

Altered Xylem-Phloem Transfer of Amino Acids Affects Metabolism and Leads to Increased Seed Yield and Oil Content in *Arabidopsis* ^W

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Seed development and nitrogen (N) storage depend on delivery of amino acids to seed sinks. For efficient translocation to seeds, amino acids are loaded into the phloem in source leaves and along the long distance transport pathway through xylem-phloem transfer. We demonstrate that *Arabidopsis thaliana* AMINO ACID PERMEASE2 (AAP2) localizes to the phloem throughout the plant. AAP2 T-DNA insertion lines showed changes in source-sink translocation of amino acids and a decrease in the amount of seed total N and storage proteins, supporting AAP2 function in phloem loading and amino acid distribution to the embryo. Interestingly, in *aap2* seeds, total carbon (C) levels were unchanged, while fatty acid levels were elevated. Moreover, branch and silique numbers per plant and seed yield were strongly increased. This suggests changes in N and C delivery to sinks and subsequent modulations of sink development and seed metabolism. This is supported by tracer experiments, expression studies of genes of N/C transport and metabolism in source and sink, and by phenotypic and metabolite analyses of *aap2* plants. Thus, AAP2 is key for xylem to phloem transfer and sink N and C supply; moreover, modifications of N allocation can positively affect C assimilation and source-sink transport and benefit sink development and oil yield.

INTRODUCTION

Nitrogen (N) and carbon (C) are two of the most important nutrients for plant growth, and in the majority of plant species, they are transported in the phloem to sink organs in the form of amino acids and sucrose, respectively. Extensive studies have been performed on long-distance transport of sucrose. Sucrose transporters (SUC/SUT) were localized to the phloem of a number of plant species, and mutant analyses and antisense approaches demonstrated that the sucrose transporters play key roles in phloem loading of carbohydrates (for review, see Sauer, 2007; Tegeder et al., 2011). By contrast, molecular mechanisms for amino acid phloem loading remain unclear, although members of the ATF amino acid transporter family were suggested to be involved in this process (Lalonde et al., 2004; Tegeder and Rentsch, 2010).

Synthesis of amino acids mainly takes place in mature roots and source leaves, which export the N to supply sinks like flowers and seeds (Lam et al., 1996). Transport of amino acids to sinks happens mainly via the phloem, and to achieve this, the leaf-synthesized N compounds are loaded into the phloem of the minor veins. In cases where amino acid synthesis occurs in roots, they are transported via the xylem with the transpiration stream, mainly to mature leaves. Here, they are imported into the mesophyll cells for transient storage or metabolism or, alternatively,

loaded into the leaf phloem of the major veins for redistribution to fruits and seeds (Rentsch et al., 2007). Transfer of root-assimilated amino acids from the xylem to the phloem also occurs along the long-distance transport pathway (e.g., root and stem) for immediate supply of rapidly growing sinks (Pate et al., 1975, 1977). Generally, all protein amino acids are transported within the phloem to the seed, but in many plant species, including *Arabidopsis thaliana*, Gln, Glu, Asp, Asn, Ser, or Ala seem to dominate (Riens et al., 1991; Lam et al., 1995; Lohaus and Möllers, 2000; Wilkinson and Douglas, 2003; Hunt et al., 2006). Within the seed sink, amino acids are released from the seed coat into the apoplast from where they are taken up by the embryo for development, storage product synthesis (i.e., enzymes for starch, protein, and fatty acid/oil biosynthesis), or storage protein accumulation (Tegeder et al., 2000, 2007; Miranda et al., 2001, 2003; Rolletschek et al., 2005; Tan et al., 2008; Sanders et al., 2009). Amino acid transporters in source and sink tissues are considered to be essential in partitioning of assimilates, and they are potentially rate limiting for distribution of organic N to, and into, seed sinks (Lalonde et al., 2004). Sink number, size, and storage product accumulation are dependent on organic N transport processes from source to sink (Crawford, 1995; Song et al., 1997; Ma et al., 1998; Forde, 2002a, 2002b; Schmidt et al., 2007; Hunt et al., 2010).

While the role of transporter function in phloem loading of amino acids has not been demonstrated up to now, it was shown in potato (*Solanum tuberosum*) that transporters play a role in long-distance transport of amino acids (Koch et al., 2003). In *Arabidopsis*, promoter-reporter gene and RNA localization studies have identified three potential phloem loaders for amino acids, AMINO ACID PERMEASE2 (AAP2), AAP3, and AAP8 (Hirner et al., 1998; Okumoto et al., 2002, 2004; Lee et al., 2007). Work by Okumoto et al. (2004) demonstrated that AAP3 is specifically

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expressed in the root phloem, but mutants of the transporter did not display any phenotype. Cellular expression of *AAP8* has not been resolved, but promoter- β -glucuronidase (GUS) studies reported *AAP8* expression in the veins of young flowers, peduncle, and siliques. The transporter is also expressed in very young seeds, and *aap8* mutants showed a reduction in seed number per silique, suggesting a function of *AAP8* in amino acid import into the seed (Schmidt et al., 2007). With respect to *AAP2*, its RNA was localized to the vascular system of *Arabidopsis* seedlings (Kwart et al., 1993), which was confirmed by promoter-GUS analyses (Hirner et al., 1998). The latter studies also indicated that *AAP2* is expressed in the vascular bundle throughout mature *Arabidopsis* plants. While the cellular localization of *AAP2* in *Arabidopsis* has not been demonstrated, expression of the *Arabidopsis* *AAP2*-promoter-GUS construct in tobacco (*Nicotiana tabacum*) showed GUS staining in the stem phloem. Analysis of the substrate selectivity of *AAP2* clarified that the transporter recognizes a wide spectrum of proteogenic amino acids when expressed in yeast mutants defective in endogenous amino acid transport systems (Kwart et al., 1993; Fischer et al., 1995), and electrophysiological transport studies in *Xenopus laevis* oocytes demonstrated that *AAP2* transports Glu and neutral amino acids with moderate or low affinity (Fischer et al., 2002).

This study addresses the significance of *AAP2* in xylem-to-phloem transfer of amino acids and its role in source and sink physiology. We first show that *AAP2* is localized to the plasma membrane and the phloem and then demonstrate that in *aap2* mutants, source-sink translocation of amino acids is changed, affecting seed protein and oil levels as well as seed yield. Phenotypic, molecular, and biochemical analyses provide support for these changes through modulations in N and C metabolism, organ development, and partitioning processes in source and sink. The possible role of *AAP2* in xylem-phloem transfer of amino acids and the implications of its function for plant growth and N/C assimilation, metabolism, and transport are discussed.

RESULTS

***AAP2* Is Localized to the Phloem throughout the *Arabidopsis* Plant**

To resolve the cellular and subcellular location of *AAP2* expression, green fluorescent protein (GFP)-*AAP2* protein localization studies were performed. First, it was tested whether the *GFP-AAP2* gene construct encoded a functional *AAP2* transporter by expressing it in yeast cells (22574D) deficient in Pro transport (Figure 1A). When plated on media with Pro as sole nitrogen source, yeast cells expressing the empty vector failed to grow, while expression of GFP-*AAP2* fusion protein restored cell growth.

We further tested functionality of the GFP-*AAP2* protein in plants by transforming two *aap2* mutant lines (see below) with an *AAP2* promoter-GFP-*AAP2* construct. Analysis of the complementation lines showed that soluble protein levels were similar for complementation lines and wild-type plants, while protein amounts in the *aap2* plants were increased, demonstrating that GFP-*AAP2* was able to restore the *aap2* phenotype (for details, see text below and Figures 7E and 7F).

After confirming that GFP-*AAP2* is a functional protein in planta, GFP-*AAP2* under the control of cauliflower mosaic virus 35S promoter was transferred into onion (*Allium cepa*) epidermal cells via particle bombardment for examination of subcellular localization (Figures 1B to 1D). The cells were plasmolyzed to distinguish between plasma membrane and vacuole. *Arabidopsis* plasma membrane and proline transporter ProT2 (Figures 1E to 1G; Grallath et al., 2005) and free GFP (Figures 1H to 1J) were used as controls as well as nontransgenic cells from red onions, in which the cytosol and vacuole can be easily differentiated due to vacuolar storage of the red pigments or anthocyanins (Figures 1K and 1L). Analysis of onion cells expressing GFP-*AAP2* showed that the transporter is primarily localized to the plasma membranes (closed arrowheads; Figures 1B and 1D). Green fluorescence was also detected in membranes surrounding the nucleus, presumably in the endoplasmic reticulum (open arrowheads; Figure 1B). Some GFP-*AAP2* and GFP-ProT2 fluorescence was also seen in structures at the plasma membranes that might represent the Golgi apparatus (asterisks; Figures 1B and 1E). Since *AAP2* and ProT2 travel via the different compartments prior to being incorporated into the plasma membrane, the localization to these compartments is probably due to the overexpression of the GFP transporter proteins in onion cells. High-magnification images showed no fluorescence in the tonoplast or cytosol in onion cells bombarded with GFP-*AAP2* or GFP-ProT2 (Figures 1D and 1G).

Promoter-GUS studies and RNA localization studies indicated that *Arabidopsis* *AAP2* is expressed in the vascular tissue (Kwart et al., 1993; Hirner et al., 1998). To resolve the cellular localization of *AAP2*, the GFP-*AAP2* fusion construct was expressed in *Arabidopsis* under the control of the endogenous *AAP2* promoter. Analysis of the transgenic roots revealed GFP-*AAP2* localization in the phloem of roots (Figure 2A). Cross and longitudinal root sections further resolved that *AAP2* is present in the companion cells (Figures 2B and 2C). Some weak fluorescence was also detected in the sieve element, and future studies using anti-*AAP2* antibodies are needed to clarify if the observed fluorescence is background or if *AAP2* is localized to both companion cells and sieve element cells as well. Expression of *AAP2* was also found in the phloem throughout the plant (Figures 2A to 2L). Using *AAP2* promoter-GUS studies, transporter expression was detected in the vascular tissue of the major but not minor veins (Figure 2D), consistent with previous work by Hirner et al. (1998). Fluorescence of *AAP2* protein in the leaf was weak, and we were only able to record GFP-*AAP2* in the phloem of the leaf petiole by confocal imaging (Figures 2E and 2F). Localization studies in stems showed that *AAP2* expression is restricted to the phloem in this tissue as well (Figures 2G to 2I). GFP-*AAP2* fluorescence was also found in the phloem of other tissues along the transport path to seed sinks such as the phloem of the silique peduncle (Figures 2J to 2L). No fluorescence was observed in tissues or cells other than phloem, consistent with a role of *AAP2* in phloem loading of amino acids.

