A transcriptional complex of FtMYB102 and FtbHLH4 coordinately regulates the accumulation of rutin in *Fagopyrum tataricum*

Running title: Transcriptional complex FtMYB102 and FtbHLH4 regulates rutin accumulation

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Abstract

Tartary buckwheat is rich in flavonoids, which not only play an important role in plant-environment interaction, but are also beneficial to human health. Rutin is a therapeutic flavonol which is massively accumulated in Tartary buckwheat. It has been demonstrated that transcription factors control rutin biosynthesis. However, the transcriptional regulatory network of rutin is not fully clear. In this study, through transcriptome and target metabolomics, we validated the role of FtMYB102 and FtbHLH4 TFs at the different developmental stages of Tartary buckwheat. The elevated accumulation of rutin in the sprout appears to be closely associated with the expression of FtMYB102 and FtHLH4. Yeast two-hybrid,

transient luciferase activity and co-immunoprecipitation demonstrated that FtMYB102 and FtbHLH4 can interact and form a transcriptional complex. Moreover, yeast one-hybrid showed that both FtMYB102 and FtbHLH4 directly bind to the promoter of chalcone isomerase (*CHI*), and they can coordinately induce *CHI* expression as shown by transient luciferase activity assay. Finally, we transferred the FtMYB102 and FtbHLH4 into the hairy roots of Tartary buckwheat and found that they both can promote the accumulation of rutin. Our results indicate that FtMYB102 and FtbHLH4 can form a transcriptional complex by inducing *CHI* expression to coordinately promote the accumulation of rutin.

Keywords: Tartary buckwheat (*Fagopyrum tataricum*), secondary metabolism, rutin, transcription factors, transcriptional regulation, medicinal plants

INTRODUCTION

Flavonoids are major constituents of polyphenol in plant secondary metabolites. They consist mainly of anthocyanins, proanthocyanidins (PAs), flavonols, and flavones, which are present in almost all higher plants

(Williams and Grayer, 2001). Flavonoids play critical roles in plant–environment interactions, including those involved in being uvioresistant, anti-herbivore and anti-pathogen (Emiliani *et al.* 2013; Barbehenn *et al*. 2011). Additionally, they also contribute to human nutrition and health (Williams *et al*. 2004). Rutin (quercetin-3-O-rutinoside), known as vitamin P, is a therapeutic flavonol bearing cytoprotective effects (Negahdari *et al*. 2021), which is massively accumulated in the seeds and leaves of Tartary buckwheat (Jiang *et al*. 2007). Previous studies have primarily focused on seeds, with few investigations on seedlings. The gene encoding flavonoid biosynthetic enzymes for the major flavonoid skeleton has been identified decades ago. Briefly, the pathway begins with phenylalanine and forms an important intermediate product, naringenin chalcone. Several key enzymes catalyze this precursor, such as phenylalanine aminolyase (PAL), cinnamic acid-4-hydroxylase (C4H), 4-coumarate-CoA ligase (4CL), and chalcone synthase (CHS) (Russell, 1971; Dixon & Lamb, 1979; Koes *et al*. 1989). Then, chalcone isomerase (CHI) catalyzes the resulting naringenin chalcone to form naringenin (Mona & Christopher, 1987). Furthermore, naringenin can be converted to flavones under the action of flavone synthase (FNS). Flavanone 3-hydroxylase (F3H), flavanone 3′-hydroxylase (F3′H), and flavonoid 3′5′-hydroxylase (F3′5′H) are responsible for producing dihydroflavonols which are precursors to flavonol and anthocyanidin branch (Hagmann *et al*. 1983; Cheng *et al*. 2014). The dihydroflavonols are subsequently catalyzed by flavonol synthase (FLS) to produce flavonols (Holton *et al*. 1993), among which rutin is a trademarked compound of Tartary buckwheat. *FtUGT73BE5* was characterized to be involved in rutin's subsequent glycosylation (Yin *et al*., 2020).

Additionally, the enzyme dihydroflavonol reductase (DFR), which is following anthocyanidin synthase (ANS) and anthocyanidin reductase (ANR), contribute to converting dihydroquercetin to proanthocyanidin (e.g., epicatechin) (Liew *et al*. 1998). Another pathway is the conversion of dihydroquercetin to catechin catalyzed by DFR and leucoanthocyanidin reductase (LAR).

Transcription factors (TFs) are the key factors to realize the spatiotemporal regulation patterns of secondary metabolites (Yang *et al*. 2012). Many TFs that regulate flavonoids synthesis have been reported, of which MYB is a crucial factor (Du *et al*. 2010; Vimolmangkang *et al*. 2013; Zhou and Memelink, 2016; Luo *et al*. 2018). MYB is one of the largest members of the plant transcription factor families and is classified into four types according to the number of MYB domain repeats (represented by R). The classes comprise of one MYB domain (R1/ R2-MYB), two (R2R3-MYB), three (R1R2R3-MYB), and four MYB domains (4R-MYB), among which R2R3 MYB is essential for flavonoids regulation (Dubos *et al*. 2010). Several MYB genes involved in flavonoids synthesis were first identified in *Arabidopsis thaliana*. For example, *PAP1*/*AtMYB75*, *AtMYB90*, *AtMYB113*, and *AtMYB114* in Arabidopsis from MYBA/SG6 play a positive regulatory role for anthocyanin accumulation by regulating *UFGT* and *DFR* expression (Zimmermann *et al*. 2004; Teng *et al*. 2005; Gonzalez *et al*. 2008; Lotkowska *et al*. 2015). The flavonol branch of the flavonoid biosynthesis is controlled in Arabidopsis by *MYB11*, *MYB12*, and *MYB111* from subgroup 7 of the R2R3-MYB family, which activates *CHS*, *CHI*, *F3H*, and *FLS*. However, *MYB3*, *MYB4*, *MYB7*, and *MYB32* with C2 repressors belonging to SG4 play an inhibitory role in the flavonoid synthesis pathway (Jin *et al*. 2000; Teng *et al*. 2005; Gonzalez *et al*. 2008; Zhou *et al*. 2015, 2017). In Tartary buckwheat, several MYBs have been reported to be involved in regulating the synthesis of flavonoids. Bai *et al*. (2014) reported that the overexpression of *FtMYB1* and *FtMYB2* significantly enhanced the accumulation of proanthocyanidins in *Nicotiana benthamiana* (*N. benthamiana). FtMYB31* is highly expressed in seeds and

has been reported to positively regulate the biosynthesis of rutin in *N. benthamiana* (Hou *et al.*, 2021). Likewise, FtMYB11, and FtMYB13-16 act as negative regulators of rutin biosynthesis (Zhou *et al.* 2017; Zhang *et al.* 2018). Zhang *et al.* (2019) reported that light-induced *FtMYB116* can promote the accumulation of rutin. SG4-like *FtMYB18* and *FtMYB8* with tissue-specific expression patterns were recently identified as repressors of accumulating anthocyanin and proanthocyanidin (Huang *et al.*, 2019; Dong *et al*., 2020). However, the fine molecular mechanism of MYB's regulation of flavonoids synthesis in Tartary buckwheat is still not well understood.

