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Development of *Agrobacterium tumefaciens*-mediated transformation of *Solanum nigrum* and expression of *AcMYB110* in *S. nigrum*

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Abstract

Solanum nigrum L. is an annual herb of *Solanaceae* and *Solanum* genus. *S. nigrum* produces a small, spherical, black, highly nutritious edible fruit and is an orphan crop. To realize the potential of large-scale production of *S. nigrum* fruits, it is necessary to improve undesirable characteristics, such as tiny fruits, by using available methods of genetic engineering and gene editing. The genetic transformation system of *S. nigrum* is the basis for genetic improvement through transgenic and gene-editing technology. In this study, an efficient *Agrobacterium tumefaciens*-mediated transformation method was established. The R2R3-MYB transcription factor *AcMYB110* from kiwifruit was used as a foreign gene to transform *S. nigrum*. The results showed that anthocyanin accumulation was accumulated in the transformed cells and tissues, which could visually track and monitor each stage of the transformation process. *AcMYB110* could be used as a reporter gene in the genetic transformation of *S. nigrum*. The petals of *AcMYB110* transgenic *S. nigrum* plants are pink, and anthocyanins were accumulated in the pericarp from young fruit to maturity, especially in the central part of the flesh of the *S. nigrum* fruit. The phenotype produced by overexpression of *AcMYB110* in kiwi and *S. nigrum* is consistent. It indicates that *AcMYB110* is relatively conservative in regulating plant anthocyanin synthesis.

Keywords

Solanum nigrum L., *Agrobacterium tumefaciens*-mediated transformation, MYB transcription factor, Kiwi, anthocyanin

Introduction

Solanum nigrum L. (Black nightshade) is an annual herb of *Solanaceae* and

Solanum genus. Its fruit is a spherical berry and has a unique flavor after ripening. The mature fruit is rich in anthocyanins and has high nutritional value. In addition, the whole plant of *S. nigrum* can be utilized in traditional medicine. Lipid-soluble extract of *S. nigrum* has the effects of dispersing blood stasis, detumescence, clearing away heat, and detoxification (Zakaria et al. 2006; Hsieh et al. 2008; Lin et al. 2007). Polyphenols in *S. nigrum* fruit can reduce body weight and body fat by reducing the number and lipid content of adipocytes and changing lipid metabolism (Peng et al. 2020). Therefore, *S. nigrum* has the potential as a special fruit crop as a medicinal and edible plant, which can provide additional sources of income for farmers (Sangija et al. 2021). Up to date, *S. nigrum* has not been domesticated, and many agronomic traits of *S. nigrum* have wild characteristics, such as the fruit is very small and harboring many seeds. The weight of one mature fruit of *S. nigrum* is only 0.3 grams. *S. nigrum* will become a potential fruit in the future by improving undesirable traits of *S. nigrum*, such as plant type and fruit size. At present, orphan crops and wild plants have been rapidly domesticated through gene editing technology, such as groundcherry (*Physalis pruinosa*), wild tomato (*Solanum pimpinellifolium*), wild allotetraploid rice (*Oryza alta*). (Lemmon et al. 2018; Li et al. 2018; Yu et al. 2021). *Agrobacterium tumefaciens*-mediated transformation is the basis of gene editing. Although Park et al. (2020) reported the *A. tumefaciens*-mediated transformation method of *S. nigrum*, we did not obtain regenerated plants when using this method for genetic transformation. Therefore, we want to establish an efficient *A. tumefaciens*-mediated transformation method for *S. nigrum* based on genetic transformation method of tomato and groundcherry, which also belong to the *Solanum* genus.