Identification of *aap2* Mutants and *AAP2* Expression Analysis

To analyze the function of *AAP2* in *Arabidopsis* plants, a reverse genetics approach was applied. Two independent *AAP2* T-DNA

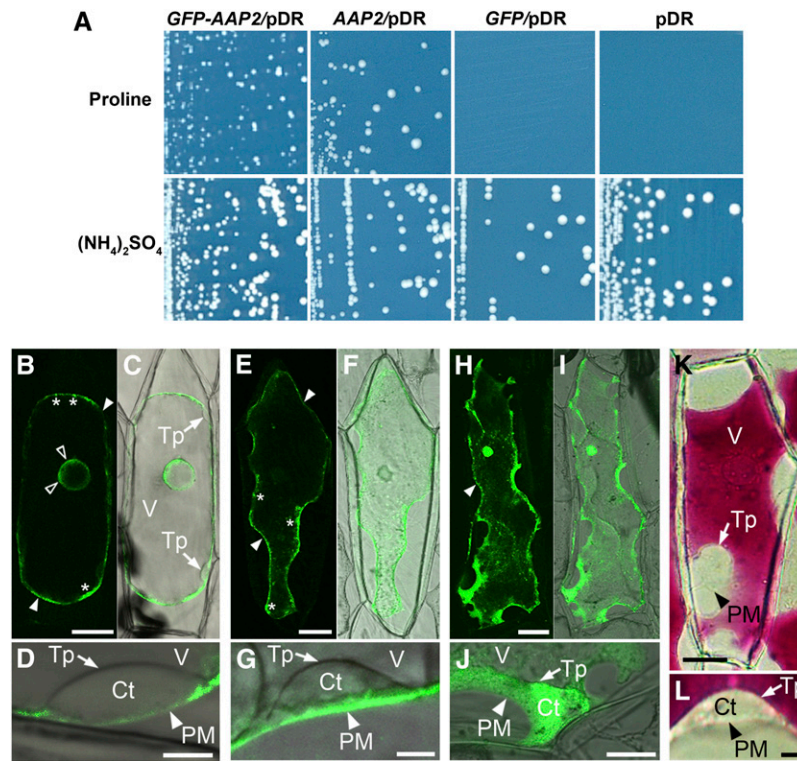


Figure 1. Expression of GFP-AAP2 in Yeast and Onion Cells.

(A) Yeast complementation by GFP-AAP2 fusion protein. Pro uptake-deficient yeast mutant (22574d) was transformed with *GFP-AAP2* in pDR. *AAP2* (positive) and *GFP* in pDR and empty pDR (negative) were used as controls for growth under selective conditions. Minimal media was supplemented with Pro (0.3 g/L) (top images) or ammonium sulfate (5 g/L) (bottom images) as a sole nitrogen source. GFP-AAP2 restores yeast growth on Pro containing medium similar to the AAP2 control, indicating that GFP-AAP2 is a functional protein.

(B) to (L) Subcellular localization of GFP-AAP2 fusion protein in onion cells following particle bombardment. Shown are confocal images (**[B]**, **[E]**, and **[H]**), overlaid bright-field images (**[C]**, **[D]**, **[F]**, **[G]**, **[I]**, and **[J]**), and bright-field images (**[K]** and **[L]**). The cells were plasmolyzed to distinguish between plasma membrane and vacuole. asterisks, Golgi apparatus; Ct, cytosol; open arrowhead, endoplasmic reticulum; PM/closed arrowhead, plasma membrane; Tp, tonoplast; V, vacuole. Bars = 50 μm in **(B)**, **(C)**, **(E)**, **(F)**, **(H)**, **(I)**, and **(K)** and 10 μm in **(D)**, **(G)**, **(J)**, and **(L)**.

(B) to (D) GFP-AAP2 expression.

(E) to (G) *Arabidopsis* amino acid transporter ProT2 (Grallath et al., 2005) was used as control for membrane localization.

(H) to (J) Localization of free GFP (control).

(K) and (L) Nontransgenic cells from red onions, illustrating that the cytosol and vacuole can be easily detected due to storage of the red pigments or anthocyanins in the vacuole (control).

insertion lines (*aap2-1*, SALK_047237; *aap2-2*, SALK_143597) were obtained from the ABRC at Ohio State University (<http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/index.html>), screened for homozygosity, and then analyzed. Allele *aap2-1* contains an insertion in the 1st intron, 164 nucleotides downstream of the start codon of the *AAP2* gene, and *aap2-2* carries a T-DNA insertion in the 4th exon at 863 nucleotides (Figure 3A). To analyze *AAP2* expression in the mutants, RT-PCR analysis was performed and revealed that *AAP2* mRNA is absent in *aap2-1* and *aap2-2* plants (Figure 3B).

Phloem Gln Levels Are Decreased and Long-Distance Transport of [¹⁴C]L-Gln Is Altered

Localization of *AAP2* in the leaf major veins and in the phloem throughout the plant suggests a function of the transporter in phloem loading along the transport pathway. Amino acids syn-

thesized in the root are translocated in the xylem mainly to the leaf. For further partitioning to the sinks, they may then be transferred in the leaf major veins from the xylem to the phloem. Leaf phloem exudates from *aap2-1* and *aap2-2* plants were analyzed to determine if *AAP2* might play a role in this process. The results showed a decrease in total phloem amino acid levels in the mutants by 20 to 25% (Figure 4A). Under the growth conditions used, Gln was found to be the main phloem amino acid transport form and presented ~45% of the total amino acids in the wild type (Figures 4A and 4B; compare with Lam et al., 1995; Hunt et al., 2006, 2010). The observed decrease in total amino acids in the mutants was solely due to a significant reduction in Gln levels. Other amino acids were present in much lower amounts than Gln (up to 25 times lower), but their phloem concentration in mutants versus the wild type was unchanged (see Supplemental Figure 1A online).

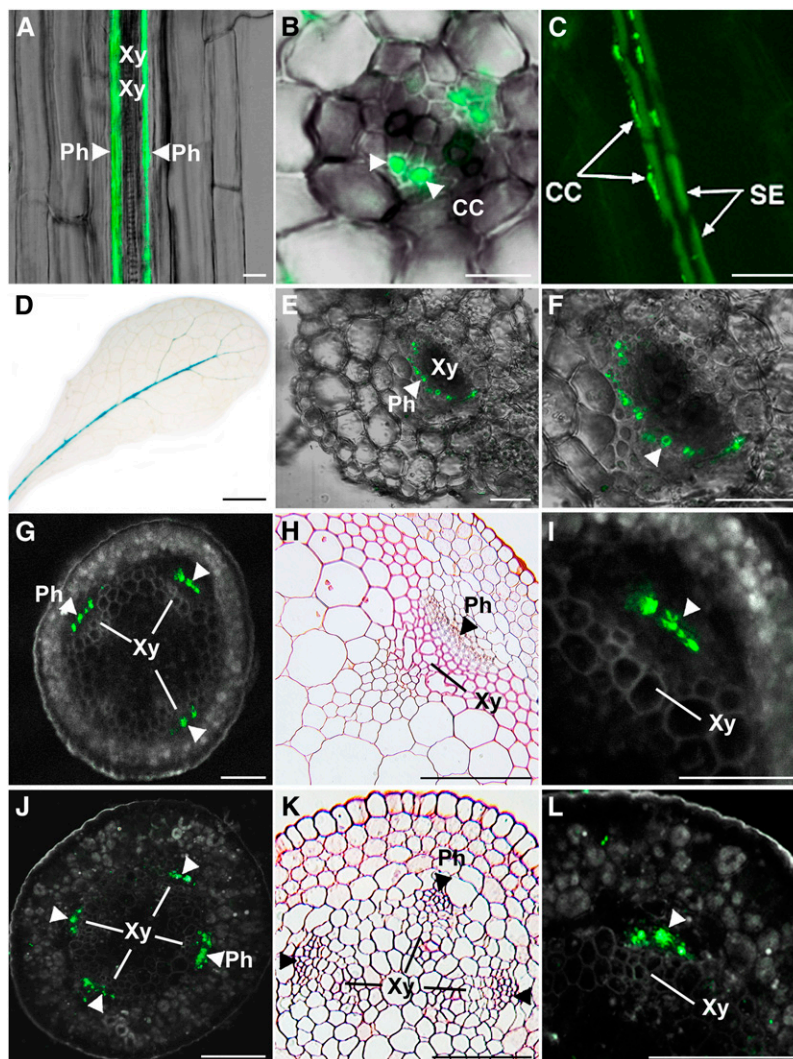


Figure 2. Localization of GFP-AAP2 in *Arabidopsis* Plants and AAP2 Promoter-GUS Expression Analysis in Leaves.

(A) to (C) GFP-AAP2 localization in roots under control of the AAP2 promoter. Longitudinal sections [(A) and (C)]; cross section (B).

(D) AAP2 promoter-GUS studies in leaves. GUS staining was detected in the major vein.

(E) to (F) Leaf petiole cross sections.

(G) to (I) Stem cross sections.

(J) to (L) Cross sections of silique peduncle.

GFP fluorescence is confined to the phloem along the transport pathway. Resin sections stained with safranin O [(H) and (K)]. CC, companion cells; Ph, phloem; SE, sieve element; Xy, xylem. Arrowheads point to the phloem. Bars = 7.5 μm in (B), 15 μm in (A), 20 μm in (E) and (F), 30 μm in (C), 100 μm in (G) to (L), and 4 mm in (D).

Transport studies with [^{14}C]-amino acids were performed to further analyze if xylem to phloem transfer and source-to-sink translocation of amino acids are altered in the *aap2* mutants. We first resolved which [^{14}C]-labeled amino acids should be used for these studies. Xylem sap was examined to determine which amino acids are transported in the xylem (and at what concentrations) and might be moved by AAP2 from the xylem to the phloem. The results showed that around 60% of the total amino acids in the xylem were present as Gln (Figures 4C and 4D). Levels of most other protein amino acids are relatively

low (10 to 100 times lower than Gln) and range from 5 to 100 μM dependent on the amino acid (see Supplemental Figure 1B online).

Based on the xylem sap and the phloem exudate analyses (see above) and since Gln is a predicted substrate for AAP2 (Fischer et al., 1995, 2002; see also Rentsch et al., 2007), [^{14}C]-labeled Gln was used for the transport studies. Gln was also chosen because previous experiments had shown that Gln is slowly metabolized when fed to the transpiration stream (Sharkey and Pate, 1975). Roots of 3-week-old plants that were in the vegetative stage

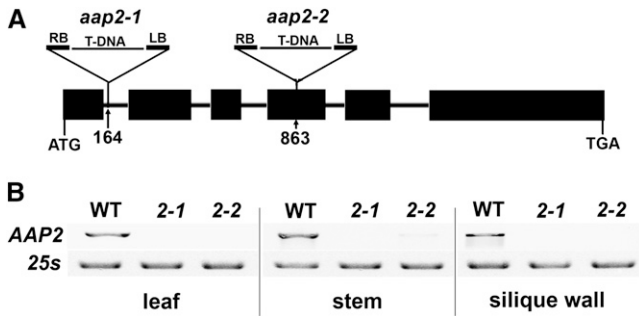


Figure 3. Molecular Characterization of *aap2* Mutants.

(A) Structure of the *AAP2* gene and positions of the T-DNA insertions in *aap2-1* and *aap2-2* mutants. Boxes and lines represent exon and intron regions, respectively. The triangles indicate the sites of T-DNA insertions. The *aap2-1* allele contains a T-DNA insertion in the first intron, 164 nucleotides downstream of the ATG. *Aap2-2* carries an insertion in the fourth exon in position 863. LB, left border of T-DNA; RB, right border. **(B)** RT-PCR analysis of *AAP2* expression in rosette leaf, stem, and silique wall of wild-type (WT), *aap2-1*, and *aap2-2* plants. *25S rRNA* was amplified as control for equal amounts of RNA in the different samples.

