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Rapid Paper

Arabidopsis Bile Acid:Sodium Symporter Family Protein 5 is Involved in Methionine-Derived Glucosinolate Biosynthesis

Yuji Sawada[1,2], Kiminori Toyooka[1], Ayuko Kuwahara[1,2], Akane Sakata[1], Mutsumi Nagano[1,2], Kazuki Saito[1,3] and Masami Yokota Hirai[1,2,*]

[1]RIKEN Plant Science Center, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa, 230-0045 Japan
[2]JST, CREST, 4-1-8 Hon-chou, Kawaguchi, Saitama, 332-0012 Japan
[3]Graduate School of Pharmaceutical Sciences, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba, Chiba, 263-8522 Japan

Glucosinolates (GSLs) are a group of plant secondary metabolites that have repellent activity against herbivore insects and pathogens, and anti-carcinogenic activity in humans. They are produced in plants of the Brassicaceae and other related families. Biosynthesis of GSLs from precursor amino acids takes place in two subcellular compartments; amino acid biosynthesis and side chain elongation occur mainly in the chloroplast, whereas the following core structure synthesis takes place in the cytosol. Although the genes encoding biosynthetic enzymes of GSLs are well known in *Arabidopsis thaliana*, the transporter genes responsible for translocation of biosynthetic intermediates between the chloroplast and cytosol are as yet unidentified. In this study, we identified the *bile acid:sodium symporter family protein 5* (*BASS5*) gene in Arabidopsis as a candidate transporter gene involved in methionine-derived GSL (Met-GSL) biosynthesis by means of transcriptome co-expression analysis. Knocking out *BASS5* resulted in a decrease of Met-GSLs and concomitant increase of methionine. A transient assay using fluorescence fusion proteins indicated a chloroplastic localization of BASS5. These results supported the idea that BASS5 plays a role in translocation across the chloroplast membranes of the biosynthetic intermediates of Met-GSLs.

Keywords: *Arabidopsis thaliana* • Bile acid:sodium symporter family protein • Co-expression • Glucosinolate • Methionine chain elongation • Widely targeted metabolomics.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid; BASS, bile acid:sodium symporter family protein; BCAT, branched-chain aminotransferase; cTP, chloroplast transit peptide; GSL, glucosinolate; MAAT, methionine analog aminotransferase; MAM, methylthioalkylmalate synthase; MAM-D, methylthioalkylmalate dehydrogenase; MAM-IL, a large subunit of methylthioalkylmalate isomerase; MAM-IS, a small subunit of methylthioalkylmalate isomerase; Met-GSL, Met-derived glucosinolate; MS, mass spectrometry; MTA, 5′-*S*-methyl-5′-thioadenosine; RT–PCR, reverse transcription–PCR; SAM, *S*-adenosylmethionine; SMM, *S*-methylmethionine; UPLC, ultra performance liquid chromatography; YFP, yellow fluorescent protein.

Introduction

Glucosinolates (GSLs) are sulfur- and nitrogen-containing plant secondary metabolites derived from amino acids and sugars (**Fig. 1**). At least 120 GSLs with different side chains have been identified in plants of the Brassicaceae and other related families of the order Capparales (Fahey et al. 2001). GSLs are hydrolyzed into a variety of bioactive products including isothiocyanates (ITCs), which play roles in the defense against generalist herbivores and pathogens. From the human view point, sulforaphane (4-methylsulfinylbutyl-ITC) and its analogs, which are found in Brassicaceae crops, have been shown to prevent the formation of mammary tumors in animal models (Zhang et al. 1994). For successful

*Corresponding author: E-mail, myhirai@psc.riken.jp; Fax, +81-45-503-9489.