Anthocyanins are water-soluble natural pigments that widely exist in flowering plants (angiosperms). Anthocyanin is one of the main pigments that constitutes the color of petals and fruits. Anthocyanins, as a convenient biomarker, have been used in genetics and epigenetics. Anthocyanins are flavonoids synthesized from a branch of phenylpropane pathway. Phenylpropane metabolism in plants is mainly controlled by MBW (MYB–bHLH–WDR) protein complex at the transcriptional level (Xu et al. 2015), in which MYB is a key regulator of anthocyanin synthesis. For example,

MYB75/PAP1 of *Arabidopsis* is a R2R3-MYB transcription factor, which mainly regulates anthocyanin biosynthesis in *Arabidopsis* (Onkokesung et al. 2014). Overexpression of *AtMYB75* in tomato increased the anthocyanin content in leaves, stems, flowers and fruits of tomato (Zuluaga et al. 2008). Liu et al. (2019) applied *AtMYB75* as a reporter gene to the CRISPR/Cas9 gene editing vector for the screening of transgenic and non-transgenic plants in the offspring. *AtMYB75* is ectopic expressed in the hairy roots of leguminous plants such as soybean, *Lotus corniculatus* Linn. and *Medicago truncatula* mediated by *Agrobacterium rhizogenes*, which can induce the accumulation of anthocyanins in hairy roots and show the purple color visible to the naked eye (Fan et al. 2020a). Overexpression of *SIMYB75* in tomato can significantly increase the anthocyanin content in various organs and tissues of tomato (Jian et al. 2019). The *SLANT1* gene, which controls anthocyanin synthesis, was used as a reporter gene in hybrid seed production (Du et al. 2020).

In the previous work, we used *AtMYB75* as a reporter gene for identifying transgenic hairy roots in the plant hairy root transformation (Fan et al. 2020a). By comparing different R2R3-MYB transcription factors that have been reported, *AcMYB110* from kiwifruit caused the highest anthocyanin accumulation through transiently heterologously expressed in tobacco leaves (Peng et al. 2019). Therefore, we tried to use *AcMYB110* as a reporter gene in the genetic transformation process. In this study, *AcMYB110* was transformed into *S. nigrum* with an improved *A. tumefaciens*-mediated transformation method, in order to establish a highly efficient transformation system of *S. nigrum* and analyze the expression characteristics of *AcMYB110* in *S. nigrum*.

Materials and methods

Plant material and in vitro seed germination

The seeds of *S. nigrum* were collected in the experimental field of Liaocheng University and preserved by our laboratory, and named Snlc1.

Binary vector constructed

AcMYB110 gene (GenBank No. KF311107) was synthesized by Beijing Qingke Biotechnology Co., Ltd. The complete sequence of *AcMYB110* gene is 729bp. Primers

110Bam (5'-CGGGATCCATGGAAACCGTTCCACTTG-3', *Bam*HI restriction site underlined) and 110Sac (5'-CGCTGAGCTCAATCATCACCAAGCAAATTCC-3', *Sac*I restriction site underlined) were used to amplify the *AcMYB110* gene with synthesized *AcMYB110* as template. The PCR product digested with *Bam*HI and *Sac*I was cloned into the expression vector pBI121 between enzyme digestion sites of *Bam*HI and *Sac*I. The new vector was verified by Sanger sequencing and named pBM110, and transformed into *A. tumefaciens* LBA4404 by electroporation.

Plant transformation

Park et al. (2020) reported the transformation method of *S. nigrum*. According to this method, we transformed the expression vector carrying *AcMYB110* into *S. nigrum*. However, the explants could not form adventitious buds in the process of meristem culture and there were a large number of roots on the explants. We think this method is not suitable for the *S. nigrum* transformation.

An efficient *A. tumefaciens*-mediated transformation method of *S. nigrum* has been established by referring to the transformation methods of tomato (Sun et al. 2016) and groundcherry (Swartwood and Van Eck. 2019). The methods were as follows: (1) Seeds of *S. nigrum* were washed with 70% (v/v) ethanol for 1 minute, disinfected with 3% (w/v) sodium hypochlorite for 15 minutes, and then washed with sterile water five times. The seeds were placed in 1/2 Murashige and Skoog (MS) medium (1/2 MS+2% sucrose+0.7% agar). They were cultured in a growth chamber under 26°C/16h light and 22°C/8h darkness for 10 days. (2) *A. tumefaciens* LBA4404 harboring pBM110 were grown to OD₆₀₀ 0.4–0.6 and then *A. tumefaciens* cells were harvested and re-suspended in liquid MS media containing 2% sucrose and 0.1 mM acetosyringone (MSIn). Cotyledons and hypocotyls cut with 0.5-1cm were immersed into *A. tumefaciens* suspension for 20min, then dried with sterile filter paper and cultured on MSIn agar medium at 19-20°C in darkness for 48h. To wash off *A. tumefaciens*, the explants were washed with MS liquid media containing 500 mg/L cefotaxime for five times. (3) Cotyledons and hypocotyls explants washed were transferred to MS selection and differentiation culture medium (MSSD: MS+250mg/L Kanamycin+1mg/L Zeatin+400mg/L cefotaxime+2% sucrose+0.7% agar). After 3

