Connecting Source with Sink: The Role of Arabidopsis AAP8 in Phloem Loading of Amino Acids

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Allocation of large amounts of nitrogen to developing organs occurs in the phloem and is essential for plant growth and seed development. In Arabidopsis (Arabidopsis thaliana) and many other plant species, amino acids represent the dominant nitrogen transport forms in the phloem, and they are mainly synthesized in photosynthetically active source leaves. Following their synthesis, a broad spectrum of the amino nitrogen is actively loaded into the phloem of leaf minor veins and transported within the phloem sap to sinks such as developing leaves, fruits, or seeds. Controlled regulation of the source-to-sink transport of amino acids has long been postulated; however, the molecular mechanism of amino acid phloem loading was still unknown. In this study, Arabidopsis AMINO ACID PERMEASE8 (AAP8) was shown to be expressed in the source leaf phloem and localized to the plasma membrane, suggesting its function in phloem loading. This was further supported by transport studies with aap8 mutants fed with radiolabeled amino acids and by leaf exudate analyses. In addition, biochemical and molecular analyses revealed alterations in leaf nitrogen pools and metabolism dependent on the developmental stage of the mutants. Decreased amino acid phloem loading and partitioning to sinks led to decreased silique and seed numbers, but seed protein levels were unchanged, demonstrating the importance of AAP8 function for sink development rather than seed quality. Overall, these results show that AAP8 plays an important role in source-to-sink partitioning of nitrogen and that its function affects source leaf physiology and seed yield.

Nitrogen (N) is an essential macronutrient, and many studies have demonstrated that the amount of N supplied to the plant positively correlates with fruit or seed development and overall crop yield (Sinclair and deWit, 1976; Muchow, 1988; Dobermann and Cassman, 2002; Ferrante et al., 2010). Amino acids represent the main transport forms of N in most plant species. Generally, root cells take up inorganic N through the activity of nitrate (Cerezo et al., 2001; Filleur et al., 2001; Kiba et al., 2012) and ammonium transporters (Kaiser et al., 2002; Loqué et al., 2006). The nitrate and ammonium might then be reduced in the roots to amino acids followed by translocation in the xylem to photosynthetically active source leaves. Alternatively, the inorganic N may move in the transpiration stream from roots to source leaves, where it is used for amino acid synthesis (Lalonde et al., 2003; Tegeder and Rentsch, 2010). A broad spectrum of leaf amino acids in varying concentrations is finally translocated in the phloem to developing sink organs such as young leaves, fruits, and seeds that rely on the organic N for their growth (Riens et al., 1991; Büssis and Heineke, 1998; Tilsner et al., 2005; Hunt et al., 2010). Ultimately, source-to-sink translocation of amino acids controls sink development and seed yield (Koch et al., 2003; Tan et al., 2010; Ruan et al., 2012; Zhang et al., 2015).

There are at least two bottlenecks in the leaf-to-seed distribution of N: transporter-mediated loading of amino acids into the phloem and amino acid import into the developing embryo (Tegeder, 2012, 2014). The importance of membrane proteins for amino acid uptake into the embryo was described recently in Arabidopsis (Arabidopsis thaliana; Sanders et al., 2009). However, the molecular function of transporters involved in amino acid phloem loading has not been demonstrated to date, in contrast to the wealth of information available for organic carbon/Suc phloem loaders (Ayre, 2011; Braun et al., 2014).

Arabidopsis, like most crop plants, is considered to be an apoplastic phloem loader, as it lacks functional plasmodesmata between the sieve element/companion cell complex of the phloem and the surrounding cells (van Bel, 1993; Haritatos et al., 2000; Rennie and Turgeon, 2009). Amino acids that are exported from the leaf move from mesophyll cells through plasmodesmata toward the phloem parenchyma/bundle sheath cells of the minor veins, where they are released into the cell wall space via a passive transport step (Dündar and Bush, 2009; Ladwig et al., 2012). Subsequent import of amino acids into the phloem occurs against their concentration gradient and requires the activity of membrane-localized transporters (Bush, 1993). While transporters responsible for phloem-loading function have not yet