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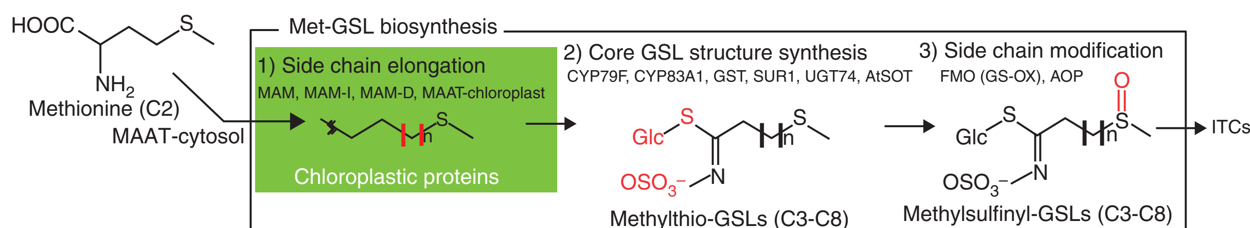

Fig. 1 Biosynthetic pathway of Met-GSLs in Arabidopsis. The three biosynthetic steps of Met-GSLs from methionine are shown. The genes encoding biosynthetic enzymes are shown; MAM, methylthioalkylmalate synthase; MAM-I, methylthioalkylmalate isomerase; MAM-D, methylthioalkylmalate dehydrogenase; MAAT, methionine analog aminotransferase; CYP, cytochrome P450; GST, glutathione *S*-transferase; SUR1, super root1, UGT, glycosyltransferase; AtSOT, sulfotransferase; FMO(GS-OX), flavin monooxygenase (glucosinolate oxygenase). A green box indicates the chloroplastic localization of MAM, MAM-I, MAM-D and MAAT-chloroplast.

biotechnological engineering of Brassicaceae crops from agronomical and nutraceutical aspects, it is important that we improve our understanding of GSL metabolism.

Most of the genes involved in GSL biosynthesis (**Fig. 1**) have been identified in *Arabidopsis thaliana*. Owing to massive accumulation of microarray data for this plant, transcriptome co-expression analysis has recently accelerated functional elucidation of Arabidopsis genes (Aoki et al. 2007, Saito et al. 2008). Novel genes responsible for GSL biosynthesis were successfully identified based on or with the help of co-expression relationships with previously identified GSL biosynthesis genes (Hirai et al. 2005, Schuster et al. 2006, Hirai et al. 2007, Sønderby et al. 2007, Sawada et al. 2009b). Nevertheless, genes responsible for transport of GSL-related metabolites have not yet been identified.

Several types of transporters have been suggested to be involved in GSL biosynthesis and accumulation. Amino acids and their analogs with elongated side chains are supposed to be synthesized in the chloroplasts and transported to the cytosol where the core structure of GSLs is synthesized. GSLs are then transported into the vacuoles for storage or exported to the phloem for long-distance transport to accumulating organs (Chen and Halkier 2000, Grubb and Abel 2006, Textor et al. 2007, Nour-Eldin and Halkier 2009). Although indirect evidence for transport has been reported, the actual transporter gene is as yet unidentified (Grubb and Abel 2006).

In this study, we focused on identification of transporter(s) involved in the process of methionine side chain elongation. In Arabidopsis, methionine is first deaminated by cytosolic branched-chain aminotransferase 4 [AtBCAT4, also referred to as methionine analog aminotransferase (MAAT)-cytosol in this paper] protein to form α-keto acid with the C2 chain [2-keto-4-methylthiobutyrate (KMTB)] (Schuster et al. 2006) (**Fig. 1**). KMTB is subjected to chain elongation cycles catalyzed by chloroplastic methylthioalkylmalate synthase (MAM), methylthioalkylmalate isomerase (MAM-I) and methylthioalkylmalate dehydrogenase (MAM-D). In the case of Arabidopsis accession Columbia, these enzymes are encoded by *MAM1* (At5g23010) and *MAM3* (At5g23020), *MAM-IL1* (MAM-I large subunit; At4g13430) and *MAM-D1* (At5g14200), respectively (Kroymann et al. 2001, Field et al. 2004, Sawada et al. 2009b). α-Keto acids with an elongated chain (C3–C8) are aminated by chloroplastic AtBCAT3 (also referred to as MAAT-chloroplast in this paper) protein and presumably additional aminotransferase(s) (Knill et al. 2008), and then transported back to the cytosol for the following core structure synthesis. Thus, the methionine derivatives must be transported at least twice across the chloroplast membranes. This idea made us interested in identifying candidate genes for chloroplast-localized transporters involved in methionine chain elongation. Here, based on the transcriptome co-expression analysis with known Met-GSL biosynthesis genes, we predicted that the novel gene *BASS5* (At4g12030) acts as a transporter of methionine derivative(s) across chloroplast membranes. Predicted function was verified by means of omics, i.e. transcriptomics and widely targeted metabolomics that we have recently established (Sawada et al. 2009a). This is one of the first papers, along with a recently published paper by Gigolashvili et al. (2009), that reports the transporter gene involved in GSL biosynthesis.

