Novel Insights Into the Function of Arabidopsis R2R3-MYB Transcription Factors Regulating Aliphatic Glucosinolate Biosynthesis

Yimeng Li1,2, Yuji Sawada2,3, Akiko Hirai2, Munee Sato2,3,4, Ayuko Kuwahara2,3,4, Xiufeng Yan1,4 and Masami Yokota Hirai2,3,4,*

1Alkali Soil Natural Environmental Science Center, Northeast Forestry University; Key Laboratory of Saline-Alkali Vegetation Ecology Restoration in Oil Field, Ministry of Education, Harbin, 150040 China
2RIKEN Plant Science Center, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, 230-0045 Japan
3RIKEN Center for Sustainable Resource Science, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, 230-0045 Japan
4JST, CREST, 4-1-8 Honcho, Kawaguchi, 332-0012 Japan
*Corresponding authors: Masami Yokota Hirai, E-mail, masami.hirai@riken.jp; Fax, +81-45-503-9489; Xiufeng Yan, E-mail, xfy@nefu.edu.cn; Fax, +86-451-82190052.
(Received May 5, 2013; Accepted June 4, 2013)

Aim: To study the role of MYB transcription factors in aliphatic glucosinolate (AGSL) biosynthesis in Arabidopsis.

Method: The study used transgenic Arabidopsis plants expressing MYB28, MYB29, and MYB76 genes. MYB28 and MYB29 positively regulated AGSL biosynthesis, while MYB76 negatively regulated it.

Results: MYB76 expression was induced by sulfur deficiency, while MYB28 and MYB29 expression was induced by normal transcriptional regulation. MYB76 and MYB29 acted synergistically to regulate AGSL biosynthesis.

Conclusion: The study illustrated how the individual MYB factors work in regulating AGSL biosynthesis when expressed alone under normal transcriptional regulation.

Keywords: Aliphatic glucosinolates • Arabidopsis thaliana • MYB transcription factors • Organ specificity • Promoter • Sulfur stress.

Abbreviations: (A)GSL, (aliphatic) glucosinolate; ANOVA, analysis of variance; BCAT4, branched-chain aminotransferase 4; CaMV 35S promoter, Cauliflower mosaic virus 35S RNA promoter; FMO, flavin-monooxygenase; GTR1, ARABIDOPSIS THALIANA GLUCOSINOLATE TRANSPORTER-1; LC-MS, liquid chromatography–mass spectrometry; MAM, methylthioacylmalate synthase; 4MS, 4-methylsulfinylbutyl GSL; 8MS, 8-methylsulfinylectyl GSL; 4MT, 4-methylthiobutyl GSL; OAS, O-acetyl-L-serine; qRT-PCR, quantitative reverse transcription–PCR; Sultr1;1, sulfate transporter 1;1; UPLC-TQMS, ultra performance liquid chromatography–tandem quadrupole mass spectrometry; UTR, untranslated region.

Introduction

Glucosinolates (GSLs) constitute an essential part of plant defense secondary metabolites in the order Brassicales, including the model plant Arabidopsis and agriculturally valuable Brassica plants (Grubb and Abel 2006, Halkier and Gershenzon 2006). Because of their repellent activity against insect herbivory and their cancer-preventing properties in humans, research on GSLs has been extensively conducted.

GSLs are synthesized from amino acids, such as methionine, tryptophan and phenylalanine. In Arabidopsis, aliphatic glucosinolates (AGSLs) derived from methionine confer plants with defensive properties against various biotic stresses because of their diverse chemical structures (Burow et al. 2010). In the biosynthetic pathway, the precursor methionine is subject to three processes, i.e. side chain elongation, core structure biosynthesis and secondary modification of the side chain, and is eventually formed into AGSLs with various side chains (Sønderby et al. 2010b; Supplementary Fig. S1). There are

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two major enzymatic reactions in the biosynthetic pathway that can lead to AGSL profile diversity. First, methylthioalkylmalate synthase 1 (MAM1) and methylthioalkylmalate synthase 3 (MAM3) are involved in elongation of the side chains and give rise to chain length diversity. AGSLs with 3C–5C side chains are commonly called short-chain AGSLs, whereas those with 6C–8C side chains are called long-chain AGSLs (Kroymann et al. 2001, Textor et al. 2007). Secondly, in the secondary modification step, flavin monoxygenases (FMOs) convert methylthioalkyl to methylsulfinylalkyl GSLs (Hansen et al. 2007, Li et al. 2008). It is reported that plants with distinct GSL profiles exhibit different resistance to generalist herbivores (Schanz et al. 2009), and structural variation in the GSL profile is associated with plant fitness (Manzaneda et al. 2010). In addition, a specific GSL molecular species (i.e. methylsulfinylalkyl GSL with a 4C chain) has a cancer-preventing property (Zhang et al. 1992). In this context, understanding of the regulatory mechanisms of GSL accumulation is of interest in basic biology as well as in application studies aimed at alteration of GSL profiles in crops.

