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Phloem loading of S-methylmethionine

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Increased phloem transport of S-methylmethionine positively affects sulfur and nitrogen metabolism and seed development in pea plants

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ABSTRACT

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 is important for the metabolism and growth of pea (Pisum sativum L.) plants. Transgenic plants were produced in which expression of a yeast SMM transporter, S-MethylMethionine Permease 1 (MMP1) was targeted to the phloem and seeds. Phloem exudate analysis showed that concentrations of SMM are elevated in MMP1 plants suggesting increased phloem loading. Further, 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 is 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 is 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 cysteine and methionine, as well as the antioxidant glutathione (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 quality of seeds (Higgins et al., 1986; Ufaz and Galili, 2008). Acquisition of S occurs predominantly via uptake of sulfate (SO₄²⁻) 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; Hopkins et al., 2005; Bourgis et al., 1999; Lappartient et al., 1999), though in legumes, S reduction also takes place in other organs. For example in pea (Pisum sativum L.) S assimilation has been localized to roots, as all the enzymes involved in sulfate reduction to cysteine 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).

Glutathione (GSH), cysteine, and methionine 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-methylmethionine (SMM) is a key contributor to phloem S transport in wheat, canola, legumes, and other plant species. In these plants, it is suggested that SMM is synthesized from methionine by the enzyme methionine 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 methionine via homocysteine 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 sucrose transporters in phloem loading and carbon movement to sinks (Tegeder et al., 2010). However, no information is available on the function of transporters in long distance movement of organic N and S, although a variety of amino acid, GSH, GSH-derivates, and GSH conjugates transporters have been identified (Jasinski et al., 2003; Zhang et al., 2004; Bouvier et al., 2006; Rentsch et al., 2007; Tegeder et al., 2007; Tan et al., 2008; Tegeder et al., 2010). Since there are no known plant SMM transporters, in this study, a high affinity yeast SMM transporter, S-MethylMethionine Permease 1 (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 only 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 S-MethylMethionine Permease 1 (MMPI, Rouillon et al., 1999) under the control of the Arabidopsis AAP1 promoter (Hirner et al., 1998). In pea, this promoter targets gene expression to the phloem throughout the plant and to seeds (Tegeder 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 MMPI is expressed throughout the transgenic plants including roots, leaves, and developing seeds (Fig. 1B), consistent with AAP1p-GUS studies in pea (Tegeder et al., 2007). Phenotypic analysis of vegetative growth illustrated that MMPI plants were taller and displayed a bushier appearance compared to wild-type (Fig. 1C). When examining the shoot biomass of 5-week-old MMPI plants, fresh and dry weight were increased by up to 35% and 44%, respectively compared to wild-type (Fig. 1D and E).

SMM Phloem Loading and Sulfur Metabolism Are Increased in MMPI Plants

Phloem exudates from MMPI leaflets were analyzed and SMM concentrations were 27-40% higher in MMPI-1 and 1-2 plants compared to wild-type (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, expression levels of genes involved in S assimilation in leaves were analyzed using quantitative RT-PCR. Surprisingly, genes important for synthesis of cysteine from sulfate such as adenosine 5′-phosphosulfate reductase (APR) and OAS (thiol)-lyase (OASTL) were downregulated (Fig. 2C). Further, transcript levels of methionine methyltransferase (MMT), the enzyme converting methionine to SMM were unchanged, whereas expression of homocysteine S-methyltransferase (HMT), responsible for the synthesis of methionine 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 MMPI 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 MMPI versus wild-type roots (Fig.
Further, *HMT* expression was slightly down-regulated, while *MMTI* 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 methionine via HMT. When analyzing expression levels of sulfate transporters, *SST* (Krusell et al., 2005) and *ST* (Smith et al., 1995) it was found that both transporters are upregulated 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 results showed that SMM was unchanged in whole *MMP1* root tissue (Fig. 2E). However, the SMM concentration in the xylem sap was increased by 64-70% in *MMP1* compared to wild-type plants (Fig. 2F) indicating increased S uptake and assimilation in roots, and translocation to the shoot.