Generally, MYB recruits basic helix-loop-helix (bHLH) and WD40 proteins to form canonical MYB–bHLH–WDR (MBW) regulatory complexes to control flavonoid synthesis *in planta*. Several bHLHs modulating the anthocyanin and proanthocyanidin synthesis have been identified in dicots, including *AN1* and *JAF14* in Petunia (Spelt et al. 2000; Montefiori et al. 2015), *Delila* and *Mutabilis* in snapdragon (Shang et al. 2011), *TT8* and *GLABRA3* in *Arabidopsis* (Nesi et al. 2000; Payne et al. 2000), *MtTT8* in *Medicago truncatula* (Li et al. 2016). The pleiotropic bHLHs regulate the binding affinity of MYB to the *cis*-element of the target gene (Hichri et al., 2011) via interaction with the R3 repeat domain in R2R3 MYB proteins (Grotewold et al., 2000). Interestingly, MYB regulating flavonol synthesis requires bHLH in maize (Goff *et al*., 1992), however, in Arabidopsis and grapevine it is independent of bHLH (Czemmel *et al*. 2009). However, relatively few bHLHs have been reported in Tartary buckwheat, with only FtTT8 reported to interact with other MYB TFs to regulate anthocyanin/proanthocyanidin synthesis (Huang *et al.* 2019; Dong *et al.* 2020; Wang *et al.* 2022). To date, the mechanism of bHLH regulation of flavonoids, as well as how MYB and bHLH synergistically regulate flavonoid synthesis remain largely elusive in Tartary buckwheat.

In this study, we identified and characterized two novel regulators, FtMYB102 and FtbHLH4, forming a transcriptional complex that co-regulates flavonoid synthesis, particularly flavonol, from sprout to seedling

development. These results may have profound impacts on the understanding of precise transcriptional regulatory mechanisms of flavonoid production and even other secondary metabolites.

MATERIALS AND METHODS

Plant material and growth conditions

Two Tartary buckwheat varieties, TB115 and TB128, were used in this study. The growth conditions of Tartary buckwheat were previously reported (Zhang *et al*., 2019). In brief, Tartary buckwheat seed coats were peeled off by soaking seeds in water for 20 min. Peeled seeds were then sterilized by washing with 75% ethanol for 1 min followed by 10 % sodium hypochlorite for 8 min and six 1-min washes with sterilized water. Next, the seeds were inoculated into sterile medium (Murashige and Skoog (MS) medium containing 0.8% agar and 1% sucrose). Subsequently, the sprouts and seedlings were harvested 3 and 8 days after sowing while kept at a photoperiod of 16h (light)–8h (dark) (~150μmolm^{-2} s^{-1}) at 25°C, respectively. The plant samples were frozen immediately using liquid nitrogen and stored at −80°C for further use.

RNA sequencing

The total RNA of Tartary buckwheat was extracted according to the manufacturer's instructions (Tiangen, Beijing, China). RNA quality was then evaluated and the integrity number (RIN) of the samples was >8.0 using a Bioanalyzer 2100 instrument (Agilent Technologies, Palo Alto, CA, US). RNA sequencing was next conducted after establishing sequencing libraries using an IlluminaXten sequencing system (Illumina Inc., San Diego, CA, USA) as per the manufacturer's instructions.

RNA-seq data and phylogenetic analysis

After removing the low-quality bases and Illumina adapter sequences, approximately 46.9 G bases of clean data were utilized for this analysis. The STAR v.2.5 software was used to map these reads to the published Tartary buckwheat reference genome (Zhang *et al.* 2017a). Based on their FPKM (reads per kilobase of

126 exon per million mapped reads) values (Mortazavi *et al*. 2008) differentially expressed genes (DEGs) were
127 then sifted. FPKM values and DEG analysis ($\log_2$ Fold change $\geq$ 1) were analyzed using Htseq and
128 DESeq2 software, respectively. The alignment of amino acid sequences with the default parameters and
129 construction of phylogenetic tree was performed by using the neighbor-joining method via MEGA V.6
130 (Kumar *et al.* 2016). Bootstrap analyses with 1000 replications were used to assess the phylogenetic tree's
131 reliability nodes.

132 **Coexpression analysis**

133 TFs were identified by using hmmscan software. Four genes, *C4H* (FtPinG0001575100), *F3H*
134 (FtPinG0006662600), *F3'H* (FtPinG0002353900), and *CHI* (FtPinG0002790600), were used in
135 coexpression analysis with a default value 0.05 to screen TFs that participated in the regulation of rutin
136 synthesis. The paired genes were considered significantly coexpressed if the Pearson correlation coefficient
137 (*r*) was greater than 0.95 (Ariani & Gepts, 2015).

138 **Quantitative RT-PCR**

139 After the total RNA of Tartary buckwheat was isolated, the first-strand cDNA was synthesized using a
140 commercial kit (TransGen, Beijing, CN) and then diluted to a concentration at 1 μg/ml for qRT-PCR using
141 TransStart® Green qPCR SuperMix UDG (TransGen, Beijing, China) according to the manufacturer's
142 instruction. Additionally, qRT-PCR was conducted at Rotor-Gene Q（Qiagen, Hilden, Germany）. The
143 experiment was conducted in triplicates, and the data were normalized to that of the reference gene, actin.
144 The primers for qRT-PCR are listed in Table S1.

145 **Gene amplificaiton and vector construction**

146 Using high fidelity DNA polymerase and cDNA as the template, transcriptional factors were amplified.
147 Target gene promoters were amplified using the genome DNA of Tartary buckwheat as the template. The

primers are listed in Table S1. These genes were constructed in a Blunt-Zero cloning vector (TransGen, Beijing, China) for further use.

Yeast two-hybrid

The pLexA-FtbHLH4 reporter was cotransformed with the p8op-LacZ plasmid into the yeast strain EGY48, and positive clones were screened using the SD/-His/-Ura dropout media. After these clones were verified to be nonself-activated and non-toxic to yeast, they were mixed with the yeast strain, YM4271 transformed pB42AD-FtMYB102. Positive clones were obtained using SD/-His/-Ura/-Trp media with the addition of 20-mg/mL X-gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for color development.

Yeast one-hybrid

The AD-FtMYB102 effector and AD-FtbHLH4 were cotransformed with the *CHIp: LacZ* reporter into the yeast strain EGY48. After the transformants were screened for SD/-Trp-Ura dropout media, they were further inoculated on the dropout media containing X-gal for color variation.

Co-immunoprecipitation

Briefly, bHLH4 and Myb102 fusions were transiently expressed in 5-week-old leaves of *Nicotiana benthamiana*, and infiltrated leaves were harvested 72 h after inoculation. Two grams of the corresponding leaves were ground in liquid nitrogen and inoculated in lysis buffer (50-mM Tris-MES, pH 8.0, 500-mM sucrose, 1-mM MgCl2, 10-mM EDTA, 5-mM dithiothreitol, 1-mM PMSF, and 1-mM Cocktail protease inhibitors) with end over end shaking for 30 min on ice. After centrifugation at 15,000 g for 1 h at 4°C, the supernatant was filtered and collected using MiraCloth. The protein concentrations of co-immunoprecipitation mixtures were then diluted to 1-mg/mL. Next, 500 µL of the above samples were precleared by incubating with protein G agarose beads at 4°C. After centrifugation, the precleared supernatant was incubated with protein G agarose beads conjugated rabbit-anti-GFP antibody (Yeason, CN)

overnight at 4°C. Subsequently, the beads were collected by five times centrifugation-wash and eluted with 50-μL glycine aqueous solution (pH 2.5) for 1 min, and then 5-μL Tris pH 9.0 was added to neutralize. The eluted fractions and crude extracts were run in 10% SDS-PAGE gel and subjected to immunoblots with the corresponding antibodies. For the immunoblots, the primary monoclonal antibodies mouse-anti-Myc and mouse-anti-GFP (TransGen Biotech, CN) were used followed by secondary antibodies Goat-anti-Mouse-IgG horseradish peroxidase. An enhanced chemiluminescent reagent (StarLighter, CN) was used to detect the signal.