Three transcriptional factors, MYB28, MYB29 and MYB76, which belong to the R2R3-MYB family, have been identified to participate in the regulation of the AGSL biosynthetic pathway in Arabidopsis (Gigolashvili et al. 2007, Hirai et al. 2007, Sønderby et al. 2007, Gigolashvili et al. 2008, Malitsky et al. 2008). It has been revealed that three MYBs have similar function in up-regulation of AGSL biosynthetic genes by the studies of ectopic overexpression of these MYB genes using the Cauliflower mosaic virus 35S RNA promoter (CaMV 35S promoter) and transactivation assays of AGSL biosynthetic genes by these MYB genes. In addition, these MYB genes were suggested to regulate each other mutually, based on the studies of the knock-out lines and the ectopically overexpressing lines of these genes, as well as transactivation assays (Gigolashvili et al. 2007, Beekwilder et al. 2008, Gigolashvili et al. 2008, Sønderby et al. 2010a). However, the regulatory relationship has not yet been fully elucidated because these studies sometimes gave contradictory results. For instance, mutual induction of MYB29 and MYB76 was suggested by ectopic overexpression experiments of (Gigolashvili et al. 2007), while MYB29 knock-out did not affect the expression of MYB76 (Sønderby et al. 2010a).

To clarify the individual function of the three MYB genes, double knock-out lines were generated by genetic crosses between the insertional mutants, myb28, myb29 and myb76. A double knock-out myb29myb76 could hardly be obtained by genetic cross as these genes are located adjacent to each other on chromosome 5. Some transgenic studies have been conducted in the myb28myb29 and myb28myb76 backgrounds. However, it remains to be clarified how these MYB genes regulate the accumulation of AGSLs with different side chain length. Accumulation of long-chain AGSLs did not alter in leaves of myb29 and myb76 single mutants, suggesting that MYB29 and MYB76 were not involved in the regulation of long-chain AGSL accumulation (Sønderby et al. 2007, Beekwilder et al. 2008, Gigolashvili et al. 2008). On the other hand, the ectopic overexpression of MYB29 in the myb28myb76 mutant and that of MYB76 in the myb28myb29 mutant recovered long-chain AGSL accumulation, implying that MYB29 and MYB76 could induce long-chain AGSL accumulation on their own (Sønderby et al. 2010a). Thus, overlapping function and interplay of three MYB genes complicate our understanding of their individual function.

In this study, we constructed transgenic ProMYB:MYB lines in the myb28myb29 background in which MYB28, MYB29 or MYB76 genes were expressed under the control of their own promoters. The myb28myb29 mutant provides a good opportunity to investigate the individual functions of the three MYB genes, because it has drastically decreased expression levels of MYB28, MYB29 and also MYB76, and is almost devoid of AGSLs. In general, promoters play a key part in the gene regulatory network and determine the biological function of genes. In AGSL biosynthesis, MYB28 expression is induced by glucose as its promoter contains several glucose-regulated motifs (Gigolashvili et al. 2007). On the other hand, MYB29 expression is regulated by the hormones methyl jasmonate and salicylic acid (Hirai et al. 2007, Gigolashvili et al. 2008). As AGSLs are sulfur-rich metabolites, sulfur plays a vital role in the regulation of AGSL biosynthesis (Falk et al. 2007, Yan and Chen 2007). Secondly, in the secondary modification step, flavin monoxygenases (FMOs) convert methylthioalkyl to methylsulfinylalkyl GSLs (Hansen et al. 2007, Li et al. 2008). It is reported that plants with distinct GSL profiles exhibit different resistance to generalist herbivores (Schanz et al. 2009), and structural variation in the GSL profile is associated with plant fitness (Manzaneda et al. 2010).

