

Methionine and *S*-methylmethionine exhibit temporal and spatial accumulation patterns during the *Arabidopsis* life cycle

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Abstract Methionine is a nutritionally essential sulfur-containing amino acid found at low levels in plant tissues. Yet, the factors that regulate its synthesis and accumulation in seeds are not fully known. Recent genetic studies demonstrate that *Arabidopsis* seeds are able to synthesize methionine *de novo* through the aspartate family pathway similarly to vegetative tissues; however, additional biochemical studies suggest that the *S*-methylmethionine (SMM) cycle also plays a major role in methionine synthesis in seeds. To better understand the contribution of these two pathways to methionine synthesis, we have sampled various vegetative and reproductive tissues during the *Arabidopsis* life cycle and determined the contents of soluble and protein-incorporated methionine, SMM, as well as the expression levels of the key genes involved in these two pathways. Our results strengthen the hypothesis that SMM that is produced in the rosette leaves from methionine contributes to methionine accumulation in seeds. However, the SMM cycle may have additional functions in plant tissues

since its key genes were expressed in all of the examined tissues, although at different rates. The accumulation patterns of soluble and protein-incorporated methionine during the *Arabidopsis* life cycle were found to be similar to most of the other amino acids, especially to those belonging to the branched-chain and aromatic amino acids that are produced in chloroplasts together with methionine. This indicates that similar factors regulate the levels of amino acids during development.

Keywords Amino acids · *Arabidopsis* life cycle · Methionine metabolism · *S*-methylmethionine cycle · Seed development

Introduction

Methionine is a nutritionally essential sulfur-containing amino acid found at low levels in plants and in their seeds. Therefore, its level limits the nutritional value of plants as a source of dietary protein for humans and animals. In plants, methionine not only serves as a building block for protein synthesis, but through its main derivative, *S*-adenosyl-methionine (SAM), controls the levels of several key metabolites such as ethylene, polyamines and biotin. SAM is also the primary methyl group donor that regulates various essential processes in plants [reviewed by (Amir 2010; Galili and Amir 2013; Roje 2006)]. For these reasons, studies were performed to assess the factors that regulate methionine metabolism and its accumulation in vegetative tissues and seeds, though most of them focused on factors regulating its synthesis [reviewed by Amir et al. 2012; Galili and Amir 2013; Hesse and Hoefgen 2003; Jander and Joshi 2010)]. The level of methionine in vegetative tissues is regulated mainly by the expression level of its first

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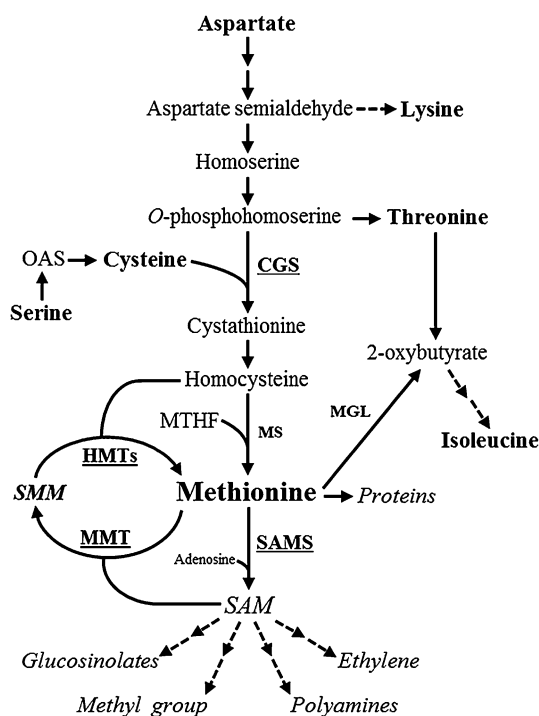


Fig. 1 Metabolic pathways and genes associated with Met metabolism. Key enzymes in the aspartate family pathway and the SMM cycle are underlined. Methionine catabolic products are presented in *italics*. Dashed arrows represent several metabolic steps. *OAS* *O*-acetylserine, *MTHF* methyltetrahydrofolate, *SMM* *S*-methylmethionine, *SAM* *S*-adenosylmethionine, *CGS* cystathionine- γ -synthase, *MS* methionine synthase, *MGL* methionine- γ -lyase, *HMTs* homocysteine *S*-methyltransferases, *MMT* Met *S*-methyltransferase, *SAMS* SAM synthase

committed enzyme, CYSTATHIONINE γ -SYNTHASE (*CGS*), which combines the carbon/amino skeleton derived from aspartate with the sulfur moiety derived from cysteine (Kim et al. 2002; Ravanel et al. 1998) (Fig. 1). Recent studies performed in legumes, tobacco and Arabidopsis seeds show that seed-specific expression of feedback-insensitive mutated forms of Arabidopsis *CGS* (*AtCGS*) leads to significantly higher levels of soluble methionine (Hanafy et al. 2013; Matityahu et al. 2013; Song et al. 2013; Cohen et al. 2014), indicating that methionine can be synthesized *de novo* in seeds via the aspartate family pathway through *CGS* activity similarly to vegetative tissues.

On the other hand, several other studies suggest that methionine is synthesized in seeds through an additional and/or alternative pathway in which methionine that is produced in vegetative tissues is converted to *S*-methylmethionine (*SMM*) by the activity of METHIONINE *S*-METHYLTRANSFERASE (*MMT*) and then transported via the phloem into the developing seeds at the onset of the reproductive period. In the seeds, the transported *SMM* is then re-converted back to soluble methionine by

HOMOCYSTEINE *S*-METHYLTRANSFERASE (*HMT*), thus contributing to the synthesis of methionine in seeds (Fig. 1) (Bourgis et al. 1999; Lee et al. 2008). This assumption is based on several observations. (1) Transgenic Arabidopsis plants overexpressing *AtCGS* and methionine over-accumulating Arabidopsis mutants exhibited relatively high levels of soluble methionine in their young rosette leaves. When these mutants began to flower, the contents of methionine decreased in the rosette leaves and significantly increased in the inflorescence apex region, immature siliques and seeds. Interestingly, the expression levels of *AtCGS* were found to be similar in leaves during these two developmental stages (Bartlem et al. 2000; Inaba et al. 1994; Kim et al. 2002), suggesting that methionine is transferred from the leaves toward the reproductive organs including the seeds. (2) Radioactive experiments showed that methionine is transported from leaves to seeds in the form of *SMM* (Bourgis et al. 1999), which was found to be the second major sulfur metabolite after glutathione in Arabidopsis and wheat phloem sap (Tan et al. 2010). (3) Flux experiments in wheat suggested that the major conversion in leaves is from methionine to *SMM*, and in seeds from *SMM* back to methionine. These researchers defined that the activity levels of *HMT* in Arabidopsis seeds are higher than that those of *MMT*, and the incorporation of ^{35}S into the protein was the same whether ^{35}S -methionine or ^{35}S -*SMM* was supplied (Ranocha et al. 2001). (4) Arabidopsis *hmt2* mutants exhibited significant accumulation of methionine in their seeds, suggesting that *SMM* that is transported to the seeds is re-converted to methionine by *HMT* isoenzymes other than *HMT2*. All the above lines of evidence strongly imply that soluble methionine is translocated as *SMM* towards the sink organs at the onset of the reproductive stage, thus exhibiting temporal and spatial accumulation patterns.