Dual-luciferase reporter assay

The *35S-Ren-CHIp:LUC* reporter plasmid, construct *pGreen-62SK-FtMYB102,* and *pGreen-62SK-FtbHLH4* effector mixture were transformed into *Agrobacterium tumefaciens* strain EHA105, which were subsequently injected into the leaves of 5-week-old *N. benthamiana*. The tobacco leaves were harvested after growing under dark conditions overnight and subsequently under a 14 h light /10 h dark photoperiod for 4 days. The samples were handled as per instruction of the Dual-luciferase® Reporter Assay System (E1920, Promega, USA), and the signal was detected using SpectraMax i3x (Molecular Devices, USA).

Luciferase complementation assay

The FtMYB102 and FtbHLH4 were constructed into plasmids of pCAMBIA1300-NLuc and pCAMBIA1300-CLuc, respectively. Both fusion proteins were then expressed in *N. benthamiana* by Agrobacterium-mediated transient expression. After the plants were placed in the dark for 1 day and photoperiod for 48 h, luciferase substrate and beetle luciferin (E1602, Promega, USA) at 150-μg/ml in saline were evenly sprayed on the back of *N. benthamiana* leaves. Luminescence was observed using NightSHADE LB985 (Berthold, Germany) after placing these leaves in the dark for 7 min.

Transformation of the hairy roots of Tartary buckwheat

When the two pieces of cotyledon of Tartary buckwheat seedlings were unfolded, the hypocotyls and cotyledons, which were cut into ~0.5-cm segments and sheared into 0.5-cm pieces, respectively, were selected as explants. Then, these explants were infected with *Agrobacterium rhizogenes ACC10060* strain transformed target gene plasmids for 10 min after preculturing them on an MS solid medium for 1 day. Next, the explants were placed on an MS solid medium containing 500-mg/ml cefotaxime under 14 h light/10 h dark photoperiod at 25°C after coculturing them with bacteria for 3 days in the dark. The hairy roots were cut into 2–3 cm pieces and propagated in liquid MS medium (containing 30 g/L sucrose) at 25°C with a rotation speed of 80 rpm when they occurred and grew approximately 1–2 weeks later.

Sample treatment and LC–MS analysis

Samples stored at −80°C were ground into fine powder. Then, 0.1 g of each sample was extracted in 1.0-mL 70% methanol aqueous at 4°C overnight and ultrasonicated for 30 mins. After centrifugation, 0.2 mL of each supernatant was filtered via a 0.22 μm membrane for liquid chromatography–mass spectrometry (LC–MS) analysis. The chromatographic conditions used for LC–MS were as the previous investigation (Yang, et al, 2020).

RESULTS

Flavonoid content varies in sprouts and seedlings

The secondary metabolites of plants have spatiotemporal characteristics, which means that the accumulation of secondary metabolites varies in different tissues and at different developmental stages. In addition to its seeds, Tartary buckwheat sprouts are also of great value. Therefore, we focused on the dynamic change in flavonoid content in Tartary buckwheat after germination. The sprouts and seedlings were harvested after sowing for 3 and 8 d, respectively. As shown in Figure 1A, the etiolated sprouts just

emerged with their cotyledons still closed, yellow, and an apical hook. After 8 days, the cotyledons were fully open and green and the hypocotyls were fully grown. Target metabolomics showed more flavonoids, such as kaempferol, quercitrin, rutin, catechin, and epicatechin existed in the sprouts compared with the seedlings from two representative varieties TB115 and TB128 (Figure 1B & Table S2).

Comparative analysis of transcriptional profiles

RNA sequencing was performed using the two varieties (TB115 and TB128) at the sprout and seedling stage to reveal the molecular mechanism that accounts for the differences in flavonoid contents of Tartary buckwheat at different developmental stages (accession number: PRJNA762576). We calculated the upregulated and downregulated genes of sprouts compared with seedlings. There were 3,215 and 3,084 genes upregulated in TB115 and TB128, respectively, with a total of 2,152 genes upregulated in both varieties (Figure 2A). The number of genes downregulated in TB115 and TB128 were 3,059 and 2,876, respectively, of which 1,855 genes were in common (Figure 2B). Following that, because flavonoid content was higher in sprouts than in seedlings, GO analysis was performed on the 2,152 upregulated genes (Figure 2C). The majority of the genes were discovered to be related to cell growth, which is consistent with the expectation that the seedlings were in the rapid growth stage. Interestingly, we discovered that the phenylpropanoid and secondary metabolic pathways both had a substantial number of annotated genes, which confirmed our previous metabolome results, indicating that a large number of secondary metabolites change in plants during these two stages.

Expression of genes related to flavonoid biosynthesis

We specifically analyzed the expression of genes related to flavonoid synthesis. The results showed that the

236 expression of most of these genes in sprouts was higher in sprouts than in seedlings (Figure 3 & Table S3).
237 For example, two out of the five transcripts of *PAL* (FtPinG0001546000 and FtPinG0005713400), three
238 *CHS* (FtPinG0000551600, FtPinG0000551900, and FtPinG0008806400) and two *4CL* (FtPinG0003957300
239 and FtPinG0005072700) displayed a much higher expression level in the sprouts compared to seedlings.
240 Additionally, *C4H* (FtPinG0001575100), *F3'H* (FtPinG0002353900), *F3H* (FtPinG0006662600 and
241 FtPinG0008251700), *DFR* (FtPinG0002371500), *FLS* (FtPinG0006907100), *CHI* (FtPinG0002790600),
242 and *GTR* (FtPinG0006606900) displayed a similar pattern. The expression patterns of these flavonoid
243 structure genes were consistent with the flavonoid content described previously (Figure 1B), indicating that
244 sprouts accumulate more flavonoids than seedlings. After that, we performed real-time quantitative
245 RT-PCR for further validation of the expression of flavonoid structure genes. The results showed that the
246 expression levels of these genes in the sprouts were significantly higher than those in seedlings (Figure S1),
247 which was also consistent with our transcriptome results.
248
249 **Coexpression revealed that FtMYB102 directly regulated *CHI* expression**
250 To obtain the potential TFs involved in flavonoid biosynthesis, coexpression was performed to identify the
251 TFs, which are tightly coexpressed with four highly expressed structural genes: *C4H* (FtPinG0001575100),
252 *F3H* (FtPinG0006662600), *F3'H* (FtPinG0002353900), and *CHI* (FtPinG0002790600). We identified 19,
253 29, 29, and 41 TFs that were coexpressed among *C4H*, *CHI*, *F3H*, and *F3'H*, respectively, through this
254 analysis (Table S4 and S5). Among these TFs, 14 were tightly coexpressed with all the four genes (Figure
255 4A). Similarly, all these 14 TFs exhibited higher expression in sprouts than in seedlings in a pattern similar
256 to *C4H*, *CHI*, *F3H*, and *F3'H* (Figure 4B), where the largest gene family (4 MYBs out of 14 TFs) were
257 proposed to directly regulate the synthesis of flavonoids.

