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Inducing Hairy Roots by Agrobacterium rhizogenes-mediated Transformation in Tartary buckwheat (Fagopyrum tataricum)

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TITLE:

Inducing Hairy Roots by *Agrobacterium rhizogenes*-Mediated Transformation in Tartary Buckwheat (*Fagopyrum tataricum*)

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KEYWORDS:

Tartary buckwheat; hairy roots; genetic transformation; GFP; *Agrobacterium rhizogenes;* secondary metabolite; gene function

SUMMARY:

We describe a method of inducing hairy roots by *Agrobacterium rhizogenes*-mediated transformation in Tartary buckwheat (*Fagopyrum tataricum*). This can be used to investigate gene functions and production of secondary metabolites in Tartary buckwheat, be adopted for any genetic transformation, or used for other medicinal plants after improvement.

ABSTRACT:

Tartary buckwheat (TB) [*Fagopyrum tataricum* (L.) Gaertn] possesses various biological and pharmacological activities because it contains abundant secondary metabolites such as flavonoids, especially rutin. *Agrobacterium rhizogenes* have been gradually used worldwide to induce hairy roots in medicinal plants to investigate gene functions and increase the yield of secondary metabolites. In this study, we have described a detailed method to generate *A. rhizogenes*-mediated hairy roots in TB. Cotyledons and hypocotyledonary axis at 7–10 days were selected as explants and infected with *A. rhizogenes* carrying a binary vector, which induced adventitious hairy roots that appeared after 1 week. The generated hairy root transformation was identified based on morphology, resistance selection (kanamycin), and reporter gene expression (green fluorescent protein). Subsequently, the transformed hairy roots were self-propagated as required. Meanwhile, a myeloblastosis (MYB) transcription factor, *FtMYB116*, was transformed into the TB genome using the *A. rhizogenes*-mediated hairy

roots to verify the role of *FtMYB116* in synthesizing flavonoids. The results showed that the expression of flavonoid-related genes and the yield of flavonoid compounds (rutin and quercetin) were significantly ($p < 0.01$) promoted by *FtMYB116*, indicating that *A. rhizogenes*-mediated hairy roots can be used as an effective alternative tool to investigate gene functions and the production of secondary metabolites. The detailed step-by-step protocol described in this study for generating hairy roots can be adopted for any genetic transformation or other medicinal plants after adjustment.

INTRODUCTION:

Tartary buckwheat (TB) (*Fagopyrum tataricum* (L.) Gaertn) is a type of dicotyledon belonging to the genus *Fagopyrum* and the family Polygonaceae[1]. As a type of Chinese medicine homologous food, TB has been receiving considerable interest owing to its distinctive chemical composition and diverse bioactivities against diseases. TB is primarily rich in carbohydrates, proteins, vitamins, and carotenoids as well as in polyphenols such as phenolic acids and flavonoids[1]. Various biological and pharmacological activities of flavonoids, including antioxidative, antihypertensive[2], and anti-inflammatory as well as anticancer and antidiabetic properties, have been demonstrated[3].

Agrobacterium rhizogenes is a soil bacterium that contributes to the development of hairy root disease in several higher plants, especially dicotyledons, by infecting wound sites[4,5]. This process is initiated by the transfer of the T-DNA in the root-inducing (Ri) plasmid[5–6] and is commonly accompanied by the integration and expression of an exogenous gene from the Ri plasmid and the subsequent steps of generating the hairy root phenotype[7]. *A. rhizogenes*-mediated transgenic hairy roots, as a powerful tool in the field of plant biotechnology, have been most widely used owing to their stable and high productivity and easy obtainment in a short period. Moreover, hairy roots induced by *A. rhizogenes* are efficiently distinguished by their plagiotropic root development and highly branching growth in a hormone-free medium[8]. They can be used in several fields of research, including artificial seed production, root nodule research, and in studying the interactions with other organisms such as mycorrhizal fungi, nematodes, and root pathogens[7,9]. In addition, hairy root transformation cultures have been extensively used as an experimental system to investigate the biochemical pathways and chemical signaling and to produce plant secondary metabolites that are used as pharmaceuticals, cosmetics, and food additives[8,10]. The valuable secondary metabolites, including indole alkaloids, aconites, tropane alkaloids, terpenoids, and flavonoids, synthesized in wild-type hairy roots have been investigated for several decades in numerous species, such as ginsenoside in *Panax ginseng*[11], coumarine in *Ammi majus*[12], and phenolic compounds in TB[2,13].

Hairy roots have been produced using *A. rhizogenes* in 79 plant species from 27 families[14]. For instance, *A. rhizogenes*-mediated hairy root transformation has been reported in soybean[15,16], *Salvia*[17], *Plumbago indica*[18], *Lotus japonicus*[19], and chicory (*Cichorium intybus* L.)[20]. TB hairy root transformation has also been investigated[2]. Few detailed protocols are available regarding the development of hairy roots mediated by *A. rhizogenes* either carrying a binary vector or not. For instance, Sandra et al.[21] introduced a method of producing transgenic potato hairy roots

sustained in wild-type shoots. The fully developed hairy roots could be visualized 5-6 weeks after the injection of *A. rhizogenes* carrying the gus reporter gene into the stem internodes of potato plants. Another study had also reported a transgenic hairy root system induced by *A. rhizogenes* harboring the gusA reporter gene in jute (*Corchorus capsularis* L.)[22]. Furthermore, Supaart et al.[23] obtained transgenic tobacco hairy roots using *A. rhizogenes* transformed with the expression vector *pBI121* carrying the gene of Δ^1-tetrahydrocannabinolic acid (THCA) synthase to produce THCA.

However, a step-by-step process for an effective generation of hairy root transformation, especially in TB, has been relatively less demonstrated. In this study, we have described a detailed protocol using *A. rhizogenes* carrying the reporter gene (*GFP*), a selective marker (*Kan*), and a gene of interest (*b4*, an identified from our group but unpublished gene from basic helix-loop-helix (*bHLH*) family) to generate hairy root genetic transformation in TB. The experiment lasted for 5-6 weeks, from the inoculation of seeds to generation of hairy roots, involving the explant preparation, infection, coculturing, subculturing, and subsequent propagation. Furthermore, *A. rhizogenes* containing a binary plasmid carrying the TB transgene of myeloblastosis transcription factor 116 (*FtMYB116*) was used to determine whether *FtMYB116* can promote accumulation of flavonoids, particularly rutin, in TB at the gene and metabolic level through the TB hairy root transformation. *FtMYB116*, which is a light-induced transcriptional factor, regulates the synthesis of rutin under different light conditions[5]. Chalcone synthase (*CHS*), flavanone-3-hydroxylase (*F3H*), flavonoid-3'-hydroxylase (*F3'H*), and flavonol synthase (*FLS*)[24] are key enzymes involved in the metabolic pathway of rutin biosynthesis. Therefore, this study demonstrates the overexpression of *FtMYB116* in TB hairy roots and the expression of key enzyme genes as well as the content of rutin and other flavonoids such as quercetin.

PROTOCOL:

The TB used in this study was named as BT18, which originated from the breed of "JinQiao No.2" cultivated by the Research Center of Small Miscellaneous Grain of Shanxi Academy of Agricultural Science. The primary steps of this protocol are illustrated in **Figure 1**.

NOTE: Operate explants-related manipulation rapidly, and when possible, keep the Petri dishes closed to avoid wilting and contamination. Unless otherwise stated, all the explant incubations were conducted under the condition of a 14-h light and a 10-h dark photoperiod at 25 °C. Unless otherwise stated, all explants- or bacteria-related operations were performed under aseptic conditions in a laminar flow hood. All the media ingredients for *A. rhizogenes* and in vitro plant cultures are provided in **Table 1**. After adjusting the pH, all media were autoclaved at 120 °C for 20 min. Solidified media were prepared by filling 25 mL of medium into a Petri dish of 9-cm diameter and allowing it to solidify.

CAUTION: Deposit all the genetically modified bacteria and plants into the appropriate waste container. Operate all hazardous chemicals in a fume cupboard and deposit them in the hazardous waste container.

