Genetics and Molecular Research

Characterization of *RsMYB28* and *RsMYB29* transcription factor genes in radish (*Raphanus sativus* L.)

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ABSTRACT. Glucosinolates (GSLs) are important secondary metabolites in Brassicaceae plants. Previous studies have mainly focused on GSL contents, types, and biosynthesis-related genes, but the molecular characterization patterns of GSL biosynthesis-related transcription factors remain largely unexplored in radish (*Raphanus sativus* L.). To isolate transcription factor genes regulating the GSL biosynthesis, genomic DNA and cDNA sequences of *RsMYB28* and *RsMYB29* genes were isolated in radish. Two R2R3-MYB domains were identified in the deduced amino acid sequences. Subcellular localization and yeast-one hybrid assays indicated that both the

RsMYB28 and *RsMYB29* genes were located in the nucleus and possessed transactivation activity. Reverse transcription quantitative analysis showed that the *RsMYB28* and *RsMYB29* genes were expressed in seeds, leaves, stems, and roots at the seedling, taproot thickening, and mature stages. Both genes were highly expressed during the seedling and taproot thickening stages. The expression level of *RsMYB28* was found to be up-regulated following wounding, glucose, and abscisic acid treatments, whereas *RsMYB29* was up-regulated following wounding and methyl jasmonate treatments. These results provide insights into the biological function and characterization of the *RsMYB28* and *RsMYB29* genes, and facilitate further dissection of the molecular regulatory mechanism underlying the GSL biosynthesis in radish.

Key words: Radish; Glucosinolate; MYB transcription factor; *RsMYB28*; *RsMYB29*; RT-qPCR

INTRODUCTION

Glucosinolates (GSLs) are amino acid-derived natural plant products in the Brassicaceae family, which are well known for their role as a degradation product that induces resistance against generalist herbivores (Kos et al., 2012) and microorganisms (Brader et al., 2006) in plants, as well as for their cancer-preventing properties in humans. In addition, GSLs have gained increasing significance as flavor compounds and potential biopesticides (Mithen et al., 2003).

Recently, many studies have revealed that the GSL biosynthesis is a tripartite pathway consisting of three stages: side-chain elongation, development of the core structure, and secondary modification of the amino acid side chain (Chen et al., 2011; Augustine et al., 2013a). The most prominent GSL biosynthetic precursor amino acids include methionine, tryptophan, and phenylalanine, which result in aliphatic, indolic, and aromatic GSLs, respectively (Kos et al., 2012).

In recent years, a great number of functional genes involved in the GSL biosynthetic pathway have been successfully identified in several plant species including *Arabidopsis thaliana* (Sønderby et al., 2010), *Brassica juncea* (Augustine et al., 2013a), and *B. rapa* (Kim et al., 2013). The R2R3-MYB proteins are plant specific and constitute one of the largest transcription factor families in plants (Wilkins et al., 2009). Many R2R3-MYB genes have been found to play important roles in a variety of biological functions, including biosynthesis of secondary metabolites (Gigolashvili et al., 2008; Verdier et al., 2012), regulation of biotic and abiotic stressors by increasing hypersensitivity to abscisic acid (ABA) (Shan et al., 2012), precise establishment of the root epidermal pattern, and control of cell cycle progression (Kang et al., 2009; Xie et al., 2010). In *Arabidopsis*, the *AtMYB28*, *AtMYB29*, and *AtMYB76* genes positively regulate aliphatic GSL biosynthesis (Gigolashvili et al., 2008; Sønderby et al., 2010; Li et al., 2013), whereas the *AtMYB34*, *AtMYB51*, and *AtMYB122* genes have been shown to regulate indolic GSL biosynthesis (Gigolashvili et al., 2007a). In *B. juncea*, four homologous genes encoding *MYB28* were found to participate in regulating the aliphatic GSL biosynthesis (Augustine et al., 2013a). Moreover, both the *MYB28* and *MYB29* genes showed high expression levels in flowers, seeds, and stems of Chinese cabbage (Kim et al., 2013). *AtMYB28* transcripts were nearly absent, whereas *AtMYB29* and *AtMYB76* transcripts

showed no significant decrease at aliphatic GSL biosynthesis induction in knockout mutants (Gigolashvili et al., 2009; Sønderby et al., 2010). This indicates that *AtMYB28* is an important regulator of aliphatic GSL biosynthesis, whereas *AtMYB29* and *AtMYB76* may play minor roles (Gigolashvili et al., 2009; Sønderby et al., 2010). These findings have provided useful information for the characterization of the *MYB28* and *MYB29* genes in other vegetable crops.

Radish (*Raphanus sativus* L., 2n = 2x = 18), belonging to the Brassicaceae family, is an important root vegetable crop worldwide. It contains large amounts of anti-oxidants and GSLs (Malik et al., 2010). Three candidate genes, *RsMAM3*, *RsIPMDH1*, and *RsBCAT4* have been reported as being involved in the 4-methylthio-3-butenyl GSL biosynthesis in radish roots, based on a QTL region synteny analysis of *A. thaliana* and *B. rapa* genome sequences (Zou et al., 2013). With *de novo* transcriptome sequencing, eight genes were found to be involved in the GSL metabolism-related pathways in radish (Wang et al., 2013). Two cDNA clones of myrosinase, a hydrolyzing GSL enzyme, were isolated and characterized in radish (Pan et al., 2014). However, the MYB transcription factors involved in regulating the GSL biosynthesis remain largely unexplored in radish.