**Amino Acid Phloem Loading and Leaf Nitrogen 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 is altered, N metabolism and transport is also likely to be affected. Consequently, analysis of phloem amino acid levels was performed and showed that amounts of total amino acids in the *MMP1* phloem were elevated by about 30% (Fig. 3A), with an increase in arginine, homoserine, isoleucine, leucine, lysine, methionine, threonine, and valine (Fig. 3B). In addition, expression of *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 upregulated in leaves (Fig. 3F), supporting increased phloem loading of amino acids.

To resolve if 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. Results showed that amounts of total N and free amino acids were increased in *MMP1* leaves by up to 9% and 53%, respectively (Fig. 3C and E). Analysis of single amino acid concentrations demonstrated that 8 out of 20 amino acids were significantly elevated in both transgenic lines, with the strongest increase in
asparagine by up to 400% (Fig. 3D). Along with this, there was a slightly increased expression of genes involved in the synthesis of glutamine (GS) and glutamate (GOGAT), which are the first products of N assimilation, an up to 6-fold upregulation of AS1, important for asparagine synthesis, and downregulation of ASNase, involved in asparagine 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 was found that amounts and profiles of free amino acids, as well as total N were unchanged in *MMP1* roots compared to wild-type (Fig. 4A-C). Expression analysis demonstrated that genes involved in the initial assimilation of inorganic N, GS and GOGAT were upregulated 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 downregulated in both lines (Fig. 4D).

Xylem sap analysis revealed that total amino acid levels were similar in overexpressors versus wild-type, but concentrations of single amino acids were altered (Fig. 4E and F). Glutamine was decreased in both overexpressors, and asparagaine and lysine were reduced or not changed dependent on the *MMP1* line. Glutamate, GABA, alanine, leucine, valine, isoleucine, methionine, and threonine levels were upregulated in *MMP1* xylem sap compared to wild-type (Fig. 4F), suggesting increased export of these amino acids out of the root.

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

In general, about 65-85% 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, 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 were 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 changes were observed (Fig. 5E), which concurs with unchanged levels of S-rich seed proteins in *MMP1* plants. Analysis of methionine and SMM in developing *MMP1* seeds showed that methionine concentrations were unchanged while SMM levels were decreased (Fig. 5F). However, expression of *HMT*, encoding for the enzyme converting SMM back to methionine, was unchanged in developing *MMP1* seeds (Fig. 5A).

**Increased SMM Phloem Loading Positively Affects Seed Number**

While SMM phloem levels are 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 to wild-type plants, but seed number per plant was positively affected by 20-27% (Fig. 6B and C).
As described above, *MMP1* seed S levels were unaltered, whereas N and protein levels were increased (see Fig. 5B-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 to 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. 6E 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 Sulfur 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 (see 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 exhibit feedback is supported by (i) upregulation of gene expression of S uptake and assimilation in the root; (ii) increased levels of SMM in the xylem; and (iii) downregulation of genes involved in leaf S assimilation (see Fig. 2C, D, and F). Most enzymes involved in S assimilation are transcriptionally regulated, with high S levels downregulating 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 downregulation of S assimilation genes (see Fig. 2B and C). On the other hand, it was shown that S deficiency leads to upregulation of S transporter and biosynthesis genes (Hirai et al., 2003; Vauclare et al., 2002; 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 upregulation of uptake and synthesis to keep up with enhanced export rates (Fig. 2D-F). Elevated levels of SMM in \textit{MMP1} xylem, leaves, and phloem (Fig. 2A, 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 also 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 (c.f. 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.

\section*{SMM Phloem Levels Influence Nitrogen 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. 2D 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 \textit{MMP1} roots and leaves as indicated by upregulation of N assimilation genes in root and leaf and increased levels of specific amino acids in the xylem, leaf and phloem (Fig. 3 and 4). Root N and amino acid steady state levels are unchanged in \textit{MMP1} plants compared to 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 aspartate family, but only those of the homoserine branch, including threonine, isoleucine and methionine, were increased while amounts of other amino acids of the aspartate family, asparagine
and lysine, were not changed or decreased (Fig. 4F). This coincides with the observed upregulation of SMM production and export in roots, which also requires the homoserine path for its synthesis (Fig. 2D and F). In addition, export of glutamate, GABA, and amino acids of the pyruvate family (alanine, valine, and leucine) that rely on glutamate for synthesis, were increased. On the other hand, xylem levels of arginine, proline, glutamine and asparagine were unchanged or decreased (Fig. 4F). Together, this suggests that in MMP1 plants increased N assimilation in roots and export of amino acids is primary focused on glutamate, the pyruvate family, and the homoserine branch of aspartate family including SMM (Fig. 2F), rather than synthesis and export of amides, aspartate, lysine, and amino acids of the glutamate family.