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been characterized in planta, it was suggested that proton-coupled amino acid permeases (AAPs) are involved, as they transport a broad spectrum of amino acids (Fischer et al., 1995; Tegeder et al., 2012; Tegeder and Ward, 2012; Zhang et al., 2015). In addition, six of eight members of the Arabidopsis AAP family have been localized to the vasculature or phloem of leaves and other organs. AAP1 and AAP4 seem to be present in the phloem of leaf minor and major veins (Fischer et al., 1995; Chen, 2006; Liu and Bush, 2006; Elashry et al., 2013), while AAP2 and AAP3 were localized to the transport phloem of leaf major veins, stem, and/or roots (Hirner et al., 1998; Okumoto et al., 2004; Zhang et al., 2010; Tegeder and Ward, 2012). Furthermore, promoter-reporter gene studies suggest AAP8 expression in the vasculature of floral buds and silique (Okumoto et al., 2002) and AAP6 localization to the vascular parenchyma throughout the plant (Okumoto et al., 2004; Hunt et al., 2010). A potential role of AAPs in phloem loading also supports findings from studies in pea (Pisum sativum) plants, where overexpression of the endogenous AAP1 transporter in the leaf phloem led to increased source-to-sink allocation of amino acids and to improved seed yield (Zhang et al., 2015). Furthermore, Arabidopsis AAP2 was shown to function in the xylem-to-phloem transfer of root-synthesized amino acids in leaf major veins (Zhang et al., 2010).

In recent Arabidopsis work, repression of the AAP8 amino acid transporter led to significant seed abortion (Schmidt et al., 2007), and because AAP8 is expressed during early embryo development (Okumoto et al., 2002), it was concluded that the seed phenotype was due to reduced amino acid import into the seed endosperm/embryo (Schmidt et al., 2007). However, based on the observations that (1) not all seeds were aborted in aap8 siliques (Schmidt et al., 2007), (2) generally, seed set is dependent on the amount of N supplied to the plant and translocated in the phloem to sinks (see above), and (3) sink tissues compete for the N delivered from the source, especially during N-limited conditions (Peoples et al., 1985; Uhart and Andrade, 1995), it seemed highly likely that, in the aap8 plants, the source-to-sink translocation of amino acids also was altered and contributed to the observed phenotype and that AAP8 is the long sought after phloem loader. Therefore, the role of AAP8 in amino acid phloem loading was examined using RNA and protein localization studies as well as physiological analyses of three aap8 mutant lines. The results demonstrate that AAP8 is indeed fundamental for the loading of a broad spectrum of amino acids into the phloem to supply vegetative and reproductive sinks with the essential N.

RESULTS

AAP8 Is Localized to the Plasma Membrane of the Phloem

To determine the localization of AAP8 function in source/rosette leaves, in situ RNA hybridization experiments were performed (Fig. 1). Using AAP8 mRNA antisense probes and a color detection procedure, the transporter transcripts were localized to both leaf minor and major veins (Fig. 1, A and B). Higher magnification imaging resolved AAP8 expression in the phloem (Fig. 1B). No signal was detected on leaf sections hybridized with AAP8 sense probes (Fig. 1C).

AAP8 expression was further analyzed at the tissue level in source and sink leaves at the vegetative and reproductive phases, suggesting a function in the phloem throughout Arabidopsis development. In addition, AAP8 expression was detected in sink leaves as well as in siliques. At least for siliques, this is consistent with previously published AAP8 promoter-GUS studies (Okumoto et al., 2002).

Using AAP8-GFP fusions and transient expression studies in Nicotiana benthamiana leaf epidermal cells, it was further resolved that AAP8 is localized to the plasma membrane (Fig. 1, D and F). Aquaporin PIP2A fused with mCherry was used as a plasma membrane control (Fig. 1, E and F). Together, the membrane localization and mRNA hybridization studies suggest that AAP8 functions in the import of amino acids into the source leaf phloem.

Identification of aap8 Mutants and AAP8 Expression Analysis

To determine the physiological function of AAP8 in phloem loading, three independent homozygous AAP8 T-DNA insertion lines (aap8-1 [SALK_092908], aap8-2 [SALK_081076C], and aap8-3 [SALK_092853]) were isolated and analyzed. Mutant alleles aap8-1 and aap8-3 contain T-DNA insertions in the first and fifth introns, respectively (Fig. 2A). Allele aap8-2 harbors an insertion in the sixth exon. The T-DNA insertions were confirmed by PCR using gene-specific primers in combination with the T-DNA left border primers followed by gel electrophoresis (Fig. 2A).

Expression analysis of AAP8 in source and sink leaves of 4-week-old wild-type and mutant plants, and in source leaves and siliques of 6-week-old plants, using RT-PCR revealed that the three mutants are AAP8 knockout lines (Fig. 2B).