The relative contribution of the aspartate family and *SMM* pathways to methionine synthesis in seeds is probably more complex than assumed. Integrated comparative proteomic and transcriptomic analyses of different sections of *Medicago truncatula* seeds suggest that these two pathways contribute to methionine synthesis in seeds at different stages of seed development (Gallardo et al. 2007). The researchers imply that methionine is synthesized through the aspartate family pathway in endosperm and seed coat up to the mid-stages of seed filling, but in the late stages of seed filling methionine is synthesized from the *SMM* pathway at the seed coat (Gallardo et al. 2007). This assumption is based on the expression levels of the three genes/proteins of *HMT*, *MS* (METHIONINE SYNTHASE) and *SAMS* (SAM SYNTHASE) (Fig. 1). Despite the fact that these results are based on transcripts and protein levels rather than on distinct metabolic changes, they suggest roles played by these two pathways in methionine synthesis in seeds.

In spite of these lines of evidence, the role of SMM in methionine synthesis in seeds requires additional studies, since it was shown that the lack of SMM cycle in Arabidopsis and maize *mnt* mutants does not alter the levels of methionine, thiols and total sulfur compared to wild-type seeds (Kocsis et al. 2003). Additionally, transgenic tobacco plants overexpressing the AtCGS together with the bacterial feedback-insensitive form of AK(ASPARTATE KINASE), which exhibited 176- and 39-fold higher levels of methionine and SMM, respectively, showed only slightly higher methionine contents in their seeds (Hacham et al. 2008). Similarly, an examination of the uptake, transport and conversion of ^{13}C -SMM and ^{13}C -methionine in Arabidopsis *hmt2* and *mnt* mutants revealed that SMM is a non-essential intermediate in the movement of methionine from vegetative tissues into the seeds (Lee et al. 2008).

In the current study, we aimed at gaining more knowledge about the relative contribution of these two pathways to methionine synthesis and accumulation in Arabidopsis seeds. To achieve this, we measured the levels of methionine and SMM, as well as the expression levels of genes encoding the key enzymes involved in these two pathways (*CGS*, *MMT*, and *HMTs*) during the development of Arabidopsis wild-type plants in its various organs. We also studied how soluble and protein-incorporated levels of other amino acids were changed in these organs during development, and compared them with those of methionine to reveal whether methionine possesses unique patterns of accumulation.

Materials and methods

Plant material and growth conditions

Arabidopsis thaliana (ecotype C-24) plants were used in this study. In all experiments, the plants were first grown on Petri dishes containing 1 % MS (Murashige and Skoog, Duchefa), 1 % sucrose (Baker) and 7 mg/mL plant agar (Duchefa), and then the young seedlings were planted on fertilized soil at 22 ± 3 °C under a 16/8 h light/dark cycle at $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ with 50–70 % relative humidity.

All analyses performed in this study were done on the vegetative and reproductive tissues during the Arabidopsis life cycle as follows: imbibed seeds at 0, 1 and 5 days after imbibition (DAI), rosette leaves at 10, 20, 30, 40 and 44 DAI, cauline leaves at 40 and 44 DAI, stems at 40 DAI, flowers at 40 DAI, siliques at 40 and 44 DAI, and seeds at 40, 44 and 55 DAI. Seeds at 55 DAI represent mature dry seeds. All of the samples were collected at 10:00 a.m. and immediately snap-frozen in liquid nitrogen, cryo-lyophilized for 3 days and stored at -80 °C until further analysis.

RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

For the expression level analysis of *AtCGS*, *AtHMT1*, *AtHMT2*, *AtHMT3*, *AtMMT* and *AtSAMS3*, total RNA was extracted from 20 mg of the different tissues. All RNA samples were extracted and analyzed as previously described (Hacham et al. 2013). To normalize the variance among samples, the PP2A-A3 transcript level was used as endogenous control (Czechowski et al. 2005) and the relative gene expression was normalized to the values determined in rosette leaves at 30 DAI. The values presented are the mean of three biological replicates, each with three technical replicates. The primers used for the expression analysis of *AtCGS* were 5'-GGCGGCTGCTGCTACCTCGTC-3' (forward) and 5'-CCCTGATCCCCTCCTCGGCC-3' (reverse); for *AtHMT1* 5'-CGCAAATTCGCCAAGCTGACG-3' and 5'-GCTCCAAGGTCACGCAATTCG-3'; for *AtHMT2* 5'-AGTGTACCCAAACAGCGGAGAAAGC-3' and 5'-TGGTTGGTGTGTGCGGCAGC-3'; for *AtHMT3* 5'-TTTGCGACGGAGCTACAGCG-3' and 5'-TCC CACC GATAGACCTTTTGCCA-3'; for *AtMMT* 5'-TGAACCTCAGCTGGCAGCAAA-3' and 5'-GCCAGCTCCTCGG AAGTTGTCG-3'; and for *AtSAMS3* 5'-GGAGATGCA GGGCTTA CCGGC-3' and 5'-TGCACAATGACCCGC CTCGC-3'.

Amino acids and S-methylmethionine (SMM) determination using GC-MS and HPLC

For soluble and protein-incorporated amino acid determinations, tissue samples throughout the Arabidopsis life cycle were collected as four distinct biological pools. Following extraction, amino acids were detected using the single ion method (SIM) as previously described (Matityahu et al. 2013). Protein-incorporated amino acids were analyzed following protein hydrolysis (Matityahu et al. 2013). The levels of SMM were detected using HPLC as previously described (Hacham et al. 2002).

Statistical and correlation analyses

Statistical analyses were performed using the GraphPad Prism 5.01 scientific software (<http://www.graphpad.com/>). In all experiments throughout the manuscript, significance was calculated using the one-way ANOVA test of $p < 0.05$. Principal component analysis (PCA), and correlations and patterns analyses (Spearman's rank correlation) of GC-MS data were done using MetaboAnalyst 2.0, a comprehensive tool suite for metabolomic data analysis [<http://metaboanalyst.ca/>; (Xia et al. 2009, 2012)], following data \log_{10} transformation and pareto scaling (mean

centered and divided by the square root of standard deviation of each variable) manipulations.

Results

Soluble methionine contents vary between different tissues during the life cycle of *Arabidopsis*

To better understand the factors that regulate methionine accumulation in *Arabidopsis* seeds and the changes in methionine levels during plant development, we collected samples from vegetative and reproductive tissues during the different developmental stages of the *Arabidopsis* ecotype C-24 life cycle. These include three stages of germination, rosette leaves during five developmental stages up to their senescence, stems, two stages of cauline leaves, flowers, two stages of siliques, and developing seeds in three different stages including dry seeds (total of 17 samples). The samples were analyzed for their soluble methionine content using GC-MS (Table S1).