(prebolting) as well as of 6-week-old plants with fully developed branches and siliques were fed with [^{14}C]L-Gln for 45 min followed by a 15-min chase with cold Gln (Figures 4E to 4G). Levels of ^{14}C -label were determined in fully expanded mature leaves as well as in developing, small sink leaves of the 3-week-old plants. The results showed an increase in ^{14}C -label in *aap2* versus wild-type source leaves by 23% (Figure 4E) and a decrease in *aap2* sink leaves by 26 to 35% (Figure 4F). When analyzing [^{14}C]L-Gln translocation to siliques during the reproductive phase, results showed an ~50% reduction in label in *aap2* silique sinks (Figure 4G).

We used an azygous (wild-type) line as well as wild-type plants as controls for our tracer studies (Figures 4E to 4G). The azygous plants were generated via the same procedure as transgenic plants and were the progeny from the heterozygous transgenic *aap2-1* line that had lost the transgene through segregation. By contrast, wild-type plants had not gone through the transformation and tissue culture process. No significant differences were detected between wild-type and azygous lines in any of the tracer experiments, demonstrating that wild-type controls can be considered as reliable controls and that the two mutant alleles do have different properties than wild-type (and azygous) plants.

Seed Nitrogen and Carbon Contents Are Affected When *AAP2* Mediated Phloem Loading of Amino Acids Is Repressed

Seed development and N storage are dependent on phloem loading and delivery of amino acids to seed sinks, and feeding experiments showed a reduction in source-to-sink transport of Gln. To resolve effects on N nutrition of mutant seeds, N levels were determined in desiccated seeds of wild-type and *aap2* plants (Figures 5A to 5C). While the total N amounts in *aap2-1* and *aap2-2* seeds were significantly reduced compared with the wild type by 13 and 10%, respectively, the concentrations and

composition of free amino acids were not changed, neither in developing nor in dry seeds (Figures 5A and 5B; see Supplemental Figures 2A to 2C online). However, compared with the wild type, the amount of protein was significantly decreased in desiccated seeds of both mutants by 12 to 15% (Figure 5C). Using SDS-PAGE, we further examined if the reduction in total protein content in *aap2* seeds reflects a decrease in seed storage proteins (Figure 5D). The stained protein gel revealed a decrease in storage proteins in *aap2* seeds, specifically in 12S globulin and 2S albumin. This was further analyzed by expression studies showing a reduction in expression of storage protein genes *2S1* (albumin; Guerche et al., 1990) and *CRU3* (globulin; Pang et al., 1988) (Figure 5E). Analysis of seed amino acid transporters demonstrated that expression of the embryo-localized *AAP1* (Sanders et al., 2009; Figure 5E) was unchanged in the mutant seeds, but RNA levels of endosperm transporter *AAP8* (Schmidt et al., 2007) were strongly decreased, indicating downregulation of *AAP8* function when N supply is reduced (see also Sanders et al., 2009).

While total N and protein amounts were decreased in *aap2* seeds, no differences were found in seed weight between mutants and the wild type (Figure 10E), suggesting that other seed compounds might be elevated. Based on the strong interaction of C and N metabolism (Foyer et al., 2006; Weigelt et al., 2008) total C levels were examined. No changes were detected in total elemental C levels in *aap2* seeds, leading to an increased seed C:N ratio of up to ~15% depending on the mutant (Figures 6A and 6B; see also Figure 5A). Derived from these results and the fact that oils can contribute up to 40% of the storage compound content in *Arabidopsis* seeds (Li et al., 2006), fatty acid levels in *aap2* seeds were tested. The results demonstrated that the total fatty acid content was significantly increased in mutant seeds by up to 14% (Figure 6C) and that the increase was due to elevated levels of C18:1, 18:2, and 20:1 fatty acids (Figure 6D). Analysis of transcript levels of fatty acid synthesis genes, specifically *FAE1* (James et al., 1995), *FAD2*, (Covello and Reed, 1996), *FAD3* (Browse et al., 1993), and *KAS1* (Olsen et al., 2004), showed an increase in expression for all genes tested consistent with the biochemical analyses (Figure 6E). In addition, transcript levels of sucrose transporters *SUC2* (Sauer and Stolz, 1994), *SUT2/SUC3* (Barker et al., 2000; Meyer et al., 2004), and *SUT4/SUC4* (Weise et al., 2000) were upregulated in *aap2* seeds, supporting increased seed C delivery for fatty acid synthesis (Figure 6E).

Leaf N Metabolism and Transport Processes Are Altered in *aap2* Plants

Transport studies using [^{14}C]L-Gln indicated increased uptake of amino acids into source leaves of *aap2* plants (Figure 4E). To further analyze this, we determined the leaf RNA levels of *Lysine Histidine Transporter 1* (*LHT1*), a transporter involved in import of amino acids into mesophyll cells (Chen and Bush, 1997; Hirner et al., 2006) and found that expression of *LHT1* was strongly increased in the mutant leaves (Figure 7A). However, total N amounts did not change in *aap2* source leaves, but levels of most amino acids were decreased (Figures 7B to 7D). Plants store surplus amino acids in the vacuole. To analyze if transient

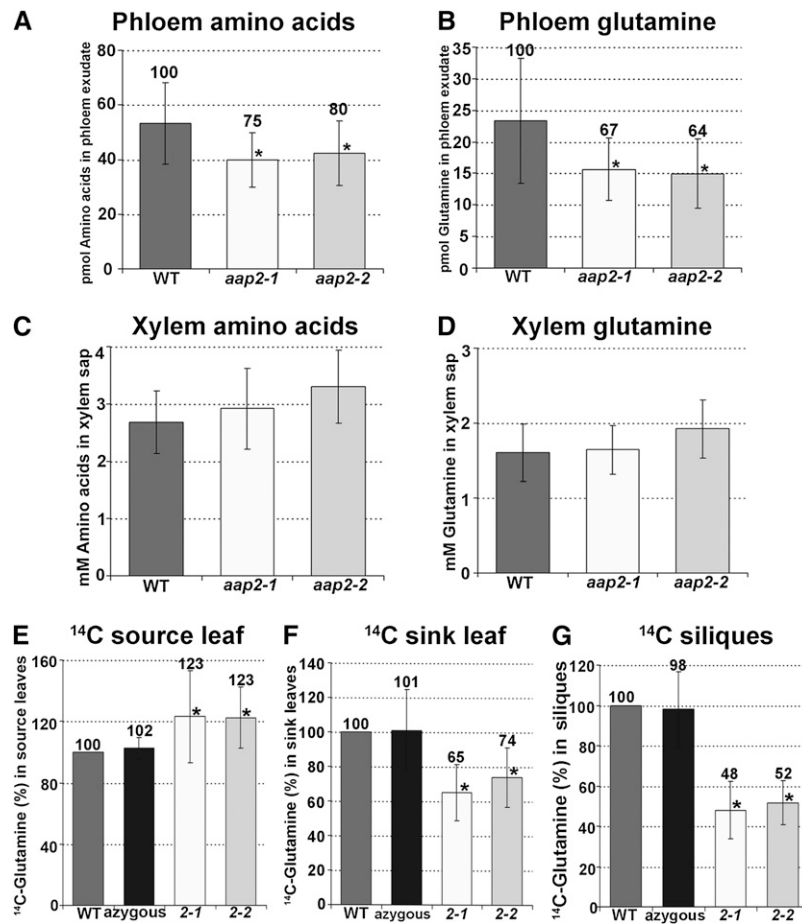


Figure 4. Amino Acid Concentrations in Phloem Exudate and Xylem Sap of *aap2* Mutants and Altered Levels of ^{14}C -Label in Source and Sink Leaves and Siliques after Feeding of $[^{14}\text{C}]\text{-L-Gln}$ via the *aap2* Roots.

Wild-type (WT) plants and an azygous (wild-type) line were used as controls for the tracer studies. Error bars depict SD. Asterisks indicate significant differences from the wild type ($P < 0.05$). Values above the columns indicate the percentage compared with the wild type, which was set to 100%.

(A) Total free amino acid levels in phloem exudates from source leaves of 6-week-old plants ($n = 8$).

(B) Gln levels in phloem exudates from source leaves of 6-week-old plants ($n = 8$).

(C) Total free amino acid levels in the xylem sap of 6-week-old plants ($n = 10$).

(D) Gln levels in the xylem sap of 6-week-old plants ($n = 10$).

(E) Accumulated radioactivity in source leaves of 3-week-old plants after $[^{14}\text{C}]\text{-L-Gln}$ feeding to the root ($n = 6$).

(F) Radioactivity in sink leaves of 3-week-old plants after $[^{14}\text{C}]\text{-L-Gln}$ root feeding ($n = 6$).

(G) Radioactivity in siliques of 6-week-old plants after $[^{14}\text{C}]\text{-L-Gln}$ root feeding ($n = 6$).

vacuolar storage pools of organic N might be decreased in *aap2* leaves, expression of *Arabidopsis* amino acid (*ANT*, At3g30390; *CAT2*, Su et al., 2004) and peptide (*PTR2*; Steiner et al., 1994) transporters that are predicted to be involved in import of the organic N into the leaf vacuole was examined (Figure 7A; Carter et al., 2004). RNA amounts of all transporters tested were decreased in *aap2* leaves.

In contrast with the decreased amino acid levels, leaf protein amounts were strongly increased by up to 30% depending on the mutant (Figure 7E). This indicates that in *aap2* source leaves compared with wild-type leaves, more amino acids are channeled into protein synthesis for metabolism or for transient storage. SDS-PAGE analysis and immunoblot results using antibodies against

the large and small subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) showed that this primary enzyme in CO_2 assimilation was upregulated in *aap2* leaves (Figure 7G). When analyzing the leaf protein levels, wild-type and azygous plants were used as controls, and no significant differences were detected between the control plants (Figure 7E). We further complemented the *aap2-1* and *aap2-2* lines using the *AAP2* promoter-*GFP-AAP2* construct and determined leaf protein levels in the resulting plants. It was found that the amounts of soluble protein were similar for complementation lines and wild-type plants, respectively (Figure 7F). Together with the tracer studies (see Figures 4E to 4G), these results further confirm that wild-type plants can be considered as true controls.

