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ORIGINAL RESEARCH PAPER

Enhancement of rutin in *Fagopyrum esculentum* hairy root cultures by the *Arabidopsis* transcription factor AtMYB12

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Abstract Common buckwheat (*Fagopyrum esculentum* Moench) is rich in phenolic compounds and may be useful for the treatment of metabolic syndrome in humans. To improve the production of rutin in buckwheat, we overexpressed the flavonol-specific transcription factor, AtMYB12 using *Agrobacterium rhizogenes* into hairy root culture systems. This induced the expression of flavonoid biosynthetic genes encoding phenylalanine ammonia lyase, cinnamate 4-hydroxylase, 4-coumarate:CoA ligase, chalcone synthase, chalcone isomerase, flavone 3-hydroxylase, flavonoid 3′-hydroxylase, and flavonol synthase. This led to the accumulation of rutin in buckwheat hairy roots up to 0.9 mg/g dry wt. PAP1 expression, however, did not correlate with the production of rutin.

Keywords Buckwheat · Flavonoids · Hairy root · Rutin · Transcription factor

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Introduction

Many plants contain various antioxidant components, such as phenolic compounds, with flavonoids being the most common and widely distributed group of plant phenolics. Apart from being rich in phenolic compounds, the seeds of common buckwheat (*Fagopyrum esculentum* Moench), in particular, are a good source of rutin (quercetin-3-rutinoside)—a flavonol glycoside that protects plants from ultraviolet radiation and diseases (Hinneburg and Neubert 2004; Kalinova et al. 2006). Rutin is an antioxidant with many pharmacologically properties in humans, such as anti-inflammatory, anticarcinogenic, antithrombotic, cytoprotective, and vasoprotective effects (Li and Zhang 2001; Kreft et al. 2002). Rutin possesses health-promoting effects, such as attenuating the metabolic syndrome induced by high-carbohydrate/high-fat diets (Panchal et al. 2011). Buckwheat could, thus,

be an important industrial source of rutin; a product that should be considered for the treatment of metabolic syndrome in humans.

Various strategies can be employed to increase the production of polyphenolic compounds. For example, overexpression of genes encoding individual biosynthetic enzymes can lead to an increase in the levels of a target compound (Park et al. 2011). This method, however, cannot induce the entire biosynthetic pathway, and, although there is an increase in the amount of end products, this is not enough for the purpose of practical application. An alternative method could use the regulatory genes that directly induce these pathways. For example, members of the MYB transcription factor family are involved in secondary metabolism. *AtMYB75/PAP1* and *AtMYB90/PAP2* control biosynthesis in vegetative tissues (Borevitz et al. 2000; Gonzalez et al. 2008), while *AtMYB123* controls the biosynthesis of proanthocyanidins in the seed coat of *Arabidopsis* (Lepiniec et al. 2006). Moreover, *AtMYB12* is a flavonol-specific activator of flavonoid biosynthesis and a transcriptional regulator of chalcone synthase (CHS) and flavonol synthase (FLS), which are indispensible for the biosynthesis of flavonol (Mehrtens et al. 2005). *AtMYB12* could also activate the caffeoylquinic acid (CQA) biosynthetic pathway, when expressed in a tissue-specific manner in tomato (Luo et al. 2008).

Because Luo et al. (2008) indicated that transcription factors might have different specificities for target genes in different plants, we hypothesized that it might be possible to accumulate high levels of rutin in buckwheat, through the overexpression of *AtMYB12* and *AtMYB75/PAP1*. In this study, the hairy root culture system was used to observe the activity of transcription factors. Using transgenic buckwheat hairy roots, we show that flavonoid biosynthetic genes are, indeed, regulated by transcription factors.

Materials and methods

Seed sterilization and germination

De-hulled seeds from common buckwheat were surface-sterilized with 70% (v/v) ethanol for 1 min and 4% (v/v) sodium hypochlorite solution for 10 min. They were then rinsed thrice in sterilized water. Six seeds were placed on 25 ml agar-solidified culture medium in Petri dishes (100 × 15 mm^2). The basal medium consisted of MS medium (Murashige and Skoog 1962), solidified with 0.8% (w/v) agar. Prior to addition of agar, the medium was adjusted to pH 5.8, and the complete medium was then sterilized by autoclaving at 121°C for 20 min. The seeds germinated in a growth chamber at 25°C under standard, cool, white fluorescent tubes with a flux rate of 35 μmol/(sm^2) and a 16 h photoperiod.