MYB TFs that regulated flavonoid synthesis were also identified using the yeast one-hybrid assay (Y1H). The promoters of *C4H*, *CHI*, *F3H*, and *F3'H* were cloned into pLacZ-2μ reporter vectors in fragments (2,000 bp), while the four MYB TFs were cloned into pB42AD (GAL4 activation domain [AD] fused). FtPinG0007148200.01, which we termed FtMYB102, is bound to the promoter of *CHI*, but not to the promoters of the other three examined genes, as shown in Figure 5A (data not shown). Sprouts accumulated more *FtMYB102* transcripts than seedlings (Figure 5B). Further analysis showed FtMYB102 coded for 265 amino acids and the phylogenetic relationship revealed that FtMYB102 clustered with AtMYB5 (Figure 6A & Figure S2A), regulating the accumulation of PA (Deluc *et al.* 2008) together with PhPH4(*Petunia hybrida*), MdMYB12(*Malus domestica*), and VvMYB5b(*Vitis vinifera)*, which belonged to classic R2R3 MYB TFs (Figure 6B).

bHLH candidate genes involved in flavonoid synthesis

Generally, bHLH TFs are able to interact with MYB TFs to form a transcription complex that regulates the expression of flavonoid synthesis genes. It was previously reported in *Arabidopsis thaliana* that TT8 can form a complex with the MYB transcription factor to regulate the synthesis of flavonoids (Zhou *et al.* 2012). In this study, we identified seven homologous genes named *FtbHLH1–FtbHLH7* in Tartary buckwheat based on the TT8 sequence alignment in *A. thaliana*. Then, we performed a phylogenetic analysis of these seven genes to construct an evolutionary tree with other Arabidopsis bHLH TFs (Figure S2B). Among them, FtbHLH1 and FtbHLH3–4 were classified as IIIf, while FtbHLH2 and FtbHLH5–7 were classified as III(d+e). Interestingly, many bHLH genes reported in *A. thaliana* and rice that were involved in flavonoid synthesis belonged to the IIIf subfamily, (Ludwig *et al.* 1989; Feyissa *et al.* 2009; Zhou *et al.* 2012), which indicated that FtbHLH1, FtbHLH3-4 were involved in the synthesis of flavonoids.

To further verify whether the three bHLH TFs participated in the regulation of flavonoids, Y1H was used to clarify whether they can directly bind to the promoters of the flavonoid synthesis genes. As described above, fragments (2,000 bp) of the promoters of *C4H*, *CHI*, *F3H*, and *F3'H* were cloned into pLacZ-2μ reporter vectors, whereas the three bHLH TFs were cloned into pB42AD. FtbHLH4 bound to the *CHI* promoter but not to the other three promoters as shown in Figure 5A, whereas FtbHLH1 and FtbHLH3 could not bind to any promoters (data not shown). As a result, we focused our research on FtbHLH4. Similar to FtMYB102, we found that the expression of FtbHLH4 was significantly higher in sprouts than in seedlings (Figure 5C). As shown, the expression patterns of FtMYB102 and FtbHLH4 genes were found to be consistent with those of flavonoid synthesis genes, suggesting that the two genes directly regulated the expression of flavonoid synthesis genes.

FtbHLH4 physically interacts with FtMYB102

To identify whether MYB102 and bHLH4 can form a complex, yeast two-hybrid experiment (Y2H) was performed by fusing FtbHLH4 with the LexA DNA-binding domain and the FtMYB102 with the B42 activation domain (AD), respectively. Y2H results showed that LacZ reporter expression was strongly induced when both FtbHLH4 and FtMYB102 were simultaneously transformed into yeast, but either individual or none was effective, indicating FtbHLH4 could interact with FtMYB102 in yeast (Figure 7A). Next, a transient luciferase activity assay was performed in *N. benthamiana* to further examine the interaction between FtbHLH4 and FtMYB102. As shown in Figure 7B, no or weak luciferase fluorescence signal was observed in leaves of *N. benthamiana* injected with water, nLUC+cLUC, nLUC+ FtbHLH4-cLUC, and FtMYB102-nLUC+cLUC. However, when leaves were injected with FtMYB102-nLUC+ FtbHLH4-cLUC, there was a significant increase in the fluorescence value of leaves.

The signal of FtMYB102-nLUC+ FtbHLH4-cLUC increased more than 400 times when compared to the control groups (Figure 7C). Furthermore, we conducted co-immunoprecipitation assays to verify the FtbHLH4–FtMYB102 interaction. To achieve this assay, 35S:FtMYB102-MYC was coinfiltrated with 35S:GFP and 35S:FtbHLH4-GFP inserted into *N. benthamiana* leaves. Afterward, the total plant proteins were extracted. Immunoprecipitation was conducted with the GFP antibody linked to agarose beads, where the FtMYB102-MYC fusion protein was pulled down in samples coexpressing FtbHLH4-GFP, but not GFP alone (Figure 7D). Therefore, these results indicate that FtbHLH4 interacted with FtMYB102.

FtMYB102 and FtbHLH4 Coordinate Activation the Expression of *CHI*

Previous studies have suggested that MYB and bHLH formed complexes that regulated the expression of flavonoids synthesis genes. To study the regulation pattern of downstream gene expression by FtMYB102 and FtbHLH4, a dual-luciferase reporter assay was performed in *N. benthamiana* (Figure 8A). Figure 8B showed that there was no LUC signal difference between 62SK (*Agrobacterium tumefaciens* strain GV3101 harboring recombinant plasmids), FtMYB102-62SK, FtbHLH4-62SK, and FtMYB102-62SK+ FtbHLH4-62SK co-transformed with empty *pGreenII 0800-LUC* vector into leaves, respectively. However, when co-transformed with *ProFtCHI*:*LUC*, the LUC signal in the individual transformation with FtMYB102-62SK and FtbHLH4-62SK was increased significantly than the 62SK control. In addition, the LUC signal in the co-transformation of FtMYB102-62SK and FtbHLH4-62SK was further increased. Therefore, the results indicated that FtbHLH4 and FtMYB102 can individually and coordinately activate *CHI* expression via a complex.

Overexpression of both FtMYB102 and FtbHLH4 can promote flavonoids biosynthesis

To verify whether FtMYB102 and FtbHLH4 promoted the synthesis of flavonoids, we generated transgenic Tartary buckwheat hair roots that overexpressed *FtMYB102* (OE-FtMYB102) and *FtbHLH4* (OE-FtbHLH4). Three independent transgenic hairy root lines were used for further functional analysis. The expression of *FtMYB102* or *FtbHLH4* in the three lines was significantly higher than that in the WT, showing that both the genes were overexpressed in the transgenic hair roots according to qRT-PCR results (Figure S3). Concomitantly, LC–MS analysis showed OE-FtMYB102 and OE-FtbHLH4 produced more flavonoids than WT (except catechin in MYB102OX-4 and MYB102-OX-6) (Figure 9A). The overexpressing lines displayed a significant increase in rutin, indicating that FtMYB102 and FtbHLH4 had greatly promoted rutin synthesis. The qRT-PCR analysis consistently revealed enhanced expressions of *CHS*, *CHI*, *F3H*, and *FLS* in OE-FtMYB102 and OE-FtbHLH4 lines compared with the control (Figure 9B), suggesting that FtMYB102 and FtbHLH4 promote flavonoid synthesis through these four genes.

DISCUSSION

Tartary buckwheat is rich in flavonoids, especially its trademark rutin, and has a high nutritional and medicinal value. This study showed that in addition to Tartary buckwheat seeds which are commonly consumed (Yang *et al.* 2020), the flavonoid content of its sprouts and seedlings is considerably high. Given that this vegetable has been gaining popularity in China, understanding its edible and medicinal value is important. Secondary metabolite biosynthesis in plants is not only tissue-specific but also dependent on the plant's developmental stages. According to Czechowski *et al*. (2016), the content of artemisinin increased gradually during the development of a young leaf to its mature form. Arce-Rodríguez *et al.* (2017) showed that capsaicinoid accumulation is dependent on the developmental stage of chili pepper fruits. As a result, two Tartary buckwheat varieties were used in this study to investigate the flavonoid content during two developmental stages (sprouts and seedlings). The results of the metabolome showed that the content of

