

Increased Phloem Transport of S-Methylmethionine Positively Affects Sulfur and Nitrogen Metabolism and Seed Development in Pea Plants^{1[W][OA]}

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Seeds of grain legumes are important energy and food sources for humans and animals. However, the yield and quality of legume seeds are limited by the amount of sulfur (S) partitioned to the seeds. The amino acid S-methylmethionine (SMM), a methionine derivative, has been proposed to be an important long-distance transport form of reduced S, and we analyzed whether SMM phloem loading and source-sink translocation are important for the metabolism and growth of pea (*Pisum sativum*) plants. Transgenic plants were produced in which the expression of a yeast SMM transporter, *S-Methylmethionine Permease1* (*MMP1*, YLL061W), was targeted to the phloem and seeds. Phloem exudate analysis showed that concentrations of SMM are elevated in *MMP1* plants, suggesting increased phloem loading. Furthermore, expression studies of genes involved in S transport and metabolism in source organs, as well as xylem sap analyses, support that S uptake and assimilation are positively affected in *MMP1* roots. Concomitantly, nitrogen (N) assimilation in root and leaf and xylem amino acid profiles were changed, resulting in increased phloem loading of amino acids. When investigating the effects of increased S and N phloem transport on seed metabolism, we found that protein levels were improved in *MMP1* seeds. In addition, changes in SMM phloem loading affected plant growth and seed number, leading to an overall increase in seed S, N, and protein content in *MMP1* plants. Together, these results suggest that phloem loading and source-sink partitioning of SMM are important for plant S and N metabolism and transport as well as seed set.

Sulfur (S) is a critical macronutrient for plant growth, and its assimilation is important for the production of the essential amino acids Cys and Met as well as the antioxidant glutathione (GSH; Saito, 2004; Jander and Joshi, 2010). Besides being a component of functional and structural molecules, S is crucial to the development of sink tissue, such as flowers and fruits, and its availability affects protein synthesis and the quality of seeds (Higgins et al., 1986; Ufaz and Galili, 2008). Acquisition of S occurs predominantly via the uptake of sulfate (SO_4^{2-}) from the soil, which is mediated by sulfate transporters (Smith et al., 1995; Davidian and Kopriva, 2010). Following uptake, sulfate is either transiently stored in vacuoles or assimilated into reduced S compounds. The S reduction

pathway is well characterized and conserved among different plant species. However, the major site of S reduction varies and might be dependent on environmental conditions and plant species (Rennenberg et al., 1979; Brunold and Suter, 1989; Hopkins et al., 2005; Hawkesford and De Kok, 2006; Hell et al., 2010). Following translocation of sulfate in the xylem transpiration stream, S reduction predominantly occurs in mature source leaves (Rennenberg et al., 1979; Bourgis et al., 1999; Lappartient et al., 1999; Hopkins et al., 2005), although in legumes, S reduction also takes place in other organs. For example, in pea (*Pisum sativum*), S assimilation has been localized to roots, as all the enzymes involved in sulfate reduction to Cys are present (Brunold and Suter, 1989). Regardless of the location of primary S assimilation, sink translocation of reduced S predominantly occurs via the phloem. It might be loaded into the phloem of source leaf minor veins following synthesis, or it may derive from xylem-phloem transfer along the translocation path when S assimilation takes place in roots (Buchner et al., 2004).

GSH, Cys, and Met have been suggested to be long-distance transport forms of organic S (Rennenberg et al., 1979; Bonas et al., 1982; Lappartient et al., 1999; Davidian and Kopriva, 2010), but in a study by Bourgis et al. (1999), it was shown that the amino acid S-methyl-Met (SMM) is a key contributor to phloem S transport in wheat (*Triticum aestivum*), canola (*Brassica napus*), le-

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gumes, and other plant species. In these plants, it is suggested that SMM is synthesized from Met by the enzyme Met methyltransferase (MMT) in source leaves and loaded into the phloem for transport to seeds (Bourgis et al., 1999; Lee et al., 2008). In the seed coat, SMM is recycled back to Met via homo-Cys methyltransferase (HMT; Gallardo et al., 2007) and amino acids are finally released into the seed apoplast, from where they are taken up by the embryo/cotyledons for development and storage product accumulation.

This work addresses the importance of phloem loading and source-sink partitioning of SMM for plant metabolism and growth. In recent years, much work has been invested in elucidating the role of Suc transporters in phloem loading and carbon movement to sinks (Tegeger et al., 2011). However, no information is available on the function of transporters in long-distance movement of organic nitrogen (N) and S, although a variety of amino acid, GSH, GSH derivative, and GSH conjugate transporters have been identified (Jasinski et al., 2003; Zhang et al., 2004; Bouvier et al., 2006; Rentsch et al., 2007; Tegeger et al., 2007, 2011; Tan et al., 2008). Since there are no known plant SMM transporters, in this study, a high-affinity yeast SMM transporter, *S-Methylmethionine Permease1* (*MMP1*; Rouillon et al., 1999), was expressed in the pea phloem and seeds to determine the importance of SMM partitioning in plant physiology. We demonstrate that increasing phloem loading of SMM affects S metabolism in leaves as well as S uptake and assimilation in roots. In addition, N assimilation and translocation to sinks were altered in *MMP1* plants. Changes in S and N metabolism and partitioning resulted in higher seed numbers and influenced the S-N ratio and storage protein profile in *MMP1* seeds. Together, the data suggest that increasing long-distance transport of organic S positively affects plant metabolism and productivity. Our study raises the possibility that SMM might play a key role in balancing plant S and N status.

RESULTS

Molecular and Phenotypic Analyses of *MMP1* Plants

In general, transformation of pea plants is extremely difficult and has been successful in very few laboratories worldwide (Grant et al., 1995; McPhee et al., 2004; Rolletschek et al., 2005). Nonetheless, we were able to produce transgenic pea lines, *MMP1-1* and *MMP1-2*, expressing yeast *MMP1* (Rouillon et al., 1999) under the control of the Arabidopsis (*Arabidopsis thaliana*) Amino Acid Permease1 (*AAP1*) promoter (Hirner et al., 1998). In pea, this promoter targets gene expression to the phloem throughout the plant and to seeds (Tegeger et al., 2007). Southern-blot analysis resolved that both lines carry a single copy of the gene construct (Fig. 1A). RNA expression analysis established that *MMP1* is expressed throughout the transgenic plants, including roots, leaves, and developing seeds (Fig. 1B), consistent with *AAP1p-GUS* studies in