Results

Identification of a candidate transporter gene involved in methionine chain elongation based on co-expression analysis

To identify candidate genes encoding chloroplast-localized transporters involved in methionine chain elongation, we carried out co-expression analysis by ATTED-II (Obayashi et al. 2009) using the known methionine chain elongation genes as queries. We found that a transporter gene (At4g12030), which is annotated as bile acid:sodium symporter family protein (BASS), was highly co-expressed with the queries. This gene, hereinafter called *BASS5*, was also

highly co-expressed with other genes involved in Met-GSL biosynthesis (**Fig. 2A**). The developmental expression pattern of this gene was quite similar to those of Met-GSL biosynthesis genes (**Fig. 2B**). In the Arabidopsis genome, there are six homologous genes belonging to the BASS family (**Supplementary Fig. S1**). The ChloroP algorithm (Emanuelsson et al. 1999) predicted the presence of chloroplast transit peptides (cTPs) in the N-terminus of these six genes. However, the developmental expression patterns were different from each other (**Supplementary Fig. S1**). No gene other than *BASS5* was co-expressed with known Met-GSL biosynthesis genes (data not shown). Based on these results, we considered that *BASS5* is solely involved in Met-GSL biosynthesis among six *BASS* members.

Knockout of BASS5 resulted in a decrease of Met-GSL accumulation

The predicted function of *BASS5* was confirmed by analyzing the contents of GSLs and amino acids in the leaves of two independent T-DNA insertion lines of *BASS5* (SALK_041259/*bass5-1* and SALK_126525/*bass5-2*), in which the expression of *BASS5* was repressed (**Fig. 3A, B**). These plants did not show apparent morphological changes (data not shown). The total content of Met-GSLs was significantly decreased compared with the wild type, whereas that of tryptophan-derived indole GSLs did not change (**Fig. 3C**). On the other hand, the content of methionine was significantly increased, which was negatively correlated with that of Met-GSLs (**Fig. 3D**). The contents of other amino acids did not change significantly (**Supplementary Table S1**). These results strongly supported the hypothesis that *BASS5* is involved in Met-GSL biosynthesis.

Subcellular localization of BASS5

To confirm the chloroplastic targeting of BASS5, a fusion construct of predicted BASS5 cTP with the yellow fluorescent protein (YFP) gene was expressed in Arabidopsis cultured cells (**Fig. 4**). Chloroplasts could be identified by the red autoflorescence emitted by their chlorophylls (**Fig. 4B**). The YFP fluorescence (**Fig. 4A**) was mostly associated with the chloroplasts (**Fig. 4C**), indicating that BASS5 is a chloroplast-localized protein.

Widely targeted metabolomics and transcriptomics of a BASS5 knockout line

We elucidated the effects of knocking out *BASS5* on the metabolite and transcript profiles by means of widely targeted metabolome analysis (Sawada et al. 2009a) and microarray analysis using *bass5-2*, which showed a stronger phenotype in Met-GSL and methionine accumulation (**Fig. 3C, D**). **Table 1** shows the metabolites which were increased, more than double, or decreased, less than half, in *bass5-2*.