1. Preparation of TB explants

1.1 Preparation of TB seeds

1.1.1 Select plump and undamaged TB seeds (**Figure 1A1**) that have been preserved at a temperature less than−20 °C for no more than 2 years.

1.1.2 Soak the seeds in water at 28 °C for approximately 20 min so that the seed coat can be easily peeled off (**Figure 1A2**). If necessary, use scissors to cut a slot on the seeds to facilitate the peeling.

1.2 Sterilization of TB seeds

1.2.1 Place 100–200 peeled seeds into a 100 mL sterilized conical flask.

1.2.2 Disinfect the seeds using 75% ethanol for 30 s.

1.2.3 Replace the ethanol with 5% sodium hypochlorite and disinfect for 15 min.

NOTE: Disinfection using mercury bichloride at a concentration of 1 g/L for 8 min may be used as an alternative sterilizer to replace sodium hypochlorite in any case of inadequate sterilization.

CAUTION: Mercury bichloride is hazardous and not an environment-friendly material. Operate it in the fuming cupboard and deposit it into the hazardous waste container, if mercury bichloride is used in any case.

1.2.4 Pour out the sodium hypochlorite.

1.2.5 Wash the seeds using sterile deionized water 5 times.

1.2.6 Blot the seeds dry with a sterile bibulous paper.

1.3 Preparation of TB seedlings

1.3.1 Prepare 50 mL Murashige and Skoog (MS) basal medium (1962) supplemented with 30 g/L sucrose and 7 g/L agar powder (MSSA) (**Table 1**) in a 300 mL plant tissue culture bottle.

1.3.2 Adjust the pH to 5.8 before autoclaving.

1.3.3 Distribute 10 seeds evenly per bottle of MSSA medium.

1.3.4 Germinate the seeds in a culture room at 25 °C ± 1 °C under the light condition for 7–10

177 days.
178
179 1.4 Preparation of sterile explants
180
181 1.4.1 Select robust seedlings of TB (**Figure 1C**), when the 2 pieces of cotyledons are unfolded.
182
183 1.4.2 Cut off the seedlings from the roots (**Figure 1C red-dash arrowheads**), avoiding contact
184 with the medium.
185
186 1.4.3 Place them in a sterile Petri dish.
187
188 1.4.4 Cut the hypocotyls into 0.8–1 cm segments, and shear the cotyledons into
189 approximately 0.5 cm pieces.
190
191 1.4.5 Preculture these explants on MSSA medium under the light condition for 24 h.
192
193 1.4.6 Transfer them into a 100 mL sterilized conical flask, which are now ready for infection.
194
195 **2. Preparation of *A. rhizogenes* for transformation**
196
197 NOTE: The *A. rhizogenes* strain *ACCC10060* was kindly provided by the Institute of Medicinal
198 Plant Development and preserved at −80 °C. *A. rhizogenes* was transformed with the binary
199 vector *pK7GWIWG2D (II)* that harbors a T-DNA carrying the *b4* gene accompanying a *GFP* as an
200 indicator gene and the Kan resistance gene as a selectable marker. The gene *b4* is a member of
201 the transcription factor *bHLH* family, which has not yet been published. To evaluate the
202 potential of TB hairy roots, *A. rhizogenes* was transformed with the binary vector *pK7WG2D*
203 containing the *MYB116* gene to investigate its effect on the production of secondary
204 metabolites such as flavonoids at the level of gene expression and by metabolic analyses.
205 Activated *A. rhizogenes* should be well prepared at the same time with the explants.
206
207 2.1 Activation of *A. rhizogenes*
208
209 2.1.1 Thaw *A. rhizogenes* on ice
210
211 2.1.2 Dip the bacteria and line them evenly onto yeast mannitol medium (YEB) supplemented
212 with 15 g/L agar powder, 50 mg/L rifampicin, and 50 mg/L spectinomycin (YEBARS, pH 7.0).
213
214 2.1.3 Incubate the bacteria at 28 °C for 12–16 h.
215
216 2.1.4 Pick a monoclonal colony and culture it in another Petri dish in the same above-
217 described manner.
218
219 2.1.5 Select monoclonal colonies and culture them in a 100 mL sterilized conical flask
220 containing 20 mL of YEB medium supplemented with 50 mg/L rifampicin and 50 mg/L

spectinomycin (YEBRS, pH 7.0) at 28 °C and 200 rpm for 16–18 h until the OD_{600} value reaches 2.0.

2.1.6 Incubate 2%–4% of the abovementioned culture in another 100-mL conical flask containing 20 mL of YEBRS medium at 28 °C and 200 rpm for 4-5 h until the OD_{600} value reaches approximately 0.5 (**Figure 1D**).

3. Infection and screening of TB explants

NOTE: The objective of this protocol is to obtain genetically transformed hairy roots. The wild-type roots were used as the negative control to assess the transgenic expression. In this protocol, *A. rhizogenes* was transformed with binary vector either *pK7WG2D* carrying the gene of *FtMYB116* or *pK7GWIWG2D (II)* carrying the gene of b4 in advance.

3.1 Resuspension of *A. rhizogenes*

3.1.1 Transfer the culture obtained in step 2.1.6 into a 50-mL sterilized centrifugal tube.

3.1.2 Spin at 4,000 x *g* for 10 min at 20 °C.

3.1.3 Remove the supernatant and resuspend the bacterial pellet with MS medium supplemented with 30 g/L sucrose and 300 µM acetosyringone (AS) (MSSAS, pH 5.8) to OD_{600} ≈ 0.2.

3.2 Infection of explants

3.2.1 Infuse the bacterial suspension obtained in step 3.1.3 into a conical flask containing the explants prepared in step 1.4.6 for 10 min (**Figure 1E**).

3.2.2 Take out the explants, and blot them dry using a sterile bibulous paper.

4. Coculture of explants with *A. rhizogenes*

4.1 Place a sterile 9 cm diameter filter paper on the MS medium, which is solidified using 7 g/L agar powder supplemented with 30 g/L sucrose and 100 µM AS (MSSAAS medium, pH 5.2).

4.2 Overlay the explants on the filter paper at 25 °C for 3 days in the dark (**Figure 1F**).

5. Induction and selective culture

5.1 Place approximately 20 infected explants on the MSSA medium supplemented with 500 mg/L cefotaxime and 50 mg/L kanamycin (Kan) (MSSACK, pH 5.8) (**Figure 1G**).

5.2 Incubate them vertically under the light condition at 25 °C ± 1 °C. The hairy roots occur

approximately 1 week after incubation (**Figure 1H** black-dash arrowheads indicate occurrence of hairy roots).

NOTE: Replace MSSACK medium every 15 days if necessary.

6. Subculturing TB hairy roots

NOTE: This procedure aims to harvest vigorous hairy roots. Regularly observe the growth of hairy roots during propagation, and remove the contaminated and inactivated ones in a timely manner. If necessary, repeat the following steps to propagate more hairy roots. It takes approximately 10–14 days from subculturing to harvest.

6.1 Select the hairy roots showing white appearance and rapid growth.

6.2 Cut them into pieces of 2-3 cm.

6.3 Clearly number them on a clean bench.

6.4 Subculture them in a 100 mL sterilized conical flask containing 5 mL of MS medium supplemented with 30 g/L sucrose and 50 mg/L Kan (MSSK, pH 5.8) at a rotary speed of 80 rpm at 25 °C in the dark until they overspread to the bottom of the flask (**Figure 1I**).

7. Identification of transformed hairy roots and conservation

NOTE: Transformed hairy roots can be identified based on the aspects of morphology and gene level. Identification can also be conducted according to the hairy root genome and resistance, which are not covered in this protocol. This procedure primarily focuses on reporter gene and target gene identification.

7.1 Remove tawny and contaminated hairy roots and select those with white appearance.

7.2 Evaluate if there is green fluorescence under a blue/light dual ultraviolet transilluminator.

7.3 Select the hairy roots exhibiting a strong fluorescence signal in the numbered tubes or wrapped using a marked tinfoil after drying them out with an absorbent paper.

