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Expression of a Brassica isopropylmalate synthase gene in *Arabidopsis* perturbs both glucosinolate and amino acid metabolism

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Abstract

Isopropylmalate synthase (IPMS) is a key enzyme in the biosynthesis of the essential amino acid leucine, and thus primary metabolism. In *Arabidopsis*, the functionally similar enzyme, methythiolalkylmalate synthase (MAM), is an important enzyme in the elongation of methionine prior to glucosinolate (GSL) biosynthesis, as part of secondary metabolism. We describe the cloning of an IPMS gene from *Brassica*, BatIMS, and its functional characterisation by heterologous expression in *E. coli* and *Arabidopsis*. Over expression of BatIMS in *Arabidopsis* resulted in plants with an aberrant phenotype, reminiscent of mutants in GSL biosynthesis. Metabolite analyses showed that these plants had both perturbed amino acid metabolism and enhanced levels of GSLs. Microarray profiling showed that BatIMS over expression caused up regulation of the genes for methionine-derived GSL biosynthesis, and down regulation of genes involved in leucine catabolism, in addition to perturbed expression of genes involved in auxin and ethylene metabolism. The results illustrate the cross talk that can occur between primary and secondary metabolism within transgenic plants.

Introduction

Secondary metabolic pathways do not exist in isolation. For example, in *Arabidopsis* there is a complex network of interactions between glucosinolate (GSL) biosynthesis and primary metabolism (Hansen *et al.*, 2001; Reintanz *et al.*, 2001; Tantikanjana *et al.*, 2001; Field *et al.*, 2004; Mikkelsen *et al.*, 2004). Understanding such interactions is important both for the specific engineering of secondary metabolic pathways, and for contributing to our knowledge on the evolution and function of secondary metabolism.

In *Arabidopsis* Col-0 there are four genes which initiate the elongation of 2-oxo acids by catalysing their condensation with acetyl CoA for either leucine biosynthesis or the elongation of methionine in GSL biosynthesis (Figure 1). Two, MAML-3 (At1g74040) and MAML-4 (At1g18500), are likely to participate in leucine biosynthesis *in planta*. Both genes have high homology to known IPMS genes, and heterologously expressed MAML-3 has IPMS activity (Field *et al.*, 2004). The two remaining IPMS-like genes, MAM1 (At5g23010) and MAML (At5g23020), are tandem duplicates which encode most of the highly conserved N-terminal IPMS domain (MAM1 aa85-474; MAML-3 aa87-507) but lack a significant portion of the partially conserved C-terminal domain found in other IPMS proteins (MAML-3 aa477-631). In yeast this C-terminal, or 'R-' region is involved in both feedback inhibition by leucine and Zn^{2+}-mediated inactivation by CoA (Cavalieri *et al.*, 1999). Deletion of the R-region results in loss of activity. Nevertheless, MAM1 and MAML encode functional enzymes which are responsible for initiating the first step of aliphatic

glucosinolate (GSL) biosynthesis, the elongation of methionine (de Quiros *et al*., 2000; Kliebenstein *et al*., 2001; Field *et al*., 2004). MAM1 and MAML do not have identical functions. Genetic and biochemical evidence suggests that MAM1 initiates the elongation of only 2-oxo-4-methylthiobutanoate and 2-oxo-5-methylthiopentanoate to produce 3C and 4C GSLs (Kroymann *et al*., 2001). In contrast, MAML appears to be able to only initiate the elongation of oxo acids which have already undergone at least one round of elongation, and produces 5C, 6C, 7C and 8C GSLs (Field *et al*., 2004). However, the full contribution of the *Arabidopsis* MAM-like gene family to GSL biosynthesis and its interactions with leucine biosynthesis is not fully understood. For example, plants with non-functional MAM1 alleles (Ler, TU1 and TU5) are still able to accumulate 3C GSLs (Haughn *et al*., 1991; Kroymann *et al*., 2001). Furthermore, we have recently shown that overexpression of MAML, usually responsible for methionine elongation, results not only in increased levels of long chain GSLs but also in the formation of novel elongated leucine homologues, homo-leucine and dihomo-leucine (Field *et al*., 2004).

Polymorphisms at MAM alleles may explain a significant part of the high natural variation in methionine-derived GSL content and profile across the Brassicaceae. However, until now, no plant MAM-like genes outside of *Arabidopsis* have been characterized. Here, we describe the isolation and functional analyses of a MAM-like gene from *Brassica atlantica* (Coss.) OE Schulz (syn *Brassica oleracea* var. *insularis* subvar. *atlantica* Coss), a member of the *B. oleracea* $n=9$ species complex from Tunisia that accumulates large amounts of 2-propenyl GSL (Mithen *et al*., 1987). Heterologous expression of this gene showed it to have IPMS activity. However, overexpression in *Arabidopsis* resulted in plants with abnormal morphology, enhanced levels of methionine-derived GSLs and perturbed amino acid metabolism.

