Plant Biotechnology 30, 403–406 (2013)
DOI: 10.5511/plantbiotechnology.13.0523a

Note

Response of aliphatic glucosinolate biosynthesis to signaling molecules in *MAM* gene knockout mutants of *Arabidopsis*

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Received March 27, 2013; accepted May 23, 2013 (Edited by M. Yamaguchi)

Abstract Aliphatic glucosinolates (AGSLs) in *Arabidopsis thaliana* are synthesized from methionine derivatives with various side-chain length that are formed from methionine via chain elongation cycle involving MAM1 and MAM3. Biosynthesis of AGSLs is highly affected by defense signaling molecules, including jasmonic acid, salicylic acid and ethylene. In response to exogenously-applied these phytohormones, *MAM* genes exhibited different induction patterns in Wild-type of *Arabidopsis*. The changing patterns of AGSL contents were distinctive in single knockout mutants of *MAM* genes compared with those in Wild-type, suggesting that *MAM1* and *MAM3* play an important role in the diversity of AGSL profiles in response to hormonal changes.

Key words: *Arabidopsis thaliana*, aliphatic glucosinolates, *MAM1*, *MAM3*, signaling molecules.

Aliphatic glucosinolates (AGSLs) are a group of nitrogen- and sulfur-containing secondary metabolites in Brassicaceae plants including *Arabidopsis thaliana*. AGSLs are biosynthesized from methionine derivatives with elongated side chains. Methionine is deaminated by BCAT4 (Schuster et al. 2006) to form 4-methylthio-2-oxobutyrate, which is converted to a variety of 2-oxo acids with different lengths of side chain by a repetitive three-step chain elongation cycle. 2-Oxo acids with different side chains are transaminated to form respective methionine derivatives, a variety of direct precursors for AGSL biosynthesis. The methionine derivatives with the lengths of side chain ranging from 3C to 5C, which undergo one to three times of chain elongation cycle, are eventually converted into commonly called short-chain AGSLs, and those with 6C to 8C side chains, which undergo four to six times of chain elongation cycle, are converted into so-called long-chain AGSLs. In *Arabidopsis*, there are two kinds of methylthioalkylmalate synthase (MAM) involved in the acetyl-CoA condensation, the first reaction of the chain elongation cycle. MAM1 is only responsible for the condensation reaction of the first three elongation cycles (Kroymann et al. 2001), while MAM3 can catalyze all six condensation reactions (Textor et al. 2007). The diversity of AGSL profiles is important in plant fitness and resistance to generalist herbivores (Schranz et al. 2009; Manzaneda et al. 2010).

Plant secondary metabolism is highly affected by defense signaling molecules, including jasmonic acid (JA), salicylic acid (SA) and ethylene. JA regulates plant responses to biotic and abiotic stresses (Delker et al. 2006). SA is a phenolic phytohormone involved in responses of plant to insect (Moran and Thompson 2001). Ethylene also participates in plant defense responses (Lin et al. 2009). In this respect, AGSL profiles could be greatly influenced through MAM by plant hormones. In this study, we focused on the influence of signaling molecules on AGSL profiles in Wild-type and single knockout mutants of *MAM* genes.

Homozygous plants of T-DNA insertion mutants *mam1* (SALK_057539) and *mam3* (SALK_007222c) were selected by genotyping. Two mutant lines together with Wild-type *Arabidopsis* (Col-0) were grown on soil in a greenhouse at 22°C under fluorescent light with a light/dark cycle of 16 h/8 h. Four weeks after germination,

Abbreviations: AGSL, aliphatic glucosinolate; MAM, methylthioalkylmalate synthase; JA, jasmonic acid; MeJA, methyl jasmonate; SA, salicylic acid; ACC, 1-aminocyclopropane-1-carboxylate.

