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EFFECT OF 5-AMINOLEVULINIC ACID AND GENISTEIN ON ACCUMULATION OF POLYPHENOL AND ANTHOCYANIN IN 'QINYANG' APPLES

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ABSTRACT

Consumers are highly interested in purchasing apples with red color. However, the early-ripening apples always show poor color, so it is particularly important to improve apple color. The study was aimed to investigate effects of 5-Aminolevulinic acid (ALA) and/or Genistein (GNT) on apple peel color of 'Qinyang', polyphenol compounds and regulation of the gene related to anthocyanin synthesis. 300 $mg \cdot L^{-1}$ ALA and/or 21.62$\mu g \cdot L^{-1}$ GNT were sprayed on 'Qinyang' apples every five days after their bags were removed. The concentrations of anthocyanin and total polyphenol were determined with spectrophotometer and the concentrations of anthocyanin and polyphenol components were determined by HPLC. The relative expressions of six anthocyanin-related structural genes and regulation factor *MYB10* were measured by RT-PCR. Spraying ALA and/or GNT had significant promoting effects on anthocyanin accumulation of apple peel. The contents of anthocyanin with ALA, GNT, and ALA plus GNT sprayed were 1.12, 1.37, and 3.00 times as much as that with water sprayed, respectively. ALA and/or GNT played an important role in promoting total polyphenol accumulation, and the flavonoids concentrations of apple peel were significantly increased. The RT-PCR results revealed that ALA and/or GNT up-regulated the expressions of *PAL*, *CHS*, *DFR*, *F3H*, *UFGT*, *LDOX* and *MYB10*.

Keywords: Apple, 5-Aminolevulinic acid, Genistein, Anthocyanin, Polyphenol, Gene expression

INTRODUCTION

The acceptance of apple is largely dependent upon its skin color; the better colored, the higher acceptance (BOYER and LIU, 2004; SAURE, 1990). The red color of apple skin is mostly determined by its anthocyanin accumulation and distribution (BAE and LEE, 1995; LANCASTER *et al*., 1994). Anthocyanin, belongs to the diverse classes of secondary metabolites, known as flavonoids (TREUTTER, 2005). The main anthocyanins of apple peel is Cy-3-gal (cyanidin-3-galactoside) (BEN-YEHUDAH *et al*., 2005), while Cy-3-ara (cyanidin-3-arabinoside) and Cy-3-glu (cyanidin-3-glucoside) are present in very small quantities (JAKOPIC *et al*., 2007).

Anthocyanin biosynthesis is positively related to the degree of gene expression, and is controlled at the transcriptional level (BAN *et al*., 2007; ESPLEY *et al*., 2007). A group of genes is known as involving in the biosynthesis of anthocyanin, including structural genes and genes transcription factors (SINGH *et al*., 2010; XIE *et al*., 2011). Some of structural genes, including *phenylalanine ammonialyase* (*PAL*), *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), *flavanone-3-hydroxylase* (*F3H*), *dihydroflavonol-4-reductase* (*DFR*), and *UDP-glucose*: *flavonoid-3-O-glucosyltransferase* (*UFGT*), *flavonol synthase* (*FLS*), *leucoanthocyanidin dioxygenase (LDOX), anthocyanidin reductase* (*ANR*), encode corresponding enzymes responsible for the biochemical reactions in the synthesis of anthocyanin (HONDA *et al*., 2002; TAKOS *et al*., 2006; WEI *et al*., 2011). It is reported that of the various transcription factors of apple, *MYB10* probably regulated both skin colors and anthocyanin accumulations in fruit fleshes of certain apple genotypes and *MYB1* and *MYBA* are expressed in red parts of fruit skin of other genotypes (ESPLEY *et al*., 2009; ESPLEY *et al*., 2007; LI *et al*., 2014; LIN-WANG *et al*., 2010).

Anthocyanin biosynthesis of apple is highly affected by many factors, such as variety, light (HUGHES *et al*., 2005; HUGHES *et al*., 2012) and temperature (UBI *et al*., 2006). Since early-ripening apples always show poor color, some measures of promoting anthocyanin accumulation are needed to improve their color. Fruit bagging benefitted for anthocyanin accumulation and had become a common practice in many orchards in Asia (JU, 1998). However fruit bagging is time and labor cost, the application of plant growth regulators has been proposed as an economically viable alternative. So Many plant growth regulators have been tested for controlling anthocyanin biosynthesis in plant tissues, such as auxins (JEONG *et al*., 2004; MORI *et al*., 1994), cytokinins (KIM *et al*., 2006), gibberellins (MARTINEZ *et al*., 1996), abscisic acid (MORI *et al*., 2005), ethylene (EL - KEREAMY *et al*., 2003), and jasmonate (AYALA-ZAVALA *et al*., 2005). Unfortunately, most of these regulators are unable to induce anthocyanin accumulation in apple fruits, except 5-aminolevulinic acid (WANG *et al*., 2006b; XIE *et al*., 2013) and genistein (WANG *et al*., 2006b).