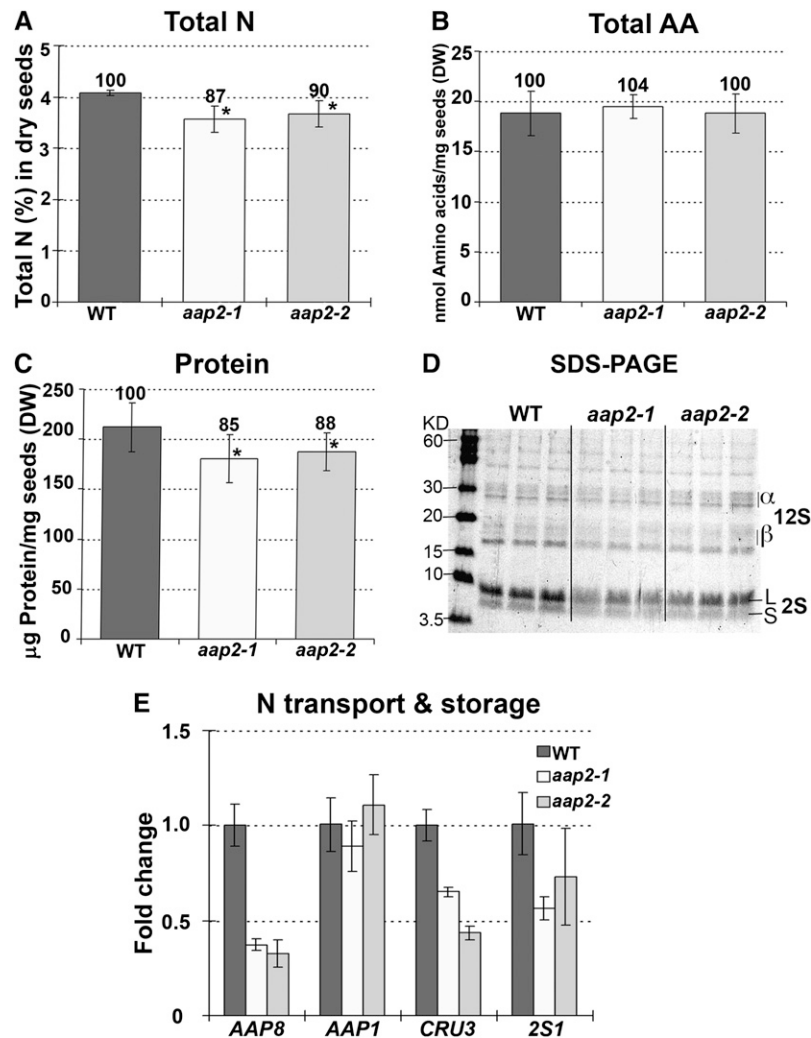


Figure 5. Seed N Levels and Expression of Genes Involved in N Transport and Metabolism in Seeds of Wild-Type and *aap2* Plants.

Error bars depict SD. Asterisks indicate significant differences from the wild type (WT; $P < 0.05$). Values above the columns indicate the percentage compared with the wild type, which was set to 100%. DW, dry weight.

(A) Total N levels (%) in dry seeds ($n = 4$).

(B) Total free amino acids (AA) in dry seeds ($n = 4$).

(C) Total amount of soluble proteins in dry seeds ($n = 5$).

(D) SDS-PAGE analysis of soluble proteins in dry seed. Three technical repetitions from independent extractions were done for each line as shown. α - and β -subunits of storage protein 12S globulin. S and L, small and large subunits of the 2S albumin storage protein.

(E) Real-time PCR analysis of genes of N transport and storage in developing seeds. Expression of amino acid transporter *AAP8* (At1g10010) and *AAP1* (At1g58360) as well as *CRU3* (At4g28520) and *2S1* (At4g27140) encoding for 12S globulin and 2S albumin, respectively, were analyzed. Three technical repetitions were done for each line using *Arabidopsis* ubiquitin 11 (*UBQ11*, At4g05050) and tubulin β -9 chain (*TUB9*, At4g20890), respectively, as control genes. Shown is the fold change in gene expression relative to *UBQ11* expression, determined from the C_T values using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

Leaf C Metabolism and Transport Processes Are Altered in *aap2* Plants

Elevated levels of Rubisco in *aap2* leaves, as well as increased sucrose transporter expression and fatty acid levels in mutant seeds, point to elevated C assimilation and sucrose export out of *aap2* leaves. We therefore determined the levels of products of C assimilation as well as the photosynthetic rate in *aap2* leaves. It

was found that the amount of starch in *aap2* leaves was slightly elevated, while glucose levels were strongly increased by up to 169% (Figures 8A and 8B). Sucrose levels, on the other hand, were decreased (Figure 8C). Significant changes were also observed for the net photosynthetic rate, which was increased by 70 to 75% in the mutants (Figure 8D).

To analyze if changes in photosynthesis and metabolite levels are consistent with expression of genes of C metabolism and

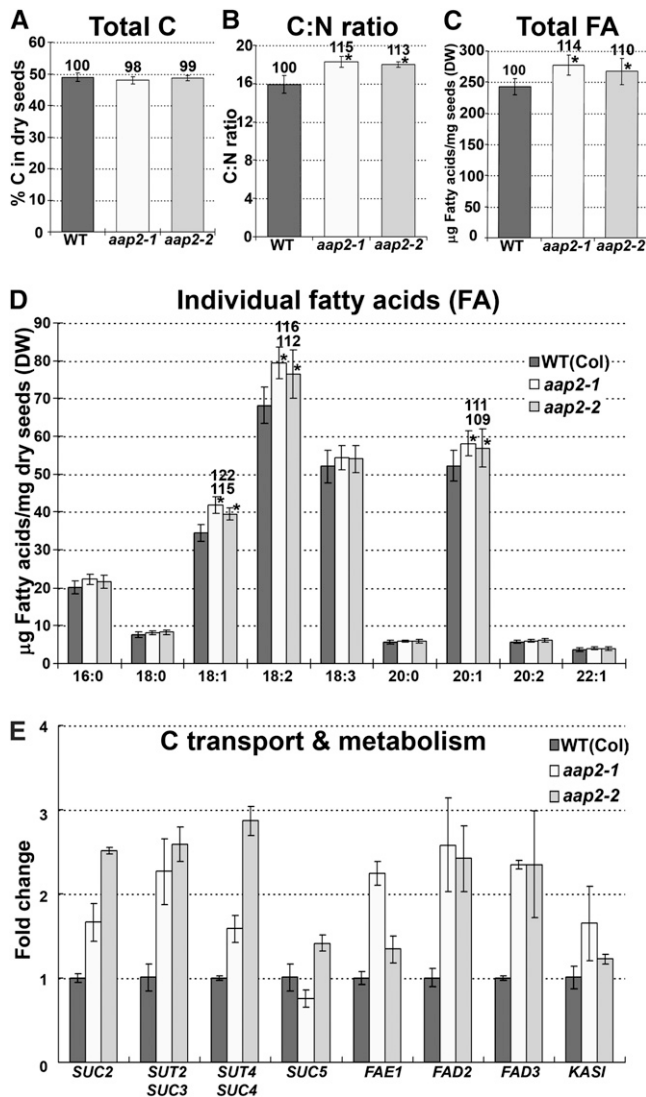


Figure 6. Analysis of Seed C Levels and Expression of Genes Involved in C Transport and Metabolism in Seeds of Wild-Type and *aap2* Plants.

Error bars depict SD. Asterisks indicate significant differences from the wild type (WT; $P < 0.05$). Values above the columns indicate the percentage compared with the wild type, which was set to 100%. DW, dry weight.

(A) Total C levels in dry seeds ($n = 4$).

(B) C:N ratio in dry seeds based on total amounts of C (A) and N (see Figure 5A) ($n = 4$).

(C) Total fatty acid (FA) levels in dry seeds ($n = 4$).

(D) Concentrations of individual fatty acids in dry seeds ($n = 4$).

(E) Real-time PCR analysis of genes of C transport and metabolism in developing seeds harvested 9 to 11 d after flowering. *SUC2* (At1g22710), *SUT2/SUC3* (At2g02860), *SUT4/SUC4* (At1g09960), and *SUC5* (At1g71890), sucrose transporters; *FAE1* (At4g34520), fatty acid elongation 1; *FAD2* (At3g12120), fatty acid desaturase 2; *FAD3* (At2g29980), fatty acid desaturase 3; *KASI* (At5g46290), β -ketoacyl-acyl carrier protein synthase I. Three technical repetitions were done for each line using *Arabidopsis* ubiquitin 11 (*UBQ11*, At4g05050) and tubulin β -9 chain (*TUB9*, At4g20890), respectively, as control genes. Shown is the fold change in gene expression relative to *UBQ11* expression, determined from the C_T values using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

transport in leaf cells, RNA levels of At1g74910 encoding for ADP-glucose pyrophosphorylase/AGPase (controlling starch synthesis; Villand et al., 1993), *DPE2* (releases glucose from maltose; Chia et al., 2004), *HXX3* (associated with the chloroplast outer envelope; phosphorylation of glucose, probably before it enters the cytosol; Wiese et al., 1999; Karve et al., 2008), *PGM* (α -D-glucose 1-phosphate and α -D-glucose 6-phosphate interconversion; Fettke et al., 2008), and *SPS4F* (conversion of UDP-glucose + D-fructose 6-phosphate to UDP + sucrose 6-phosphate; Strand et al., 2000), as well as of C transporters were examined (Figures 8E and 8F; see Supplemental Table 1 online). Expression of the genes examined and involved in synthesis of sugars was upregulated. In addition, transcript levels of transporters exporting triose phosphate (*TPT*; Schneider et al., 2002), maltose (*MEX1*; Niittylä et al., 2004), and glucose (*pGlcT*; Weber et al., 2000) from the chloroplasts into the cytosol are increased, as well as expression of transporters involved in phloem loading of sucrose *SUC2* (Sauer and Stolz, 1994; Truernit and Sauer, 1995) and *SUC3/SUT2* (Barker et al., 2000; Meyer et al., 2004). Expression of vacuolar glucose importers (*TMT2*, Wormit et al., 2006; *VG72*, Aluri and Büttner, 2007) were also increased in *aap2* plants, indicating that the observed high glucose levels might be due to increased import and accumulation of glucose in the vacuole. Together, the results support increased C assimilation and export from the leaf for sink C supply when N partitioning within the plant is altered.

Leaf Development Is Affected in *aap2* Plants

To determine if leaf development is affected in *aap2* mutants, we analyzed if changes occurred in leaf number, leaf size, and leaf chlorophyll content. Leaf numbers of *aap2* and wild-type plants did not significantly alter during the growth period (Figure 9A), and plants developed up to 11.6 leaves per plant, including very tiny leaves. If only sufficiently developed leaves were included, an average of nine leaves per wild-type and mutant plant was counted (Figure 9C). While no differences could be found in the leaf size of 4-week-old plants (Figure 9B), an increase was identified in *aap2* after 6 weeks of growth (Figure 9C). The leaf surface was significantly increased by up to 35% dependent on the mutant (Figure 9D). When analyzing the chlorophyll content of the rosette leaves of 4- and 6-week-old plants, no differences were detected between mutants and the wild type (see Supplemental Figures 3A and 3B online). We further tested leaves of 7-week-old plants that started to senesce and found that the chlorophyll content was generally higher in the mutant compared with wild-type leaves (Figure 9E). By week 8, senescence and desiccation were strongly progressed in both wild-type and mutant plants, making further measurements impossible.