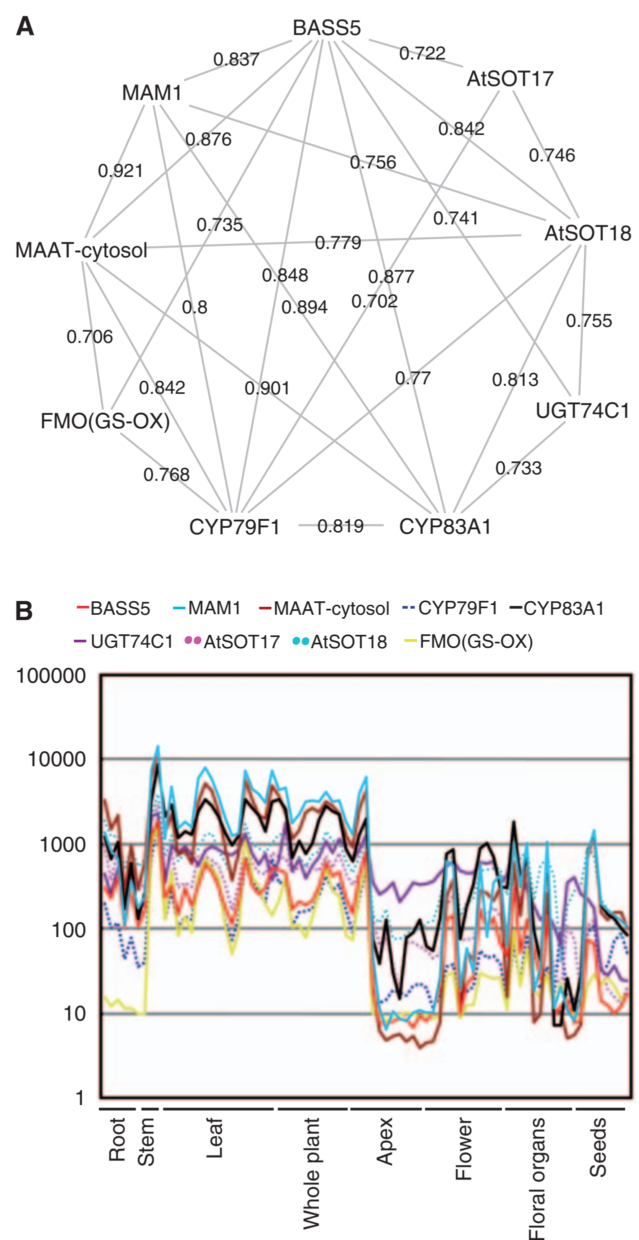

Fig. 2 Expression pattern of Met-GSL biosynthesis genes. (A) The co-expression relationship of *BASS5* with Met-GSL biosynthesis genes. Pearson's correlation coefficients between gene pairs are shown on the edges. Only the genes connected directly to *BASS5* were shown in the network (see Materials and Methods). (B) Developmental expression pattern of the genes shown in (A). The figure was illustrated by AtGenExpress Visualization Tool (http://jsp.weigelworld.org/expviz/expviz.jsp).

Methionine and methionine-derived metabolites, namely methionine sulfoxide, 1-aminocyclopropane-1-carboxylic acid (ACC), *S*-methylmethionine (SMM), *S*-adenosylmethionine (SAM) and 5′-*S*-methyl-5′-thioadenosine (MTA), were increased, whereas four Met-GSLs were decreased in *bass5-2* compared with the wild type. The result indicated that