5-aminolevulinic acid (ALA) is an essential biosynthetic precursor of all tetrapyrrole compounds such as, chlorophyll, heme and vitamin B_{12} (JAHN and HEINZ, 2009). Xie (XIE *et al*., 2013) reported that ALA could promote anthocyanin accumulation in 'Fuji' apple in commercial field production. However, there was no research reports about effects of ALA on early-maturity apple under field conditions.

Genistein (GNT) is a kind of isoflavone, which naturally exists in soybean and a kind of tyrosine protein kinases inhibitor, which is always involved in researching on cells G protein signal transduction (BOWLER *et al*., 1994). GNT could promote anthocyanin accumulation in 'Fuji' peel in vitro in growth incubator (WANG *et al*., 2006b). Besides, it would improved fruit colorations of peach (ZHU *et al*., 2007b), flat peach (ZHU *et al*., 2007a) and grape (ZHU *et al*., 2010) under field conditions. However, there was not research reports about the effect of GNT on apple under field conditions.

Although there were researches done on effects of ALA or GNT applications on apple fruits, however there had been no research done on effects of combined applications of ALA and GNT on early-maturity apple. Besides, more detailed research still need to be done on effects of ALA and/or GNT treatments on polyphenol compounds in the apple peel. The study systematically investigated the effects of ALA and/or GNT on anthocyanin and polyphenol compounds of apple peel in order to gain a comprehensive understanding of effects of ALA, GNT, and ALA plus GNT on 'Qinyang' apples.

MATERIALS AND METHODS

Experimental sites, treatments and design: An experiment was conducted with ten-year-old trees of 'Qinyang' apple (*Malus* x *domestica* Borkh.) on the Farm of Baishui County, Shannxi Province, China in 2013. The young fruits of the trees were bagged approximately 6 weeks after flowering. The paper bags had two-layer which the inner layer was red and the outer layer was transparent with its outer surface yellow and its inner surface black. The experiment had five treatments: bagging treatment in which the apple fruits were bagged with paper bags until the sampling time (August 10, 2013) (DT); and other four spraying treatments in which water (containing 0.01% Tween-20 as the surfactant) (CT), 300 $mg \cdot L^{-1}$ ALA (containing 0.01% Tween-20 as the surfactant) (AT), 21.62$\mu g \cdot L^{-1}$ GNT (containing 0.01% Tween-20 as the surfactant) (GT), and 300 $mg \cdot L^{-1}$ ALA (containing 0.01% Tween-20 as the surfactant) plus 21.62 μg·L-1 GNT (containing 0.01% Tween-20 as the surfactant) (Volume ratio of 1:1) (AGT) were separately sprayed on the apple fruits every five days after the bags that held them were removed debagging (July 22, 2013), respectively. The ALA and GNT were made by Sangon Biotech, Shanghai, China. 'Qinyang' generally ripens in late July/early August in middle of Shaanxi, China. The fruit bags were removed 20 days prior to harvesting (July 22, 2013). All the test fruits were collected on south-sides of the trees receiving sufficient sunlight. The five treatments (including CT) were replicated with five times and a completely randomized design was adopted for them. The sample sizes of all treatments were 30 fruits (six fruits per tree). The peel (0.50 mm thick) of the sample fruits were immediately frozen in liquid nitrogen and stored at -80℃ for future analysis.

Total anthocyanin and total polyphenols measurements: Peels from 30 apples per replicate and sampling's time were collected and directly frozen in -80 °C. Peel tissues were ground to powder and anthocyanin was extracted as followed: 4.0 mL 1 % HCl in methanol was added to 1.0 g of lyophilized peel powder into 5.0 mL tubes at 4.0℃ for 24 hours without light. After centrifugation for 20 min at 12000 rpm at 4.0℃, the supernatants were collected in another tubes and determined by spectrophotometry at 553 nm and 600 nm absorbance value using UV/VIS Spectrometer (TU-1810, PERSEE), which was the difference between the two cyanine glycosides the relative content. Difference between each additional 0.01 was defined as a unit of U (WANG *et al*., 2012; XIE *et al*., 2013).

Peel tissues were ground to powder and polyphenol was extracted as followed: 8.0 mL 65% ethanol were added to 2.0 g of lyophilized peel powder into 10.0 mL tubes at 58.0℃ under the water bath, extracting for 35 min. After centrifugation for 20 min at 12000 rpm at 4.0 °C, the supernatants were kept without light at 4.0℃. 1.0 mL of the supernatants was diluted with sodium carbonate solution 12.0 mL, 1.0 mL Folin-Ciocalteu, and 12.0 mL 65% ethanol constanting volume to 25.0 ml and then the mixtures were incubated for 1 h at dark room temperature. The absorption was measured at 765 nm using UV/VIS Spectrometer. Calibration curves were established using a gallic acid standard in the range of 10-50 $\mu g \cdot mL^{-1}$, and results were presented as mg gallic acid equivalent per g dry matter(LI *et al*., 2013).

