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Ectopic overexpression of *AcMYB110* causes significantly increased content of anthocyanins in *Solanum nigrum*

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Abstract

Solanum nigrum L. is an annual undomesticated berry plant of *Solanaceae*. The fruits of *S. nigrum* are tiny, but there are about 25 seeds in a single fruit. The total number of seeds produced in one plant can reach more than 3 000. The height is about 30 - 40 cm, and the whole growth cycle is two months when *S. nigrum* was cultivated in the light incubator of the laboratory. The *Agrobacterium tumefaciens*-mediated transformation has been established in *S. nigrum*. So *S. nigrum* has the characteristics of model plants. *AcMYB110*, an R2R3-MYB transcription factor from kiwi (*Actinidia* spp.), was transformed into *S. nigrum* mediated by *A. tumefaciens*. The results indicated that the petals of 35S:*AcMYB110 S. nigrum* plants are pink compared with white petals in wild-type plants, and content of anthocyanins was significantly increased in the pericarp from young fruit to its maturity, especially in the central part of the fruit flesh. The results showed that the ectopic expression of *AcMYB110* in *S. nigrum* is consistent with the expression of *AcMYB110* in kiwi. This suggests that *AcMYB110* plays a conserved role in regulating anthocyanins synthesis in fruits and can be potentially applied for improvement of the anthocyanins content in horticulture fruits breeding.

Keywords: *AcMYB110*, *Actinidia* spp. (kiwi), anthocyanin, R2R3-MYB, *Solanum nigrum*.

Introduction

Solanum nigrum is an annual undomesticated berry plant of *Solanaceae.* The wild *S. nigrum* in northern China is a dwarf plant, about 30 - 40 cm high, and has small fruits (about 3 g), but each fruit has a large number of seeds about 20 - 30 (25 on average). The average number of fruit in one plant is about 100, and about 3 000 seeds can be produced. It takes about one month from sowing to flowering and another one month from flowering to fruit ripening. Therefore, the whole growth cycle is two months or so. The genetic transformation mediated by *A. tumefaciens* has been established in *S. nigrum* (Schmidt *et al*. 2004, Bhattacharya *et al*. 2012, Zou *et al*. 2017, Sharada *et al*. 2019, Chhon *et al*. 2020, Peng *et al*. 2023). The growth characteristics and research attributes make *S. nigrum* a model plant.

Anthocyanins are water-soluble pigments, which are widely distributed in many plants. Anthocyanins are synthesized through flavonoid biosynthesis pathway. There are two kinds of genes involved in the anthocyanin biosynthesis pathway in plants, one is the genes encoding the key enzyme of anthocyanin biosynthesis, and the other is the genes encoding the transcription factors (TFs) regulating the expression of functional genes. Among TFs, three transcription factors R2R3-MYB, bHLH (basic helix-loop-helix), and WD-repeat regulate the anthocyanin biosynthesis pathway by forming a protein complex (Gonzalez *et al*. 2008). However, some studies have shown that transcription factors R2R3-MYB can independently regulate anthocyanin biosynthesis, so MYB factors play a crucial role in controlling anthocyanin biosynthesis (Gonzalez *et al*. 2008, Jian *et al*. 2019). In our previous study, *AtMYB75*/*PAP1*, an R2R3-MYB

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Abbreviations: bHLH - basic helix-loop-helix; MYB - V-myb avian myeloblastosis viral oncogene homolog; OE - overexpression; R2R3-MYB - MYB consisting of two adjacent conserved N-terminal DNA-binding domain repeats R2R3; TFs - transcription factors; WD-repeat - highly conserved repeating units usually ending with Trp-Asp (WD).
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transcription factor from *Arabidopsis*, was used as a reporter gene in *Agrobacterium rhizogenes*-mediated hairy root transformation in soybean, *Lotus japonicus*, *Medicago truncatula*, and tomato (Fan *et al*. 2020). The transient expression of several R2R3-MYB transcription factors from different plants was compared in tobacco leaves, and the results showed that *AcMYB110* from kiwi (*Actinidia* spp.) caused the highest anthocyanin accumulation (Peng *et al*. 2019). 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). Therefore, we would like to know the accumulation of anthocyanins in different organs of transgenic lines of *S. nigrum* with *AcMYB110* overexpression.

Materials and methods

Solanum nigrum L. was collected in the Liaocheng City, China and preserved in our laboratory under the name Snlc1. *S. nigrum* was cultivated in a growth chamber at a temperature of 24 - 26°C, air humidity of 70%, a 16-h photoperiod, and an irradiance of 100 μmol m^{-2} s^{-1}.

