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Enhanced Salt Stress Tolerance in Transgenic Potato Plants Expressing IbMYB1, a Sweet Potato Transcription Factor

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IbMYB1, a transcription factor (TF) for R2R3-type MYB TFs, is a key regulator of anthocyanin biosynthesis during storage of sweet potatoes. Anthocyanins provide important antioxidants of nutritional value to humans, and also protect plants from oxidative stress. This study aimed to increase transgenic potatoes' (*Solanum tuberosum* cv. LongShu No.3) tolerance to environmental stress and enhance their nutritional value. Transgenic potato plants expressing *IbMYB1* genes under the control of an oxidative stress-inducible peroxidase (SWPA2) promoter (referred to as SM plants) were successfully generated through *Agrobacterium*-mediated transformation. Two representative transgenic SM5 and SM12 lines were evaluated for enhanced tolerance to salinity, UV-B rays, and drought conditions. Following treatment of 100 mM NaCl, seedlings of SM5 and SM12 lines showed less root damage and more shoot growth than control lines expressing only an empty vector. Transgenic potato plants in pots treated with 400 mM NaCl showed high amounts of secondary metabolites, including phenols, anthocyanins, and flavonoids, compared with control plants. After treatment of 400 mM NaCl, transgenic potato plants also showed high DDPH radical scavenging activity and high PS II photochemical efficiency compared with the control line. Furthermore, following treatment of NaCl, UV-B, and drought stress, the expression levels of IbMYB1 and several structural genes in the flavonoid biosynthesis such as CHS, DFR, and ANS in transgenic plants were found to be correlated with plant phenotype. The results suggest that enhanced IbMYB1 expression affects secondary metabolism, which leads to improved tolerance ability in transgenic potatoes.

Keywords: IbMYB1, potato, salt tolerance, secondary metabolite, transgenic

Introduction

Plants have to fight against abiotic stresses from the environment, such as drought, extreme temperature, salt, and UV irradiation, for growth and crop production [36, 52]. Low-molecular-weight antioxidants and antioxidant enzymes play important roles in scavenging and controlling the production and accumulation of reaction oxygen species (ROS) when plants are exposed to environmental stresses [5, 22]. Therefore, various metabolic pathways using secondary metabolites have evolved in plants to help them adapt to changing environments [16]. Flavonols, anthocyanins, and proanthocyanidins are secondary plant metabolites that are known collectively as flavonoids. Flavonoids are found to directly scavenge Q^{2-} and $\cdot OH$ through single electron transfer [54], and the total flavonoid level was found to be highly correlated to a plant's antioxidant capacity [2]. Anthocyanin is also linked to stronger abiotic stress resistance [38, 48, 46]. Phenols, including flavonoids (flavonols, flavones, anthocyanins, *etc*.) and several classes of non-flavonoids (phenolic acids, lignins, stilbenes, terpenoids, *etc*.), have been found to determine the antioxidant activity of plants [13]. The flavonoid biosynthetic pathways appear to be mostly regulated at the transcriptional level [39, 47].

The MYB regulators are divided into four types, MYB1R, R2R3-MYB, R1R2R3 MYB (MYB 3R), and 4R MYB, based on the repeats of their specific DNA-binding domains. Numerous R2R3-MYB proteins, which were mainly found in plants, have been proved to be involved in the control of plant-specific processes, including secondary metabolism and response to biotic and abiotic stresses [12, 44]. In many plant species, MYB proteins were reported to be capable to regulate the biosynthesis of flavonoids and anthocyanin, and be induced by abiotic stress, exemplified by *Arabidopsis MYB75* (*PAP1*), *AtMYB90* (*PAP2*) [4], *MYB12*[35], and *MYBL2* [34], petunia *AN2* [40] and *PH4* [39], grape *MYBA1* and *MYBA2* [27, 28], *MYB5a* [11], and *MYBF1* [9], sweet potato *MYB1* [33], apple *MYB10*/*MYB1*/*MYBA* [3, 15, 45], legume *LAP1* [37], Persimmon *MYB4* [1], and *Epimedium sagittatum MYBA1* [19].

In this study, we aimed to develop transgenic potatoes (cv. LongShu No.3) that express *IbMYB1*, which was isolated from sweet potato by Kim *et al.* [23] and under control of an oxidative stress-inducible peroxidase (SWPA2) promoter [24], in order to increase the anthocyanin content in leaf and in tuber, and to subsequently enhance the resistant ability of potato to abiotic stress.