On the contrary, synthesis of asparagine, aspartate and glutamine seems to be upregulated in MMP1 leaves as indicated by their increased levels and by gene expression studies (Fig. 3D and F). In MMP1 plants, leaf N supply and content is increased (Fig. 3E, see also below), and much of the N seems to be channeled into asparagine synthesis. Asparagine 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 homoserine (Bauer et al., 1977; Murray and Cordova-Edwards, 1984) and other amino acids of the aspartate family including lysine for translocation to sinks (Fig. 3B and D). Homoserine is one of the main phloem amino acids in pea (Urquhart and Joy, 1981; Rochat and Boutin, 1991; Fig. 3D). While homoserine levels in MMP1 compared to 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 methionine, isoleucine, threonine, lysine, valine, and leucine. With the exception of lysine, accompanied increased levels of these amino acids in the xylem support 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 also van Bel, 1990). However, it cannot be excluded that some improved assimilation of the aspartate and pyruvate family amino acids in leaves might also contribute to their increased phloem content. In any case, increased loading of valine, leucine, isoleucine, threonine, and methionine into the phloem is further consistent with the upregulation of CAT6 (Fig. 3F), which in Arabidopsis is expressed in companion cells.
(Brady et al., 2007) and mediates 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 upregulation of the N inducible amino acid importer AAP2 (Tegeder et al., 2007) as well as increased seed total N and protein levels (Fig. 5A-C).

Taken together, the results support 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. 6E and F) further point to an increase in N uptake by MMP1 roots.

Sulfur and Nitrogen Metabolism and Transport Are Important for Seed Development

Studies with Arabidopsis hmt2 mutants, in which the conversion of methionine to SMM is inhibited, demonstrated that these plants over-accumulate 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 methionine 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 methionine and protein synthesis in seeds (Gallardo et al., 2007). It was found that during development of Medicago seed coats, de novo synthesis of methionine was decreased, while conversion from SMM to methionine was upregulated, suggesting that the methionine required for protein synthesis during seed filling was mainly derived from SMM. In addition, the synthesis of S-adenosylmethionine, 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 methionine 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. 5D 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).
further supports that phloem SMM levels are a function of MMP1 transport activity in the leaf phloem rather than in the seeds (c.f. Tilsner et al., 2005). In addition, decreased SMM and unchanged methionine levels were observed in developing MMP1 seeds (Fig. 5F), indicating that probably more SMM was converted to methionine to obtain sufficient methionine 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 methionine, was unchanged in MMP1 seeds (Fig. 5A). However, near-normal HMT enzyme activity has been reported in Arabidopsis hmt2 mutant seeds, suggesting post-transcriptional control of HMT genes or alternative pathways for SMM conversion to methionine (Lee et al., 2008).

While the mechanism is unclear, increased SMM phloem transport seems to affect seed number in MMP1 plants (Fig. 6A-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 applications 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). It could, however, also be caused by other factors such as changes in C:N ratio, which has been hypothesized to be important for sink development (Lawlor, 2002). Further, a number of studies indicate that SMM plays very diverse roles in plant physiology, and might also function in methylation of plant metabolites, maintenance of S-adenosylmethionine homeostasis and in 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 signal to regulate plant processes including metabolism and growth. However, this is somewhat in disagreement with studies performed with Arabidopsis and maize 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 to the wild-type. These findings suggest that SMM is not essential for plant growth and development and that other S compounds may substitute 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 methionine; 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.

CONCLUSIONS

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 upregulated. 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 finally leading 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 upregulated by the higher N levels.

Since the discovery of SMM as 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 to 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 \textit{MMP1} pea plants. Perhaps one potential benefit of using SMM as a long distance S transport compound lies in that SMM might provide the signal for regulating plant S and N metabolism. The homeostasis of SMM, or a tightly co-regulated key metabolite, might serve as a sensitive switch, which when tampered, will trigger complex responses in whole-plant S and N uptake, assimilation, and transport to regain a balanced optimum for plant growth and development.