The *pBM110* was transformed into *S. nigrum* Snlc1 following previously described methods (Peng *et al*. 2023). The transgenic lines of *S. nigrum* were cultivated in a growth chamber at conditions mentioned above.

Fruits of *AcMYB110* transgenic and wild-type plants of *S. nigrum* were collected for anthocyanin measurement every fifth day after flowering. Anthocyanin content was measured as previously described by Neff and Chory (1998). Anthocyanin was extracted from the fruit tissue of *S. nigrum* with 600 μL of acidified methanol (1% in concentrated HCl) overnight at 4°C and in the dark. The extracting solution was diluted to a 60% solution by addition of 400 μL of ddH_2O. The samples were centrifuged at 4°C and 2 000× *g* for 5 min. The supernatant was moved to a new centrifuge tube, and an equal volume of chloroform was added. The samples were vortexed and centrifuged at 4°C and 2 000× *g* for 5 min again. The supernatant (200 μL) was diluted 1:6 with 1 000 μL fresh 60% acidified methanol. Total of 1 200 μL samples were used for determination of absorbance (A) at 530 and 657 nm. Relative anthocyanin content was calculated as $[(A_{530} - A_{657}) \times 6/\text{mg(FM)}] \times 1\,000$ (Wang *et al*. 2022).

Results

Five *AcMYB110* overexpressing (OE) transgenic lines transformed with *pBM110* were confirmed with RT-PCR (Peng *et al*. 2023). The leaf and flower of wild type (WT) *S. nigrum* are shown in Fig. 1*A*, and the vein of the leaf was green. All OE plants showed purple-colored veins in the leaf (Fig. 1*B*). Two types of flower color of the OE lines were found. One type found in OE line Ac110-1 had purple apex of petals, and purple spots on anthers (Fig. 1*C*). Another type found in OE lines, Ac110-2/-3/-4/-5, had light red petals, and darker yellow stamens (Fig. 2*A*,*B*). In the young fruit stage, the fruit peel of OE lines was light red (Fig. 2*C*), the center of the fruit pulp was red, and it became dark purple at maturity (Fig. 2*D*). When *S. nigrum* fruit was almost mature at 30 d after flowering, its peel began to turn purple, and the fruit ripens within the next five days (Fig. 2*C*). The fruit of *S. nigrum*, like the fruit of the tomato, showed respiratory climacteric behavior during the ripening.

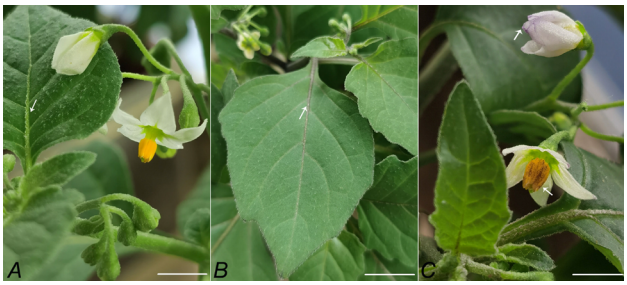

Fig. 1. The appearance of wild type (WT) and *AcMYB110* overexpression (OE) line Ac110-1 of *Solanum nigrum*. *A* - leaf and flowers of WT, *arrows* show green colored vein; *B* - leaf of Ac110-1, *arrows* show purple colored vein; *C* - flowers of Ac110-1, *arrows* show purple colored petal apex and spotted anthers. *Bars* = 1 cm.

The fruits of 5, 10, 15, 20, 25, 30, and 35 d after flowering of *AcMYB110* (pBM110) OE line Ac110-2 and WT of *S. nigrum* were used for anthocyanin content measurement (Fig. 3*A*,*B*). The results showed that anthocyanins were accumulated already in young fruits of OE transgenic plants. The anthocyanin content in mature fruits of OE lines was about 50% higher than that of WT fruits (Fig. 4). When the fruit was about to mature, the anthocyanin content rose sharply.

Discussion

Tomato (*Solanum lycopersicum* L.) belongs to family *Solanaceae* and it is an important vegetable and nutrient crop in the world. Because of genome size (about 950 MB), short life cycle, easy transformation, different mature phenotypes and abundant bioinformatics resources, tomato has become a model plant for studying fruit ripening and biotic and abiotic stresses (Klee and Giovannoni 2011). However, low light intensity is particularly unfavorable and can cause abortion in tomato (Kinet 1977). When *S. nigrum* and tomato variety Moneymaker were cultivated in the light incubator of the laboratory, there was no seed in fruits of tomato, but the seeds in fruit of *S. nigrum* were normal. This is very important for genetic research. The genome of *S. nigrum* has not been sequenced yet. There is a report about chloroplast sequencing of *S. nigrum* (Khan *et al*. 2017). The evolutionary relationships in phylogenetic analysis showed that *S. nigrum* is closer to *Solanum tuberosum* when compared with other species belonging to *Solanum* genus (Khan *et al*. 2017). The genetic transformation mediated by *A. tumefaciens* has been established in *S. nigrum*. So *S. nigrum* has the potential to become a model plant.

