Short communication

Identification and quantification of anthocyanins in transgenic purple tomato

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ABSTRACT

Anthocyanins are natural pigments derived from the phenylpropanoid pathway. Most tomatoes produce little anthocyanins, but the transgenic purple tomato biosynthesizes a high level of anthocyanins due to expression of two transcription factors (*Del* and *Ros1*). This study was to identify and quantify anthocyanins in this transgenic tomato line. Seven anthocyanins, including two new anthocyanins [malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside and malvidin-3-(feruloyl)-rutinoside-5-glucoside], were identified by LC-MS/MS. Petunidin-3-(trans-coumaroyl)-rutinoside-5-glucoside and delphinidin-3-(trans-coumaroyl)-rutinoside-5-glucoside were the most abundant anthocyanins, making up 86% of the total anthocyanins. Compared to undetectable anthocyanins in the wild type, the contents of anthocyanins in the whole fruit, peel, and flesh of the *Del/Ros1*-transgenic tomato were 5.2 ± 0.5, 5.1 ± 0.5, and 5.8 ± 0.3 g/kg dry matter, respectively. Anthocyanins were undetectable in the seeds of both wide-type and transgenic tomato lines. Such novel and high levels of anthocyanins obtained in this transgenic tomato may provide unique functional products with potential health benefits.

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1. Introduction

The natural pigments produced in plants, including chlorophylls, carotenoids, and anthocyanins, are generally synthesized via phenylpropanoid and terpenoid pathways (Gonzali, Mazzucato, & Perata, 2009). Anthocyanins [derived from Greek *anthos* (flower) and *kyanos* (dark blue)] are one of the most important water-soluble plant pigments (Delgado-Vargas & Paredes-López, 2003). They are synthesized by the flavonoid branch of the phenylpropanoid pathway through secondary metabolism in higher plants. Among over 600 types of anthocyanins (Xu & Howard, 2012), the majority of anthocyanin aglycones found in nature consist of six anthocyanidins, i.e., cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin. They share a 2-phenylbenzopyrilium (flavyl-ium) skeleton hydroxylated in 3, 5, and 7 positions, with different substitutions at R1 and R2 (Fig. 1). In comparison with other flavonoids, anthocyanins possess a positive charge on its C-ring, which leads to different colors in response to various pH (Wang & Stoner, 2008).

Anthocyanins present in human foods have received considerable attention due to their possible health-promoting properties such as antioxidant and anti-inflammatory effects (Bowen-Forbes, Zhang, & Nairb, 2010; Lim et al., 2013). Based on food intake data from NHANES 2001–2002, the daily intake of anthocyanins was estimated to be 12.5 mg/day/person in the United States (Wu et al., 2006). The predominant dietary anthocyanins are malvidin, delphinidin, and peonidin glycosides (Bognar et al., 2013), which can be found in many plant foods, including berries, purple sweet potatoes, grapes, and wine.

Tomato (*Solanum lycopersicum L.*) is one of the most important food crops in the world. Its rich red color is due to accumulation of the carotenoid pigments, i.e., lycopene and phytoene, in the peel and flesh (Khachik et al., 2002; Pannellini et al., 2010). However, when compared to anthocyanin-enriched plants, tomatoes generally produce little anthocyanins. Genetic engineering is a powerful approach to induce and enhance biosynthesis of anthocyanins in plants (Schijlen, Ric de Vos, van Tunen, & Bovy, 2004), which has been successfully applied in food crops such as potato and rice (Lukaszewicz et al., 2004; Tanaka & Ohmiya, 2008). Several

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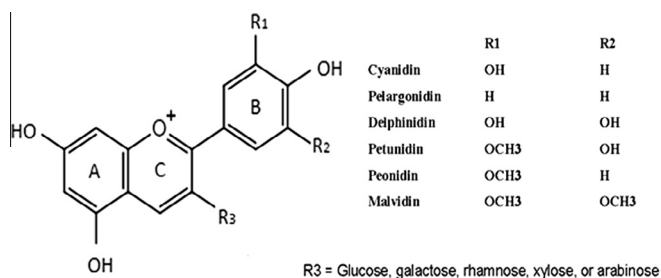   E-mail address: wwang@ksu.edu (W. Wang).