7.4 Lyophilize them in liquid nitrogen, followed by storing all the harvest at −80 °C for further investigation.

7.5 Gene identification

7.5.1 Triturate 0.1 g of the hairy roots into fine powder in liquid nitrogen.

7.5.2 Prepare the genomic DNA of independent transgenic lines of TB using the modified

cetyltrimethylammonium bromide (CTAB) method[25] according to the instruction of the manufacturer of the plant genomic DNA kit.

7.5.3 Perform polymerase chain reaction (PCR) using 100 ng of genomic DNA template and primers listed in **Table 2**.

7.5.4 Perform the amplification cycle as follows: predenaturation at 94 °C for 5 min, denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s, and primer extension at 72 °C for 30 s. After 36 cycles and a final extension step at 72 °C for 10 min, analyze the amplification products on 1% agarose gels.

7.5.5 Stain the gels with nucleic acid staining and visualize them under UV light.

REPRESENTATIVE RESULTS:

Agrobacterium rhizogenes-mediated TB hairy root transformation

This study describes the step-by-step protocol that was established to obtain genetically transformed hairy roots using *A. rhizogenes*. It took approximately 5-6 weeks from the inoculation of TB seeds to the harvesting of the identified hairy roots, and some key steps are depicted in **Figure 1** (**A-H**). Briefly, sterilized shelled seeds were inoculated (**Figure 1B**) to achieve faster sterile germination. *A. rhizogenes* (**Figure 1D**) and sterile explants should be activated and prepared in advance, respectively. This is followed by some key steps, including infection of explants with activated *A. rhizogenes* (**Figure 1E**), coculture (**Figure 1F**), and selective culture (**Figure 1G**). The infected explants should be placed evenly on the solidified MS medium and space must be maintained between them to readily separate the different transgenic lines. Hairy roots appear with a fluffy white color in a plagiotropic manner in the wound sites of the explants (**Figure 1H**). The hairy roots form a highly branched and an interlocked matrix and can be propagated as required (**Figure 1I**). The harvested hairy roots can be used to investigate the gene function or the gene– or protein–protein interaction. Alternatively, the TB hairy roots can be massively propagated to yield secondary metabolites such as rutin in designated bioreactors.

The method to induce transgenic hairy roots in TB has been substantiated using a binary vector (*pK7GWIWG2D (II)*) carrying the genes *GFP* and *b4* (a member of the transcriptional factor *bHLH* family, not yet published). The reporter gene *GFP* was used to easily distinguish the transgenic hairy roots from the nontransgenic ones by visualizing the signal under a blue/light dual ultraviolet transilluminator (**Figure 2**) or by identifying the target gene (**Figure 3**). The transformed hairy roots exhibited green fluorescence when illuminated under blue or ultraviolet light (represented using black arrowheads in **Figure 2A**), whereas the untransformed hairy roots did not exhibit the green fluorescence (**Figure 2B**). The hairy roots with a high GFP signal were propagated for a fortnight, as illustrated in **Figure 2C**.

To further identify whether the binary vector has been successfully transformed into the TB genome, gene identifications were conducted. Briefly, plant genomic DNA of the TB hairy roots was prepared for PCR analysis based on the modified CTAB method[25]. PCR was performed by

amplifying the genes (*Kan*, *GFP*, and *b4*), which was present in **Figure 3**, respectively. The primers are listed in **Table 2**. The presence of the 3 genes in all the transgenic lines (**Figure 3, lanes 5–11**) indicated that the binary vector has been successfully transformed into the TB genome. *Kan* and *GFP* were absent in the wild-type roots (**Figure 3, lane 3**) and experimental negative control (**Figure 3, lane 4**), whereas *b4* was detected in the wild-type roots. These 3 genes were undoubtedly presented in the positive control (**Figure 3, lane 2**) but were apparently absent in the negative control (**Figure 3, lane 4**).

Evaluation of the light-induced transcription factor *FtMYB116* in TB using the aforementioned hairy root system

FtMYB116 was expressed by employing the abovementioned protocol of hairy root induction. This was accomplished by preinserting the gene *FtMYB116* into the binary vector *pK7WG2D* and then infecting with *A. rhizogenes* to achieve gene overexpression. Briefly, hairy roots of 0.1 g were triturated into fine powder by using liquid nitrogen. Total RNA was extracted by following the instructions of manufacturer of plant RNA isolation kit[26]. Then reverse-transcription PCR and real time PCR were performed to amplify FtMYB116 and rutin synthesizing pathway related genes. Subsequently the regulatory effects of *FtMYB116* on rutin synthesis-related gene expression and the yield of rutin were verified.

Figure 4A shows the relative expression of *FtMYB116* in the transgenic lines of TB hairy roots. Compared with the control group, the relative expression of *FtMYB116* exhibited a considerable increase in all 3 independent transgenic lines. **Figure 4B** and **Figure 4C** illustrate the promotion of the biosynthesis of rutin and quercetin at the metabolic level through *FtMYB116* overexpression. The contents of rutin and quercetin in the transgenic were significantly ($p < 0.01$) increased compared with those in the wild-type, reaching 40 and 0.5 mg/g FW, respectively, which were 8 times those in the wild-type. The relative gene expressions of *CHS*, *F3H*, *F3'H*, and *FLS* in all 3 transgenic lines were remarkably higher than those in the control group (**Figure 4D**). Together, these results confirmed that the strategy described in this study could be successfully used to generate hairy root transformation in TB and investigate the gene expression and metabolic yield of secondary metabolites.

FIGURE AND TABLE LEGENDS:

Figure 1: Processes to induce *A. rhizogenes*-mediated transgenic hairy roots in TB. Representative images of critical stages are displayed: (**A1**) and (**A2**) represent before and after peeling off the seed coats; (**B**) represents each 10 seeds inoculated in a tissue bottle containing MSSA medium; (**C**) denotes the seedlings of TB at 7–10 days after inoculation, and the red-dash arrowheads show the cutting points; (**D**) and (**E**) indicate the preparation of *A. rhizogenes* (OD_{600} = 0.5) and the infection of explants, respectively; (**F**) and (**G**) symbolize coculturing with activated *A. rhizogenes* on MSSAAS medium and selective culturing on MSSACK medium, respectively; hairy roots emerge from (**H**)**,** as shown by the black-dash arrowheads; and (**I**) shows the propagation of hairy root formation; the black-dash arrowheads indicate the induced hairy roots.

Figure 2: Transformation of the binary vector carrying the *GFP* reporter gene. (**A**) denotes the induced hairy roots after selective culture examined under the blue/light dual ultraviolet transilluminator. (**B**) and (**C**) represent wild-type root and propagation of transformed hairy roots, respectively.

Figure 3: PCR amplification of genes (*Kan*, *GFP*, and *b4*) from genomic DNA isolated from wild-type root and hairy roots of TB in 7 independent transgenic lines. (**A**): Kan, (**B**): GFP, (**C**): b4. **Lane 1**: molecular size markers (white arrowhead indicates 750 bp), **lane 2**: plasmid (binary vector *pK7GWIWG2D (II)* carrying *Kan*, *GFP*, and *b4* genes) as the positive control, **lane 3**: wild-type root, **lane 4**: purified H_2O as the negative control, and **lanes 5–11**: the 7 independent transgenic lines.

Figure 4: Relative expression of *FtMYB116* in the transgenic lines of TB hairy roots. (**A**) and the promotive effect of the overexpression of *FtMYB116* on the biosynthesis of (**B**) rutin and (**C**) quercetin (This figure has been modified from Dong et al.[5]). Experiments were performed in triplicate and conducted 3 times. "**" indicates a significant difference at $p < 0.01$ using Student's *t*-test. (**D**) Expression of genes related to flavonoid synthesis pathways in transgenic lines. The relative expression level was normalized to that of the actin control. Data are presented as mean ± standard deviation (n = 3).

Table 1: Media and their ingredients.

Table 2: Primer sequence.