Seed Yield Is Increased in *aap2* Plants

To resolve if changes in C and N partitioning in *aap2* plants affect sink set and growth, the number of side branches, flowers, and siliques that developed after 4, 5, and 6 weeks of growth was determined. Wild-type and mutants bolted after 3 weeks of growth and developed a main stem. Side branches started to grow after 4 weeks of plant growth. The branch number per *aap2*

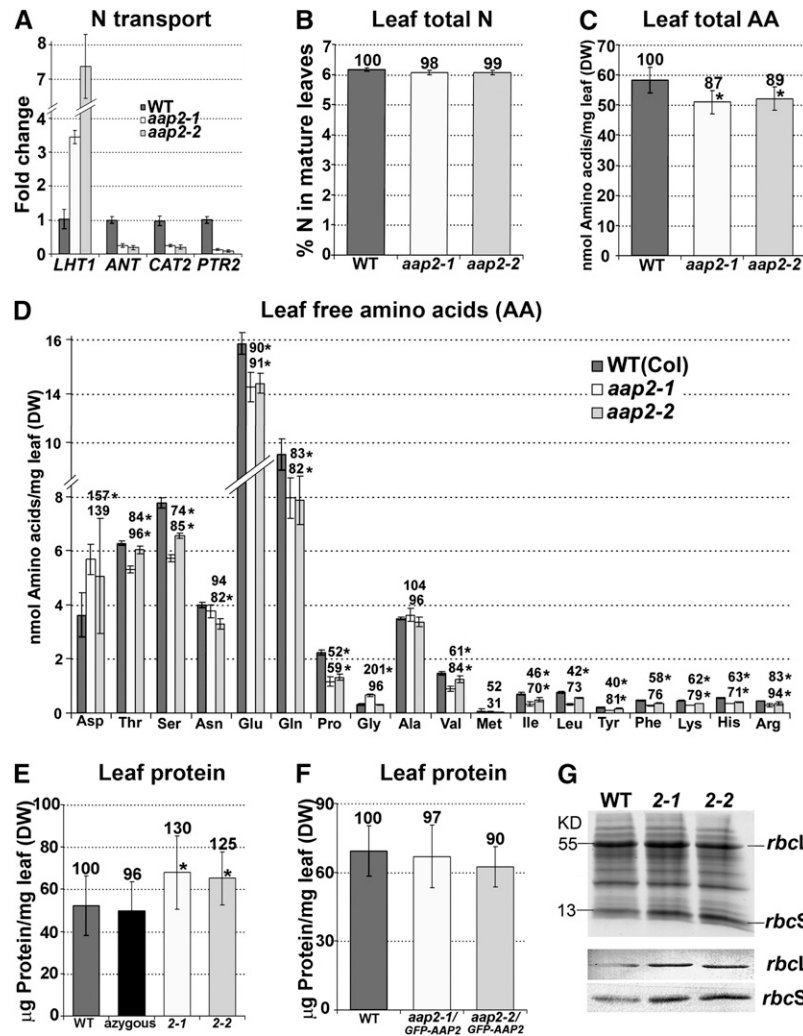


Figure 7. Expression of Amino acid Transporters and Analysis of Total N, Free Amino Acids, and Soluble Protein in Source Rosette Leaves of Wild-Type and *aap2* Plants.

Error bars depict SD. Asterisks indicate significant differences from the wild type (WT; $P < 0.05$). Values above the columns indicate the percentage compared with the wild type, which was set to 100%. DW, dry weight.

(A) Real-time PCR analysis of amino acid transporter *LHT1* (At5g40780; plasma membrane localized), *ANT* and *CAT2* (At3g30390 and At1g58030, respectively; both in tonoplast), and vacuolar peptide transporter *PTR2* (At2g02040; see Rentsch et al., 2007). Three technical repetitions were done for each line using *Arabidopsis* ubiquitin 11 (*UBQ11*, At4g05050) and tubulin β -9 chain (*TUB9*, At4g20890), respectively, as control genes. Shown is the fold change in gene expression relative to *UBQ11* expression, determined from the C_T values using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

(B) Total leaf N levels ($n = 4$).

(C) Total free amino acid levels in leaves ($n = 4$).

(D) Free amino acid composition and concentration in leaves ($n = 4$).

(E) Amounts of soluble leaf protein. Wild-type and azygous plants were used as controls ($n = 5$).

(F) Amounts of soluble leaf protein in wild-type and complementation lines *aap2-1/GFP-AAP2* and *aap2-2/GFP-AAP2* ($n = 8$).

(G) SDS-PAGE analysis of leaf proteins (top image) and immunoblot analysis using antibodies against small and large subunits of Rubisco (bottom image).

plant was increased after 5 and 6 weeks by around 13% (Figure 10A). Analysis of flower and silique set on the main stem revealed an increase of 50 to 100% in the mutants after 4 weeks of growth (Figure 10B). After 5 weeks, flower and silique development was improved by up to 35% dependent on the *aap2* lines. Differences in

silique set up to 20% between mutant and the wild type were observed after a growth period of 6 weeks. The increased silique set was not only due to the increase in branch number in *aap2* plants (Figure 10A) but also to an increase in silique number per main (9 to 12%) and side branch (14 to 19%), respectively (Figure 10C).

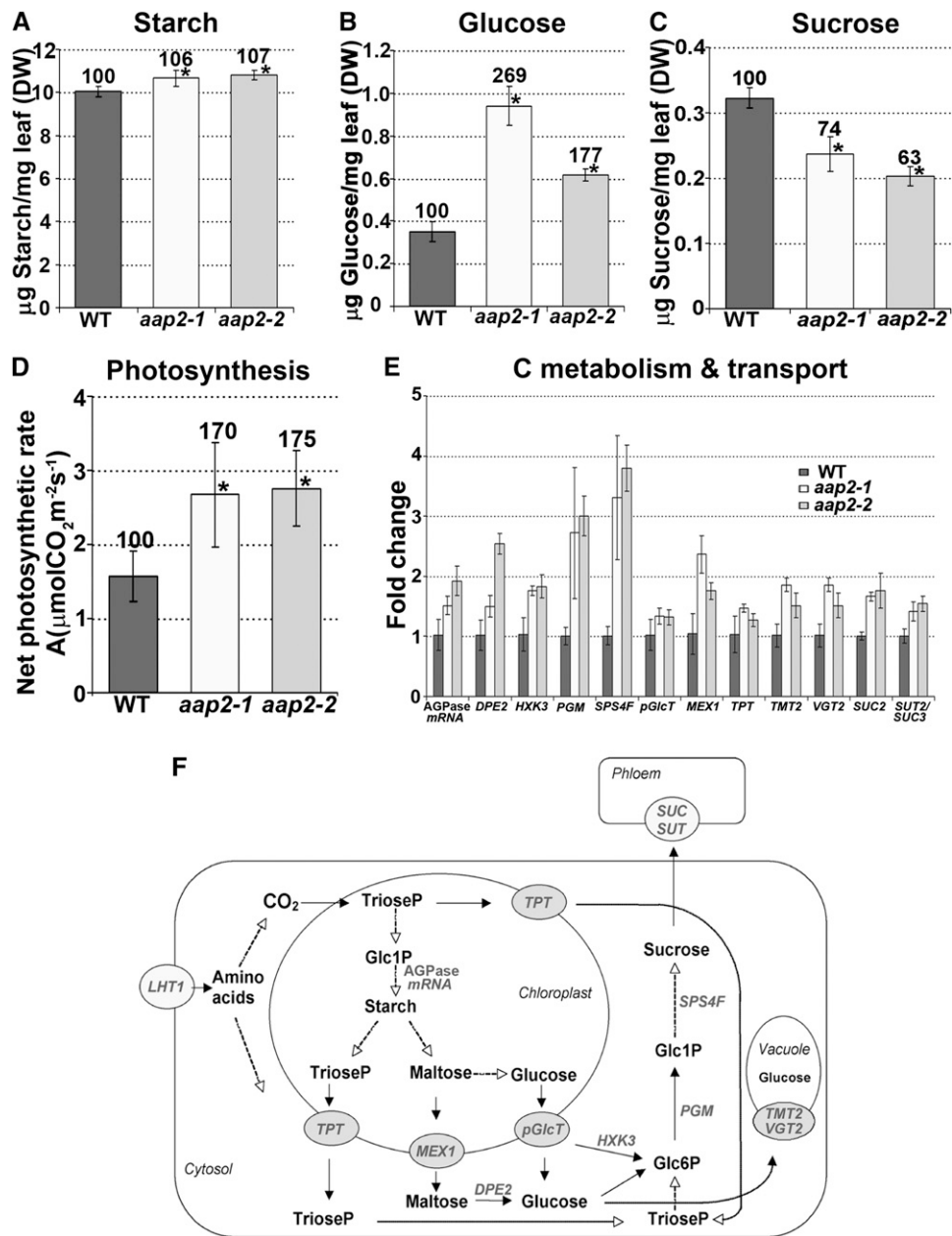


Figure 8. Analysis of Starch, Sugar, and Gene Expression Levels of N and C Transport and C Metabolism in Source Rosette Leaves of Wild-Type and *aap2* Plants.

Error bars depict SD. Asterisks indicate significant differences from the wild type (WT; $P < 0.05$). Values above the columns indicate the percentage compared with the wild type, which was set to 100%. DW, dry weight.

(A) Leaf starch levels ($n = 5$).

(B) Leaf glucose levels ($n = 5$).

(C) Leaf sucrose levels ($n = 5$).

(D) Net photosynthetic rate ($n = 4$). Photosynthetic rates were determined at ambient [CO₂].

(E) Real-time PCR analysis of genes of C transport and metabolism in leaves. *pGlcT*, plastidic glucose transporter (At5g16150); *MEX1*, plastidic maltose transporter (At5g17520); *TPT*, plastidic triose phosphate/phosphate translocator (At5g46110); *TMT2* (At4g35300) and *VGT2* (At5g17010), vacuole hexose importer; *SUC2* (At1g22710) and *SUT2/SUC3* (At2g02860), plasma membrane sucrose transporters (phloem loading); RNA levels of At1g74910 encoding for AGPase (ADP glucose pyrophosphorylase); *HXK3*, hexokinase (At1g50460); *DPE2*, cytosolic transglucosidase (At2g40840); *PGM*, phosphoglucomutase (At1g70730); *SPS4F*, sucrose phosphate synthase (At4g10120). Three technical repetitions were done for each line using *UBQ11* (At4g05050) and *TUB9* (At4g20890), respectively, as control genes. Shown is the fold change in gene expression relative to *UBQ11* expression, determined from the C_T values using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

(F) Overview of carbon metabolism and transport processes in source leaves and genes analyzed in *aap2* plants. For description of the specific genes and references, see Supplemental Table 1 online.