Materials and methods

Plant growth

Plants were routinely grown in *Arabidopsis* mix (2 parts Levington's M3 potting compost:1 part grit/sand) under standard glasshouse conditions at approximately 20 °C. For aseptic growth, seeds were surface-sterilized and plated on growth medium (1×MS plus vitamins (Duchefa, the Netherlands), 1% sucrose, 0.8% agar, 2.5 mM MES pH 5.7). The seeds were stratified for 2 days at 4 °C in the dark before germination in a growth room (16 h light, 8 h dark, 20 °C). For amino acid and microarray analysis segregating T1 seedlings of two independent 35S:BatIMS lines (E1 and E8) and two independent empty vector lines were aseptically grown in the presence of kanamycin (75 ug/ml). Resistant seedlings were harvested after 9 days.

RNA extraction and cDNA synthesis

Total RNA extractions and cDNA synthesis were performed as described previously (Field *et al*., 2004).

Cloning of a Brassica atlantica *MAM homologue*

Degenerate primers were designed to regions conserved across the MAM-like gene family in *Arabidopsis thaliana* (IPMS-F and IPMS-R). 3′ RACE PCR was performed on *Brassica atlantica* leaf cDNA using the following primer pairs: IPMS-F/3′ adapter and IPMS-F/IPMS-R. A MAML-4 like product was isolated. The 5′ region of the *MAML-4* like product could not be isolated, so BatIMSL1, a primer based on the 5′ regions of *MAML-3* and *MAML-4*, and BatIMSR1 were used to successfully amplify a 1880 bp *B. atlantica* MAML-4 like ORF, BatIMS, which was cloned into pGEM TEasy (Promega, Southampton, UK). Several independent clones were sequenced to ensure that no errors had been introduced. The BatIMS sequence was submitted to GenBank where it was assigned the accession number DQ143886.

E. coli *complementation experiments*

The BatIMS ORF lacking its predicted chloroplast target peptide (aa1-52) was amplified from the pGEM TEasy plasmid containing the BatIMS ORF using BatIMS/53-KpnI and BatIMS/TGA-KpnI. After amplification the product was digested with *KpnI* and ligated into the inducible expression vector pQE30 (Qiagen, Crawley, UK) digested with *KpnI* to give pQE-BatIMS. Complementation

of the ΔLeuA (IPMS-null) CV512 *E. coli* strain was then tested as described previously (Field *et al*., 2004).

Construction of plant transformation vectors

A plant binary vector was constructed from pGreenII 000-35S, which contains a cauliflower mosaic virus (CAMV) 35S promoter/terminator cloning cassette in the original multiple cloning site of pGreenII 000 (Hellens *et al*., 2000). pGreenII 000-35SII was created by removal of redundant restriction sites by a SpeI/StuI digestion and religation. An *HpaI* fragment containing the *nptiiA* gene, under the control of a CAMv 35S promoter and terminator, was introduced to the *HpaI* site of the vector to create pGreenII 049-35SII. Ligation sites were sequenced to confirm correct sequence and orientation. An *EcoRI* fragment containing BatIMS was excised from pGEM TEasy and ligated into *EcoRI* digested pGreenII 049-35SII to give 35S:BatIMS. Vectors with the correct insert and orientations were then co-transformed with the partner plasmid pSOUP into *Agrobacterium tumefaciens* AGL1 by electroporation.

Arabidopsis *transformation*

Transgenic *Arabidopsis* were generated by *Agrobacterium tumefaciens* mediated transformation with 35S:BatIMS and empty vector through floral dipping of ecotype Col-0 (Clough and Bent, 1998). Eight T0 transformants were recovered from each transformation after selection for kanamycin resistance (E1–E8). Southern blot analysis indicated that each line appeared to contain single insertions at independent loci (data not shown).

Soluble amino acid analysis

Seedling material was prepared as above. Two extractions were performed for each of three biological replicates and amino acids were quantified using the AccQ Tag system (Waters) as previously described (Field *et al*., 2004). Average amino acid amounts between the two 35S:BatIMS lines, and between the two empty vector lines was not significantly different, so the data was merged to give 35S:BatIMS vs empty vector. ANOVA (part of the GenStat 8.0 package) was used for calculating the significance of differences between amino acids in 35S:BatIMS and empty vector lines (and glucosinolates, below).