Fig. 1. Structures of common anthocyanidins and anthocyanins.

transgenic tomatoes with increased flavonoid levels have also been developed. A transgenic tomato line created by expressing two maize regulatory genes, *Lc* and *C1*, was reported to produce a high level of flavonols rather than anthocyanins (Bovy et al., 2002). Overexpression of the *ANT1* gene encoding a MYB transcription factor was further reported to induce a purple spotting on the epidermis of tomatoes (Mathews et al., 2003). In addition to the *ANT1* gene, combining the *atv* gene with either *Aft* or *Abg* was found to generate anthocyanin petunidin-3-(p-coumaryl)-rutinoside-5-glu coside predominantly in the epidermis of tomatoes up to 0.1% in fresh weight (Mes, Boche, & Myers, 2008). Furthermore, expression of two snapdragon (*Antirrhinum majus*) transcription factors, i.e., Delila (*Del*) and Rosea1 (*Ros1*), in the fruit of transgenic tomatoes activated multiple anthocyanin biosynthesis-related genes, including *phenylalanine ammonia-lyase* (*PAL*) and *flavonoid 3'5'-hydroxylase* (*F3'5'H*) (Butelli et al., 2008). The *Del/Ros1* transgenic tomato grew normally during the green stage and then started to accumulate purple pigments during the ripening stage, exhibiting an intense and uniform purple color both in the peel and flesh (Butelli et al., 2008).

According to the reported methods by Butelli et al. (2008), the Park lab has engineered and produced the *Del/Ros1* transgenic tomato line (Lim, Miller, Park, & Park, 2014). Fig. 2 shows the whole, cross-section, and freeze dry of ripe wild-type and transgenic *Del/Ros1* fruits.

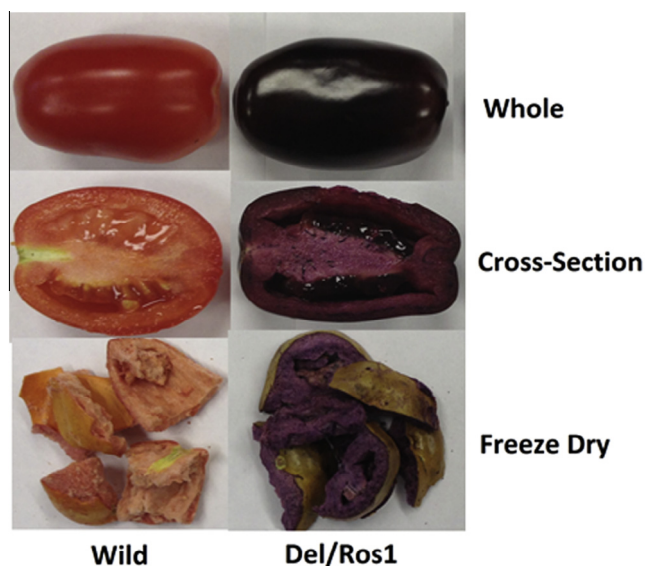The objectives of this study were to identify and quantify the anthocyanin profile in this transgenic tomato, and to determine the distribution of anthocyanins in the peel, flesh, and seed of the fruits.

2. Materials and methods

2.1. Materials

Acetonitrile, methanol (MeOH), and formic acid used in this study at either HPLC grade or analytic grade were purchased from Thermal Fisher Scientific (Suwanee, GA). Water used was purified through Barnstead E-Pure Deionization System (Dubuque, IA) and filtered by Millpore 0.45 µm membrane (Bedford, MA). A standard of Peonidin-3-glucoside chloride was purchased from Sigma–Aldrich (St. Louis, MO).

2.2. Sample preparation and extraction

Wild type (*Solanum lycopersicum* L. cv Rubion) and *Del/Ros1* transgenic purple tomatoes generated in previous studies (Lim et al., 2014) were harvested in the Kansas State University Department of Horticulture greenhouses. For each line, ripe tomatoes were washed with tap water, diced into approximately 0.5 cm cubes, freeze-dried (Labconco, FreeZone 2.5), and ground by a food processor into powder. Prepared powder was then stored at $-80\ ^{\circ}C$ until further extraction. For preparation of anthocyanin extracts, 0.05 g of the powder was extracted with 4 mL of acidified MeOH with 1 N formic acid at 9:1 (v/v). The flasks containing powder/solvent mixture were wrapped with aluminum foil to avoid light exposure. After a 12-h extraction, the samples were centrifuged at 2800 rpm for 30 min and then the supernatant was collected and dried by vacuum drier at $25\ ^{\circ}C$ overnight. One milliliter of the acidified MeOH was added and then the dissolved extract was filtered by Whatman syringe filter (Whatman 0.45um PVDF) for LC-MS/MS analysis.