346 most flavonoids was higher in the sprouts than in the seedlings. Most previous studies on the flavonoid
347 content of Tartary buckwheat have focused on seeds. As a result, this study is critical in determining the
348 flavonoid content of Tartary buckwheat in the different developmental stages. Follow-up consumption
349 studies might be an interesting research topic.
350 To reveal the transcriptional mechanism of flavonoid accumulation at the sprout and seedling stages, RNA
351 sequencing and coexpression analysis was performed. TFs coexpressed with four flavonoid synthetic genes
352 (*CHI*, *F3H*, *F3′ H*, and *C4H*) were screened. The observations showed that one of the MYB TFs, namely
353 FtMYB102, showed a consistent expression pattern with all the four genes. Y1H showed that FtMYB102
354 directly binds to the promoter of *CHI*. Then, a transient luciferase activity assay was conducted in *N.*
355 *benthamiana* to demonstrate that FtMYB102 promoted *CHI* expression (Figure 8B). A new MYB
356 transcription factor was discovered here that regulated flavonoid synthesis at different Tartary buckwheat
357 development stages. FtMYB102 clustered with AtMYB5, which does not belong to the SG7 group of
358 MYBs that particularly control flavonol accumulation, in an unusual phylogenetic relationship (Wang *et al.*
359 2017). AtMYB5 and its homology in grape, VvMYB5b, have generally been considered a regulator of PA
360 (Deluc *et al.* 2008). The overexpression of FtMYB102 not only significantly promoted PA accumulation
361 but also flavonol accumulation in this study (Figure 9A), which further broaden the roles of MYB5 genes
362 on flavonoid synthesis regulation.
363 Generally, bHLH proteins act as transcriptional factors, subtly controlling flavonol metabolism via the
364 affinity of *cis*-regulatory element of downstream genes (Li *et al.* 2020), broad or restricted expression
365 pattern, influenced by their dimerization properties with MYB TFs (Feller *et al.* 2011). They directly bind
366 to the *cis*-elements (G-box and E-box) of structural genes via the basic region, located at the N-terminal
367 end of the domain, while the HLH region, at the C-terminal end, is involved in homo- and

368 hetero-dimerization (Toledo-Ortiz et al. 2003). In this study, we detected seven homologs of AtTT8 in the
369 Tartary buckwheat genome, which exert distinct DNA-binding functions, i.e., only FtbHLH4 binds to *CHI*
370 promoter but others are not able to, which could explain the specific binding of bHLH members to
371 *cis*-elements to regulate flavonoid synthesis. FtbHLH4 was found to be more highly expressed in sprouts
372 than in seedlings, and it resembled FtMYB102, suggesting that FtbHLH4 regulates sprout and seedling
373 flavonoid biosynthesis. The regulation of flavonoids in Tartary buckwheat was also confirmed by the
374 overexpression of *FtbHLH4* in hairy roots, which leads to the accumulation of flavonoids.
375 It has also been reported that MYB TFs can interact with bHLH to form a complex that regulates gene
376 expression in *Arabidopsis thaliana* (Gonzalez *et al.* 2008); however, this has not been reported in Tartary
377 buckwheat. In this study, Y2H, transient luciferase activity assay, and CoIP demonstrated that FtbHLH4 can
378 interact with the FtMYB102 (Figure 7). Furthermore, through transient luciferase activity assay, it was
379 shown that when both MYB102 and bHLH4 TFs were present, the activation of *CHI* was significantly
380 stronger than when they were present independent of each other (Figure 8). These results indicate that
381 MYB102 and bHLH4 interacted with each other to form a complex that directly bound to the *CHI* promoter
382 and actively triggered *CHI* gene expression. Our results strongly suggest that the combined action of
383 FtMYB102 and FtHLH4, which enhances target gene *CHI* expression, is critical for high flavonoid
384 production (Figures1, 7–9). These findings highlight the importance of the interaction specificity between
385 the cooperative partners of FtMYB102 and FtHLH4 proteins in regulating flavonoid metabolism at the
386 sprout and seedling stages.
387 Based on the results, we developed a working model (Figure 10). In the sprout stage, the highly expressed
388 MYB102 and bHLH4 interact to form a transcriptional complex that directly binds to the promoter of *CHI*
389 and induces its expression. *CHI* is an essential catalytic enzyme in the rutin synthesis pathway. Under its

390 catalysis, naringenin chalcone is transformed into chalcone, and rutin is further synthesized under the
391 action of several other catalytic enzymes. Therefore, when the expression level of *CHI* is high, the
392 synthesis of rutin is increased, resulting in the formation of high rutin in sprouts. In the seedling stage, the
393 expression of MYB102 and bHLH4 decreased, resulting in decreased *CHI* expression, ultimately reducing
394 the content of rutin in the seedlings. It remains unknown what factors cause the difference in the
395 transcription levels of MYB102 and bHLH4 when Tartary buckwheat is in different developmental stages.
396 Since many differences are observed when plants are at different stages of development, the changes in the
397 expression of these two TFs could have been produced by a hormone or a single signal molecule. Hence,
398 further research is needed to address this issue.
399

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406

407 **Conflict of interests**

408 The authors declare that there is no conflict of interests regarding the publication of this article.
409

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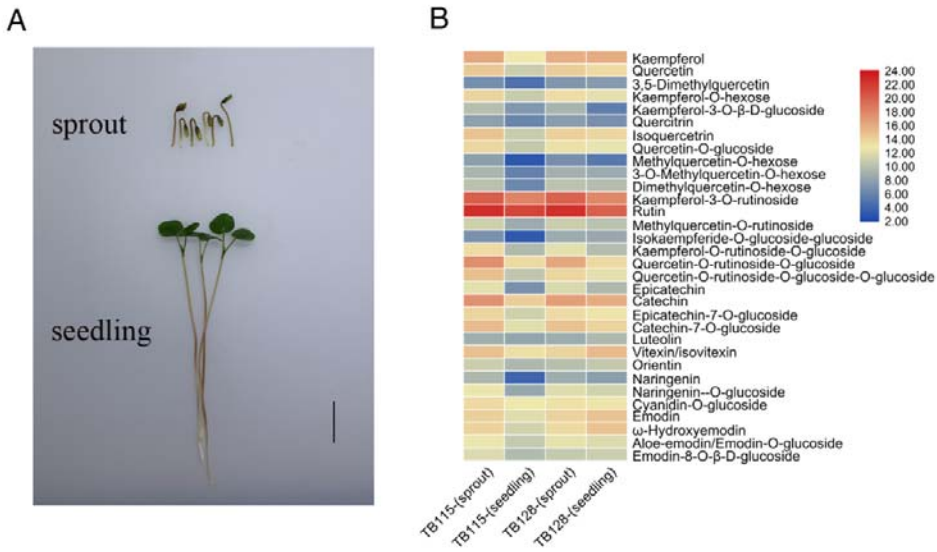


561

562 **Figure 1. Flavonoid content in Tartary buckwheat at the sprout and seedling stages.** (A) Photographs

563 of Tartary buckwheat at the sprouting and seedling stages. Scale bar = 2□cm. (B) Heat map of the content

564 of various flavonoids at the sprout and seedling stages of the TB115 and TB128 varieties. The depths of

565 color in the red and blue rectangles indicate higher and lower flavonoid content level.