In this study, two R2R3-MYB transcription factors, *RsMYB28* and *RsMYB29*, were isolated from radish and their expression patterns under different treatments and in different tissues at three developmental stages were investigated. Moreover, subcellular localization and transactivation activity analyses of these two transcription factors were performed. The results of this study provide valuable information for functional validation of the *RsMYB28* and *RsMYB29* genes, and facilitate further dissecting of the molecular regulatory mechanism of the GSL biosynthesis in root vegetable crops.

MATERIAL AND METHODS

Plant materials

The advanced inbred radish line 'NAU-ZQH' was grown in a growth chamber at 25°C/14-h light and 18°C/10-h dark. At the four-true leaf stage, a wounding treatment was adopted by crushing 90% of the area of a leaf using a hemostat (Jun et al., 2011; Miao et al., 2013). The leaves were treated with methyl jasmonate (MeJA, 100 μM), ABA (100 μM), glucose (100 μM), and sorbitol (as an osmotic control) (100 μM) (Jun et al., 2011). Three individuals were used for each treatment, and three replicates of leaves for each treatment were collected at different time points (0, 1, 2, 4, 6, 8, 12, and 24 h). For the spatial-temporal expression in different tissues, roots, leaves, and stems were sampled at the seedling, taproot thickening, and mature stages, respectively. Three replicates were obtained for each tissue. At the mature stage, samples of flesh and skin were separated. All samples were frozen immediately in liquid nitrogen and stored at -80°C until further use.

Isolation of cDNA and genomic DNA sequences

Genomic DNA was isolated from radish root using a modified CTAB method (Liu et al., 2003). Total RNA was isolated from radish samples using a Simple P total RNA extraction kit (Tiangen Biotech Co., Ltd., Beijing, China), following the manufacturer instructions. First-strand cDNA was synthesized with M-MLV (RNase H-) (Takara, Dalian, China) and oligo-dT primers. Based on previously published radish transcriptome sequences (Wang et al.,

2013), two gene-specific primer pairs (*RsMYB28*F1/R1 and *RsMYB29*F1/R1) were designed to amplify the open reading frame (ORF) of *RsMYB28* and *RsMYB29*, respectively. The polymerase chain reaction (PCR) contained 20 ng genomic DNA or cDNA, 0.2 mM dNTPs, 2.0 mM $MgCl_2$, 0.8 U Taq DNA polymerase, and 0.4 μM each primer. The PCR products were purified and cloned into the pMD18-T vector (Takara). The sequence analysis was performed on an ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, Forster City, CA, USA).

Phylogenetic analysis

The amino acids of the *RsMYB28* and *RsMYB29* genes were predicted using the DNASTAR software (Lasergene). A BLASTx of the coding sequences was performed on NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The retrieved protein sequences that were used for the analysis are listed in **Table S1**. Sequence alignment was determined by ClustalW (http://align.genome.jp/). The neighbor-joining method, including bootstrap analyses with 1000 replicates, was used to construct a phylogenetic tree using MEGA6 (Tamura et al., 2007).

Subcellular localization of *RsMYB28* and *RsMYB29*

To generate *pJIT166-RsMYB28(29)-GFP* (green fluorescent protein) containing an *RsMYB28(29)-GFP* fusion plasmid under the control of cauliflower mosaic virus 35S (CaMV 35S) promoter, the coding sequences of *RsMYB28* and *RsMYB29* were cloned into pJIT166-GFP vectors with *Bam*HI/*Xba*I and *Hin*dIII/*Xba*I, respectively. After sequence confirmation, the recombinant plasmids were transformed into onion (*Allium cepa*) epidermal cells using a gene gun (PDS 1000, Bio-Rad, Hercules, CA, USA) (Yang et al., 2012). The *RsMYB28-GFP* and *RsMYB29-GFP* fusion proteins in the transformed onion cells were cultured on Murashige and Skoog medium for 24 h in darkness and observed under a confocal laser scanning microscope (Leica SP2, Leica, Wetzlar, Germany). The images were obtained by automatic recording.

Transactivation activity analysis of *RsMYB28* and *RsMYB29*

The ORF of *RsMYB28* and *RsMYB29* were inserted into the *Nco*I/*Sma*I and *Eco*RI/*Bam*HI cloning sites of the yeast expression vector pGBKT7 to produce *pBD-RsMYB28* and *pBD-RsMYB29*, respectively. Both the controls (positive control: pCL1 and negative control: pGBKT7) and the recombinant plasmids were transformed into *Saccharomyces cerevisiae* strain Y2HGold (Clontech, Palo Alto, CA, USA) (Gao et al., 2012). pCL1 transformants were selected on SD/-Leu medium, while the other transformed yeast strains were plated on SD/-Trp medium. After 3 days, all transformed cell lines were streaked onto SD/-His-Ade and SD/X-α-gal, to investigate the growth response.