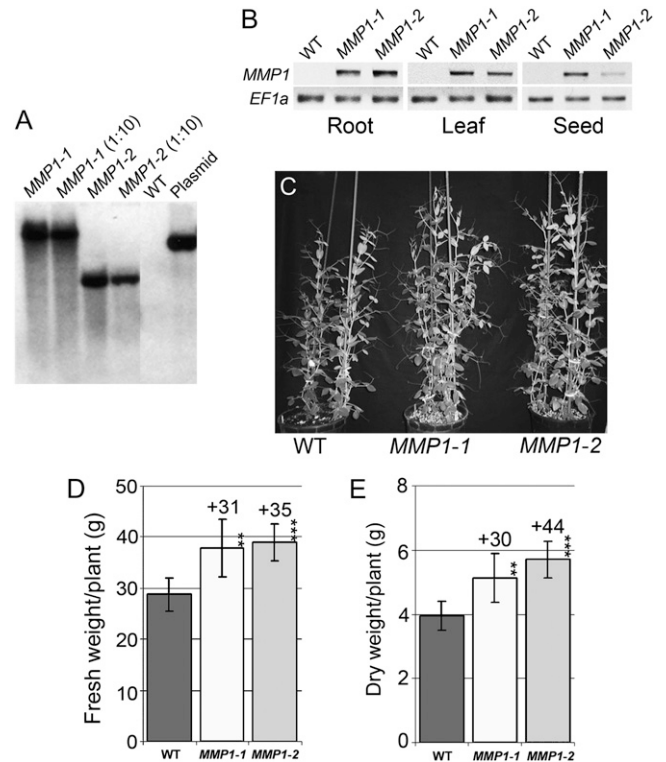


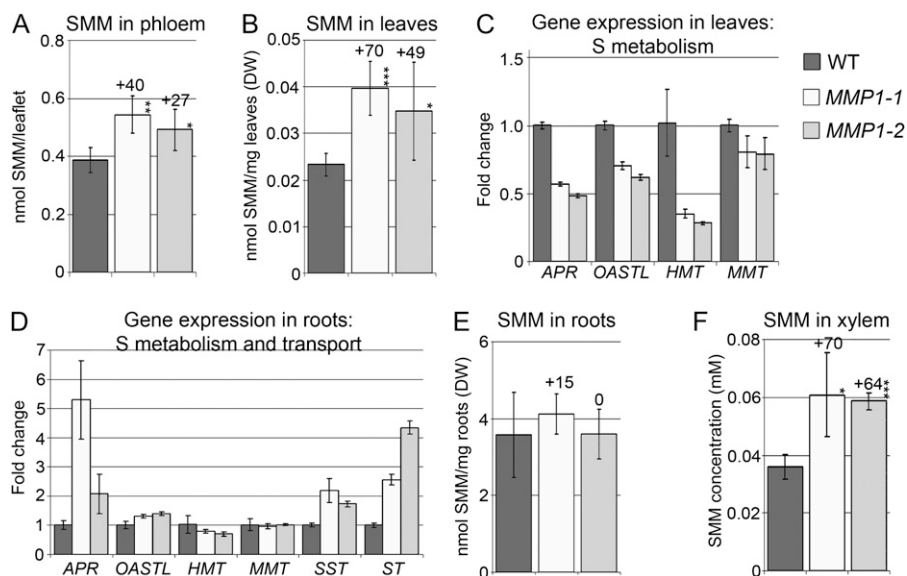
Figure 1. Molecular and phenotypic analyses of *MMP1* plants. A, Southern-blot analysis using *Hind*III-digested genomic DNA from *MMP1-1*, *MMP1-2*, and wild-type (WT) plants. Vector pTKan with the gene construct *AAP1p-MMP1* (plasmid) was used as a positive control. Both transgenic lines carry a single copy of the gene construct. B, Analysis of *MMP1* expression in *MMP1-1* and *MMP1-2* lines using RT-PCR. *EF1a* was amplified as a control for equivalent amounts of RNA used and even gel loading. C, Photograph of 5-week-old *MMP1* and wild-type plants. Two plants were grown in each pot. D, Shoot fresh weight of 5-week-old *MMP1* and wild-type plants ($n = 6$). E, Shoot dry weight of 5-week-old *MMP1* and wild-type plants ($n = 6$). Results are presented as means \pm SD. Asterisks indicate significant differences from the wild type detected by Student's *t* test: ** $P < 0.01$, *** $P < 0.001$.

pea (Tegeger et al., 2007). Phenotypic analysis of vegetative growth illustrated that *MMP1* plants were taller and displayed a bushier appearance compared with wild-type plants (Fig. 1C). When examining the shoot biomass of 5-week-old *MMP1* plants, fresh and dry weight were increased by up to 35% and 44%, respectively, compared with the wild type (Fig. 1, D and E).

SMM Phloem Loading and S Metabolism Are Increased in *MMP1* Plants

Phloem exudates from *MMP1* leaflets were analyzed, and SMM concentrations were 27% to 40% higher in *MMP1-1* and *MMP1-2* plants compared with wild-type plants (Fig. 2A), suggesting an increase in SMM phloem loading and source-sink translocation in transgenic plants. Concurrently, whole leaf tissue SMM levels were increased by up to 70% (Fig. 2B). To decipher whether the positive changes in SMM content in leaves are due to increased SMM synthesis,

Figure 2. SMM levels and expression of genes involved in S metabolism and transport in *MMP1* plants. A, SMM concentrations in phloem exudates of *MMP1* and wild-type (WT) plants ($n = 4$). B, SMM levels in leaves ($n = 4$). DW, Dry weight. C and D, Fold change in gene expression of *APR*, *OASTL*, *HMT*, *MMT*, and sulfate transporters *SST* and *ST* in leaves (C) and roots (D). For *OASTL*, *MMT*, *HMT*, *SST*, and *ST* expression analyses, degenerated primers were used, since pea homologs are unknown. E, SMM levels in roots ($n = 4$). F, SMM concentrations in xylem sap ($n = 3$). Results are presented as means \pm SD. Asterisks indicate significant differences from the wild type detected by Student's *t* test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.



expression levels of genes involved in S assimilation in leaves were analyzed using quantitative reverse transcription (RT)-PCR. Surprisingly, genes important for the synthesis of Cys from sulfate, such as adenosine 5'-phosphosulfate reductase (*APR*) and OAS (thiol)-lyase (*OASTL*), were down-regulated (Fig. 2C). Furthermore, transcript levels of *MMT*, the enzyme converting Met to SMM, were unchanged, whereas the expression of *HMT*, responsible for the synthesis of Met from SMM, was decreased (Fig. 2C). These findings indicate that reductive S assimilation in the leaf tissue does not account for the increased SMM content in *MMP1* leaves and that the organic S compounds might be derived from roots.