Measurements of seeds following 48 h of imbibition [0 days after imbibition (DAI)] showed relatively high levels of soluble methionine, suggesting the degradation of seed storage proteins (Fig. 2a). The levels of soluble methionine continued to increase up to 5 DAI, when the cotyledons appeared in the young seedlings. The relatively high level of methionine at this stage is apparently the result of continuous degradation of the seed proteins and/or de novo synthesis of soluble methionine (Fig. 2a). The levels of soluble methionine in the rosette leaves at 10 DAI showed significantly lower levels compared to imbibed seeds. The level of methionine continued to decrease up to 40 DAI, when the reproductive tissues emerged (Fig. 2a). At this stage, stems carrying the cauline leaves were already developed, including flowers with green siliques

and immature seeds. While the levels of soluble methionine decreased in the rosette leaves at 40 DAI, the contents of methionine in the reproductive tissues of flowers and developing seeds significantly increased up to 10- and 21-fold, respectively, compared to rosette leaves (Fig. 2a). We also measured the levels of soluble methionine in seeds at three different maturation stages: 40 DAI (immature green seeds), 44 DAI (seeds at their full size at the beginning of the desiccation processes) and 55 DAI (mature dry seeds). The results strongly indicate a significant reduction (up to 66-fold) in soluble methionine contents as the seeds desiccate and mature (Fig. 2a).

Total methionine levels in different tissues during *Arabidopsis* development

The levels of soluble methionine and of other soluble amino acids are relatively low compared to their total levels, which include protein-incorporated amino acids (Hacham et al. 2008). Thus, to have a broader view of the level of methionine, we also analyzed total methionine levels following protein hydrolysis in all tissues examined (Table S2).

Total methionine contents were about 10- to 100-fold higher than the soluble amino acid (Fig. 2a, b). Generally, total methionine showed different patterns of change in the various tissues during the *Arabidopsis* life cycle compared to those observed for soluble methionine. The highest levels were found in dry seeds at the beginning of imbibition and in mature dry seeds (Fig. 2b), most likely due to the accumulation of high levels of total methionine within the seed storage proteins. Following imbibition, a consistent moderate reduction in total methionine contents was observed up to 10 DAI, indicating that storage proteins were being degraded, thus explaining the higher levels of soluble methionine found at these stages (Fig. 2a). In the

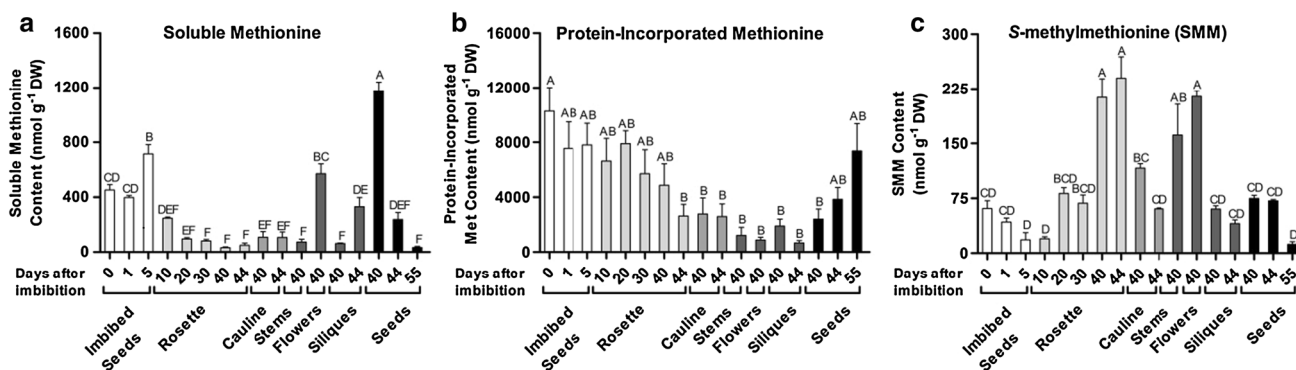


Fig. 2 The levels of methionine and S-methylmethionine (SMM) in various tissues during the life cycle of *Arabidopsis*. **a** Soluble methionine contents. **b** Protein-incorporated methionine contents. **c** SMM contents. Soluble and protein-incorporated methionine contents were

determined via GC-MS analysis, while SMM contents were determined via HPLC analysis. Data shown are mean \pm SE of four independent biological replicates. Significance was calculated using the two-way ANOVA test of $p < 0.05$ and identified by different letters

rosette leaves, there was a slight reduction in total methionine content, from 10 to 40 DAI. Between 40 and 44 DAI, when the reproductive tissues are fully developed, the total methionine content was significantly reduced in the rosette leaves. At that stage, the levels of methionine in the developing seeds significantly increased (Fig. 2a).

SMM contents exhibit inverted accumulation patterns from soluble methionine

To assess the role of SMM in methionine synthesis in seeds, the accumulation patterns of SMM were determined during the Arabidopsis life cycle using HPLC (Table S3). The results show a significant increase in SMM levels in the rosette leaves from 10 DAI, when the leaves were formed, up to 44 DAI, when the reproductive organs were fully mature and contained the developing seeds (Fig. 2c). The largest increase occurred in the rosette leaves between 30 and 40 DAI, indicating that SMM was accumulated massively in these tissues at this stage, apparently before being transferred to the reproductive organs (Fig. 2c). Supporting this transfer are the relatively high levels of SMM found in the stems and flowers (Fig. 2c). Higher levels of SMM (up to fourfold than in leaves 10 DAI) were also detected in the cauline leaves. A significant reduction was observed in the siliques and the developing seeds compared to the rosette leaves during the reproductive stage, implying that in these reproductive tissues SMM were converted back to soluble methionine to supply methionine toward its incorporation into the seed storage proteins (Fig. 2c). Indeed, mature dry seeds (55 DAI) had the lowest SMM contents during the Arabidopsis life cycle (Fig. 2c). It seems that the conversion from SMM to methionine occurred less in the flowers, as the SMM levels were found to be relatively high in these tissues.

Transcript levels of key genes involved in methionine synthesis through the aspartate family pathway and the SMM cycle

To gain a broader picture of the synthesis and accumulation patterns of methionine and SMM, we conducted a quantitative real-time PCR (qRT-PCR) analysis to measure the transcript levels of *AtCGS* that operate in the aspartate family pathway, and of genes encoding enzymes that operate in the SMM cycle. There is only one gene in Arabidopsis for *CGS* and *MMT* and three genes encoding *HMTs*.

CGS (At3g01120) was expressed in all tissues examined. The highest levels were measured in the rosette leaves, from 5 DAI up to 40 DAI (Fig. 3a). However, the expression level decreased in these tissues at 44 DAI, displaying similar expression levels to those detected in the reproductive tissues. The elevation in levels of *CGS* at the

early stages of seed germination from 1 DAI up to 5 DAI suggests that methionine is synthesized de novo in addition to methionine that is released from the degradation of seed storage proteins. The newly synthesized methionine is required to support what is needed for soluble methionine and its associated derivatives (such as ethylene, polyamines, glucosinolates) that are apparently required less in seeds, where most of the soluble methionine is incorporated into the seed storage proteins. Notably, the observed expression patterns of *CGS* are not compatible with the levels of methionine (Fig. 2a, b), suggesting that additional regulatory metabolic factors are involved in methionine accumulation. These factors may include methionine catabolic enzymes, which lead to the synthesis of SAM and its associated metabolites, incorporation into proteins and/or conversion into SMM (Amir 2010; Roje 2006).

