Early Steps in the Biosynthesis of NAD in Arabidopsis Start with Aspartate and Occur in the Plastid[1]

Akira Katoh, Kazuya Uenohara, Mitsuru Akita, and Takashi Hashimoto*

Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 630–0192, Japan (A.K., K.U., T.H.); and Faculty of Agriculture and Venture Business Laboratory, Ehime University, Matsuyama 790–8577, Japan (M.A.)

NAD is a ubiquitous coenzyme involved in oxidation-reduction reactions and is synthesized by way of quinolinate. Animals and some bacteria synthesize quinolinate from tryptophan, whereas other bacteria synthesize quinolinate from aspartate (Asp) using L-Asp oxidase and quinolinate synthase. We show here that Arabidopsis (*Arabidopsis thaliana*) uses the Asp-to-quinolinate pathway. The Arabidopsis L-Asp oxidase or quinolinate synthase gene complemented the *Escherichia coli* mutant defective in the corresponding gene, and T-DNA-based disruption of either of these genes, as well as of the gene coding for the enzyme quinolinate phosphoribosyltransferase, was embryo lethal. An analysis of functional green fluorescent protein-fused constructs and in vitro assays of uptake into isolated chloroplasts demonstrated that these three enzymes are located in the plastid.

NAD is a coenzyme that switches between oxidized and reduced forms without any net consumption. NAD is converted to its reduced form, NADH, mainly in catabolic reactions, whereas NADH is mostly oxidized by the mitochondrial electron transfer chain. NAD is also utilized irreversibly as a substrate by several distinct classes of enzymes (Hunt et al., 2004; Katoh and Hashimoto, 2004). For example, NAD is consumed in a deacetylation reaction catalyzed by the silent information regulator (Sir2) family enzymes, which couple the degradation of NAD to nicotinamide and the deacetylation of a substrate, such as acetylated histones (Fig. 1; Tanner et al., 2000). Recent emerging interest in Sir2 and its mammalian ortholog Sirt1 stems from their role in promoting longevity due to calorie restriction (Chen et al., 2005). Although plant Sir2 homologs have not been characterized functionally, resveratrol, a polyphenol found in red wine, is drawing attention as a potent activator of human Sirt1 (Howitz et al., 2003).

NAD is synthesized de novo from amino acid precursors in two alternate pathways: the Asp pathway and the kynurenine pathway (Fig. 1; Rongvaux et al., 2003; Katoh and Hashimoto, 2004). In many bacteria, including *Escherichia coli*, L-Asp is the starting amino acid. The first enzyme in this pathway, L-Asp oxidase (AO; EC 1.4.3.16), oxidizes L-Asp to give α-iminosuccinic acid. In the next step, α-iminosuccinate is condensed with glyceraldehyde-3-P and cyclized to produce quinolinic acid by quinolinate synthase (QS). The third step is catalyzed by quinolinic acid phosphoribosyl transferase (QPT; EC 2.4.2.19), which forms nicotinic acid mononucleotide (NaMN) from quinolinic acid and phosphoribosyl pyrophosphate. The two subsequent enzymatic steps then convert NaMN to NAD. In contrast to this prokaryotic pathway, mammals and fungi couple Trp catabolism with NAD biosynthesis. Recently, several bacteria were found to synthesize NAD from Trp (Kurnasov et al., 2003). In this kynurenine pathway, L-Trp is degraded via kynurenine to quinolinic acid in five enzymatic steps. The steps leading from quinolinic acid to NAD are conserved among prokaryotes and eukaryotes and catalyzed by the same set of enzymes. In many organisms, including plants, nicotinamide and nicotinic acid, degradation products of NAD and NADP, are reutilized for the synthesis of pyridine nucleotides by salvage pathways (Ashihara et al., 2005).

Previous studies have not provided a clear consensus on the de novo pathways leading to the formation of NAD in plants. The feeding of corn (*Zea mays*) seedlings with [benzene ring-U-^{14}C]DL-Trp and [5-^{3}H]L-Trp resulted in significant levels of radioactivity being incorporated into niacin (a generic term for nicotinamide and nicotinic acid), whereas the incorporation into niacin of tracer from [U-^{14}C]L-Asp was negligible (Tarr and Arditti, 1982). In contrast, the feeding of [3-^{14}C]Asp to tobacco (*Nicotiana rustica*) plants afforded nicotine, which was labeled at C-2 and C-3 in the pyridine ring (Jackanicz and Byerrum, 1966). The pyridine ring of nicotine is derived from an intermediate of NAD biosynthesis in tobacco and related species (Dawson et al., 1956, 1958; Yang et al., 1965).