**Results**

**Expression level of MYB genes in the ProMYB:MYB lines**

A double knock-out mutant myb28myb29 was transformed with each MYB gene driven by its own promoter. The promoter fragments used in this study spanned 2–3 kbp (Supplementary Fig. S2) and contained the promoter regions used in the previous studies (Gigolashvili et al. 2007, Gigolashvili et al. 2008). Thus we considered that the respective promoters were sufficient for analyzing the expression of these MYB genes. Among approximately 20 transformants of each line, we selected for further experiments three, two and two transformants of ProMYB29:MYB28, ProMYB29:MYB29 and ProMYB76:MYB76, respectively, which showed representative AGSL profiles. Expression levels of MYB genes were analyzed by quantitative reverse transcription–PCR (qRT–PCR) in these transgenic lines (Fig. 1). To distinguish between endogenous and transgenic expression of MYB genes, two kinds of primer sets were designed: 3′-untranslated region (UTR) primers, which can only...
MYB genes for aliphatic glucosinolate biosynthesis

Fig. 1 The relative expression levels of MYB genes in leaves. The expression of (A) MYB28, (B) MYB29 and (C) MYB76 in 3-week-old rosette leaves was analyzed by qRT–PCR. The expression level relative to that of the wild type is shown. Col, ProMYB28_1, ProMYB28_2, ProMYB28_3, ProMYB29_1, ProMYB29_2, ProMYB76_1, ProMYB76_2 represent Columbia wild type, ProMYB28, ProMYB29, ProMYB76, respectively. For each line, three plants were independently analyzed. The means ± SD are shown. Pink columns represent the transcript levels derived from both transgenic and endogenous genes amplified with 3′-UTR primers. Blue columns represent total MYB transcript levels derived from both transgenic and endogenous genes amplified with exon primers.

Fig. 2 Glucosinolate profiles

GSL contents in leaves of these ProMYB:MYB lines were analyzed by liquid chromatography–mass spectrometry (LC-MS) (Fig. 2; Supplementary Table S1). As expected, AGSLs were hardly detected in myb28myb29 (below 0.1% of the wild-type level), leaving the accumulation of tryptophan-derived indole GSLs unchanged.

The total amount of AGSLs was recovered in ProMYB28:MYB28 and ProMYB29:MYB29 lines (Fig. 2A). ProMYB28:MYB28 showed a higher recovery rate, ranging from 34% to 42%, while ProMYB29:MYB29 only recovered about 14–16% of the total AGSL content in the wild type. The non-linear relationship between the levels of MYB transcripts and AGSLs suggested a different capability of MYB genes to induce AGSL biosynthesis. There was no significant difference in the total AGSL content between ProMYB:MYB76 and myb28myb29, indicating that slight MYB76 expression in ProMYB:MYB76 lines (Fig. 1C) was not sufficient to recover AGSL accumulation.

We also analyzed accumulation of the individual AGSL molecular species (Fig. 2; Supplementary Table S1). Accumulation of short-chain AGSLs was recovered to a higher extent in ProMYB28:MYB28 than in ProMYB29:MYB29 (Fig. 2B). The major constituents of short-chain AGSLs, 4-methylthiobutyl (4MT) and 4-methylsulfinylbutyl (4MS) GSLs, were recovered similarly in both lines (Fig. 2D, E), suggesting that the ratio of methylsulfanylalkyl to methiolthioalkyl GSLs did not significantly change. The accumulation of long-chain AGSLs including 8-methylsulfynylcoctyl (8MS) GSL was barely found in all ProMYB:MYB76 lines (Fig. 2C, F). These results suggest that the own promoter-driven MYB genes could not recover the accumulation of long-chain AGSLs in leaves.

To investigate the MYB function in other organs, we analyzed the AGSL content in seeds of ProMYB:MYB lines. The recovery patterns were different from those in leaves (Fig. 3). Interestingly, ProMYB29:MYB76 recovered the accumulation of short-chain AGSLs (Fig. 3B, D, E). Their recovery rates were higher in ProMYB76:MYB76 than in ProMYB29:MYB29. In addition, the accumulation of long-chain AGSLs was recovered in ProMYB29:MYB28 (Fig. 3C, F). The results suggested that the MYB genes work differently in regulating AGSL profiles in an organ-specific manner. GSLs are synthesized in source organs such as leaves and transported into seeds as a sink via long-
distance phloem transport (Chen et al. 2001, Nour-Eldin et al. 2012). Little accumulation of AGSLs in seeds of ProMYB29:MYB29 lines suggested a loss of the transport system in the absence of both MYB28 and MYB76 (see below).