Quantitative real-time PCR (RT-qPCR)

Differences in expression patterns of *RsMYB28* and *RsMYB29* were analyzed in various tissues from different radish developmental stages. In addition, the different leaf treatments were analyzed using the SYBR Green Master ROX (Roche, Basel, Switzerland). The amplification reactions were incubated at 95°C for 30 s, followed by 40 cycles of 95°C

for 5 s, 58°C for 15 s, and 72°C for 20 s. The *Actin* gene was used as internal control (Xu et al., 2012). Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The primers for the selected transcripts are shown in Table 1.

Table 1. Primer sequences used in this study.

Primer name	Sequence (5'-3')	Purpose
RsMYB28F1	GGGACCATCACACAATTCATTTCTC	Genomic DNA and cDNA isolation
RsMYB28R1	TTTCTTACTTCTTGCGGTGTCTTA	Genomic DNA and cDNA isolation
RsMYB29F1	GAAGAAGAAGCAAAAAAATGTCAAG	Genomic DNA and cDNA isolation
RsMYB29R1	AACCACGAATTTAACCAGTAAGCAA	Genomic DNA and cDNA isolation
RsMYB28F2	CGCAAGCTTATGTCAAGAAAACCAT	Subcellular localization
RsMYB28R2	TGCTCTAGATATGATTTGCTTCTCG	Subcellular localization
RsMYB29F2	CCCAAGCTTATGTCAAGAAAGGCAT	Subcellular localization
RsMYB29R2	TGCTCTAGAATCTTGGTTCGTATCA	Subcellular localization
RsMYB28F3	CATCCATGGCGAAGAAAATGTCAAG	Transcriptional activation ability
RsMYB28R3	TCCCCCGGGCTCGTTCAGGAAAGTT	Transcriptional activation ability
RsMYB29F3	CCGGAATTCATGTCAAGAAAGGCAT	Transcriptional activation ability
RsMYB29R3	CGCGGATCCCTAATCTTGGTTCGTA	Transcriptional activation ability
RsMYB28F4	CTCTTACATCCACGAACA	RT-qPCR
RsMYB28R4	CTCCTCCGAACTAAACTC	RT-qPCR
RsMYB29F4	GGACCATTGAAGAAGAC	RT-qPCR
RsMYB29R4	CAAGTAGTTAGCCCATCG	RT-qPCR
Actin2/7F	GCATCACACTTTCTACAAC	RT-qPCR
Actin2/7R	CCTGGATAGCAACATACAT	RT-qPCR

RESULTS

Cloning and characterization of *RsMYB28* and *RsMYB29* genes

Using the specific primer pairs *RsMYB28*F1/R1 and *RsMYB29*F1/R1, the genomic DNA and cDNA sequences of *RsMYB28*/*RsMYB29* genes were isolated from radish, respectively. The genomic DNA of *RsMYB28* (Figure 1A) contained three exons and two introns spanning 1836 bp. The *RsMYB28* cDNA clone was 1614 bp in length and consisted of a 354-bp 5'-untranslated region (UTR), 168-bp 3'-UTR, and an ORF of 1092 bp. The ORF encoded 363 amino acids with a calculated molecular mass of 40.8 kDa and a predicted pI of 5.64 (Figure 1B). The genomic DNA of *RsMYB29* spanned 1493 bp, with three exons and two introns (Figure 1A). The full-length *RsMYB29* cDNA was 1093 bp, which was flanked by a 17-bp 5'-UTR and a 53-bp 3'-UTR. *RsMYB29* contained a 1023-bp ORF encoding 340 amino acids with a calculated molecular mass of 38.2 kDa and a pI of 4.67 (Figure 1C).

The amino acid sequence alignment indicated that *RsMYB28* and *RsMYB29* had similar sequence identity with *AtMYB28* and *AtMYB29*, respectively. Our results indicated that *RsMYB28* and *RsMYB29* have two imperfect sequence repeats (R2: W-X_{19}-W-X_{19}-W and R3: F-X_{18}-W-X_{18}-W) at the N-terminal region, whereas there was a low level of sequence conservation at the C-terminal region. *RsMYB28* and *RsMYB29* also had a nuclear localization signal sequence, LKKRL, which is also present in the *AtMYB28* and *AtMYB76* sequences, respectively, followed by an R3 domain (amino acid residues 112-116) (Figure 2).

The phylogenetic tree was constructed based on *RsMYB28*, *RsMYB29*, and 18 MYBs from other plant species (Figure 3). The tree indicated the formation of three different groups; *RsMYB28* and *RsMYB29* were located in the first and second groups, respectively. *RsMYB28* was grouped most closely with *Bju_MYB28-1*, *Bo_MYB28-1*, and *Bra_MYB28-3*, whereas *RsMYB29* grouped with *Bju_MYB29-2*. Thus, they showed close evolutionary relationships with MYB28 and MYB29-like sequences from *Brassica* species, respectively.