Therefore, S assimilation in root tissue was examined. In fact, transcript levels of S assimilatory genes, especially *APR*, were increased in *MMP1* versus wild-type roots (Fig. 2D). Furthermore, *HMT* expression was slightly down-regulated, while *MMT* RNA levels were unchanged. This suggests that de novo S assimilation is increased in *MMP1* roots and that lower amounts of SMM are converted back to Met via *HMT*. When analyzing the expression levels of sulfate transporters, *SST* (Krusell et al., 2005) and *ST* (Smith et al., 1995), it was found that both transporters are up-regulated by up to 4-fold in *MMP1* roots (Fig. 2D), indicating increased S uptake.

HPLC analysis was then performed to determine SMM levels in roots and in the xylem, and the results showed that SMM was unchanged in whole *MMP1* root tissue (Fig. 2E). However, the SMM concentration in the xylem sap was increased by 64% to 70% in *MMP1* compared with wild-type plants (Fig. 2F), indicating increased S uptake and assimilation in roots and translocation to the shoot.

Amino Acid Phloem Loading and Leaf N Metabolism Are Increased in *MMP1* Plants

As S and N assimilation are tightly coordinated in plants (Wang et al., 2003; Dubousset et al., 2009) and S

metabolism and partitioning in *MMP1* plants are altered, N metabolism and transport are also likely to be affected. Consequently, analysis of phloem amino acid levels was performed and showed that the amounts of total amino acids in the *MMP1* phloem were elevated by about 30% (Fig. 3A), with an increase in Arg, homo-Ser, Ile, Leu, Lys, Met, Thr, and Val (Fig. 3B). In addition, expression of *Cationic Amino Acid Transporter6* (*CAT6*), a putative companion cell transporter (Brady et al., 2007) that uses most of the above-listed amino acids as substrates (Hammes et al., 2006), was strongly up-regulated in leaves (Fig. 3F), supporting increased phloem loading of amino acids.

To resolve whether the increased amounts of phloem amino acids derive from elevated assimilation in the leaf, total N and amino acid levels and expression of genes involved in N assimilation in leaves were examined. The results showed that amounts of total N and free amino acids were increased in *MMP1* leaves by up to 9% and 53%, respectively (Fig. 3, C and E). Analysis of single amino acid concentrations demonstrated that eight out of 20 amino acids were significantly elevated in both transgenic lines, with the strongest increase in Asn, by up to 400% (Fig. 3D). Along with this, there was a slightly increased expression of genes involved in the synthesis of Gln (*GS*) and Glu (*GOGAT*), which are the first products of N assimilation, an up to 6-fold up-regulation of *AS1*, important for Asn synthesis, and down-regulation of *asparaginase* (*ASNase*), involved in Asn catabolism (Fig. 3F).

Xylem But Not Root Amino Acid Levels Are Affected in *MMP1* Plants

To determine whether alterations in root amino acid metabolism and export contribute to the observed increase in phloem and leaf amino acid contents in *MMP1* plants, we analyzed total root N levels and amino acid concentrations in the root and xylem. It

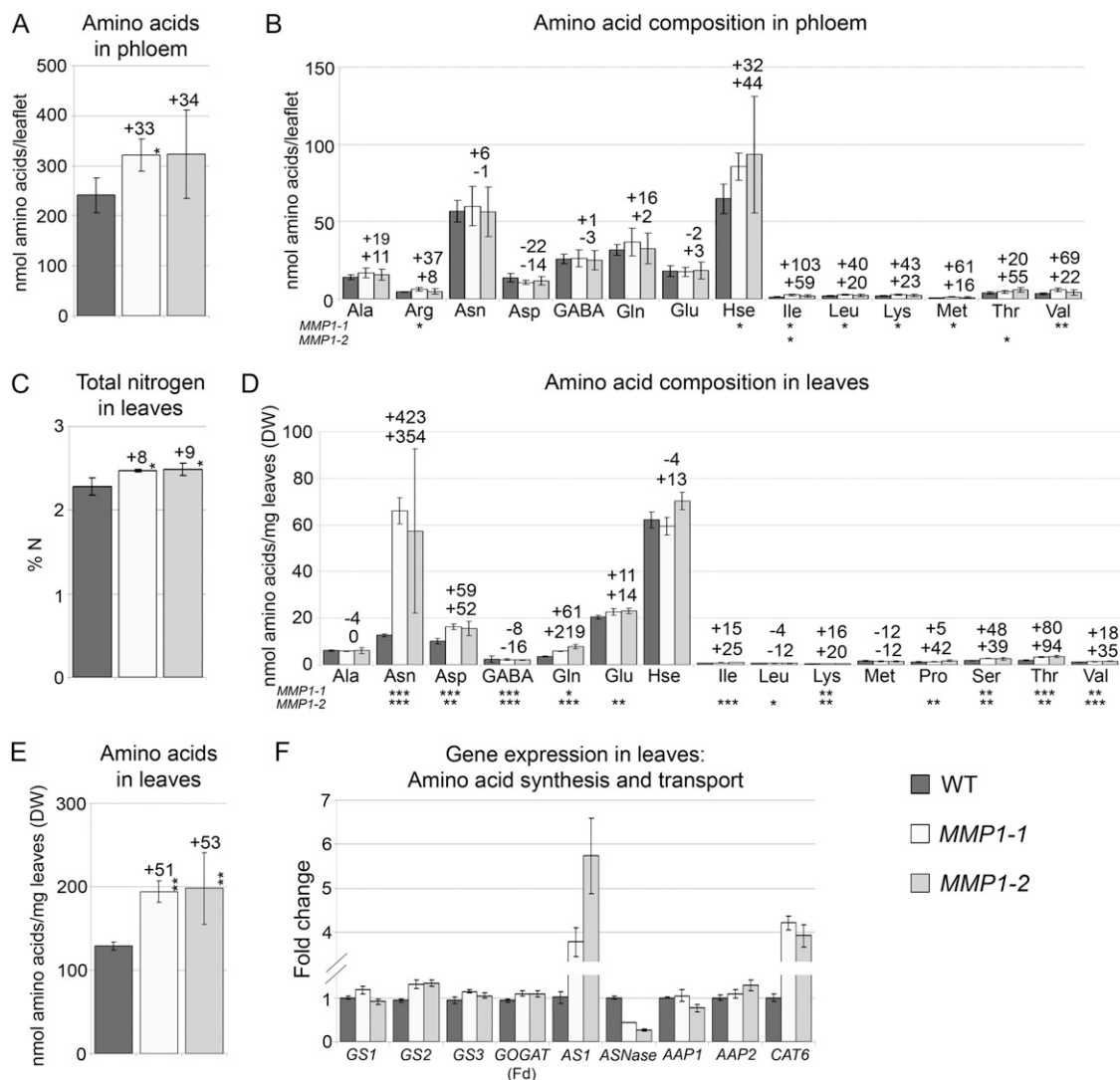


Figure 3. N levels in *MMP1* phloem exudate and leaves, and expression of genes involved in amino acid synthesis and transport in leaves. A, Total free amino acid levels in the phloem of *MMP1* compared with wild-type (WT) plants. B, Amino acid composition and concentration in the phloem exudates. Hse, Homo-Ser. C, Total N levels in leaves. D, Total free amino acid levels in leaves. DW, Dry weight. E, Amino acid composition and concentration in leaves. F, Fold change in gene expression of pea *GS1*, *GS2*, and *GS3*, *AS1*, and amino acid transporters *AAP1*, *AAP2*, and *CAT6*. Degenerated primers were used for ferredoxin-dependent Glu synthase [*GOGAT* (Fd)] and *ASNase*. Results are presented as means \pm SD. Asterisks indicate significant differences from the wild type detected by Student's *t* test ($n = 4$): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