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* Corresponding author; e-mail hasimoto@bs.naist.jp; fax 81–743–72–5529.

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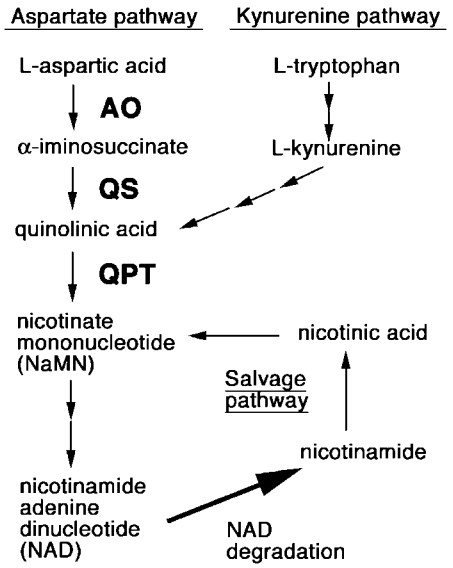

Figure 1. Alternate pathways for NAD biosynthesis. Some bacteria, such as *E. coli*, synthesize quinolinic acid from Asp using AO and QS, whereas animals, fungi, and other bacteria produce quinolinate from Trp via kynurenine. Quinolinate is converted to NAD in three steps, with the first step catalyzed by QPT. In some biochemical reactions, NAD is metabolized to nicotinamide, which is recycled back to NaMN via a salvage pathway.

In addition, feeding radiolabeled Trp to Nicotiana species consistently resulted in a negligible incorporation of the tracer into nicotine (Leete, 1957; Henderson et al., 1959). AO, an enzyme in the Asp pathway, has been partially purified from cotton (*Gossypium hirsutum*) callus (Hosokawa et al., 1983).

To clarify the de novo NAD biosynthetic pathway leading to quinolinic acid in plants, we first examined the enzymatic functions of Arabidopsis (*Arabidopsis thaliana*) AO and QS homologs by complementing *E. coli* mutants. The Arabidopsis AO and QS genes, as well as the QPT gene, are essential for plant growth and development because their T-DNA insertion mutants died during embryonic development. We further demonstrate that these three enzymes are located in the plastids. Our studies show that Arabidopsis synthesizes NAD from Asp as *E. coli* does.

RESULTS

Identification of Arabidopsis AO and QS

Computer searches of the Arabidopsis genome database revealed that the protein encoded by At5g14760 is significantly homologous to *E. coli* AO (42% identity and E-value of $1 \times e^{-104}$), whereas the protein encoded by At5g50210 is a homolog of *E. coli* QS (24% identity and E-value of $1 \times e^{-10}$). In contrast, no significant homology with the enzymes in the kynurenine pathway was found, one exception being the protein encoded by At5g15860, which was moderately homologous to mouse arylformamidase (26% identity and E-value of $4 \times e^{-14}$; Katoh and Hashimoto, 2004).

To test whether At5g14760 and At5g50210, respectively, encode AO and QS, these Arabidopsis genes were introduced and expressed in *E. coli* mutants deficient in AO or QS (Fig. 2). Because the *E. coli* strains *nadB*$^-$ and *nadA*$^-$ do not express AO and QS, respectively, and cannot synthesize NAD via the de novo pathway, they require a supplement of nicotinic acid in minimal medium to synthesize NAD via the salvage pathway. When At5g14760 was expressed in the *nadB*$^-$ genotype, *E. coli* cells were able to form colonies on minimal medium lacking nicotinic acid. Likewise, expression of At5g50210 in the *nadA*$^-$ genotype also complemented the requirement of an exogenous source of nicotinic acid for growth. These results suggest that At5g14760 and At5g50210 indeed encode AO and QS, respectively.