Total RNA extraction and quantification of gene expression

Total RNA was isolated from different lines of transgenic buckwheat hairy roots using the RNeasy Plant Mini Kit (Qiagen; Valencia, CA, USA). For qRT-PCR, 1 μg total RNA was reverse-transcribed using the Superscript II First Strand Synthesis Kit (Invitrogen; Carlsbad, CA, USA) and oligo $(dT)_{20}$ primers.

Transcription levels were analyzed by real-time PCR. The gene-specific primer sets were designed for real-time PCR, as previously described (Li et al. 2010) (Supplementary Table 1). Gene expression was normalized to that of the histone H3 gene, which, in this case, was used as a housekeeping gene (Timotijevic et al. 2010). Real-time PCR was performed in triplicate on a MiniOpticon system (Bio-Rad Laboratories; Hercules, CA) with the Quantitect SYBR Green PCR Kit (Qiagen). The PCR protocol was as follows: denaturation for 5 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C, annealing for 15 s at 56°C, and elongation for 20 s at 72°C. The results of PCR were calculated as the mean of 3 replicate treatments. Statistical differences between treatments were evaluated by calculating the standard deviation.

Plasmid construction for the transformation of buckwheat hairy roots

The *PAP1* and *AtMYB12* overexpression constructs consisted of a cauliflower mosaic virus (CaMV) 35S promoter, the respective gene sequences, and the *bar* (*bialaphos resistance*) gene as a selectable marker. PCR was used to amplify the full-length sequences of *PAP1* and *AtMYB12*, which were previously published (Borevitz et al. 2000; Mehrtens et al. 2005). The PCR primers and cloning vectors used for these experiments are shown in Supplementary Table 1. The

recombinant genes were PCR amplified with gene specific primer sets, according to the Gateway cloning strategy (Karimi et al. 2002). After a second round of PCR using adapter primer sets, each amplified product was subcloned into the pDONR221 vector (Invitrogen) by recombination. These vectors were then transformed into the *E. coli* strain TOP10 (Invitrogen), and clones were selected with 50 mg/l kanamycin. Finally, the inserts were shuttled from the entry vectors into the pB7FWG2 binary destination vector (obtained from the Functional Genomics unit of the Department of Plant System Biology; VIB-Ghent University) using Gateway LR Clonase II Plus Enzyme Mix (Invitrogen). The clones were transformed in the *E. coli* strain TOP10 (Invitrogen) and selected with 50 mg spectinomycin/l.

Transformation of buckwheat hairy roots

The resulting binary vectors, pPAP1 and pAtMYB12, were transformed in the *Agrobacterium rhizogenes* strain, R1000. A β-glucuronidase overexpression vector (pGUS) was used for the control hairy roots. Young stems of common buckwheat were collected from the plants grown in vitro and cut at the ends into 7 mm sections. The excised stems were dipped into the *A. rhizogenes* R1000 cultures in liquid inoculation medium for 10 min, blotted dry on sterile filter paper, and incubated in the dark at 25°C on agar-solidified MS medium. After 2 days of co-cultivation, the explanted tissues were transferred to a hormone-free medium containing MS salts and vitamins (0.5 mg nicotinic acid/l, 0.5 mg pyridoxine–HCl/l, 0.1 mg thiamine-HCl/l, and 2 mg glycine/l), 30 g sucrose/l, 500 mg cefotaxime/l, 1 mg glufosinate/l and 8 g agar/l. Numerous hairy roots were observed emerging from the wound sites within 2 weeks. The hairy roots were separated from the explanted tissues and subcultured in the dark at 25°C on agar-solidified MS medium. After repeated transfer to fresh medium, rapidly growing hairy root cultures were obtained. Isolated roots (0.5 g/l) were transferred to 30 ml MS liquid medium, containing 30 g sucrose/l, in 100 ml flasks. The root cultures were maintained at 25°C on a shaker (100 rev/min) in a growth chamber under standard, cool, white fluorescent tubes with a flux rate of 35 μmol/(s/m^2) and a 16 h photoperiod. After 30 days, hairy roots were harvested and their dry weight and phenolic compound content was determined. Three flasks were used for every culture condition, and the experiments were performed in duplicate.