DISCUSSION:

TB has been used in several studies related to secondary metabolites at genetic and metabolic levels[1,2,5,27–28]. Hairy root culture, as a unique source for metabolite production, plays a pivotal role in metabolic engineering[29] and can be used to alter metabolic pathways by inserting the related genes. Kim et al.[2] initially introduced the establishment of TB hairy root cultures by *A. rhizogenes*-mediated transformation to achieve the production of phenolic compounds. The content of rutin that they obtained in the TB hairy roots was more than 10 times higher than that in the wild-type roots. In the present study, the introduction of *FtMYB116* led to a higher expression of rutin-related genes and surged the production of rutin in the TB hairy roots. This technique has been confirmed to be apt for phenotypic characterization and expression of phenylpropanoid-related genes such as *FtF3H and FtFLS* in TB hairy roots[5,30,31]. Zhang et al.[32] used TB hairy roots to investigate the production of rutin by overexpressing a series of *FtMYB* transcriptional factors. Zhou et al.[33] observed a decrease in the content of rutin owing to the overexpression of *FtMYB11* in TB hairy roots. These results together with our findings indicate the feasible effects of hairy root transformation on the interaction between *FtMYB* transcriptional factors and rutin biosynthesis-related genes.

Although there are limited data regarding a step-by-step protocol for the induction of TB hairy roots, we describe herein the step-by-step protocol for the first time to obtain transgenic TB hairy roots in an efficient and stable manner using *A. rhizogenes* carrying a binary vector.

During these experimental processes, numerous factors have to be carefully considered to obtain the optimal induced hairy roots. First, the selection of explants is a determining factor. TB cultivars are known to affect the morphology of hairy roots and the production of phenolic compounds. Thwe et al.[30] illustrated that gene expression in the phenylpropanoid biosynthetic pathway and the contents of phenolic compounds varied among TB cultivars. They also found hairy roots in one cultivar, which was deep reddish-purple owing to its anthocyanin content[13]. In our study, 2 just unfolded cotyledons and hypocotyls were selected as the explants. This is because young and tender leaves favor a high hairy root induction rate[2,30], whereas highly differentiated and old plant cells adversely affect the hairy root induction. Second, the strain of *A. rhizogenes* has a significant impact on hairy root induction. Different bacterial strains exhibit different transforming abilities in terms of morphologies and induction efficiency of hairy roots, which can be illuminated by the different plasmids harbored by the strains[34]. Aye et al.[35] compared the effects of several *A. rhizogenes* strains (R1000, R1200, 15834, LBA9402, and A4) on TB hairy root induction and phenylpropanoid biosynthesis and found that the most promising strain for hairy root production in TB was R1000. This finding has been supported by Kim et al.[2] Nevertheless, the strain ACCC10060 that was excluded in the study of Aye et al. but used in our study exhibited satisfactory infection efficiency. The fluffy white appearance of hairy roots obtained using our protocol is in agreement with the hairy roots generated in *Salvia miltiorrhiza*[36], wherein the same strain ACCC10060 carrying the binary vector *pK7GWIWG2D (II)* was used to silence the target gene. Third, degerming including pretreatment of materials and a concentration of cefotaxime in selective culture also play vital roles in hairy root induction. Incomplete disinfection in any step could lead to the failure of hairy root transformation. In addition, the bacterial concentration has a significant influence on the production of transformed roots. High concentrations may reduce the plant cells by competitive inhibition, whereas low concentrations may cause low availability[4].

Furthermore, culture conditions such as the growth medium, appropriate preculturing and coculturing time, and other biotic or abiotic factors play an important role in hairy root inducton. Huang et al.[37] recommended 1/2 MS medium containing sucrose at a concentration of 30 g/L for cocultivation to achieve maximum TB hairy roots. This can be explained by the high salt medium that is suitable for hairy root formation, whereas a low salt medium favors excessive bacterial multiplication[34]. AS is a type of phenolic compound that can facilitate *A. rhizogenes*-mediated transformation in a number of plant species by the transcription of the *vir* region of *Agrobacterium*[34,38], and *vir* could be effectively induced in a medium with a pH of < 5.7[39,40]. Therefore, we recommend a coculture medium with pH 5.2 supplemented with 100 µM of AS. Huang et al.[37] reported that TB hairy roots turned to brown after day 24 from white and pale yellow. Therefore, they subcultured hairy roots every 24 days; however, we recommend subculturing every fortnight to avoid browning of hairy roots. In addition, environmental conditions such as light, hormones, temperature, and UV radiation appear to affect the expression of flavonoid biosynthesis-related genes by highly stimulating or depressing signal transduction[41,42]. The previous study has demonstrated the significance of far-red light in monitoring rutin-related gene expression in TB hairy roots[5].

The *A. rhizogenes*-mediated transformation has the advantage that any exogenous gene of

interest inserted in a binary vector can be transferred to the transformed hairy root clone[34] to achieve overexpression, loss-of-function via RNA silencing[43], or discovery of new metabolic genes by transcriptome analyses[5]. Hairy roots have great potential to produce secondary metabolites, recombinant proteins, and evenantibodies[44]. This is primarily owing to their easy and rapid growth in hormone-free medium, being less expensive, no requirement for regeneration into complete plants[21], and the relatively high yield of secondary metabolites compared to that from the starting plant material[31]. These roots can also be separated from the original explant to establish long-term, stable, and characterized root clones maintaining their biosynthetic capacity and phenotypes. Altogether, based on these findings, this protocol provides a rapid, distinct, and efficient method to produce transformed hairy roots to investigate the production of secondary metabolites and puts forward a reference for hairy root induction in other plants. However, the potential to explore hairy root cultures to generate massive yields of bioactive compounds depends on the appropriate bioreactor system in which certain parameters such as the supply of oxygen must be concerned[4,8]. This protocol is limited to the production of secondary metabolites derived in hairy roots and to investigate the visualized phenotype of functional genes such as the variance of color and the contents of secondary metabolites; however, the phenotypic changes in the entire plant regardless of the obtainment of regenerated plants from the hairy roots could not be evaluated in this study.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

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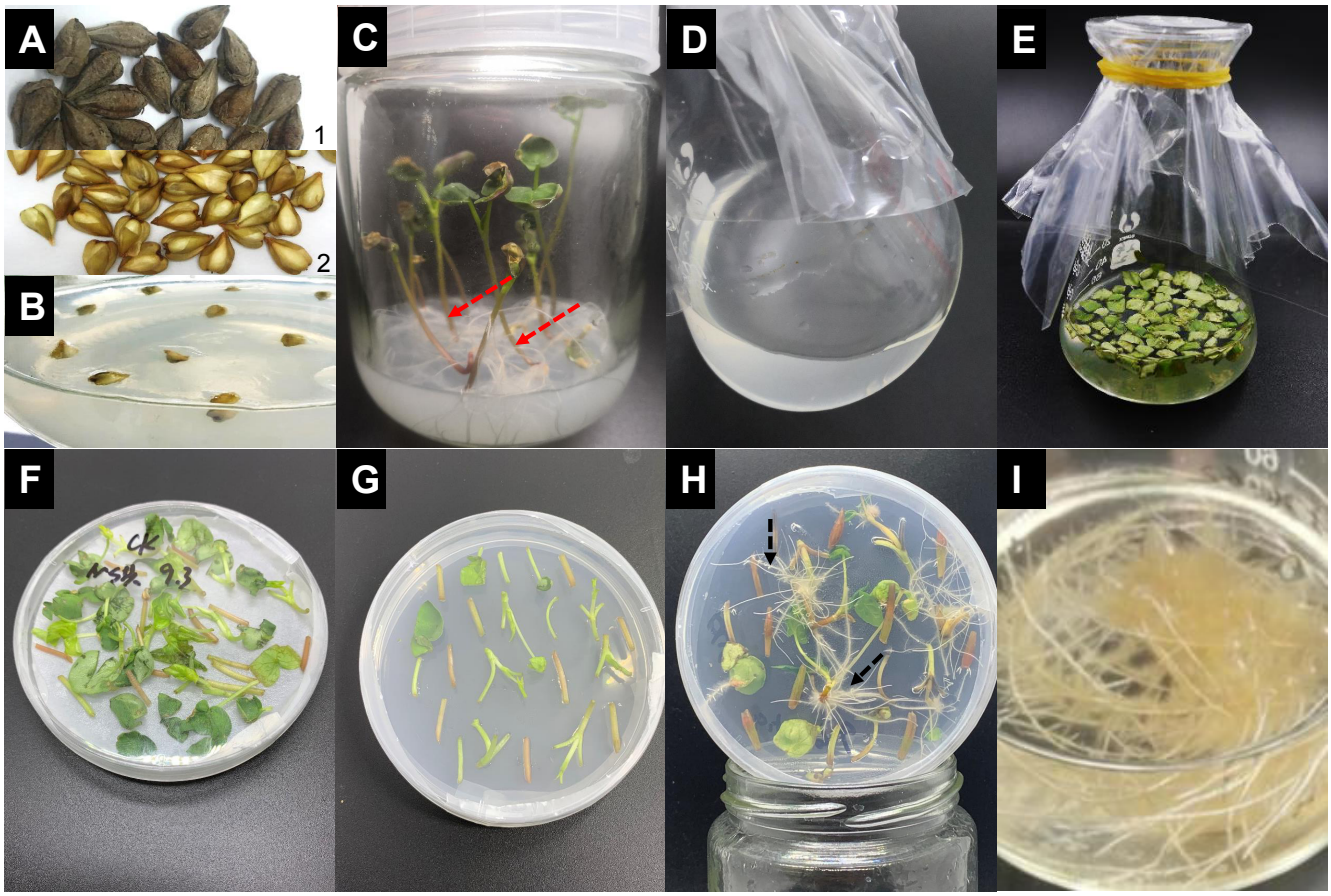