was found that amounts and profiles of free amino acids, as well as total N, were unchanged in *MMP1* roots compared with the wild type (Fig. 4, A–C). Expression analysis demonstrated that genes involved in the initial assimilation of inorganic N, *GS* and *GOGAT*, were up-regulated in *MMP1* roots, suggesting increased N reduction (Fig. 4D). Transcript levels of *AS1* were unchanged or decreased depending on the *MMP1* line, and *ASNase* expression was down-regulated in both lines (Fig. 4D).

Xylem sap analysis revealed that total amino acid levels were similar in overexpressors versus the wild type, but concentrations of single amino acids were altered (Fig. 4, E and F). Gln was decreased in both

overexpressors, and Asn and Lys were reduced or not changed dependent on the *MMP1* line. Glu, γ -aminobutyric acid (GABA), Ala, Leu, Val, Ile, Met, and Thr levels were up-regulated in *MMP1* xylem sap compared with the wild type (Fig. 4F), suggesting increased export of these amino acids out of the root.

N Levels Are Increased in *MMP1* Seeds, While S Content Is Unchanged

To investigate if increased phloem SMM and amino acid levels result in increased S and N distribution to individual pea seeds, *MMP1* cotyledons were analyzed. Expression of *AAP2*, a transporter in-

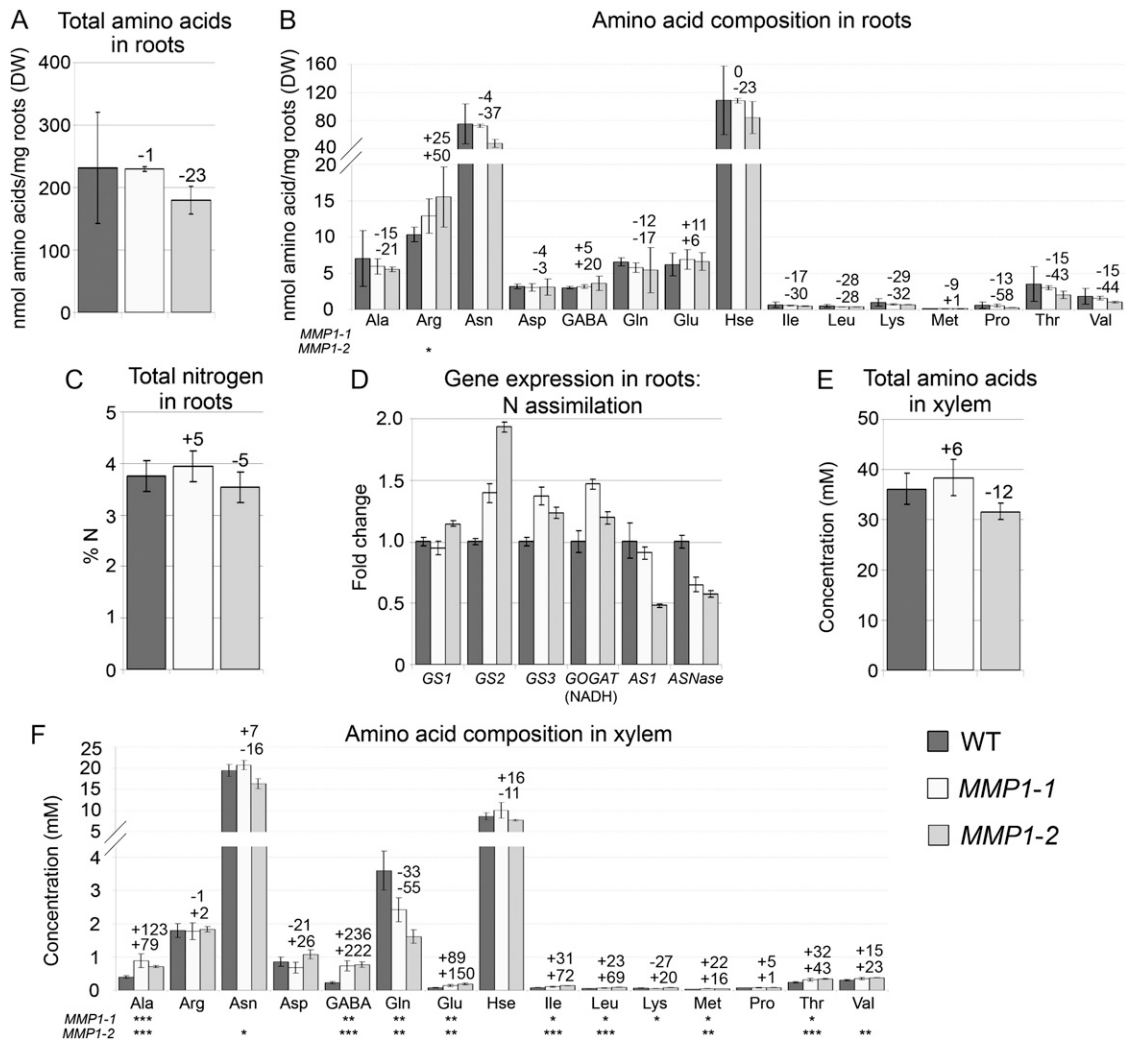


Figure 4. Analysis of N levels in root and xylem of *MMP1* plants, and root gene expression studies. A, Total free amino acid levels in roots of *MMP1* compared with wild-type (WT) plants ($n = 4$). DW, Dry weight. B, Amino acid composition and concentration in roots ($n = 4$). Hse, Homo-Ser. C, Total N levels in roots ($n = 4$). D, Fold change in gene expression of pea *GS1*, *GS2*, and *GS3*, and *AS1*. Degenerated primers were used for NADH-dependent Glu synthase [*GOGAT* (NADH)] and *ASNase*. E, Total free amino acid levels in xylem sap ($n = 3$). F, Amino acid composition and concentration in xylem sap ($n = 3$). Results are presented as means \pm SD. Asterisks indicate significant differences from the wild type detected by Student's *t* test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

involved in the import of amino acids into pea cotyledons (Tegeder et al., 2000), was up-regulated in *MMP1* seeds (Fig. 5A), indicating increased N uptake. This is consistent with changes in the total N content, which was elevated by up to 10% in *MMP1* compared with wild-type seeds (Fig. 5B). In addition, total soluble protein levels were elevated by 6% to 7% in *MMP1* seeds (Fig. 5C).