weeks the explants harboring new organogenic callus or buds were transferred to new MSSD. In this selection and differentiation stage, the explants were cultured in a growth chamber under 16h light and 8h darkness at 25°C. (4) When the bud grew to 2-3cm, the bud should be cut off explants and transferred to MS rooting medium (MSRo: MS+200mg/L Kanamycin+1mg/L IAA+2% sucrose+0.7% agar). (5) After the roots of the regenerated plants grew to about 2cm, the plants were moved from the tissue culture bottle to the nutrient soil/vermiculite (1:1), and grew in a growth chamber under 26°C/16h light and 22°C/8h darkness.

DNA extraction and PCR analysis

Genomic DNA was extracted from fresh young leaves of each regenerated plant and PCR amplification was performed according to the previous method (Fan et al.2020b). To isolate plants carrying *AcMYB110*, each regenerated plant with a purple stem was genotyped with primers designed to amplify *AcMYB110* and NOS terminator. The forward primer M110F (5'-ACGGAGAAGGAAAGTGGTACC-3') is located in *AcMYB110* and reverse primer NOS-R (5'-GATAATCATCGCAAGACCGG-3') from the NOS terminator were used.

Results

Construction of overexpression vector carrying *AcMYB110* gene

The binary vector pBI121 (Chen et al. 2003) was selected for *A. tumefaciens*-mediated transformation, because it contains the commonly used selective marker gene *NPTII* in dicotyledons. The expression of *AcMYB110* gene was driven by 35S promoter after replacing GUS gene with *AcMYB110* gene. The vector with correct enzyme digestion and Sanger sequencing was named pBM110 (Fig. 1).

Establishment of efficient transformation method of *S. nigrum*

A. tumefaciens harboring pBM110 was used to transform *S. nigrum* according to transformation method reported by Park et al. (2020). However, the explants could not form adventitious buds in the selection and differentiation medium (MS+Zeatin+IAA), and there were many roots on the explants (Figure 2). The explants transformed with pBM110 formed purple roots and purple callus (Fig. 2). This indicates that *AcMYB110* promotes anthocyanin accumulation in callus and roots and can be used as a reporter

gene in *A. tumefaciens*-mediated transformation of *S. nigrum*. The role of IAA in plant regeneration is to induce the production of roots. Therefore, we adjusted the hormone formula of the differentiation medium. Only zeatin was added to the differentiation medium of *S. nigrum*.

An efficient *A. tumefaciens*-mediated transformation method of *S. nigrum* has been established, which is more straightforward than methods of tomato and groundcherry. In this study, cotyledons and hypocotyls of *S. nigrum* were all used for meristematic culture, and there were no difference between them in the efficiency of callus producing adventitious buds (Fig. 3. A). So cotyledons and hypocotyls of *S. nigrum* can be used for *A. tumefaciens*-mediated genetic transformation. Cefotaxime needs to be added to the selection and differentiation medium to inhibit the growth of *A. tumefaciens* and does not affect the regeneration of adventitious buds of *S. nigrum* explants. When buds and true leaves of explants are transferred to MS rooting medium, it is generally not necessary to add Cefotaxime. The explants transformed with pBM110 formed purple adventitious buds (Fig. 3 B) and transgenic plants with purple stems (Fig. 3 C).