Glucosinolate analysis

The aerial parts of *Arabidopsis* plants (approximately 20 plants per line) were individually harvested at transition to flowering and freeze dried. GSLs were extracted from the freeze dried material or seeds, converted to desulphoglucosinolates and analysed by LC-MS with APCI, as previously described (de Quiros *et al*., 2000).

Microarray analysis

Seedling material was prepared as above. RNA was extracted from one empty vector line and one 35S:BatIMS line (E8) across three biological replicates. The six RNA samples were cleaned using Qiagen RNAeasy mini-columns. Total RNA quality was assessed by running 1 μl of each RNA sample on Agilent RNA6000nano LabChips® (Agilent Technology 2100 Bioanalyzer Version A.01.20 SI211). cRNA was prepared essentially as described in the Affymetrix Manual II, starting with 5 μg of total RNA. Twenty micrograms of cRNA was then hybridised to an ATH1 genechip array (Affymetrix, Santa Clara, CA) containing probes to more than 22 000 different *Arabidopsis* genes and the signal detected according to manufacturers instructions. Raw data was then submitted to the FunAlyse pipeline (www.gla.ac.uk/functionalgenomics/rp/affy_analysis.html), where it was normalized using RMA (Irizarry *et al*., 2003) and differentially expressed genes identified using the rank products method (Breitling *et al*., 2004a). Seventy percent of the top 100 genes were the same as those identified using dCHIP. Ranked data was then processed using IGA with 2210 GO annotation groups (Breitling *et al*., 2004b). These included a custom group containing all known genes of aliphatic GSL biosynthesis, and a group containing the genes of leucine catabolism as defined by KEGG (www.genome.jp/kegg). The raw microarray data were submitted in MIAME-compliant format to the ArrayExpress database (www.ebi.ac.uk/arrayexpresss/) and have been assigned the accession number E-MEXP-424.

Quantitative RT PCR

cDNA was synthesised from the same six RNA samples as used in the microarray experiment. Quantitative RT PCR was then set up using the DyNAmo™ SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland) in 20 µl reactions according to manufacturers instructions. PCR cycling was performed in a DNA Engine Opticon 2 (MJ Research). An initial denaturation step of 95 °C for 10 min was followed by 44 cycles of: 94 °C for 10 s; 53 °C for 10 s, 72 °C for 16 s, 75 °C for 1 s and a plate fluorescence read. The products were extended by incubation at 72 °C for 5 min, and finally a melting curve analysis performed to check for the presence of non-specific products. Fold changes in mRNA levels for a gene-of-interest (GOI) between 35S:BatMAM1 and the empty vector lines were calculated using the comparative method $E^{-\Delta C_T}$ where E is the PCR efficiency, C_T the cycle threshold, and ΔC_T = average $C_{T_{GOI}}$ − average $C_{T_{reference}}$. The housekeeping gene adenine phosphoribosyltransferase was used as the reference. E was calculated using LinRegPCR (Ramakers *et al.*, 2003). Non-quantitative RT PCR was used to verify expression of the BatIMS transgene in 35S:BatIMS plants using the primers BatIMSF and BatIMSR, designed to specifically amplify a 179 bp product from BatIMS. Sequences for unlisted primers are available on request.