Next, we examined the expression levels of the key genes participating in the SMM cycle. First, we measured the expression patterns of *MMT* (At5g49810) that catalyzes the formation of SMM from methionine and SAM. The results demonstrate that this gene is expressed in all tissues examined at relatively stable expression levels except in seeds at 0 DAI, which showed fourfold lower expression levels than those detected in the rosette leaves at 40 DAI (Fig. 3b). This implies that there is lower *MMT* activity when the seed storage proteins are degraded, supporting the requirements of methionine at this stage. The highest expression levels were detected in the rosette leaves at 40 and 44 DAI (Fig. 3b), when the highest SMM contents were observed (Fig. 2c).

MMT is also expressed in seeds (Fig. 3b). To determine if the SMM in seeds is synthesized de novo from *MMT* activity or transported mainly from other tissues, we examined the expression level of *SAMS3* (At3g17390), which represents the expression of *SAMSs* in seeds (according to <http://bar.utoronto.ca>). This enzyme produces SAM, the second substrate of *MMT*. The analysis showed that the expression levels were significantly reduced during the late two stages of seed development (Fig. 3f), suggesting a low level of SAM production and thus lower activity of *MMT* in seeds at these stages.

Expression analysis of the three *HMT* genes revealed that their expression levels were relatively high in the late stage of developing seeds, especially during the desiccation and maturation stage, compared to the first two stages of seed development (Fig. 3c–e). Among these three isoforms, the expression level of *HMT3* (At3g22740) decreased significantly from 0 DAI up to 20 DAI and then increased by about 7.5-fold in leaves at 44 DAI (Fig. 3e). Its expression level increased greatly in the maturing seeds (about 3.3- and 26-fold compared to rosette leaves at 44 and 20 DAI, respectively) (Fig. 3e). These findings imply that this isoform may play a more functional role in the conversion of

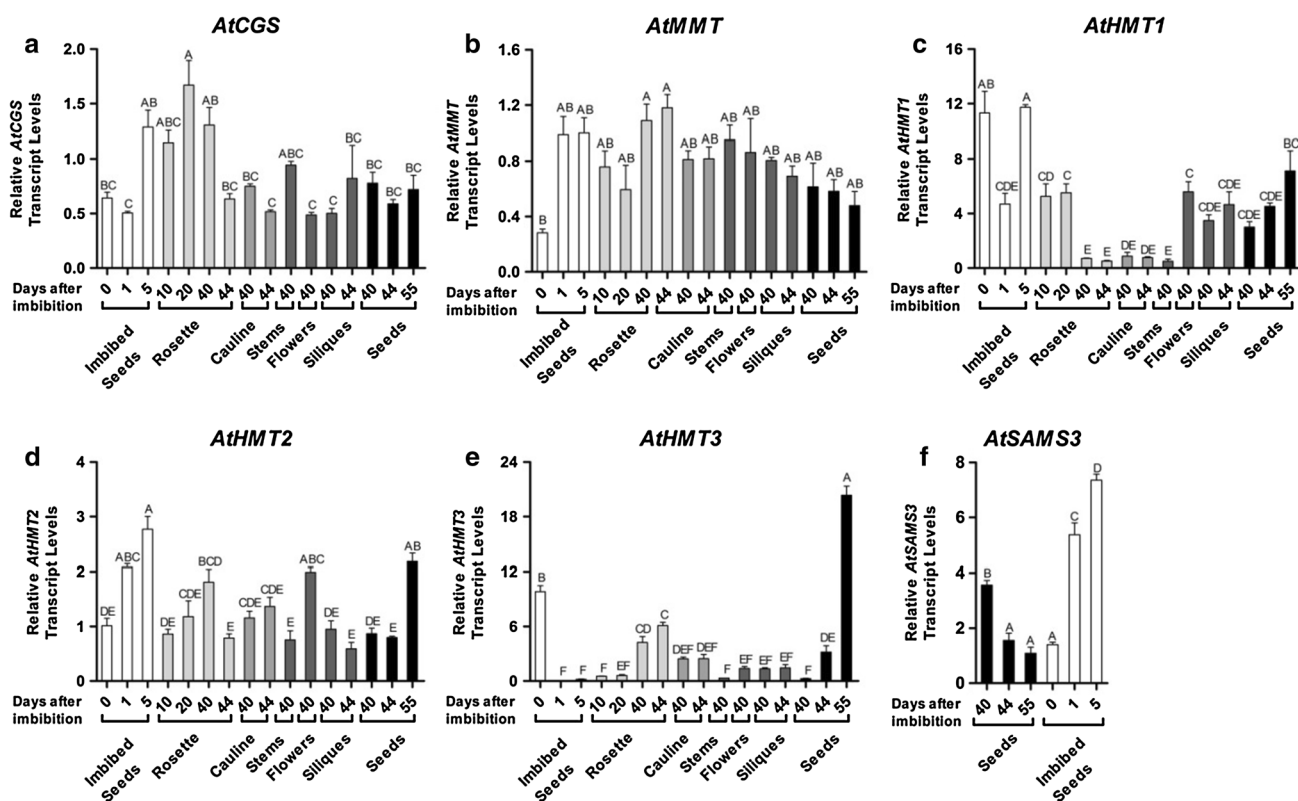


Fig. 3 Transcript levels of key genes involved in methionine synthesis through the aspartate family pathway and the SMM cycle. Transcript levels of: **a** *AtCGS*; **b** *AtMMT*; **c** *AtHMT1*; **d** *AtHMT2*; **e** *AtHMT3* and **f** *AtSAMS3*. The expression levels of all investigated genes were determined following qRT-PCR analysis and normalized

to the PP2A gene and the relative gene expression in rosette leaves 30 DAI. Data shown are mean \pm SE of three independent biological replicates. Significance was calculated using the two-way ANOVA test of $p < 0.05$ and identified by different letters

SMM into methionine at the late stages of seed development. Expression analysis of *HMT1* (At3g25900) revealed that its levels decreased in the rosette leaves from 5 DAI up to 44 DAI, reaching similar levels to those detected in the cauline leaves and the stems. However, its expression level increased greatly in the flowers, siliques and developing seeds (up to fourfold) compared to the levels observed in the rosette leaves at 40 DAI (Fig. 3c). Its expression levels are quite similar to the levels of soluble methionine (compare Fig. 3c to Fig. 2a). Finally, the expression level of *HMT2* (At3g63250) showed only minor fluctuations during the whole life cycle compared to *HMT1* and *HMT3* (Fig. 3d) and were not consistent with those observed for soluble methionine.

The accumulation patterns of other amino acids during the Arabidopsis life cycle

Soluble and protein-incorporated amino acids levels were measured to assess the similarity of their accumulation patterns with those of methionine and to reveal whether methionine exhibits unique independent patterns (Tables

S1 and S2). When measuring total soluble amino acid contents, we could clearly observe higher contents in the rosette leaves at 5 DAI, flowers at 40 DAI, siliques at 44 DAI and developing seeds at 40 DAI (Fig. 4a), which also showed distinct separation compared to all other tissues examined when plotted on principal component analysis (PCA) (Fig. S1).