The Phenotype of transgenic *S. nigrum* with *AcMYB110*

Five regenerated plants with purple stems were transferred into the nutrient soil for growth. *AcMYB110* was inserted into the *S. nigrum* genome by PCR assay using primer set M110F and NOS-R (Fig. 4). To test inheritance of the *AcMYB110* transgene, T1 generation seedlings were grown on 1/2 MS medium containing kanamycin (200 mg/L). About 3/4 of T1 plants grew well and showed purple stem, indicating dominant inheritance of the *AcMYB110* gene in the segregated progenies.

About 6 weeks later, transgenic *S. nigrum* began to flower. At the flowering stage, the petals of transgenic *S. nigrum* were light red (Fig. 5 A), the anthers were orange, and the sepals and styles were slightly reddish (Fig. 5 B). In the young fruit stage, the peel of transgenic *S. nigrum* was light red (Fig. 5 C), the center of the fruit pulp was red, and it become dark purple at maturity (Fig. 5 D).

Discussion

We learned from the regeneration and transformation methods of *Solanaceae*

plants, tomato and groundcherry, and found that the MS medium with hormone ratio from these methods is suitable for the genetic transformation of *S. nigrum* (Sun et al. 2006; Swartwood and Van Eck. 2019). For the selection of transformed explants, cotyledons are generally selected in tomato for genetic transformation (Van Eck et al. 2019). However, only hypocotyl explants regenerated buds in the transformation of groundcherry (Swartwood and Van Eck. 2019). Here, we used cotyledons and hypocotyls for genetic transformation simultaneously. As a result, cotyledons and hypocotyls of *S. nigrum* can regenerate into plants, and their transformation efficiency is the same.

Plant selection marker gene is a critical component of developing efficient transformation methods. In dicotyledonous plants, the commonly used selection gene is the *NPTII* gene. Theoretically, only transgenic cells can obtain resistance to kanamycin and survive and form a complete plant after adding kanamycin to the meristematic medium. However, some plants are not sensitive to antibiotic selection, and some nontransgenic plants can grow in antibiotic medium, which requires PCR analysis to amplify the selection marker gene to determine which plants are genuinely transgenic. In this study, we used the anthocyanin regulation gene *AcMYB110* to select transgenic plants. The expression of *AcMYB110* makes transgenic cells or tissues produce anthocyanin accumulation. This visual anthocyanin with naked eyes enables us to track different stages of genetic transformation. Therefore, *AcMYB110* is very efficient, convenient, and time-saving as a reporter gene for selecting transgenic plants in *S. nigrum*.

Overexpression of *AcMYB110* causes the petals of kiwi to be red, and the flesh and skin of kiwi fruit are red (Wang et al. 2022). In this study, the accumulation pattern of anthocyanins in different tissues and organs of plants overexpressing *AcMYB110* in *S. nigrum* are consistent with the phenotype produced by overexpression of *AcMYB110* in kiwi, which indicates that the *AcMYB110* transcription factor is a relatively conservative in regulating anthocyanin synthesis.

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Author contributions

YF and SL designed the experiments and wrote the paper. WP, XW, HW, and ZZ carried out binary vector construction and *A. tumefaciens*-mediated transformation of *S. nigrum*; CT, QL and KL carried out PCR analysis and T0/T1 plants management. All authors read and approved the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Legends to figures