Primers

IPMSF, 5′-TCTTCGATACWACTCTCCGWGACGG-3′; IPMSR, 5′-ATYWACTGCCTTGTAAG

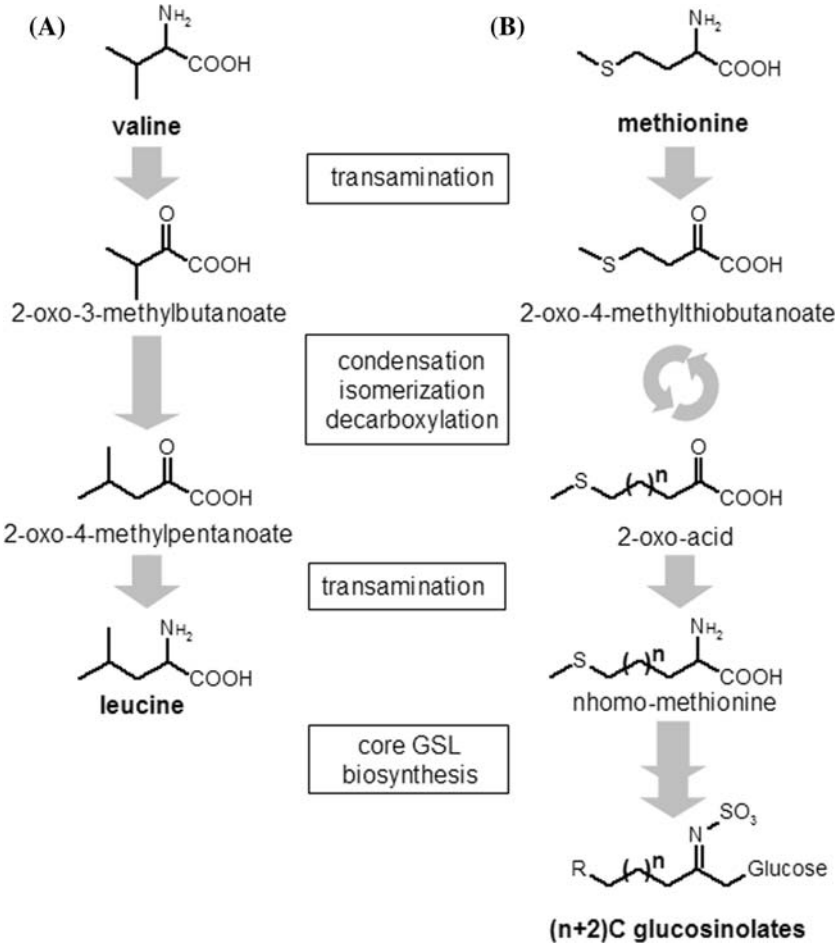

Figure 1. Parallels between leucine biosynthesis (a) and aliphatic GSL biosynthesis (b). Condensation of a 2-oxo acid and acetyl CoA by an IPMS or MAM enzyme followed by isomerization and decarboxylation results in elongation of the 2-oxo acid by a single methyl group. In GSL biosynthesis only the elongated 2-oxo acid may then undergo further rounds of elongation.

CTG-3′; BatIMSL1, 5′-CCAGGCCATGGAGTC TTCGATTC-3′; BatIMSR1, 5′-TTCAGGCAGG TACTTTGTTG-3′; BatIMS/53-KpnI, 5′-TTGGT ACCTGCTCCCTCTCAGATCCCT-3′; BatIMS/ TGA-KpnI, 5′-CTGGTACCTCCGATT TTGAT TCAGGCAGGT-3′; BatIMSF, 5′-GAGCCACC CTCACCTCCAA-3′; BatIMSR, 5′-GCA GATG ACGGGGACGTA AC-3′

Results

Cloning of a Brassica atlantica *MAM homologue*

B. atlantica is a member of the *B. oleracea* $n=9$ species complex. It accumulates large quantities of 2-propenyl GSLs, resulting from a single round of methionine elongation, but does not accumulate any longer chain GSLs. Degenerate RT PCR was used with the aim of isolating highly processive MAM homologues from *B. atlantica*. A MAML-4 like ORF, BatIMS, was cloned from *B. atlantica* cDNA by RACE PCR (GenBank accession DQ143886). BatIMS has 90% amino acid identity to MAML-4 (At1g18500) and 86% identity to MAML-3 (At1g74040). Southern analysis and isolation of genomic DNA from bacterial artificial chromosome (BAC) libraries indicates that there are only two genes with homology to MAML-3 and MAML-4 in the *B. oleracea* genome (data not shown).

BatIMS possesses IPMS activity

An expression construct was designed to heterologously express the predicted mature peptide encoded by BatIMS, with the addition of an N-terminal 6× HIS tag. The *E. coli* strain CV512 has a non-functional IPMS (Δ*Leu*A) rendering it unable to grow on media lacking leucine (Somers *et al*., 1973). Upon induction CV512 containing pQE-BatIMS produced protein of the predicted size and became able grow on minimal media lacking leucine (Figure 2).

Overexpression of BatIMS in Arabidopsis *causes abnormal development*

BatIMS was constitutively overexpressed in *A. thaliana*. Seven out of eight independent 35S:BatIMS lines displayed an abnormal phenotype which varied in severity: plants were dwarfed, and mature rosette leaves were pale with crinkled margins (Figure 3). No morphological phenotype was observed in lines transformed with an empty vector. The phenotype became milder or was lost