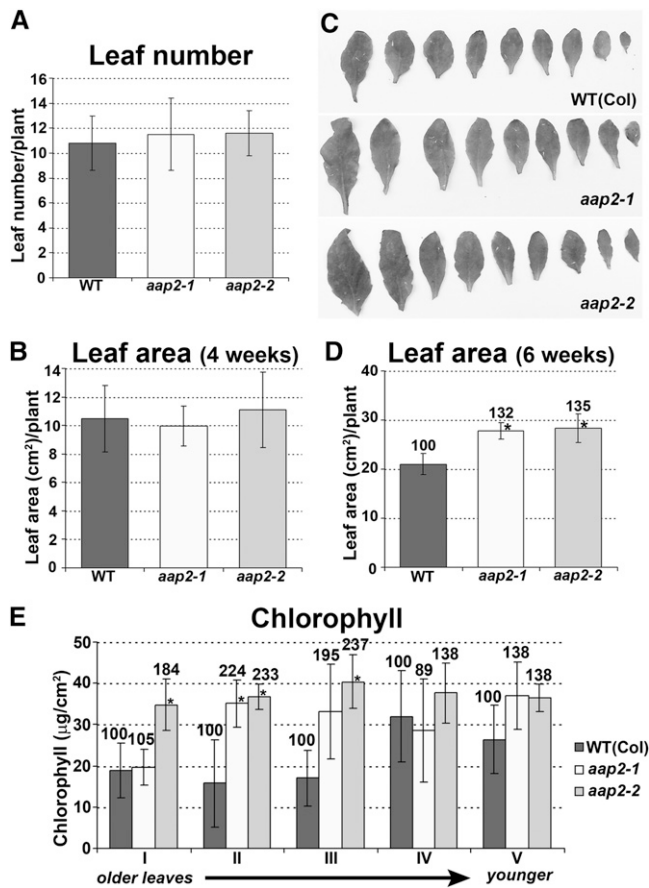


Figure 9. Analysis of *aap2* Leaf Development.

Error bars depict SD. Asterisks indicate significant differences from the wild type (WT; $P < 0.05$). Values above the columns indicate the percentage of each value in *aap2* plants compared with the wild type, which was set to 100%.

- (A) Leaf number of 6-week-old plants ($n = 8$).
 (B) Leaf surface area of 4-week-old leaf area ($n = 6$).
 (C) Images of rosette leaves of 6-week-old plants.
 (D) Leaf surface area of 6-week-old leaf area ($n = 6$).
 (E) Chlorophyll content of the 7-week-old plants ($n = 5$). Ten developed leaves in wild-type and *aap2* plants were analyzed in groups of 2 along their age. Leaves 1 and 2 represent leaf group I. They were the oldest leaves of the rosette, and senescence was evident (yellowing of leaves). Leaf group V consisted of fully developed (mature) green leaves that were the youngest within the rosette.

When analyzing yield characteristics, no effect was observed for seed set per silique, and the weight per seed was unchanged in mutants compared with the wild type (Figures 10D and 10E). Nevertheless, the improved number of siliques per plant led to an increase in total seed yield per mutant plant of 15 to 16% (Figure 10F). As a consequence, the total seed protein yield per plant was similar in wild-type and mutant plants (Figure 10G), even though the protein content per *aap2* seed was decreased (Figure 5C). Even more notably, the total amount of oil in the mutants compared with the wild-type plant was significantly increased by 32 and 19% for *aap2-1* and *aap2-2*, respectively (Figure 10H).

DISCUSSION

AAP2 Functions in Xylem-Phloem Transfer of Amino Acids

Amino acids are found in both xylem and phloem, but seeds or other sinks receive most of their organic N from the phloem, which requires efficient transfer of the N between the xylem and the phloem (Pate et al., 1975, 1977). It has been demonstrated, mainly in legumes, that movement of amino acids from xylem to phloem is quantitatively relevant in supplying fruit sinks with N (Atkins et al., 1979; for review, see van Bel, 1990; Atkins, 2000). Here, we provide evidence that AAP2 functions in xylem-phloem transfer of amino acids. It was established that AAP2 is a plasma membrane transporter that is expressed in the phloem and specifically in the companion cells (Figures 1B to 1L and 2), which is consistent with recent microarray studies identifying AAP2 as a companion cell transporter (Brady et al., 2007; Zhang et al., 2008). Our results further show that AAP2 is expressed in the phloem throughout the plant. The majority of xylem sap amino acids end up in the leaf due to high transpiration rates, and reallocation of amino acids from the xylem to the phloem occurs in the major veins, the location of AAP2 function in leaves (Figures 2D to 2F).

A more direct demonstration of AAP2 function in xylem to phloem movement and phloem loading is provided by xylem sap and leaf phloem exudate analyses (Figures 4A to 4D). Gln was the predominant amino acid in both transport streams, and in *aap2* mutants, the Gln phloem concentration was strongly decreased. Furthermore, phloem and xylem levels of other amino acids, which are present at much lower concentrations than Gln, were not affected in *aap2* plants. In heterologous expression systems (yeast and *Xenopus oocytes*), AAP2 transports a broad spectrum of amino acids (including Gln) when present in relatively high concentrations (Fischer et al., 1995, 2002; see also Rentsch et al., 2007). The observed change in only Gln in *aap2* phloem exudate is consistent with the predicted role of AAP2 in moderate/low affinity but high capacity transport. It further suggests that under physiological conditions, AAP2 might preferentially transfer Gln from the xylem to phloem. A role in movement of amino acids between the different vascular systems has also recently been demonstrated for AAP6, a xylem parenchyma amino acid transporter (Okumoto et al., 2002). Differing from our studies, phloem sap analysis of the *Arabidopsis aap6* mutant showed reduced levels of less (Lys, Phe, and Leu) and more (Asp) abundant amino acids (Hunt et al., 2010). However, in contrast with AAP2, AAP6 transports Asp and has a much higher affinity for neutral and acidic amino acids than AAP2 (Fischer et al., 2002).

Strong support for the predicted function of AAP2 in xylem-phloem transfer is further provided by short-term pulse-chase experiments. [^{14}C]L-Gln fed to *aap2* roots accumulated in source leaves, and allocation of label to sink leaves was decreased compared with the wild type. Similarly, flux of [^{14}C]L-Gln to *aap2* siliques was strongly reduced (Figures 4E to 4G). It cannot be excluded that some of the transported [^{14}C]L-Gln might have been converted to other N compounds or incorporated into proteins. However, our experiments were performed over a very short time frame, and Gln seems to be slowly metabolized when fed to the transpiration stream (Sharkey and Pate, 1975).

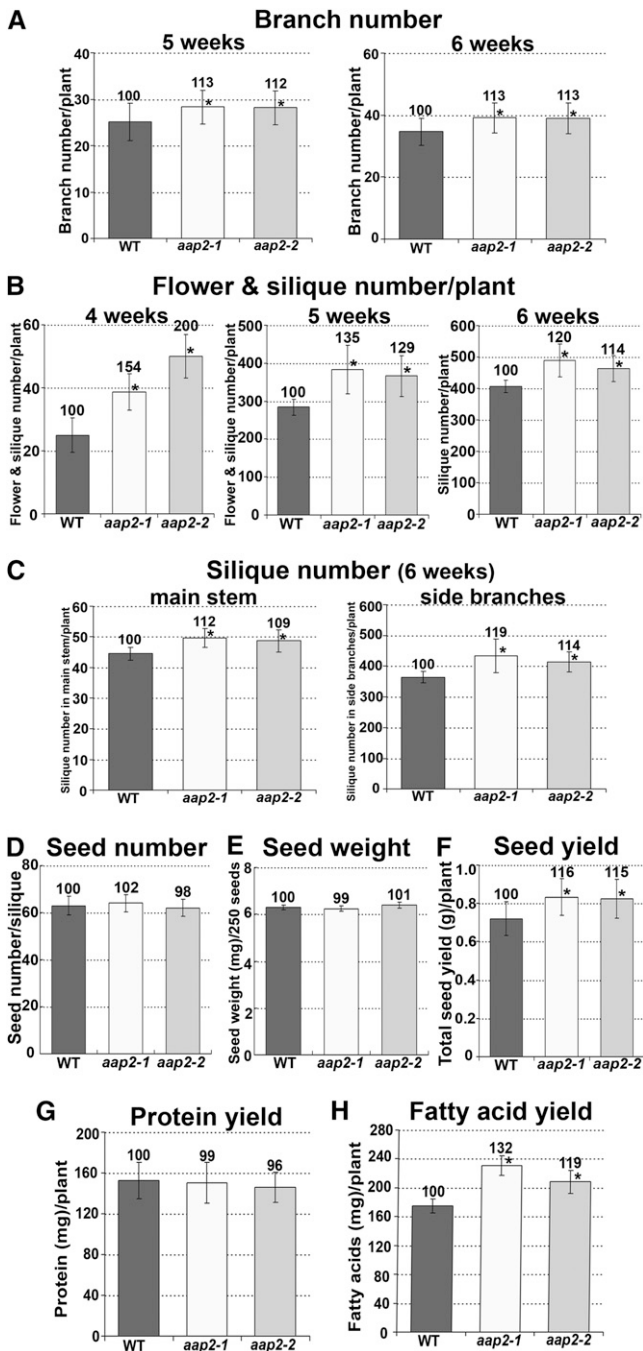


Figure 10. Yield Characteristics for *aap2* and Wild-Type Plants.

Error bars depict SD. Asterisks indicate significant differences from the wild type (WT; $P < 0.05$). Values above the columns indicate the percentage compared with the wild type, which was set to 100%.

(A) Number of branches per plant after 5 and 6 weeks of plant growth ($n = 22$).

(B) Number of flower and siliques per plant after 4, 5, and 6 weeks of growth ($n = 16$).

(C) Number of siliques on the main stem or on all side branches in 6-week-old plants ($n = 8$).

(D) Seed number per silique ($n = 60$).

Importance of AAP2 Function for Seed Development and Storage Reserve Accumulation

Arabidopsis seeds contain high amounts of proteins and oils (Baud et al., 2002) requiring a tight control of C/N partitioning and metabolism. Amino acids levels were unchanged in *aap2* seeds, while amounts of protein were decreased (Figures 5B and 5C; see Supplemental Figures 2A to 2C online). Similar results were obtained with mutants of *AAP1*, an amino acid transporter responsible for amino acid import into the embryo (Sanders et al., 2009), suggesting that in seeds/embryos, a steady pool of amino acids is maintained before the N is channeled into protein synthesis. While protein amounts were decreased as a consequence of reduced N delivery, the oil content in *aap2* seeds was strongly increased (Figures 5C, 6C, 6D, and 10H). This was further confirmed by an increased C:N ratio, which generally accounts for elevated oil levels in seeds (Figures 5A, 6A, and 6B; Li et al., 2006). The negative relationship between seed N levels and oil content is consistent with other reports (see Rathke et al., 2004 and references within) and has been related to competition for C skeletons during C/N metabolism (Bhatia and Rabson, 1976). Since total N amounts were decreased in *aap2* seeds, more C was presumably channeled into oil synthesis. In addition, the higher C:N ratio in *aap2* seeds points to increased delivery of carbohydrates, specifically sucrose, at reduced amino acid supply. This coincides with the upregulation of sucrose transporters in seeds and induction of fatty acid synthesis related gene expression (Figure 6E).

Another surprising result was that total seed yield was increased by ~15% in *aap2* plants (Figures 10A to 10F), suggesting that, while xylem-phloem transfer by AAP2 is important for seed N storage (see above), abolishment of AAP2 function does not cause N deficiency leading to reduced sink development (Crawford, 1995; Ma et al., 1998; Song et al., 1997; Forde, 2002a, 2002b; Schmidt et al., 2007; Sanders et al., 2009; Hunt et al., 2010). In *Arabidopsis*, the majority of amino acids are synthesized in source leaves (Coruzzi, 2003), and loading of the amino N into the minor vein phloem might be essential for sink initiation and growth rather than xylem-phloem transfer. However, such phloem loaders still need to be identified and their physiological function determined (Tegeder and Rentsch, 2010).