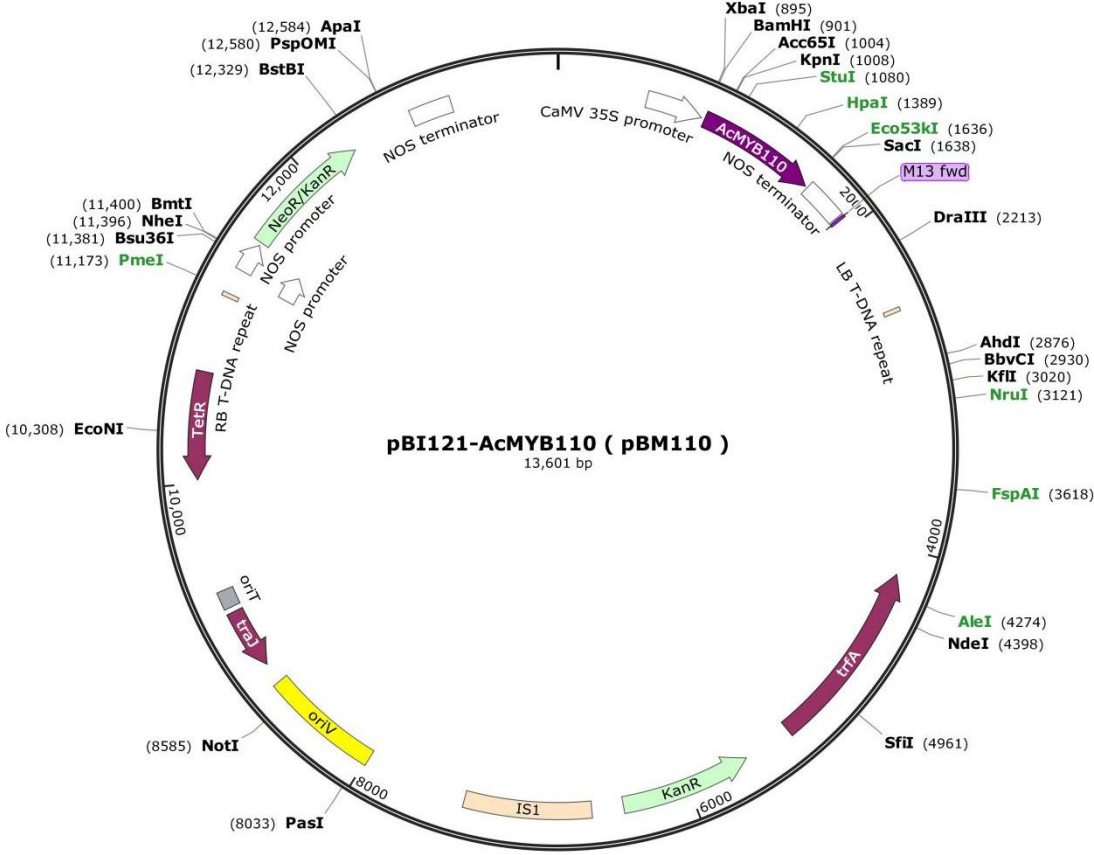
Fig.1 Map of the binary vector pBM110 harboring *AcMYB110* driven with 35S promoter.

Fig.2 A: Determination of explants of *Solanum nigrum* infected with *Agrobacterium tumefaciens* containing pBM110 in MS with ZT and IAA . B: the enlarged explant in the circle in A. Arrowheads mark anthocyanin accumulation in the roots and callus.

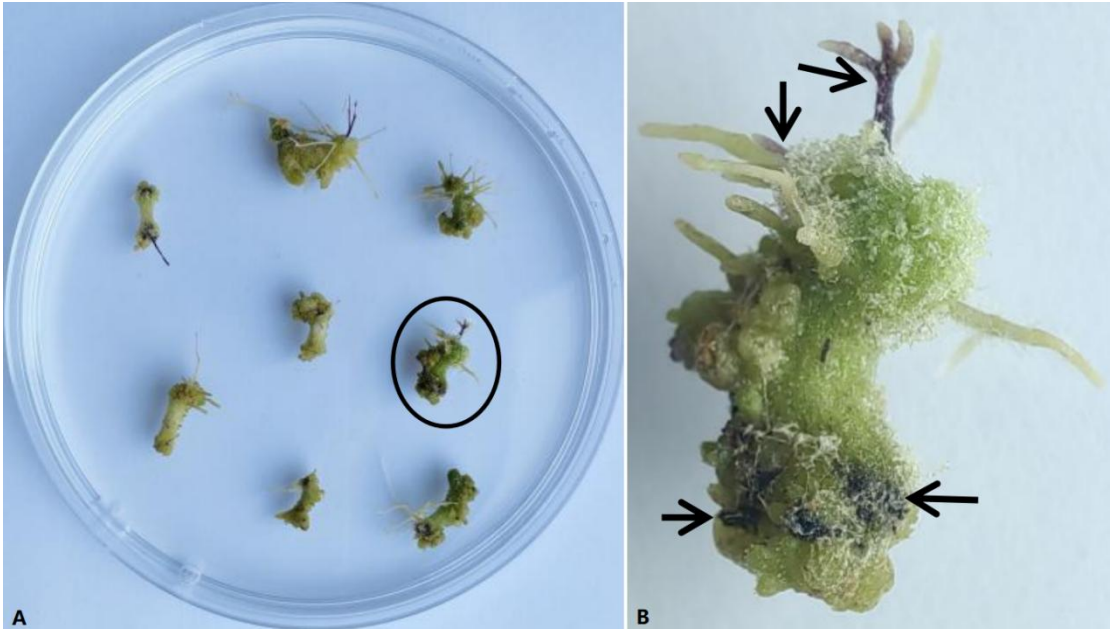
Fig.3 Establishment of an efficient transformation method of *Solanum nigrum*. A: First stage of determination and selection of explants of *S. nigrum* (The explants on the left side of the vertical line is the hypocotyls of *S. nigrum*, and the cotyledons is on the right side). B: Second stage of determination and selection and anthocyanins accumulated in some callus cells and buds. C: Rooting culture.