2.3. Identification and analysis of anthocyanins by LC-MS/MS

LC coupled Electrospray Ionization tandem Mass Spectrometry (LC-MS/MS) was used to carry out anthocyanin identification and quantification. A Shimadzu HPLC system (Kyoto, Japan) was used for chromatographic analysis and separation. This system employed a DGU-20A3 built in degasser, a LC-20AB solvent delivery pump, a SIL-20ACHT auto-sampler, a CTO-20AC column-holding oven, a CBM-20A communicator module, and a SPD-M20A Photodiode Array Detectors. A Waters (Milford, MA) C_{18} reversed phase column (250 mm length, 4.6 mm diameter) was used for anthocyanin separation. Data was analyzed using LC solution software (Kyoto, Japan). Elution was performed with mobile phase A (5% formic acid in de-ionized water) and mobile phase B (5% formic acid in acetonitrile/water 1:1 v: v). An optimum column temperature was set at $25\ ^{\circ}C$. At a flow rate of 0.8 mL/min, the gradient conditions were set as follows: solvent B volume at 5–20% for 35 min, 20–50% for 10 min, and held at 50% for 10 min before returning to 5% at 60 min. The detector performed a full spectrum scan between 190 and 800 nm, where 520 nm was used for monitoring anthocyanins. Peonidin-3-glucoside was used as an internal standard for quantitation of extraction recovery and the anthocyanin contents were expressed as peonidin 3-glucoside equivalent (PN3GE). Based on a signal-to-noise ratio of 3:1 and the standard deviation of the lowest concentration of PN3G/slope of the calibration line, the detection limit was estimated to be 2 pmol.

Mass spectrometric scan was performed on a Bruker Esquire 3000 in positive mode with a scanning interval 500–1200 m/z. Nebulization was conducted at $350\ ^{\circ}C$ aided by concurrent N_2 flow at 10 psi; capillary and cone voltages were set at 3.5 kV and 40 V;

Fig. 2. Representative images of the whole, cross-section, and freeze dry of the ripe wild-type (left column) vs. the transgenic *Del/Ros1* tomato fruit (right column).

drying gas flow rate was 5 L/min. Mass of precursor ions and reactions of fragments loss were evaluated. Data were analyzed using Bruker Hystar Post Processing software (Bruker Daltonics, GmbH, Billerica, MA). The ESI/MS data was used to confirm the mass of each anthocyanin HPLC peak. The mass spectrometry instrument was controlled by the esquire control 5.3 software (Bruker Daltonics, GmbH, Billerica, MA) and the data were processed with Data analysis 3.3 software (Bruker Daltonics, GmbH, Billerica, MA). Individual identification of each anthocyanin was accomplished by comparison of HPLC retention time, absorbance spectra, and MS spectra with our previously published anthocyanin data (Lim et al., 2013; Xu et al., 2015). The new anthocyanins were identified by matching the mass spectral data with those from the National Institute of Standards and Technology Mass Spectra Library data (NIST08, National Institute of Standards and Technology, Gaithersburg, MD, USA).

2.4. Statistical analysis

Data were analyzed using SAS statistical software, version 9.3 (SAS Institute, Cary, NC, USA). Results were evaluated by one-way ANOVA using a general linear model procedure followed by Tukey's post-hoc test. The results were presented as means ± SD, and a probability at $p \leqslant 0.05$ was considered significant.