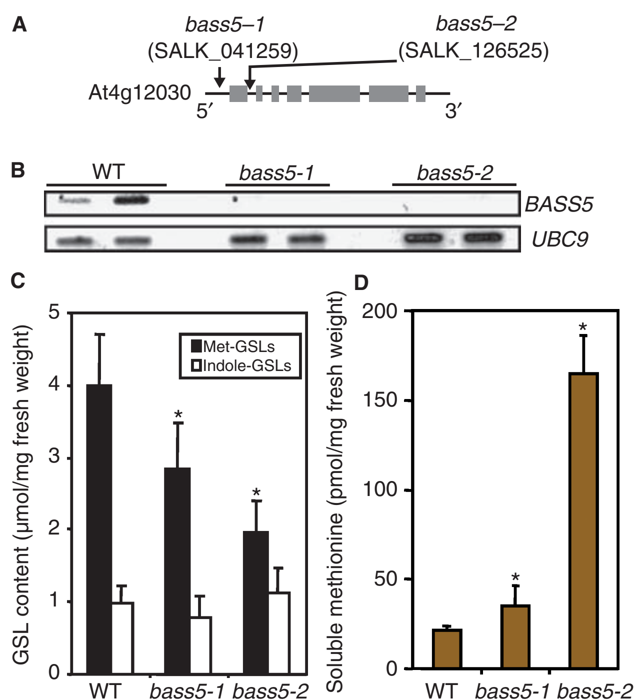

Fig. 3 Effects of *BASS5* knockout on the metabolite accumulation pattern. (A) Diagrams of the *BASS5* gene with exons shown as gray boxes. The arrows indicate the T-DNA insertion sites. (B) Expression levels of *BASS5* examined by RT–PCR using duplicate samples. *UBC9* was used as the control. (C, D) The contents of GSLs (C) and methionine (D) in the leaves of *bass5*. The mean and standard deviation ($n = 6$) are shown. Asterisks indicate a statistically significant change compared with wild-type (WT) plants by Welch's *t*-test ($P < 0.05$).

knocking out *BASS5* affected specifically methionine metabolism and Met-GSL biosynthesis. KMTB, a putative substrate of BASS5, is occasionally detected in Arabidopsis leaves (Sawada et al. 2009b). Under the experimental conditions in this study, however, KMTB was not detected in the leaves of the wild type and *bass5-2* (data not shown).

The genes whose expression changed remarkably in *bass5-2* are listed in **Supplementary Table S2**. Although methionine metabolism was perturbed in *bass5-2* (**Table 1**), expression of the genes that are apparently involved in methionine metabolism (such as enzymes of methionine and methionine-derived metabolite biosynthesis) did not change in *bass5-2*. The expression of known Met-GSL biosynthesis genes also did not change in *bass5-2*.

Discussion

Mammalian bile acid transporters expressed in the liver and the intestine play a critical role in driving the enterohepatic circulation of bile acids (Alrefai and Gill 2007). Although bile acids have not been found in plants to date, ATP-dependent bile acid transport into plant vacuoles has been reported (Hortensteiner et al. 1993). A BASS gene *OsSbf1* was isolated in deep-water rice (*Oryza sativa* L.) as an ethylene-induced gene, and the BASS genes were shown to exist in monocot and dicot plant species (Rzewuski and Sauter 2002). However, the actual function of BASS in plants remains unclear.

In this study, we successfully predicted the function of Arabidopsis *BASS5*, based on the co-expression patterns of this gene with other genes of known function. The specific decrease of Met-GSLs in the leaves of *BASS5* knockout lines (*bass5-1* and *bass5-2*) clearly showed that *BASS5* plays a role in Met-GSL biosynthesis (**Fig. 3C**). A coordinate increase of methionine and methionine-derived metabolites (**Table 1**) also supported the idea that the BASS5 functions in the biosynthetic process from methionine to Met-GSLs. The transcriptomic data of *bass5-2*, in which no remarkable change was observed in the expression of methionine and methionine-derived metabolite biosynthesis genes (**Supplementary Table S2**), suggested that the remarkable change in metabolite accumulation was not due to changes in gene expression pattern as a consequence of *BASS5* being knocked out. In the course of Met-GSL biosynthesis, methionine is first deaminated by AtBCAT4 (MAAT-cytosol) to form KMTB (a keto acid with a C2 chain) in the cytosol (Schuster et al. 2006). KMTB undergoes chain elongation in the chloroplasts to form elongated keto acids with C3–C8 chains (Grubb and Abel 2006). Elongated keto acids with C3 and C4 chains are aminated by chroloplastic AtBCAT3 (MAAT-chloroplast), while others presumably are aminated by other aminotransferase(s) (Knill et al. 2008). Aminated keto acids, namely methionine analogs with elongated side chains, are subjected to GSL core synthesis in the cytosol. Considering the above-mentioned compartmentation of the GSL biosynthetic pathway, the chloroplastic localization of BASS5 (**Fig. 4**) suggested that BASS5 may be responsible for influx of KMTB into the chloroplast and/or efflux of methionine analogs from the chloroplast (**Fig. 5**).

