Arabidopsis Mutants Define an *in Vivo* Role for Isoenzymes of Aspartate Aminotransferase in Plant Nitrogen Assimilation

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ABSTRACT

Arabidopsis contains five isoenzymes of aspartate aminotransferase (AspAT) localized to the cytosol, chloroplast, mitochondria, or peroxisomes. To define the *in vivo* function of individual isoenzymes, we screened for Arabidopsis mutants deficient in either of the two major isoenzymes, cytosolic AAT2 or chloroplastic AAT3, using a native gel activity assay. In a screen of 8,000 M_2 seedlings, three independent mutants deficient in cytosolic AAT2 (*aat2*) and two independent mutants deficient in chloroplastic AAT3 (*aat3*) were isolated. Mapping of *aat2* and *aat3* mutations and the five AspAT genes (*ASP1–ASP5*) established associations as follows: the mutation affecting *aat2* maps with and cosegregates with *ASP2*, one of two expressed genes for cytosolic AspAT; the mutation affecting *aat3* maps to the same location as the *ASP5* gene encoding chloroplastic AspAT. Phenotypic analysis of the *aat2* and *aat3* mutants revealed a dramatic aspartate-related phenotype in one of the mutants deficient in cytosolic AAT2. The *aat2-2* mutant displays an 80% reduction in levels of aspartate transported in the phloem of light-grown plants, and a 50% reduction in levels of asparagine transported in dark-adapted plants. These results indicate that cytosolic AAT2 is the major isoenzyme controlling aspartate synthesized for nitrogen transport in the light, and that this aspartate pool is converted to asparagine when plants are dark adapted.

MANY enzymes involved in plant metabolism exist as multiple isoenzymes, some of which are targeted to distinct subcellular compartments (Wendel and Weeden 1989). Understanding whether these isoenzymes play overlapping or distinct roles *in vivo* is a question that remains open for many isoenzyme families. For some, the putative *in vivo* roles of individual isoenzymes have been addressed using molecular and transgenic approaches. For example, chloroplastic and cytosolic isoenzymes of glutamine synthetase (GS) are encoded by multiple genes in all higher plants studied including Arabidopsis (Peterman and Goodman 1991). Promoter-GUS fusions and immunocytochemistry have shown that cytosolic and chloroplastic isoenzymes of GS are each expressed in distinct cell types in several species examined, implying distinct *in vivo* functions (Forde *et al.* 1989; Edwards *et al.* 1990; Carvalho *et al.* 1992). As traditional biochemical analyses of isoenzymes cannot address the *in vivo* significance of cell-specific or subcellular compartmentation, the function of individual isoenzymes *in planta* has remained largely unaddressed.

An isoenzyme family that has received particular attention at the biochemical level is aspartate aminotransferase (AspAT, E.C. 2.6.1.1), which plays a key role in both nitrogen and carbon metabolism in many organisms. In plants, distinct AspAT isoenzymes have been localized to each of four subcellular compartments: the cytosol, chloroplasts, mitochondria, and peroxisomes, as shown for several plant species including Arabidopsis (Liu and Huang 1977; Weeden and Marx 1987; Schultz and Coruzzi 1995; Wilkie *et al.* 1995). These distinct AspAT isoenzymes are believed to be involved in shuttling reducing equivalents between subcellular compartments, or between cells, and to be involved in the assimilation of nitrogen into aspartate which serves as an important nitrogen donor and nitrogen-transport compound in plants (Ireland and Joy 1985; Givan 1990).

To attempt to address the function of the distinct AspAT isoenzymes in plants, we initiated a molecular-genetic study of the *ASP* gene family and AAT isoenzymes in Arabidopsis. Using native gel assays combined with subcellular fractionation, we showed that mitochondrial AAT1 is a minor component of Arabidopsis extracts, while cytosolic AAT2 and chloroplastic AAT3 predominate in all tissues examined (leaves, roots, flowers, and cotyledons) (Schultz and Coruzzi 1995). At the molecular level, Arabidopsis has been shown to contain five genes for AspAT (*ASP1–ASP5*) encoding isoenzymes localized to distinct subcellular compartments. The *ASP2* and *ASP4* genes each encode cytosolic isoenzymes, with *ASP2* being the most highly expressed gene (especially in roots) based on the analysis of steady state mRNA levels (Schultz and Coruzzi 1995). The

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ASP1 gene was predicted to encode a mitochondrial isoenzyme, while *ASP3* was predicted to encode either a plastid or peroxisomal enzyme based on transit peptide sequence analysis (Schultz and Coruzzi 1995). A fifth *ASP* gene was identified in Arabidopsis (pcAtAAT1, referred to herein as *ASP5*) and appears to encode a chloroplastic AspAT isoenzyme based on *in vitro* chloroplast uptake experiments (Wilkie *et al.* 1995).

To uncover the *in vivo* role of specific AspAT isoenzymes, we developed a screen to identify Arabidopsis mutants deficient in either of the two major isoenzymes of AspAT, cytosolic AAT2, and chloroplastic AAT3. Subsequent phenotypic analysis of the mutants was used to provide insights into the *in vivo* function of each isoenzyme. This mutant approach, outlined herein, has enabled us to determine that the cytosolic AAT2 isoenzyme controls the major flux of nitrogen assimilated into aspartate, which is used to transport nitrogen from sources to sinks.

MATERIALS AND METHODS

Plant lines and growth conditions: Plant lines used in all experiments were of the Columbia (Col) ecotype of *Arabidopsis thaliana*. The Landsberg (Ler) ecotype was used for mapping purposes only. Plants were grown in tissue culture or soil (as noted) in EGC growth chambers (Environmental Growth Chambers, Chagrin, OH) set on a 16-hr light (65 $\mu E \cdot m^{-2} \cdot sec^{-1}$) / 8-hr dark cycle, unless otherwise noted. Mutagenized M_2 Arabidopsis Columbia seeds treated with ethylmethane sulfonate (EMS) or nitrosourea were kindly donated by Robert Last (Boyce Thompson Institute, Cornell University). For screening, M_2 seeds were surface sterilized and germinated on Murashige and Skoog (MS) media containing 3% sucrose and 0.05% aspartate, to enable the isolation of putative aspartate auxotrophs.