Materials and Methods

Plant Transformation and Gene Expression Analysis

The transformation construct was obtained by inserting the 750 bp *IbMYB1* gene (Accession No. AB258984) fragment into the *Agrobacterium* tumefaciens strain EHA 105 harboring the pCAMBIA2300 binary vector. The T-DNA region also contained the kanamycin resistance gene of phosphotransferase II (*nptII*) and the *IbMYB1* gene was driven by the oxidative stress-inducible *SWPA2* promoter [24].

Potato plants (*Solanum tuberosum* L. cv. LongShu No.3) were taken into *Agrobacterium*-mediated transformation, where the protocols of potato propagation, transformation, and gene expression analysis all followed the method described by Kim *et al.* [25]. For the transgenic lines selection, the primers for genomic DNA of the *IbMYB1* gene were 5′-CTTAGGCAACAGGTGGTCGCTT-3′ and 5′-GTGAATTTAACGCTTAGCTTAACA-3′ and the primers of RT-PCR were 5′-CCATGGCTTCTTCAAGGCAAGA-3′ and 5′-ACGTTGTTTCCGGTTGTG TCGT-3′.

Treatments

Sterile potato seedlings were cultured in 0 or 100 mM NaCl-containing MS medium, root fresh weight and dry weight were measured at 20 days, and the expression of gene *IbMYB1* was assayed. Potato seedlings from *in vitro* bottles were then transplanted to pots in a greenhouse with normal growth condition (a 16 h photoperiod with light intensity of 100 µmol photons m^{-2} s^{-1}, 60% (w/v) relative humidity at day 25ºC/ night 20ºC. Four-week-old healthy potato plants with similar height in pots were watered with 0 or 400 mM NaCl for 6 days. Samples were taken at 0, 2, 4, and 6 days after treatment and kept in a deep freezer at –80ºC for further analysis and RNA extraction. For UV-B stress, four-week-old potato plants were supplied with normal growth light plus 8 h UV-B radiation (308 nm; +2.7 kJ m^{-2} day^{-1}) according to the method of Yin *et al.* [53], and for drought stress, plants were restricted from water for 6 days.

Physiological Analysis for Abiotic Stress Tolerance

The determination of total phenols and flavonoids were performed according to the methods described previously [7]. The extraction and quantification of the total anthocyanin were performed according to the pH differential method [30] with slight modification; two reagents of pH 1.0 buffer (potassium chloride, 0.025 M) and pH 4.5 buffer (sodium acetate, 0.4 M) were prepared in advance, the extracts were mixed with 4-fold each reagent in volume, the mixture was incubated in dark for 15 min, and then the absorbance of 530 nm and 700 nm were measured immediately.

The assay of DPPH radical scavenging ability followed the method of Erkan *et al.* [14], where 100 µl of 5 times diluted 80% acidized methanol extracts was incubated with 0.2 mM methanolic DPPH solution for 15 min, and the absorbances at 515 nm were recorded. The ferric-reducing antioxidant power (FRAP) assay was according to Zhang *et al.* [54]. The assay of the chlorophyll content followed the method of Daneshmand *et al.* [10] and in the meantime the content of carotene was calculated. The determination of Fv/Fm and photosynthetic electron transport rate (ETR) was done using the by pulse amplitude modulated chlorophyll fluorescence system (Imaging PAM, Walz, Effeltrich, Germany).

Statistical Analysis

Data were statistically analyzed with Duncan's multiple range test using Statistical Package for the Social Sciences (SPSS 12). Means refer to statistical significance at *P* < 0.05.

Results

Generation of Transgenic Potato Plants and Their Effects on Root Growth of Potato *In Vitro*