Source-to-Sink Transport of Amino Acids Is Reduced in aap8 Plants

In Arabidopsis leaves, amino acid loading into the phloem of minor veins represents a key step in the long-distance transport of N to sink organs. Glu is the dominant N transport form in Arabidopsis during the day, and it is metabolized slowly in the phloem transport path (see Fig 4 below; Sharkey and Pate, 1975). In addition, Glu is present in relatively high amounts when compared with other amino acids (see Fig. 4). As
AAP8 transports both acidic and neutral amino acids, including Glu and Gln (Okumoto et al., 2002; Schmidt et al., 2007), in planta transport studies were performed using radiolabeled Glu and Gln (Fig. 3) to further dissect AAP8 function in phloem loading. Mutant and wild-type plants at the vegetative stage were initially used for the experiments to exclude fruit/seed effects on the source-to-sink allocation process (Okumoto et al., 2002; Schmidt et al., 2007). [14C]Glu or [14C]Gln was fed to source leaves, and label translocated to sink leaves was measured by scintillation counting (Fig. 3, A and B). The results showed a decrease in radioactivity in the mutant sink leaves compared with the wild type for both Glu and Gln feeding. Similarly, when radiolabeled Glu or Gln was fed to source leaves of plants at the reproductive stage, accumulated label was reduced significantly in the *aap8* siliques (Fig. 3, C and D). Together, these results support that AAP8 is important for the phloem loading of amino acids throughout plant development.

These results were further confirmed by analyses of leaf exudates from plants at the vegetative or reproductive growth stage (Fig. 4). Generally, exudates released from the cut leaf petioles contain phloem sap but also may consist of some xylem sap, apoplastic fluid, and other cell saps (Fiehn, 2003; Zhang et al., 2012). Analysis of the leaf exudates of *aap8* mutant lines versus the wild type during vegetative growth revealed a significant reduction in total amino acid levels of 41% to 51% (Fig. 4A). Amino acid amounts also were reduced in the *aap8* leaf exudates at the reproductive stage by up to 63% (Fig. 4B), indicating that, in the mutants, far fewer amino acids were delivered via the phloem to the sink organs throughout the life cycle. Analysis of individual amino acids revealed that a broad spectrum of amino acids was reduced in the *aap8* phloem during the growth period (Fig. 4). Together with the leaf-feeding experiments, the results demonstrate that AAP8 is responsible for the source-to-sink translocation of a variety of amino acids and that its function is required during both vegetative and reproductive growth.

As other members of the Arabidopsis AAP family also might be expressed in the phloem (or vascular parenchyma) and play a role in moving amino acids toward or into the phloem (Fischer et al., 1995; Hirner et al., 1998, Okumoto et al., 2002, 2004; Tegeder et al., 2007, 2012; Hunt et al., 2010; Tan et al., 2010; Zhang et al., 2010; Tegeder and Ward, 2012; Elashry et al., 2013), the leaf expression of different AAPS was analyzed in the mutants and the wild type using quantitative reverse transcription (qRT)-PCR. The results showed no change in *AAP1*, *AAP2*, *AAP4*, *AAP5*, and *AAP6* transporter expression profiles during the vegetative stage (Fig. 5A; Supplemental Fig. S1A). During
the reproductive phase, AAP5 expression was still unchanged; however, transcript levels were reduced for AAP1, AAP2, AAP4, and AAP6 in mutant versus wild-type leaves (Fig. 5B; Supplemental Fig. S1B). These results suggest that leaf amino acid transporter expression is dependent on the developmental, and potentially metabolic, status.

Source Leaf N Status and Metabolism Are Altered in aap8 Mutants

In further experiments, it was analyzed if and how the reduced amino acid phloem loading affects source leaf N status and metabolism during plant development (Fig. 6). First, leaf amino acid levels were measured in the wild type, and the results showed that total free amino acid pools were generally higher (46%) during the reproductive versus the vegetative phase (Fig. 6, A and B). When comparing aap8 with wild-type leaves, total amino acids were increased significantly, by up to 65%, depending on the mutant and the developmental stage (Fig. 6, A and B). The increase was due to elevated amounts of a broad spectrum of amino acids, although during the reproductive growth period, Gln contributed most strongly to the elevated amino acid levels (Fig. 6B). Furthermore, analysis of soluble protein levels demonstrated significant increases in aap8 leaf protein of up to 18% and 27% during vegetative and reproductive growth, respectively (Fig. 6, C and D). Together, these results suggest that decreased source-to-sink amino acid allocation leads to the accumulation of N metabolites during the vegetative and reproductive stages.

The N and/or amino acid status has been shown to regulate N assimilation (Deng et al., 1991; Li et al., 1995; Aslam et al., 2001). To determine the effects of altered leaf N metabolite levels and export in aap8 plants on the
expression of genes involved in N assimilation, qRT-PCR was performed (Fig. 7). During the vegetative phase, no differences were detected in transcript levels of genes encoding for nitrate reductase, Gln synthetase, and Gln 2-oxoglutarate aminotransferase between mutants and the wild type (Fig. 7A; Supplemental Fig. S2A). In contrast, after transitioning to the reproductive stage, the expression of N assimilation genes was decreased in \textit{aap8} plants compared with the wild type (Fig. 7B; Supplemental Fig. S2B). The observed variation in expression patterns during vegetative versus reproductive phases points to differences in the regulation of N metabolism during the development of Arabidopsis plants.