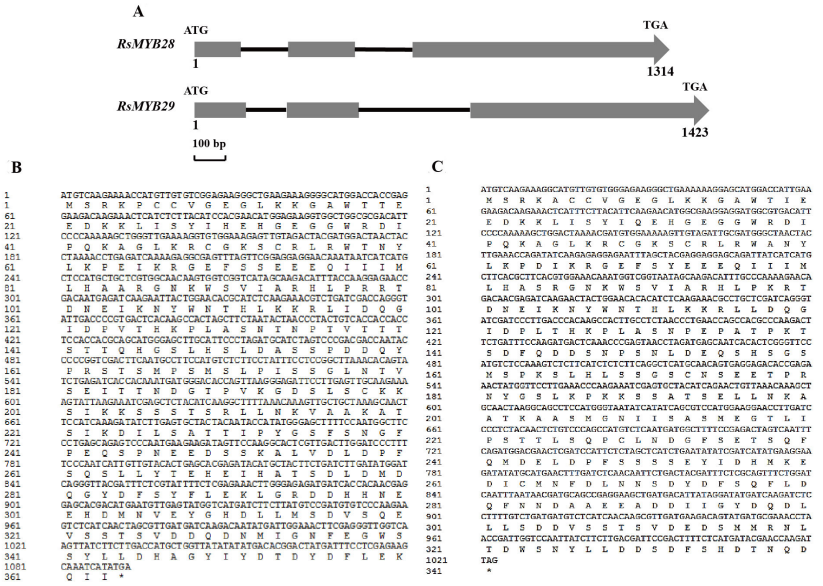

Figure 1. DNA structure and deduced amino acid sequences of *RsMYB28* and *RsMYB29*. **A.** DNA structure of *RsMYB28* and *RsMYB29.* Exons are highlighted in gray boxes and introns by black lines, the start and stop codons are shown for the entire open reading frame. Nucleotide and deduced amino acid sequences of the *RsMYB28* (**B**) and *RsMYB29* (**C**).

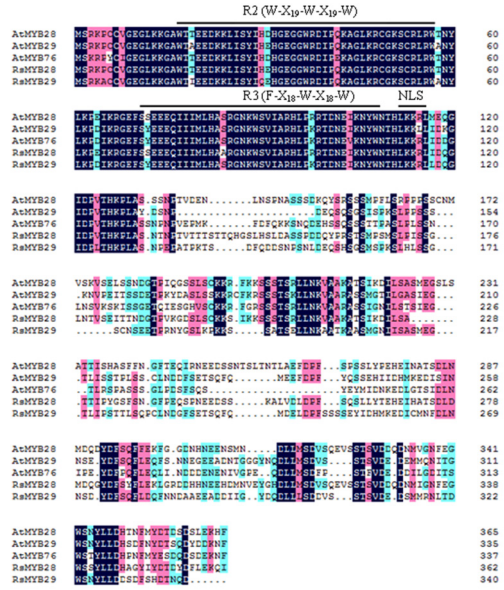

Figure 2. Deduced peptide sequences of RsMYB28 and RsMYB29 and three aliphatic glucosinolate-regulating *Arabidopsis thaliana* MYB proteins: AtMYB28, AtMYB29, and AtMYB76. Their GenBank accession Nos. are NP_196386, NP_200950, and NP_196387, respectively. Features of the sequence include R2 and R3 domains and a putative nuclear localization signal (NLS, LKKRL), which are indicated by solid lines. Purple, blue and pink background represents amino acid identity of 100, 75 and 50%, respectively.

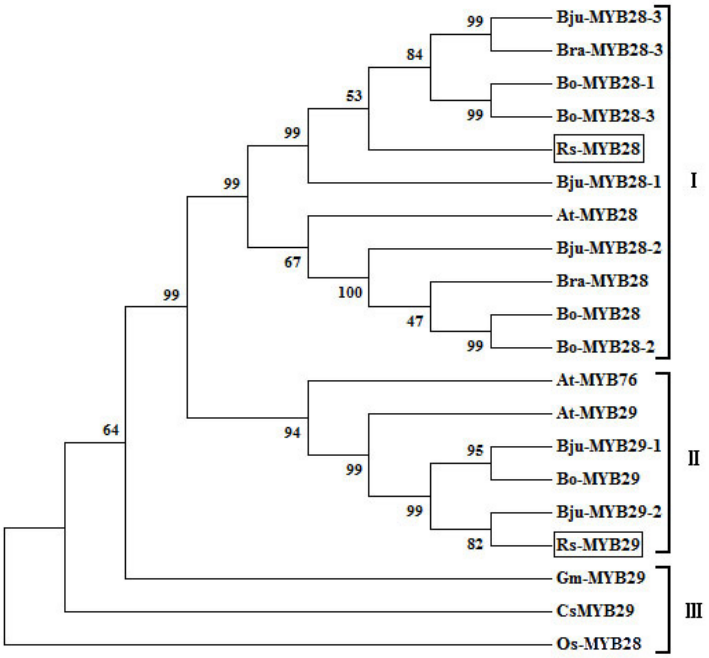

Figure 3. Phylogenetic tree based on *RsMYB28*, *RsMYB29*, and homologous sequences from different plant species. Information about which proteins are included in the tree is presented in **Table S1**. The percentage of replicate trees in which the associated proteins clustered together in the bootstrap test (1000 replicates) is shown next to the branches.