In this study, we used *Agrobacterium*-mediated transformation to develop transgenic potatoes (cv. LongShu No.3) with enhanced tolerance to environmental stress and enhanced nutritional value. We employed transgenic potato plants that expressed the *IbMYB1* gene under control of an oxidative stress-inducible peroxidase (*SWPA2*) promoter (referred to as SM plants). After 12 transgenic lines were created, the potatoes were run through a high temperature selection and RT-PCR analysis. Two lines, namely, SM5
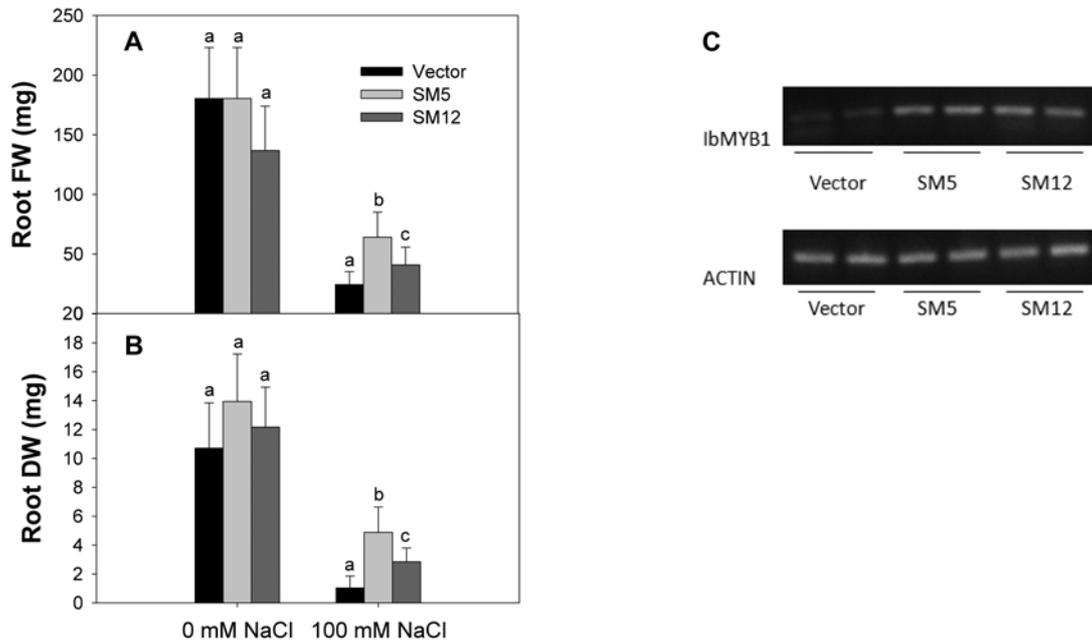
Fig. 1. Overexpression of IbMYB1 in transgenic potato lines of vector, SM5, and SM12 confers enhanced tolerance to salt (0 and 100 mM) for 20 days after treatment.

(**A**) The root fresh weight and (**B**) root dry weight. (**C**) RT-PCR analysis of the expression of IbMYB1 of leaves from vector, SM5, and SM12. β-Actin was used as an internal control. Data are expressed as the mean ± SE of 15 replicates. Bars carrying the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

and SM12, with higher transcription levels were investigated (data not shown). A transgenic potato line with an empty pCAMBIA2300 vector was used as the control.

Since potatoes are considerable sensitive to salt, three transgenic potato lines (including an empty vector as a control line) were used for salt tolerance analysis. The vector lines SM5 and SM12 were cultured in the 0 or 100 mM NaCl-containing MS medium to assay the root growth during salt stress. At the 20th day, 15 seedlings of each line were taken for determination of root fresh weight and root dry weight; the results in Figs. 1A and 1B revealed the transgenic lines obtained significantly higher root FW and DW compared with vector under salt stress. Total RNA was extracted from leaves and the expression of *IbMYB1* was analyzed by RT-PCR at the same time; it could be apparently found that the expression of *IbMYB1* was much higher in SM5 and SM12 lines compared with control lines (Fig. 1C). Moreover, owing to the higher expression of *IbMYB1* in SM5 than in SM12, the salt resistance ability in SM5 was stronger than SM12. It also proved the positive correlation between the gene expression level and the physiological function.

Effects of *IbMYB1* Overexpression on the Secondary Metabolite Accumulation and Antioxidant Ability in Potato