Figure 2

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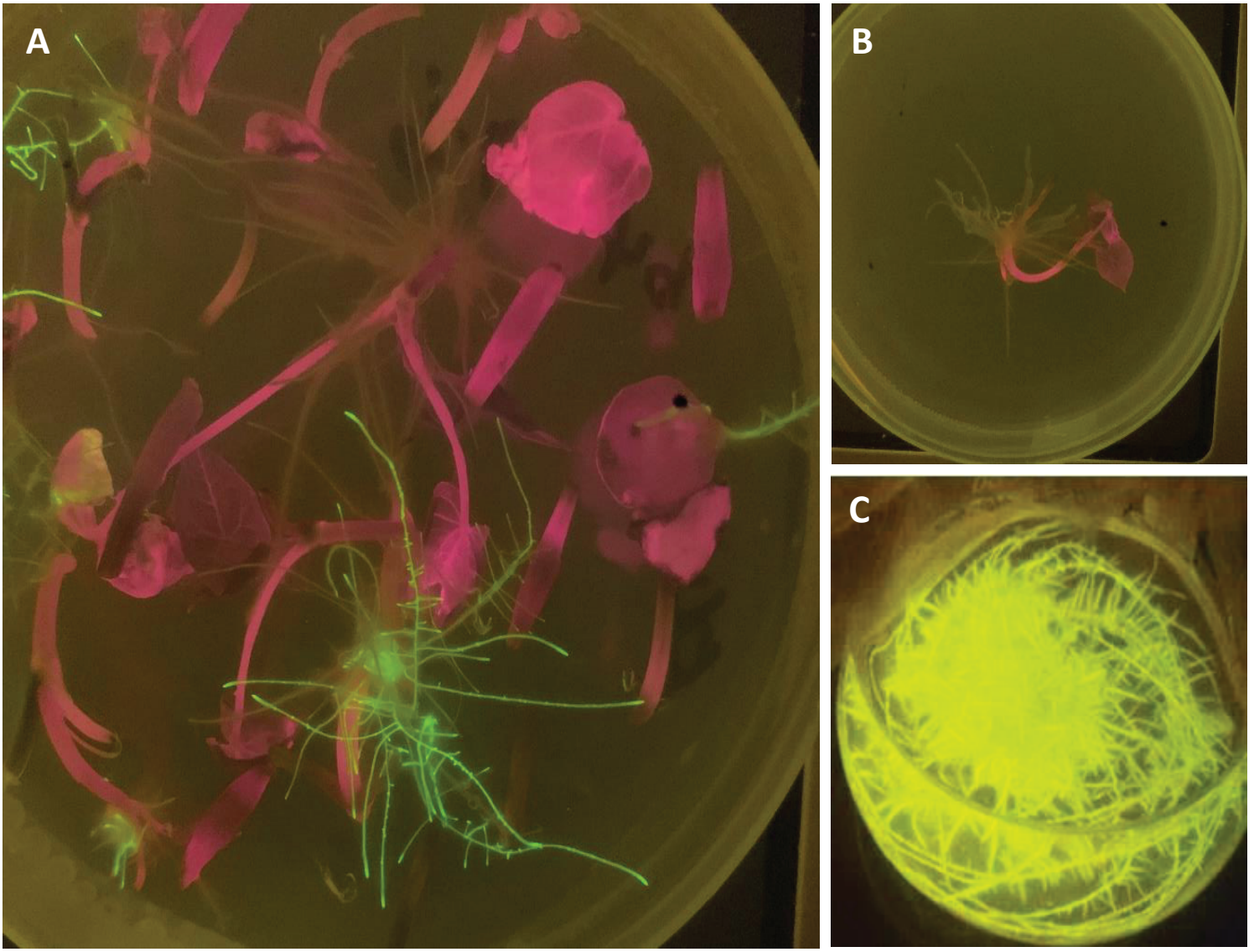

Figure 3

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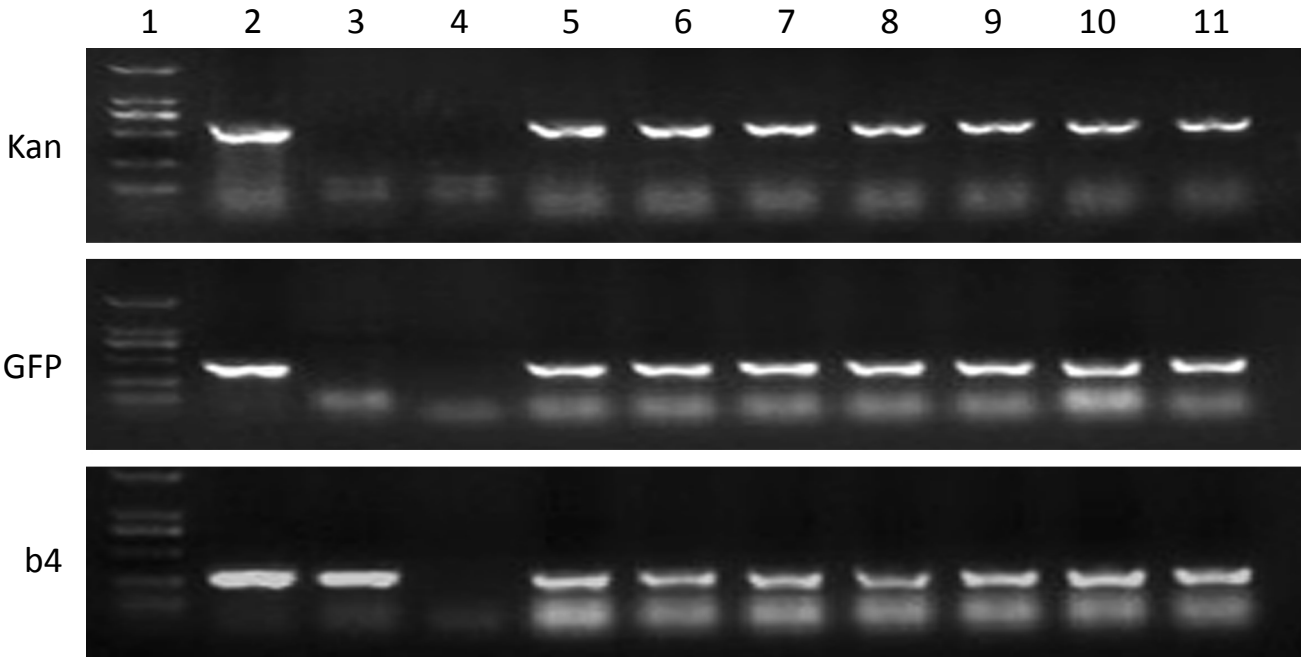