Quantitative analysis of rutin using HPLC

Rutin was determined by HPLC. The extract was filtered through a 0.45 μm poly-filter, diluted twofold with methanol. Analysis used a C18 column (250 × 4.6 mm^2, 5 μm) at 30°C with a gradient of 0.15% acetic acid and methanol as elutant at 1 ml/min. Detection was at 280 nm. The results were quantified using rutin from Sigma as standard.

Results

Establishment of transgenic hairy root lines

Explants of buckwheat stems were infected with *A. rhizogenes* strain R1000 harboring the pPAP1, pAtMYB12, or pGUS (β-glucuronidase, for control hairy roots) binary vector. The transgenic hairy root cultures were examined by PCR, with primers for the *bar* (*bialaphos resistance*) gene and the *rol* genes (data not shown). The *rol* genes of the Ri-plasmid in *A. rhizogenes* are responsible for the induction of hairy roots from plant species. By using qRT-PCR to measure the expression levels of *PAP1* or *AtMYB12* in the transgenic hairy root lines and *GUS*-control hairy root lines, 6 *PAP1*-overexpressing (P) and 6 *AtMYB12*-overexpressing (M) hairy root lines were identified. *PAP1*-overexpressing hairy root lines were designated as P-1, 3, 4, 6, 7, and 8, while *AtMYB12*-overexpressing (M) hairy root lines were designated as M-1, 4, 7, 8, 10, and 14 (Figs. 1a, 2a).

Effect of AtMYB12 on the expression levels of flavonoid biosynthetic genes

To analyze the effect of AtMYB12 on the expression of flavonoid biosynthesis genes in buckwheat hairy roots, real-time qRT-PCR analyses were carried out. Figures 1b and 2b depict the results of the qRT-PCR analyses. AtMYB12 was strongly overexpressed in AtMYB12-overexpressing hairy root lines with the relative expression levels ranging from 35 to 60-fold more than that of GUS-overexpressing hairy root lines. The flavonoid biosynthetic genes encoding phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase

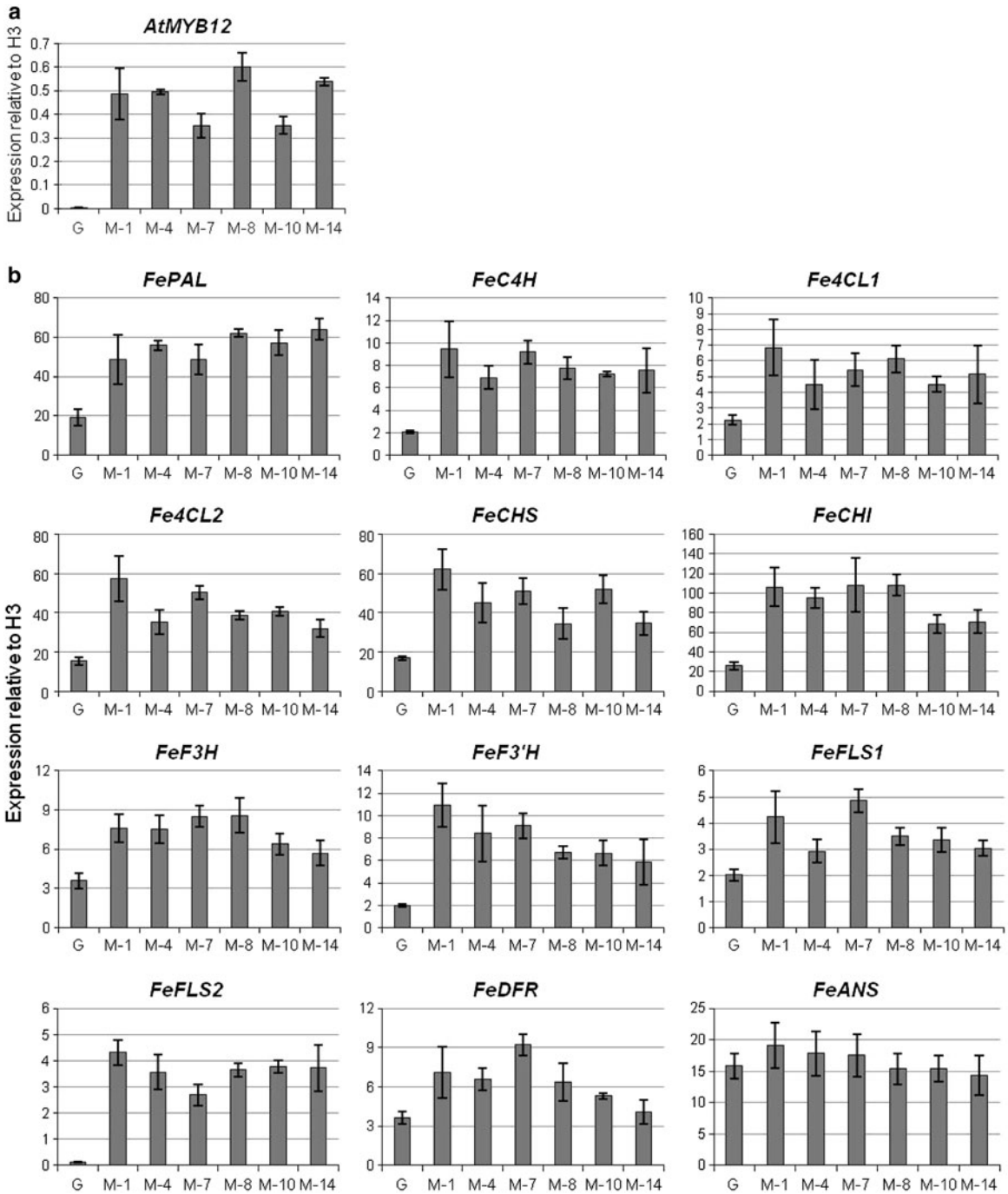

Fig. 1 Analysis of gene expression in buckwheat hairy root lines overexpressing *AtMYB12*. **a** The expression level of *AtMYB12* relative to H3 in buckwheat hairy roots overexpressing *AtMYB12* was confirmed by quantitative real-time PCR. **b** The expression level of flavonoid biosynthetic genes (*FePAL*, *FeC4H*, *Fe4CL*, *FeCHS*, *FeCHI*, *FeF3H*, *FeF3′H*, *FeFLS*, *FeDFR*, and *FeANS*) relative to H3 in buckwheat hairy roots overexpressing *AtMYB12* was confirmed by quantitative real-time PCR. The top ends of the *bars* and the *error bars* indicate the mean and standard deviation ($n = 3$), respectively. *G* a control hairy root line transformed with the β-glucuronidase (GUS) overexpression vector pGUS; *M-n* transgenic hairy root lines ("*n*" indicates the *line number*) transformed with the AtMYB12 overexpression vector, pAtMYB12