Subcellular localization of RsMYB28 and RsMYB29

Using predict protein software (http://www.predictprotein.org/), we found that the RsMYB28 and RsMYB29 proteins had a conserved SV40-type putative nuclear localization motif, LKKRL, which is a nucleus-localized protein. To examine the subcellular localization of RsMYB28 and RsMYB29 in living cells, the recombinant constructs of *Pro35S:RsMYB28-GFP* and *Pro35S:RsMYB29-GFP* fusion genes were introduced into onion epidermal cells by particle bombardment. As compared with the GFP-positive control vector (Figure 4A), both *RsMYB28-GFP* and *RsMYB29-GFP* transiently transformed cells showed a strong green fluorescence signal in the nucleus (Figure 4B and C), implying that they might be localized in the nucleus.

Transcriptional activation of *RsMYB28* and *RsMYB29*

To investigate the transcriptional activity of *RsMYB28* and *RsMYB29*, they were fused into the pGBKT7 vector (Figure 5A and B) and then transformed into the yeast strain Y2HGold (Clontech). As shown in Figure 5C and D, the yeast cells co-transformed with pCL1 (positive control), *pGBKT7-RsMYB28*, and *pGBKT7-RsMYB29* grew normally in SD/-His-Ade medium and SD/X-α-gal medium with a clear blue polyline (Figure 5D and E). In contrast,

the co-transformed yeast cells containing pGBKT7 (the negative control) failed to grow on both SD/-His-Ade and SD/X-α-gal media. These results indicated that both the *RsMYB28* and *RsMYB29* genes have transcriptional activity in the yeast cell. This transcriptional activity may be indispensable for the roles that these genes play in regulating gene expression in radish.

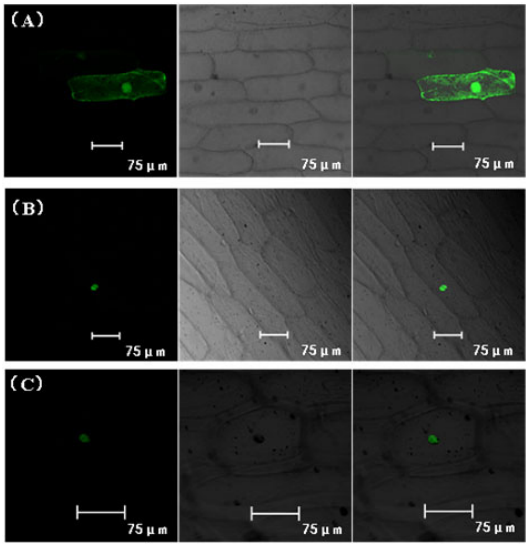

Figure 4. Subcellular localization of RsMYB28 and RsMYB29 proteins in onion (*Allium cepa*) epidermis cells. Onion epidermis cells transformed with GF-positive control vector (**A**), *Pro35S:RsMYB28-GFP* (**B**), and *Pro35S:RsMYB29-GFP* (**C**).

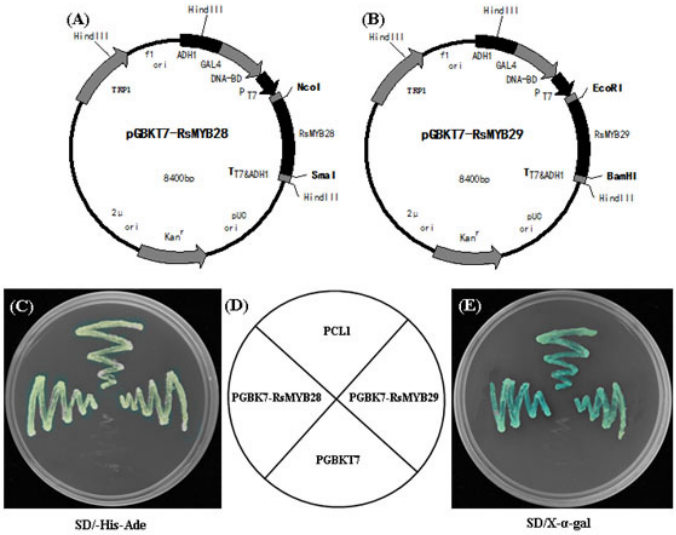

Figure 5. Structure of the *RsMYB28* (**A**) and *RsMYB29* (**B**) vectors; growth of transformed yeast cells grown on SD without histidine and adenine (**C**); position of each yeast strain (**D**); and X-α-gal activity (**E**).

Expression analysis of *RsMYB28* and *RsMYB29* in response to different treatments

RT-qPCR was carried out to analyze the dynamic expression patterns of *RsMYB28* and *RsMYB29*. The differential expression patterns of *RsMYB28* and *RsMYB29* in different organs at three main developmental stages, compared with the constitutively expressed *Actin* gene, are shown in Figure 6. *RsMYB28* and *RsMYB29* were expressed in most of the tissue types and showed similar expression patterns. In the stem, the expression of *RsMYB28* and *RsMYB29* at the taproot thickening stages was 13.6- and 97-fold higher than that at the mature stage, respectively.