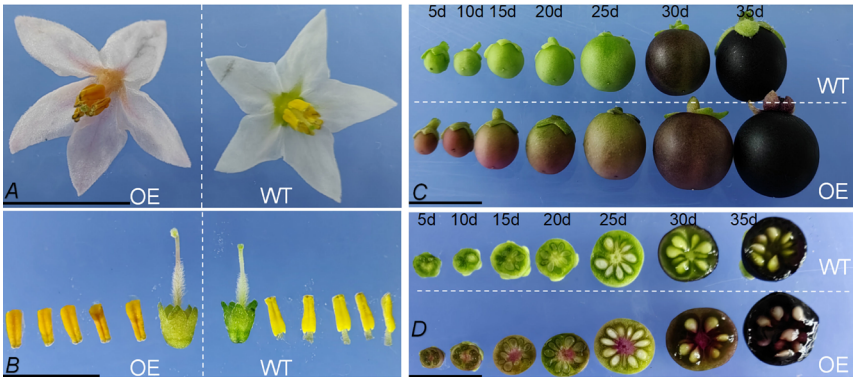

Fig. 2. The appearance of flowers and fruits in *AcMYB110* (pBM110) OE line Ac110-2 and wild type (WT) of *S. nigrum*. *A* - flower; *B* - stamens and pistils; *C* - fruits of 5, 10, 15, 20, 25, 30, and 35 d after flowering; *D* - cross-section of fruits. *Bars* = 1 cm.

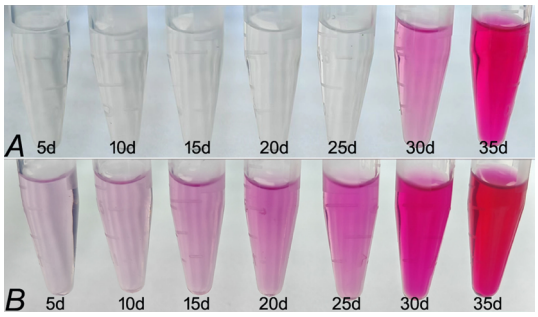

Fig. 3. Anthocyanin content in fruits of WT (*A*) and OE line Ac110-2 (*B*) of *Solanum nigrum*.

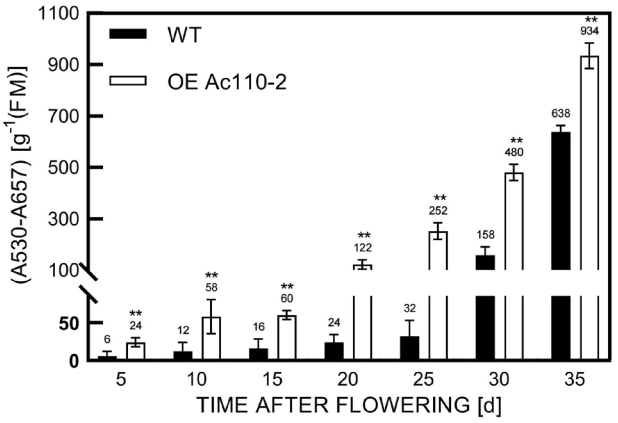

Fig. 4. Anthocyanin content in fruits of WT and OE line Ac110-2 of *Solanum nigrum*. Means ± SEs, $n = 6$. *Asterisks* indicate significantly statistical difference: $^{**}p<0.01$.

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* was 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. The fruit of *S. nigrum* and the fruit of kiwi are both berries, thus *S. nigrum* can be used as a model plant for studying berries.

There are many reports that anthocyanins can enhance the tolerance of plants to drought, low temperature, and other stresses, and improve the resistance of plants to diseases and pests (Xie *et al*. 2016). Accumulation of anthocyanins reduces susceptibility to *Botrytis cinerea* in tomatoes, and shelf life can significantly be extended (Zhang *et al*. 2013). Therefore, the accumulation of anthocyanins is considered a visual molecular marker to judge whether plants encounter adverse environments during their growth (Xie *et al*. 2016).

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