Next, we were interested in investigating the individual patterns of the amino acids (Fig. 5). The results point to two global trends. (1) The levels of all soluble amino acids were dramatically reduced during seed development from 40 DAI until full maturation and desiccation at 55 DAI, most likely due to the massive incorporation of soluble amino acids into seed storage proteins that occurs during seed development and maturation. (2) High levels of total soluble amino acids were detected in rosette leaves at 5 DAI and flowers at 40 DAI (Figs. 4a, 5). Notably, only threonine and serine showed different profiles, since their levels remained relatively stable in the vegetative tissues even when the reproductive stage had already begun, and flowers, siliques and developing seeds operate as strong sink organs (Fig. 5).

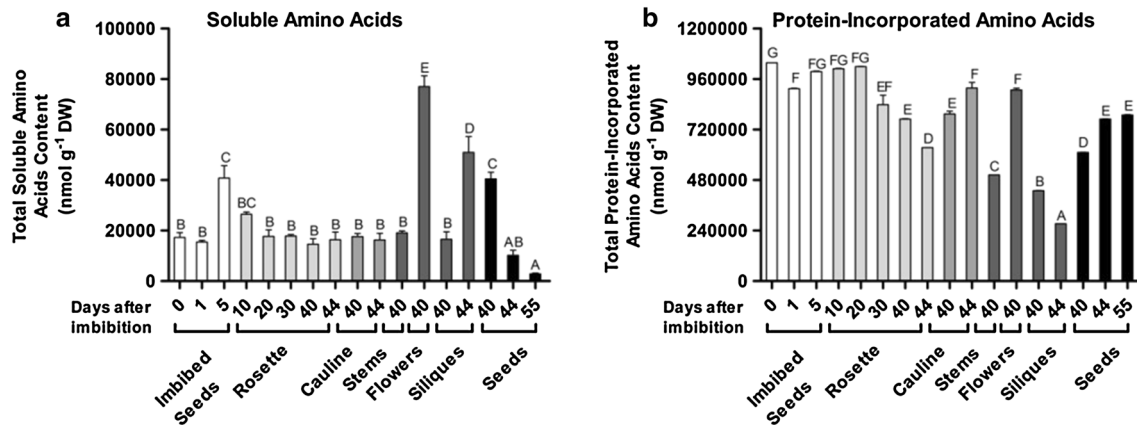


Fig. 4 GC–MS analysis of amino acids in various tissues during the life cycle of Arabidopsis. **a** Total soluble amino acids; **b** total protein-incorporated amino acids. All soluble and protein-incorporated amino

acid contents were determined via GC–MS analysis. Data shown are mean \pm SE of five independent biological replicates

Next, we performed the same manipulations on the data obtained from the GC–MS analysis of the protein-incorporated amino acids, as we did for the soluble amino acid data, allowing us to compare the changes during the Arabidopsis life cycle between soluble and protein-incorporated amino acids. Analysis of the total protein-incorporated amino acids showed a relatively consistent high level in imbibed seeds at 0 DAI up to 20 DAI for rosette leaves. From this point up to 44 DAI, a gradual decline was observed (Fig. 4b). The cauline leaves and flowers exhibited relatively high levels, while the lowest levels were observed in the stems and siliques (Fig. 4b). Finally, a consistent increase in total protein-incorporated amino acids content was observed during seed development up to full maturation at 55 DAI (Fig. 4b), implying that amino acids were incorporated into the seed storage proteins. All these tissue samples also showed clear separation according to PCA (Fig. S2).

The accumulation patterns of individual protein-incorporated amino acids are shown in Fig. 6. The results reveal several trends. (1) Most of the amino acids accumulate highly in the rosette leaves and flowers. (2) The levels of most amino acids are reduced in the rosette leaves when the reproductive organs appear at 40 and 44 DAI. (3) The contents of all amino acids increase during seed development as expected due to massive synthesis of seed storage proteins at these stages.

Do other amino acids exhibit an accumulation patterns similar to methionine?

The data described above suggest that methionine is transferred from the mature leaves toward the

reproductive tissues in the form of SMM. To assess whether other amino acids display similar patterns, or whether this behavior is unique to methionine during development, we performed correlation tests. These tests were performed between methionine levels and the levels of the other soluble and protein-incorporated amino acids during the entire developmental stage using the statistical tools embedded in the MetaboAnalyst 2.0 metabolomic data analysis software (see “Materials and methods”). The seven highest correlation values were found to be between soluble methionine and the levels of the branched-chain amino acids, leucine, valine and isoleucine, the aromatic amino acids phenylalanine, tryptophan and tyrosine, and asparagine (Fig. 7a). Apart from serine, threonine and homoserine that did not exhibit significant correlation with methionine, all the other amino acids showed significant correlation, although their values of significance were relatively low during the developmental processes.

The levels of soluble amino acids are approximately 25-fold lower than those incorporated in the proteins. Thus, the correlation values between protein-incorporated methionine and other incorporated amino acids were measured as well. The tests revealed that phenylalanine and tyrosine showed the highest correlation values to methionine (Fig. 7b), as observed in their soluble forms (Fig. 7a). Interestingly, serine also showed high correlation values to methionine, even though its soluble form exhibited the lowest correlation values (Fig. 7a). Lysine, glutamate, proline and threonine also showed relatively high correlation values to methionine (Fig. 7b), while the branched-chain amino acids of leucine, isoleucine and valine showed lower levels (Fig. 7b), even though they were highly correlated to methionine in their soluble form (Fig. 7a).