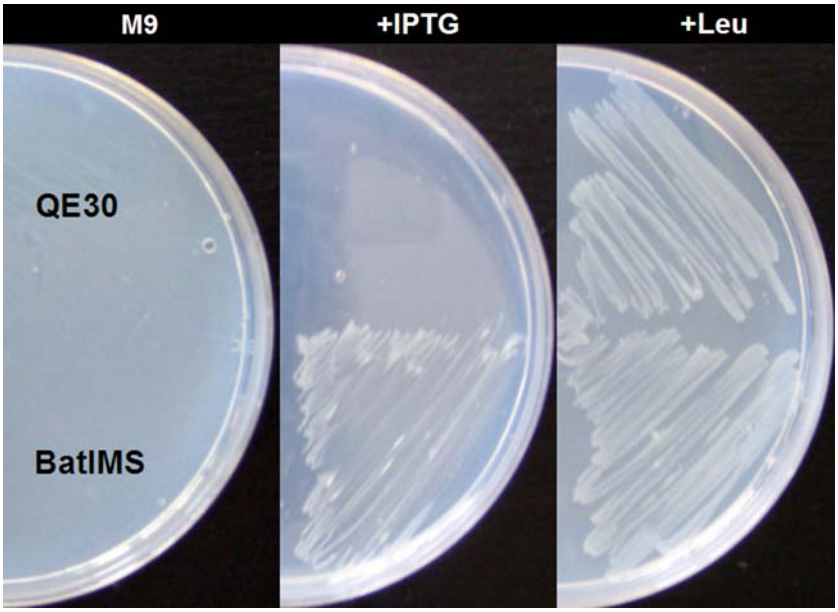

Figure 2. BatIMS complements the *E. coli* leucine auxotroph CV512. *E. coli* harbouring pQE-BatIMS or an empty vector control, pQE-30, grown on solid minimal media (left), +0.1 mM IPTG (centre) or + 30 mM leucine (right).

after several generations. RT PCR and Northern experiments showed that loss of phenotype was accompanied by loss of BatIMS transgene expression, presumably due to silencing. Therefore all further characterization was performed on the segregating T1 generation. RT PCR was used to confirm that the BatIMS transgene, MAM1, MAML, MAML-3 and MAML-4 were being expressed in all T1 lines, in both seedlings and mature plants (data not shown). Thus, the observations described here are not the consequence of transgene silencing, or co-suppression of a homologous *Arabidopsis* gene.

Overexpression of BatIMS perturbs amino acid and glucosinolate metabolism

We predicted that the aberrant phenotype of 35S:BatIMS plants was due to leucine overproduction, as high concentrations of branched chain amino acids can be toxic (Miflin, 1969; Bourgin, 1978; Schulze-Siebert *et al*., 1984; Relton *et al*., 1986). Therefore we measured levels of free amino acids in 35S:BatIMS seedlings and empty vector controls (Figure 4). Unexpectedly, levels of free leucine actually decreased in the seedlings of 35S:BatIMS lines. This was accompanied by a more than 2-fold increase in