Sink Development Is Decreased But Seed N Content Is Unchanged

To analyze if and how sink development is affected when phloem loading and source-to-sink delivery of amino acids are decreased, silique number and seed set were examined (Fig. 8, A and B). The total silique number of \textit{aap8} compared with wild-type plants was decreased significantly, by up to 40% (Fig. 8A), and seeds per silique were reduced between 7% and 16% depending on the mutant (Fig. 8B). Seed weight was unchanged (Fig. 8C). This led to an overall decrease in seed yield of up to 40% in the mutants (Fig. 8D). Analysis of \textit{aap8} and wild-type plants showed no significant differences in either total elemental N content (Fig. 8E) or storage protein amounts (Fig. 8F). This demonstrates that AAP8 function in source-to-sink amino acid transport affects sink set but not seed N storage pools.

**DISCUSSION**

AAP8 Is Essential for Phloem Loading of Amino Acids

Following uptake from the soil, inorganic N is reduced in roots and leaves to amino acids. In Arabidopsis and many other plant species, the synthesis of amino acids occurs primarily in mature source leaves (Noctor et al., 2002), and a large proportion of the produced amino acids are exported from the leaves in the phloem of minor veins for sink N nutrition (Tegeder et al., 2012). In contrast, the leaf major veins seem to function mainly in the xylem-to-phloem transfer of root-derived amino acids (Atkins, 2000; Zhang et al., 2010). In this study, AAP8 was localized to the plasma membrane, and its expression was found in the phloem of both minor and major veins (Fig. 1), supporting its role in the cellular uptake of amino acids and in the phloem loading of leaf as well as root-synthesized amino acids.

AAP8 belongs to the AAP family of active, proton-coupled amino acid importers (Fischer et al., 1995, 2002; Tegeder and Ward, 2012), and it mediates the transport of acidic and neutral amino acids when expressed in yeast (Saccharomyces cerevisiae) strain 22Δ8AA (Okumoto et al., 2002). AAP8 function in phloem loading is in line with the facts that (1) Arabidopsis...
requires an apoplastic phloem-loading mechanism (Rennie and Turgeon, 2009), (2) phloem loading of amino acids occurs against their concentration gradient (Bush, 1993; Tegeder et al., 2012), (3) almost all protein amino acids can be detected in the Arabidopsis phloem (Fig. 4; Zhang et al., 2010), and (4) a broad-spectrum specific transporter is needed to import the large spectrum of proteinogenic amino acids into the sieve element-companion cell complex (Okumoto et al., 2002; Tegeder and Ward, 2012).

To resolve the physiological function of AAP8, three aap8 mutant lines were analyzed. When feeding source leaves of aap8 plants at vegetative and reproductive growth stages with radiolabeled Glu, partitioning of label to sink leaves and siliques, respectively, was reduced significantly (Fig. 3, A and C). Similarly, in aap8 plants at the reproductive phase, root-feeding experiments with Asp resulted in a reduction of radioactivity in siliques (Schmidt et al., 2007). As AAP8 was localized previously to immature seeds (Okumoto et al., 2002), the latter was interpreted to be due to decreased uptake into the early embryo (Schmidt et al., 2007). However, our results provide strong support that a lack of AAP8 function in phloem loading causes, or at least contributes to, the seed phenotype, as (1) AAP8 is localized to the phloem (Fig. 1), (2) the transport studies here were done at vegetative stage to exclude the seed regulation of source-to-sink amino acid transport (Fig. 3A), and (3) phloem Glu and Asp levels were decreased during the vegetative and reproductive stages (Fig. 4).

Leaf-feeding studies with Gln (Fig. 3, B and D), leaf exudate analyses (Fig. 4), and the biochemical characterization of AAP8 in yeast (Okumoto et al., 2002) further support that AAP8 is essential for phloem loading of both neutral and acidic amino acids. A function in the loading of low-abundance basic amino acids into the phloem seems less clear, as some variation in their amounts was observed dependent on the aap8 mutant and the developmental stage (Fig. 4). The import of basic amino acids into the leaf phloem might generally be assumed by other members of the AAP family, such as AAP5 (Fig. 5; Fischer et al., 1995, 2002).