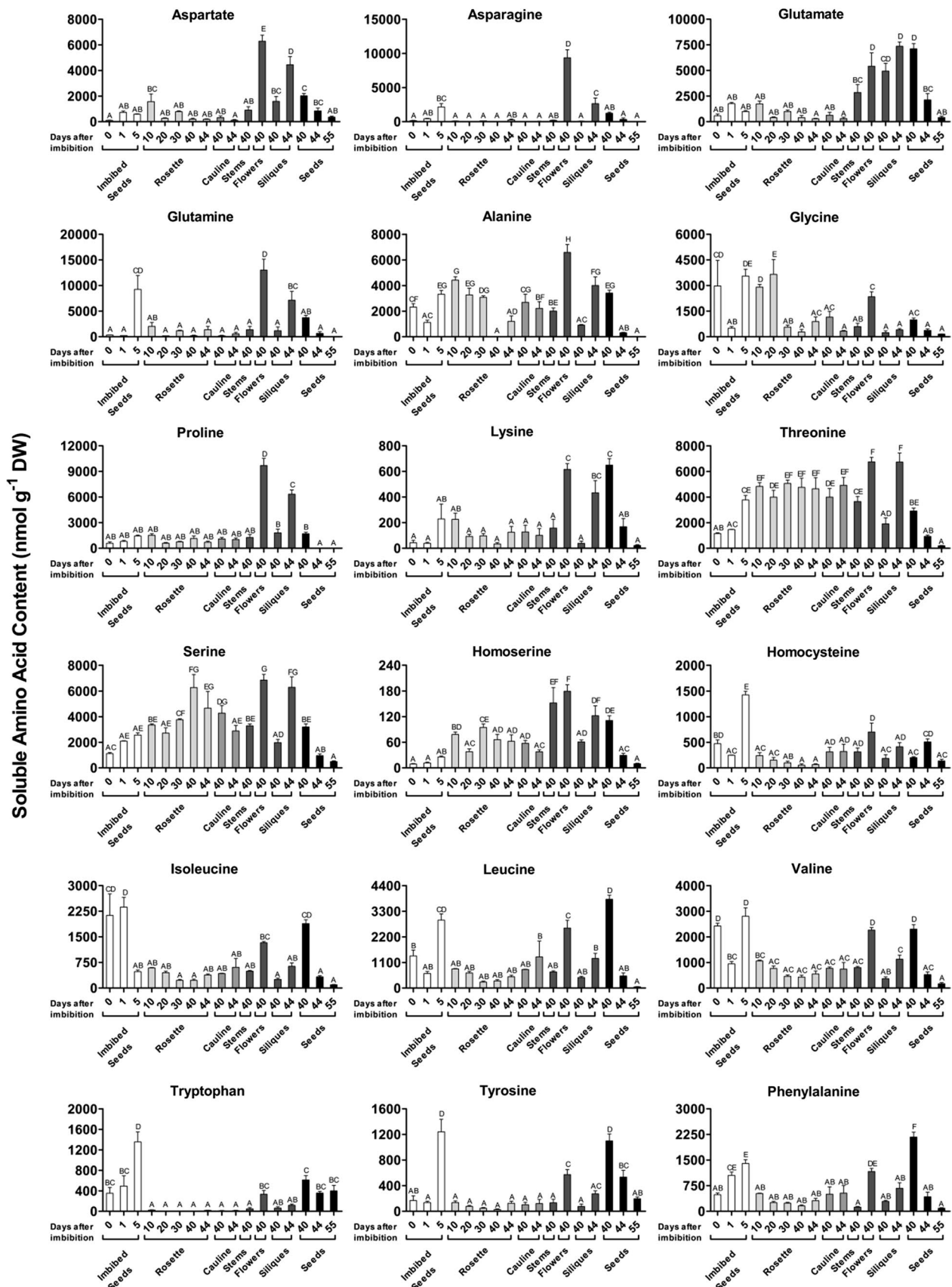


Fig. 5 The accumulation patterns of individual soluble amino acids in various tissues during the life cycle of *Arabidopsis*. Soluble amino acid contents were determined via GC-MS analysis. Data shown are mean \pm SE of five independent biological replicates

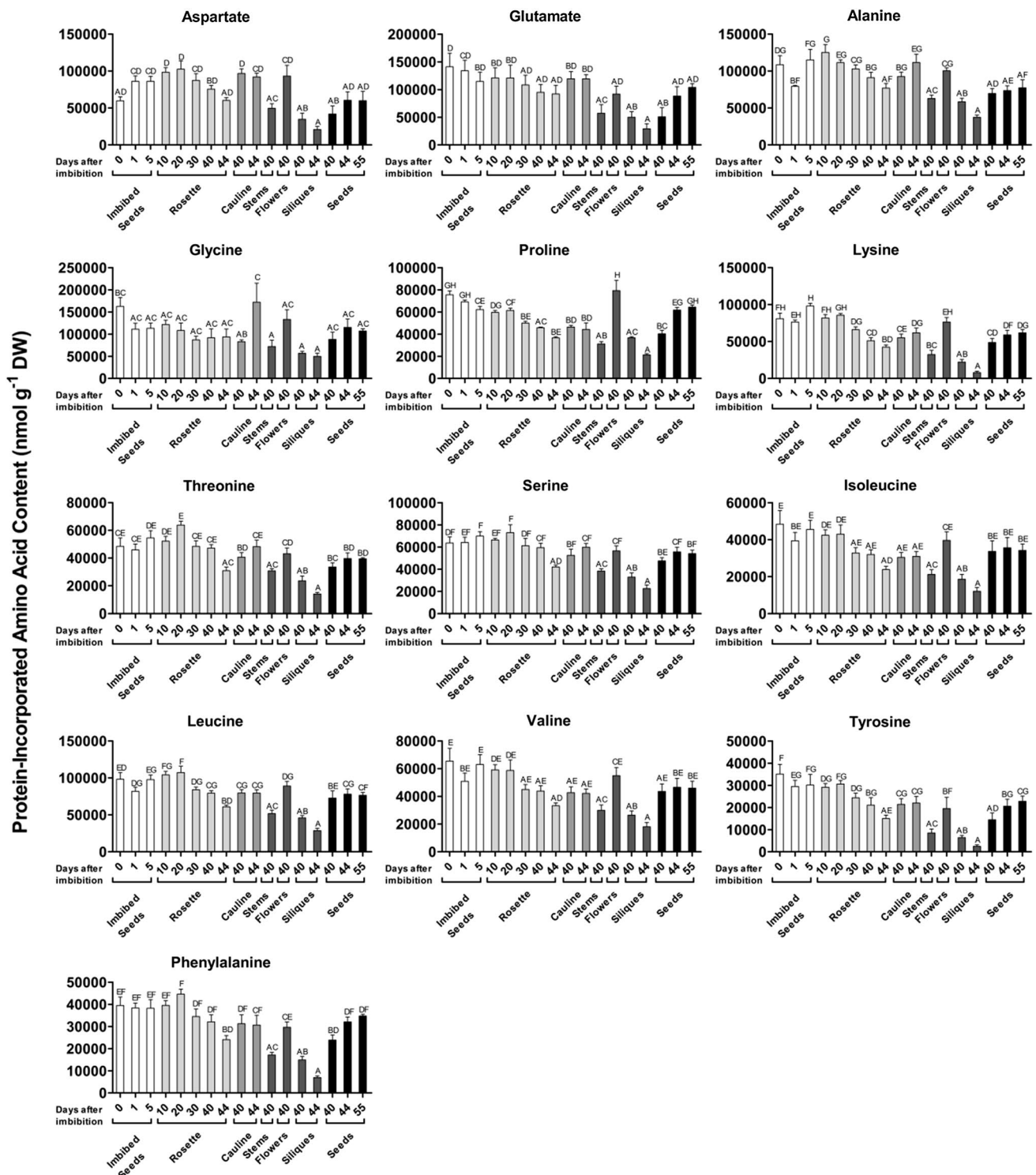


Fig. 6 The accumulation patterns of individual protein-incorporated amino acids in various tissues during the life cycle of Arabidopsis. Protein-incorporated amino acid contents were determined via GC–MS analysis. Data shown are mean \pm SE of five independent biological replicates

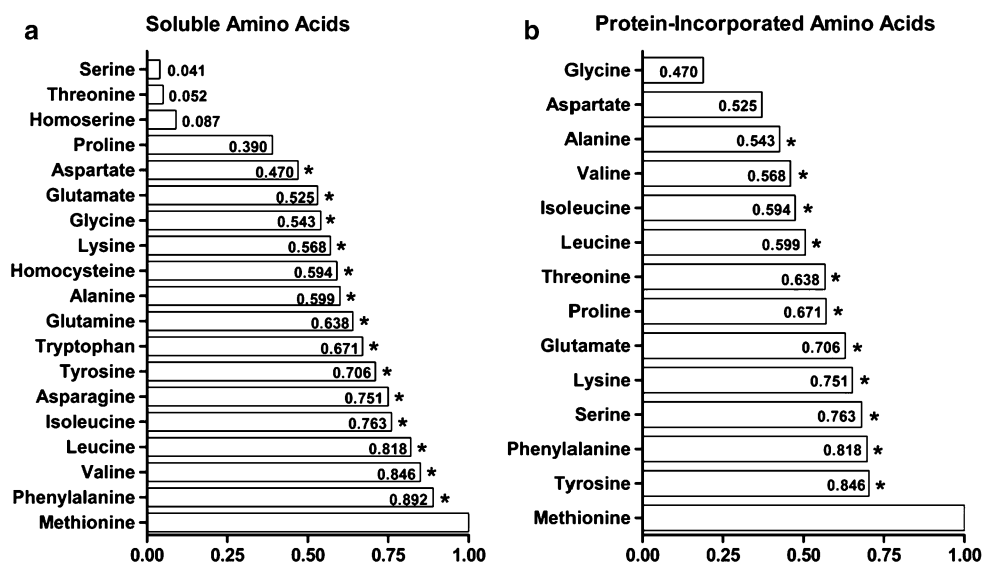


Fig. 7 Correlation analyses between amino acids and methionine. **a** Soluble amino acids; **b** protein-incorporated amino acids. The analyses were performed according to the Spearman's rank correlation