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This article can be found at http://www.jspcmb.jp/
Published online September 11, 2013

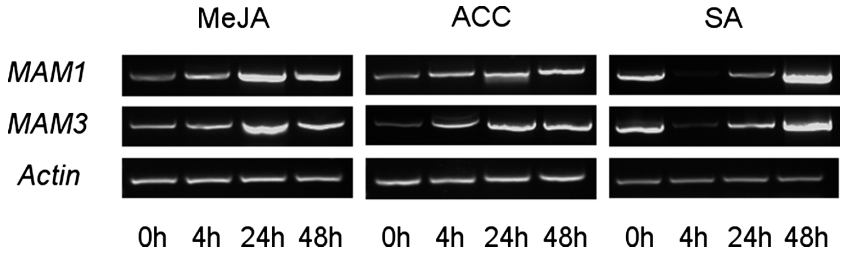

Figure 1. Expression of *MAM* genes in Wild-type (Columbia) of *Arabidopsis* after treatment with exogenous hormones. Plants were sampled at 0 h (untreated control), 4 h, 24 h and 48 h after treatment with MeJA, ACC and SA, respectively. Expression of *MAM1* and *MAM3* in 4-week-old rosette leaves was analyzed by semi-quantitative RT-PCR using *Actin* as a reference gene.

plants were sprayed with 0.2 mmol l^{-1} methyl jasmonate (MeJA), 2.0 mmol l^{-1} SA or 2.5 mmol l^{-1} 1-aminocyclopropane-1-carboxylate (ACC), an ethylene precursor. At 0 h (untreated control), 4 h, 24 h and 48 h after treatments, leaves were harvested into pre-frozen sample tubes and stored at −80°C until use. AGSLs in mutant lines and Wild-type were extracted and analyzed by high-performance liquid chromatography (HPLC) (Waters) (Chen et al. 2003; Petersen et al. 2001; Petersen et al. 2002).

To reveal the responses of *MAM* gene expression to exogenously-applied hormones, relative expression levels of *MAM1* and *MAM3* in Wild-type were analyzed by semi-quantitative reverse transcriptase (RT)-PCR, using the *Actin* gene as a control. The primers for *MAM1* were 5′-ATG GCT TCA TCG CTT CTG AC-3′ (forward) and 5′-TTA CAC ATT CGA TGA AAC CT-3′ (reverse), those for *MAM3* were 5′-ATG GCT TCG TTA CTT CTC AC-3′ (forward) and 5′-TTA TAC AAC AGC GGA AAT CT-3′ (reverse), and those for *Actin* were 5′-TGG AAC TGG AAT GGT TAA GG-3′ (forward) and 5′-TCT CCA GAG TCG AGC ACA AT-3′ (reverse). Different hormone treatments resulted in distinctive induction patterns of *MAM* genes. *MAM1* and *MAM3* had the same changing trend under each condition (Figure 1). The expression levels of *MAM1* and *MAM3* increased slightly at 4 h after MeJA treatment and peaked at 24 h. Then the expression levels at 48 h declined but still higher than 4 h. Induction patterns of *MAM* genes by SA were opposite to MeJA. The expression levels drastically decreased at 4 h after treatment, then recovered slowly and reached the highest level at 48 h. The expression levels at 48 h were higher than those at 0 h. After ACC treatment, the expression levels of both *MAM1* and *MAM3* increased and peaked at 48 h.

