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Changes of phenolic compounds in LebZIP2-overexpressing transgenic plants

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The bZIP gene is a transcription factor that plays various roles in relation to plant stress and hormone signaling. This gene is also involved in plant environmental stress and herbicide tolerance. We generated *Nicotiana benthamiana* transgenic plants with LebZIP2-encoding gene isolated from tomatoes using *Agrobacterium*-mediated transformation. Transgenic seeds harvested from these T_0 transgenic plants were grown and examined for gene transfer and changes in phenolic compounds in the T_1 generation. RT-PCR analysis using a primer specific to the LebZIP gene confirmed that the gene was transferred to the T_1 generation. We analyzed the increase and decrease tendency for 30 phenolic compounds using the T_1 generation-transgenic plants and investigated the mechanism between the specifically increased compound and LebZIP2 gene. Gallic acid, homogentisic acid, protocatechuic acid, myricetin, t-cinnamic acid, and b-resorcyclic acid were identified as the phenolic compounds that increased in T_1 transgenic plants overexpressing the LebZIP gene. Among these, homogentisic acid at 246.75-1055.19 µg/g, was increased by 2-5 fold in the T_1 transgenic plants compared to the control. Protocatechuic acid was found at 1640.54-2456.00 µg/g and was increased by 2-4 fold in T_1 transgenic plants. t-Cinnamic acid was present in a small amount of 23.14 µg/g in the control, whereas it was 102.19-135.47 µg/g in T_1 transgenic plants, showing an increase of 4-5 folds. These results indicated that homogentisic acid, protocatechuic acid, and t-cinnamic acid among the 30 phenolic compounds analyzed, were significantly increased in LebZIP2-overexpressing T_1 transgenic plants, and support the evidence that the LebZIP2 gene is significantly involved in the increment of three phenolic compounds.

Keywords: Homogentisic acid, LebZIP2, Phenolic compounds, Protocatechuic acid, t-cinnamic acid, T_1 transgenic plants

The basic leucine zipper protein (bZIP) is one of the largest transcription factors (TF) in plants; there are 75 bZIPs in *Arabidopsis* and 92 bZIPs in rice[1,2]. The bZIP family proteins contain a DNA binding domain adjacent to the basic amino acid enriched leucine zipper dimer domain and these TFs have been categorized into 11 groups (I-XI)[3]. The bZIP protein is rich in proline and glutamine, and is involved in germination processes, flower development, reactive stress biomedical signals, and Abscisic Acid (ABA) signaling[4,5]. Transgenic plants overexpressing AREB1/ABF2 from *Arabidopsis* showed ectopic phenotypes of resistance to multiple stresses, and expression of bZIP genes was found to play an important role in the glucose signaling process[6]. Transgenic plants overexpressing OsbZIP72, a gene related to ABA signaling, have been reported to demonstrate increased drought tolerance[7]. *Nicotiana benthamiana* with the LebZIP2 gene has also been investigated regarding enhanced resistance to herbicides[8].

Phenolic compounds exist in more than 8000 groups throughout the plant kingdom and have aromatic rings with one or more hydroxyl groups attached[9]. Phenolic compounds are secondary metabolites associated with phenylpropanoid pathways in plants[10-12]. Plant phenolic compounds are composed of simple phenols, coumarins, lignins, tannins, phenolic acids, and flavonoids based on a number of phenolic units at the molecular level[13]. These compounds play important roles in physiological and morphological factors such as plant growth, reproduction, and protection from pathogens[14].

Phenolic compounds act as an important signal for plant resistance to biotic and abiotic stress[15]. The antioxidant properties of polyphenols arise from hydrogen or electron donors and polyphenol-derived radicals[16]. Antioxidant compounds synthesized in

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transgenic plants can be increased by expression of cDNAs encoding key enzymes of polyphenol biosynthesis and these plant polyphenols form a group including flavonoids, phenolic acids, phenols, lignans, and tannins[17]. Transgenic plants with *Pssu-ipt* (promoter sequence of the gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase/ oxygenase the gene for isopentenyl transferase) gene showed increased antioxidant enzymes and peroxidases, and presence of the pathogenic PR protein[18]. Salicylic acid (SA), which is known to regulate signal transduction mechanisms of the PR protein, occurs in various groups of secondary metabolites and is normally synthesized during plant growth and development[19].