In general, about 65% to 85% of pea seed proteins are present in the form of globulin storage proteins, with predominant localization to the seed cotyledons (Tzitzikas et al., 2006). Depending on the S content, globulins are further categorized into S-rich legumins and S-poor vicilins and convicilins, and increased availability of S positively affects seed levels of S-rich

proteins (Higgins et al., 1986; Sexton et al., 1998; Tabe et al., 2002; Ufaz and Galili, 2008). To examine if S-poor and S-rich proteins were affected, soluble proteins including globulins were analyzed by SDS-PAGE. The stained gel showed that levels of convicilins and vicilins were increased in *MMP1* seeds, while S-rich legumins were unaltered (Fig. 5D). For further confirmation, the expression of storage protein genes for legumins (*LegA* and *LegB*), convicilin (*Cvc*), and vicilin (*Vic*) was examined using RNA from developing cotyledons. While *LegA* and *LegB* expression was decreased, transcript levels of both *Cvc* and *Vic* were increased in *MMP1* cotyledons (Fig. 5A), consistent with the changes in *MMP1* convicilin and vicilin levels. When testing the total S content in seeds, no

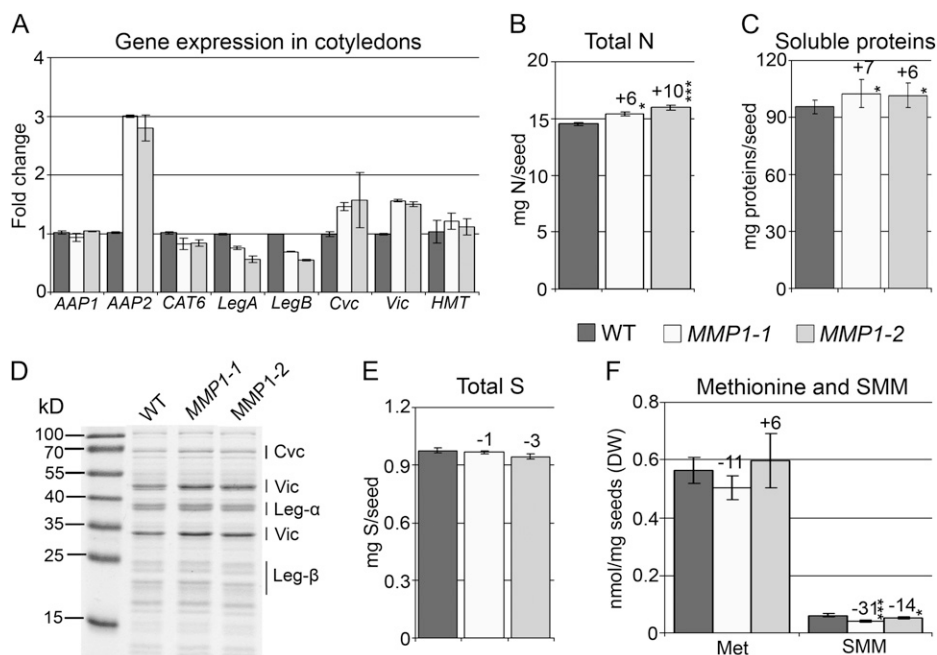


Figure 5. Expression of genes involved in seed amino acid transport and seed storage protein synthesis, and total seed N, S, Met, SMM, and protein levels. A, Fold change in the expression of amino acid transporters *AAP1*, *AAP2*, and *CAT6* and of the storage protein genes *LegA*, *LegB*, *Cvc*, and *Vic* as well as *HMT* in developing cotyledons of *MMP1* compared with wild-type (WT) plants. B, Total N levels in dry seeds. C, Amounts of soluble seed proteins using a SDS-PAGE gel. Leg- α , Legumin α -subunits; Leg- β , legumin β -subunits. E, Total S levels in dry seeds. F, Concentrations of Met and SMM in developing *MMP1* seeds. DW, Dry weight. Results are presented as means \pm SD. Asterisks indicate significant differences from the wild type detected by Student's *t* test ($n = 4$): * $P < 0.05$, *** $P < 0.001$.

changes were observed (Fig. 5E), which concurs with unchanged levels of S-rich seed proteins in *MMP1* plants. Analysis of Met and SMM in developing *MMP1* seeds showed that Met concentrations were unchanged while SMM levels were decreased (Fig. 5F). However, expression of *HMT*, encoding for the enzyme converting SMM back to Met, was unchanged in developing *MMP1* seeds (Fig. 5A).

Increased SMM Phloem Loading Positively Affects Seed Number

While SMM phloem levels were increased in *MMP1* plants, seed S levels were unchanged. This raises the question of where the S is partitioned to. Analyses of yield-related parameters showed an increase in total seed yield of up to 19% in *MMP1* plants (Fig. 6A). Seed weight was unchanged in transgenic compared with wild-type plants, but seed number per plant was positively affected by 20% to 27% (Fig. 6, B and C).

As described above, *MMP1* seed S levels were unaltered, whereas N and protein levels were increased (Fig. 5, B–E). When calculating total seed S amount per plant, it was found that the overall S levels per *MMP1* plant were increased by 16% compared with the wild type (Fig. 6D), whereas total seed N and protein yields per plant were elevated by up to 31% and 34%, respectively, dependent on the transgenic line (Fig. 6, E and F).

DISCUSSION

While the role of transporters in source-sink translocation of organic S in plants still needs to be addressed, it has recently been suggested that SMM

is a major player in S phloem transport (Bourgis et al., 1999; Gallardo et al., 2007; Lee et al., 2008). This work links the function of organic S transporters with plant N and S metabolism and provides evidence for the importance of SMM phloem loading and source-sink distribution for plant growth and seed development.