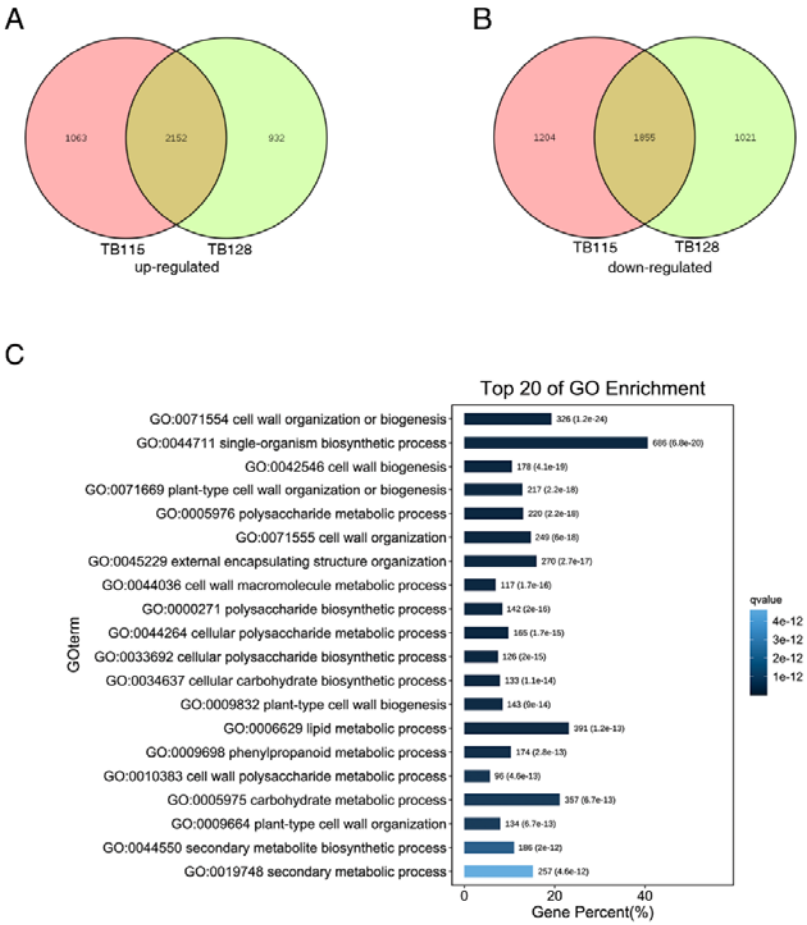
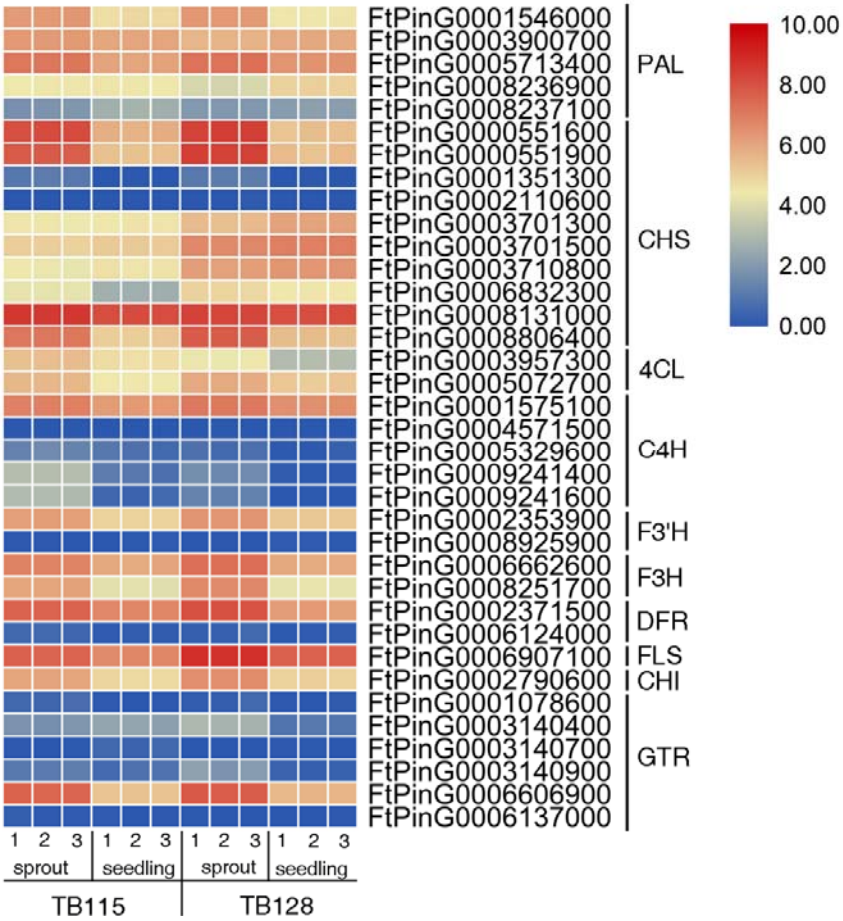


Figure 2. Gene expression varied in sprouts from seedlings in two varieties TB115 and TB128, and GO analysis among these differential genes. (A) Upregulated genes in the two varieties. Among these genes, 2,152 genes (brown) were upregulated in both varieties. (B) 3,059 and 2,876 genes were downregulated genes in both varieties. 1,855 genes (brown) were overlapped. (C) GO analysis on the 2,152 upregulated genes. Top 20 of the GO enrichment were listed.



572

573 **Figure 3. Heat map of the expression levels of flavonoid synthesis genes at sprout and seedling stages.**

574 For each variety, the sprouting and seedling level were studied, the numbers (1-3) represent three biological

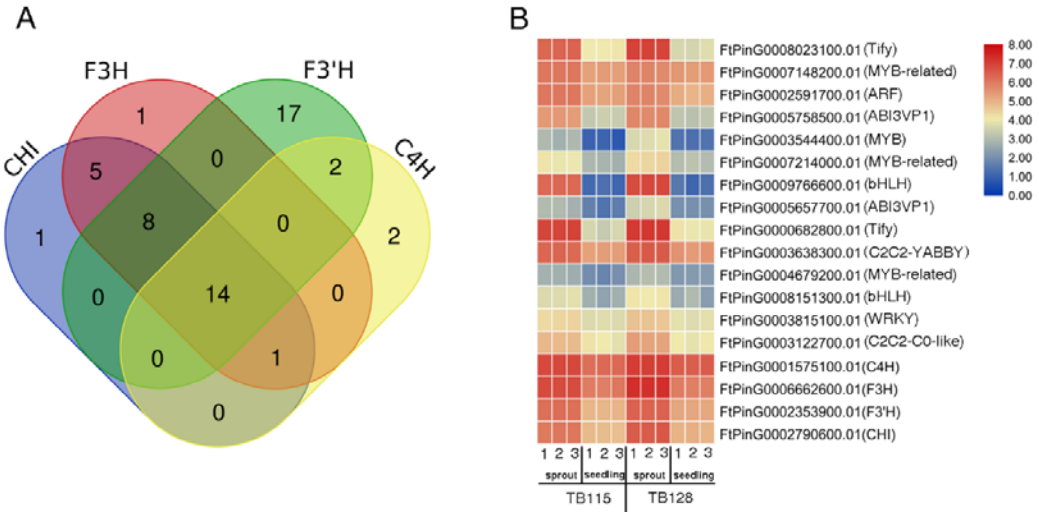
575 repeats. The depths of color in the red and blue rectangles indicate higher and lower z scores of RNA

576 expression level. PAL: phenylalanine ammonialyase; CHS: chalcone synthase; 4CL: 4-coumarate:CoA

577 ligase; C4H: cinnamate-4-hydroxylase; F3′ H: flavonoid-3′- hydroxylase; F3H: flavanone-3-hydroxylase;