In our previous study, we found that the bZIP transcription factor responds to a variety of environmental stresses and has transgenic plants overexpressing bZIP have been reported[8,20]. The morphological characteristics of the plant, response to environmental stress, and herbicide resistance were studied in tobacco transgenic plants overexpressing the bZIP gene[20].

The purpose of this study was to analyze the phenol contents of transgenic tobacco plants overexpressing the bZIP gene, and to investigate the association between the bZIP gene and phenolic compounds.

Materials and Methods

Preparation of plant material

The transgenic and control plants used in this study were cultivated for 3 weeks or more after production of transgenic plants using the bZIP gene[20]. Two transgenic lines with good germination from seeds among the transgenic plants inserted with the bZIP gene were propagated and used as analysis material with the transgenic T_1 generation.Total RNA was extracted from two proliferating T_1 generation lines and cDNA synthesis was performed. Insertion of the bZIP gene was confirmed using specific primers used in the previous study[20]. The ground parts of the transgenic tobacco were collected in the fresh state, placed in completely sealed polyethylene plastic bags, and stored at −80 °C (Ultra Low, Sanyo). The collected samples were homogenized (40-mesh) after rapid freeze-drying (Biotron, Cleanvac 8B) at −80°C.

RT-PCR analysis using the T_1 generation of transgenic plants overexpressing the LebZIP2 gene

T_0 plants of transgenic *N. benthamiana* with the previously studied LebZIP2 gene were propagated and harvested[20]. The *N. benthamiana* seeds were sown and T_1 transgenic plants were grown in the greenhouse for 4 weeks or more. Total RNA was isolated from harvested transgenic leaves grown over 4 weeks[21]. RT-PCR was performed using the specific primers of the LebZIP gene used in the previous study to confirm the transfer of the LebZIP2 gene in T_1 generation-transgenic plants (Table 1)[20].

Analysis of phenolic compounds by HPLC

Using 2 g of the plant samples, 10 mL of 99.9% acetonitrile (HPLC grade) and 0.1 N HCl were added and the mixture was extracted at room temperature for 2 h. The mixture was concentrated using a vacuum condenser and filtered through a 0.45 μM syringe filter. HPLC analysis was performed using a Shimadzu HPLC system (SPD-M10A Diode Array Detector, Kyoto, Japan) and a YMC-Pack ODS AM-303 (4.6 × 250 mm ID, YMC Co., Kyoto, Japan). Solvent A, a mixture of distilled water and tertiary distilled water containing 0.1% acetic acid, and solvent B, which was a mixture of acetonitrile and tertiary distilled water containing 0.1% acetic acid, were used in the gradient program to measure the mobile phase at a UV absorbance of 280 nm. The flow rate was 1 mL/min. Analysis of the phenolic compounds revealed 30 kinds of substances including gallic acid (Fig. 3). Qualitative and quantitative analysis of these phenolic compounds was performed by diluting the respective standard materials at constant concentrations of 0 ppm, 1 ppm, 50 ppm, and 100 ppm, respectively, followed by calculation of the regression curves of the phenol compounds. The area obtained in the HPLC analysis was substituted as Y and assigned to calculate the X value, which was then converted to the amount in μg/g (Table 2).

3D structure construction and phylogenetic tree

3D structure and phylogenetic tree construction: A phylogenetic tree was constructed using the EMBL-EBI method (http://ebi.ac.uk/), and a 3D structure model of LebZIP2 was constructed using Swiss-Model (http://swissmodel.expasy.org/).