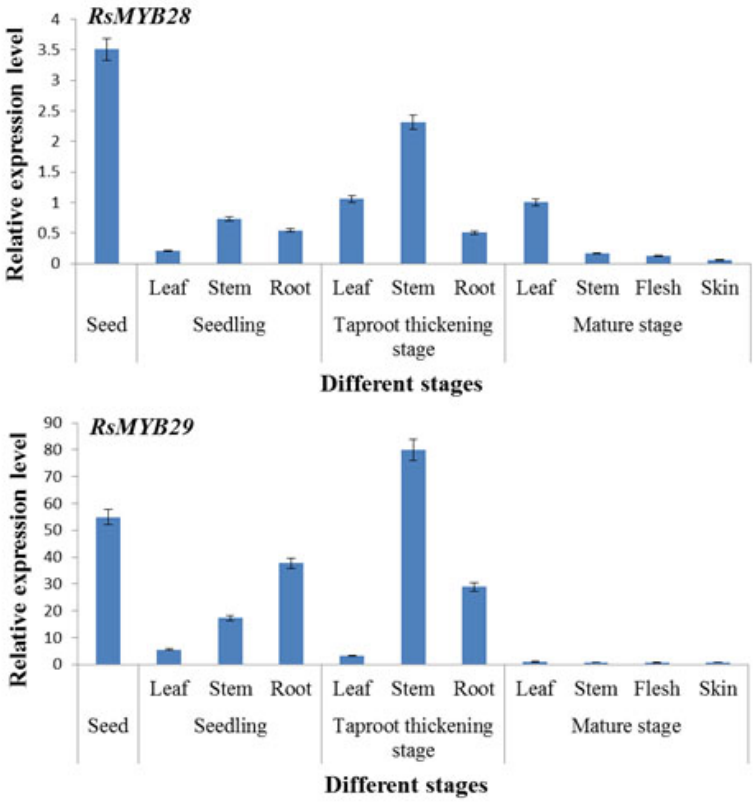

Figure 6. Expression levels of *RsMYB28* and *RsMYB29* genes in different tissues at different developmental stages. *Actin2/7* was used as a constitutively expressed *Actin* gene. Independent data were analyzed in triplicate, and the error bars represent standard deviations.

The expression of *RsMYB28* and *RsMYB29* under the different treatments wounding, MeJA, ABA, and glucose were profiled using RT-qPCR (Figure 7). In the wounding treatment, the expression level of the *RsMYB28* gene showed a gradual increase until 24 h, whereas the *RsMYB29* gene dramatically increased at 4 h followed by a strong decrease at 6 h. In the MeJA treatment, the expression level of *RsMYB29* steadily increased with a peak at 8 h, followed by a steady decrease. In contrast, *RsMYB28* expression remained constant. Under

ABA stress, the *RsMYB28* gene showed a dramatic rise in expression level with a peak at 8 h, followed by a decrease at 10 h. *RsMYB29* expression was not markedly affected by ABA stress. In the case of the exogenous glucose treatment (using sorbitol as osmotic control), the expression level of *RsMYB28* dramatically increased after 4 h, with a peak at 8 h, followed by a decrease. In contrast, *RsMYB29* only showed a slight increase at 6 h. Thus, the expression of *RsMYB28* appeared to be induced in the wounding, glucose, and ABA treatments, whereas *RsMYB29* expression was triggered in the wounding and MeJA treatments. This indicates that the regulatory role of *RsMYB28* may be greater than that of *RsMYB29* under some treatments.