578 DFR: dihydroflavonol reductase; FLS: flavonol synthase; CHI: chalcone isomerase; GTR: glucosyl

579 transferase



580

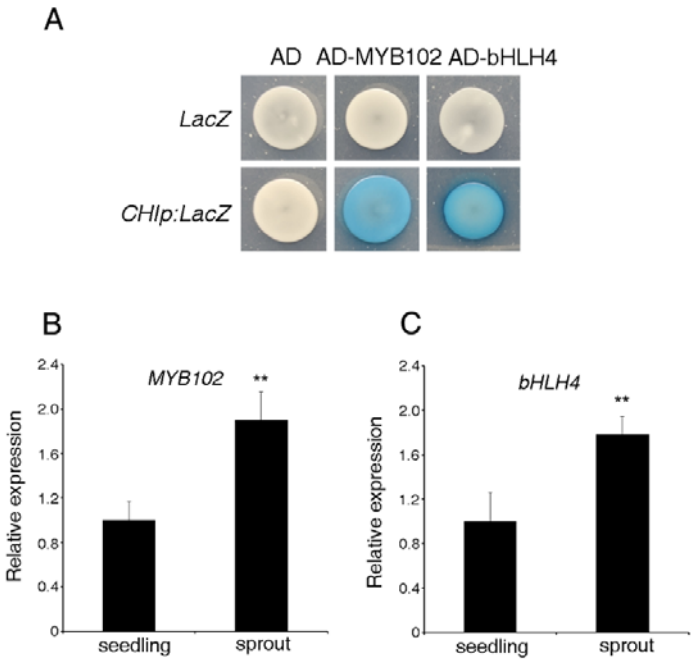
581 **Figure 4. Identification of transcription factors coexpressed with flavonoid synthesis genes.** (A) The

582 Venn diagram shows the number of genes coexpressed with CHI, F3H, F3′H, and C4H. (B) Heat map of

583 the expression levels of the 14 TFs indicated between brackets, including CHI, F3H, F3′H, and C4H. The

584 numbers one, two, and three represent three biological repeats. The depths of color in the red and blue

585 rectangles indicate higher and lower z scores of RNA expression level.



586

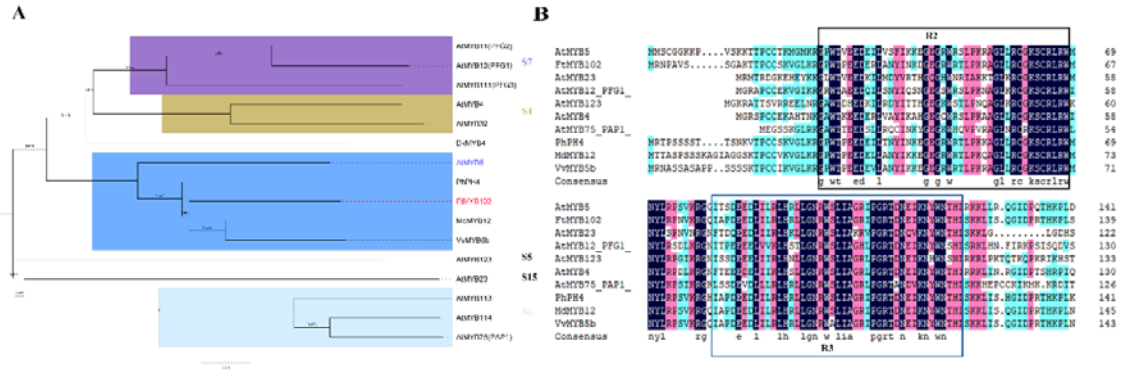
587 **Figure 5. FtMYB102 and FtbHLH4 are candidate transcription factors that regulate rutin synthesis**.

588 (A) Yeast one-hybrid assay showing that AD-MYB102 and AD-bHLH4 bind to the promoter regions of

589 *CHI*. (B) and (C) MYB102 and b*HLH4* expression in sprout and seedling. The relative expression levels

590 were normalized to those of the actin control. Data represent the means ± standard deviation of biological

591 triplicates.



592

593 **Figure 6. Phylogenetic and structural domain analysis of FtMYB102.** (A) Phylogenetic analysis of

594 FtMYB102 with MYB TFs in different subgroups and species. Three S7 group members: AtMYB11-12

595 and AtMYB111 in *A. thaliana*; two S4 group members: AtMYB4 and At MYB32; one S5 and S15 group

596 member AtMYB123 and AtMYB23, respectively; three S6 group members AtMYB75 and AtMYB113-114.

597 FtMYB102, PhPH4(*Petunia hybrida*), MdMYB12(*Malus domestica*) and VvMYB5b(*Vitis vinifera)* were

598 classified as AtMYB5. (B) The structural domain analysis of FtMYB102 showed it was a classic R2R3

599 MYB TF.

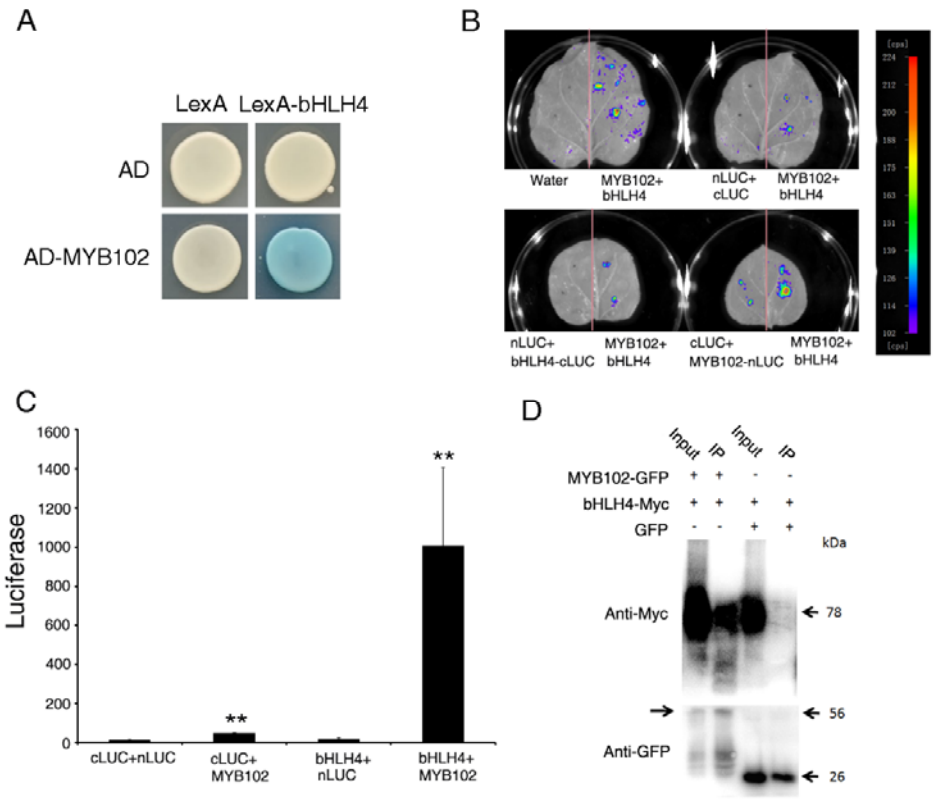


Figure 7. FtbHLH4 physically interacts with FtMYB102. (A) Yeast two-hybrid assay using bHLH4 and MYB102 constructs with AD: the B42 activation domain alone; AD-MYB102: MYB102 fused with the B42 activation domain; LexA: the LexA DNA-binding domain alone; LexA-bHLH4: the LexA DNA-binding domain fused to bHLH4. (B) Luciferase Complementation Imaging Assay (LCI assay) of MYB102–nLUC with bHLH4-cLUC in tobacco leaves with Water, nLUC+ cLUC, nLUC+ bHLH4-cLUC and cLUC+ MYB102–nLUC served as controls; MYB102–nLUC + bHLH4-cLUC in the four leaves were four biological repeats. (C) The interaction between MYB102 and bHLH4 was quantitatively detected using LCI. Data represent the means ± standard deviation of biological triplicates. (D) Co-IP assay with MYB102-GFP and bHLH4-Myc coexpression in tobacco leaves. +: the corresponding component was added to the reaction system; −: no corresponding component added; the number on the right represents the size of the proteins.