Table 1 — Specific primers used in RT-PCR of *N. benthamiana* transgenic plants

Primer name	Primer sequence (5′→3′)
NbActin-F	CAGCTCATCCGTGGAGAAGA
NbActin-R	AGGATACGGGGAGCTAATGC
LebZIP2-F	ATGGCTTCGTCAAGTGGT
LebZIP2-R	TCAGTACTGCAAGACATC

Results

Based on sequence data from a previous study, the 3D structure of LebZIP2 was analyzed through the alignment of amino acid sequences[8]. The 3D structures were determined by target sequences that were obtained from different plants and exhibited >64% similarity in amino acid sequence. The 3D structures of LebZIP2 were predicted to four different types (Fig. 1). The 4h22.1.B template was predicted as a leucine-rich repeat flightless-interacting protein 1 formatted homodimer. The 6ds9.1.A template was predicted as the monomer form of a *de novo*-designed three-helix bundle GRa3D. The 6g1l.1.A template was predicted as a microphthalmia-associated transcription factor, homo-dimer, and putative vacuolar protein sorting-associated protein, as well as the monomer state of the 6 h 7w.1.1 template. The 3D structure of LebZIP2 may be related to its different amino acid sequence. A phylogenetic tree of bZIP proteins from different plants was constructed using the EMBL-EBI method. LebZIP2 exhibited the shortest evolutionary distances with NtbZIP and CcbZIP, whereas, LebZIP1 and VvZIP exhibited the longest.

The transgenic plants transformed with the LebZIP2 gene were transferred into pots and insertion of the LebZIP2 gene from their T_1 generation was again confirmed using the gene-specific primers usedpreviously (Fig. 2)[20].

Comparative HPLC analysis in the control and transgenic *N. benthamiana* plants overexpressing the LebZIP2 gene showed gallic acid to be at 54.67 µg/g in the control, 72.21 µg/g in transgenic plant #1 and 97.72 µg/g in the transgenic plant #2. Homogentisic acid was shown to be at 171.05 µg/g in the control, 246.75 µg/g in transgenic plant #1 and 1055.19 µg/g in transgenic plant #2. Transgenic plant #2 was also measured at more than 5 times in compared to the control plant. Protocatechuic acid was found to be at 596.22 µg/g in the control, and 1640.54 µg/g and 2465.00 µg/g in transgenic plants #1 and #2,

Table 2 — Calibration curves of 30 phenolic compounds

Standards chemicals	Equation
Gallic acid	y = 47.638x + 144.05
Pyrogallol	y = 2.2087x - 2.1338
Homogentisic acid	y = 9.8339x - 23.401
Protocatechuic acid	y = 25.447x - 2.2837
Gentisic acid	y = 3.3267x + 11.674
Chlorogenic acid	y = 4.8652x - 12.034
p-Hydroxybenzoic acid	y = 32.158x + 4.2742
(+)Catechin	y = 11.682x - 3.996
Vanillic acid	y = 29.469x + 11.278
Syringic acid	y = 55.324x + 27.508
Caffeic acid	y = 14.737x - 26.154
Vanillin	y = 78.013x - 8.6281
p-Coumaric acid	y = 39.421x + 14.495
Rutin	y = 14.096x - 9.1985
Ferulic acid	y = 18.57x + 3.1248
m-Coumaric acid	y = 104.83x + 177.01
Salicylic acid	y = 10.43x + 2.0793
Hespsridin	y = 33.61x - 13.632
Benzoic acid	y = 6.9618x + 2.3859
o-Coumaric acid	y = 147.58x - 310.61
Myricetin	y = 23.69x - 85.77
Resveratrol	y = 26.775x - 12.57
Quercetin	y = 20.767x - 65.419
t-Cinnamic acid	y = 160.71x + 1.8274
Naringenin	y = 45.344x - 36.433
Hesperetin	y = 49.722x + 7.4397
b-Resorcylic acid	y = 24.161x - 11.12
Naringin	y = 21.468x - 16.187
Kampferol	y = 26.652x - 24.209
Veratric acid	y = 38.525x - 32.961