3. Results and discussion

3.1. Chromatographic separation

The objectives of this study were focused on characterizing the anthocyanin profile in a transgenic purple tomato and quantifying the anthocyanin content in each part of the tomato using HPLC-MS/MS. The profile of the anthocyanin peaks from transgenic tomato Del/Ros1 was shown by HPLC chromatogram in Fig. 3. While no anthocyanin peaks were detectable in the wild type, a total of seven peaks were eluted at the retention times between 23 and 38 min in the transgenic Del/Ros1 fruits. Of these, peaks 2 and 4 were the major anthocyanins and their peak areas appeared to be more than half of the total anthocyanin peak areas.
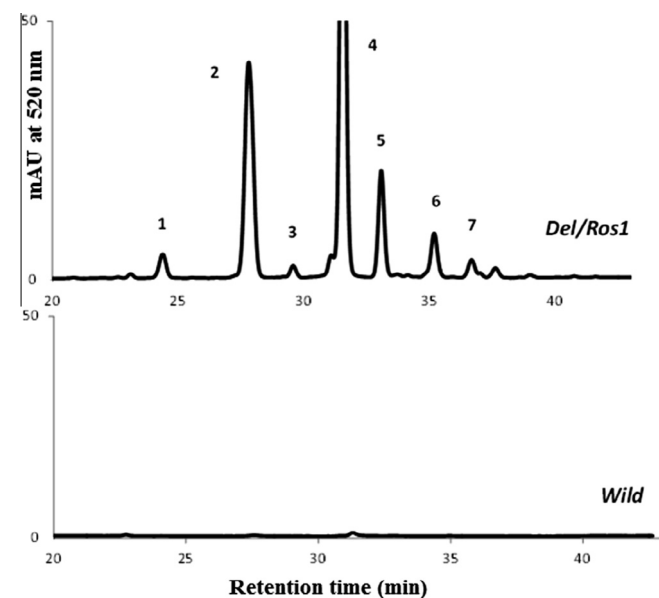

Fig. 3. Representative HPLC chromatograms of anthocyanins in the wild-type and the transgenic Del/Ros1 tomatoes (the peak number corresponding to anthocyanin name is shown in Table 1).

3.2. Mass spectrometric identification

Following HPLC separation, LC-MS/MS data were characterized by monitoring the molecular ion characteristics for each peak. The m/z ratio of each intact anthocyanin and its daughter fragments are listed in Table 1. As shown in Table 1, delphinidin (Dpd m/z 302), petunidin (Ptd m/z 316), and malvidin (Mv m/z 331) were the three anthocyanidin aglycones detected in the transgenic tomato line. Five of the seven anthocyanins including delphinidins (peaks 1–3) and petunidins (peaks 4–5) were reported previously by Butelli et al. (2008). However, two new malvidins (peaks 6–7) were found in the transgenic tomato line for the first time. As shown in Fig. 4a, the ions of peak 6, i.e., malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside (m/z 947), produced three fragments of m/z 785, 493, and 331. Transition 947–785 and 947–493 implied the loss of glucose (m/z 162) and p-coumaroyl (m/z 454), respectively. Transition 947–331 produced malvidin aglycone (m/z 331) caused by the loss of glucose and p-coumaroyl. In Fig. 4b, malvidin-3-(feruloyl)-rutinoside-5-glucoside (peak 7) produced transitions of 977–815, 493, and 331 m/z. Transition 977–815 and 947–493 indicated the loss of glucose (m/z 162) and feruloyl (m/z 484), respectively, while transition 947–331 produced malvidin aglycone (m/z 331).

When compared with other glycosylated anthocyanidins, malvidins have been found to have stronger inhibitory effects on nitric oxide production in LPS/IFN-γ -activated RAW 264.7 mouse macrophage cells due to better absorption and better free radical scavenging activity (Wang & Mazza, 2002). In addition, possible health benefits of dietary malvidins have been reported because of anti-proliferative (Hyun & Chung, 2004; Seeram, Zhang, & Nair, 2003) and anti-inflammatory activities (Jing et al., 2008; Wedick, Pan, Cassidy, & Rimm, 2012).

The reason why two new anthocyanins could be detected in the transgenic line may relate to the modified extraction and HPLC method that allowed for a distinct peak separation. In this study, 10% of formic acid was added to the methanol before extraction, creating a low pH environment for anthocyanin stabilization. The decrease of flow rate and solvent B ratio during the gradient elution might also provide better peak separation. Lastly, with a C18 stationary phase column, more polar solvent A and less polar solvent B mobile phase may carry out a better gradient elution.