Effects of N Reallocation on Source Leaf Metabolism

Since both organic N and C compounds are important for sink development, alterations in leaf C assimilation, metabolism, and source-sink transport might be responsible for the increased seed and oil yields in *aap2* plants (Figures 10A to 10H). These changes are most probably related to modifications in N uptake into *aap2* mesophyll cells, as suggested by upregulation of amino acid importer *LHT1* (Figure 7A) and increased uptake of ^{14}C -label

(E) Weight of 250 seeds ($n = 5$).

(F) Total seed yield (g) per plant ($n = 16$).

(G) Seed protein yield per plant. Calculated based on seed yield per plant **(F)** and total seed protein levels (see Figure 5C).

(H) Seed fatty acid yield per plant. Calculated based on seed yield per plant **(F)** and total seed fatty acid levels (see Figure 6C).

in leaves following root feeding with [^{14}C]L-Gln (Figure 4E). Strangely, the free amino acid levels in whole *aap2* leaf extracts were decreased, while protein levels were increased (Figures 7C to 7G). Future studies need to resolve if amino acid concentrations are altered in the different cellular compartments of *aap2* leaves (e.g., in the vacuole; Figure 7A) and if changes in levels of specific amino acids or other signaling compounds might trigger increased usage of amino acids for protein and enzyme synthesis.

Generally, N partitioning to leaves positively regulates photosynthesis and consequently improves allocation of carbohydrates to sink tissues for vegetative and reproductive growth (Sage, 1994; Nakano et al., 1997; Martin et al., 2002; Krapp et al., 2005; Smith and Stitt, 2007). Indeed, levels of Rubisco and rate of photosynthesis were increased in mature *aap2* leaves, and expression of genes encoding for enzymes involved in C assimilation and transport were upregulated (Figures 7G, 8E, and 8F). Furthermore, starch concentrations in *aap2* leaves were moderately increased and glucose concentrations strongly elevated (Figures 8A and 8B), which is in conflict with the assumption that photosynthesis and sugar metabolism are upregulated by low rather than high sugar concentrations (Rolland et al., 2006). However, many studies have described that C and N act jointly and that the C-N balance might regulate C assimilation rather than the specific C metabolite concentration (Oliveira and Coruzzi, 1999; Stitt and Krapp, 1999; Coruzzi and Zhou, 2001; Martin et al., 2002; Sato et al., 2009). On the other hand, glucose might be transiently stored in the vacuole to avoid repression of C photosynthesis as supported by upregulation of vacuolar hexose importers in *aap2* leaves (Figure 8E). By contrast, sucrose levels in *aap2* leaves were decreased compared with the wild type, probably caused by increased export of sucrose due to increased sink strength (Figure 8C). This coincides with upregulation of sucrose transporter expression in *aap2* leaves and agrees with the increased sink numbers in *aap2* plants as well as a higher C:N ratio and elevated fatty acid levels in *aap2* seeds (Figures 6, 8C, and 10). Sucrose levels might also be kept low to further prevent feedback inhibition of photosynthesis (Stitt, 1991; Rolland et al., 2006), including expression of photosynthesis genes (Dijkwel et al., 1996, 1997).

Effects of N Reallocation on Source and Sink Development

When addressing leaf development, it was found that after 6 weeks of growth, the leaf surface in *aap2* plants was strongly increased (Figures 9C and 9D). In addition, senescence was slightly delayed (Figure 9E). These observed effects might be due to changes in sugar levels in *aap2* leaves and associated sugar-sensing mechanisms as part of a complex network regulating development and senescence of leaves (Riou-Khamlichy et al., 2000; Hanson et al., 2001; for review, see Paul and Pellny, 2003). Moreover, our data suggest that a combination of elevated photosynthesis (see above), increased leaf area for photosynthesis, and senescence (prolongation of photosynthesis) affects C partitioning from source to sink in *aap2* plants, resulting in improved sink development and seed oil levels.

Decreased movement of N to seeds and increased allocation to *aap2* leaves also led to increased development of side branches, flowers, and siliques in *aap2* plants (Figures 10A to 10F). These differences were due to changes in the rate of sink development

rather than to extended growth (Figures 10A to 10C). While timing of flowering was similar in the wild type and mutants, within the first week of inflorescence growth, flower set on the main stem was strongly increased in *aap2* plants. In addition, the speed of branching was increased and more flowers and siliques developed per branch after 5 and 6 weeks of growth, respectively. These results demonstrate that alterations in N transport and subsequent changes in leaf metabolism affect the speed of progression through a developmental series finally leading to increased seed yield. While altered C levels in the phloem might provide the signal for the observed changes, modulation of N transport processes in the phloem might be equally important for the improved sink set and growth in *aap2* mutants (see above) as an elevated C:N ratio in the phloem sap seems to be essential for flower induction and fruit development in *Arabidopsis* (Corbesier et al., 2002) in addition to the accurate amount of allocated C (Goetz et al., 2001; Zhang et al., 2001; Chen et al., 2004).

Conclusions and Perspectives

Based on the data, we can conclude that AAP2 is functioning in xylem to phloem transfer of amino acids with consequences for seed organic N and C levels and that its knockout leads to changes in C to N balance rather than causing N limitations for sink development. The *aap2* plants further demonstrate the influence of N partitioning processes on C/N metabolism and transport and reinforce evidence for tight interactions of C/N metabolic pathways between source and sink. Finally, they provide a link between N allocation, source and sink growth, and the speed of development.

Several approaches have been followed with varying success to improve N use efficiency for plant growth under sufficient and limited N supply, such as manipulation of N uptake and N metabolism (for review, see Hirel et al., 2007). Our work reveals the possibility that AAP2 may control sink growth and storage compound accumulation and provides evidence that fruit development might depend upon amino acid allocation to the photosynthetic machinery and for carbohydrate synthesis. This in turn has consequences for source leaf development and C export and partitioning to sinks, affecting sink development (number and pace) and seed oil levels. Future studies might help to resolve if manipulation of N allocation and fine-tuning of N long-distance transport processes can provide a strategy for maintaining or increasing seed number and storage compounds under N limitation. Considering the complex interplay of the different pathways and organs, high-resolution analyses of the localization and concentrations of amino acids (and sugars) are needed to discover the signals that trigger the observed responses and to advance the field of amino acid transport and its importance for cell and plant physiology.

METHODS

Materials

Arabidopsis thaliana ecotype Columbia (Col-0) lines with T-DNA insertion in AAP2 (At5g09220) were obtained from the ABRC (<http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/abrc/home.htm>). Information about these lines was acquired from the SIGnal database (Alonso et al., 2003).

Homozygous plants of *aap2* mutant lines (*aap2-1*, SALK_047237; *aap2-2*, SALK_143597) were identified by PCR using T-DNA left border primer SALK_LBa1 5'-TGGTTCACGTAGTGGCCATCG-3' and gene-specific primers for *aap2-1* (5'-CCGCTGCCGCCAATAACCACC-3' and 5'-CCAAAACAGAGTGAGAAAACAGAG-3') and *aap2-2* (5'-GACAAAATGCGGTTTTGCTCCAGG-3' and 5'-TGCAACTTGAACGATTCCAAGAGC-3'). The PCR products were sequenced to confirm the positions of the T-DNA insertions.

Plant Growth Conditions and Harvest

Arabidopsis plants were grown in a growth chamber in 36-well Com-Packs or single pots (T.O. Plastics) of 10 cm (xylem and phloem sap and phenotypic analyses) or 20 cm (photosynthesis measurements) in diameter. The soil was a mixture of peat (60%), pumice (20%), and sand (20%). Growth conditions were generally 16 h light at 20°C during the day and 16°C at night. Light conditions were around 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, and humidity was 50%. Plants were given 1.75 g/L Peters 20-20-20 all-purpose plant food (Scotts-Sierra Horticultural Products) once a week. Under these growth conditions, the life cycle of the *Arabidopsis* plants from seeds (planting) to seeds (harvest of desiccated seeds) was ~8 weeks. Two independent sets of mutant and wild-type plants were grown for analyses and confirmation of the results.

For gene expression and most biochemical analyses, pools of source rosette leaves (first six fully developed leaves) of at least 15 plants per line were harvested after 6 weeks of growth or germination (WAG). At this time point, side branches carrying a large number of siliques (Figures 10A to 10C) were developed. Some experiments were performed with younger plants (e.g., tracer studies and phenotypic analyses). At 3 WAG, the *Arabidopsis* plants were in the prebolting phase and grew up to nine rosette leaves. Four of these leaves were fully expanded and were considered as source leaves, and the two smallest were used as sink leaves. Bolting and development of the main stem occurred 3 to 4 WAG, and the first side branches started to appear 4 WAG (Figure 10A). Developing seeds were collected from 6-week-old plants. Flowers were marked and seeds were collected 9 to 11 d after flowering. Alternatively, desiccated seeds were collected. For gene expression and biochemical analyses, materials were immediately frozen in liquid nitrogen and stored at -80°C or lyophilized and then stored in the dark in a desiccator. To determine the seed number per silique, fully developed siliques from the main stem (bottom 15 siliques) were transferred to 70% ethanol to remove the chlorophyll. Seeds per cleared silique were counted under the dissecting microscope (Wild Heerbrugg). For collecting dry seeds, the Arasystem was used (Lehle Seeds). Plants used for phenotypic analyses were grown without any growth restrictions.

Preparation of GFP Constructs

Constructs were prepared for GFP-AAP2 expression and localization studies in yeast cells, onion (*Allium cepa*) cells, and *Arabidopsis*, respectively. The constructs for yeast complementation experiments and particle bombardment of epidermal onion cells were made by replacing the *AAP1* cDNA (*NotI*) in previously described constructs (Lee et al., 2007) with *AAP2* cDNA, resulting in *PMA1* promoter-*GFP-AAP2* term/pDR196 (yeast) and cauliflower mosaic virus 35S promoter-*GFP-AAP2*/pDH51 (onion). For localization of GFP-AAP2 in *Arabidopsis*, the *AAP2* promoter (Hirner et al., 1998) was amplified by PCR and cloned (*HindIII*, blunt) into the *SmaI* site of *GFP-AAP2*/pDH51. The *AAP2* promoter-*GFP-AAP2* cassette was cut with *KpnI*, blunted, and transferred into the binary vector pCB302 (Xiang et al., 1999). As controls, GFP constructs were used (Lee et al., 2007).

GFP-AAP2 Expression in Yeast, Onion Cells and *Arabidopsis* Plants

To analyze if the GFP-AAP2 encodes a functional fusion protein, functional complementation of *Saccharomyces cerevisiae* strain 22574d

(*MAT- α* , *ura 3-1*, *gap 1-1*, *put 4-1*, *uga 4-1*) expressing GFP-AAP2 was performed as described by Lee et al. (2007). Selection for yeast growth was performed on nitrogen-free medium supplemented with the amino acid Pro (0.3 g/L) as sole nitrogen source. For nonselective conditions, minimal media was supplemented with ammonium sulfate (0.3 g/L).