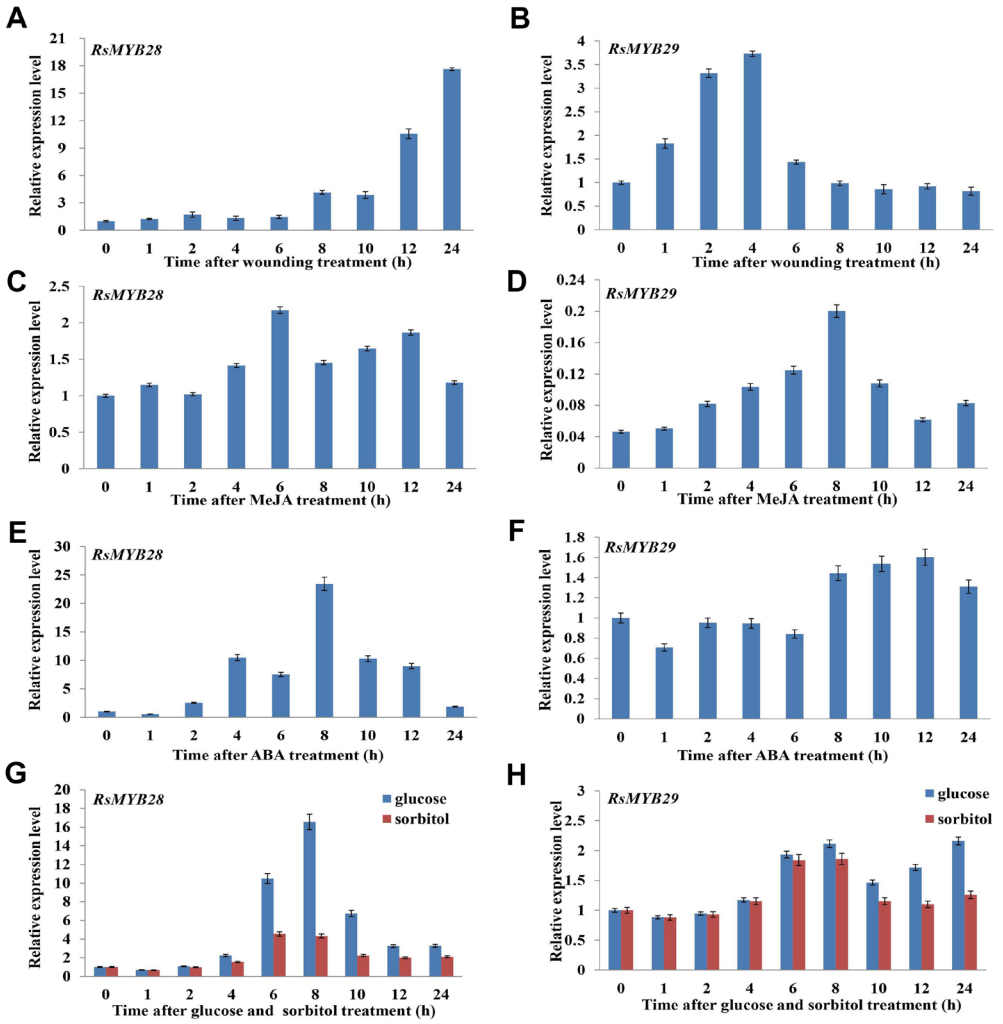

Figure 7. Expression patterns of *RsMYB28* and *RsMYB29* subjected to various treatments in radish, as assayed by RT-qPCR. **A.** and **B.** Wounding treatment, **C.** and **D.** treated with 100 μM methyl jasmonate (MeJA), **E.** and **F.** treated with 100 μM abscisic acid (ABA), and **G.** and **H.** treated with 100 μM glucose. *Actin2/7* was used as a constitutively expressed *Actin* gene. Independent data were analyzed in triplicate and the error bars represent the standard deviations.

DISCUSSION

Genomic DNA and phylogeny of *RsMYB28* and *RsMYB29* genes

Two R2R3-MYB transcriptional factors, *MYB28* and *MYB29*, along with *MYB76*, have been identified as regulators of aliphatic GSL biosynthesis in *A. thaliana*, *B. juncea*, and *B. rapa* (Hirai et al., 2007; Gigolashvili et al., 2007b, 2008; Li et al., 2013). However, there has been no study on the *MYB28* and *MYB29* coding sequences in radish. In this study, the genomic DNA and cDNA of *RsMYB28* and *RsMYB29* were isolated. Like the *BjuMYB28* gene of other R2R3-MYB members (Augustine et al., 2013a), both genes were found to be conserved with three separated exons and two introns (Figure 1). The amino acid sequence alignment showed that both *RsMYB28* and *RsMYB29* contained two highly conserved domains, and showed high homology with *AtMYB28* and *AtMYB29*, respectively. This indicates a potential similarity in function (Figure 2). In contrast, the RsMYB28 and RsMYB29 proteins showed structural variation compared with the AtMYB28, AtMYB29, and AtMYB76 proteins in C-terminal region, which will lead to differential gene functions in the regulation of the aliphatic GSL biosynthesis.

Our phylogenetic analysis showed that *RsMYB28* clustered together with *MYB28* from different Brassicaceae species, whereas *AtMYB76* clustered with *RsMYB29* (Figure 3). This is in line with what has previously been reported (Araki et al., 2013). A previous comparison of the *R. sativus* genome structure with that of the A, B, and C genomes of *Brassica* species revealed extensive chromosome homology among Brassicaceae species (Li et al., 2011). In our study, the RsMYB28 and RsMYB29 proteins were grouped with the Bju_MYB28s and Bju_MYB29s proteins, respectively, indicating that *RsMYB28* and *RsMYB29* have a high level of sequence conservation with their homologues from the *B. juncea* genomes. Silencing of the *BjMYB28* transcription factor gene caused a reduction in the GSL content in *B. juncea* (Augustine et al., 2013b). *BoMYB29* has been shown to be a regulator for increasing methylsulfinyl GSL content in *B. oleracea* (Araki et al., 2013). Therefore, these two genes may have similar biological functions in Brassicaceae species in the aliphatic GSL biosynthesis.