Figure 4. Analysis of amino acid concentrations in leaf exudates in aap8 plants at vegetative (4-week-old plants) and reproductive (6-week-old plants) growth stages. Total and individual amino acid (AA) levels of 4-week-old plants at the vegetative stage (n = 4; A) and 6-week-old plants at the reproductive stage (n = 4; B) are shown. Error bars depict sd. Asterisks indicate significant differences from the wild type (WT; P < 0.05). Numbers above the columns show the percentage change in aap8 plants compared with the wild type.
Besides AAP8, other members of the Arabidopsis AAP family are expressed in rosette/source leaves, and among those, AAP1, which shares a close homology with AAP8, and AAP4 also seem to be present in the phloem of minor and major veins, most probably contributing to amino acid phloem loading (Fischer et al., 1995; Chen, 2006; Liu and Bush, 2006; Elashry et al., 2013). In addition, AAP2, AAP3, and AAP6 were localized to the transport phloem (e.g. leaf major vein, root, and stem; Hirner et al., 1998; Okumoto et al., 2004; Zhang et al., 2010; Tegeder and Ward, 2012) or to the vascular parenchyma (Hunt et al., 2010), where they play a role in the xylem-to-phloem transfer of root-derived amino acids (Tegeder, 2014). Like AAP8, all the other AAP proteins transport neutral and acidic amino acids (Fischer et al., 1995, 2002; Okumoto et al., 2002). However, at the vegetative stage, when amino acid levels were strongly reduced in the aap8 phloem, no changes were observed in the expression of AAPs (Fig. 5A). This suggests that the AAPs do not, or only partially, compensate for the loss of AAP8 function and that AAP8 is an important player in amino acid phloem loading in Arabidopsis. The lack, or the insufficiency, of compensation for AAP8 function in the mutants also may indicate that AAP8 interaction with other AAPs or transport proteins is needed for complete amino acid phloem loading, potentially through oligomerization, as shown for Arabidopsis ammonium transporters (Loqué et al., 2007; Yuan et al., 2013). Interestingly, in contrast to the vegetative phase, the transcript levels of AAPs were reduced during the reproductive phase (Fig. 5). This may hint to differences in transporter regulation depending on the leaf metabolite and/or developmental status (Fig. 6) and might involve transcriptional regulation or regulation via post-translational modifications (Guo et al., 2004; van der Graaff et al., 2006; Lalonde et al., 2010; Tegeder, 2012).

**AAP8 Function Affects Leaf N Metabolism during Reproductive Growth But Not during Vegetative Growth**

During the vegetative stage, plants take up large amounts of N and, following its reduction, transiently store some of the N as amino acids or proteins for nutrition of fruits and seeds later in development (Diaz et al., 2008; Masclaux-Daubresse et al., 2010). In aap8 plants at the vegetative growth phase, total and individual leaf amino acids, as well protein levels, were increased compared with the wild type (Fig. 6, A and D). This is most probably caused by the decreased phloem loading in the mutants leading to a buildup of amino acids, some of which seem to be channeled into proteins. Surprisingly, an analysis of N assimilation genes resolved similar transcript levels in wild-type and mutant leaves at the vegetative phase (Fig. 7A), which suggests that the accumulation of N metabolites in aap8 leaves also is a consequence of the continuation of N reduction. Generally, it is assumed that N assimilation is regulated by the N status and that high pools of amino acids provide a signal for down-regulation (Vincentz et al., 1993; Fan et al., 2006; Miller et al., 2008; Nunes-Nesi et al., 2010). However, the expression analysis of aap8 mutants during vegetative development (Fig. 7A) suggests that plants are able to adjust to the altered (metabolite) signals and that the negative feedback loop is not present when leaves are building up N reserves at the early growth phase. Nevertheless, when the aap8 plants transitioned to the reproductive phase, a down-regulation of N assimilation genes was observed (Fig. 7B). While in Arabidopsis, some N uptake from the soil and N assimilation still occur during the reproductive phase, many of the amino acids transported to fruits and seeds derive from the remobilization of leaf storage pools established earlier in development (see above; Diaz et al., 2008; Masclaux-Daubresse et al., 2010; Masclaux-Daubresse and Cardon, 2011). Similar to what was seen during vegetative growth, leaves of aap8 plants at the reproductive stage showed an accumulation of both amino acids and proteins (Fig. 6, B and D). However,
leaf transcript levels of N assimilation genes were down-regulated (Fig. 7B), suggesting that the observed high leaf N pool is the consequence of reduced N remobilization and source-sink partitioning and that they, directly or indirectly, regulate the N metabolic pathway via transcriptional repression (Oaks et al., 1977; Sakakibara et al., 1992; Vincentz et al., 1993; Campbell, 1999; Ferrario-Méry et al., 2001; Yanagisawa, 2014). Gln, the main amino acid remobilized in Arabidopsis during the reproductive phase (Figs. 4B and 6B; Guiboileau et al., 2012; Guan et al., 2015), has been discussed as a regulatory feedback signal, and the accumulation of the amide in aap8 leaves agrees with this assumption (Fig. 4B; Oaks et al., 1977; Vincentz.
used to identify differentially expressed genes.

greater than 2 or less than 0.5 between the wild type and mutants was

fruit and seeds/pods as well as the number of seeds that grow

(B) was performed on rosette leaf RNA from 4-week-old (A) and 6-week-old

growth set. The results for the second growth set are shown in

growth set. For primers used, see Supplemental Table S1. Experiments were performed on plants from at least two independently
grown sets of plants. For primers used, see Supplemental Table S1.