Phloem Loading of SMM Affects Source S Metabolism

Targeted expression of the yeast SMM transporter *MMP1* to the phloem throughout the pea plant led to an increase in SMM concentrations in the leaf phloem sap (Fig. 2A), which in turn affected S root uptake, SMM synthesis in root and leaves, and SMM translocation to the shoot. The concept that increased SMM phloem loading exhibits feedback is supported by the following: (1) up-regulation of gene expression of S uptake and assimilation in the root; (2) increased levels of SMM in the xylem; and (3) down-regulation of genes involved in leaf S assimilation (Fig. 2, C, D, and F). Most enzymes involved in S assimilation are transcriptionally regulated, with high S levels down-regulating gene expression (Lappartient et al., 1999; Vauclare et al., 2002; Davidian and Kopriva, 2010). This is consistent with the observed high levels of SMM in *MMP1* leaves and the down-regulation of S assimilation genes (Fig. 2, B and C). On the other hand, it was shown that S deficiency leads to up-regulation of S transporter and biosynthesis genes (Vauclare et al., 2002; Hirai et al., 2003; Davidian and Kopriva, 2010). In *MMP1* roots, the concentration of SMM was unchanged, suggesting a steady-state pool of the root amino acid. However, since xylem concentrations were strongly increased, the actual SMM levels in root cells might be decreased, triggering an up-regu-

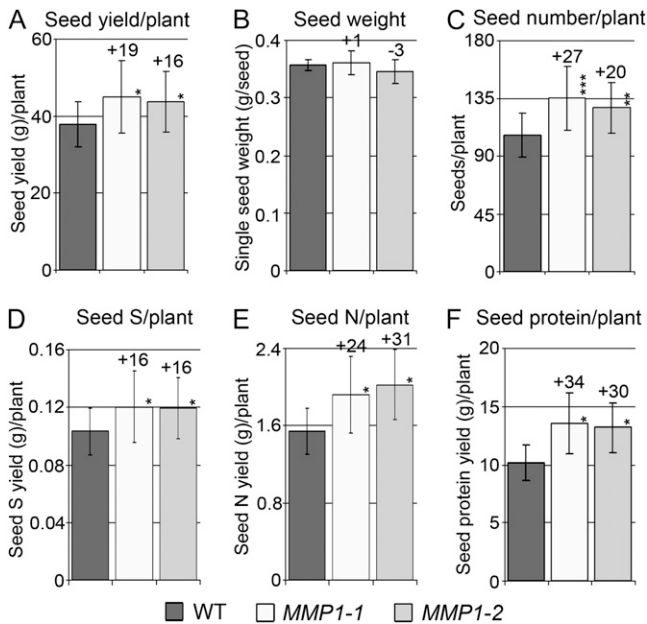


Figure 6. Analysis of yield-related parameters in *MMP1* and wild-type (WT) plants. A, Total seed yield per *MMP1* or wild-type plant. B, Average single seed weight from eight plants. C, Total number of seeds per plant. D, Total seed S yield per plant. E, Total seed N yield per plant. F, Total seed protein yield per plant. Results are presented as means \pm SD. Asterisks indicate significant differences from the wild type detected by Student's *t* test ($n = 8$): * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

lation of uptake and synthesis to keep up with enhanced export rates (Fig. 2, D–F). Elevated levels of SMM in *MMP1* xylem, leaves, and phloem (Fig. 2, A, B, and F) as well as the overall increase in seed S yield (Fig. 6D) further support an increase in S uptake, assimilation, and source-sink translocation in the transgenic plants. While it seems evident that improved SMM phloem loading or SMM phloem concentrations cause the changes in metabolism and transport in leaves and roots (see below), regulation of these processes is probably highly complex. It most likely involves nutrient (SMM) sensing and signaling mechanisms in the different plant organs as well as shoot-root communication processes (Scheible et al., 2004; Walch-Liu et al., 2005; Nikiforova et al., 2006; Hirai and Saito, 2008; Miller et al., 2009; Ruffel et al., 2010; Yi et al., 2010). Future feeding experiments with radiolabeled SMM, as well as integrated “omics” studies, might help to identify some of the regulatory components leading to the observed changes.

SMM Phloem Levels Influence N Metabolism and Transport

It is well known that plant N and S metabolisms are tightly coordinated (Wang et al., 2003; Nikiforova et al., 2006). Our data demonstrate that increased SMM source-sink partitioning goes in hand with increased root S uptake and SMM synthesis and xylem

transport to the leaf (Fig. 2, D and F). At the same time, substantial changes in leaf amino acid content and export were observed (Fig. 3). The changes in leaf and phloem N seem to be due to increased N assimilation in both *MMP1* roots and leaves, as indicated by the up-regulation of N assimilation genes in root and leaf and the increased levels of specific amino acids in the xylem, leaf, and phloem (Figs. 3 and 4). Root N and amino acid steady-state levels are unchanged in *MMP1* plants compared with the wild type, suggesting that, consistent with the expression data, increased synthesis is keeping up with enhanced export rates of amino acids (Fig. 4). Xylem levels of members of the Asp family, but only those of the homo-Ser branch, including Thr, Ile, and Met, were increased, while amounts of other amino acids of the Asp family, Asn and Lys, were not changed or decreased (Fig. 4F). This coincides with the observed up-regulation of SMM production and export in roots, which also requires the homo-Ser path for its synthesis (Fig. 2, D and F). In addition, export of Glu, GABA, and amino acids of the pyruvate family (Ala, Val, and Leu) that rely on Glu for synthesis was increased. On the other hand, xylem levels of Arg, Pro, Gln, and Asn were unchanged or decreased (Fig. 4F). Together, this suggests that in *MMP1* plants, increased N assimilation in roots and export of amino acids is primarily focused on Glu, the pyruvate family, and the homo-Ser branch of the Asp family, including SMM (Fig. 2F), rather than on the synthesis and export of amides, Asp, Lys, and amino acids of the Glu family.

On the contrary, the synthesis of Asn, Asp, and Gln seems to be up-regulated in *MMP1* leaves, as indicated by their increased levels and by gene expression studies (Fig. 3, D and F). In *MMP1* plants, leaf N supply and content are increased (Fig. 3E; see below), and much of the N seems to be channeled into Asn synthesis. Asn might be transiently stored until usage (Genix et al., 1990; Tsai and Coruzzi, 1990; Lam et al., 1994) or it may be needed for improved synthesis of homo-Ser (Bauer et al., 1977; Murray and Cordova-Edwards, 1984) and other amino acids of the Asp family, including Lys for translocation to sinks (Fig. 3, B and D). Homo-Ser is one of the main phloem amino acids in pea (Urquhart and Joy, 1981; Rochat and Boutin, 1991; Fig. 3D). While homo-Ser levels in *MMP1* compared with wild-type leaves (and xylem) were unchanged, phloem levels were increased, suggesting higher synthesis and export of this amino acid out of the leaves. Other amino acids that were increased in the *MMP1* phloem are Met, Ile, Thr, Lys, Val, and Leu. With the exception of Lys, the accompanying increased levels of these amino acids in the xylem support the idea that they are root derived and probably transferred from the xylem to the phloem for sink supply (Pate et al., 1975; Atkins et al., 1979; for review, see Van Bel, 1990). However, it cannot be excluded that some improved assimilation of the Asp and pyruvate family amino acids in leaves might also contribute to their increased phloem content. In any case, increased load-

ing of Val, Leu, Ile, Thr, and Met into the phloem is further consistent with the up-regulation of *CAT6* (Fig. 3F), which in *Arabidopsis* is expressed in companion cells (Brady et al., 2007) and mediates the transport of the respective amino acids (Hammes et al., 2006). Elevated levels of amino acids in the phloem also suggest increased delivery of amino N to *MMP1* seeds and uptake into the pea cotyledons. This is in agreement with the up-regulation of the N-inducible amino acid importer *AAP2* (Tegeder et al., 2007) as well as the increased seed total N and protein levels (Fig. 5, A–C).