AO, QS, and QPT Are Essential for Growth in Arabidopsis

If Arabidopsis synthesizes NAD de novo exclusively from Asp, null alleles of *AO* and *QS* should be lethal. To test this hypothesis, we analyzed phenotypes of T-DNA insertion alleles of *AO* and *QS*, which are both single genes in the Arabidopsis genome (Fig. 3). We also characterized a transposon insertion allele of *QPT* (Fig. 3), a presumably essential gene utilized in both the Asp and kynurenine pathways (Fig. 1). Tobacco QPT has been shown to complement a QPT-deficient *E. coli* strain (Sinclair et al., 2000) and Arabidopsis QPT is encoded at a single locus. In the *ao* allele (SALK_013920) obtained from the SALK collection, an inverted tandem insert of T-DNA was found in the seventh exon of *AO* 2,318 to 2,365 bp downstream of the translational start site (with the first adenine in ATG numbered as 1), whereas inverted T-DNAs were

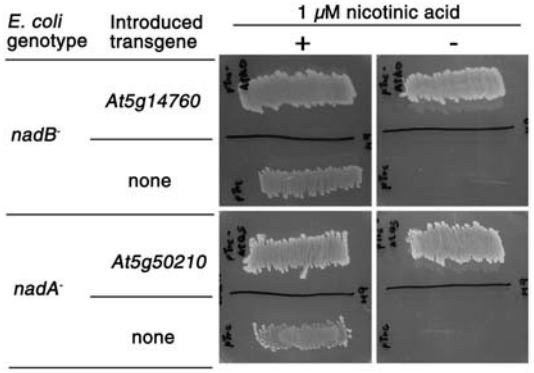

Figure 2. Complementation of *E. coli* AO and QS mutants with the Arabidopsis homologs. *E. coli* strains *nadB*$^-$ and *nadA*$^-$, which were deficient in AO and QS, respectively, were transformed with the vectors that expressed the Arabidopsis homolog of AO (At5g14760) or QS (At5g50210), or with an empty vector (none). Transformed bacteria were cultured on minimal medium containing 1 mM isopropylthio-β-galactoside and 50 μg/mL ampicilin in the presence (+) or absence (−) of 1 μM nicotinic acid.

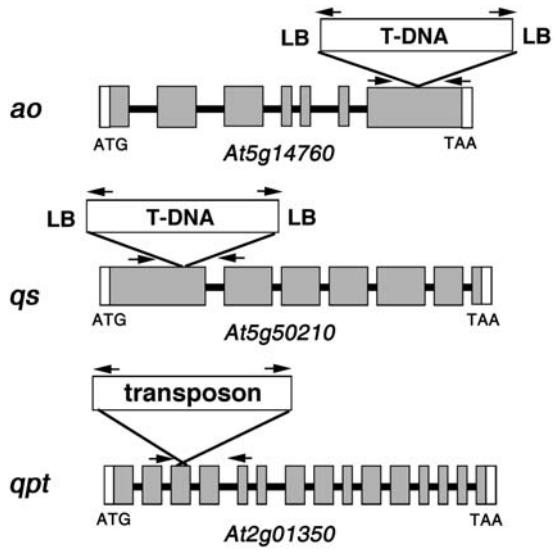

Figure 3. Gene-disrupted alleles of Arabidopsis *AO*, *QS*, and *QPT*. The positions of the T-DNA and dissociation transposon insertion in *AO* (At5g14760), *QS* (At5g50210), and *QPT* (At2g01350) are schematically shown. In *qpt*, the transposon's insertion generated a small tandem duplication of a part of the third exon. Gray and white boxes represent protein-coding regions and untranslated flanking sequences, whereas thick lines represent introns. Approximate positions of PCR primers are indicated by arrows. LB, Left border of T-DNA.

inserted in the first exon of *QS* between 592 and 622 bp in the *qs* allele (SALK_075260). The *qpt* allele (EGT_3.031643) obtained from the Cold Spring Harbor Laboratory collection contained a dissociation transposon in the third exon of *QPT* 489 bp from the translational start site. The nucleotides between 458 and 488 bp were duplicated at the insertion site.