Nomenclature: Genes encoding aspartate aminotransferase isoenzymes were named *ASP1–ASP4*, as described previously (Schultz and Coruzzi 1995). *ASP5* refers to a fifth Arabidopsis AspAT gene described by Wilkie *et al.* (1995, 1996). The mutants deficient in cytosolic or chloroplastic AspAT isoenzymes are named *aat2* and *aat3*, respectively, to distinguish them from the *ASP* genes. This nomenclature is consistent with the community standards for Arabidopsis genetics (Meinke and Koornneef 1997).

AspAT activity gels: For the AspAT native gel assays, one to three leaves of M_2 seedlings were ground in 20 μl grinding buffer (50 mm TrisCl pH 7.5, 5% glycerol, 0.1% Triton X-100) and extracts were clarified by centrifugation. Supernatants were electrophoresed through nondenaturing, discontinuous PAGE mini-gels (mini protean II; Bio-Rad, Richmond, CA) and stained for AspAT activity at room temperature with gentle shaking for 15–60 min. Stain was made fresh for each gel by adding 0.05 g of fast blue BB (F0250; Sigma, St. Louis) to 50 ml of AspAT substrate solution, pH 7.4 (Wendel and Weeden 1989). AspAT substrate solution is stable at room temperature for up to 6 months and is composed of 2.2 mm α-ketoglutaric acid (K1875; Sigma), 8.6 mm L-aspartic acid (A6683; Sigma), 0.5% polyvinyl pyrrolidone-40 (PVP-40, Sigma), 1.7 mm EDTA (disodium salt), 100 mm sodium phosphate (dibasic).

Mapping the *ASP1–ASP5* genes: Restriction fragment length polymorphisms (RFLPs) or CAPS (cleaved amplified polymorphisms, Koniecnzy and Ausubel 1993) were identified between Col and Ler ecotypes of Arabidopsis for the *ASP1–ASP5* genes. The enzymes used to generate the polymorphisms were as follows: for *ASP1*, *Dra*I or *Ava*I; for *ASP2*, *Hpa*II or *Ava*I, for *ASP3*, *Dra*I or *Ava*I, for *ASP4*, *Taq*I and for *ASP5*, *Aci* I. The gene-specific probes used to identify RFLPs for *ASP1–ASP3* were described previously (Schultz and Coruzzi 1995). The primers used to generate the CAPS marker for *ASP4* were CS-13 (5′ *GAGAGTTGGAGCTGAG* 3′) and CS-51 (5′ *CGGCTA CAAACATACGAACC* 3′). The primers used to generate the PCR probe for RFLP analysis of *ASP5* were BM24 (5′ *CAAT CAATGTCGTGTGCTCC* 3′) and BM17 (5′ *TCGCATCAGCAA GATACTCG* 3′). The cDNA and genomic clones representing *ASP5* were described by Wilkie *et al.* (1995, 1996). *ASP1*, *ASP2*, *ASP3*, and *ASP5* were mapped with the Lister and Dean Recombinant Inbred lines generated from the Columbia and Landsberg ecotypes (Lister and Dean 1993) using 22, 28, 23, and 20 individuals, respectively. The segregation data were analyzed and placed on the genetic map by C. Lister (John Innes Centre, Norwich, UK) or since July 1996, by M. Anderson (Nottingham Arabidopsis Stock Centre, WWW server http://nasc.nott.ac.uk/). *ASP4* was mapped relative to known CAPS or SSLP (simple sequence length polymorphisms) markers using 27 plants (Bell and Ecker 1994).

Mapping the *aat2* and *aat3* mutant alleles: CAPS and SSLP analysis was used to map the genes affected in the *aat2-4* and *aat3-3* mutants. Mapping populations were generated for *aat2-4* or *aat3-3* from the following crosses, respectively: *aat2-4*/*aat2-4* (Col) × *AAT2*/*AAT2* (Ler) or *aat3-3*/*aat3-3* (Col) × *AAT3*/*AAT3* (Ler). Homozygous mutants were identified by screening approximately 120 individuals from the appropriate segregating F_2 population. Note, the *aat3-3* mutant is not described here in detail because the mutation affects the electrophoretic mobility of chloroplastic AAT3 rather than causing a loss of activity (Schultz 1994).

Genetic characterization of the *aat* mutants: The individual *aat* mutants were outcrossed to wild-type Columbia to eliminate background mutations. *aat2-1* was outcrossed over five generations, *aat2-4* was outcrossed over three generations, and *aat2-2*, *aat3-1*, and *aat3-2* were outcrossed over one generation. To show that the *aat2-1* mutant gene segregated in a semidominant manner, three putative heterozygotes and four putative homozygous mutants from the F_2 generation (from the cross *aat2-1*/*aat2-1* × *AAT2*/*AAT2*) were selfed. At least 10 (and up to 37) F_3 individuals were analyzed from each of the seven F_2 individuals. The following crosses were performed to test for allelism. The pollen recipient is listed first and the number of seeds obtained (and analyzed) is given after each cross; *aat2-1* × *aat2-2*, 10 seeds; *aat2-1* × *aat2-4*, 13 seeds; *aa3-2* × *aat3-1*, two crosses, 11 and 13 seeds, respectively. To minimize the risk of self-fertilization the flowers chosen as pollen recipients were at a stage where the pollen on the attached anthers was not mature. All anthers were removed prior to touching the stigma with mature donor pollen. The plants chosen as pollen recipients were just starting to send up flowering bolts and the surrounding flowers (not used in the crosses) were removed, to minimize the possibility of self-pollination from a neighboring flower. Since the frequency of spontaneous outcrossing in Arabidopsis is very low (approximately 0.05%, Redei and Koncz 1992) it is very unlikely that the results of the crosses are due to spontaneous self-fertilization by a neighboring flower. To determine whether any of the *aat2* mutants cosegregate with any of the *ASP* genes, the *aat* mutants (Col) were outcrossed to Landsberg and the F_2 progeny were analyzed.

Analysis of growth rate: To compare growth rate of *aat* mutants and wild-type Col, *aat2* or *aat3* mutants and wild-type (Col) seeds were sown side-by-side in a row on MS media

containing 3% sucrose. The plates were incubated vertically using a 16-hr light/8-hr dark regimen, and root length was assessed by visual inspection as an indicator of growth rate.