Figure 4

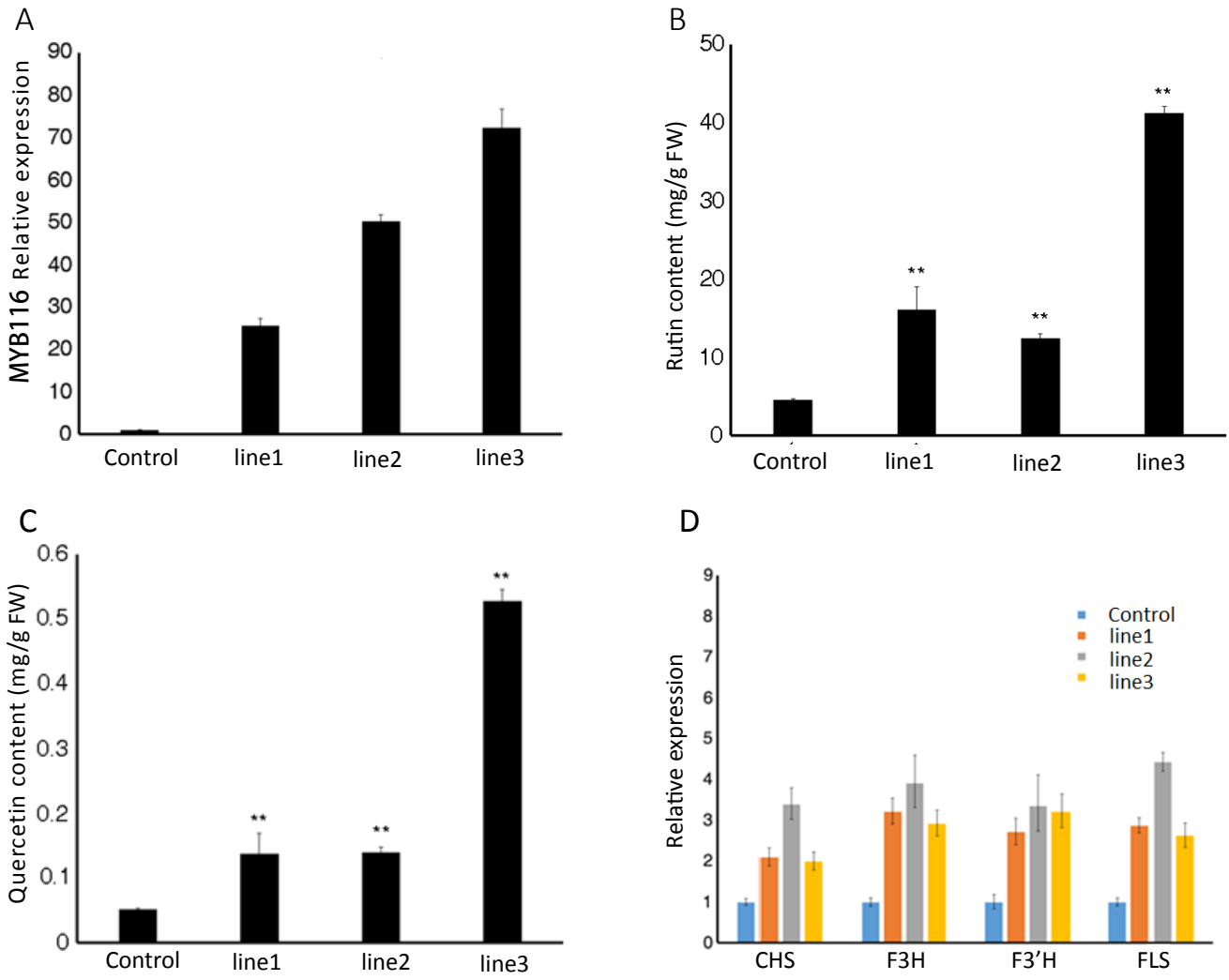

Table 1

Media	Medium ingredients
MSSA	Murashige and Skoog (MS) medium containing sucrose in 30 g/L, and agar powder in 7 g/L, pH 5.8
YEBARS	Yeast Mannitol Medium (YEB) containing agar powder at 15 g/L, rifampicin at 50 mg/L, and spectinomycin at 50 mg/L, pH
YEBRS	YEB containing rifampicin at 50 mg/L, and spectinomycin at 50 mg/L, pH 7.0
MSSAS	MS medium containing sucrose at 30 g/L, and acetosyringone (AS) at 300 µM, pH 5.8
MSSAA	MS medium containing sucrose at 30 g/L, agar powder at 7g/L, and AS at 100µM, pH 5.2
MSSACK	MS medium containing sucrose at 30 g/L, agar powder at 7 g/L, cefotaxime at 500 mg/L, and kanamycin (kan) at 50
MSSK	MS medium containing sucrose at 30 g/L, and kan at 50 mg/L, pH 5.8

Table 2

Primer	Sequence (5'-3')
GFP-F	CCACAAGTTCAGCGTGTCCG
GFP-R	AAGTTCACCTTGATGCCGTTC
b4-F	AAATCTTTTCCCTGTGG
b4-R	ATGCCATCATTGCCAAG
Kan-F	ATTCGGCTATGACTGGGCAC
Kan-R	TGAATCCAGAAAAGCGGCCA

Name of Material/ Equipment	Company	Catalog Number
2*Taq PCR MasterMix	Aidlab, China	PC0901
Agar powder	Solarbio Life Science,Beijing, China	A8190
Applied Biosystems 2720 thermo cycler	ThermoFisher Scientific, US	A37834
AS	Solarbio Life Science,Beijing, China	A8110
binary vectors	ThermoFisher Scientific (invitrogen), US	/
Cefotaxime,sodium	Solarbio Life Science,Beijing, China	C8240
CF15RXII high-speed micro	Hitachi, Japan	No. 90560201
Diposable Petri-dish	Guanghua medical instrument factory, Yangzhou, China	/
DYY-6C electrophoresis apparatus	Bjliuyi, Beijing China	ECS002301
EASYspin Plus Plant RNA Kit	Aidlab, China	RN38
ELGA purelab untra bioscience	ELGA LabWater, UK	82665JK1819
Epoch Microplate Spectrophotometer	biotek, US	/
Gateway BP/LR reaction enzyme	ThermoFisher Scientific (invitrogen), US	11789100/11791110
HYG-C multiple-function shaker	Suzhou Peiying Experimental Equipment Co., Ltd. China	/
Kan	Solarbio Life Science,Beijing, China	K8020
MLS-3750 Autoclave sterilizer	Sanyo, Japan	/
MS salts with vitamins	Solarbio Life Science,Beijing, China	M8521
NaCl	Solarbio Life Science,Beijing, China	S8210
Other chemicals unstated	Beijing Chemical Works, China	
PHS-3C pH meter	Shanghai INESA Scientific Instrument CO.,Ltd, China	a008
Plant Genomic DNA Kit	TIANGEN BIOTECH (BEIJING) CO., LTD	DP305
Rifampin	Solarbio Life Science,Beijing, China	R8010
Spectinomycin	Solarbio Life Science,Beijing, China	S8040
Sucrose	Solarbio Life Science,Beijing, China	S8270
Trans2K DNA Marker	TransGen Biotech, Beijing, China	BM101-01
Tryptone	Solarbio Life Science,Beijing, China	LP0042
Whatman diameter 9 cm Filter paper	Hangzhou wohua Filter Paper Co., Ltd	/
Yeast Extract powder	Solarbio Life Science,Beijing, China	LP0021

Comments/Description
Diluted in DMSO, 100 mM pK7WG2D/pK7GWIWG2D (II) Diluted in Water, 200 mg/mL
Diluted in Water, 100 mg/mL
ethanol, mercury bichloride, etc.
Diluted in DMSO, 50 mg/mL Diluted in Water, 100 mg/mL

Dear Editor and Reviewers:

We have revised the previous manuscript thoroughly and fully addressed concerns both from editor and reviewers. The line-by-line response for each of the editorial and peer review comments has been displayed as follow.