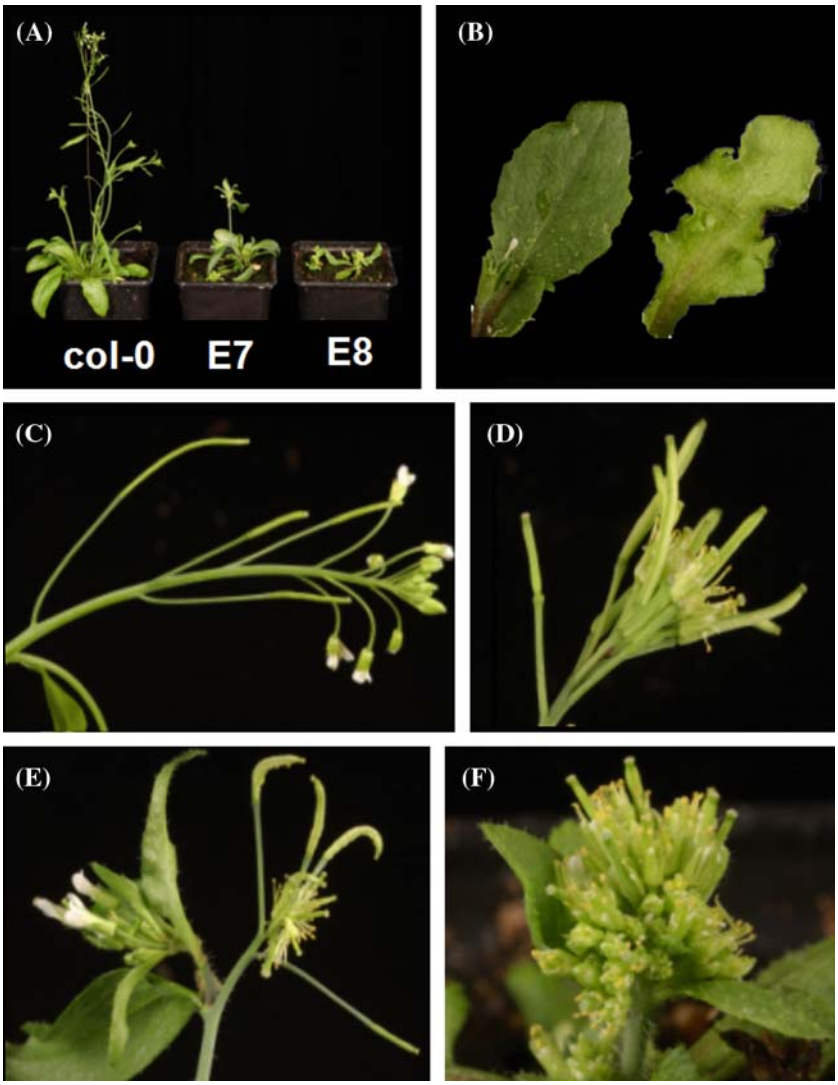

Figure 3. 35S:BatIMS transgenics exhibited a strong developmental phenotype with incomplete penetrance. (A) Wild type Columbia (left) and two independent transformants (right), (B) a mature wild type leaf (left) compared to a mature 35S:BatIMS leaf (right), (C) wild type florette, (D–F) 35S:BatIMS florettes. Note both normal and abnormal development on a single plant (E).

the related amino acid valine (164 ± 27 pmoles/g FW in the transgenics versus 74 ± 3 pmoles/g FW in the empty vector controls; $P < 0.001$; $n = 12$) as well as increases in serine, glycine, threonine, methionine and histidine (Figure 4). Total levels of amino acids were unchanged ($P > 0.05$). Levels of amino acids between the two independent 35S:BatIMS lines tested were not significantly different, except for alanine which was present at higher levels in one line ($P < 0.01$).

We also measured GSL content in 35S:BatIMS and empty vector plants to determine whether BatIMS overexpression also affected GSL metabolism. In the leaves of T1 plants expressing BatIMS there were two fold higher levels of aliphatic GSLs (Figure 5). This increase was not observed in the seeds of T1 plants, nor in the T2 or T3 generations (data not shown). The profile of aliphatic GSLs was not significantly different in any of the plants analysed, whether GSL levels had increased or not.

Microarray analysis of BatIMS plants

To help determine how BatIMS overexpression perturbs the metabolism and development of *Arabidopsis* plants we carried out global transcript profiling using the Affymetrix ATH1 genechip. Segregating T1 seedlings were grown on kanamycin plates and resistant seedlings were sampled after nine days to avoid potential variation from the unstable and pleiotropic phenotype of mature 35S:BatIMS plants. Raw data was analysed using the FunAlyse pipeline at the Sir Henry Wellcome Functional Genomics Facility. Eighty-one genes were upregulated and 105 downregulated with false discovery rate (FDR) < 1 (Supplementary Table 1). The microarray data was validated by quantitative RT PCR (Supplementary Table 2). Iterative group analysis (IGA) was then performed to identify changes in functionally related groups of genes, including those below the arbitrary FDR cut-off (Breitling *et al.*, 2004b). Several groups of genes were found to be significantly up or down regulated in 35S:BatIMS seedlings (Table 1). These included at least 4 of the 10 known genes of the aliphatic GSL biosynthetic pathway (AOP2, AtST5b, AtST5c, and CYP79F1/CYP79F2), and components of leucine catabolism. The members of each group detected are listed in Supplementary Table 3, along with rank and fold change.

Discussion

IPMS activity

We have demonstrated that the *B. atlantica* MAML-4 homologue, BatIMS, has IPMS activity when expressed in *E. coli*, and thus is likely to function as an IPMS *in planta*. However, overexpression of BatIMS in *A. thaliana* resulted in a significant decrease in leucine levels ($P = 0.004$) and a highly significant increase in the related amino acid valine ($P < 0.001$). It is striking that we

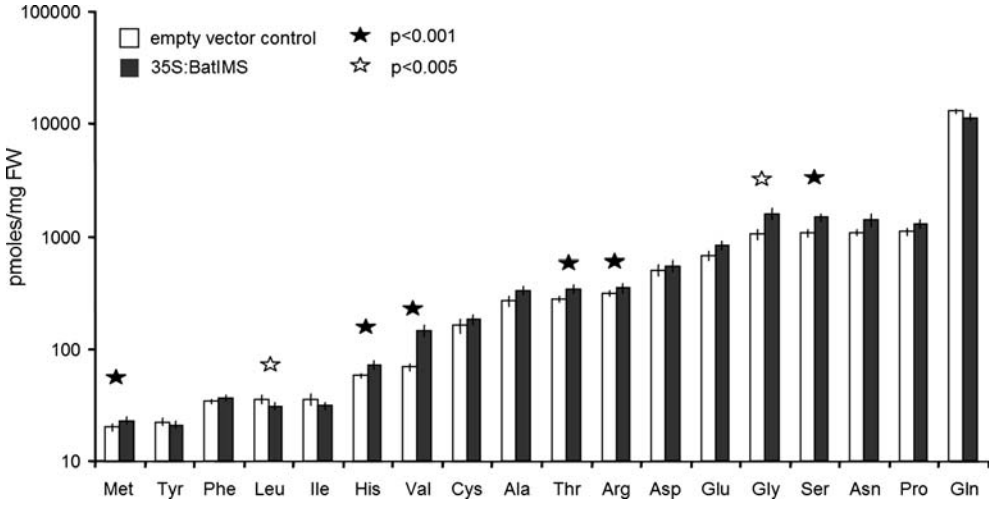

Figure 4. Soluble amino acid content of transgenic seedlings. Expressed as the average of 6 experiments ± 1 SE. Note the logarithmic scale.