We could not identify lines with a homozygous disruption for *AO*, *QS*, or *QPT* among the seeds obtained from the stock center. When plants heterozygous for the T-DNA or transposon insertion were self pollinated, we found wild-type and heterozygous plants at a 1:2 ratio, but no homozygous plants in the next generation (Table I). These segregation data are consistent with the hypothesis that homozygous null alleles for the AO, QS, and QPT genes do not grow into viable seedlings. Indeed, when immature siliques of the self-pollinated heterozygous plants were cut open, roughly 25% of the seeds they contained were found to be small and unviable (Fig. 4B; data not shown).

To confirm that the observed embryo-lethal phenotypes are caused by disruption of AO, QS, and QPT, we transformed the heterozygous plants with a vector that constitutively expresses the wild-type AO, QS, or QPT protein fused to green fluorescent protein (GFP) at the C terminus. Among the progeny of the transformants, we identified several plants that were homozygous for the T-DNA or transposon insertion alleles of AO, QS, and QPT and possessed the relevant transgenes (Fig. 4C). Expression of the transgenes was confirmed by detection of GFP fluorescence in the transgenic plants (see below). The newly introduced transgenes efficiently complemented the presumed embryo-lethal phenotypes of the homozygous null alleles, as shown by the similar growth and development of the complemented plants compared with the wild type (Fig. 4A).

Table I. *Genetic analysis of ao, qs, and qpt mutants*

X^2 values were calculated based on a 2:1 ratio of heterozygous and wild-type plants.

Mutant	*n*	Wild Type	Heterozygote	Homozygote	X^2	*P*
ao	98	34	64	0	0.073	0.79
qs	124	43	81	0	0.090	0.76
qpt	123	42	81	0	0.034	0.85

AO, QS, and QPT Are Present in the Plastid

Arabidopsis AO, QS, and QPT contain extra amino acid sequences at their N termini relative to their *E. coli* counterparts. The computer programs Predotar (Small et al., 2004; http://urgi.infobiogen.fr/predotar/predotar.html) and TargetP (Emanuelsson et al., 2000; http://www.cbs.dtu.dk/services/TargetP), respectively, predicted that Arabidopsis AO and QS are targeted to plastids (0.98 and 0.33 for AO and 0.98 and 0.94 for QS), whereas Arabidopsis QPT is localized to mitochondoria (0.70 and 0.60).

To determine experimentally the subcellular distribution of these Arabidopsis proteins, we first analyzed transgenic Arabidopsis seedlings expressing AO-GFP, QS-GFP, or QPT-GFP in the respective homozygous null backgrounds as described above. Complementation of the embryo-lethal phenotypes by expression of

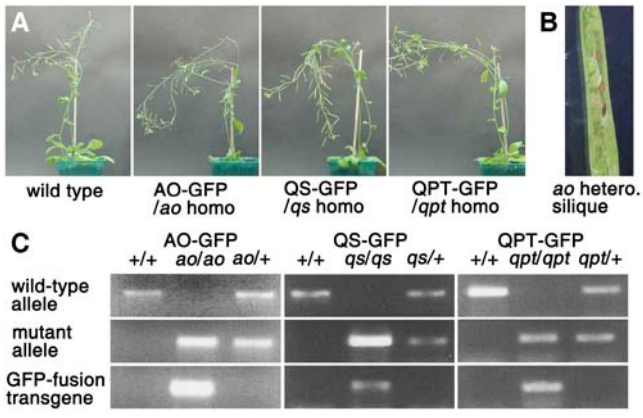

Figure 4. Functional complementation of homozygous null mutants with the constitutive expression of AO-GFP, QS-GFP, or QPT-GFP. A, Two-month-old adult wild-type plants (ecotype Columbia) and homozygous mutant plants expressing GFP-fusion transgenes. B, A young silique of a self-pollinated heterozygous *ao* mutant plant. Development was affected in about 25% of seeds. C, Genomic PCR analysis. The wild type (+/+), heterozygotes (mutant allele/+), and homozygotes (mutant allele/mutant allele) expressing relevant GFP-fusion transgenes were analyzed for the presence of wild-type alleles, T-DNA/transposon insertion mutant alleles, and GFP-fusion transgenes.