is established, seeds are able to compete for the N re-
mobilization and export (Guan et al., 2002; Lawlor, 2002; Ferrante et al., 2010). When analyzing sink set in aap8 plants, Schmidt et al. (2007) ob-
served a strong reduction in seed number per siliquie. Since AAP8 also is expressed during early embryo-
genesis, decreased uptake of amino acids into the endosperm and reduced embryo N nutrition most probably contribute to the aap8 phenotype (Okumoto et al., 2002; Schmidt et al., 2007). However, in this study, not only seed development was decreased in aap8 mutants but also the siliquie number per plant, leading to an overall reduction in seed yield of up to 40% (Fig. 8, A–D). Similar effects on sink development were ob-
served for an Arabidopsis mutant of a Gln synthetase important for leaf N remobilization and export (Guan et al., 2015). The reduction in fruit number in aap8 plants supports that decreased amino acid remobilization and phloem loading causes N limitation conditions early in reproductive development. The data further suggest that sink set in Arabidopsis is determined by nutrient supply during reproduction (Schulze et al., 1994) and that, when N levels are low, the filling of siliques with seeds has priority over flower and fruit development (Bennett et al., 2012). The importance of amino acid phloem loading for sink development receives further support from studies in pea demonstrating that over-
expression of an amino acid transporter in the phloem results in increased pod and seed set (Zhang et al., 2015). This is also in line with, for example, the work of Allen and Morgan (1972) showing that increasing root N supply and source-to-sink N allocation leads to more pods per plant. However, overexpression of orthophosphate dikinase, and subsequently increased Gln/amino acid remobilization and export from leaves, resulted in enhanced seed weight and N levels rather than improved seed number (Taylor et al., 2010).

When analyzing aap8 seed N content, no significant changes were found in total elemental N (Fig. 8E; Schmidt et al., 2007) and protein content (Fig. 8F) despite the reduced source-sink partitioning of amino acids and the reduction in siliquie number and seed set (see above). Similar results were observed for the Gln synthetase mutants mentioned above (Guan et al., 2015). Together, these findings suggest that sinks ac-
climate to low N supply and that, once sink strength is established, seeds are able to compete for the N re-
sources. Obviously, the survival strategy of Arabidopsis comprises fewer seeds but with sufficient N/storage protein (Bennett et al., 2012). These results further support that seed filling with N mostly depends on seed sink strength and N import into the embryo, rather than on phloem loading by amino acid transporters in source organs. This corroborates with work in Arabidopsis where the repression of amino acid import into the embryo led to reduced seed protein levels (Sanders et al., 2009). It is also in agreement with studies over-
expressing amino acid transporters in legume cotyledons, resulting in increased total seed N and protein con-
tents (Rolletschek et al., 2005; Zhang et al., 2015).

AAP8 Function in Phloem Loading of Amino Acids Is
Essential for Sink Development

During the transition from the vegetative to the repro-
ductive phase, flowers, pods (siliques), and finally
seeds become dominant sinks for N (and carbon) as-
similates. As sinks develop, they compete for available
N metabolites, and many studies have shown that N availability affects the number of flowers and finally
fruits/pods as well as the number of seeds that grow
within the fruit (Allen and Morgan, 1972; Sinclair and
deWit, 1976; Muchow, 1988; Dobermann and Cassman,
2002; Lawlor, 2002; Ferrante et al., 2010). When analy-
zing sink set in aap8 plants, Schmidt et al. (2007) ob-
served a strong reduction in seed number per siliquie.
demonstrated recently in pea plants, to improve both seed number and seed N levels, phloem loading and seed loading of amino acids need to be enhanced simultaneously (Zhang et al., 2015).

**CONCLUSION**

This study provides direct genetic evidence for the physiological importance of an amino acid transporter in phloem loading. The broad-spectrum specific amino acid permease AAP8 is important for the import of neutral and acidic amino acids into the phloem and N partitioning to the developing sinks. The data presented further suggest that the relationship between phloem loading and leaf N metabolism varies depending on the developmental stage of the plant. During the vegetative phase, AAP8-mediated N export seems to exert little control over N metabolism, as N assimilation and transient N accumulation in source organs continue, independent of changes in phloem loading. However, during reproductive development, when sink N demands are high, reduced phloem loading and subsequent increases in leaf N pools seem to negatively affect N reduction in leaves, probably via feedback regulation. Overall, AAP8 function in the phloem is important for source-to-sink partitioning of N and sink development but does not play a role in seed N filling.