Analysis of anthocyanin and polyphenol compounds: Both of anthocyanins and polyphenol were carried out by HPLC on Dionex® P680 HPLC pump equipped with a Dionex® UV-vis detector (UVD170U) and a Dikma® C18 column (250×4.6 mm I.D., 5 μm).

The anthocyanin supernatant was filtered through a 0.22 μm syringe filter prior to HPLC analysis (WANG *et al*., 2012). The HPLC analysis anthocyanins conditions were as follows: column temperature, 30.0℃; injection volume, 20.0 μL; flow rate, 1.0 $mL \cdot min^{-1}$; solvents: A, acetonitrile; B, 1‰ trifluoroacetic acid in water; gradient, 0 min, 88% B; 1 min, 88% B; 15 min, 70% B; 30 min, 68% B; 32 min, 5% B; 38% min, 5% B; 40 min, 88% B;

48 min, 88% B; detection wavelength, 500 nm. The concentrations of polyphenols were calculated by peak area.

The polyphenol supernatant was filtered through a 0.45 μm syringe filter prior to HPLC analysis. The HPLC analysis polyphenol conditions were as follows: column temperature, 30℃; injection volume, 20 μL; flow rate, 1.0 mL·min^{-1}; solvents: A, acetonitrile; B, 3‰ trifluoroacetic acid in water; gradient, 0 min, 90% B; 10 min, 90% B; 12 min, 85% B; 20 min, 85% B; 22 min, 80% B; 40 min, 80% B; 42 min, 68% B, 50 min, 68% B; 52 min, 60% B; 60 min, 60% B; 62 min, 55% B; 70 min, 55% B; 72 min, 50% B; 80 min, 50 min; 82 min, 90% B; 90 min, 90% B; detection wavelength, 280 nm. The concentrations of polyphenols were calculated by peak area.

Anthocyanins and polyphenols standard samples were produced by Sigma. The concentrations of anthocyanins and polyphenols were calculated based on peak area.

RNA-extraction: Peels from 30 apples per replicate and sampling's time were collected and directly frozen in -80 ℃. The peel tissues were ground and RNA was extracted according to GASIC *et al*., 2004; HARB *et al*., 2013 with some modifications for analysis. 0.50 grams of powdered tissues were added to 800 μL pre-warmed (65.0℃) extraction buffer (100 mM Tris–HCl, pH 8.0; 25.0mM EDTA; 2.0 M NaCl, 3% CTAB; 4% PVP 40; 3% β-mercaptoethanol) and incubated for 30 min at 65.0℃. The liquid volume of chloroform: isoamylalcohol (24:1) was added when they were cooled to room temperature. The centrifugal tubes were upside down thoroughly incorporated, standing for 10 min and centrifuged at 12000 rpm for 20 min at 4.0℃. The above steps were repeated. RNA was precipitated 2 h at -20 °C by the addition of LiCl at a final concentration of 2.0 M. Following centrifugation 12000 rpm at 4.0℃ for 20 min, the pellet was washed twice with a volume of 70% ethanol, centrifuged for 10 min, and air-dried at room temperature. Finally, the pellet was suspended in 1‰ DEPC H_2O. The quality and quantity of the extracted RNA were assessed by Spectrometer (Thermo NANODROP 2000), respectively.

qPCR analysis: The frist-strand cDNA was synthesized using an M-MuLV Frist Strand cDNA Synthesis Kit (Sangon Biotech, Shanghai) according to the manufacturer's instructions. To assess the quantitative changes in the expression of the selected genes, gene-specific primers were designed (Table 1) based on sequences obtained from the NCBI database using Primer 5.0 software and those used in previous studies (FENG *et al*., 2014; HARB *et al*., 2013; XIE *et al*., 2013). Subsequent qRT-PCRs were carried out using the Maxima SYBR Green qPCR Master Mix (Thermo, USA) on a PIKO REAL 96 (Thermo, USA). The SYBR green chemistry was used for gene expression analysis. Dissociation curves were run to check whether primer dimers and other amplification by-products were present. The qPCR reaction conditions were as follows: pre-treatment at 50 ℃ for 2 min, initial denaturation at 95℃ for 10 min, amplifications through 40 cycles at 95℃ for 15 s, annealing at n℃ for 60 s, extension at n℃ for 30 s, melt curve n-95℃ and a final extension at 20℃ for 10 s, where the N was 59.0 in *PAL*, 54.0 in *CHS*, 56.5 in *DFR*, 60.0 in *F3H*, 61.0 in *UFGT*, 62.5 in *LDOX* and 59.5 in *MYB10*. The expression levels of the tested genes were calculated relative to transcript abundance of the *MdActin* gene as reference gene employing relative quantification with efficiency correction. Data after reaction was recorded and analyzed by the Piko Real Software 2.0 (Thermo, USA), using the ddCT method (LIVAK and SCHMITTGEN, 2001).