GFP-AAP2/pDH51 and *GFP*/pDH51 were introduced into onion epidermal cells by particle bombardment according to Chiu et al. (1996) and the manufacturer's protocol (Bio-Rad). Bombarded cells were incubated overnight at room temperature on moist filter paper. Epidermal onion cells were plasmolyzed using 1 M sucrose solution prior to the microscopy analysis.

Arabidopsis plants (the wild type and *aap2-1* and *aap2-2* lines) were transformed with *AAP2* promoter-*GFP-AAP2*/pCB302 constructs (Clough and Bent, 1998), and transgenic plants were selected as mentioned by Lee et al. (2007). Hand sections were prepared from different organs of transgenic *Arabidopsis* plants as described previously (Mano et al., 2002), and the GFP-AAP2 proteins were localized using confocal microscopy (Carl Zeiss).

Analysis of Phloem Exudates and Xylem Sap, and [¹⁴C]-Gln Transport Studies

Phloem exudates were obtained from 6-week-old *Arabidopsis* plants as previously described (Deeken et al., 2008). Aliquots of 100- μL phloem exudates were lyophilized and resuspended in 33 μL water, and 1/10 volume 1 N HCL was added to precipitate the EDTA. The samples were incubated on ice for 30 min, followed 30 min centrifuging at 4°C and 13,000 rpm. The supernatants were diluted 1:1 with double deionized water. Xylem sap was obtained from 6-week-old plants as previously described (Bauer et al., 1997). Briefly, the soil was saturated and plants were cut right above the root crown. Xylem sap was collected for 30 min (first drops were discarded) from 10 *aap2* or wild-type plants and stored at -80°C. For HPLC analysis, xylem sap was diluted 10 times. The sap was diluted 1:50 with double deionized water. Diluted phloem and xylem sap was used for amino acid analyses by HPLC (see below).

For transport studies, 3- and 6-week-old plants grown in single pots were removed from the soil and their main root was cut ~1 cm below the stem-root transition area in 5 mM EDTA buffer (270 mosmol, pH 7.5). The plants were transferred to a microcentrifuge tube with 1 mL of a solution containing 2.5 mM MES, 5 mM EDTA, and 2 mM cold Gln and [¹⁴C]-Gln (1 μCi , 37 kBq; Moravek Biochemical and Radiochemicals), and fed for 45 min at 300 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ illumination, followed by feeding for 15 min using a chase solution (same as above but without [¹⁴C]-Gln). The 3-week-old plants contained up to nine leaves. The first four mature source leaves and two developing sink leaves were harvested separately from a total of at least six plants and their surface was scanned. The radioactivity was determined by liquid scintillation spectrometry (Packard Instruments) and calculated per cm^2 leaf area. From the 6-week-old plants, 30 siliques were harvested from the main stem of six single plants, pooled and treated as described by Schmidt et al. (2007) before the radioactivity was determined.

RNA Extraction and Gene Expression Analysis

Total RNA was extracted from seeds and rosette leaves as previously described (Pélissier and Tegeger, 2007). Following DNA digestion with TURBO DNase (Applied Biosystems/Ambion), reverse transcription was performed using 1.5 μg RNA and Moloney murine leukemia virus reverse transcriptase (MMLV-RT; Invitrogen). The resulting cDNAs were diluted 1:4 or 1:10 with water for quantitative RT-PCR according to Sanders et al. (2009). Three technical replicates were run for each primer pair using a reaction mix of Platinum Taq Buffer (Invitrogen), 200 μM deoxynucleotide triphosphate (Fisher Bio-Reagents), 2.5 mM MgCl_2 , 0.25 \times SYBR Green I, 50 nM ROX, 0.5 units Platinum Taq DNA polymerase (all Invitrogen), and 0.25 μM primers. Real-time amplification data were collected with

an Applied Biosystems 7500 thermal cycler. Genes related to N or C transporters and metabolism as well as seed storage proteins were analyzed (see Supplemental Table 1 online). Gene specific primers for quantitative RT-PCR were designed in the VECTORNTI Suite, version 9, and data analysis was performed as explained earlier (Sanders et al., 2009). Primer sequences can be found in Supplemental Table 1 online.

Elemental N and C, Amino Acid and Fatty Acid Analyses

Lyophilized material of rosette leaves or dry seeds (1 to 2 mg) was used for total C and N quantification with the Costech ECS 4010 elemental analyzer as recently described (Sanders et al., 2009). To determine the amino acid content and composition, extracts were prepared from 30 mg of lyophilized seeds or rosette leaves and taken for HPLC according to Sanders et al. (2009). For oil content quantification, extracts were obtained from 250 mg of dry seeds (Sanders et al., 2009) and analyzed by gas chromatography with an Agilent 6890 series gas chromatograph outfitted with a 30 × 0.53-mm EC-WAX column and a flame ionization detector. The peak areas of all individual fatty acids were determined to establish the amount of total and single fatty acids.

Protein Extraction and Quantification

Extraction buffer (350 μL of 100 mM Tris-HCl, pH 8, 0.5% [w/v] SDS, 10% [v/v] glycerol, and 2% [v/v] 2-mercaptoethanol) was added to 1 mg lyophilized leaf or seed tissue followed by grinding and two centrifugation steps at 14,000 rpm and 4°C for 10 min. The supernatant was taken for protein quantification as well as SDS-PAGE and immunoblot analysis. The amount of protein was determined using the NanoOrange protein quantification kit according to the manufacturer's protocol (Invitrogen) and a Bio-Tek Synergy HT microplate reader (excitation, 480 nm; emission, 590 nm) equipped with KC4 v.3.4 software (<http://www.biotek.com>). For SDS-PAGE and immunoblot analyses, 20 μL and 10 μg of protein extracts, respectively, were added to 6× loading buffer (62.5 mM Tris-HCl, pH 6.8, 10% [v/v] glycerol, 2% [w/v] SDS, 5% [v/v] 2-mercaptoethanol, 8 M urea, and 0.002% [w/v] bromophenol blue), boiled for 10 min, loaded onto 12% (leaf extract) or 15% (seed extract) polyacrylamide gels, and separated for 2 h at 100 V using a minigel apparatus (Bio-Rad). For visualization of proteins, gels were stained with Coomassie Brilliant Blue R 250 overnight and destained in 40% (v/v) methanol and 10% (v/v) acetic acid solution. For immunoblot analysis, gels were incubated for 20 min in buffer (25 mM Tris, 192 mM glycine, 20% [v/v] methanol, and 0.02% [w/v] SDS) followed by protein transfer to a nitrocellulose membrane according to the instructions (Bio-Rad). The membrane was blocked with TBST buffer (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, 1% BSA, and 5% dry milk powder) for 30 min at room temperature with gentle shaking and then incubated overnight at 4°C in blocking buffer with an antibody against the large and small Rubisco subunit, respectively. Rubisco antibodies were provided by G. Edwards (anti-spinach [*Spinacia oleracea*] Rubisco LSU [1:10,000; courtesy of B. McFadden], *anti-Amaranthus hypochondriacus* Rubisco SSU [1:5000; courtesy of J. Berry]). After washing in TBST, the membrane was incubated for 2 h at room temperature in a 1:10,000 diluted secondary antibody conjugated to alkaline phosphatase (Sigma-Aldrich), washed with TBS (0.01 M Tris and 0.5 M NaCl), and equilibrated with detection buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 50 mM MgCl₂) for 5 min, and Rubisco was visualized using NBT/BCIP (Roche) in detection buffer for 15 to 30 min.

Carbohydrate Analyses

For analyses of glucose, sucrose, and starch content in leaves, 0.5 mL ethanol (80%) was added to 3 mg lyophilized rosette leaves, the mix was incubated at 75°C for 30 min, centrifuged at 13,000 rpm for 5 min (Eppendorf), and the supernatant was transferred to a fresh tube. Another

0.5 mL 80% ethanol was added to the pelleted plant tissue and proceeded as described. The supernatant was collected, dried under vacuum at 17,000 rpm (Savant), and dissolved in 200 μL distilled water. One hundred microliters of this solution was used for determining the amount of glucose with Amplex Red Glucose/Glucose Oxidase Assay according to the manufacturer's protocol (Invitrogen). In this assay, glucose oxidase reacts with D-glucose to form D-gluconolactone and H₂O₂, and the latter then reacts with horseradish peroxidase and a specific kit reagent to generate the red fluorescent oxidation product. This product was measured at wavelength 530 nm (excitation) and 590 nm (emission) with a Bio-Tek Synergy HT microplate reader equipped with KC4 v3.4 software, and the glucose concentration was determined using a standard curve. To quantify sucrose, 120 units of invertase (Sigma-Aldrich) were added to 100 μL of the sugar extract and incubated at 50°C for 10 min to hydrolyze sucrose to glucose, which then was measured. For starch quantification, the remaining pellets from the extraction described above were washed with water, dissolved in 500 μL buffer (0.2 N KOH and 0.1 N HAC, pH 4.5), and incubated at 95°C for 40 min. Three units of amylogucosidase (Sigma-Aldrich) and 0.25 units of α-amylase (Sigma-Aldrich) were added for hydrolysis of starch to glucose at 40°C overnight. The reaction mix was centrifuged (13,000 rpm, room temperature), and the supernatant was taken for determining starch-derived glucose.

Photosynthetic Gas Exchange and Chlorophyll Analysis

Gas-exchange measurements were performed using a LI-6400XT photosynthesis system (LI-COR) and six plants per *aap2* mutant line and wild type. They were grown in an environmentally controlled chamber under conditions described above but at a 12-h daylength to ensure that leaves were large enough for the leaf chamber. Plants used for analysis were 8 weeks old. Two or three fully developed, mature leaves from each plant were measured. A light response curve was used to determine the PPFD at which the plants reached the highest photosynthetic rate. The PPFD used for the reported data was 300 μmol m⁻² s⁻¹ at a partial pressure (CO₂) of 385 ppm.

To determine the chlorophyll content of mutant and wild-type leaves, a standard protocol for ethanol extraction was used. Leaf disks were harvested with a cork-borer from six leaves of five 4-week-old plants. For the 6- and 7-week-old plants, 10 mature leaves from five wild-type and *aap2* plants, respectively, were analyzed in groups of two along their age. Leaves 1 and 2 represent leaf group I. They were the oldest leaves of the rosette, and in 7-week-old plants, senescence was evident for these leaves (yellowing of leaves). Leaf group V consisted of mature, green leaves that were the youngest within the rosette.

Statistical Analysis

Results are shown for one growth set but are representative of two independently grown sets of plants and presented as a mean standard deviation (±SD) of at least four biological repetitions, each from a pool of a minimum of 15 plants. Statistical significance was determined with a one-way analysis of variance using SigmaStat 3.0 (Systat Software).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number At5g09220 (*AAP2*) and in Supplemental Table 1 online.

Supplemental Data

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

Supplemental Figure 1. Amino Acid Concentrations and Composition in the Phloem and Xylem of *aap2* Plants.

Supplemental Figure 2. Amino Acid Levels in Developing and Desiccated *aap2* Seeds.

Supplemental Figure 3. Chlorophyll Levels in Four and 6-Week-Old *aap2* Plants.

Supplemental Table 1. Overview of Primers Used for Expression Analyses of Genes of N and C Metabolism and Transport.

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