Comments:

• Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg). Please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

Answer:

Thanks for the comments. We have modified the images in the form of .sv in the high solution. In the meantime, the tables we uploaded have been changed to .xlsx files.

Editorial Comments:

Comments:

• Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Answer:

Thanks for the advises and reminding. We really appreciate the significant concerns raised by the reviewers, and we have addressed every comment as much as we can. As for some comments that are not addressed, we also provide explanations in this letter.

Comments:

• The language in the manuscript is not publication grade. Please employ professional copy-editing services.

Answer:

Thanks for the advice. We have employed professional copy-editing services in terms

of language and academic aspects.

Comments:

• Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video.

Answer:

Thanks for the comments. The thing we would like to show in the video has been highlighted in the submission.

Comments:

• Please add more specific details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:
1) 1.3.1: unclear
2) 4.1: Mention filter paper specifications

Answer: "

Thanks for the comments. We have added more specific details to our protocol steps.

For "1.3.1", we added more information and divided it into 3 steps, 1.3.1 to 1.3.3 as follow. '1.3.1. Prepare 50 ml MS basal medium (Murashige and Skoog 1962) supplemented with 30 g/L sucrose and 7 g/L agar powder (MSSA) (Table 1) in each 300 ml plant-tissue-culture bottle.

1.3.2. Adjust pH to 5.8 before autoclaving.

1.3.3. Distribute 10 seeds each evenly on per bottle of MSSA medium.'

For "4.1", we modified "…to filter paper of diameter 9 cm on…". In addition, we detailed all medium used in this protocol, including steps 1.3.1, 2.1.2, 2.1.5, 3.1.3, 4.1, 5.1, 6.4.

In addition, we added more details to the protocol. For example, we added the specification of plant-tissue-culture bottle to step "1.3.1", "avoiding contact with the medium" to step 1.4.2 and "Thaw *A.rhizogenes* on ice" to 2.1.1, as well as "To prepare solidified the media plates in petri dish, fill with each 25 mL of medium to a petri dish of 9 cm diameter, and let them solidify." in "NOTE under Protocol".

Furthermore, some other addition or changes were presented in other comments.

Comments:

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Answer:

Thanks for the comments. We have rearranged the discussion part to make it more scientifically logic and cover the modifications and troubleshooting, critical steps within the protocol, significance with respect to existing methods (paragraphs 1-3), future applications and limitations of the technique (paragraph 4). For instance, the limitation "However, the potential to explore hairy root cultures to generate massive yields of bioactive compounds depends on the appropriate bioreactor system in which certain parameters such as the supply of oxygen must be concerned[5,9]. This protocol is limited to the production of secondary metabolites derived in hairy roots and to investigate the visualized phenotype of functional genes such as the variance of color and the contents of secondary metabolites; however, the phenotypic changes in the entire plant regardless of the obtainment of regenerated plants from the hairy roots could not be evaluated in this study." has been extended in the last paragraph in the discussion part.

Comments:

• Figures: Please remove the embedded figures from the manuscript. Figure legends, however, should remain within the manuscript text, directly below the Representative Results text.

Answer:

Thanks for the comments. We have removed embedded figures from the manuscript and remained the figure legends directly below the representative results text.

Comments:

• Tables: Please remove the embedded Tables from the manuscript. All tables should be uploaded to the Editorial Manager site in the form of Excel files. A description of the table should be included with the Figure legends.

Answer:

Thanks for the comments. We have removed embedded tables from the manuscript and remained the figure legends directly below the representative results text. In addition, the tables formed in Excel are uploaded to the Editorial Manager site along with the re-submission.

Comments:

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Answer:

Thanks for the comment. We combined the figure 8 and Figure 4s from the published article “The light-induced transcription factor FtMYB116 promotes accumulation of rutin in *Fagopyrum tataricum*” as the Figure 4 in our article. We have uploaded the permission as a word document to the editorial manager site in the “supplemental files”. In the meantime, we cite the figure as “This figure has been modified from Zhang et al.[18].”.

Peer-Reviewer #1:

The manuscript sounds and is well written. However, the following major revisions should be conducted;

- Introduction:

Comments:

• The introduction should be extended to cover the research questions, especially the second and fourth paragraphs.

Answer:

Thanks for the comments. We have extended the second and fourth paragraphs of introduction. In the second paragraph, we focus on the principle of the method of *A. rhizogenes*-mediated transformation and its application in secondary metabolite

biosynthesis not only TB, but also other medicinal plants. In the fourth paragraph, we list the basic steps of this method, introduce the related gene in rutin metabolic pathway, and present their relevance.

Comments:

• Additionally, the authors should revise English and correct the few mistakes in the grammar of introduction

Answer:

Thanks for the comments. In spite of the language limitation, we have sought professional copy-editing service for the academic proofreading. Meanwhile, we have addressed some scientifically imprecise information to avoid misunderstanding.

- Protocol:

Comments:

• The data are well represented. However, some figures should be explained in details. For example, Figure 1 and Figure 3 should be explained in details and show up the most significant conclusions obtained.

Answer:

Thanks for the comments. We have added more detailed information of figures in the representative results. For example, "It takes approximately 5-6 weeks from inoculating TB seeds to harvesting the identified hairy roots, while some key steps are depicted in **Figure 1 (A-H)**. Briefly, the sterilized shelled seeds were inoculated **(Figure 1B)** to obtain sterile germination faster. *A. rhizogenes* **(Figure 1D)** and sterile explants should be activated and prepared in advance, respectively. Then some key steps subsequently follow, including infection of explants with activated *A. rhizogenes* **(Figure 1E)**, co-culture **(Figure 1F)** and selective culture **(Figure 1G)**. The infected explants should be placed evenly on solidified MS medium and maintain space between them to separate different transgenic lines readily. Hairy roots occur in wound sites of explants **(Figure 1H)** and are propagated as required **(Figure 1I)**."

We have elaborated the figure 3. For example, "Briefly, plant genomic DNA of TB hairy roots for PCR analysis was prepared based on the modified CTAB method[28]. PCR was performed by amplifying gene with DNA from 7 independent transgenic lines (Figure

3, lanes 5-11) of hairy roots and primers listed in Table 2. *Kan*, *GFP* and *b4* were conducted in **Figure 3**, respectively. The presence of the three genes in all transgenic lines indicated the binary vector has been transformed into TB genome. *Kan* and *GFP* were absent in wild type roots (lane 3), while *b4* was detected in wild type roots. Certainly, three genes were presented in positive control (lane 2) and were apparent absent from the experimentally negative control (lane 4)."

Comments:

• The current data should be linked with the previous findings and reasons should be given and discussed.

Answer:

Thanks for the comments. Because this a protocol-based journal, discussion did not focus on the representative results. However, we briefly discussed previous research on transcriptional factors in TB via hairy roots, and drew forth why the hairy root transformation is important, and elaborately compared the difference among these methods.

Comments:

• The conclusion section should be extended to cover the significant outcomes of this work.

Answer:

Thanks for the comments. We have extend the significant outcomes of our work in the last paragraph of introduction and discussion, as well as in the summary.

Peer-Reviewer #2:

Major Concerns:

Comments:

• The main problem I found with the manuscript is the writing. I did not mark any corrections or suggestions on the files. In any case, as a result of usage problems and lack of clear scientific writing, it is often difficult to understand exactly what the authors mean. I strongly recommend the authors seek an academic proofreading service.

Answer:

Thanks for the comments. We have employed an academic proofreading service to improve the writing. At the same time, we revised thoroughly the manuscript in

scientific aspects to address some imprecise information.

Comments:

• (note: the pdf file I received has several yellow highlighted texts, but apparently are not to indicate correction made or to be made).

Answer：

Thanks for the comments. The highlighted texts made in this article are based on the JoVE submission instruction to film afterwards.