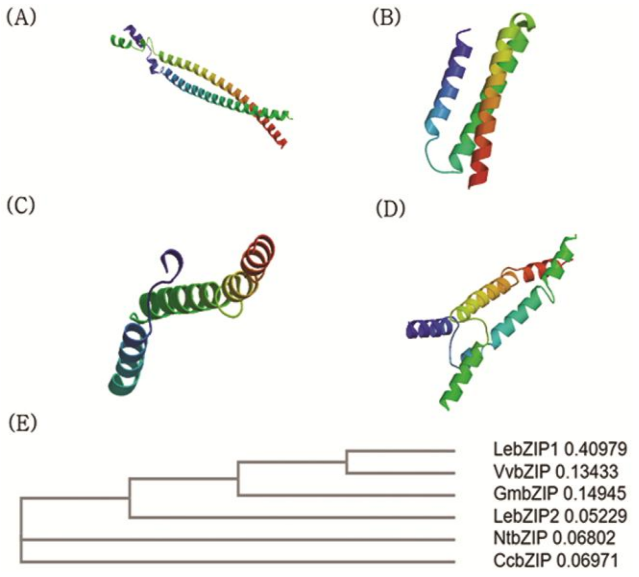

Fig. 1 — 3D structure prediction and phylogenetic tree for alignment sequences of LebZIP2. Alignment sequences are represented here by Swiss-model program to (A) 4h22.1.B; (B) 6ds9.1A; (C) 6h7w.1.1; (D) 6g1l.1.A; and (E) Phylogenetic tree of amino acid sequences of the bZIP proteins from different plants. LebZIP1 (GenBank Accession No. AF176641), VvbZIP (CAN73127), GmbZIP (ABI34666), NtbZIP (AAK92213) and CcbZIP (AAD21199)

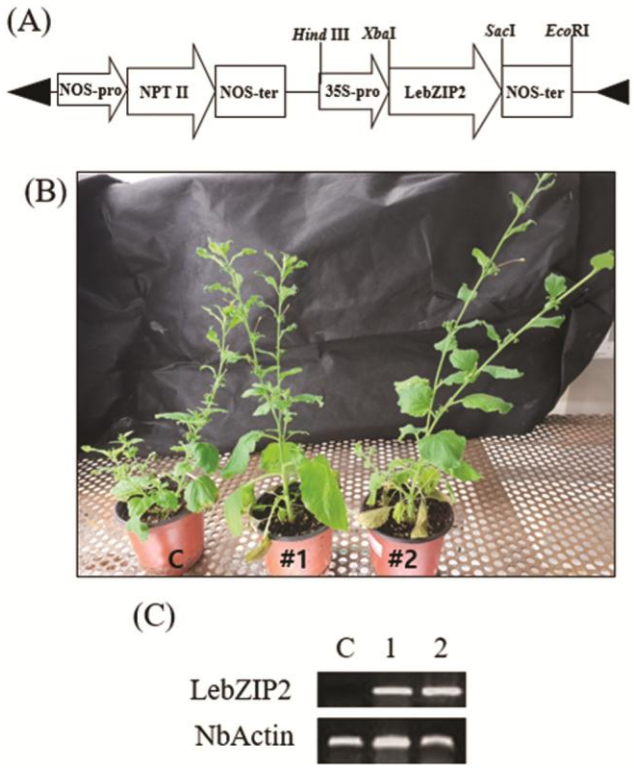

Fig. 2 — Propagation of T_1 generation for transgenic *N. benthamiana* plants using LebZIP2 gene and molecular confirmation. (A) Vector construction using pMBP1[20]; (B) photo of control; and (C) and T_1generation of transgenic plants (#1 and #2); (c) RT-PCR analysis using cDNA made from isolated total RNA for plant samples.

respectively. Myricetin was measured at 31.11 µg/g in the *N. Benthamiana* control and was found at 35.16 µg/g and 33.37 µg/g in transgenic plants #1 and #2, respectively. The content of t-Cinnamic acid was 23.14 µg/g in the control, 102.19 µg/g in transgenic plant #1, and 135.47 µg/g in transgenic plant #2. The t-cinnamic acid content in the transgenic plant overexpressing the LebZIP2 gene was more than doubled. The content of b-resorcylic acid was found to be zero in the control, but was measured at more than 100 µg/g in all the transgenic plants (Table 3 & Fig. 3).