In our previous study (Sawada et al. 2009b), we analyzed the change in metabolite accumulation caused by knocking out *MAM-IL1*, one of the methionine chain elongation genes (**Figs. 1, 5**). A significant increase in methionine and methionine-derived metabolites (methionine sulfoxide, SMM, MTA and SAM) and a significant decrease in Met-GSLs were observed. Given that AtBCAT4 (MAAT-cytosol) is known to catalyze the reverse reaction, amination of KMTB to form methionine, this result suggested that the blocking of methionine chain elongation leads to redirection of metabolic flow to primary methionine metabolism (Sawada et al. 2009b). The loss-of-function phenotype for *BASS5* was similar to that of *MAM-IL1*. Because we did not analyze the contents of elongated keto acids and elongated methionine analogs, it remains to be clarified whether BASS5 is responsible for influx of KMTB or efflux of methionine analogs.

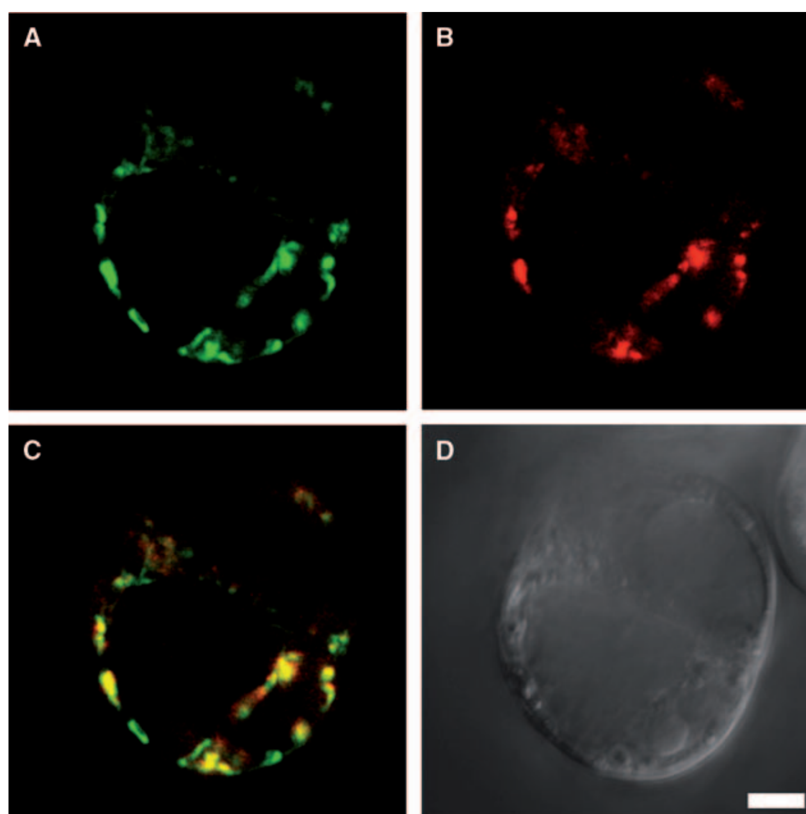

Fig. 4 Micrographs showing the chloroplast localization of a cTP_BASS5–YFP fusion protein in Arabidopsis cells. (A) The YFP signals of a cTP_BASS5–YFP fusion, (B) the autofluorescence of chlorophylls, (C) an overlay of cTP_BASS5–YFP and autofluorescence signals and (D) a differential interference contrast image. Bar = 10 µm.