Comments:

• The described method seems efficient, as shown in Figure 1. The manuscript, however, is not sufficiently detailed, lacking information on several steps.

Answer:

We have supplemented as much details in the manuscript as possible. For example, we added the filter paper specification; we added note and caution to make the operation much clearer.For “1.3.1”, we added more information and divided it into 3 steps, 1.3.1 to 1.3.3 as follow. ‘1.3.1. Prepare 50 ml MS basal medium (Murashige and Skoog 1962) supplemented with 30 g/L sucrose and 7 g/L agar powder (MSSA) (Table 1) in each 300 ml plant-tissue-culture bottle.

1.3.2. Adjust pH to 5.8 before autoclaving.

1.3.3. Distribute 10 seeds each evenly on per bottle of MSSA medium.’

For “4.1”, we modified “…to filter paper of diameter 9 cm on…”. In addition, we detailed all medium used in this protocol, including steps 1.3.1, 2.1.2, 2.1.5, 3.1.3, 4.1, 5.1, 6.4.

In addition, we added more details to the protocol. For example, we added the specification of plant-tissue-culture bottle to step “1.3.1”, “avoiding contact with the medium” to step 1.4.2 and “Thaw *A.rhizogenes* on ice” to 2.1.1, as well as “To prepare solidified the media plates in petri dish, fill with each 25 mL of medium to a petri dish of 9 cm diameter, and let them solidify.” in “NOTE under Protocol”.

Furthermore, we have added more details in step 7 as follow.

7.1. Remove tawny and contaminated hairy roots and select hairy roots with white appearance.

7.2. Evaluate if there is green fluorescence under blue/light dual ultraviolet transilluminator.

7.3. Select the hairy roots harboring strong fluorescence signal into numbered tubes or wrapped by using marked tinfoil after drying them out with absorbent paper.

7.4. Lyophilize them in liquid nitrogen, followed by storing all the harvest in -80 ℃for the further investigation.

7.5. Gene identification

7.5.1. Triturate hairy roots of 0.1 g into fine powder in liquid nitrogen.

7.5.2. Prepare the genomic DNA of independent transgenic lines of TB by using the modified cetyltrimethyl ammonium bromide (CTAB) method28 as per manufacturer's instructions of plant genomic DNA kit.

7.5.3. Perform polymerase chain reaction (PCR) with 100 ng genomic DNA template and primers listed in Table 2.

7.5.4. Perform the amplification cycle as follow: pre-denaturation at 94 ℃for 5 min, denaturation at 94 ℃for 30 s, and primer annealing at 55 for 30s and primer extension at 72 for 30 s. After 36 cycles and final extension 72 for 10 min, analyze amplification products on 1 % agarose gels.

7.5.5. Stain gels with nucleic acid staining and visualize the gels under UV light.

Comments:

• There are several mistakes and imprecise information (an example: "…the binary vector has been successfully transformed into hairy roots…"), that may require extensive scientific review, besides additional general copy-editing.

Answer:

Thanks for the comments. We have corrected to" …the binary vector has been transformed into TB genome…". In addition, we have also corrected some other imprecise information based on scientific proofreading.

Comments:

• Part of the results describes the expression of a transcription factor and the effect on

the expression of other genes involved in flavonoid biosynthesis and the flavonoid yield. No protocol is presented and no references are found on the method used to obtain this data.

Answer:

Thanks for the comments. The transcriptional factor *FtMYB116* has been inserted a binary vector, which has been transformed into *A. rhizogenes* in advance. Because the expression of genes is not the primary objective, we did not involve this part in our protocol. However, we have extended the brief protocol of the expression of *FtMYB116 and related genes,* "Briefly, hairy roots of 0.1 g were triturated into fine powder by using liquid nitrogen. Total RNA was extracted by following the instructions of manufacturer of plant RNA isolation kit[15]. Then reverse-transcription PCR and real time PCR were performed to amplify FtMYB116 and rutin synthesizing pathway related genes. Subsequently the regulatory effects of *FtMYB116* on rutin synthesis-related gene expression and the yield of rutin were verified." in the representative results.

Comments:

• There is no information on the genes tested (what is FSH, CHS, etc.?).

Answer:

Thanks for the comments. We previously explained CHS (chalcone synthase), F3H (flavanone 3-hydroxylase) and FLS (flavonol synthase) as key enzymes involved in flavonoid biosynthesis in the discussion part. To make these tested genes much clearer, we defined these genes in introduction part and figure 4 legends.

Comments:

• Discussion is lengthy and not focused on the results presented. No discussion of potential applications to increase flavonoid production, for example. The authors also make several statements for which no references are provided. The protocol presented is not compared with previously published results on buckwheat hairy roots induction and flavonoid production (e.g. Kim et al 2009 - quoted but not discussed). For sure there would be much more to comment.

Answer:

Thanks for the comments. To increase flavonoid production is one of the potential applications of hairy roots, but is not the main point that this article should focus on. However, we analyzed some reasons to affect flavonoid production such as the cultivar, light, time *etc.* TB hairy roots induction is the main point that has been elaborately

discussed in this study in light of published methods.

Minor Concerns:

Comments:

• The protocol presented uses mercury bichloride (MB) to disinfest the seeds ("sterilize", according to the text). Mercury bichloride is extremely poisonous. Still, the authors made no comments on its hazards, or on procedures for handling and disposing of. They did not provide any explanation to justify using this compound. In fact, Kim et al (2009) (reference 3) disinfested buckwheat seeds with 70% (v/v) ethanol for 1 min and 4% (v/v) sodium hypochlorite solution for 10 min. This should be quite effective for disinfesting seeds and is not nearly as hazardous and MB.

Answer:

Thanks for the comments. We totally agree mercury bichloride (MB) is a relatively more hazardous compounds than sodium hypochlorite. In fact, we also do use sodium hypochlorite to disinfect buckwheat seeds in our lab. The reason we use MB in our lab is because inadequate disinfection happens sometimes when we use sodium hypochlorite so that the seeds were infected by some fungus. Therefore, we put sodium hypochlorite in the first place to sterilize seeds in this protocol, and we added NOTE to indicate MB is an alternative sterilizer after step 1.2.3 and caution for MB handle, operation and discard subsequently.

Comments

• The protocol presented for PCR detection of the transgenes uses RNA extraction and cDNA. OK, one can do that. But why not just prepare a DNA extraction - much simpler and less expensive?

Answer:

Thanks for the comments. This part actually was a mistake. Thanks for pointing it out. We indeed performed DNA extractions for PCR detection of the transgenes. We conducted RNA extraction and cDNA to indicate our target gene expression in the representative results. In the revision, we corrected this mistake.

Comments:

• What is gene 2b, for which there is a primer pair in Table 2?

Answer:

Thanks for the comments. We did not mention gene 2b in this article. Probably reviewer meant gene b4, of which we listed a primer pair in Table 2. Gene b4 is a newfound from our group but unpublished gene belonging to *bHLH* family. In order to not cause necessary trouble in the later publication, we name this gene as gene b4 in this article. Furthermore, we add some more detailed information about b4 in the introduction part.

Zhihui Zhu, a in progress master student, is doing transgenic medicinal-plant in HuaiBei Normal University and is joint-supervised in Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences.

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Jianping Xue, Professor, Ph.D. in genetic breeding, is working in College of Life Science, Huaibei Normal University. He is mainly working on biomedical technology, resources in medicinal plants.

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Yaolei Mi is working on gene identification and metabolomics of medicinal plants in Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences. He obtained master degree in biomedical engineering from the University of New south wales in Sydney. He has published a article in *Advanced Functional material.*

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Best regards.

Sun Wei, PhD
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Title of Article: Method of Inducing Hairy Roots by Agrobacterium rhizogenes-mediated transformation in Tartary Buckwheat (Fagopyrum tataricum)

Author(s): Yaolei Mi, Zhihui Zhu, Guangtao Qian, Yu Li, Xiangxiaomeng, Qingyuchen Jianpingxue, Wei Sun, Yuhua Shi

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