Statistical analysis: All data were expressed as mean±SD and SD shown by error bar. Unless otherwise stated, experiments were performed in duplicate and repeated three times. Data were subjected repeated measures ANOVA with SPSS version 18.0 software. Duncan values were calculated in cases where significant variance was found at $P < 0.05$.

RESULTS

Concentrations of anthocyanin and anthocyanin compounds in five treatments: Fig.2 presents the anthocyaninin contents in the five treatments, which showed that anthocyanin accumulations in of 'Qinyang' apple peel with treatment of AGT, AT and GT the were significantly enhanced (Fig.1; Fig.2).The anthocyanin contents of treatments decreased in the order of AGT, GT, AT, CT and DT. This indicated that spraying ALA and/or GNT on apple fruits caused their anthocyanin to reach a high level, whereas shading apple fruits lowered their anthocyanin level, and exposing-light apple fruits' anthocyanin at intermediate level. The mean of the anthocyanin contents of the apple fruits treated with ALA plus GNT, ALA and GNT solutions were 300%, 137% and 112% of those of the apple fruits exposed to Light, respectively. Tab.2 and Fig.3 present the anthocyanin components of cyaniding-3-galactoside (Cy-3-gal), cyaniding-3-glucoside (Cy-3-glu) and cyaniding-3-arabinoside (Cy-3-ara) in the five treatments. For AGT, GT and AT, Cy-3-gal and Cy-3-glu accumulations were significantly enhanced in apple peel and the components tended to vary in the same patterns as the anthocyanin contents did, whereas, the Cy-3-ara content of CT was higher than that of AGT, GT, and AT.

Concentrations of total polyphenols and polyphenol compounds in five treatments: Fig. 4 shows the contents of total polyphenols in 'Qinyang' apple peel with five treatments, which indicated that the total polyphenol accumulations of AGT, AT and GT were significantly enhanced in 'Qinyang' apple peel. The total polyphenols of DT apple peel were lower than that of other treatments, and The total polyphenols of AT and GT showed no differences. The total polyphenols in the treatments decreased with the order of AGT, GT, AT, WT and DT.

Fig. 1 'Qinyang' apples in five treatments

Tab.3 presented polyphenol components of five treatments, which showed that, the flavonoids contents of AGT, AT and GT increased significantly in 'Qinyang' apple peel. The concentrations of AGT's hyperin, rutin, catechin and quercitrin appeared higher than that of the other four treatments. All polyphenol compounds but chlorogenic acid of DT were lower than that of the other treatments. The concentrations of CT's chlorogenic acid was the highest, which showed no difference among the AGT, AT and DT. The phloridzin concentrations of CT and AT were higher than that of AGT and GT, which showed no differences between latters

Gene expression related to apple peel color: Figure 5 shows expressions of anthocyanin-related genesin 'Qingyang' apples at the harvest time (20 days after applying ALA and/or GNT). The relative expressions of *PAL* in the AGT, GT, AT and CT were significantly higher than that of DT, while significantly lower in the

AGT and GT than in the AT. The relative expressions of *CHS* in the AGT, GT and AT were significantly higher than that of CT. Meanwhile, comparison of productions of mRNA *F3H*, *DFR* and *LDOX* in ALA involved treatments and CT indicated that ALA could induce mRNA *F3H*, *DFR* and *LDOX* productions but presented no significantly effect on *UFGT*. GNT presented similar effects to those of ALA but did not significantly induced *LDOX*. ALA plus GNT significantly promoted all the genes except *PAL*. The relative expression of *MYB10* in the AGT, GT and AT were also promoted by 128%, 122% and 117% compared with its expression in the CT, respectively.