AGSL biosynthetic gene expression

To comprehend the direct cause of the recovery of AGSL accumulation in leaves, the expression levels of the genes involved in the AGSL biosynthetic pathway (Supplementary Fig. S1) were investigated by qRT–PCR. We analyzed the expression of branched-chain aminotransferase 4 (BCAT4) (Schuster et al. 2006), MAM1 (Kroymann et al. 2001) and MAM3 (Textor et al. 2007) committed to side chain elongation; the Cyt P450 monooxygenases, CYP79F1, CYP79F2 (Reintanz et al. 2001, Chen et al. 2003, Tantikanjana et al. 2004) and CYP83A1 (Hemm et al. 2003; Naur et al. 2003) involved in the formation of the AGSL core structure; and FMO genes that convert methylthioalkyl to methylsulfinylalkyl AGSLs (Hansen et al. 2007, Li et al. 2008) (Fig. 4).

The expression levels of FMOGSOX2, FMOGSOX4 and FMOGSOX5 were not affected by MYB gene manipulation (data not shown). The expression of the other genes was repressed in myb28myb29 to <1% of the wild-type level (Fig. 4). The expression of these genes was not recovered in ProMYB28:MYB28 and ProMYB29:MYB29 to a similar extent (Fig. 4A, B). This result was consistent with the recovery pattern of total AGSLs in these two lines. The expression of the other genes was recovered in ProMYB28:MYB28 and ProMYB29:MYB29 to a similar extent (Fig. 4D–H). The recovery rates of CYP79F1 and CYP79F2 were very low in the ProMYB28:MYB28 and ProMYB29:MYB29 lines (<10% of the wild-type level), probably because the functions of the oxidases were redundant. The expression of MAM3, an essential gene for the formation of long-chain AGSLs, was not recovered in any of the ProMYB:MYB lines (Fig. 4C). This probably led to the inability to recover long-chain AGSL accumulation. The results for ProMYB28:MYB28 and ProMYB29:MYB76 were almost consistent with the microarray results of myb28 and myb76 except that
MAM3 expression was repressed in myb28 (Hirai et al. 2007, Sønderby et al. 2010a).

As the seeds of ProMYB:MYB29 hardly recovered AGSL accumulation (Fig. 3), we analyzed the expression levels of ARABIDOPSIS THALIANA GLUCOSINOLATE TRANSPORTER-1 (GTR1) and -2 (GTR2), which are essential for long-distance GSL transport (Nour-Eldin et al. 2012). However, their expression levels were not significantly changed in myb28myb29 and ProMYB:MYB lines compared with those in the wild type (data not shown).

**Function of own promoter-driven MYB genes under altered sulfur condition**

To investigate how the MYB gene expression responds to sulfur stress, their expression was analyzed in leaves under mild sulfur deficiency (one-tenth of the usual sulfate concentration; 1/10 S) and sulfur deprivation (0 S). The O-acetyl-L-serine (OAS) content and the sulfate transporter 1;1 (Sultr1;1) expression level, which are the indicators of sulfur-deficient stress (Hirai et al. 2003), were increased under altered sulfur conditions, showing that the plants were suffering from sulfur stress (Fig. 5).

Under the 1/10 S condition, total AGSLs underwent a moderate decrease in the wild type (Fig. 6A). The AGSL contents under 1/10 S were less distinguishable from those under normal, sulfur-replete condition (Full S) in the ProMYB:MYB lines (Fig. 6A). In all lines, AGSLs decreased to a nearly undetectable level under sulfur-deprived conditions (Fig. 6A).

In the wild type, the expression levels of MYB29 and MYB76 decreased under sulfur-deficient and -deprived conditions. They were positively correlated with sulfur concentrations (Fig. 6C, D). The expression level of MYB28 slightly increased under sulfur-deficient conditions, but decreased under sulfur-deprived condition, suggesting that MYB28 is apt to be constitutively expressed to maintain the basic accumulation of AGSLs under mild stress (Fig. 6B). Interestingly, the induction level of MYB28 expression under sulfur deficiency was enhanced in ProMYB28:MYB28, indicating that in the absence of MYB29 and/or MYB76, MYB28 was more sensitive to variations in the levels of sulfur.