3.3. Anthocyanin quantification in transgenic tomato

While anthocyanins were undetectable in the wild-type, the content of anthocyanins in the Del/Ros1 transgenic tomato is equally distributed, with 5.1 ± 0.5 g/kg DW in the peel and 5.8 ± 0.3 g/kg DW in the flesh. Total anthocyanin contents in Del/Ros1 are near 5.2 ± 0.5 g PN3GE/kg DW, or 0.5% of dry weight, which is higher than some of the anthocyanin-enriched foods such as red raspberry (3.9 g/kg DW by Wang & Lin, 2000), strawberry (3.2 g/kg DW by Wang & Lin, 2000), and mulberry (2.1 g/kg DW by Bae & Suh, 2007). Anthocyanins were undetectable in the seeds of both wide-type and transgenic tomato lines.

Table 2 lists the content profile of individual anthocyanin in the whole, peel, and flesh of the transgenic tomato line. The predominant anthocyanins were delphinidin-3-(trans-coumaroyl)-rutinoside-5-glucoside and petunidin-3-(trans-coumaroyl)-rutinoside-5-glucoside, which contributed to nearly 86% of the total anthocyanins. The reason why they are the highest among all the anthocyanins is not clear, but it may be due to the transgenic Del/Ros1-induced overexpression of the genes that relate to the specific anthocyanin biosynthesis pathway in which delphinidin-3-(trans-coumaroyl)-rutinoside-5-glucoside is an immediate precursor for petunidin-3-(trans-coumaroyl)-rutinoside-5-glucoside (Holton & Cornish, 1995). Two new anthocyanins, malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside and malvidin-3

Table 1
Mass spectrometric data of anthocyanins in the transgenic Del/Ros1 tomato.

Peak #	Retention time (min)	Anthocyanins	$[M+H]^+$ (m/z)	Detected fragments*
1	24.67	Delphinidin-3-(caffeoyl)-rutinoside-5-glucoside	935	773 (Dpd+3CR), 465 (Dpd+Glc), 302 (Dpd)
2	28.17	Delphinidin-3-(trans-coumaroyl)-rutinoside-5-glucoside	919	757 (Dpd+3TR), 465 (Dpd+Glc), 302 (Dpd)
3	29.80	Delphinidin-3-(feruloyl)-rutinoside-5-glucoside	949	787 (Dpd+3FR), 464 (Dpd+Glc), 303 (Dpd)
4	31.77	Petunidin-3-(trans-coumaroyl)-rutinoside-5-glucoside	933	772 (Ptd+3TR), 479 (Ptd+Glc), 316 (Ptd)
5	33.26	Petunidin-3-(feruloyl)-rutinoside-5-glucoside	963	800 (Ptd+3FR), 478 (Ptd+Glc), 316 (Ptd)
6	34.39	Malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside	947	785 (Mv+3PR), 493 (Mv+Glc), 331 (Mv)
7	36.81	Malvidin-3-(feruloyl)-rutinoside-5-glucoside	977	815 (Mv+3FR), 493 (Mv+Glc), 331 (Mv)