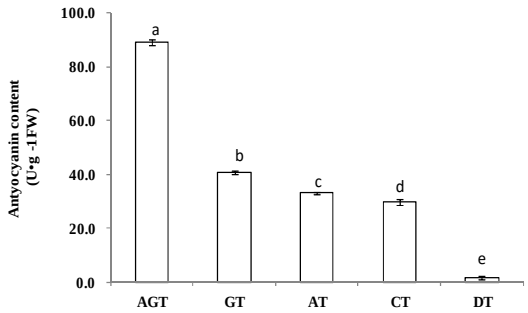
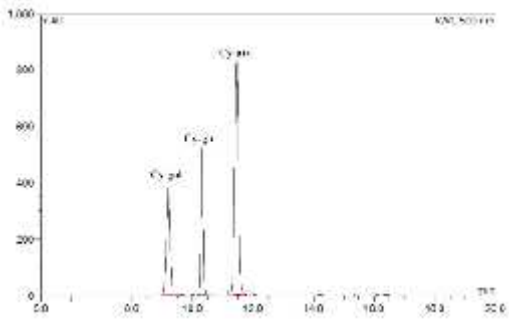
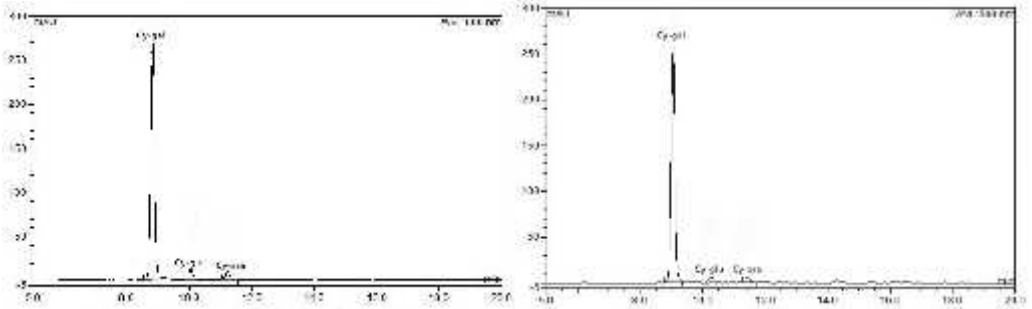


Fig. 2 Anthocyanin content of 'Qinyang' apple peels in five treatments.

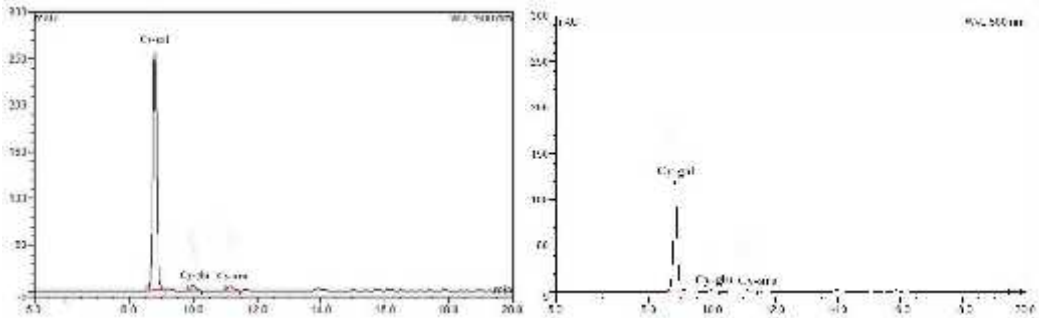
AGT: 300 mg•L^{-1} ALA plus 21.62 μg•L-1 GNT; AT: 300 mg•L^{-1} ALA; GT: 21.62 μg•L-1 GNT; CT: water spraying treatment; and DT: bagging treatment. Data are mean ± SD (n = 6) and the same letters mean insignificant differences at $p < 0.05$.



(Standard samples)



(AGT) **(AT)**



(GT) **(CT)**

Fig. 3 HPLC Chromatogram of anthocyanin in the peels of 'Qinyang' apples.