method (see “Materials and methods”). Calculated correlation values appear within bars; asterisks represent statistical significance of P value < 0.001

Discussion

Accumulation patterns of methionine and SMM during the Arabidopsis life cycle

The current study supports the assumption that in addition to the de novo synthesis of methionine in seeds (Cohen et al. 2014; Hanafy et al. 2013; Matityahu et al. 2013; Song et al. 2013), methionine is also transferred from vegetative tissues in the form of SMM and contributes to methionine accumulation in seeds. The assumption is based on several observations. (1) The levels of soluble and protein-incorporated methionine decreased in the rosette leaves at 40 and 44 DAI when the reproductive tissues of flowers and developing seeds were developed, whereas the contents of methionine increased significantly by 10- and 21-fold, respectively, in these organs compared to the rosette leaves (Fig. 2a, b). (2) The level of SMM is significantly higher in the rosette leaves (from 30 DAI up to 44 DAI) and stems and flowers, indicating that SMM is formed massively in the rosette leaves at these stages and then transferred into the reproductive organs through the stems (Fig. 2c). (3) The lower levels of SMM in the siliques and developing seeds compared to the levels detected in flowers (about 2.75-fold lower level; Fig. 2c) imply that SMM is converted back to soluble methionine in these tissues and is available for the synthesis of the seed storage proteins. Accordingly, mature dry seeds (55 DAI) exhibited the lowest SMM contents during the Arabidopsis life cycle (Fig. 2c). (4) The fact that

SMM showed inverted accumulation patterns than those of methionine strengthens the notion that methionine synthesized in the leaves is converted to SMM mainly during the senescence of these leaves. (5) The expression levels of *MMT* are slightly, but significantly higher in leaves at 40 and 44 DAI, indicating that the production of SMM is elevated at these developmental stages. This observation, together with the reduction in the soluble and total methionine contents at these stages, strengthens the assumption that SMM is formed in the mature rosette leaves before being transferred into the developing reproductive tissues and that *MMT* plays a prominent role in converting methionine to SMM. (6) The transcript pattern of *HMT1*, which is similar to that of soluble methionine and the higher expression level of *HMT3* in the late stages of seed development, supports the assumption that SMM is converted to methionine in the developing seeds through the activity of these isoforms. (7) The lower expression level of *CGS* in the reproductive tissues compared to the rosette leaves and the higher levels of methionine found in the reproductive tissues suggest that methionine is transferred to these tissues and synthesized less in situ. (8) The expression levels of the three isoforms of *HMT* are significantly increased at the last stage of seed development, suggesting that re-conversion of SMM back to soluble methionine occurs at these stages. Higher levels of *HMT* were also observed in the last stages of seed development of *Medicago truncatula*, suggesting that it contributes to methionine accumulation at this stage of development (Gallardo et al. 2007).

Our observations stand in agreement with previous studies (Bourgis et al. 1999; Kocsis et al. 2003; Lee et al. 2008; Ranocha et al. 2001), suggesting that the SMM cycle is an important contributor to methionine synthesis in seeds, even though the tissues contributing to SMM production have not yet been discovered. Due to the lower levels of soluble and protein-incorporated methionine at 40 and 44 DAI and the higher levels of methionine in the reproductive tissues, we assume that the rosette leaves play an important role in transporting SMM toward the seeds, as suggested previously by Bourgis et al. (1999). However, since relatively high levels of SMM were also detected in the flowers and stems, we cannot exclude the possibility that these tissues are also significant contributors to SMM production. This proposal was raised previously by Lee et al. (2008), suggesting that SMM was produced mainly at the top of the flower stalk (cauline leaves, stems, flowers, silique hulls and seeds) and less in the rosette leaves. However, the higher SMM contents in the flowers and stems might also result from SMM transfer through these tissues rather than de novo synthesis of SMM. Thus, the precise functional role and contribution of the SMM cycle to methionine synthesis in seeds requires additional studies (Kocsis et al. 2003; Hacham et al. 2008).

Roles of the SMM cycle

Our gene expression analyses indicated that the enzymes constituting the SMM cycle (*HMTs* and *MMT*) were expressed in all of the tissues examined. These findings are in agreement with a previous study, which showed that Arabidopsis leaves, roots and developing seeds can metabolize ^{35}S -methionine to ^{35}S -SMM and vice versa (Ranocha et al. 2001). Principally, the tandem action of *MMT* and *HMTs*, together with those of *SAMS* and *S-ADENOSYL-HOMOCYSTEINE HYDROLASE* (*ADOHCY HYDROLASE*), set up a futile cycle in which methionine is converted to SMM and vice versa (Mudd and Datko 1990). In each turn of the cycle, a molecule of ATP is hydrolyzed into adenosine, pyrophosphate and phosphate (Mudd and Datko 1990). Thus, it might be that methionine formed from SMM will be re-converted back to SMM by the activity of *MMT* in these tissues, leading to a loss of energy. The observation that *MMT* and *HMTs* are detected in these tissues suggests that the contribution of the SMM cycle to methionine synthesis in seeds is only one of this cycle's roles in the plant's metabolism. Indeed, several previous studies have proposed other roles for the SMM cycle. Among them are a short-term control of SAM levels (Ranocha et al. 2001), stabilization of the free methionine pool (Mudd and Datko 1990), donation of methyl groups to other plant metabolites by SMM (Giovanelli 1987) and

repair of SAM suffering from chemical damage reactions under physiological conditions (Bradbury et al. 2014).

The observation that a higher expression level of *HMT3* was found in the rosette leaves 40 and 44 DAI simultaneously with an elevated expression of *MMT* suggests other roles of this cycle at this stage. Also, the expression levels of *HMT2* that remain relatively stable through all of the developmental stages imply that this isoform may have additional yet unknown roles other than the contribution to methionine synthesis in seeds.

However, most probably other factors are involved in the control of this cycle in addition to the expression levels of the genes encoding to enzymes within the cycle. One of these factors could be the availability and the contents of SAM, the second substrate for *MMT*. An indication of this possibility comes from the observation that the expression level of *SAMS3* was significantly reduced in the late stages of seed development. This strongly implies that the level of SAM decreased at this stage and probably also decreased the activity of *MMT* at these stages in seeds. Low expression levels of this gene were also detected in the endosperm and seed coat of *M. truncatula* seeds during seed filling (Gallardo et al. 2007), suggesting that the SMM pool within seeds is derived mainly from the phloem rather than inter-production in seeds. Additional studies are required to reveal the different roles of this cycle in metabolism and its important functions in plants.