Table 1 Metabolite levels changed in the leaves of *bass5-2*

Compound name	Fold change	*P*-value	Q-value
Methionine sulfoxide	9.9	0.001	0.05
Methionine	7.5	0.004	0.09
ACC	4.6	0.003	0.08
SMM	3.4	0.003	0.08
SAM	3.3	0.004	0.08
MTA	2.9	0.001	0.06
6-Methylsulfinyl-*n*-hexyl-glucosinolate	0.4	0.0004	0.04
8-Methylsulfinyl-*n*-octyl-glucosinolate	0.3	0.00005	0.01
3-Methylsulfinyl-*n*-propyl-glucosinolate	0.3	0.002	0.08
7-Methylthio-*n*-heptyl-glucosinolate	0.2	0.003	0.08

Metabolite levels were analyzed in 4–6 replicates. In total, 216 metabolites were detected by means of widely targeted metabolomics (see **Supplementary Table S1**). Fold change of the average metabolite content in the knockout line compared with that in the wild type was calculated. Metabolites that differentially accumulated in bass5-2 compared with the wild type were identified based on the following criteria: fold change >2 or <0.5, and Q-value (Storey and Tibshirani 2003) of Welch's *t*-test < 0.1.

Concerning the transport of GSLs across membranes, limited data are available such as uptake of GSLs into Brassica leaf protoplasts mediated by a proton-coupled symporter (Grubb and Abel 2006, Halkier and Gershenzon 2006). Because the mechanism of this uptake across the plasma membranes is different from that of sodium-dependent BASS (Nour-Eldin and Halkier 2009), *BASS5* does not seem to be responsible for GSL uptake across the plasma membranes involved in long-distance transport of Met-GSLs, e.g. from leaves (GSL-producing organs) to other sink organs.

This study indicated that co-expression analysis is useful to identify candidate transporters involved in secondary metabolism. Although the mechanism of transport and substrate(s) of BASS5 remain to be clarified, further metabolome analysis could narrow down the candidate substrates.

Materials and Methods

Plant growth conditions

Wild-type *A. thaliana* (accession Colombia-0) and T-DNA insertion lines were grown in a pre-fabricated room-type chamber at 22°C and a 16 h photoperiod on agar-solidified

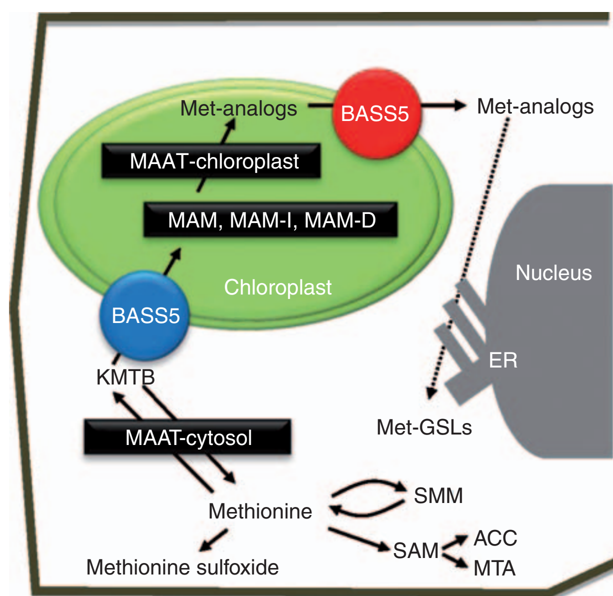

Fig. 5 A model of Met-GSL biosynthesis associated with a predicted BASS5 function. Met-GSL biosynthetic enzymes are boxed. The dashed arrow indicates multiple biosynthetic reactions. The BASS5 is presumably responsible for the influx of KMTB (shown as a blue circle) or the efflux of methionine analogs (shown as a red circle) on the chloroplast membranes.

1/2 MS (Murashige–Skoog) medium containing 1% sucrose for transcriptome and metabolome analyses.

Co-expression analysis

The co-expression relationship was analyzed by using Correlated Gene Search in PRIMe (Platform for RIKEN Metabolomics, http://prime.psc.riken.jp/) (Akiyama et al. 2008) using the following 22 genes as queries: *Myb28*, *Myb29*, *Myb76*, *AtBCAT4*, *AtBCAT3*, *MAM1*, *MAM3*, *MAM-IL1*, *MAM-D1*, *CYP79F1*, *CYP79F2*, *CYP83A1*, *SUR1*, *UGT74B1*, *UGT74C1*, *AtSOT17*, *AtSOT18*, *FMO(GS-OX1)*, *AOP1*, *AOP2*, *AOP3* and *BASS5*. Parameter setting was as follows: Matrix, all data sets v.3 (1,388 data of AtGenExpress); Method, interconnection of sets. The correlation data used in PRIMe have been released by ATTED-II (Obayashi et al. 2009). A co-expression network comprised of *BASS5* and the genes directly connected to this gene was illustrated using Cytoscape (Cline et al. 2007) (**Fig. 2A**).