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**Fig. 3** The relative AGSL contents in seeds. The graphs show contents of total AGSLs (A), total short-chain AGSLs (B), total long-chain AGSLs (C), 4MT (D), 4MS (E) and 8MS (F) in seeds of wild type, myb28myb29 and ProMYB:MYB lines. For each line, five seeds were analyzed three times. The vertical axes indicate the relative peak area of AGSLs in LC-MS analysis normalized by the peak area of the standard compound 10-camphorsulfonic acid (per five seeds). The means ± SD are shown. Data were processed by one-way multiple ANOVA with Duncan’s test. Different letters within a graph represent the statistical difference (P < 0.05).
Discussion

Novel insights into interplay of MYB28, MYB29 and MYB76

In the previous studies exploring the function of these MYB genes, the use of the CaMV 35S promoter to overexpress these genes often resulted in transgene silencing. In addition, these studies did not provide the information on transcriptional regulation of the transgenic MYB genes. In this study, we used the transgenic lines expressing a single MYB gene under the control of its own promoters in the myb28myb29 background, where endogenous MYB28, MYB29 and MYB76 were not expressed. Our experiments showed that MYB genes driven by their own promoters had different impacts on regulation of AGSL biosynthesis from those in the previous transgenic studies. It is noteworthy that the expression level of MYB28 in the ProMYB28:MYB28 lines reached only approximately 20% of that in the wild type under sulfur-replete condition. The MYB28 promoter and coding region used in this study spanned from position -2,682 to position +1,321 and contained the promoter region used in the previous study for tissue-specific expression and induction by glucose (position -1,995 to position +157, Gigolashvili et al. 2007). The promoter used in this study was thus considered to confer an expression level of the

Fig. 4 The relative expression levels of AGSL biosynthetic genes. The expression levels in 3-week-old rosette leaves were analyzed by qRT–PCR and normalized using an endogenous reference gene, UBC9. The expression levels of BCAT4 (A), MAM1 (B), MAM3 (C), CYP79F1 (D), CYP79F2 (E), CYP83A1 (F), FMOGSOX1 (G) and FMOGSOX3 (H) relative to those of the wild type are shown. For each line, three plants were independently analyzed. The means ± SD are shown. Data were processed by one-way multiple ANOVA with Duncan’s test. Different letters within a graph represent the statistical difference (P < 0.05).
transgenic MYB28 comparable with that of the endogenous MYB28. The simplest explanation for 20% recovery of MYB28 expression was that MYB28 requires MYB29 and/or MYB76 for its ‘normal’ level of expression. On the other hand, MYB28 expression was induced by 2- to 4-fold under mild sulfur deficiency in ProMYB28::MYB28 lines. The induction was not very obvious in the wild type, suggesting that MYB29 and/or MYB76 inhibited the induction of MYB28 expression. This was consistent with the report that the MYB28 transcript was significantly increased in the myb29 mutant, suggesting that MYB28 expression was repressed by MYB29 (Sønderby et al. 2010a). These apparently contradictory results (i.e. up- or down-regulation of MYB28 expression by MYB29) suggest unknown transcriptional regulation of MYB28. One of the possible scenarios is as follows: under mild sulfur deficiency, the MYB29 level is decreased because MYB28 expression is repressed. At the same time, a hypothetical transcription factor is induced to up-regulate MYB28 expression. Assuming that this transcription factor does not fully work in the presence of MYB29—for instance, this factor competes against MYB29 for the same binding site in the MYB28 promoter—MYB28 expression is much more induced by mild sulfur deficiency in the absence of MYB29 (myb29) than in the presence of MYB29 (the wild type). MYB29 also seemed to require MYB76 and/or MYB28 for its ‘normal’ level of expression, as suggested by Sønderby et al. (2010a). Based on our result, MYB76 expression seemed to require both MYB28 and MYB29. The interplay and epistasis among MYB28, MYB29 and MYB76 will be further clarified by genetic crosses between ProMYB28::MYB28, ProMYB29::MYB29 and ProMYB76::MYB76.

**Contribution of the respective MYB genes to determination of AGSL profiles**

The content of total AGSLs had a non-linear relationship to the recovery rate of MYB genes. For instance, approximately 20% recovery of MYB28 expression resulted in an approximately 35% recovery of AGSL accumulation in ProMYB28::MYB28 lines. However, in ProMYB29::MYB29, approximately 50% recovery of MYB29 expression only led to an approximately 15% recovery of AGSL accumulation. Interestingly, among the AGSL biosynthetic genes tested, the expression of only MAM1 and BCAT4 was recovered to a greater extent in ProMYB28::MYB28 than in ProMYB29::MYB29. As these two genes are located upstream in the AGSL biosynthetic pathway, the expression levels of these genes may determine the AGSL chemotypes, suggesting that MYB28 plays a larger role in promoting AGSL biosynthesis than MYB29.