\textbf{MATERIALS AND METHODS}

\textbf{Plant Materials and Growth Conditions}

Pea (\textit{Pisum sativum} L. cv. Bohatyr) plants were grown in 2-gallon pots with a mixture of peat (60%), pumice (20%), and sand (20%) (SunGro Horticulture, Bellevue, WA) in a greenhouse or growth chamber under 14-h light and with photosynthetically active radiation between 400-500 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) at the soil surface. Greenhouse temperatures ranged from 18°C to 22°C during the day and 15°C to 18°C at night. Growth chamber were at a 22°C/18°C during the day/night. Plants were fertilized weekly with 20-20-20 (N, P, K) fertilizer (J.R. Peters, Allentown, PA). 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 six hours after beginning of the light period and over maximum three hours. Tissues were sampled concurrently for wild-type and transgenic plants. Dry seeds were harvested from eight desiccated plants.

\textbf{Construct Preparation and Plant Transformation}

The coding sequence of the \textit{S-MethylMethionine Permease 1} gene (\textit{MMP1}, YLL061W; Rouillon et al., 1999) from \textit{Saccharomyces cerevisiae} was amplified using
PCR (polymerase chain reaction) with MMP1 specific primers (Table S1), and cloned into pTKan (Hajdukiewicz et al., 1994) carrying the AAP1 promoter from Arabidopsis (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 primers listed in 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 (reverse transcriptase-PCR) with two independent sets of primers (Tan et al., 2008; Table S1). Amplification products were analyzed by gel electrophoresis. Pea elongation factor 1α (EF1α, X96555) reverse transcription products were amplified (for primers see Table S1) as a control for even amounts of total cDNAs used for the different PCR reactions.

cDNA synthesis and quantitative RT-PCR was 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 (NCBI, 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. Table S2 lists the genes analyzed (including accession numbers), 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 (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 (Table S2). Fold changes in gene expression were calculated by comparison to expression of pea elongation factor 1a (EF1α, X96555) and ubiquitin (PUBL-4, L81139, L81140, L81141, L81142). Representative results of three independent experiments using EF1α 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 content 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). SO₂ gases were separated with a 0.8 m gas chromatography (GC) column at 105°C and analyzed for total area (Brenna et al., 1997). Protein extractions and SDS-PAGE gel electrophoresis was done after Sanders et al. (2009). Protein concentrations were determined using the NanoOrange® kit (Invitrogen, Carlsbad, CA).

Free amino acids were extracted from 3 mg of lyophilized tissue with 80% methanol at 70°C for 15 min shaking at 1000 rpm, and with alpha-aminobutyric acid (7 nmol·mg⁻¹ tissue) added as an internal standard. After centrifugation and collection of supernatants, the pellets were re-extracted 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, Farmingdale, NY). The resulting pellet was dissolved in high performance liquid chromatography (HPLC)-grade water. Samples for amino acid measurements were derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) using the AccQ-fluor kit (Waters, Milford, MA) according to the manufacturer’s instructions. Derivatized samples were filtered (0.22 μm; SunSri, Duluth, GA) to remove debris and particles. An amino acid mixture (Catalog No. A2407, Sigma, St. Louis, MO) with added glutamine, SMM, ornithine, γ-aminobutyric acid (GABA), arginine, lysine, histidine, ammonium chloride, and homoserine was used as 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 X 150 mm, 3.5 \( \mu \)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 \( \mu \)L/L triethylamine, 250 \( \mu \)L/L phosphoric acid, pH 5.8) were used at 1.35 mL/min flow rate with the following gradient: 0-0.5min, 100% C; 0.5-16.8 min, linear gradient to 96.5% C and 3.5% B; 16.8-29.85 min, to 96.5% D and 3.5% B; 29.85-34.2 min, to 95% C and 5% B; 34.2-35.68 min, to 91% C and 9% B; 35.68-49 min, to 83% C and 17% B; 49-57.35 min, immediate change to 60% B and 40% A; 57.35-64.5 min, immediate change to 100% C; 64.5-72 min, 100% C. Amino acids were detected using a Waters 2475 multi \( \lambda \) fluorescence detector with excitation at 280 nm and emission at 395 nm. Amino acid concentrations were calculated by comparing peak areas to amino acid standards.