The R2R3-MYB family is known to be strongly related to the plant secondary metabolism [6]. In order to better understand the effects that *IbMYB1* might have on the potato secondary metabolism, the accumulations of total phenols content (TPC) (Fig. 2A), total flavonoids content (TFC) (Fig. 2B), total anthocyanin content (TAC) (Fig. 2C), and proanthocyanidins content (PAs) (Fig. 2D) were determined at 0, 2, 4, and 6 days after the potato plants were poured with 400 mM NaCl-containing water. Healthy four-weeks-old potato plants in pots were taken for this determination. The TPC started with the similar level at day 0 in three potato lines, increased significantly in SM5 while dropping significantly in vector from days 2 to 6, whereas in SM12 the TPC remained in the same level throughout the test. The TFC in the three lines showed no significant difference at day 0, stayed at the same level in SM5 and SM12 until day 6, but declined significantly in vector under salt stress. The TAC remained at the same level in three potato lines before stress, then all increased significantly under stress, while the accumulations in SM5
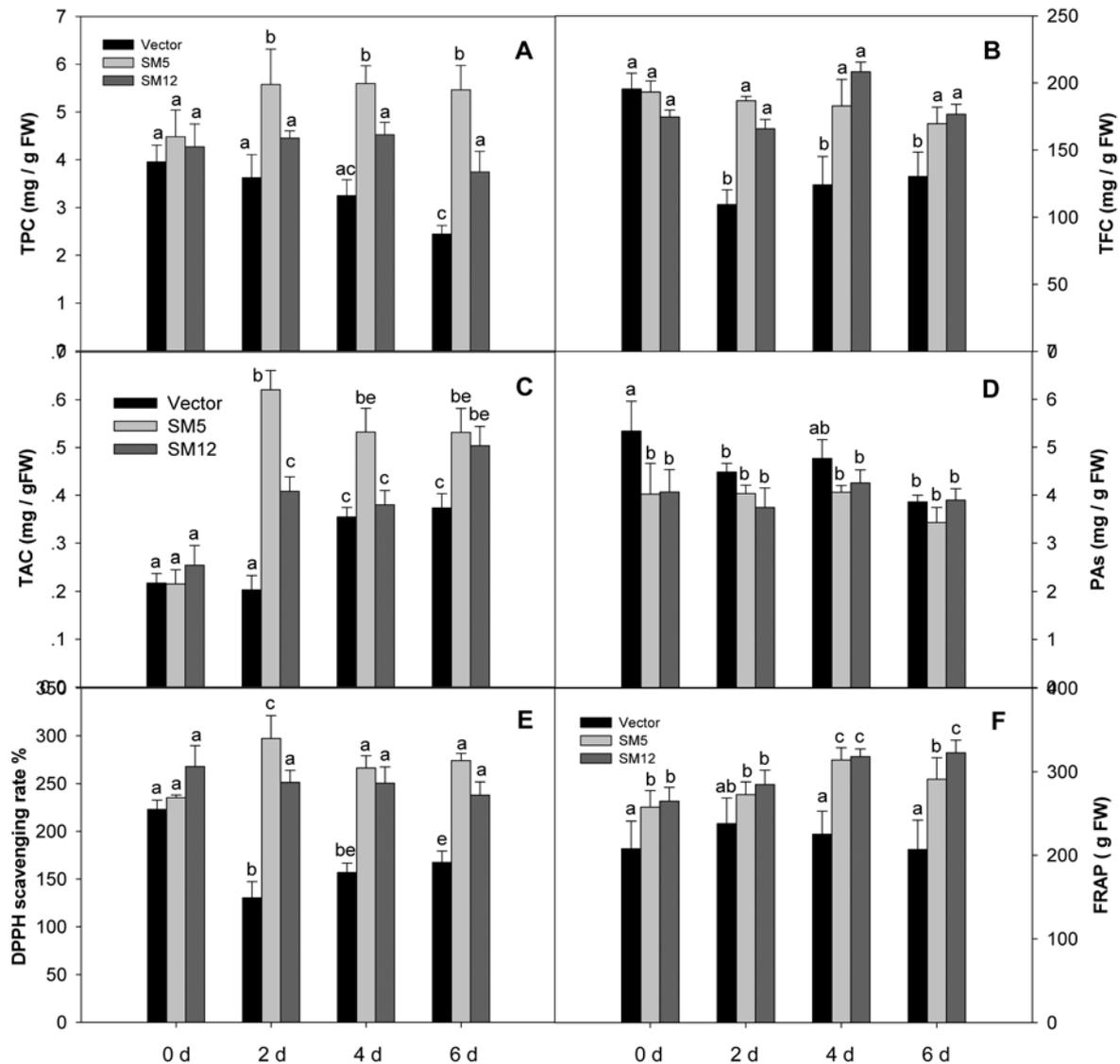
Fig. 2. Overexpression of *IbMYB1* in transgenic potato lines of vector, SM5, and SM12 confers increased contents of secondary metabolite after poured with salt (0 mM and 400 mM), and samples were collected at 0, 2, 4, and 6 days.
(**A**) TPC: total phenols content. (**B**) TFC: total flavonoids content. (**C**) TAC: total anthocyanins content. (**D**) PAs: Proanthocyanidins contents. (**E**) DPPH radical scavenging rate % represents the scavenging ability per g FW. (**F**) FRAP represents the ferric-reducing antioxidant power per g FW. Data are expressed as the mean ± SE of five replicates. Bars carrying the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

and SM12 were much higher than that in vector. Moreover, at days 2 and 4, the TAC of SM5 also was significantly higher than that of SM12. The change of the contents of PAs was quite mild, being slightly lower in transgenic lines compared with control lines, with no significant difference except at day 0. Moreover, transgenic lines of SM5 and SM12 had significantly stronger DPPH radical scavenging ability and ferric reducing antioxidant power compared with vector (Figs. 2E, 2F).