Taken together, these results support the notion that changes in SMM transport and metabolism affect N assimilation in root and leaves, probably by feedback regulation mechanisms, leading to improved source-sink translocation of N. Elevated levels of N in *MMP1* leaves, phloem, and single seeds, as well as the enhanced overall seed N and protein yields (Fig. 6, E and F), further point to an increase in N uptake by *MMP1* roots.

S and N Metabolism and Transport Are Important for Seed Development

Studies with *Arabidopsis hmt2* mutants, in which the conversion of Met to SMM is inhibited, demonstrated that these plants overaccumulate SMM in source leaves and subsequently load more SMM into the phloem for transport to the seeds (Lee et al., 2008). In *hmt2* seeds, the Met levels were increased due to almost normal enzymatic activity of HMTs. *Medicago* transcriptome and proteome studies provided further evidence for the importance of SMM long-distance transport for Met and protein synthesis in seeds (Gallardo et al., 2007). It was found that during the development of *Medicago* seed coats, de novo synthesis of Met was decreased while conversion from SMM to Met was up-regulated, suggesting that the Met required for protein synthesis during seed filling was mainly derived from SMM. In addition, the synthesis of S-adenosyl-Met, a precursor for SMM synthesis, was dramatically down-regulated during seed development, indicating that SMM synthesis might not occur in the seed coat at a later seed stage and that the SMM required for Met synthesis is mainly phloem derived (Gallardo et al., 2007). Surprisingly, in mature *MMP1* seeds, the amounts of total S and S-rich proteins were unchanged, although SMM phloem levels were increased (Fig. 5, D and E). This suggests that S uptake into *MMP1* seeds is not changed even though *MMP1* is expressed in seeds (Fig. 1B; Tegeder et al., 2007). It further supports the idea that phloem SMM levels are a function of *MMP1* transport activity in the leaf phloem rather than in the seeds (Tilsner et al., 2005). In addition, decreased SMM and unchanged Met levels were observed in developing *MMP1* seeds (Fig. 5F), indicating that probably more SMM was converted to Met to obtain sufficient Met for the higher demand for storage protein synthesis in *MMP1* seeds.

Somewhat contradictory, gene expression of *HMT*, encoding the enzyme responsible for the conversion of SMM to Met, was unchanged in *MMP1* seeds (Fig. 5A). However, near-normal HMT enzyme activity has been reported in *Arabidopsis hmt2* mutant seeds, suggesting posttranscriptional control of *HMT* genes or alternative pathways for SMM conversion to Met (Lee et al., 2008).

While the mechanism is unclear, increased SMM phloem transport seems to affect seed number in *MMP1* plants (Fig. 6, A–C). This is consistent with field experiments showing that fertilization of S alone increases seed yield (Malhi et al., 2007). In addition, responses to S application are greater when sufficient amounts of N are applied (Hocking et al., 1987; Zhao et al., 1999; Schonhof et al., 2007). Therefore, in *MMP1* plants, the synergistic increase of S and N assimilates in the phloem or a change in the S-N ratio might trigger increased seed set (Fismes et al., 2000). However, it could also be caused by other factors, such as changes in carbon-N ratio, which has been hypothesized to be important for sink development (Lawlor, 2002). Furthermore, a number of studies indicate that SMM plays very diverse roles in plant physiology and might also function in the methylation of plant metabolites, maintenance of S-adenosyl-Met homeostasis, and cell membrane damage control under abiotic stress (Giovanelli et al., 1980; Kocsis et al., 2003; Rácz et al., 2008). Therefore, in addition to being an essential long-distance transport form of reduced S, SMM may act as a signal to regulate plant processes, including metabolism and growth. However, this is somewhat in disagreement with studies performed with *Arabidopsis* and maize (*Zea mays*) *mmt* mutants, in which the SMM cycle was abolished and no SMM was produced (Tagmount et al., 2002; Kocsis et al., 2003). The *mmt* plants grew and reproduced normally, with similar seed S content compared with the wild type. These findings suggest that SMM is not essential for plant growth and development and that other S compounds may substitute for SMM in S transport (Kocsis et al., 2003). Nevertheless, when SMM phloem levels are increased by either repressing *HMT2* in the leaves or expressing SMM transporters in the phloem, changes in seed number and protein amounts (this study) or protein quality (higher Met, no growth effects; Lee et al., 2008) can potentially be made. The observed differences in alterations with respect to seeds in *hmt2* mutants and *MMP1* expressors might be species dependent or may be based on other factors that could potentially be identified by a detailed comparison of *Arabidopsis* lines (*hmt2* versus *MMP1*) using combined transcriptome and metabolome studies.

CONCLUSION

Plant response to increased phloem loading and source-sink distribution of SMM seems to be managed by a number of strategically important processes. First,

to accommodate the elevated demand for S caused by improved phloem loading, acquisition of sulfate by the root is increased and the S assimilatory machinery is up-regulated. Second, N metabolism in root and leaf is induced, probably to provide the precursors for the synthesis of organic S compounds and to deliver the required N for increased growth and sink development. Third, higher SMM phloem levels lead to increased seed/sink development and S partitioning to the increased number of seeds per plant, rather than to individual seeds. Whether altered SMM phloem transport positively affects seed numbers directly or indirectly is unknown. However, modifications in phloem metabolite levels might provide feed-forward regulation signals that finally lead to improved sink set. Fourth, elevated amino acid levels in the phloem lead to increased N uptake into seeds (and finally more protein), facilitated by amino acid importers that are up-regulated by the higher N levels.