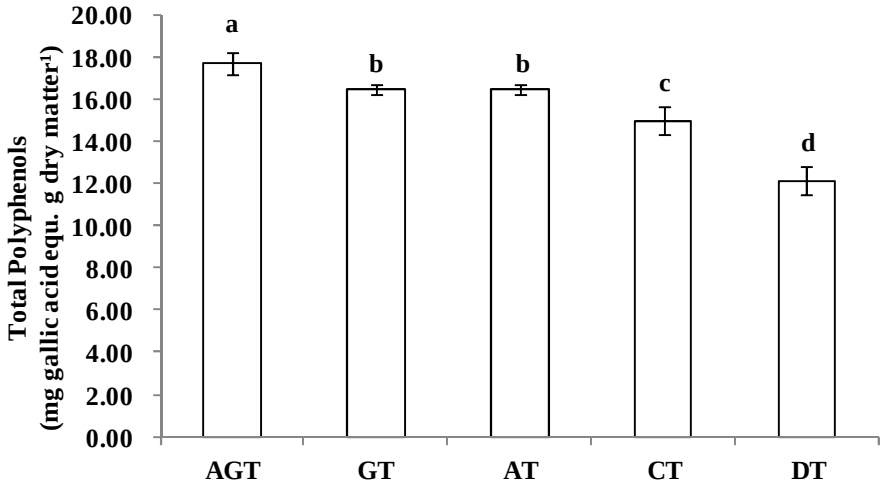
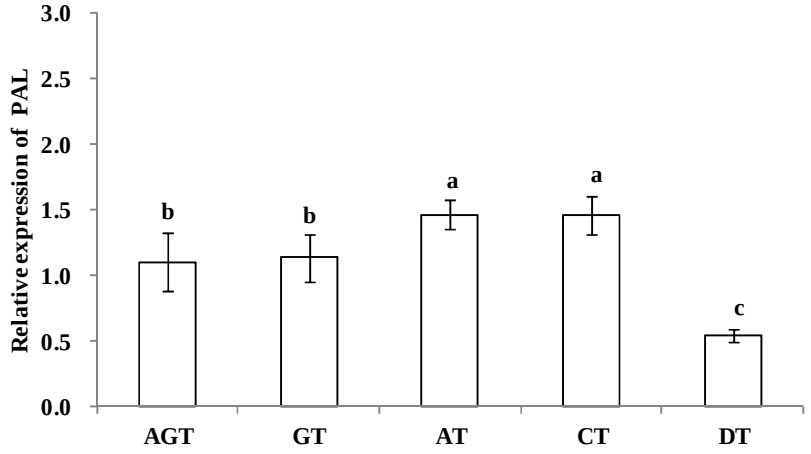
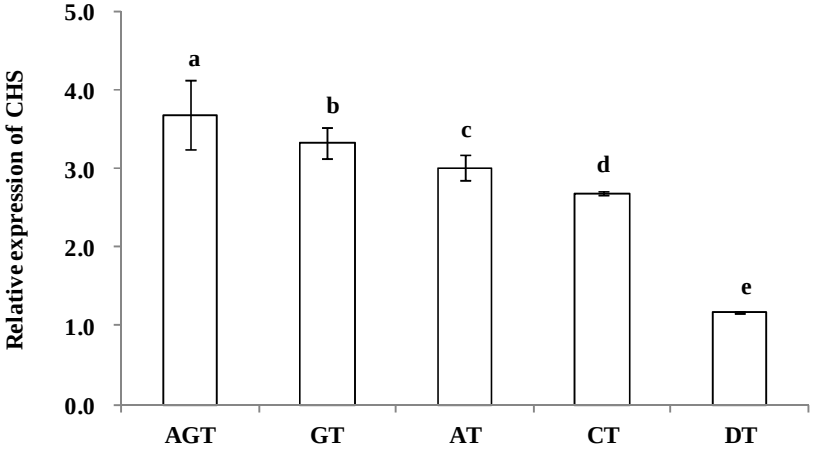


Fig. 4 Polyphenols content of 'Qinyang' apple peels in five treatments.

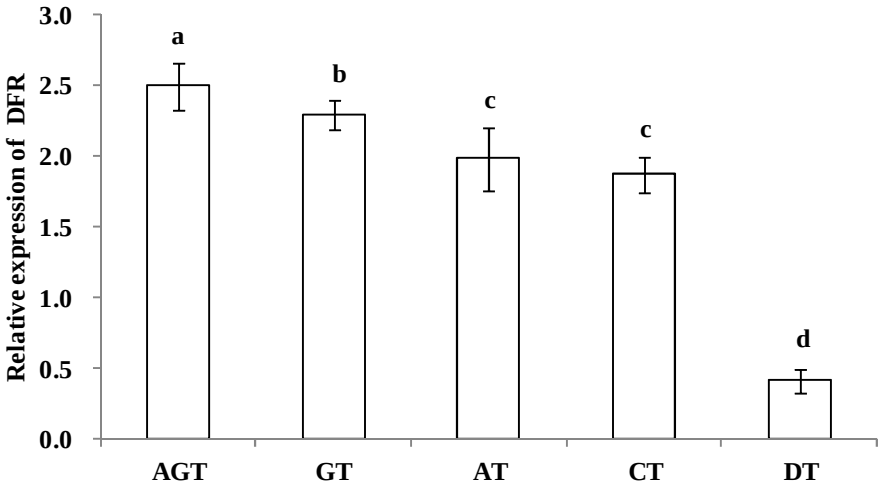
AGT: 300 mg•L^{-1} ALA plus 21.62 μg•L-1 GNT; AT: 300 mg•L^{-1} ALA; GT: 21.62 μg•L-1 GNT; CT: water spraying treatment; and DT: bagging treatment. Data are mean ± SD (n = 6) and the same letters mean insignificant differences at $p < 0.05$.