Genotyping of T-DNA insertion mutants

Extraction of genomic DNA and PCRs were carried out with Ampdirect Plus (Shimazu) using T-DNA insertion lines of *BASS5* (SALK_041259/*bass5-1* and SALK_126525/*bass5-2*). T-DNA insertion sites were confirmed by nucleotide sequencing.

Reverse transcription–PCR (RT–PCR) analysis

RT–PCR was performed with cDNAs synthesized from total RNAs of wild-type and T-DNA insertion lines using the SuperScript III First-Strand Synthesis System (Invitrogen). The primer sequences were 5′-CCATGGGCTGACACAAATACT-3′ and 3′-CCAAATAATATGAGCCTTGATAAAC-5′ for *UBC9* (At4g27960), and 5′-CACTGGTTTCTTCTTCAGCAAGGCACC-3′ and 3′-GCCGACCATAAACAACAGCAAATTCCG-5′ for *BASS5* (At4g12030).

Metabolic profiling of BASS5 T-DNA insertion lines

Approximately 50–100 mg of the leaves of wild-type and T-DNA insertion lines (*bass5-1* and *bass5-2*) 3 weeks after germination were used for metabolic profiling with ultra performance liquid chromatography (UPLC)-ZQ (Waters) (for GSL and amino acid analyses) and UPLC-TQD (Waters) (for widely targeted metabolomics) as previously described (Sawada et al. 2009a, Sawada et al. 2009b). Met-GSL and tryptophan-derived indole-GSL are the two major GSL classes of Arabidopsis. The sum of the contents of Met-GSL molecular species (methylthioalkyl and methylsulfinylalkyl GSLs with C4–C8 chains) was indicated as Met-GSL content in **Fig. 3C**. Similarly, the sum of the contents of indole-GSL molecular species (indol-3-ylmethyl, 1-methoxyindol-3-ylmethyl and 4-methoxyindol-3-ylmethyl GSLs) was indicated as indole-GSL content. Analytical conditions for UPLC-TQD are released in the data repository and distribution site DROP Met at our website PRIMe (http://prime.psc.riken.jp/) (Akiyama et al. 2008). The metabolites whose accumulation levels changed remarkably (fold change >2 or <0.5, Q-value by the Welch's *t*-test <0.1) were identified (**Table 1**). Q-values were calculated with the 'qvalue' package (Storey and Tibshirani 2003) in R (www.r-project.org).

Transcriptome analysis

Total RNA was extracted with an RNeasy Plant mini kit (Qiagen). Hybridization with the ATH1 microarray (Affymetrix) was conducted as described (Hirai et al. 2007). Probe sets assigned to a single locus were selected based on TAIR7 (The Arabidopsis Information Resource; http://www.arabidopsis.org/index.jsp) and analyzed statistically. Q-values were calculated with the 'qvalue' package (Storey and Tibshirani 2003) in R (www.r-project.org). The data are provided as **Supplementary Tables S2 and S3**. The raw data are available from ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/) (accession: E-MEXP-2240).

Transient expression of a BASS5 chloroplast transient peptide–YFP fusion

By using the ChloroP 1.1 algorithm (Emanuelsson et al. 1999), the presence of a cTP comprised of 57 amino acids was predicted in the N-terminus of BASS5 (**Supplementary Fig. S1**).

The cDNA fragment coding for the N-terminal 61 amino acids (including the predicted cTP) of BASS5 was fused to the YFP reporter gene. The fusion construct was transiently expressed in Arabidopsis MM1 cultured cells, and YFP fluorescence was monitored as previously described (Okazaki et al. 2009).

Supplementary data

Supplementary data are available at PCP online.

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