Subcellular localization and transactivation analysis of *RsMYB28* and *RsMYB29*

The subcellular localization of MYB28 in *A. thaliana* and *B. juncea* was found to be in the nucleus (Gigolashvili et al., 2007b; Augustine et al., 2013a). Our GFP transient expression indicated that RsMYB28 and RsMYB29 were both located in the nuclei in onion epidermal cells and also possessed transcriptional activity. This may be indicative of an expression-regulating function in the nucleus (Figures 4 and 5). Previous studies have indicated that *AtMYB28* is an important regulator and *AtMYB29* only plays a minor role, whereas *AtMYB76* has an accessory role (Gigolashvili et al., 2009; Sønderby et al., 2010).

Expression levels of *RsMYB28* and *RsMYB29* at different stages and in different organs

MYB28 and *MYB29* showed high expression levels in the flowers, seeds, and stems, but the expression was dramatically different in the stem when compared with that in other Chinese cabbage organs (Kim et al., 2013). In our study, the expression of *RsMYB28* and *RsMYB29* could be detected in all examined tissues including seeds, roots, stems, and leaves,

especially in seeds and stems (Figure 6), which is in agreement with a previous study reporting that *RsMYB28* and *RsMYB29* exhibited high expression level in the stem, and seed (Kim et al., 2013). Augustine et al. (2013a) reported that the expression levels of four *BjuMYB28* homologues were higher in seedlings, stems, and siliques than in roots and primary leaf tissues in *B. juncea*. In addition, an expression analysis using RNA-seq in sugar beet revealed that the highest number of *BvMYB* genes was expressed in inflorescences, followed by seedlings, taproots, seeds, and young leaves (Stracke et al., 2014). In the present study, both *RsMYB28* and *RsMYB29* exhibited high expression levels at the seedling and taproot thickening stage. Therefore, it could be suggested that *RsMYB28* and *RsMYB29* may play crucial roles in radish growth.

Expression patterns of *RsMYB28* and *RsMYB29* under various treatments

GSLs are important secondary metabolites for plant defense (Grubb and Abel, 2006). Various environmental stimuli such as wounding, herbivore attacks, MeJA, salicylic acid (SA), and 1-aminocyclopropane (ACC) have been reported to influence GSL metabolism (Mikkelsen et al., 2003). Interestingly, several GSL biosynthetic regulatory genes were found to be up-regulated by MeJA, SA, and ACC (Gigolashvili et al., 2007b, 2009; Sønderby et al., 2010). In *A. thaliana*, *MYB28* and *MYB29* play a vital role in aliphatic GSL biosynthesis (Gigolashvili et al., 2009). Our results showed that the expression level of *RsMYB28* increased quickly in response to wounding. *RsMYB29* showed a transient induction following wounding, as was also shown in *MYB28*, *MYB29*, and the aliphatic GSL biosynthetic genes *BCAT4* and *MAM1* in *A. thaliana* (Schuster et al., 2006). The spatial expression patterns of the *RsMYB28* and *RsMYB29* genes suggested that they might play a synergistic action in resistance against herbivores (Figure 7A and B). The expression of *RsMYB29* was up-regulated by MeJA treatment, whereas that of *RsMYB28* remained unaffected (Figure 7C and D). This result is in accordance with the *MYB28* and *MYB29* expression patterns found for aliphatic GSL synthesis regulation in Chinese cabbage after MeJA treatment (Zang et al., 2015).

There is little known about *MYB28* and *MYB29* induction after ABA treatment. Under drought, salt, or cold conditions, ABA is often regarded as the primary signal for plant resistance against abiotic stress (Shinozaki and Yamaguchi-Shinozaki, 2007). In this study, *RsMYB28* was significantly induced by ABA treatment, whereas *RsMYB29* was not affected (Figure 7E and F), implying that *RsMYB28* may be involved in plant defense against abiotic stress.

In addition, compared with sorbitol, glucose can greatly induce aliphatic GSLs through MYB transcription factors (Gigolashvili et al., 2007b; Miao et al., 2013). In this study, the expression level of *RsMYB29* was lower than that of *RsMYB28*, suggesting a predominating role of *RsMYB28* for regulation of aliphatic GSL biosynthesis by glucose induction (Figure 7G and H). Furthermore, the expression of *RsMYB28* showed a 16.6-fold increase after 8 h of glucose treatment, which is consistent with previous findings in *A. thaliana* subjected to glucose treatment (Gigolashvili et al., 2007b).

In the present study, genomic DNA and cDNA of *RsMYB28* and *RsMYB29* genes were isolated and characterized in radish. The *RsMYB28* and *RsMYB29* were found to be localized in the nucleus and possessed transactivation activity. Both the *RsMYB28* and *RsMYB29* genes were strongly expressed at the seedling and taproot thickening stages, and their expression patterns were up-regulated after wounding. The *RsMYB28* gene was induced by ABA and glucose, whereas *RsMYB29* was induced only by MeJA. It could be concluded that the

RsMYB28 and *RsMYB29* genes play a synergistic role in the resistance against biotic and abiotic stressors through different signaling pathways. These findings provide useful information for functional analysis of the R2R3-MYB transcription factor family, and facilitate the dissection of the molecular regulatory mechanisms underlying the GSL biosynthesis in radish.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

Table S1. Protein information from NCBI used for construction of the phylogenetic tree.