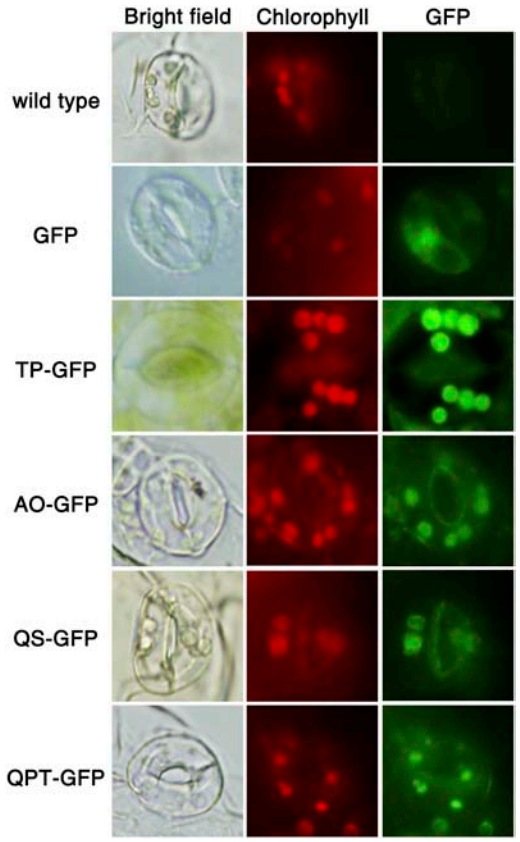

Figure 5. GFP-fused proteins of AO, QS, and QPT are localized to the chloroplasts of Arabidopsis guard cells. Chlorophyll autofluorescence (red) and GFP fluorescence (green) in guard cells were simultaneously observed. Bright-field images on the right show the shape of guard cells. Unfused GFP was found in the cytoplasm and nucleus, whereas TP-GFP (plastid-targeted positive control), AO-GFP, QS-GFP, and QPT-GFP were all found in the chloroplasts.

the transgenes suggests that these GFP-fused proteins are active enzymes targeted to their proper subcellular locations. Fluorescence microscopic observation of stomata guard cells in stable Arabidopsis transformants confirmed that nonfused GFP was present in the cytoplasm and the nucleus, whereas GFP fused to the transit peptide of the small subunit of Rubisco at the N terminus (TP-GFP; Chiu et al., 1996) was targeted to the chloroplast (Fig. 5, A and B). We found that AO-GFP, QS-GFP, and QPT-GFP are all distributed to the chloroplast (Fig. 5, D–F). In the root tissue, fluorescence of AO-GFP, QS-GFP, and QPT-GFP was observed in small, oblique organelles that resembled undifferentiated plastids (data not shown).

We next conducted an in vitro uptake assay using isolated chloroplasts (Fig. 6). Full-length forms of AO, QS, and QPT were synthesized with an in vitro transcription-translation system using reticulocyte lysates and then incubated with purified chloroplasts from pea (*Pisum sativum*) seedlings. Intact chloroplasts were recovered after the reaction and were found to contain the imported substrates that had been processed into smaller polypeptides. These processed proteins corresponded to AO, QS, or QPT upon cleavage of the predicted transit peptides.

DISCUSSION

Our results indicated that Arabidopsis synthesizes NAD from Asp, using AO, QS, and QPT, each of which is essential for plant growth and development. These enzymes are found in the plastid, suggesting that the early steps in the biosynthesis of NAD occur in this organelle.

The Aspartate Pathway in Plants

Arabidopsis AO and QS complemented *E. coli* mutants deficient in the respective enzymes, indicating that they are orthologs of *E. coli* AO and QS. That null mutations for either AO or QS are homozygous lethal during embryonic development suggests that the enzymatic steps catalyzed by AO and QS are essential for cell metabolism and that the kynurenine pathway does not contribute to the formation of quinolinic acid in Arabidopsis. In the Arabidopsis genome, the genes coding for the enzymes in the kynurenine pathway are not present, except for a distant homolog of arylformamidase catalyzing the third step in the pathway (Katoh and Hashimoto, 2004). Expressed sequence tags (ESTs) probably encoding AO or QS are found in Arabidopsis, Nicotiana species, tomato (*Lycopersicon esculentum*), and several other dicotyledonous species in which no ESTs for the kynurenine pathway are present, except for arylformamidase-like ESTs. These results suggest that Arabidopsis and other dicotyledonous plants synthesize NAD from Asp via quinolinic