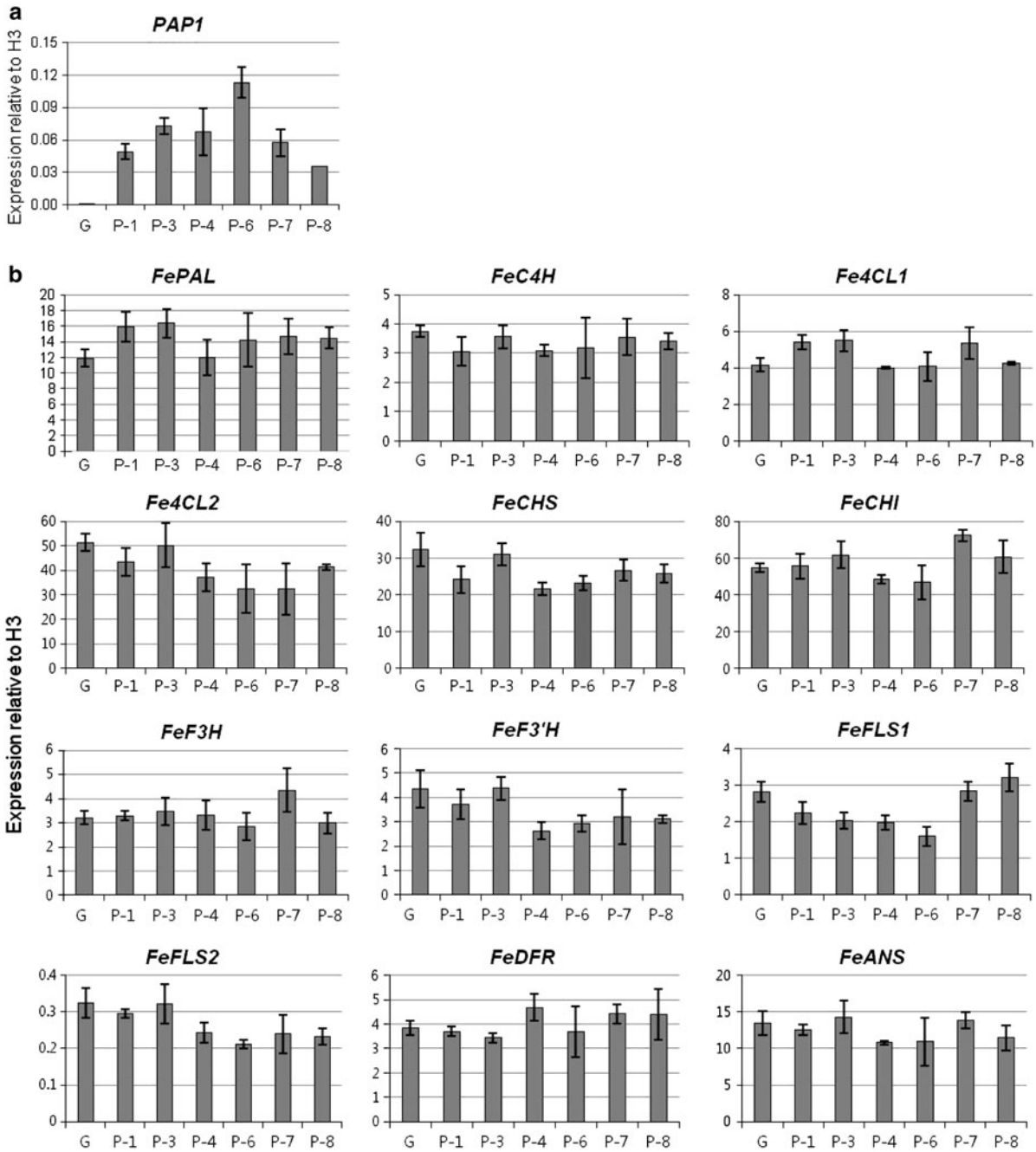

Fig. 2 Analysis of gene expression in buckwheat hairy root lines overexpressing *PAP1*. **a** The expression level of *PAP1* relative to H3 in buckwheat hairy roots overexpressing *PAP1* was confirmed by quantitative real-time PCR. **b** The expression level of flavonoid biosynthetic genes (*FePAL*, *FeC4H*, *Fe4CL*, *FeCHS*, *FeCHI*, *FeF3H*, *FeF3′H*, *FeFLS*, *FeDFR*, and *FeANS*) relative to H3 in buckwheat hairy roots overexpressing *PAP1* was confirmed by quantitative real-time PCR. The top ends of the *bars* and the *error bars* indicate the mean and standard deviation ($n = 3$), respectively. *G* a control hairy root line transformed with the β-glucuronidase (GUS) overexpression vector pGUS; *P-n* transgenic hairy root lines ("*n*" indicates the *line number*) transformed with the PAP1 overexpression vector, pPAP1

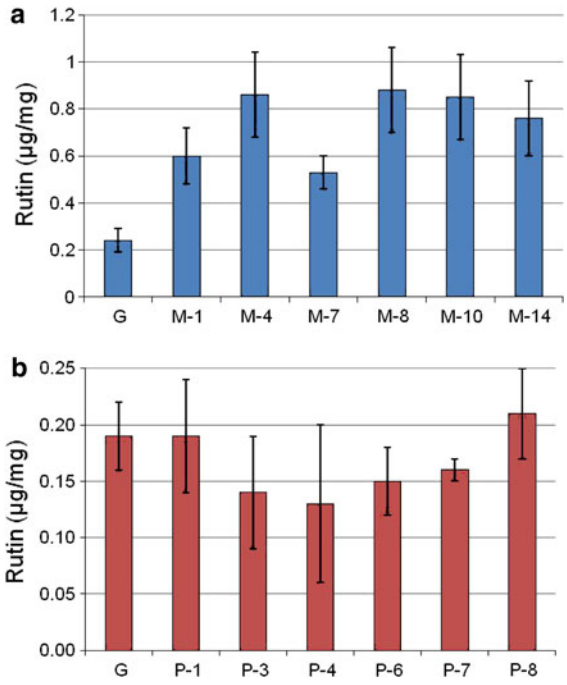

Fig. 3 Rutin production in buckwheat hairy root lines overexpressing *AtMYB12* and *PAP1*. Production of rutin in **a** *AtMYB12*- and **b** *PAP1*-overexpressing hairy root lines was analyzed by HPLC. The top ends of the *bars* and the *error bars* indicate the mean and standard deviation ($n = 3$), respectively. *G* a control hairy root line transformed with the β-glucuronidase (GUS) overexpression vector pGUS; *M-n* and *P-n* transgenic hairy root lines ("*n*" indicates the *line number*) transformed with the AtMYB12- and PAP1-overexpression vectors pAtMYB12 and pPAP1, respectively