HPLC analysis of free amino acids in phloem exudates: To assess the levels of amino acids transported via the phloem in leaves of wild-type and mutant plants, phloem exudates were obtained using a method reported for pea (Urquhart and Joy 1981) and modified for Arabidopsis as follows: single Arabidopsis rosette leaves (from soil grown plants) were cut from the plant to leave as much petiole attached to the leaf as possible. Leaves were immediately placed in a microfuge tube containing 50 μl of 20 mm EDTA pH 7.0 such that 1–2 mm of petiole was submerged. Phloem exudates were collected from either light-grown or dark-adapted plants, for two hours in the light and dark, respectively (Lam *et al.* 1995). Control exudates into water yielded minimal levels of amino acids. After 2 hr the leaves were removed from each tube and the final volume measured by pipette to account for any increase in volume due to the exudate. Samples were diluted one in three and filtered (#DDN02003NB; Micron Separations, Westborough, MA) prior to HPLC analysis. Samples were derivatized at 4° with o-phthaldialdehyde immediately prior to injection using an autosampler and then separated by reverse phase-HPLC (SCL-10A system; Shimadzu, Tokyo, Japan) on a C18 column (Supelcosil LC-18, 25 cm × 4.6 mm, 5 μm; Supelco, Bellfonte, PA) at room temperature. Amino acids were separated with a gradient of buffer A (0.1 m sodium acetate pH 7.2, 4.5% methanol, 0.5% tetrahydrofluran) starting at 27.5% and finishing with 100% buffer B (80% methanol). The gradient was determined empirically as follows: time-Buffer B(%), 0.01 min-27.5% B, 38 min-27.5% B, 39 min-33% B, 49 min-65% B, 73 min-66.3% B, 75 min-75% B, 78 min-80% B, 83 min-100% B, then hold 5 min. Flow rate was 1 ml/min. Derivatized amino acids were detected using a Perkin-Elmer LS30 fluorimeter (excitation wavelength 360 and emission wavelength 455). Amino acid standards were from Sigma. Amount of each amino acid was determined by linear comparison of five standard runs where each amino acid was present at 1000 pmol, 500 pmol, 100 pmol, 10 pmol, 5 pmol. The standards all gave linear correlation of concentration to area under the curve for each peak as determined by linear regression.

RESULTS

Use of a quantitative gel assay to screen for Arabidopsis mutants deficient in cytosolic AAT2 or chloroplastic AAT3: AspAT holoenzymes present in Arabidopsis were detected using an AspAT activity stain on leaf extracts run on nondenaturing gels (Schultz and Coruzzi 1995). AspAT activity gels show that crude leaf extracts of wild-type Arabidopsis contain two prominent AspAT isoenzymes; cytosolic AAT2 and chloroplastic AAT3 (Figure 1, lane 1). A mitochondrial form of the enzyme (AAT1) is low in abundance and rarely detected in crude extracts but is detected in preparations of partially purified mitochondria (Schultz and Coruzzi 1995). The AAT2 and AAT3 isoenzymes are the predominant AspAT isoenzymes detected in all other tissues examined including cotyledons, roots, stems, and flowers (data not shown). The native gel assay for AspAT activity was shown to be quantitative using serial dilutions of crude leaf extracts. Cytosolic AAT2 can be detected in samples with 6% of wild-type activity remaining

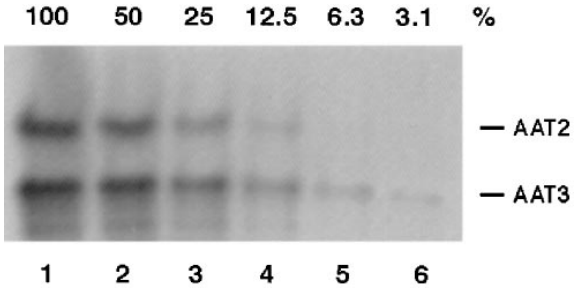

Figure 1.—A quantitative gel assay for cytosolic AAT2 and chloroplastic AAT3. An extract made from three wild-type Arabidopsis (Col) rosette leaves ground in 20 μl grinding buffer (approximately 100 mg protein) (lane 1) was serially diluted (twofold; lanes 2–6) and separated by nondenaturing PAGE and stained for AspAT activity. The top band represents cytosolic AspAT (AAT2) and the bottom band represents chloroplastic AspAT (AAT3).

(Figure 1, lane 5), while chloroplastic AAT3 activity is detectable in samples with 3% of wild-type activity remaining (Figure 1, lane 6). As the AspAT native gel assay was deemed to be quantitative, it was used to screen for Arabidopsis mutants deficient in either cytosolic AAT2 or chloroplastic AAT3. In a screen of 8000 M_2 seedlings, several independent mutants deficient in either cytosolic AAT2 or in chloroplastic AAT3 were identified. Three independent loss-of-activity mutants deficient in cytosolic AAT2 (*aat2-1*, *aat2-2*, and *aat2-4*) were identified from separate pools of EMS mutagenized seeds. Lines homozygous for each of the three *aat2* mutants contain no detectable AAT2 activity, as judged by the native gel assay (for example Figure 2A, lane 3). Thus, the *aat2* mutants each contain less than 6% of wild-type AAT2 activity, as judged by quantitations of dilutions of wild-type extracts in the native gel assay (see Figure 1). For chloroplastic AAT3, two independent loss-of-activity mutants were isolated from separate pools of nitrosourea-treated seeds (*aat3-1* and *aat3-2*). Lines homozygous for each of the two *aat3* mutants contain no detectable chloroplastic AAT3 activity in native gel assays (Figure 2B, lane 3) and therefore contain less than 3% of wild-type AAT3 activity (see Figure 1).