Among the 30 analyzed phenolic compounds, more compounds showed reduced levels in the transgenic lines than in the control. These compounds include gentisic acid, chlorogenic acid, p-hydroxybenzoic acid, caffeic acid, vallin, ferulic acid, hespsiridin, o-coumaric acid, quercetin, naringenin, hesperetin, and kampferol. The phenolic compounds reduced by a large proportion in transgenic plants compared to the phenol contents of the control were gentisic acid, chlorogenic acid, and p-hydroxybenzoic acid. Chlorogenic acid was present in the control at 13942.16 µg/g, whereas in transgenic plants overexpressing the LebZIP2 gene, it was decreased to 9212.61 µg/g and 10749.83 µg/g. Phenol compounds such as vallin, ferulic acid, hespsiridin, o-coumaric acid, quercetin, naringenin, hesperetin, and kampferol were found in small amounts in the control. However, gentisic acid was detected at 4809.13 µg/g in the control, whereas it was decreased to 1992.18 µg/g and 2637.42 µg/g in the transgenic plants. p-hydroxybenzoic acid was detected at 7409.27 µg/g in the control but was decreased in the transgenic plants to 4634.42 µg/g and 5964.01 µg/g (Table 3).

Among the 30 phenolic compounds used in the analysis, (+) catechin, vanillic acid, p-coumaric acid, rutin, salicylic acid, benzoic acid, resveratrol, and veratric acid were not detected in the non-transformed control *N. Benthamiana* plants. p-coumaric acid and resveratrol were found at 82.29 µg/g and 16.39 µg/g in transgenic plant #2, respectively. Unusually, Syringic acid decreased from 981.95 µg/g in the control to 761.83 µg/g in transgenic plant #1, but showed different results increasing to 1060.29 µg/g in transgenic plant #2. Naringin was at 52.52 µg/g in the control, whereas the results were different at 75.89 µg/g in transgenic plant #1 and 49.92 µg/g in transgenic plant #2 (Table 3).

Discussion

LebZIP2 contains 164 amino acids, including the basic region leucine zipper domain[8]. The bZIP transcription factors were found to participate in the regulation of plant developmental genes or in environmental stress mechanisms, such as NaCl, Cold, and ABA signaling[22].The four identified 3D structural predictions of LebZIP2 show different oligomerization or protein states (Fig. 1). The oligo-state of the target template was largely classified as a monomer or homodimer. Leucine-rich repeat flight-interacting protein 1 is involved in early cell responses to viruses[23]. *De novo*-designed three-helix bundle GRa3D is related to electrostatics and the structures of various hydrophobic side chains[24]. Putative vacuolar protein sorting-associated protein is involved in pathways for fungal growth and development[25]. Microphthalmia-associated transcription factor is a key element in the regulation of tyrosinase activation for melanogenesis[26].

Table 3 — Content analysis for 30 phenolic compounds in non-transgenic plant and transgenic plants overexpressed LebZIP2 gene using HPLC

	Phenolic compounds	Control plants	Transgenic plant #1	Transgenic plant #2
			μg/g	
1	Gallic acid	54.67	72.21	97.92
3	Pyrogallol	90.89	56.82	104.92
4	Homogentisic acid	171.05	246.75	1055.19
5	Protocatechuic acid	596.22	1640.54	2465.00
6	Gentisic acid	4809.13	1992.18	2637.42
7	Chlorogenic acid	13942.16	9212.61	10749.83
8	p-Hydroxybenzoic acid	7409.27	4634.42	5964.01
9	(+)Catechin	0.00	0.00	0.00
10	Vanillic acid	0.00	0.00	0.00
11	Syringic acid	981.95	761.83	1060.29
12	Caffeic acid	635.31	363.25	467.80
13	Vanillin	31.67	20.85	14.37
14	p-Coumaric acid	0.00	0.00	82.29
15	Rutin	0.00	0.00	0.00
16	Ferulic acid	100.57	52.63	70.23
17	m-Coumaric acid	125.16	0.00	0.00
18	Salicylic acid	0.00	0.00	0.00
19	Hespsridin	97.25	65.23	94.24
20	Benzoic acid	0.00	0.00	0.00
21	o-Coumaric acid	72.11	0.00	0.00
22	Myricetin	31.11	35.16	33.37
23	Resveratrol	0.00	0.00	16.39
24	Quercetin	57.63	22.83	41.29
25	t-Cinnamic acid	23.14	102.19	135.47
26	Naringenin	37.65	26.24	23.99
27	Hesperetin	30.57	16.79	28.31
30	b-Resorcylic acid	0.00	131.10	146.16
31	Naringin	52.52	75.89	49.92
32	Kampferol	42.52	21.81	29.94
33	Veratric acid	0.00	0.00	0.00