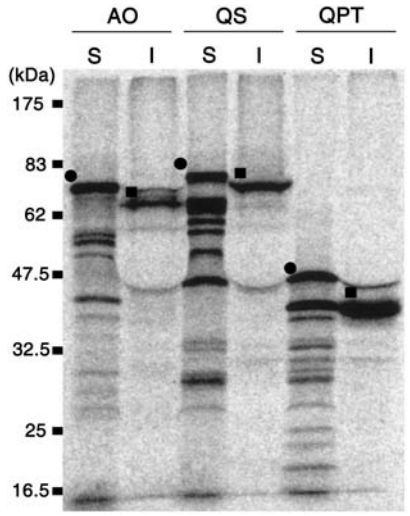

Figure 6. Import of protein into chloroplasts in vitro. After the translated radiolabeled substrates (S) of AO, QS, or QPT were incubated with isolated pea chloroplasts, imported proteins in the chloroplasts (I) were analyzed. Precursor and mature forms of AO, QS, and QPT are, respectively, indicated by black circles and black squares in the top left of the bands. The size of M_r markers is shown on the left.

acid. Classical tracer feeding experiments demonstrated that Asp, but not Trp, is an excellent precursor for the pyridine ring of nicotine in Nicotiana species (Dawson et al., 1956, 1958; Leete, 1957; Henderson et al., 1959; Jackanicz and Byerrum, 1966), and the partial purification of cotton AO (Hosokawa et al., 1983) is consistent with the general occurrence of the Asp pathway in dicots.

Interestingly, the genome sequences of rice (*Oryza sativa*) contain not only the AO and QS homologs, but also the five genes encoding putative enzymes for all five steps in the kynurenine pathway (Katoh and Hashimoto, 2004). These rice genes are apparently expressed because EST sequences are found for many of them. Several genes of the kynurenine pathway are also found in the EST databases of wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*). The efficient incorporation of labeled Trp into NAD in corn seedlings (Tarr and Arditti, 1982) supports the existence of the kynurenine pathway in the grass family. We speculate that monocotyledonous plants may synthesize NAD from both Asp and Trp and may regulate the two pathways separately. It would be worth examining the early NAD biosynthetic pathways in rice and other monocots.

Biosynthesis of NAD Precursors Occurs in the Plastid

The subcellular distribution of functional GFP-fused enzymes and import of precursor proteins into isolated chloroplasts indicated that Arabidopsis AO, QS, and QPT are all compartmentalized in the plastid. Aspartate is synthesized in the plastid by a chloroplast-localized isoform of Asp aminotransferase and is used to synthesize Lys, Thr, and Ile within this organelle (Coruzzi and Last, 2000). The chroloplast proteome databases (http://gene64.dna.affrc.go.jp/RPD/, http://proteomics.arabidopsis.info, and http://ppdb.tc.cornell.edu) do not contain enzymes in the biosynthetic pathways of NAD or these Asp-derived amino acids, probably reflecting that the proteomes of differentiated chloroplasts are biased with proteins involved in active photosynthesis (Kleffmann et al., 2004). However, enzymes involved in the biosynthesis of Asp and amino acids derived from Asp (but not NAD) have been identified in the proteomes of undifferentiated plastids from a tobacco Bright-Yellow 2 cell culture (Baginsky et al., 2004). Absence of AO, QS, and QPT in the plastid proteome indicates that levels of these enzymes are low in the plastid under standard growth conditions. It is conceivable that these enzymes are rate limiting for NAD biosynthesis and, in the plastid, compete for the common starter amino acid Asp with Asp kinase, which initiates the biosynthesis of Asp-derived amino acids.

It remains to be demonstrated whether the remaining two enzymes involved in the synthesis of NAD (nicotinate mononucleotide aminotransferase and NAD synthase) are also localized in the plastid. Although prediction tools such as Predotar and TargetP do not predict that these enzymes are located in the plastid and the current plastid proteome database does not include them, it would be worthwhile to examine experimentally their subcellular distribution.

In recent years, many plant proteins have been found to be targeted to both mitochondria and plastids (Duchêne et al., 2005; Mackenzie, 2005). Whereas we provided good evidence that Arabidopsis AO, QS, and QPT are all targeted to plastids, one or more of these proteins might also be targeted to mitochondria. However, mitochondria did not give significant fluorescent signals when these proteins were expressed as GFP fusions in leaf and root cells of stably transformed Arabidopsis plants (Fig. 5), suggesting that targeting to mitochondria is marginal, if operating at all, in Arabidopsis.