(4CL) 1 and 2, CHS, chalcone isomerase (CHI), flavone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H) and flavonol synthase (FLS) 1 and 2 showed a correlation to the AtMYB12 expression level. In general, the expression level of flavonoid biosynthetic genes (*FePAL*, *FeC4H*, *Fe4CL1*, *Fe4CL2*, *FeCHS*, *FeCHI*, *FeF3H*, *FeF3′H*, *FeFLS1*, and *FeFLS2*) increased in response to elevated AtMYB12 expression. However, the expression level of *FeDFR* and *FeANS* was more or less unaffected by changes in AtMYB12 expression; *FeDFR* expression increased slightly in AtMYB12-overexpressing hairy root lines.

We also analyzed the effect of PAP1 on the expression of flavonoid biosynthesis genes in buckwheat hairy roots. qRT-PCR analysis showed that PAP1 was overexpressed in PAP1-overexpressing hairy root lines, with the relative expression levels ranging from 50 to 100-fold more than that of GUS-overexpressing hairy root lines. However, the flavonoid biosynthetic genes did not seem to be correlated to PAP1 expression level. The expression level of all flavonoid biosynthetic genes analyzed in this study was unchanged in PAP1-overexpressing hairy root lines.

Effect of AtMYB12 on rutin levels

To investigate the effect of AtMYB12 and PAP1 on the levels of rutin, the AtMYB12- and PAP1-overexpressing hairy roots were analyzed by HPLC (Fig. 3). High levels of rutin were detected in the hairy roots lines expressing AtMYB12, compared to the control hairy roots expressing GUS. AtMYB12-overexpressing hairy root lines produced 2.2–3.7 times more rutin (0.53–0.88 μg/mg) than the GUS-control hairy root line. In contrast, no significant difference between the control and PAP1-overexpressing hairy root lines could be detected by HPLC analysis.

Discussion

The MYB transcription factor family is widely distributed in higher plants. MYB family members have been found to be involved in diverse physiological and biochemical processes, including the control of cell morphogenesis and response to environmental stress and phytochromes, as well as the regulation of secondary metabolism (Du et al. 2009).

This study demonstrates that the expression of the transcription factor AtMYB12, together with AtMYB75/PAP1 expression, enhances the levels of rutin in buckwheat hairy roots. AtMYB12 was originally identified in *Arabidopsis*, where its overexpression resulted in increased accumulation of flavonol (Mehrtens et al. 2005). AtMYB12 induced gene expression in both flavonol and CQA biosynthetic pathways in tomato as well as in the flavonoid biosynthetic pathway in tobacco. In the tomato fruit, in particular, high levels of flavonol and CQA accumulated as a result of AtMYB12 overexpression (Luo et al. 2008). In our study, AtMYB12 induced the expression of flavonoid biosynthetic genes such as the PAL gene, which was not a target of AtMYB12 in *Arabidopsis* (Mehrtens et al. 2005), but was an AtMYB12 target in both tobacco and tomato (Luo et al. 2008). In PAP1-overexpressing buckwheat

hairy roots, rutin levels and gene expression in the flavonoid biosynthetic pathway did not increase by PAP1 expression. PAP1 was reported to be an anthocyanin activator in *Arabidopsis*, and its overexpression upregulated many transcripts involved in the formation of anthocyanins (Borevitz et al. 2000; Tohge et al. 2005).

Although AtMYB12 resulted in significant enhancement of rutin levels in transgenic buckwheat hairy roots, more effective engineered metabolism is needed to identify transcription factors from buckwheat, and to ensure their appropriate specificity in this plant. We are now working to determine and characterize such transcription factors from buckwheat, with the ultimate aim to be able to increase rutin production in buckwheat.

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