Effects of IbMYB1 Overexpression on the Photosynthetic Pigments and Fluorescent Ability in Potato

Photosynthetic pigment contents and fluorescence ability have also been assayed under salt stress. The contents of chlorophylls A and B (Figs. 3A, 3B) had no significant difference in SM5 and SM12 from 0 to 6 days when poured with salt, but were sharply decreased in vector. The contents of carotene (Fig. 3C) in the three potato lines stayed the same at 0 and 2 days, significantly declined in vector while
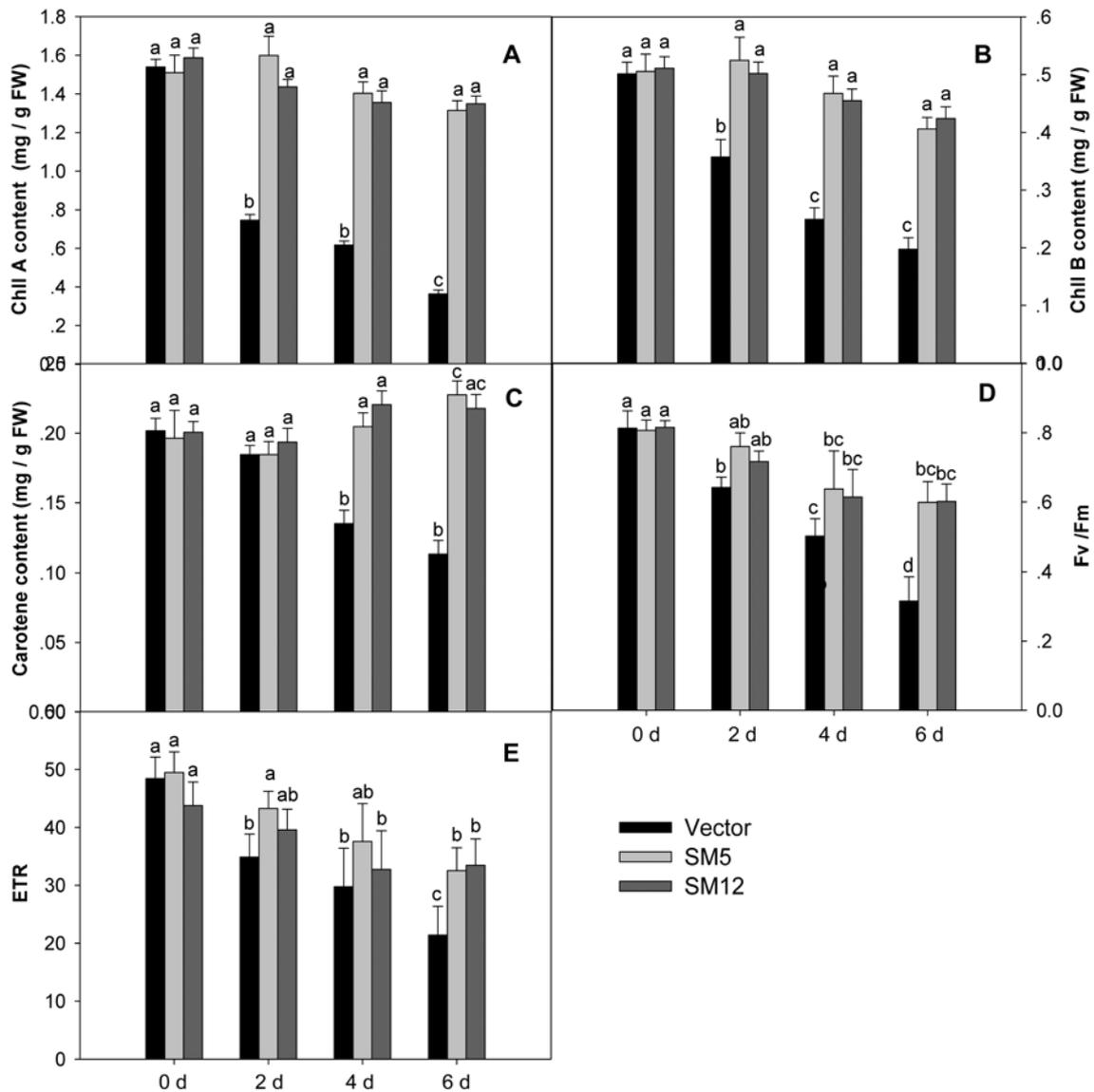
Fig. 3. Overexpression of *IbMYB1* in transgenic potato lines of vector, SM5, and SM12 confers increased photosynthetic pigments and fluorescence ability after being poured with salt (0 mM and 400 mM), and samples were collected at 0, 2, 4, and 6 days.
(**A**) Chll A represents the chlorophyll A. (**B**) Chll B represents the chlorophyll B. (**C**) Carotene content. (**D**) Fv/Fm. (**E**) ETR represents photosynthetic electron transport rate. Data are expressed as the mean ± SE of five replicates. Bars carrying the same letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