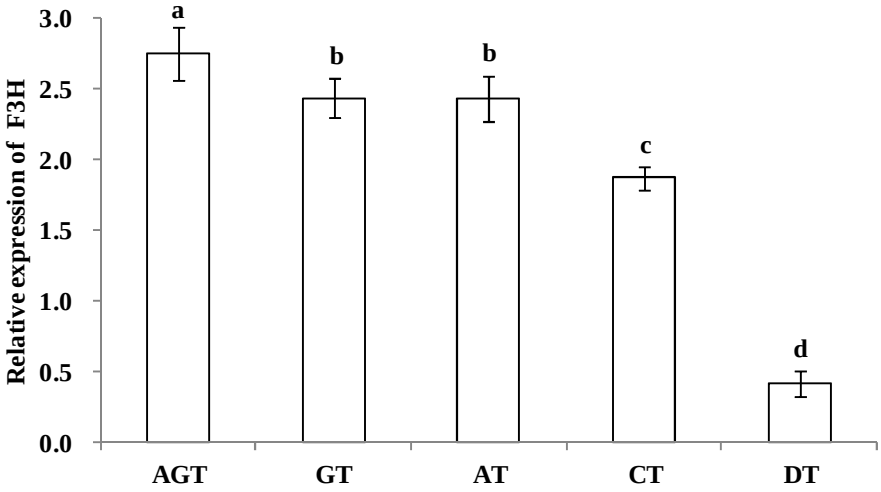
(A)



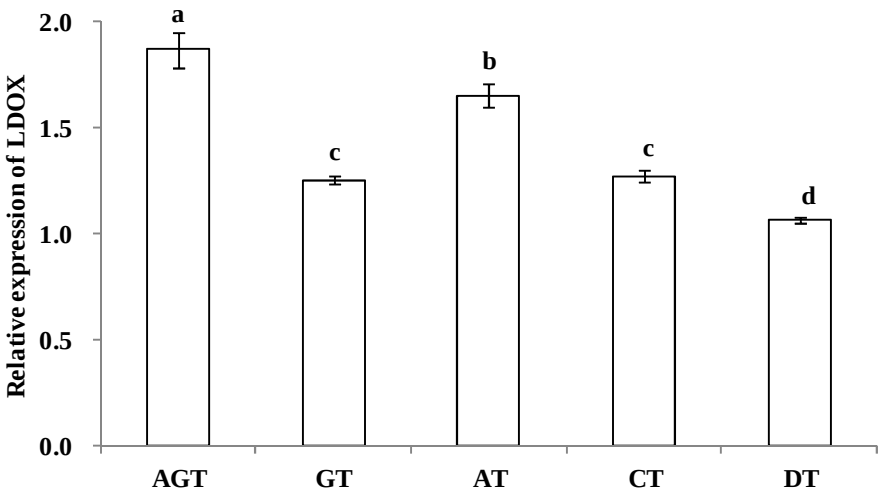
(B)


Relative expression of DFR
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
c
c
d
AGT
GT
AT
CT
DT

(C)


Relative expression of F3H
3.0
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
c
d
AGT
GT
AT
CT
DT

(D)


Relative expression of LDOX
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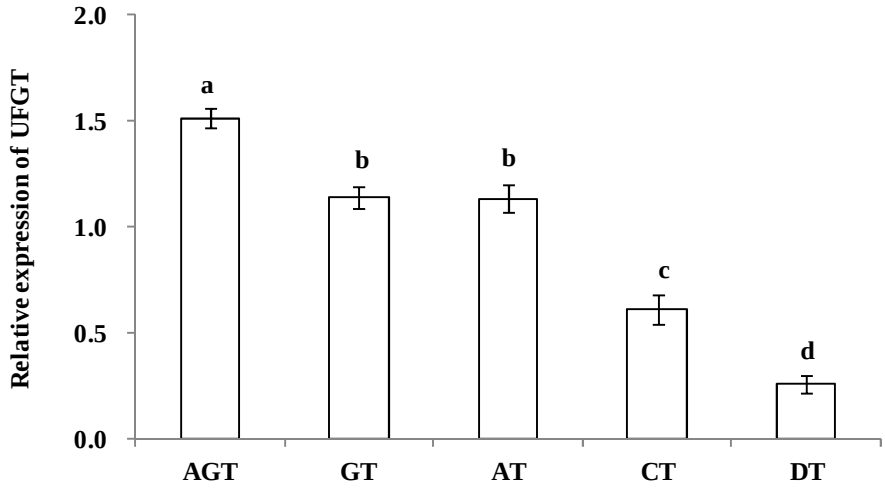
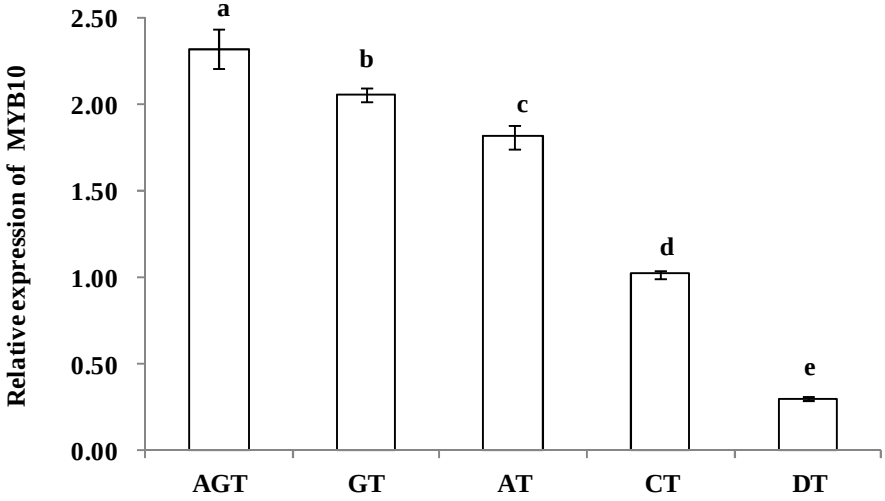
1.0
0.5
0.0
a
c
b
c
d
AGT
GT
AT
CT
DT