Genetic characterization of *aat2* and *aat3* mutants: To determine whether the loss-of-activity gel phenotype observed in the *aat2* mutants was controlled by a single nuclear gene, the F_2 generation (from crosses to wild-type Columbia plants) were analyzed for each of the three *aat2* mutants. Three distinct gel phenotypes were observed in the F_2 generation for each of the *aat2* mutants: wild-type (Figure 2A, lane 1), heterozygotes (+/−) with reduced levels of AAT2 (Figure 2A, lane 2), and homozygotes (−/−) with no detectable AAT2 activity (Figure 2A, lane 3). Each had normal levels of AAT3 activity. As the assay is quantitative, intermediate levels of AAT2 activity would be expected to occur in heterozygotes with structural gene mutations. To confirm that the F_2 individuals with the "intermediate" AAT2 gel phe-

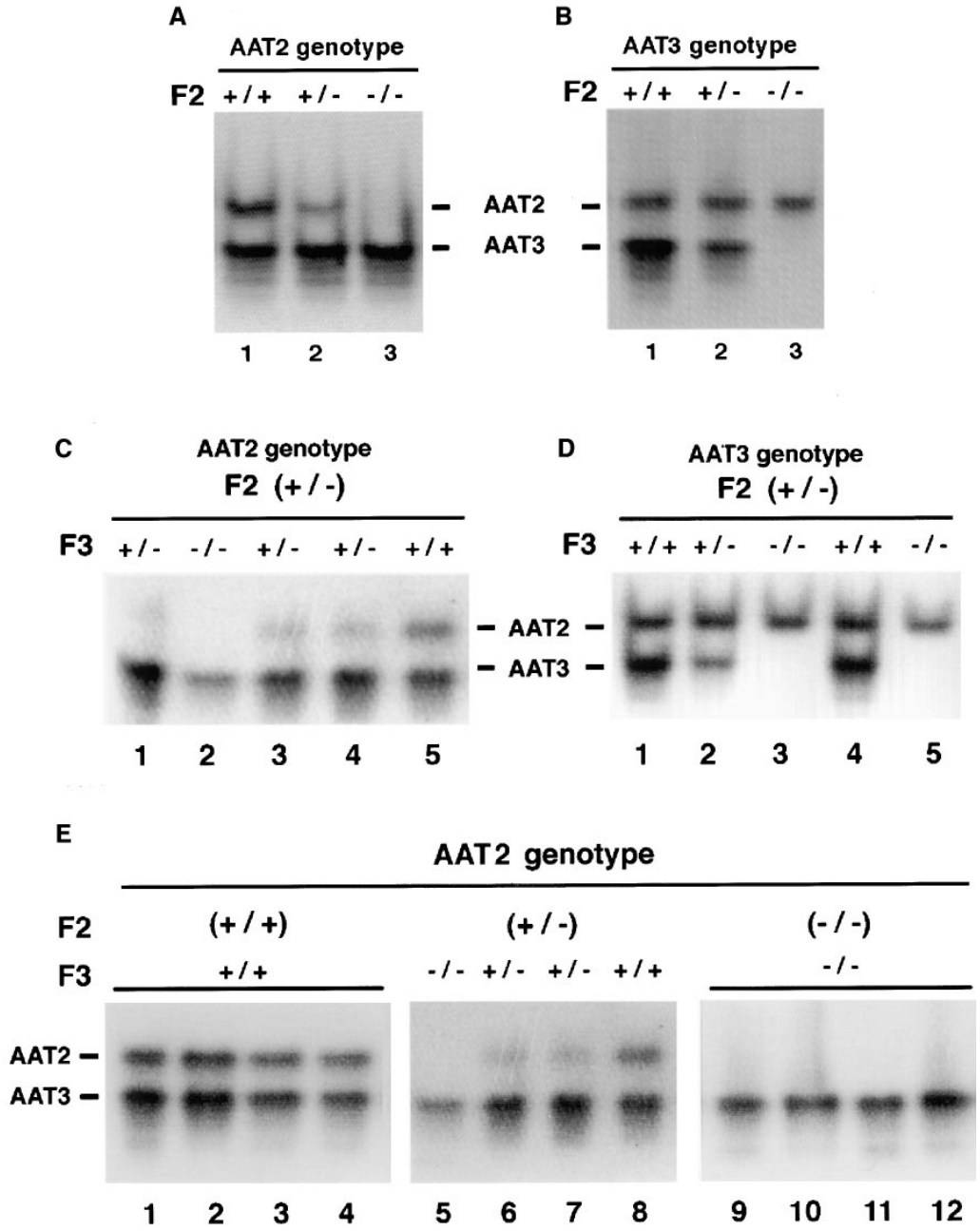

Figure 2.—Arabidopsis mutants deficient in cytosolic AAT2 or chloroplastic AAT3. (A) Gel phenotype of an *aat2* mutant deficient in cytosolic AAT2. Representative F_2 individuals from a segregating population resulting from the selfing of *AAT2*/*aat2-2*. The genotype of each F_2 individual is shown above each lane; lane 1 wild type (+/+), lane 2 heterozygote (+/−), and lane 3 homozygous mutant (−/−). (B) Gel phenotype of a mutant deficient in chloroplastic AAT3. Representative F_2 individuals from a segregating population resulting from the selfing of *AAT3*/*aat3-2*. The genotype of each F_2 individual is shown above each lane: lane 1 wild-type (+/+), lane 2 heterozygote (+/−), and lane 3 homozygous mutant (−/−). (C) Gel phenotype of five randomly selected individuals in the F_3 generation as a result of self-fertilization of a putative heterozygote (*AAT2*/*aat2-1*) from the F_2 generation. (D) Gel phenotype of five randomly selected individuals in the F_3 generation as a result of self-fertilization of a putative heterozygote (*AAT3*/*aat3-2*) from the F_2 generation. (E) Gel phenotype of F_3 individuals derived from the selfing of F_2 individuals designated +/+ (lanes 1–4); +/− (lanes 5–8); −/− (lanes 9–12) where + indicates *AAT2* and − indicates *aat2-1*.

notype were indeed heterozygotes, the putative heterozygotes were selfed and their F_3 progeny analyzed by gel assay. The F_3 generation from each of the putative heterozygotes showed the same segregation pattern as the F_2 generation, *i.e.*, three distinct gel phenotypes could be discerned (Figure 2C, lanes 1–5 and 2E, lanes 5–8, show one example). By contrast, when F_2 individuals identified as +/+ or −/− were selfed, all F_3 individuals showed the gel phenotype of the parent (Figure 2E, lanes 1–4, and 9–12, respectively). By this criterion, all three *aat2* mutants are judged to be semidominant because the phenotype (*i.e.*, reduced AAT2 activity) observed in the heterozygotes is intermediate between the wild-type and homozygous mutant plants. Furthermore, genetic analysis of the segregating F_2 population is consistent with the mutant phenotypes being caused by a mutation in a single nuclear gene (Table 1). The *aat2-1*, *aat2-2*, and *aat2-4* mutations are allelic as demonstrated by failure to complement in pairwise crosses, *i.e.*, all individuals in the F_1 generation of the crosses between the *aat2-1* mutant and the other *aat2* mutants had the *aat2* mutant gel phenotype (data not shown). For the *aat3* mutants, it is also possible to distinguish heterozygous F_2 individuals for the mutation affecting AAT3 (Figure 2B, lane 2). When these putative heterozygotes are selfed, resulting F_3 individuals segregate for the AAT3 activity as +/+; +/−; and −/− (Figure 2D, lanes 1–5). Genetic analysis of the segregating F_2 populations is consistent with the *aat3* mutant phenotypes being caused by mutations in a single nuclear gene (Table 1).