Mitochondria contain in their matrix considerable amounts of pyridine nucleotides. Localization of at least the first part of the NAD biosynthesis predominantly in plastids indicates that NAD or its immediate precursors must be imported across the permeability barrier of the inner mitochondrial membrane. Intact plant mitochondria have been shown to take up NAD in a concentration- and temperature-dependent manner (Tobin et al., 1980; Neuburger and Douce, 1983). Recently, the mitochondrial NAD transporters were identified in yeast (*Saccharomyces cerevisiae*), and homologous protein sequences are found in Arabidopsis and other plant species (Todisco et al., 2006). In plants, NAD may be synthesized outside the mitochondria and then imported into these organelles.

MATERIALS AND METHODS

Complementation of *Escherichia coli* Mutants

Escherichia coli strains (*E. coli* Genetic Stock Center nos. 7419, *lam nadB51*::*Tn10 rph-1* and 6692, and *lam nadA50*::*Tn10 relA1 rpsE2130* [*SpcR*]) have disruptions in the *nadB* gene encoding AO and in the *nadA* gene encoding QS, respectively, and were used for the complementation assay. The entire open reading frames of Arabidopsis (*Arabidopsis thaliana*) AO and Arabidopsis QS were amplified from the Institute of Physical and Chemical Research cDNA clones pda01728 and pda01949 (http://www.brc.riken.jp), respectively, by PCR using the primer pair 5′-GGTTCCATGGCGGCTCATGTTTCTACTGGAAAC-3′ and 5′-GGCCCTCGAGTGCAATCAATAAGTGAGCTGCTA-3′ for AO; and 5′-TTAACCATGGCGTTAGCTCTCTCCGTCGCACCT-3′ and 5′-GGCCCTCGAGTTCTCTTGCTCTCACAACTTAAT-3′ for QS. The forward primers contained *Nco*I sites, whereas the reverse primers contained *Xho*I sites. The PCR products were cloned into the vector pGEM-T (Promega), excised as *Nco*I-*Xho*I fragments, and cloned into the expression vector pTrcHis-2C (Invitrogen). The resultant plasmids pTrcHis-2C-AtAO and pTrcHis-2C-AtQS were introduced into the *E. coli* mutant strains Genetic Stock Center nos. 7419 and 6692, respectively, which were then grown on M9 minimal medium containing 50 μg/mL ampicillin and 1 mM isopropylthio-β-D-galactoside, with or without 1 μM nicotinic acid.

Arabidopsis T-DNA and Transposon Insertion Mutants

T-DNA insertion lines of AO (SALK_013920) and QS (SALK_075260) were generated at the Salk Institute Genomic Analysis Laboratory (http://signal.salk.edu) and obtained from the Arabidopsis Biological Resource Centre. A transposon insertion line of QPT (EGT_3.031643) was obtained from the Cold Spring Harbor Laboratory (http://genetrap.cshl.org). T-DNA insertion sites were analyzed by PCR and then by sequencing the T-DNA-genome junctions,

using as primers 5′-CCCATCAAACTACAAACTGCTA-3′ and 5′-CAAATAAGAACAAAAACTCCTG-3′ for AO; 5′-AACAGAAATCAAACAACAAATC-3′ and 5′-GACCCCTAACCCTTCTCCTAAT-3′ for QS; and 5′-GCGTGGACCGCTTGCTGCAACT-3′ for the left border sequence of T-DNA. The transposon insertion site in QPT was analyzed after amplifying the transposon-genome junction regions, using as primers 5′-TAGCTGAAGATGCTGGTCATACAGG-3′ and 5′-TGCCATTAACTGCAGGAAACAGAC-3′ for QPT; and 5′-ACCCGACCGGATCGTATCGGT-3′ and 5′-GAAACGGTCGGGAAACTAGCTCTAC-3′ for transposon terminal sequences. Arabidopsis seedlings were grown on Murashige and Skoog salt agar plates without antibiotics at 22°C under a 16-h light/8-h dark cycle, and were analyzed for the presence of the T-DNA or transposon insertions by genomic PCR using the above primers.