Accumulation patterns of other amino acids during plant development

Analyses of the soluble and protein-incorporated amino acids gave us the opportunity to define whether methionine has unique accumulation patterns during the development, or whether its patterns are like most other amino acids. Our correlation analyses revealed that patterns of soluble methionine were similar to those exhibited by most other soluble amino acids, with the exception of threonine and serine. These two amino acids are associated with the metabolism of methionine via the aspartate family pathway (Fig. 1). Threonine synthesis competes with that of methionine (Ravanel et al. 1998; Curien et al. 2003), and serine is the donor for cysteine synthesis, which is the thiol precursor required for methionine synthesis (Nikiforova et al. 2005). Their accumulation pattern suggests that these amino acids have their own patterns that are not related to methionine and to the other amino acids.

Notably, the two groups of amino acids showing the highest correlation values to soluble methionine are the branched-chain and aromatic amino acids, which are synthesized within the chloroplast together with methionine [reviewed by (Maeda and Dudareva 2012)]. A recent study had also showed high correlations between methionine and

the levels of these amino acids in transgenic *Arabidopsis* seeds expressing a feedback-insensitive form of AtCGS (Cohen et al. 2014). The branched-chain amino acids are synthesized primarily through threonine, but it was demonstrated previously that they could also be synthesized from methionine through the activity of METHIONINE γ -LYASE (MGL), particularly during times of stress (Joshi and Jander 2009). Since, in the current study, the samples were taken from non-stressed plants, it would be interesting in the future to determine if a metabolic link exists between their accumulation patterns to that of methionine, as well as with the aromatic amino acids.

Previous genetic studies showed a relationship between methionine metabolism and other soluble amino acids. *Arabidopsis thaliana* antisense lines with repressed CGS activity exhibited an overall increase in their total soluble amino acids content, although little change in the content of soluble methionine and SMM were reported (Kim et al. 2002; Kim and Leustek 2000). Contrary to these results, several recent studies strongly imply a positive correlation between AtCGS and the accumulation of methionine and other amino acids since transgenic soybean, tobacco and *Arabidopsis* expressing the AtCGS showed significant elevations in soluble methionine and significantly higher contents of other soluble amino acids (Matityahu et al. 2013; Song et al. 2013; Hacham et al. 2008; Cohen et al. 2014). In addition, *Arabidopsis* plants that suppress the expression of SAMS exhibited much higher levels of total soluble amino acids, and methionine, arginine, proline and lysine were the most increased amino acids (Kim et al. 2002). These results suggest that changes in the expression of genes that are associated with methionine metabolism might affect the levels of other amino acids. Generally, little is known about the global regulation of amino acids in plants, and it appears that the disruption of a single amino acid such as methionine can lead to pleiotropic effects on the homeostasis of all amino acids (Guyer et al. 1995). Hence, additional studies are required to determine if the accumulation patterns of soluble amino acids during plant development are related to methionine or one of the other amino acids. Alternatively, it is possible that other transcriptional and/or developmental factors play prominent regulatory roles in determining the levels of amino acids, since most exhibited similar accumulation patterns during the *Arabidopsis* life cycle. Still, a master regulatory factor involved in these processes, such as the yeast GENERAL CONTROL PROTEIN 4 (GCN4), which is responsible for the activation of more than 30 genes required for amino acids biosynthesis, has not yet been detected in plants (Guyer et al. 1995), thereby addressing the necessity for additional research to define the factors regulating the levels of amino acids during plant development.

The accumulation patterns of protein-incorporated methionine in the various tissues are also significantly correlated with other protein-incorporated amino acids. The only amino acids showing low correlation values with methionine are glycine and aspartate. In general, an examination of the patterns of protein-incorporated amino acids indicated that most of the amino acids produced in the rosette leaves were incorporated into the vegetative storage proteins and other proteins from 5 to 30 DAI. However, at later stages from 40 DAI when the reproductive tissues start their development, the vegetative proteins are degraded to release amino acids for the synthesis of these tissues. These findings are in agreement with other studies suggesting that vegetative protein degradation is the major process responsible for the dramatic increases in the remobilization of nitrogen into the sink organs during leaf senescence (Soudry et al. 2005). It was previously demonstrated that these senescence-associated processes are accompanied by a transition from anabolic to catabolic processes (Soudry et al. 2005), and that the SENESCENCE-ASSOCIATED GENES (SAGs) are the key proteins involved in the regulation of these processes (Gepstein et al. 2003). SAGs encode hydrolytic enzymes responsible for the intensive breakdown of the various macromolecules such as proteins, nucleic acids, polysaccharides and lipids (Gepstein et al. 2003), as well as for nitrogen recycling, which is the most recycled nutrient during senescence (Tegeeder and Rentsch 2010). It was shown that in the leaves of *Arabidopsis* at these late stages, glutamate, glutamine, aspartate and asparagine constitute 60–64 % of the total free amino acids that are transported in the vascular tissues, and that glutamine and asparagine are among the most modified amino acids being transported (Lam et al. 1996). Based on the results of our study, we suggest that methionine is also subjected to such modifications, being converted to SMM before being translocated into the reproductive tissues. Since methionine is a hydrophobic amino acid and SMM is a polar compound, we assume that this modification enables methionine to be transported more effectively in the phloem flux. The transportation of amino acids from leaves to seeds is prominent not only for the synthesis of seed storage proteins, but also for many other metabolic processes. Amino acids play critical metabolic roles due to their conversion to many other essential metabolites (e.g., Tzin and Galili 2010). Some of these metabolites are being used by the tricarboxylic acid (TCA) cycle as an energy source and/or as the precursors for the synthesis of many secondary metabolites (e.g., phenylpropanoids, alkaloids, glucosinolates), act as signaling molecules, (e.g., hormones), structural components (e.g., lignin) and defense substances (e.g., glucosinolates, anthocyanins, nicotine) (Maeda and Dudareva 2012; Tzin and Galili 2010). Therefore, the transportation of amino acids from leaves to seeds might play additional important roles in seed biology.

All in all, our findings strengthen the hypothesis that SMM is an important contributor to methionine synthesis in seeds and that it is produced mainly in the rosette leaves. Since *HMT3* has a higher expression level in seeds and may be the key player in the conversion of SMM to methionine in the seeds, we assume that its overexpression in a seed-specific manner might enhance the conversion of SMM in seeds and lead to higher flux of SMM toward the seeds, resulting in higher levels of methionine in the transgenic seeds. We also found that *MMT* is expressed in seeds; thus we assume that even greater amounts of methionine might accumulate in the transgenic seeds if the *HMT3* enzyme is expressed in the background of a seed-specific RNAi::*MMT* transgenic line. Future research using Arabidopsis and crop plants will determine which of these approaches is the most feasible in an effort to increase methionine contents in seeds. This is an important biotechnology goal, since the low contents of methionine limit the nutritional quality of seeds and additional approaches should be tested to increase its levels (Amir et al. 2012; Galili and Amir 2013).

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Conflict of interest The authors declare that they have no conflicts of interest.

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