In our recent study, the myb28myb29 mutant was transformed with MYB29 driven by the CaMV 35S promoter. The transcript level of MYB29 in the Pro35S::MYB29 lines exhibited a 4- to 8-fold increase compared with that in the wild type, which was much higher than that in ProMYB28::MYB29 (~50% of the wild-type level). However, the Pro35S::MYB29 lines did not give rise to higher expression levels of biosynthetic gene (10–30% of the wild-type level) than ProMYB28::MYB29, nor the full recovery of AGSL accumulation (<50% of the wild-type level). Long-chain AGSLs were also absent in Pro35S::MYB29 as well as in ProMYB29::MYB29 (Araki et al. in press). This suggests that the expression level of MYB29 does not determine its own the content and profile of AGSLs.

**Regulation of MYB28, MYB29 and MYB76 expression by sulfur stress**

In the sulfur stress experiment, mild and drastic decreases in AGSL accumulation were observed under sulfur-deficient and -deprived conditions, respectively. The expression of MYB28, MYB29 and MYB76 responded to sulfur-deficient stress in opposite directions; the expression of MYB29 and MYB76 was repressed, while that of MYB28 was induced. It is reported that Arabidopsis SLIM1, an ethylene-insensitive3-like transcriptional...
factor, is a central transcriptional regulator of sulfur deficiency response (Maruyama-Nakashita et al. 2006). In roots, the expression of BCAT4, MAM1 and MAM3 was repressed by sulfur deficiency in a SLIM1-dependent manner (Maruyama-Nakashita et al. 2006). However, the involvement of SLIM1 in down-regulation of these genes was limited, because the expression of these genes was repressed to a lesser extent even in the slim1 mutant by sulfur limitation. The effect of SLIM1 on the expression of MYB28, MYB29 and MYB76 was unclear (Maruyama-Nakashita et al. 2006). Genetic crosses between the slim1 mutant and our ProMYB:MYB lines will clarify the relationship among SLIM1 and MYB28, MYB29 and MYB76.

Conclusion

A variety of biotic and abiotic factors other than sulfur nutrition affect GSL metabolism (Grubb and Abel 2006, Halkier and Gershezon 2006, Yan and Chen 2007). Although several factors, such as hormones and transcription factors, have been identified to participate in the regulatory mechanism of GSL metabolism, the relationship among them remains to be clarified. The ProMYB:MYB lines established here will be useful to understand more clearly the individual biological functions of MYB28, MYB29 and MYB76, by breaking down the links of the complicated regulatory network.

Materials and Methods

Plant materials

A double knock-out mutant myb28myb29 was generated by crossing two single mutants, myb28 and myb29, possessing the T-DNA insertion and transposon element in At5g61420 (SALK_136312) and At5g07690 (SM_3_34316), respectively. Note that the combination of single mutants was different from that in the previous studies (Sønderby et al. 2007, Beekwilder et al. 2008). To construct the ProMYB:MYB lines, about 4,000 (for ProMYB28:MYB28 and ProMYB29:MYB29) or 3,000 (for ProMYB76:MYB76) bp fragments spanning the promoters (2,683, 2,674 and 2,000 bp for MYB28, MYB29 and MYB76, respectively) and the coding regions of MYB28, MYB29 and MYB76 were amplified by PCR by using Arabidopsis genomic DNA (Supplementary Fig. S2). Primers used for amplification of the ProMYB:MYB lines, about 4,000 (for ProMYB28:MYB28 and ProMYB29:MYB29) or 3,000 (for ProMYB76:MYB76) bp fragments spanning the promoters (2,683, 2,674 and 2,000 bp for MYB28, MYB29 and MYB76, respectively) and the coding regions of MYB28, MYB29 and MYB76 were amplified by PCR by using Arabidopsis genomic DNA (Supplementary Table S2). The 3'-UTRs of MYB genes were not included in the amplified fragments. They were then cloned into a binary vector pGW81 (Nakagawa et al. 2007). The resulting vectors were transformed into Agrobacterium tumefaciens EHA101 and then used for transformation of Arabidopsis myb28myb29. Transgenic lines were selected on agar-solidified 1/2 Murashige and Skoog medium with 50 mg l−1 kanamycin or 20 mg l−1 hygromycin. The homozygous lines were used for subsequent analyses.
Plant growth conditions