**Figure 8.** Analyses of silique number, seeds per silique, seed yield, and seed N levels in *aap8* plants. A, Total silique number per plant (*n* = 16). B, Seed number per silique (*n* = 11). C, Weight of 250 seeds (*n* = 20). D, Seed yield per plant (*n* = 15). E, Total elemental seed N (*n* = 5 plants). F, Total soluble seed protein (*n* = 6 plants). Error bars depict SD. Asterisks indicate significant differences (*P* < 0.05) from the wild type (WT). Numbers above the columns indicate the percentage change in *aap8* plants versus the wild type.
MATERIALS AND METHODS

Plant Material, Growth Conditions, and Harvest

Three AAP8 (At3g10010) T-DNA insertions lines (SALK_092980 [aap8-1; T-DNA in the first intron], SALK_081076C [aap8-2; sixth exon], and SALK_038033 [aap8-3; fifth intron]) were obtained from the Arabidopsis Biological Resource Center at Ohio State University (https://abrc.osu.edu/). The aap8 mutant lines were screened for homozygosity using standard procedures and two sets of left border and/or gene-specific primers (Supplemental Table S1). Arabidopsis (Arabidopsis thaliana) ecotype, Columbia wild-type and aap8 plants were grown in 36-well Com-Packs (T.O. Plastics) for leaf exudate, molecular, biochemical, and phenotypic analyses in pots of 7.6 cm2 for transport studies. The plants were grown in a mixture of peat (60% [w/w]), pumice (20% [w/w]), and sand (20% [w/w]; SunGro Horticulture) and in environmentally controlled growth chambers in a 16-h light regime at 200 to 250 μmol photons m−2 s−1 with day/night temperatures of 21°C/16°C at 70% relative humidity. Plants were fertilized with 1.75 g L−1 Peters 20-20-20 all-purpose plant food once per week according to Zhang et al. (2010). Under these growth conditions, the life cycle of Arabidopsis plants from planting to seed harvest was about 8 weeks.

For molecular and biochemical analyses, plants were harvested 4 weeks after germination for the vegetative stage or 6 weeks after germination for the reproductive stage. At the vegetative stage, 12 to 14 rosette leaves developed (Boyes et al., 2001); the first four fully expanded leaves were collected and considered as source leaves, while the four youngest and smallest leaves were used as sink leaves. At the reproductive stage, up to 22 rosette leaves had developed. Leaves 3 to 6 were harvested as source leaves. Independent pools of leaves from about 30 plants were analyzed. At least two independent sets of mutant and wild-type plants were grown for analyses and confirmation of the results.

RNA Extraction, Gene Expression, and In Situ RNA Hybridization Analyses

Using TRIzol Reagent (Invitrogen), total RNA was extracted from source (rosette) and sink leaves of 4-week-old as well as from source leaves and siliques of 6-week-old Arabidopsis wild-type and aap8 mutant plants (Chomczynski, 1993). First-strand synthesis was done with oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). PCR was performed on the first-strand complementary DNAs (cDNAs) with two sets of AAP8 cDNA-specific oligonucleotide primers (Supplemental Table S1). For expression analysis of genes involved in N metabolism and transport, qRT-PCR was performed according to Zhang et al. (2010). First-strand cDNAs were diluted 1:10 with double-deionized water. Three technical repetitions were run for each primer pair used (Supplemental Table S1). The reaction mix consisted of Platinum Taq buffer (Invitrogen), 200 μM deoxynucleotide triphosphate (Fisher BioReagents), 2.5 mM MgCl2, 0.25× SYBR Green I, 50 mM ROX reference dye, 0.5 units of Platinum Taq DNA polymerase, and 0.25 μM primers. Cycle threshold values were determined with the Applied Biosystems 7500 Fast Thermal Cycler. qRT-PCR experiments were performed on rosette leaves from at least two independent plant growth experiments, and each sample was measured in three technical replicates. A mean fold change of greater than 2 or less than 0.5 between the wild type and mutants was used to identify differentially expressed genes (Rajeevan et al., 2001; Guether et al., 2009).

AAP8 transcripts were localized using rosette/source leaves from 6-week-old wild-type plants embedded in paraffin and in situ RNA hybridization and color detection methods as described previously (Lee and Tegeder, 2004).

Subcellular Localization of AAP8

Subcellular localization of AAP8 was performed by transient expression of AAP8-GFP fusions in Nicotiana benthamiana epidermal cells using a leaf infiltration method (Sparkes et al., 2005). An AAP8-GFP construct was prepared and transferred into Agrobacterium rhizogenes strain 18r12v as described by Collier and Tegeder (2012). To prevent repression, coinfiltration was done with Agrobacterium tumefaciens GV3101 pMP10 containing the p19 protein gene of Tomato bushy stunt virus (Voinnet et al., 2003). As a plasma membrane control, Arabidopsis aquaporin Pip2A (At3g53420; Nelson et al., 2007) fused to mCherry (Shaner et al., 2004) was used. The fluorescing fusion proteins were localized using confocal microscopy (Carl Zeiss).