Since the discovery of SMM as a long-distance transport form of reduced S (Bourgis et al., 1999), several studies have demonstrated that SMM is important for plant S metabolism, although it might not be essential for plant growth (Kocsis et al., 2003; Gallardo et al., 2007; Lee et al., 2008). Yet, the question remains: What advantage does SMM have over other S-containing compounds in terms of S transport? In this study, the SMM concentrations in the phloem and xylem were less than 0.2% of the total amino acids, and manipulation of SMM phloem levels promoted dramatic changes in S as well as N metabolism and transport in *MMP1* pea plants. Perhaps one potential benefit of using SMM as a long-distance S transport compound lies in the fact that SMM might provide the signal for regulating plant S and N metabolism. The homeostasis of SMM, or a tightly coregulated key metabolite, might serve as a sensitive switch that, when tampered with, will trigger complex responses in whole plant S and N uptake, assimilation, and transport to regain a balanced optimum for plant growth and development.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Pea (*Pisum sativum* 'Bohatyr') plants were grown in 2-gallon pots with a mixture of peat (60%), pumice (20%), and sand (20%; SunGro Horticulture) in a greenhouse or growth chamber under 14 h of light and with photosynthetically active radiation between 400 and 500 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at the soil surface. Greenhouse temperatures ranged from 18°C to 22°C during the day and from 15°C to 18°C at night. Growth chambers were at 22°C/18°C during the day/night. Plants were fertilized weekly with 20:20:20 (N:phosphate:potassium) fertilizer (J.R. Peters). Developing side branches were removed for a defined source-sink position and easy management of the plants. For confirmation of results, materials were harvested and analyzed from independently grown sets of plants. For biochemical analyses and gene expression studies, leaves, roots, and developing seeds (75%–80% relative water content) were collected from at least six 10-week-old plants. Plant samples were collected 6 h after the beginning of the light period and over a maximum of 3 h. Tissues were sampled concurrently for wild-type and transgenic plants. Dry seeds were harvested from eight desiccated plants.

Construct Preparation and Plant Transformation

The coding sequence of the *MMP1* gene (YLL061W; Rouillon et al., 1999) from *Saccharomyces cerevisiae* was amplified using PCR with *MMP1*-specific primers (Supplemental Table S1) and cloned into pTKan (Hajdukiewicz et al., 1994) carrying the *AAP1* promoter from *Arabidopsis thaliana*; Hirner et al., 1998). The construct was introduced into the *Agrobacterium tumefaciens* strain AGL1, and pea plants were transformed (Grant et al., 1995). Homozygous transgenic plants were identified by PCR using genomic DNA and the primers listed in Supplemental Table S1. For Southern-blot analysis, standard procedures were applied.

Gene Expression Analyses

RNA was isolated from root, leaf, and seed tissue of homozygous *MMP1* plants (Péllissier and Tegeder, 2007) and used for RT-PCR with two independent sets of primers (Tan et al., 2008; Supplemental Table S1). Amplification products were analyzed by gel electrophoresis. Pea elongation factor 1 α (*EF1 α* ; X96555) reverse transcription products were amplified (for primers, see Supplemental Table S1) as a control for even amounts of total cDNAs used for the different PCRs.

cDNA synthesis and quantitative RT-PCR were performed following Sanders et al. (2009). The expression of genes of S and N transport and metabolism and storage proteins was analyzed. Primers were designed based on published pea sequences (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/>). In cases where specific pea gene sequences were unknown, degenerated primers were designed along known cDNAs from at least two legume species. Supplemental Table S2 lists the genes analyzed (including accession numbers), the primers used, and the gene accessions and legume species when degenerated primers were applied. For the pea homolog to *Arabidopsis CAT6*, a partial clone was produced using PCR with degenerated primers (Supplemental Table S1) performed on pea cotyledon cDNAs. The 921-bp partial clone was then sequenced, and gene-specific primers were designed and used for quantitative RT-PCR analysis (Supplemental Table S2). Fold changes in gene expression were calculated by comparison with expression of *EF1 α* (X96555) and ubiquitin (*PUB1-4*; L81139, L81140, L81141, L81142). Representative results of three independent experiments using *EF1 α* as the control gene are shown.

Collection and Analysis of EDTA-Phloem Exudates and Xylem Sap

Phloem exudates were obtained from a total of 16 plants using two leaflets per plant (Urquhart and Joy, 1981). The exudates of four plants were pooled and analyzed. Xylem sap was collected from nine 6-week-old plants (Bauer et al., 1977), and pools from three plants were analyzed.

Elemental Nutrient and Metabolite Analyses

Total C and N contents were analyzed according to Sanders et al. (2009). For total S measurements, seed samples were combusted with a Costech ECS 4010 elemental analyzer (Costech Analytical Technologies) with a dual reactor configuration (Fry et al., 1992). SO_2 gases were separated with a 0.8-m gas chromatography column at 105°C and analyzed for total area (Brenna et al., 1997). Protein extractions and SDS-PAGE were done after Sanders et al. (2009). Protein concentrations were determined using the NanoOrange kit (Invitrogen).

Free amino acids were extracted from 3 mg of lyophilized tissue with 80% methanol at 70°C for 15 min of shaking at 1,000 rpm, with α -aminobutyric acid (7 nmol mg^{-1} tissue) added as an internal standard. After centrifugation and collection of supernatants, the pellets were reextracted using 20% methanol as described above and centrifuged. The supernatant was collected, combined with that from the previous step, and dried to a pellet under vacuum (SpeedVac concentrator; Savant Instruments). The resulting pellet was dissolved in HPLC-grade water. Samples for amino acid measurements were derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate using the AccQ-fluor kit (Waters) according to the manufacturer's instructions. Derivatized samples were filtered (0.22 μm ; SunSri) to remove debris and particles. An amino acid mixture (catalog no. A2407; Sigma) with added Gln, SMM, Orn, GABA, Arg, Lys, His, ammonium chloride, and homo-Ser was used as a standard.

HPLC analysis was performed using a Waters 2695 separation module with column heater, autosampler, and Empower2 software. Separation was

performed on a Waters 4.6 × 150-mm, 3.5- μ m SunFire C18 column equipped with a Sentry guard column at 37°C. Solvent A (acetonitrile), solvent B (HPLC-grade water), solvent C (AccQ-Taq Eluent A, diluted), and solvent D (100 mM sodium acetate, 783 μ L L⁻¹ triethylamine, and 250 μ L L⁻¹ phosphoric acid, pH 5.8) were used at 1.35 mL min⁻¹ flow rate with the following gradient: 0 to 0.5 min, 100% C; 0.5 to 16.8 min, linear gradient to 96.5% C and 3.5% B; 16.8 to 29.85 min, to 96.5% D and 3.5% B; 29.85 to 34.2 min, to 95% C and 5% B; 34.2 to 35.68 min, to 91% C and 9% B; 35.68 to 49 min, to 83% C and 17% B; 49 to 57.35 min, immediate change to 60% B and 40% A; 57.35 to 64.5 min, immediate change to 100% C; 64.5 to 72 min, 100% C. Amino acids were detected using a Waters 2475 multi λ fluorescence detector with excitation at 280 nm and emission at 395 nm. Amino acid concentrations were calculated by comparing peak areas with amino acid standards.

Supplemental Data

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

Supplemental Table S1. Primers used for molecular analyses other than quantitative RT-PCR.

Supplemental Table S2. Primers employed for quantitative RT-PCR.

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