCAGTGGCTGTAACCC-3'; and Actin-rp, 5'-GGACGAAATGGAACCAGCCG-3'.

Cultivation of Plant Material

Wild-type and transgenic Arabidopsis plants (ecotype Columbia) were grown in a climate-controlled chamber on soil at 22°C and 100 µmol quanta m⁻² s⁻¹. Prior to germination, seeds were incubated for 2 d in the dark at 4°C for imbibition (Weigel and Glazebrook, 2002). For short-day growing conditions, the light was given for 10 h/d; for long-day conditions light was present for 16 h/d. For root growth and seedling analysis, surface-sterilized seeds were sown on half-concentrated Murashige and Skoog (1962) plates, with 1.25 µM thidiazuron. For lipid quantification, 0.1 g completely mature and air-dried seeds were subsequently transferred to the growth chamber, and growth was continued for imbibition (Weigel and Glazebrook, 2002). For short-day growing conditions, the light was given for 10 h/d; for long-day conditions light was present for 16 h/d. For root growth and seedling analysis, surface-sterilized seeds were sown on half-concentrated Murashige and Skoog (1962) plates, with 1.25 µM thidiazuron. For lipid quantification, 0.1 g completely mature and air-dried seeds were subsequently transferred to the growth chamber, and growth was continued for 24 h under long-day conditions.

Extraction of Total RNA and RNA Gel-Blot Hybridization

Total RNA was isolated from frozen tissue samples (liquid nitrogen) by using the Purescript extraction kit (Genta Systems, North Minneapolis, MN), according to the manufacturer’s instructions. For RNA gel-blot hybridization analysis, standard methods (Sambrook et al., 1989) were used. Blots were visualized by a Phospho-Imager (Packard, Frankfurt).

Histochemical Localization of GUS

Whole seedlings or tissue from transgenic plants were collected in glass centrifillation vials, filled with ice-cold 90% acetone, and incubated for 20 min at room temperature. Subsequently, the samples were stained according to standard protocols (Weigel and Glazebrook, 2002).

Immunological Analysis

Accumulation of chlorophyll-binding protein CP24 during illumination was examined by western-blot analysis. Antibodies were kindly provided by Prof. R.B. Klösgen (Pflanzenphysiologie, Martin-Luther Universität Halle, Germany). Plant tissue (0.5 g) frozen in liquid nitrogen was homogenized in 250 µL buffer A (50 mM HEPES, 5 mM MgCl₂, pH 7.5, 5% SDS, 1% Triton X-100, 15% glycerol, 1 mM EDTA, phenylmethylsulfonyl fluoride [PMSF] 1/100 [v/v]) at room temperature. SDS-PAGE, northern transfer, and immunodetection were conducted according to standard protocols.

Transmission Electron Microscopy

For chloroplast ultrastructure analysis from wild-type and RNAi mutants cotyledons from 5-d-old seedlings, grown under short-day conditions, were used. The seedlings were fixed with solution 1 (3% [v/v] glutaraldehyde, 30 mM Pipes, pH 7.0) for 1 h and subsequently washed twice for 10 min in cacodylate buffer, pH 7.0 (50 mM sodium cacodylate, 6.4 mM HCl). The samples were post-fixed in solution 2 (1% [w/v] osmium tetroxide, 50 mM sodium cacodylate, 6.4 mM HCl, pH 7.0) for 1 h and washed as described above. Subsequently, samples were incubated for 1 h in 0.5% uranyl acetate, followed by a serial dehydration with 30%, 50%, 70%, 90%, and 100% [v/v] of acetone in water. The specimens were infiltrated with a series of 25%, 50%, 75%, and 100% (v/v) of resin for 8 h to evaporate the isopropanol. Subsequently, samples were incubated for 10 min and the supernatant was transferred into preweighted 1.5-mL reaction tubes. Samples were centrifuged at 12,000 g at room temperature for 10 min. The supernatant was transferred into new 1.5-mL reaction tubes, and proteins were quantified with bichromonic acid reagent (Pierce Chemical, Rockford, IL) according to manufacturer’s instructions.

Chlorophyll Quantification

Chlorophyll quantification was carried out according to a standard protocol (Arnon, 1949).

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