AGSL contents in both Wild-type and mutant lines were analyzed. As short-chain AGSLs were the most abundant, the amounts of total and short-chain AGSLs showed the same changing patterns. In Wild-type, the changing patterns of total and short-chain AGSL contents over time after hormone treatment were consistent with those of *MAM1* and *MAM3* expression levels; after MeJA treatment the AGSL contents increased until 24 h and then declined (Figure 2A), after ACC treatment the AGSL contents raised continually (Figure 3A), and after SA treatment the AGSL contents decreased at 4 h and then recovered (Figure 4A). It was reported that genes encoding myrosinase were pathogen- and SA-inducible (Textor and Gershenzon 2009; Thangstad et al. 1993). At 48 h after SA treatment, expression levels of *MAM1* and *MAM3* were much higher than those at 0 h (Figure 1). However, AGSL contents at 48 h were less those at 0 h (Figure 4A), probably because hydrolysis of GSLs by myrosinase remained at its induced level at 48 h after SA treatment. MeJA and ACC treatments had no obvious influence on long-chain AGSL contents in Wild-type. However, at 4 h after SA treatment long-chain AGSL accumulation was repressed and then recovered (Figure 4A). The changing patterns were consistent with those of *MAM* genes expression levels. Interestingly, there was an obvious decrease of indole glucosinolate (IGSL) contents in Wild-type at 4 h after SA treatment, but the recovery degree at 24 or 48 h after treatment was much less than that of AGSLs (Figure 4A).

In the *mam1* mutant line, the expression of *MAM1* was blocked, leaving only MAM3 to catalyze all six chain elongation cycles. Long-chain AGSL contents in *mam1* were not affected by MeJA (Figure 2B) or ACC treatment (Figure 3B). At 4 h after SA treatment long-chain AGSL accumulation was repressed and not recovered (Figure 4B). Likewise, total and short-chain AGSL contents were also decreased at 4 h after SA treatment and not recovered (Figure 4B). Concerning the fact that expression of *MAM3* could be recovered at 24 h after SA treatment, this result suggested a lack of substrates for MAM3 at later period. A similar changing pattern of IGSL contents was also observed after SA treatment (Figure 4B). After MeJA treatment, the contents of total and short-chain AGSL in *mam1* had the same changing patterns as in Wild-type (Figure 2B). However, in case of ACC treatment, total and short-chain AGSL contents in *mam1* were decreased at 4 h after treatment and then recovered, changing patterns of which were different from those observed in Wild-type (Figure 3B).

In the *mam3* mutant line, the expression of *MAM3* was blocked, leaving MAM1 to catalyze only the first

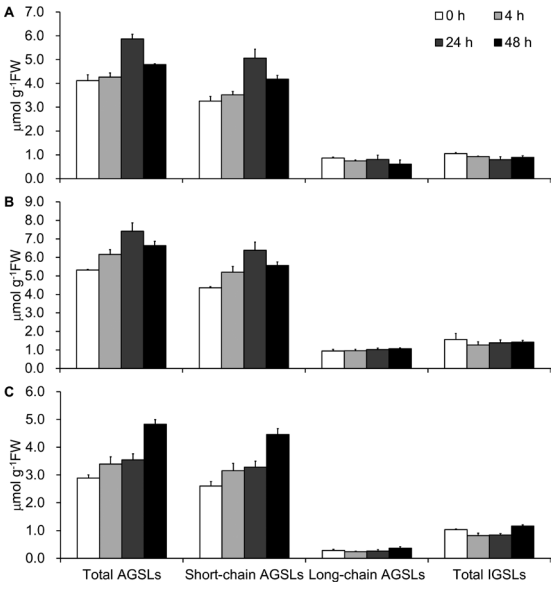

Figure 2. Contents of AGSLs and IGSLs in Wild-type (Columbia), *mam1* and *mam3* after treatment with MeJA. Four-week-old rosette leaves of (A) Wild-type, (B) *mam1* and (C) *mam3* were sampled at 0 h (untreated control), 4 h, 24 h and 48 h after treatment with MeJA. AGSLs and IGSLs were analyzed by HPLC using benzylglucosinolate as an internal standard for quantification. The mean±S.D. (n=3) are shown.