slightly raised in SM5 and SM12 at 4 and 6 days. The changes of Fv/Fm and ETR (Figs. 3D, 3E) followed the similar trend; they also generally declined from 0 to 6 days, but the downtrend was quite gentle in SM5 and SM12 while relatively sharp in vector, so Fv/Fm and ETR in SM5 and SM12 were significantly higher than those in vector. In conclusion, between SM5 and SM12, the photosynthetic pigment contents and fluorescence ability showed no significant differences and they were all significantly higher than vector.

The Expression Patterns of Flavonoid Biosynthesis-Related Genes in the Transgenic Potato Under Various Abiotic Stresses

The flavonoid biosynthesis pathway followed the early products of the phenylpropanoid pathway, which synthesizes
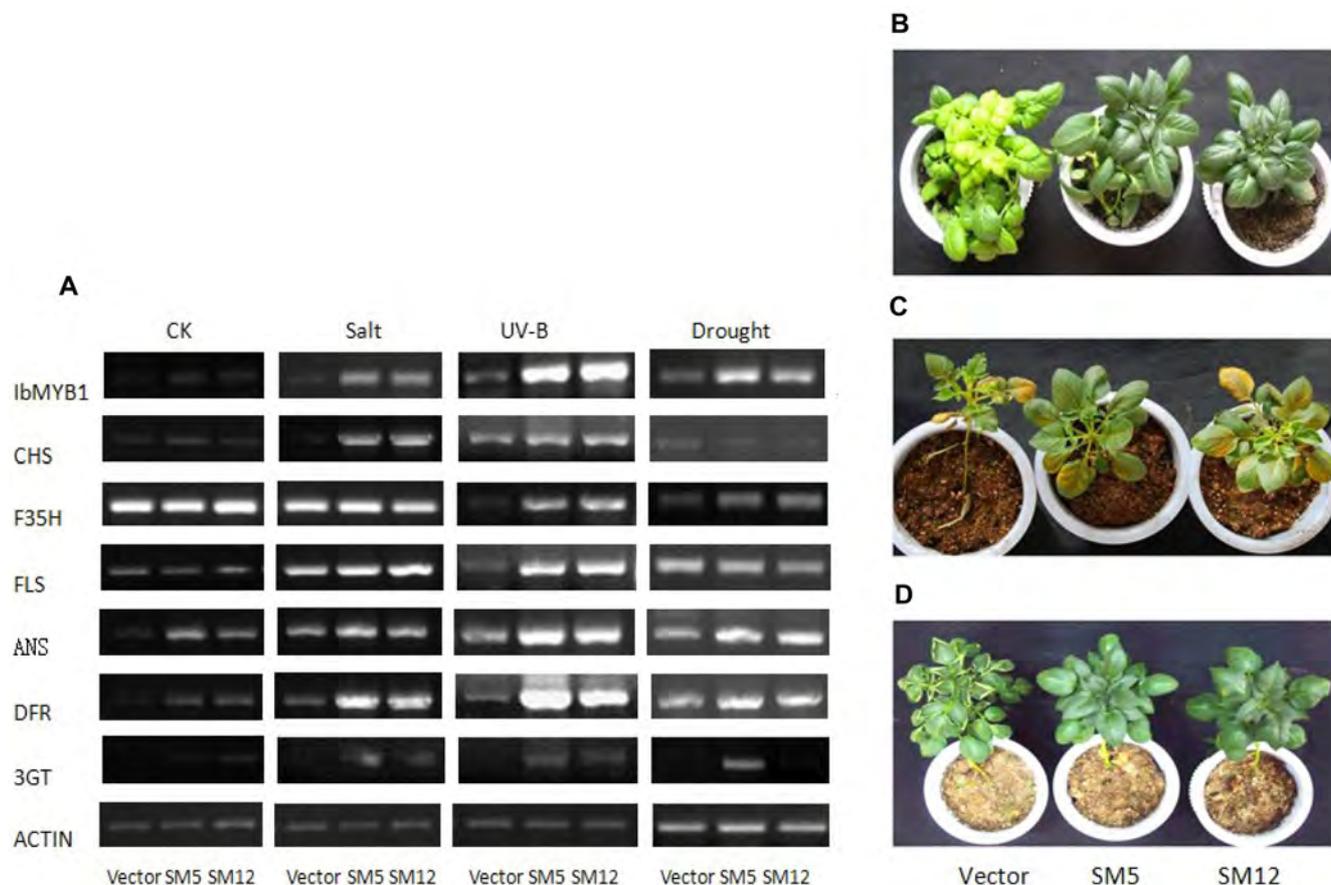
Fig. 4. The expression patterns of flavonoid biosynthesis-related genes in vector, SM5, and SM12 under salt, UV-B, and drought stresses for 6 days (**A**), and the plant growth status at 6 days after salt (**B**), UV-B (**C**), and drought (**D**).
CHS: chalcone synthase; F3′5′H: flavonoid-3′5′-hydroxylase; FLS: flavonol synthase; ANS: anthocyanidin synthase; DFR: dihydroflavonol 4-reductase; 3GT: anthocyanidin 3-O-glucosyltransferase. β-Actin was used as an internal control.