Expression of GFP-Fused Proteins

The coding regions lacking the stop codons were amplified from the Arabidopsis cDNA clones of AO, QS, and QPT, using as primers 5′-AAAAAGCAGGCTCGCGAGCTTTAAGGTCGGAAA-3′ and 5′-AGAAAGCTGGGTCGCAATCAATAAGTGAGCTGC-3′ for AO; 5′-AAAAAGCAGGCTCGACTGCTTCCAATGGCGTTAG-3′ and 5′-AGAAAGCTGGGTCTCTCTTGCTCTCACAACTTAAT-3′ for QS; and 5′-AAAAAGCAGGCTCGACAATGATCTCTGTCTCTC-3′ and 5′-AGAAAGCTGGGTCCGATGTTCTCTGTGCTCGT-3′ for QPT, and cloned into the pGWB5 Gateway binary vector (obtained from T. Nakagawa, Shimane University) by way of the pDONER221 entry vector (Invitrogen). The transgene in pGWB5 is expressed as a C-terminal GFP-fused protein under the control of the cauliflower mosaic virus 35S promoter and the nopaline synthase terminator. The constructs were introduced into the Arabidopsis lines hemizygous for the T-DNA and transposon insertion at the AO, QS, or QPT locus, using the Agrobacterium-mediated infiltration method (Clough and Bent, 1998). An Arabidopsis plastid marker line that expresses GFP fused to the plastid transit peptide of the small subunit of Rubisco was provided by Y. Niwa (University of Shizuoka).

The presence of GFP fusion transgenes in the Arabidopsis genome was confirmed by PCR using the primer pair 5′-TCCTACAACAGCTAGTTGGA-3′ and 5′-CGGCCATGATATAGACGTTG-3′ for AO-GFP; 5′-TGTCGGGTGTGAGCCGATAC-3′ and 5′-TGTGATCGCGCTTCTCGTTG-3′ for QS-GFP; and 5′-GAGGTGGGAAGAAGGACCAA-3′ and 5′-GGCGGACTTGAAGAAGTCGT-3′ for QPT-GFP.

Fluorescence Microscopy

Thin sections of the leaf epidermal cell layer were prepared by manually cutting cotyledons and leaves with sharp razor blades, and guard cells of the stomata were examined. GFP-fused proteins and chlorophyll fluorescence were detected by employing an ECLIPSE 1000 fluorescence microscope (Nikon). GFP was excited at 460 to 500 nm and detected between 510 to 560 nm, whereas chlorophyll fluorescence was excited at 510 to 560 nm and detected at above 590 nm. Pictures were taken with a C4742-95 digital camera (Hamamatsu Photonics).

In Vitro Import Assay into Isolated Chloroplasts

Import-competent chloroplasts were isolated and purified from pea (*Pisum sativum*) seedlings as described previously (Bruce et al., 1994). Isolated chloroplasts were suspended in an import buffer (50 mM HEPES-KOH [pH 8.0] and 330 mM sorbitol) to a chlorophyll concentration of 2 mg/mL. The AO, QS, and QPT genes in pDONER221 were subcloned into the vector pET-DEST42 (Invitrogen), and were used as templates for in vitro transcription/translation with a TnT-Coupled Reticulocyte Lysate system (Promega) in the presence of L-[^{35}S]Met (>37 TBq/mmol; GE Healthcare Bio-Sciences). A chloroplast suspension of 5 μL was mixed with 2.5 mM Na-ATP (pH 7.0), 0.1 mM Na-GTP (pH7.0), and 5 mM $MgCl_2$ in 45 μL of the import buffer, and then 5 μL of [^{35}S]-labeled proteins were added. The reaction mixture was incubated in the dark at room temperature for 20 min and then loaded onto 75 μL of a 40% Percoll cushion in the import buffer. Intact chloroplasts were isolated by centrifugation (1,300*g*, 4°C, 5 min). The chloroplasts in the pellet were suspended in the import buffer and recovered by centrifugation. The purified chloroplasts were suspended in the sample buffer of the Laemmli system (Laemmli, 1970). Samples were separated by SDS-PAGE and analyzed with an image analyzer BAS-2500 (Fuji Film).

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