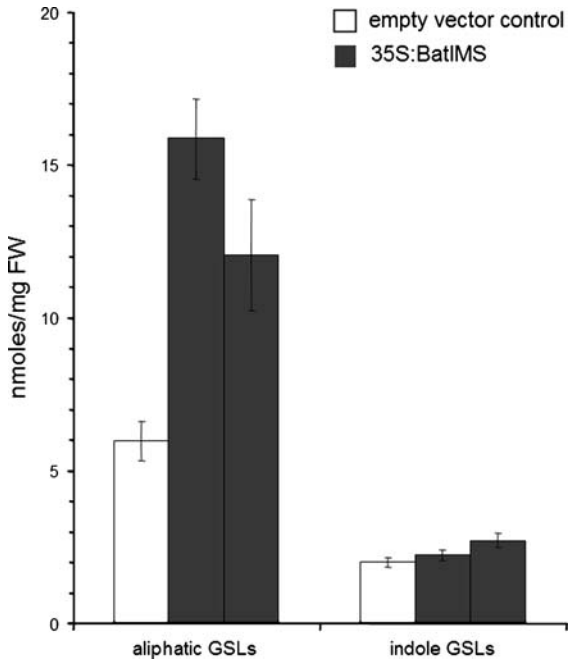

Figure 5. Aliphatic and indolic GSL content in the leaves of wild type *Arabidopsis* and two independent 35S:BatIMS lines (average of approximately 20 extractions per line ±SE).

observed very similar changes in a T-DNA insertion allele of MAML-4 (Field *et al*., 2004). Additionally, microarray analysis showed that almost all known genes of leucine catabolism were downregulated in 35S:BatIMS plants (Table 1). These results can be partly explained by feedback inhibition of BatIMS by leucine, an effect previously demonstrated on purified IPMS from spinach (Hagelstein and Schultz, 1993). However, it is not clear how feedback inhibition alone could reduce leucine to below wild type levels. Thus, it is likely that another regulatory mechanism is responsible. In *Salmonella typhimurium* IPMS is functional as a tetrameric complex. Leucine causes dissociation of the complex into its monomeric subunits and loss of activity (Kohlhaw *et al*., 1969; Leary and Kohlhaw, 1970). It is therefore possible that overexpression of an IPMS (BatIMS), or knocking out a putative IPMS (MAML-4) could disrupt the stoichiometry of a putative IPMS complex in *Arabidopsis*, resulting in reduced overall IPMS activity, and increased flux into valine biosynthesis. BatIMS overexpression also causes changes in the levels of a number of other amino acids. Such deregulation may be further evidence for a general amino acid control network in plants; a hypothesis proposed by several research groups (Zhu and Galili, 2003; Field *et al*., 2004; Voll *et al*., 2004).

GSL biosynthesis

We have shown that in 35S:BatIMS plants the genes for aliphatic GSL biosynthesis are upregulated, and that the levels of aliphatic GSLs are 2-fold higher. These results suggest that, when overexpressed, BatIMS may be able to initiate methionine elongation in addition to acting as an IPMS. The BatIMS IPMS domain has 83% amino acid homology with the corresponding domains of MAML and MAM1 (aa85-474) which both act on methionine or methionine homologues. Purification of BatIMS protein and analysis of its kinetics with various substrates would help ascertain its precise catalytic functions. Alternatively, BatIMS overexpression may cause a shift in the regulation of GSL biosynthesis, whether directly, perhaps via physical interactions with MAM1 and MAML, or indirectly, as a result of pleiotropic effects. These results demonstrate that the enzymes of the leucine and glucosinolate biosynthetic pathways may be compartmentalised under normal conditions. Overexpression of an IPMS, as described here, or overexpression of MAML (Field *et al*., 2004)

Table 1. Iterative group analysis (IGA) identified groups of functionally related genes with significantly altered expression between empty vector and 35S:BatIMS plants.