Supplemental Material

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

**Supplemental Table S1.** Primers used for molecular analyses other than qRT-PCR

**Supplemental Table S2.** Primers employed for qRT-PCR

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FIGURE LEGENDS

**Figure 1.** Molecular and phenotypic analyses of *MMP1* plants. A, Southern blot analysis using HindIII-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 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. Elongation factor 1α (*EF1α*) was amplified as a control for equivalent amount of RNA used and even gel loading. C, Photograph of 5-week-old *MMP1* and WT plants. Two plants were grown in each pot. D, Shoot fresh weight of 5-week-old *MMP1* and WT plants (*n* = 6). E, Shoot dry weight of 5-week-old *MMP1* and WT plants (*n* = 6). Results are presented as mean ± SD (standard deviation). Asterisks indicate significant differences from WT detected by a Student’s *t*-test. **P<0.01 and ***P < 0.001.

**Figure 2.** S-methylmethionine (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). C and D, Fold change in gene expression of adenosine 5’-phosphosulfate reductase (*APR*), cysteine synthase/OAS(thiol)-lyase (*OASTL*), homocysteine methyltransferase (*HMT*), methinoine methyltransferase (*MMT*), and sulfate transporter *SST* and *ST* in (C) leaves and (D) roots. 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 mean ± SD. Asterisks indicate significant differences from WT detected by a Student’s *t*-test. *P<0.05, **P < 0.01 and ***P < 0.001.
Figure 3. Nitrogen 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 to wild-type (WT) plants. B, Amino acid composition and concentration in the phloem exudates. C, Total nitrogen (N) levels in leaves. D, Total free amino acid levels in leaves. E, Amino acid composition and concentration in leaves. F, Fold change in gene expression of pea glutamine synthetase 1 (GS1), GS2 and GS3, asparagines synthetase 1 (AS1), and amino acid transporters AAP1, AAP2, and CAT6. Degenerated primers were used for ferredoxin-dependent glutamate synthase [GOGAT (Fd)] and asparaginase (ASNase). GABA, γ-aminobutyric acid; Hse, homoserine. Results are presented as mean ± SD. Asterisks indicate significant differences from WT detected by a Student’s t-test (n = 4). *P<0.05, **P < 0.01 and ***P < 0.001.

Figure 4. Analysis nitrogen levels in root and xylem of MMP1 plants, and root gene expression studies. A, Total free amino acid levels in roots of MMP1 compared to wild-type (WT) plants (n = 4). B, Amino acid composition and concentration in roots (n = 4). C, Total nitrogen (N) levels in roots (n = 4). D, Fold change in gene expression of pea glutamine synthetase 1 (GS1), GS2 and GS3, and asparagines synthetase 1 (AS1). Degenerated primers were used for NADH-dependent glutamate synthase [GOGAT (NADH)] and asparaginase (ASNase). E, Total free amino acid levels in xylem sap (n = 3). F, Amino acid composition and concentration in xylem sap (n = 3). GABA, γ-aminobutyric acid; Hse, homoserine. Results are presented as mean ± SD. Asterisks indicate significant differences from WT detected by a Student’s t-test. *P<0.05, **P < 0.01, and ***P < 0.001.
**Figure 5.** Expression of genes involved in seed amino acid transport and seed storage protein synthesis, and total seed N, S, methionine and SMM, and protein levels. A, Fold change in expression of amino acid transporters AAP1, AAP2, and CAT6, and of storage protein genes legumin A (LegA), legumin B (LegB), convicilin A (Cvc) and vicilin (Vic), as well as homocysteine methyltransferase (HMT) in developing cotyledons of MMP1 compared wild-type (WT) plants. B, Total N levels in dry seeds. C, Amounts of soluble seed proteins. D, Analysis of soluble seed proteins using SDS-PAGE gel. Cvc, convicilins; Vic, vicilins; Leg-α, legumin α subunits; Leg-β, legumin β subunits. E, Total S levels in dry seeds. F, Concentrations of methionine (Met) and SMM in developing MMP1 seeds. Results are presented as mean ± SD. Asterisks indicate significant differences from WT detected by a Student’s t-test (n = 4). *P<0.05, ***P < 0.001.

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