The wild type (Columbia), myb28myb29 and seven lines of transgenic Arabidopsis (ProMYB28MYB28_1, ProMYB28MYB28_2, ProMYB28MYB28_3, ProMYB29MYB29_1, ProMYB29MYB29_2, ProMYB29MYB29_3, ProMYB29MYB29_4) were grown for 3 weeks in a growth chamber at 22°C under fluorescent light with a light/dark cycle of 16 h/8 h. Agar-solidified 1/2 Murashige and Skoog medium with 1% sucrose was utilized for plant cultivation. For the sulfur stress experiment, the medium containing 1.5 mM sulfate (Full S) was adjusted to 0.15 mM MgCl₂.

Glucosinolate and O-acetyl-L-serine analyses

The contents of GSLs and OAS were measured by LC-MS using ultra performance liquid chromatography–tandem quadrupole mass spectrometry (UPLC-TQMS) according to Sawada et al. (2009a, 2009b).

Approximately 10–50 mg of rosette leaves was sampled from 3-week-old plants and transferred to a pre-frozen, 2 ml tube containing 5 mm zirconia beads. After chilling in liquid nitrogen, samples were stored at −80°C until use. Plant samples were lyophilized using a freeze dryer (FDU-2100, EYELA) in a vacuum, homogenized using a Shake Master (Bio Medical Science) at a speed of 1,000 r.p.m. and homogenized again following the addition of 800 μl of the extraction buffer (methanol : milliQ water = 4 : 1, 0.1% formic acid, 5 μM sinigrin as the AGSL internal standard, 1.68 μM 10-camphorsulfonic acid as the internal standard for negative ion mode analysis and 0.0336 μM lidocaine as the internal standard for positive ion mode analysis). A 200 μl homogenate was transferred to a new tube, concentrated to dryness using a Speedvac (Thermo Fisher Scientific) vacuum centrifuge, dissolved with 200 μl of ultrapure water (LC-MS grade) and filtered using Amicon Ultrafree-MC centrifugal filter devices (0.2 μm, 100 PK). For seed analysis, five seeds were extracted with 500 μl of extraction buffer without sinigrin, and diluted to 1/6 with ultrapure water.

For UPLC-TQMS analysis, samples were diluted to 1/500 with ultrapure water (LC-MS grade) using an automated liquid handling system (Microlab STARplus, Hamilton). Compounds were separated using a HSS T3 1.8 μm column (1.0 × 50 mm, Waters), and the UPLC gradient program: 0–0.25 min, 99.9% of solvent A; 0.4 min, 91% of solvent A; 0.8 min, 83% of solvent A; 1.9 min, 0.1% of solvent A; 1.9–2.1 min, 0.1% of solvent A; 2.11–2.7 min, 99.9% of solvent A; and a flow rate of 0.24 ml min⁻¹. Conditions for the TQMS (Waters) detection were capillary voltage of −0.80 kV (negative ion mode) or 0.50 kV (positive ion mode), cone voltage of 50 V (negative ion mode) or 30 V (positive ion mode), source temperature of 150°C, and desolvation gas of 1,000 l h⁻¹ at 600°C.

AGSL contents in leaves were calculated by comparing the peak area of each compound with that of sinigrin.

RNA extraction and qRT–PCR

Total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen) and treated with DNase (TURBO DNA-free). A 2 μg aliquot of total RNA was used to synthesize the first strand of cDNA using SuperScript II (Invitrogen). The cDNA was diluted to 1/25 with water for qRT–PCR analysis.

The qRT–PCR analyses were performed using Fast SYBR Green (Applied Biosystems) on an Applied Biosystems StepOnePlus (Applied Biosystems) by the method of ΔΔCt using UBC9 as the endogenous control. The primers used are listed in Supplementary Table S2.

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by National Natural Science Foundation of China [No. 31070351]; the Cultivation Program of the Northeast Forestry University for Excellent Doctoral Dissertations [to Y.L.].

Acknowledgments

The metabolite analysis using UPLC-TQMS was supported by the Japan Advanced Plant Science Network. We thank Dr. Kensuke Kawade (RIKEN Center for Sustainable Resource Science) for the critical reading of the manuscript.

Disclosures

The authors have no conflicts of interest to declare.

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