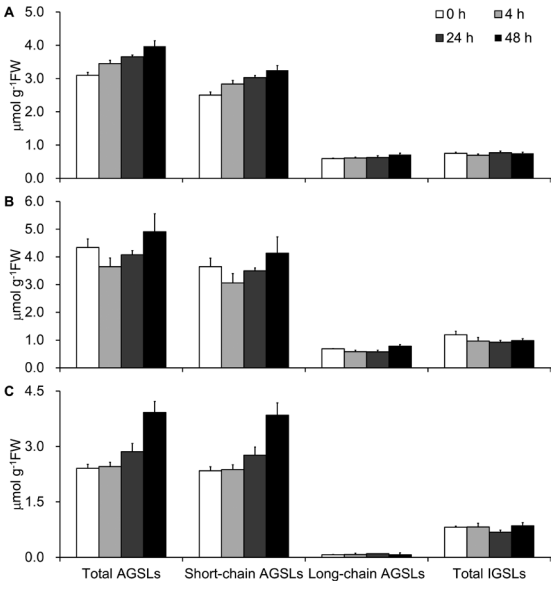

Figure 3. Contents of AGSLs and IGSLs in Wild-type (Columbia), *mam1* and *mam3* after treatment with ACC. Four-week-old rosette leaves of (A) Wild-type, (B) *mam1* and (C) *mam3* were sampled at 0 h (untreated control), 4 h, 24 h and 48 h after treatment with ACC. AGSLs and IGSLs were analyzed by HPLC using benzylglucosinolate as an internal standard for quantification. The mean±S.D. (n=3) are shown.

three chain elongation cycles, and there was almost no long-chain AGSLs accumulated in *mam3*. After ACC and SA treatment, total and short-chain AGSL contents in *mam3* had the same changing patterns as in Wild-type (Figures 3C and 4C). However, MeJA treatment resulted

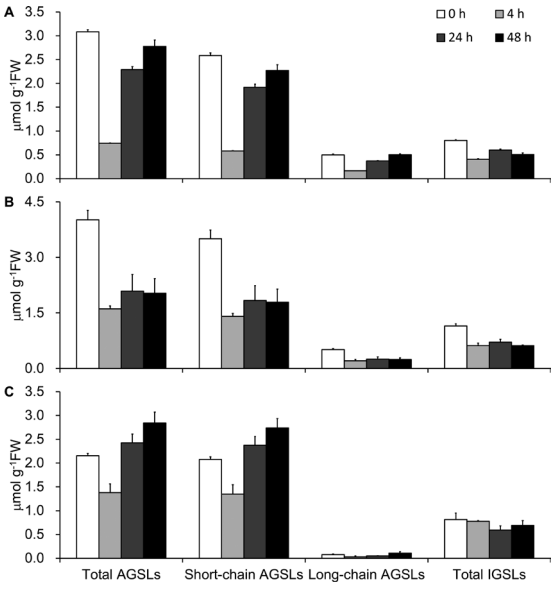

Figure 4. Contents of AGSLs and IGSLs in Wild-type (Columbia), *mam1* and *mam3* after treatment with SA. Four-week-old rosette leaves of (A) Wild-type, (B) *mam1* and (C) *mam3* were sampled at 0 h (untreated control), 4 h, 24 h and 48 h after treatment with SA. AGSLs and IGSLs were analyzed by HPLC using benzylglucosinolate as an internal standard for quantification. The mean±S.D. (n=3) are shown.

in different changing pattern in *mam3* compared to that in Wild-type. The contents of total and short-chain AGSL continuously increased in *mam3* and were not declined at 48 h after treatment (Figure 2C). The changing patterns were also different from those in *mam1*.

Exogenously-applied hormones had distinctive effects on the expression of *MAM* genes, resulting in different changing patterns of AGSL accumulation in Wild-type and single knockout mutants of *MAM* genes. This can give rise to diversity of AGSL profiles in *Arabidopsis* to adapt to complex and changing environment.

Acknowledgements

This work was supported by National Natural Science Foundation of China (No. 31070351) and the Cultivation Program of the Northeast Forestry University for Excellent Doctoral Dissertations.

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