TABLE 1

***aat2* and *aat3* mutant alleles each segregate as a single nuclear gene**

Cross	WT	Het	Mut	χ^2	*P*
AAT2/aat2-1 (selfed)	17	25	8	3.24	>0.10
AAT2/aat2-2 (selfed)	17	38	18	0.15	>0.90
AAT3/aat3-1 (selfed)	9	26	11	0.96	>0.75
AAT3/aat3-2 (selfed)	33	62	31	0.10	>0.95

Chi-square analysis (with 2 d.f.) of segregating F_2 populations testing for deviations from 1:2:1 ratio of wild-type (WT):heterozygote (Het):mutant (Mut). The deviations from expected values (*i.e.*, χ^2) are low. Thus the segregation analysis is consistent with the notion that the mutant phenotypes are caused by mutations in a single nuclear gene, and that each gene segregates in a semidominant manner.

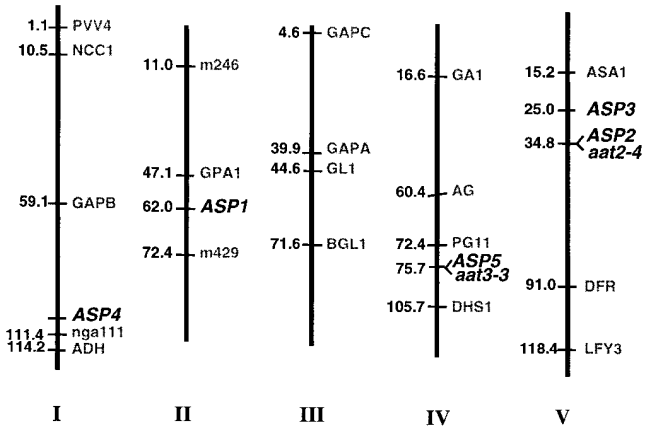

Figure 3.—Map positions of the five AspAT genes (*ASP1–ASP5*) and the mutations present in plants deficient in cytosolic AAT2 (*aat2*) or chloroplastic AAT3 (*aat3*). *ASP1–4* genes are described in Schultz and Coruzzi (1995). The *ASP5* gene is described in Wilkie *et al.* (1995, 1996). The *ASP* gene locations and *aat* mutations are shown relative to a subset of markers on the recombinant inbred line map from October 1997 (Nottingham Arabidopsis Stock Centre, WWW server http://nasc.nott.ac.uk/). CAPS and SSLP markers used for mapping purposes are not italicized.

The *aat3-1* and *aat3-2* mutations are allelic as demonstrated by failure to complement in pairwise crosses, *i.e.*, all individuals in the F_1 generation of the crosses between the *aat3-1* mutant and *aat3-2* mutant had the *aat3* mutant gel phenotype (data not shown).

Mapping of the five *ASP* genes and *aat2* and *aat3* mutant loci: Genetic mapping was performed to determine whether the *aat2* or *aat3* mutants were linked to any of the five *ASP* genes (*ASP1–ASP5*) encoding distinct AspAT isoenzymes. For mapping purposes, several of the *aat2* and *aat3* mutants (Columbia ecotype) were outcrossed to the Landsberg (Ler) ecotype. The *aat2* and *aat3* mutant loci were then mapped relative to known CAPS or SSLP markers, using approximately 30 individuals, a number deemed sufficient to map each mutation relative to one of 19 markers on a specific arm of each chromosome (Koniecnzy and Ausubel 1993). Separately, RFLP or CAPS polymorphisms were identified for the *ASP1–ASP5* genes and these *ASP* genes were mapped using Recombinant Inbred lines (for *ASP1*, *2*, *3*, and *5*) or relative to known CAPS or SSLP markers (for *ASP4*). A summary of the RFLP or CAPS markers identified for each *ASP* gene is in materials and methods. The relative map positions for the *ASP1–ASP5* genes and *aat2* and *aat3* mutants are shown in Figure 3. This mapping data along with cosegregation analysis (see below) has enabled us to predict which *ASP* gene is likely to be affected in the *aat2* or the *aat3* mutants, as outlined below.

The *aat3* mutation affecting the chloroplast AAT3 isoenzyme maps to the bottom of chromosome IV, near the PG11 CAPS marker (Figure 3). Arabidopsis contains two genes which could encode the major chloroplastic AAT3 isoenzyme: *ASP3* (Schultz and Coruzzi 1995) and *ASP5* (Wilkie *et al.* 1995, 1996). *ASP3* was predicted to encode either a plastid or peroxisomal AspAT based on the presence of a putative transit peptide (Schultz and Coruzzi 1995). *ASP5* encodes an aspartate aminotransferase polypeptide which can be imported into chloroplasts, as judged by *in vitro* uptake experiments (Wilkie *et al.* 1995). Results presented here suggest that *ASP5* most likely encodes the major chloroplast AAT3 isoenzyme in Arabidopsis, as it maps to chromosome IV at approximately 76 cM in the same location as the mutation in the *aat3* plants deficient in chloroplast AAT3 (Figure 3). By contrast, the *ASP3* gene maps to a separate chromosome (*V*) at approximately 25 cM.