various flavonoid compounds, including anthocyanins. Except for salt stress, the three lines of four-week-old potato plants were treated with UV-B and drought, to assay the change of the expression of the genes in the flavonoid biosynthesis pathway *via* RT-PCR. Besides the expression of gene *IbMYB1*, the expressions of the genes of chalcone synthase (*CHS*), flavonoid-3′5′-hydroxylase (*F3′5′H*), flavonol synthase (*FLS*), anthocyanidin synthase (*ANS*), dihydroflavonol 4-reductase (*DFR*), and anthocyanidin 3-O-glucosyltransferase (*3GT*) in the leaves of SM5 and SM12 were to varying degrees higher than those in leaves of control lines (Fig. 4A). Moreover, potato plants that contained the *IbMYB1* gene could better survive in different stresses (Figs. 4B, 4C, and 4D).

Discussion

IbMYB1 has been found to regulate anthocyanin biosynthesis [33]. The transient expression of *IbMYB1a* has been found

to cause anthocyanin pigmentation in tobacco plants [23]. Studies also show that transgenic *Arabidopsis* expressing *IbMYB1a* produces strong anthocyanin pigmentation in seedlings and generates a deep purple color in leaves, stems, and seeds [8]. Molecular breeding technology has been found to improve the resistant capacity of potato against environmental stress. However, few studies have focused on the anthocyanin enhancement in potatoes [43, 50]. Therefore, the *IbMYB1* gene was used to enhance the stress tolerance through the accumulation of anthocyanin in plants. In this study, we successfully generated transgenic potato plants expressing the *IbMYB1* gene under the stress-inducible *SWPA2* promoter. We then characterized the plants under various stress conditions such as high salinity, drought, and UV-B rays. Results show that the transgenic potato plants had enhanced resistance to abiotic stresses through the accumulation of secondary metabolites, including anthocyanin.

After the terminal buds of potato seedlings from each line were cut and transferred to the MS medium containing 0 or 100 mM NaCl for 20 days, SM5 and SM12 lines showed reduced impact of salt stress on root growth (fresh and dry weight) compared with control lines expressing the vector only (Fig. 1). The *IbMYB1* in the leaves of SM5 and SM12 lines showed high expression under 100 mM NaCl, whereas this did not occur in the control lines (Fig. 1D). Total anthocyanins in the SM5 and SM12 lines were noticeably higher than in control vector lines under salt stress (Fig. 2). These results correspond to previous reports [8, 23, 33].