Transport Studies with Radiolabeled Amino Acids

For in planta transport studies, source leaf 2 or 3 of 4-week-old plants at the vegetative stage or leaf 5 or 6 of 6-week-old plants was fed with radiolabeled amino acids following the protocol of Franceschi and Tarly (2002). Leaflets were exposed to 600 μL of a solution containing 2.5 mM MES, 5 mM EDTA, 2 mM cold Glu or Gln, and 1 μCi of 14C-Glu or 14C-Gln (Moravek Biochemical and Radiochemicals). Leaf feeding was performed for 45 min (4-week-old plants) or 12 h (6-week-old plants) at 300 μmol photons m−2 s−1 light, followed by freezing of a chase solution (same as above but without 14C-labeled amino acids) for 15 min (4-week-old plants) or 1 h (6-week-old plants). Sink leaves were harvested immediately and scanned for leaf surface area. A single leaf was placed in scintillation vials containing 2 mL of Ecoscint A (National Diagnostics). The radioactivity was determined by liquid scintillation spectrometry (Packard Instruments) and calculated per 1 cm2 of leaf area. For 6-week-old plants, 40 siliques per plant were harvested from the main shoot, incubated overnight in a vial containing 5 mL of Ecoscint A, and treated with 1 mL of acetic acid (Schmidt et al., 2007) before determining the radioactivity.

Collection of Leaf Exudates

Leaf exudates were obtained from cut petioles of 4- or 6-week-old Arabidopsis plants for 2 h as described by Deeken et al. (2008). Exudates were collected from one leaf (leaf 3 or 4 for 4-week-old plants or leaf 5 or 6 for 6-week-old plants) per plant, and aliquots (80 μL) were lyophilized followed by resuspension in 33 μL of water. To precipitate the EDTA used in the exudation buffer, 3.3 μL of 1× HCl was added to the solution. Samples were incubated on ice for 30 min, followed by centrifugation at 4°C and 10,000 g for 30 min. The supernatant was diluted 1:1 with double-deionized water and used for amino acid analysis.

Amino Acid and Total Elemental N Analyses

Free amino acid concentrations were determined using HPLC. For amino acid extraction, 300 μL of 80% methanol and the internal standard 6-amino caproic acid at 15 ps final concentration were added to 3 μg of lyophilized source leaf tissue and incubated at 70°C for 15 min while shaking (1,000 rpm). Following centrifugation at 10,000 g for 15 min at room temperature, the supernatant was collected. Pellets were reextracted with 20% methanol as described above. Supernatants were combined and dried under vacuum (SpeedVac concentrator; Savant Instruments), and the resulting pellet was made up in 160 μL of HPLC-grade water. A 20-fold dilution was prepared and used for amino acid extract derivation with 4-fluoro-7-nitro-2,1,3-benzoxadiazole according to Aoyama et al. (2004). An amino acid standard was made using a commercially available mixture (Sigma; catalog no. A2407) supplemented with Arg, γ-aminobutyrate, Gln, His, Lys, ammonium chloride, and homoser. HPLC was performed using a Waters 2695 separation module with a 2475 multi λ fluorescence detector and Empower2 software as described (Tan et al., 2010). Total N content was analyzed from dry seeds according to Sanders et al. (2009).

Protein Extraction and Quantification

Total soluble protein was extracted from leaves and seeds using a procedure described by Zhang et al. (2010) with some modifications. Protein extraction buffer (50 mM HEPES, 5 mM MgCl2, 1 mM EDTA, and 10% [v/v] ethylene glycol, pH 7.5) was added to 1 mg of lyophilized tissue in a microtuge tube followed by grinding with a micropestle. Plant debris were sedimented by centrifugation at 14,000 rpm and 4°C for 10 min. The protein in the supernatant was quantified using the NanoOrange kit (Invitrogen) as described in the manufacturer’s manual and a microplate fluorescence reader (Bio-Tek Synergy HT; excitation, 480 nm; and emission, 590 nm).

Statistical Analysis

Results are shown for one plant growth set but are representative of at least two independently grown sets of plants. Data are generally presented as means ± SD of at least three biological repetitions, with the exception of the qRT-PCR results, which are shown as means of three technical repetitions (see above). To determine the significance of results, one-way ANOVA and mean separation tests were performed using SigmaStat (Systat Software).
Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AAP8 (AH1g3010).

Supplemental Data
The following supplemental materials are available.

Supplemental Figure S1. Expression analysis of AAPs in source leaves of a second independently grown set of apq8 and wild-type plants.

Supplemental Figure S2. Expression analysis of genes involved in N assimilation in source leaves of a second independently grown set of apq8 and wild-type plants.

Supplemental Table S1. Primers used for apq8 mutant screening and expression analyses.

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