The mutation affecting the major cytosolic AAT2 isoenzyme in the *aat2* plants, mapped to the top of chromosome *V* (Figure 3). While there are two putative genes for cytosolic AAT2, *ASP2* and *ASP4*, gene expression studies suggested that *ASP2* is the likely candidate to encode the major cytosolic AAT2 isoenzyme. *ASP2* mRNA accumulates to high levels especially in roots, while *ASP4* mRNA is expressed at extremely low levels in all tissues examined (Schultz and Coruzzi 1995). In support of this, the *aat2-4* mutation and the *ASP2* gene each map to the same region of chromosome *V*. By contrast, the *ASP4* gene maps to a different chromosome (chromosome *I*). Independent genetic evidence also suggests that the *ASP2* gene and the *aat2-4* mutation are linked. All 33 homozygous *aat2-4* mutants identified from a segregating F_2 population from the cross, *aat2-4*/*aat2-4* (Col) × *AAT2*/*AAT2* (Ler), showed the Columbia-specific RFLP for *ASP2* (data not shown).

***aat2-2* mutants deficient in cytosolic AAT2 display reduced growth rate and aspartate deficiency:** Phenotypic analysis of the *aat* mutants deficient in either cytosolic

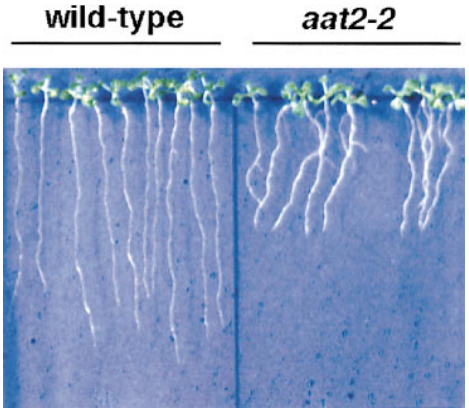

Figure 4.—*aat2-2* mutants exhibit reduced growth rates. The growth of *aat2-2* mutant plants and wild-type (Col) plants were monotored on MS medium containing 3% sucrose.

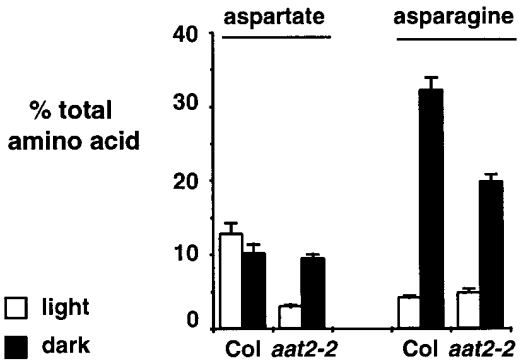

Figure 5.—*aat2-2* mutants have specific reductions in levels of aspartate in light-grown plants and asparagine in dark-adapted plants. The relative proportions of aspartate and asparagine in the phloem exudates from wild-type Columbia (Col) and *aat2-2* mutant plants grown in light (unshaded boxes) or dark adapted (shaded boxes). Each sample is the average of a single leaf from three representative plants. Plants were grown in soil in a normal day/night cycle (16 hr light/8 hr dark) for 3 wk and either light adapted (□) or dark adapted (■) for 24 hr. Error bars represent the standard error of the mean.

AAT2 (*aat2-1*, *aat2-2*, *aat2-4*) or chloroplastic AAT3 (*aat3-1* and *aat3-2*) provides a means to analyze the *in vivo* role of each of the two major AspAT isoenzymes in plant nitrogen metabolism. As all the *aat2* and *aat3* mutants were isolated and propagated on media or soil supplemented with 0.05% aspartate, we first determined whether any of these mutants were auxotrophic for aspartate. In all cases, the seed from the *aat2* and *aat3* mutants germinated and the developing plants set seed in the absence of any amino acid supplement under normal growth conditions (data not shown). These results suggest that none of the *aat2* or *aat3* mutants are auxotrophic. To determine whether any of the *aat2* or *aat3* mutants exhibit more subtle growth impairments, growth rate of the mutant plants was compared to wild type. For this, *aat2* or *aat3* mutants were sown side-by-side with wild type on tissue culture plates containing MS media supplemented with 3% sucrose. Plates were incubated vertically, and root length was measured as an indicator of growth rate. Neither of the *aat3* mutants (*aat3-1* or *aat3-2*) displayed impaired growth. Of the three *aat2* mutants, only the *aat2-2* mutant showed a reduction in root growth (20–50%), compared to wild-type Columbia controls (Figure 4). To minimize the possibility that the reduced rate of root growth in *aat2-2* was due to a background mutation, three independent homozygous mutant lines and three homozygous wild-type lines from the F_2 generation of the cross *aat2-2*/*aat2-2* × *AAT2*/*AAT2* (Col) were tested in a repeat experiment. All three of the homozygous mutant lines (*aat2-2*/*aat2-2*) showed the reduced growth rate phenotype, whereas all three of the wild-type (*AAT2*/*AAT2*) lines from the segregating population exhibited wild-type root growth rates (data not shown).

The altered growth of the *aat2-2* mutant suggested that the AAT2 isoenzyme might play a major role in assimilation of primary nitrogen into aspartate, as aspartate serves to transport assimilated nitrogen in many plant species including Arabidopsis (Urquhart and Joy 1981; Richardson and Baker 1982; Hayashi and Chino 1986; Schultz 1994; Lam *et al.* 1995). HPLC analysis showed that the *aat2-2* mutant indeed contains an 80% reduction in levels of aspartate transported in phloem exudates of light-grown *aat2-2* plants (Figure 5). Levels of free aspartate were also specifically reduced in whole leaf extracts of light-grown plants (Schultz 1994). In addition, the *aat2-2* mutant showed a specific and significant reduction (50%) in the levels of free asparagine measured in phloem exudates of dark-adapted plants (Figure 5). These results suggest that the pool of aspartate synthesized by cytosolic *AAT2* in the light is converted to asparagine when plants are dark adapted. As levels of aspartate are unaffected in dark-adapted *aat2-2* mutants compared to wild type, this suggests another AAT isoenzyme controls aspartate levels in dark-adapted plants. The other two *aat2* mutants (*aat2-1* and *aat2-3*) and the two *aat3* mutants (*aat3-1* and *aat3-2*) each showed normal amino acid profiles.