Group	Direction	Members	Changed	*P*-value	Percent changed
Aliphatic GSL biosynthesis	Up	9	4	6.7*e*−08	44
Photosynthesis light harvesting	Up	19	4	2.3*e*−06	27.7
Response to heat	Up	10	5	4.3*e*−04	50
Response to auxin stimulus	Down	32	4	3.1*e*−05	12.5
Leucine catabolism	Down	8	6	2.3*e*−04	75
Trehalose phosphate synthesis	Down	6	2	2.6*e*−04	33.3
Ethylene negative regulation	Down	9	5	2.6*e*−04	55.5

results in decompartmentalisation and crosstalk between the two pathways.

BatIMS phenotype

Plants overexpressing MAML, which has 83% amino acid identity with BatIMS across the IPMS domain, have no visible phenotype (Field *et al*., 2004). However, overexpression of BatIMS in *A. thaliana* had surprisingly wide ranging consequences: there were extensive metabolic and transcriptional changes which gave rise to a strong morphological phenotype. The phenotype is reminiscent of the *bushy*/*sps*/*cyp79F1* mutants which also have crinkled leaves and a loss of apical dominance (Hansen *et al*., 2001; Reintanz *et al*., 2001; Tantikanjana *et al*., 2001). SPS/CYP79F1 performs the second step in aliphatic GSL biosynthesis, converting elongated methionine homologues into aldoximes. BatIMS also appears to affect aliphatic GSL biosynthesis. The exact cause of the phenotype in *bushy*/*sps*/*cyp79F1* mutants is unclear, except that it involves perturbations in cytokinin and auxin homeostasis and perhaps methionine metabolism (Hansen *et al*., 2001; Reintanz *et al*., 2001; Tantikanjana *et al*., 2001; Tantikanjana *et al*., 2004). There is evidence that similar processes are also perturbed in 35S:BatIMS plants, although it is unclear whether these are as a direct consequence of BatIMS function or pleiotropic effects. Our microarray data shows that there are alterations in auxin and ethylene homeostasis (Table 1). A number of auxin responsive genes are downregulated in 35S:BatIMS plants, suggesting that auxin levels are low. Furthermore, 1-aminocyclopropane-1-carboxylate synthase 6 is upregulated (rank 11, 3.93-fold) while negative regulators of ethylene signalling are downregulated, suggesting that ethylene levels are high. Amino acid and array data also indicate that methionine and sulphur metabolism are perturbed. There is a higher level of methionine in 35S:BatIMS plants, and a number of methionine metabolising genes are upregulated including phosphoethanolamine *N*-methyltransferase 3 and nicotianamine synthase (rank 17, 3.2-fold and rank 63, 1.95-fold, respectively). Three 5′-adenylylsulfate reductase genes (APR1,2&3) are also upregulated. These genes are upstream of methionine biosynthesis and known to be induced by sulphate deficiency and by cytokinin (Koprivova *et al*., 2000; Ohkama *et al*., 2002). Toxicity due to high levels of valine is unlikely to contribute significantly to the stunted phenotype of 35S:BatIMS plants as MAML-4 KO plants have similarly increased levels of free valine yet a wild type phenotype (Field *et al*., 2004).

Regulation of GSL biosynthesis

Here we show that aliphatic GSL biosynthesis is transcriptionally regulated, and that upregulation results in a corresponding increase in levels of aliphatic GSLs. Our data also reinforces recent evidence indicating that GSL biosynthesis is under the control of transcription factors (Celenza *et al*., 2005; Levy *et al*., 2005). The transcriptional regulation of general GSL biosynthesis has already been used to identify previously unknown pathway components (Piotrowski *et al*., 2004; Gachon *et al*., 2005; Hirai *et al*., 2005). The data presented here may be used in a similar manner. For example a putative glutathione *S*-transferase (GST) gene, At3g03190, was associated with GSL biosynthesis by cluster analysis (Hirai *et al*., 2005). At3g03190 is ranked here at 102 among upregulated genes, and is identified by IGA if included in the aliphatic GSL biosynthesis group (aliphatic GSL biosynthesis, 5/11 changed, $P = 1.16e{-}009$).

Conclusions

Manipulation of the levels and chemical structures of glucosinolates in crop plants is an attractive target, and may have implications for the enhancement of pest resistance, animal feeding quality and anticarcinogenic activity. Previous studies have indicated that MAM genes may be important in determining not only the length of the aliphatic side chain but also the total level of glucosinolates (de Quiros *et al*., 2000; Mithen *et al*., 2003), and that wild *Brassica* species that accumulate high amount of glucosinolates may be sources of useful MAM alleles. In this paper we demonstrate that for this strategy to be successful, further studies are required on the spatial and temporal expression and interactions of both IPMS and MAM genes to prevent cross talk between biosynthetic pathways on primary and secondary metabolism.

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