Protocatechuic acid (PCA) is one of the naturally occurring phenolic acids and has a structure similar to antioxidative compounds such as syringic acid, gallic acid, caffeic acid, and vanillic acid[20]. Protocatechuic acid is widely used in folk remedies for skin softeners, coolants, sedatives, tonic, cancer, cough, debility, indigestion, heart disease, and hypertension. Protocatechuic acid has also been found in hibiscus, carrot, and *Agaricus bisporus*, and has been shown to induce a preventive effect on many diseases[27]. Acai oil is rich in protocatechuic acid (630 ± 36 mg/kg)[28]. In the present study, the content of protocatechuic acid in *N. benthamiana* was 596.22 μg/g, which was increased to 1640.54-2465.00 μg/g in transgenic plants transformed with LebZIP2 gene (Table 3). The LebZIP2 transcription factor increased the content of protocatechuic acid, indicating that antioxidant activity was increased.

In the present study, the amount of t-cinnamic acid was found to be four times higher in the transgenic plants than in the control plants. This result may be related to the mechanism by which the LebZIP2 gene induces t-cinnamic acid. Cinnamic acid is one of the basic phenylpropanoids with antioxidant activity produced by plants in response to environmental stress. t-cinnamic acid is known to be formed from phenylalanine ammonia lyase (PAL), the most studied enzyme in plant secondary metabolites[29]. In addition, t-cinnamic acid is a derivative of salicylic acid (SA) that causes SA to act as a signaling molecule that regulates plant stress, flowering, and development[30]. It is reported