DISCUSSION

In plants, the amino acid aspartate is used to transport assimilated nitrogen from sources to sinks. As such, aspartate is one of the four most abundant free amino acids in leaves of many plant species including Arabidopsis and is also a major amino acid transported in the phloem (Schultz 1994; Lam *et al.* 1995). Thus, understanding which isoenzymes of aspartate aminotransferase control the synthesis of aspartate in higher plants has significance for plant nitrogen use. As there are five *ASP* genes encoding isoenzymes of AspAT localized to the cytosol, chloroplast, mitochondria, or peroxisomes, we used plant mutants defective in specific

AspAT isoenzymes to determine the *in vivo* function of each in aspartate biosynthesis. Historically, the use of mutants has been a powerful tool in the study of amino acid biosynthesis in microorganisms. However, the isolation of amino acid biosynthesis mutants in plants has been hampered by issues such as gene redundancy and problems with uptake of supplemented amino acids. There are only a few examples of whole plant mutants in amino acid biosynthesis enzymes. For example, Arabidopsis tryptophan biosynthesis mutants were isolated using a positive selection for resistance to 5-methylanthranilate (5-MA) (Last and Fink 1988). In this case, gene redundancy does occur, but a mutation in the more highly expressed gene for tryptophan synthase β (*TBS1*) can lead to auxotrophy (Last *et al.* 1991). Whole plant mutants have also been isolated with specific defects in isoenzymes of glutamine synthetase (GS2) (Wallsgrove *et al.* 1987) or glutamate synthase (Fd-GOGAT) (Somerville and Ogren 1980). These mutants were isolated based on their conditional lethal phenotype: death under photorespiratory growth conditions and growth in elevated CO_2. Mutants deficient in chloroplastic isoenzymes of GS2 or Fd-GOGAT demonstrated that photorespiratory ammonia released in mitochondria is reassimilated by isoenzymes localized in the chloroplast. As the factors controlling the flow of metabolites between subcellular compartments are as yet unknown, these case studies highlight the power of using genetics to define the metabolic flux that occurs between organelles *in planta*.

In the above examples, whole plant mutants were selected or screened for on the basis of a phenotype: resistance to 5-methylanthranilate (5-MA) or photorespiratory defects. In the case of aspartate aminotransferase (AspAT) where five genes encode isoenzymes localized to four distinct subcellular compartments, it was impossible to predict whether a mutation in any one *ASP* gene would lead to an associated aspartate-related phenotype. This is especially so given that other aminotransferases such as tyrosine aminotransferase might functionally mask a genetic defect in an aspartate aminotransferase gene (Wightman and Forest 1978). Since it was impossible to predict a phenotype (if any) resulting from a mutation in any one of the five *ASP* genes, we screened for Arabidopsis mutants deficient in specific AspAT isoenzymes using a brute-force screen for loss of isoenzyme activity. In this screen, aspartate was added to the growth media to allow the isolation of putative auxotrophs. Once the *aat* mutants were identified on the supplemented media, we then performed phenotypic analysis to assay for any aspartate related defects.

In a screen of 8000 M_2 seedlings, mutants defective in either of the two major AspAT isoenzymes were isolated: mutants lacking either cytosolic AAT2 (*aat2*, three mutant alleles) or chloroplastic AAT3 (*aat3*, two mutant alleles). The mutations affecting cytosolic AAT2, map to the same location and cosegregate with the *ASP2* gene, one of two genes for cytosolic AspAT. The mutations affecting chloroplastic AAT3 map to the same local region as the *ASP5* gene, a nuclear gene coding for chloroplastic AspAT (Wilkie *et al.* 1995, 1996). This mapping and cosegregation data support the notion that the mutations affecting cytosolic AAT2 or chloroplastic AAT3 are likely to be due to lesions in the structural genes for AspAT, *ASP2* and *ASP5*, respectively. The dosage effect observed in both the *aat2* and *aat3* heterozygotes (intermediate levels of isoenzyme activity) would be expected from mutations in structural genes. Furthermore, preliminary analysis indicates that all *aat2* mutants have normal levels of *ASP2* mRNA, supporting the notion that the mutations affecting the aat2 isoenzyme are structural rather than regulatory gene mutations (data not shown). Sequencing of specific *ASP* genes in each mutant will verify whether the loss of AAT activity is due to a structural gene mutation.

Phenotypic analysis revealed that the majority of the *aat2* and *aat3* mutants (with the notable exception of *aat2-2*) do not exhibit growth impairments or aspartate deficiencies, suggesting a significant degree of functional redundancy among the AspAT isoenzymes in Arabidopsis. An alternate explanation is that most of the mutants are leaky and contain sufficient residual AspAT activity (or other aminotransferase activities) to permit normal growth. It is, however, noteworthy that the *aat3* and *aat2* mutants contain less than 3–6% wild-type activity, respectively, as determined by native gel assay. Another possibility is that there are other phenotypes that have not yet been uncovered. The finding that all three *aat2* mutants have low levels of enzyme activity detected *in vitro* (less than 6% wild type) but only one (*aat2-2*) shows an aspartate deficiency and growth phenotype is reminiscent of the Arabidopsis *trp1* mutants. *trp1* mutants have defects in the enzyme phosphoribosylanthranilate transferase (PAT). Nine allelic *trp1* mutants show undetectable levels of activity *in vitro* (less than 1%); however, only four of these *trp1* mutants require tryptophan for growth (auxotrophs) while the others do not (prototrophs) (Rose *et al.* 1997).

The *aat2-2* mutants deficient in cytosolic AAT2, display defects in growth and a specific and dramatic reduction in the levels of free and transported aspartate. It is notable that the defect in aspartate synthesis in the *aat2-2* mutant is conditional on light. That is, light-grown *aat2-2* plants exhibit an 80% decrease in levels of transported aspartate. By contrast, levels of free aspartate are unaffected in dark-adapted plants. These results suggest that the cytosolic AAT2 isoenzyme controls the bulk of aspartate synthesized in the light, and suggests that another AspAT isoenzyme controls aspartate synthesized in the dark. Moreover, the *aat2-2* mutant plants also show a dramatic decrease in levels of free and transported asparagine, specifically in dark-adapted plants. This finding indicates that aspartate synthesized in the