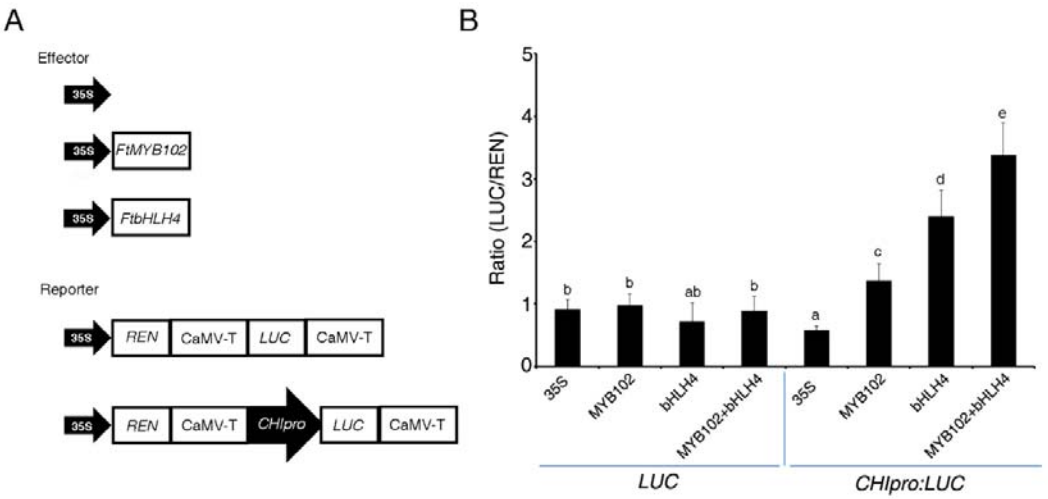


Figure 8. MYB102 and bHLH4 coordinately activate the expression of *CHI*. (A) Schematic diagrams of the effector and reporter plasmids used in dual-LUC assays. REN, Renilla luciferase; LUC, firefly luciferase. (B) Dual-LUC assay in tobacco leaves using the constructs shown in (A). The 35S effector was used as a negative control. Data represent the means ± standard deviation of biological triplicates.

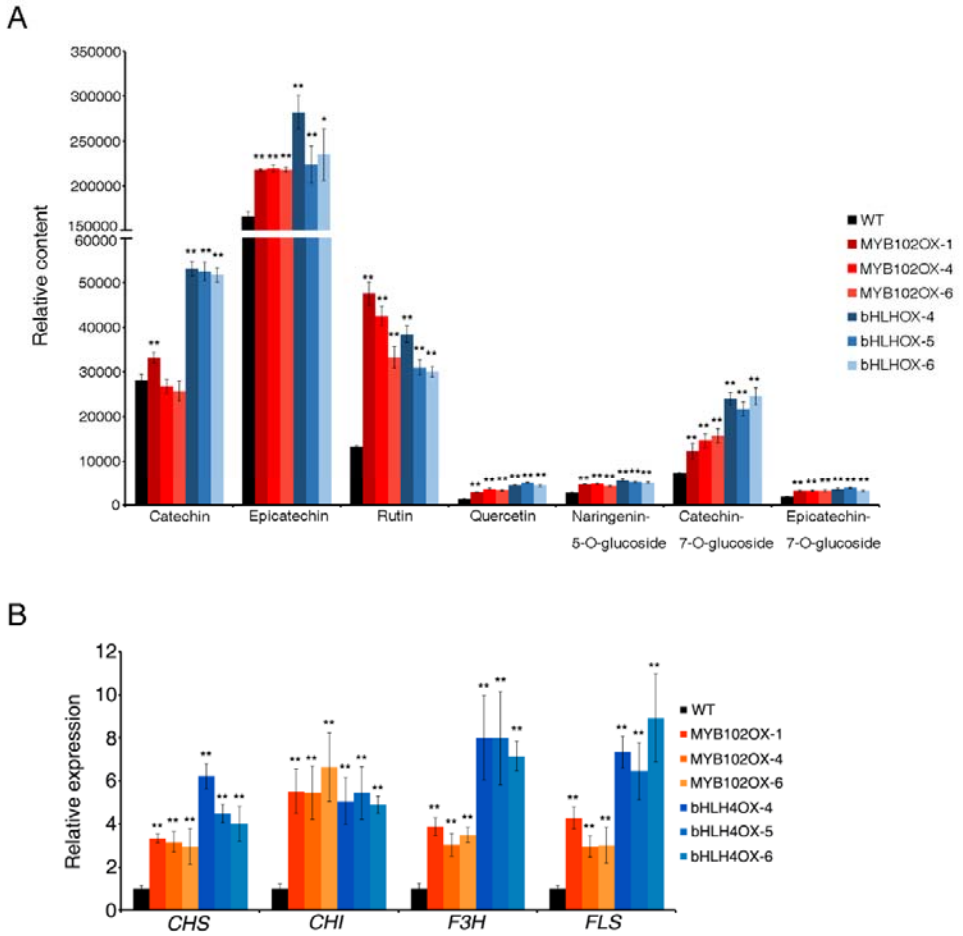


Figure 9. Overexpression of MYB102 and bHLH4 can promote flavonoid biosynthesis in Tartary buckwheat hairy roots. (A) Relative flavonoid content in MYB102 and bHLH4 overexpression lines in Tartary buckwheat hairy roots. Double asterisks indicate a significant difference at $P < 0.01$ using the Student's t test-test. (B) Detection of gene expression related to flavonoid synthesis pathways in MYB102 and bHLH4 transgenic lines. The relative expression levels were normalized to that of the actin control. Data represent the means ± standard deviation of biological triplicates.

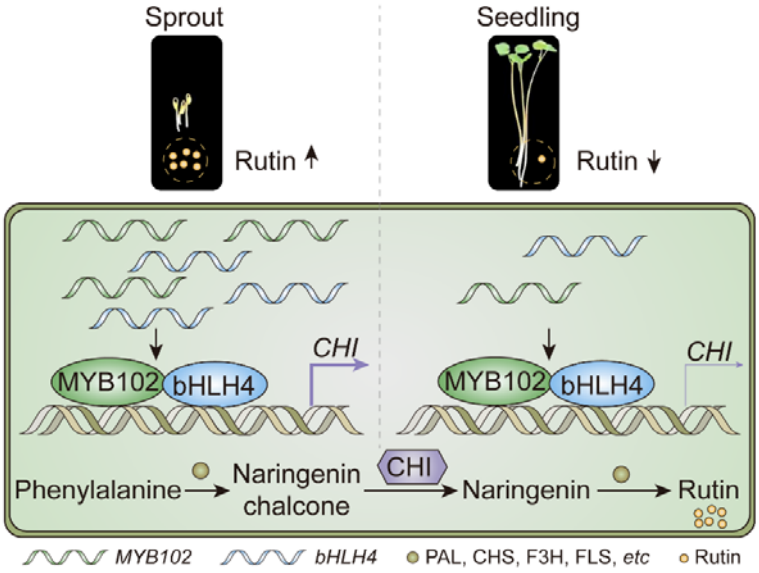


Figure 10. A working model of the MYB102 and bHLH4 form a transcriptional complex by inducing *CHI* expression to promote the accumulation of rutin. (Left) In sprouts, the transcripts of MYB102 and bHLH4 are abundant, thus more MYB102 and bHLH4 proteins form a complex to activate the high expression of *CHI*, since *CHI* is an important structural gene for rutin, this causes rutin levels to be high. (Right) In seedlings, unlike sprouts, MYB102 and bHLH4 transcripts are reduced, and *CHI* expression is downregulated as the number of MYB102 and bHLH4 complexes is less; thus, rutin levels are reduced.