* Dpd, delphinidin; Ptd, petunidin; Mv, malvidin; Glc, glucose; 3CR, 3-(caffeoyl)-rutinoside; 3TR, 3-(trans-coumaroyl)-rutinoside; 3FR, 3-(feruloyl)-rutinoside; 3PR, 3-(p-coumaroyl)-rutinoside.
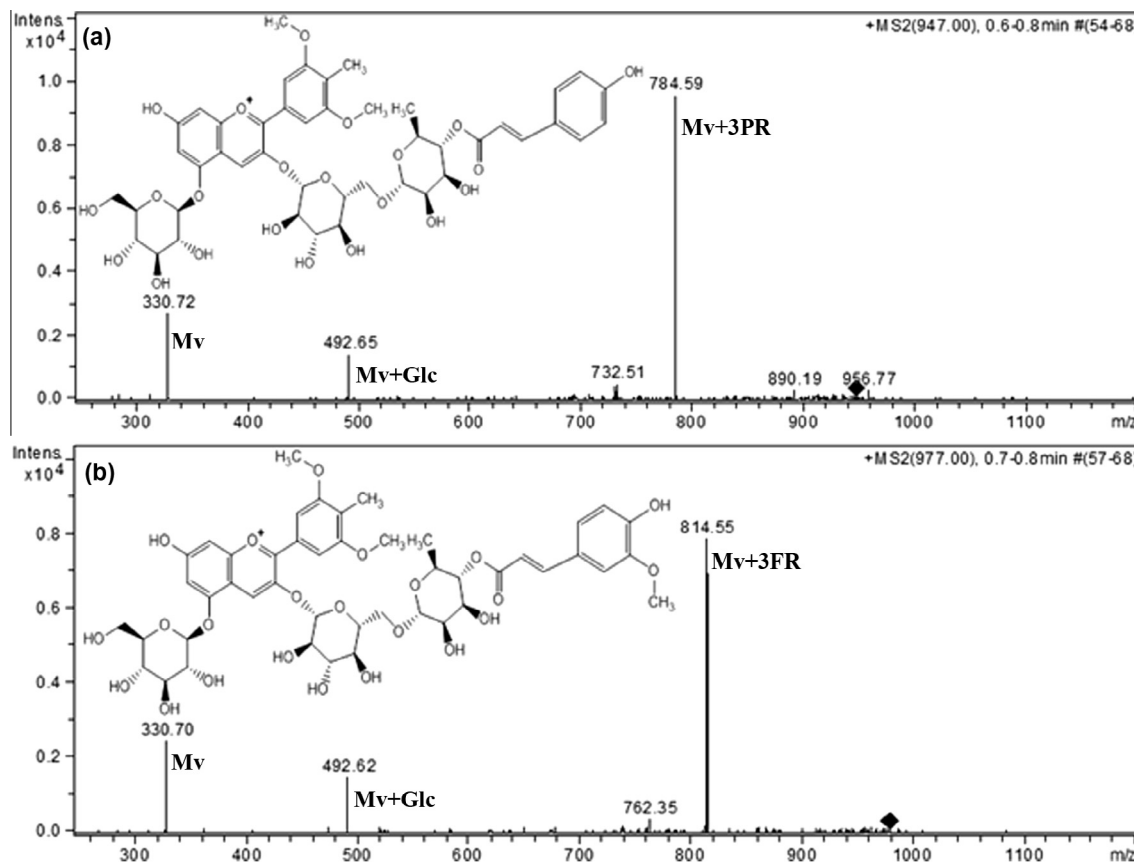

Fig. 4. Mass spectrometric data of two new malvidins detected in the transgenic Del/Ros1 purple tomato: (a) Malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside; (b) malvidin-3-(feruloyl)-rutinoside-5-glucoside (Mv, Malvidin; Glc, glucose; 3FR, 3-(feruloyl)-rutinoside; 3PR, 3-(p-coumaroyl)-rutinoside).

Table 2
The contents of individual anthocyanin (mg/kg dry matter) in the whole fruit, flesh and peel of the transgenic Del/Ros1 tomato*.

	Whole	Peel	Flesh
Delphinidin3-(caffeoyl)-rutinoside-5-glucoside	136.7 ± 10.9[a]	84.9 ± 7.0[b]	153.6 ± 11.5[c]
Delphinidin-3-(trans-coumaroyl)-rutinoside-5-glucoside	1434.6 ± 144.2[a]	1145.3 ± 115.0[b]	1624.3 ± 104.5[a]
Delphinidin-3-(feruloyl)-rutinoside-5-glucoside	53.4 ± 6.3[a]	25.5 ± 2.8[b]	64.9 ± 4.0[c]
Petunidin-3-(trans-coumaroyl)-rutinoside-5-glucoside	2849.6 ± 303.7[a]	3325.1 ± 351.4[a]	3021.6 ± 156.5[a]
Petunidin-3-(feruloyl)-rutinoside-5-glucoside	446.6 ± 75.1[a]	233.8 ± 11.9[b]	554.2 ± 30.2[c]
Malvidin-3-(p-coumaroyl)-rutinoside-5-glucoside	240.0 ± 15.4[a]	270.6 ± 23.0[a]	259.0 ± 27.7[a]
Malvidin-3-(feruloyl)-rutinoside-5-glucoside	76.2 ± 15.5[a]	34.1 ± 2.6[b]	107.0 ± 13.3[c]

* Data are expressed as mean ± SD (n = 4–6). Values marked by different letters within same row indicate significant difference (p < 0.05).

-(feruloyl)-rutinoside-5-glucoside made up 6% of the total anthocyanins.

In conclusion, seven anthocyanins, including 2 new anthocyanins, have been identified in the Del/Ros1 transgenic tomato.

Compared to undetectable anthocyanins in the wild type, the Del/Ros1 transgenic tomato produced a high level of anthocyanins that may provide unique functional products with potential health benefits.

Acknowledgments

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