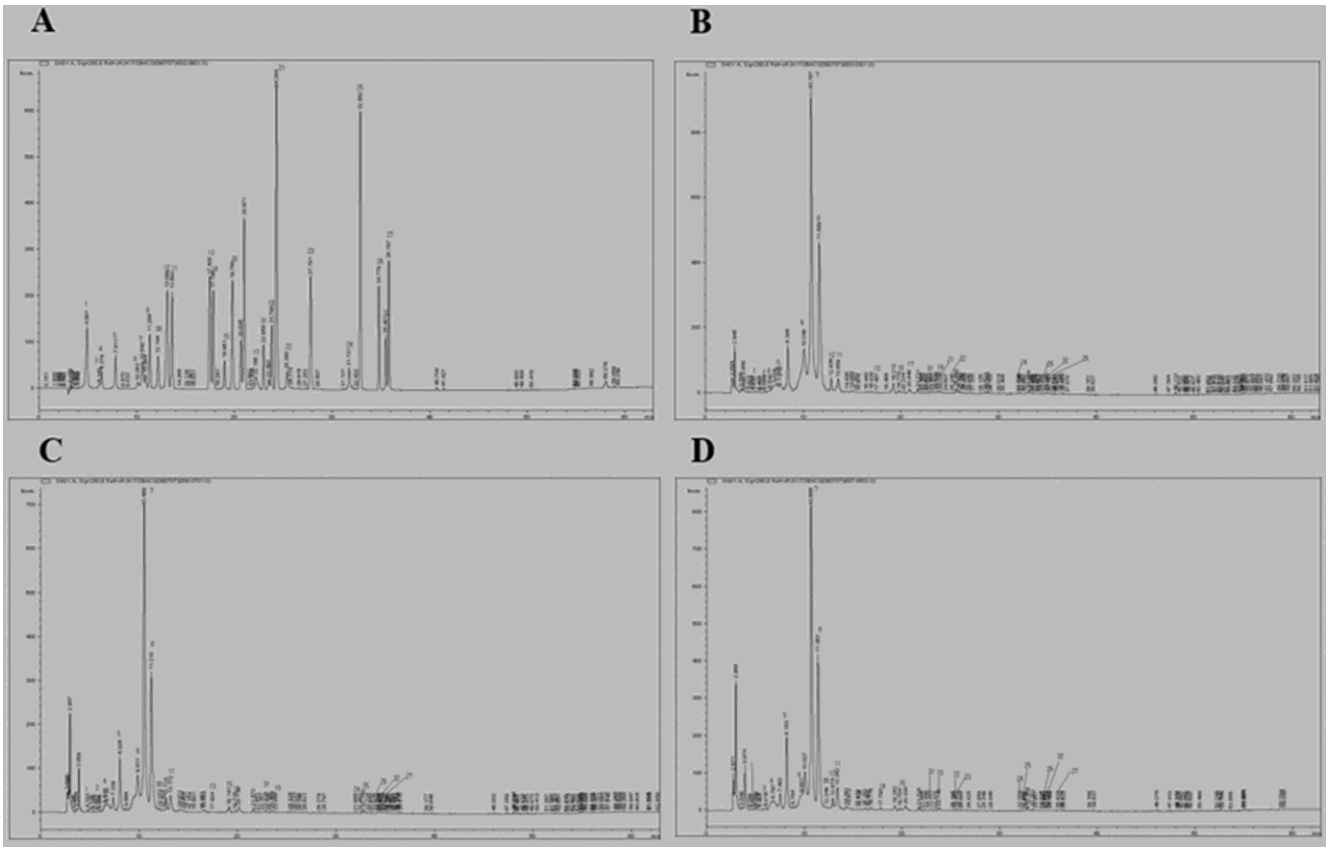

Fig. 3 — (A & B) HPLC profile of standards and control or (C & D) transgenic plants in analysis for 30 phenolic compounds. The numbers of chromatograms are shown in Table 3

that the content of cinnamic acid is increased by 29% in leaves and by 2-fold in the stem at the beginning of flowering in planting process of buckwheat[31]. The metabolic pathway from t-cinnamic acid to salicylic acid via benzoic acid is involved in stress-induced flowering, indicating an increase in salicylic acid content in the stem of buckwheat[31]. Salicylic acid is an endogenous regulatory factor involved in a wide range of plant metabolic, and stress responses[30]. Previous studies have shown that resistance to herbicides in transgenic plants overexpressing the LebZIP2 gene is correlated with the relationship for the increasing content of t-cinnamic acid and salicylic acid[20].

Accumulation of large amounts of homogentisic acid (HGA) is an important factor limiting the tocochromanol biosynthesis process and has the greatest effect on the synthesis of tocopherol[32]. Homogentisic acid phytyltransferase (HPT) associated with HGA is an enzyme that specifically acts on tocopherol synthesis in plants, and it has been reported to have little effect on the synthesis of other prenylquinone compounds in plastids[33]. Overexpression of the *Arabidopsis* HPT gene was found to double the vitamin E content detected in common seeds[34]. In HPT gene-deficient transgenic plants, leaf senescence was rapidly accelerated after the onset of flowering with a significant increase in oxidative stress[35]. Overexpression of the *Arabidopsis thaliana* AtHPT (HPT1) gene was found to increase the total tocopherol content in seeds and leaves of transgenic plants by 1.4-4.4 times compared to the control[36]. The results of the present study also show that the homogentisic acid content in transgenic plants overexpressing LebZIP2 is significantly increased, suggesting that the tocopherol content is increased, leading to a slower aging process due to reduced oxidative stress in the transgenic plants.

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