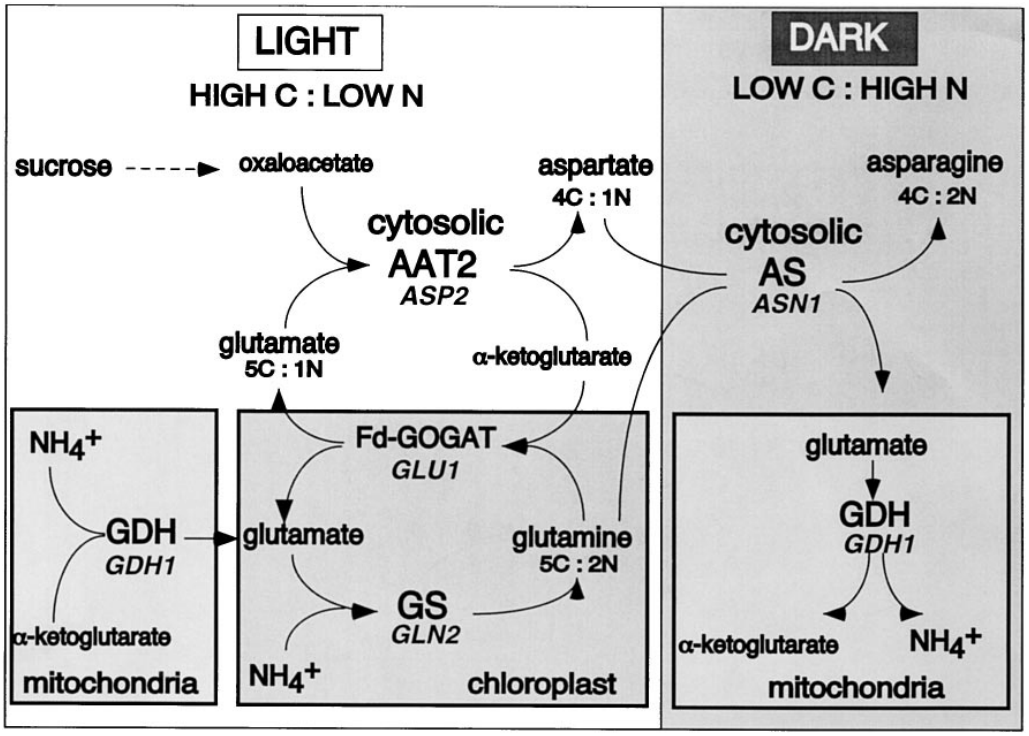

Figure 6.—Cytosolic AAT2 controls the synthesis of aspartate in the light, which is converted to asparagine in the dark. A model is depicted for the metabolic flow of nitrogen assimilation into the nitrogen-transport amino acids glutamate, glutamine, aspartate, and asparagine in the light and dark. In the light, inorganic nitrogen is assimilated initially into glutamate and glutamine by the combined actions of the plastid enzymes: chloroplastic glutamine synthetase (GS2, encoded by *GLN2*), and ferredoxin-dependent glutamate synthase (Fd-GOGAT, encoded by *GLU1*; Oliveira *et al.* 1997; Coschigano *et al.* 1998). The conversion of glutamate into aspartate in the light is controlled by cytosolic AAT2. In the dark, this pool of aspartate is converted into asparagine by asparagine synthetase (*ASN1*) (Lam *et al.* 1994, 1995).

light by the cytosolic AAT2 isoenzyme supplies the pool of aspartate used for asparagine synthesis in the dark. This metabolic conversion of aspartate to asparagine appears to reflect carbon:nitrogen economy in plants. In the dark, when carbon skeletons are limiting, asparagine (2N:4C) serves as a more carbon-efficient nitrogen transport compound compared to aspartate (1N:4C), glutamate (1N:5C), or glutamine (2N:5C) (see Figure 6). These findings provide *in vivo* support for a metabolic control model proposed by Lam *et al.* (1995) in which aspartate synthesized in the light was predicted to be the precursor to asparagine synthesized in the dark (Lam *et al.* 1994; Lam *et al.* 1995). While this model was initially developed based on the preferential synthesis of asparagine in the dark and by the differential regulation of nitrogen assimilation genes by light and metabolites, the *aat2-2* mutants provide experimental evidence suggesting this mechanism is operating *in vivo.* Moreover, the *aat2* mutants identify cytosolic AAT2, as the specific isoenzyme controlling the synthesis of this pool of aspartate. The finding that cytosolic AAT2 controls the bulk of aspartate synthesized in the light is somewhat unexpected. As nitrogen is assimilated into glutamate in plastids (by GOGAT), based on subcellular compartmentation, chloroplastic AAT3 would be a likely candidate to control aspartate synthesis in the light. The lack of a detectable aspartate-related phenotype associated with either of the two mutants defective in chloroplastic AAT3 cannot exclude a major role for this isoenzyme in aspartate synthesis, as neither of the two *aat3* mutants may contain a null allele. Nonetheless, the *aat2-2* mutants demonstrate that cytosolic AAT2, not chloroplastic AAT3, controls the bulk of aspartate synthesized in the light for nitrogen transport.

The preliminary analysis of *aat2* mutants described herein shows that cytosolic AAT2 plays an important role in nitrogen assimilation into aspartate in light-grown plants. In addition, the *aat2-2* mutant also identifies two distinct features about the genes for cytosolic AAT2. First, while there are two genes for cytosolic AspAT in Arabidopsis (*ASP2* and *ASP4*), a mutation linked to one gene leads to a phenotype. This is reminiscent of the case for duplicated genes for tryptophan synthase (Last *et al.* 1991), as *ASP2* is the major expressed gene for cytosolic AspAT, while *ASP4* is expressed at extremely low levels (based on steady state levels of mRNA; Schultz and Coruzzi 1995). Thus, the *ASP4* gene may function at low constitutive levels to provide aspartate for protein synthesis, while *ASP2* serves to synthesize aspartate used as a nitrogen trans-

port amino acid in the phloem. The *aat2* mutants also indicate that the major cytosolic AAT2 isoenzyme is functionally distinct from the AspAT isoenzymes which occur in the mitochondria, chloroplasts, and peroxisomes. While these other subcellular isoenzymes may be involved in shuttling reducing equivalents between subcellular compartments, it appears that the cytosolic AAT2 isoenzyme controls the bulk of the assimilation of nitrogen into aspartate used to transport nitrogen within the plant. Thus, these genetic studies have pinpointed the AAT2 isoenzyme as the isoenzyme controlling the synthesis of transported aspartate. As aspartate is a precursor to essential amino acids including lysine, these basic research studies using a genetic approach in Arabidopsis to identify isoenzymes controlling metabolic flux into transported aspartate may have significance for modifying levels of aspartate-derived amino acids in transgenic crop plants.

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