Interestingly, accumulation of anthocyanins did not lead to visible levels for human eyes in SM5 and SM12 lines, showing the difference from the abundant pigments that have been synthesized in transgenic *Arabidopsis* plants. This could be related to three factors. Firstly, transcription factors may have a slightly different mechanism of action in different plants [31]. *MdMYB10* from apples has been been found to induce the accumulation of anthocyanin in apples [15]. However, when it was transformed into *Arabidopsis*, no visible pigments in transgenic lines were found [17]. Moreover, the contents of anthocyanin were not mentioned, suggesting that the transgenic lines' higher tolerance to osmotic stress should be attributed to high flavonoid contents. Secondly, the cultivar of potato used in this study may be less sensitive to the synthesis of anthocyanin when the exogenous *IbMYB1* gene is introduced. Kortstee *et al.* [29] overexpressed an apple *MYB10* gene in apples, strawberries, and potatoes to discover whether this gene can be used as a visual selection marker during plant transformation. Although the strawberries and apples changed to a purple hue, there was no color change in potato seedlings, even though the gene was well expressed. Analysis of the anthocyanin content showed that the transgenic lines were higher than in the wild type. Finally, anthocyanin biosynthesis is also controlled by environmental conditions. In some plants such as apples and peaches, the accumulation of anthocyanin may be enhanced by sunlight [20, 26, 49]. However, anthocyanin content decreases under strong sunlight in pears [42]. The accumulation of anthocyanin can also be enhanced by sucrose [18]. Therefore, in this study, we also aimed to induce the accumulation of anthocyanin with the use of sucrose; however, results showed that accumulation did not rise. We surmise that the factors affecting the biosynthesis of anthocyanin in potatoes may be more complex than previously believed, and further study is warranted.

Kim *et al*. [23] evaluated anthocyanin biosynthetic genes, including *CHS, CHI, F3H, DFR,* and *ANS*. The expression levels of these genes in transgenic lines have an overall advantage over those of the control line. It is important to note that in addition to the last metabolites of anthocyanin, the biosynthesis of phenol, flavonoids, and proanthocyanin are also under the control of these genes through phenylpropanoid metabolism. Some R2R3-MYB factors have been found to be linked to flavinoid accumulation and phenylpropanoid metabolism [11, 17, 32, 44, 51]. No known research has been conducted on the total phenol content (TPC) and total flavonoid content (TFC) in terms of *IbMYB1*. Therefore, we deduce that the TPC, TFC, and proanthocyanin in transgenic lines may differ from those of the control line. Results showed high accumulations of TPC and TFC in SM5 and SM12 lines, which were obviously up-regulated by the expression of *IbMYB1* (Fig. 2). However, proanthocyanins were poorly distributed in vegetable extracts [2], so the contents of proanthocyanins in different lines showed no significant difference and variation after salt stress.

In the flavonoids biosynthesis pathway, phenylalanine was firstly catalyzed into tetrahydroxychalcone by chalcone synthase (*CHS*), and then turned into naringenin with chalcone isomerase (*CHI*). Flavonoid 3'-hydroxylase (*F3'H*) and flavonoid 3'5'-hydroxylase (*F3'5'H*) catalyzed naringenin into dihydroflavonols, which was then turned into flavonols by flavonol synthase (*FLS*) and into leucoanthocyanidins by dihydroflavonol 4-reductase (*DFR*). Leucoanthocyanidin was ultimately transformed to anthocyanin by anthocyanidin synthase (*ANS*) and anthocyanidin 3-*O*-glucosyltransferase (3GT) [51]. Therefore, we also analyzed the expression of the key proteins of *CHS, F3'5'H, FLS, ANS, DFR,* and *3GT* in each line under various abiotic stresses such as high salinity, drought, and UV-B rays (Fig. 4). Results were similar with tobacco and *Arabidopsis* plants expressing IbMYB1 [23], indicating that *IbMYB1* may explain the enhanced TPC, TFC, and total anthocyanin content through up-regulation of the expression of these genes.

High accumulations of antioxidants provide better active oxygen scavenging capability, and there were higher DPPH radical scavenging ability and ferric-reducing antioxidant power (FRAP) in SM5 and SM12 lines under salt stress compared with control vector lines. DPPH scavenging ability and FRAP have been widely used to evaluate antioxidant components in plant extracts [14, 41]. Therefore, the chloroplast and photosynthetic system in transgenic lines may protect plants from salt stress. SM5 and SM12 lines

may maintain nearly normal chlorophyll A/B and carotene content under salt stress compared with control lines (Fig. 3). Pigments of control plants are often destroyed through salt stress. Alfalfa cultivars with significantly high expressions of genes involved in flavonoid biosynthesis and high levels of flavonoids under drought stress have high antioxidant capacity compared with other cultivars. Salt-tolerant cultivars are more likely to be able to retain their color [22].

In conclusion, this study demonstrated that *IbMYB1* can regulate overall phenylpropanoid metabolism and its metabolites, therefore enhancing the abiotic stress resistance ability of transgenic potatoes. The agronomic characterization of transgenic SM5 and SM12 plants in marginal lands, including semidry lands, is an important topic for future research.

Acknowledgments

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