Fig.4 PCR analysis for detection of *AcMYB110* gene and NOS terminator. Expected product size of 730 bp. Lanes: M: molecular size marker (DL2000); 1: binary plasmid pBM110, 2: regenerated plants with a green stem; 3–7: regenerated plants with a purple stem.

Fig. 5 The appearance of flower and fruits in *AcMYB110* overexpression (OE) line and wildtype (WT) of *Solanum nigrum*. A: flower; B: stamens and stigmas; C: fruits of 5, 10, 15, 20, 25, 30, and 35 days after flowering; D: cross section of fruits in C. Bars=1cm

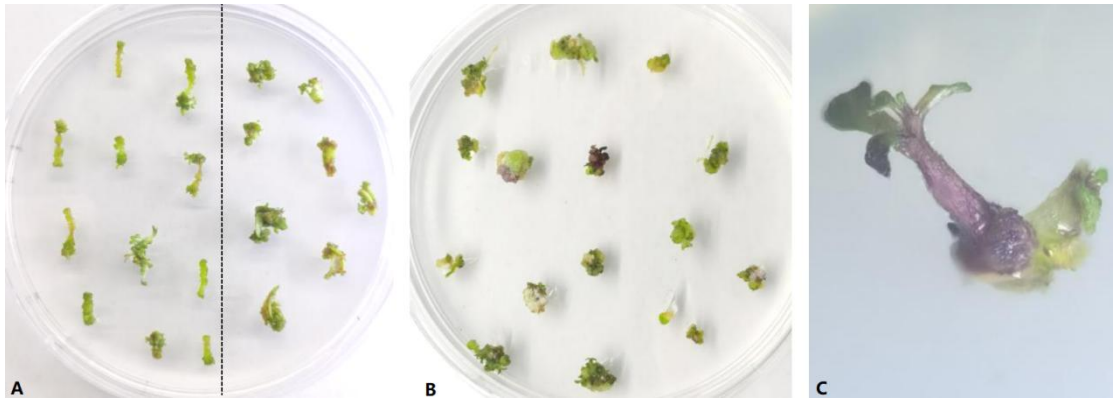


343 **Fig. 2**



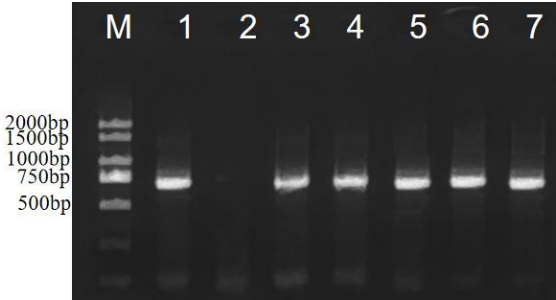
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Fig. 3



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Fig. 4



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Fig. 5