(E)



(F)



(G)

Fig. 5 Relative expression levels of (A) phenylalanine ammonia-lyase (*PAL*), (B) chalcone synthasw (*CHS*), (C) dihydroflavonol-4-reductase (*DFR*), (D) flavanone-3-hydroxylase (*F3H*), (E) leucoanthocyanidin dioxygenase (*LDOX*), (F) UDP-glucose: flavonoid-3-O-glucosyltransferase (*UFGT*), and (G) MYB transcription factor (*MYB10*) genes in 'Qinyang' apples of five treatments at harvest time.

AGT: 300 mg•L^{-1} ALA plus 21.62 μg•L-1 GNT; AT: 300 mg•L^{-1} ALA; GT: 21.62 μg•L-1 GNT; CT: water spraying treatment; and DT: bagging treatment. Data are mean ± SD (n = 6) and the same letters mean insignificant differences at $p < 0.05$.

Table 1 RT-PCR Primers of apple genes related to anthocyanin biosynthesis

Gene name	Forward primer(5'-3')	Reverse primer(5'-3')
PAL	TGCACTGTGCCAAGCTATTGA	TCAACAACCCTGAGGAGGTCT
CHS	GCAAGTGTTGTCAGATTACGG	TGATACTGGTGTCTTCAAGCAG
DFR	CAAGTACAGCTTGGAGGACAT	TCCAAGCTGGTAAATGTAAAACA
F3H	GAAGATGAGCAAGGATCTTGAG	TTCCACAAAGAGCTTTCAAGTG
UFGT	CCACCGCCCTTCCAAACACTCT	CACCCTTATGTTACGCGGCATGT
LDOX	CCAAGTGAAGCGGGTTGTGCT	CAAAGCAGGCGGACAGGAGTAGC
MYB10	TGCCTGGACTCGAGAGGAAGACA	CCTGTTTCCCAAAAGCCTGTGAA
Actin	CTCCGTGGTGGTTTTTAAGTG	AGGAGGCAGAAACAGTACCAT

Table 2 Concentrations of cyaniding-3-galactoside chloride, cyaniding-3-glucoside chloride and cyaniding-3-arabinoside chloride of 'Qinyang' apple peels in five treatments.

Handing method	Cy-3-gal	Cy-3-glu	Cy-3-ara
AGT	39.31±0.69a	2.00±0.10a	1.47±0.12a
GT	36.58±0.59b	0.83±0.21b	1.01±0.19b
AT	37.39±1.29b	0.86±0.20b	1.07±0.72b
CT	17.43±0.22c	0.37±0.16c	0.50±0.18c
DT	--	--	--

'--' show not detected. Values are means ± SD values of six replicates. Different letters (a-c) within the same column indicate significant difference at $p < 0.05$ by Duncan's test.

Table 3 Concentrations of hyperin (Quercetin-3-galactoside), rutin (Quercetin-3-o-rutinoside), catechin, quercitrin, chlorogenic acid and phloridzin of 'Qinyang' apple peels in five treatments.

Handing method	Hyperin	Rutin	Catechin	Quercitrin	Chlorogenic acid	Phloridzin
AGT	73.78±1.89a	73.44±1.82a	2.21±0.22a	31.65±2.03a	4.45±0.68b	10.19±0.53a
GT	72.87±1.51a	71.86±2.48a	1.86±0.10b	27.12±0.69b	4.15±0.05b	11.94±1.52a
AT	69.89±0.95ab	67.89±0.95b	1.77±0.04b	22.63±0.55c	4.63±0.29b	7.43±0.34b
CT	37.62±1.36c	37.95±1.40c	1.36±0.12c	18.87±0.64d	10.59±0.87a	7.60±0.12b
DT	10.89±1.51d	11.22±1.51d	0.29±0.16d	18.41±0.79d	2.12±0.11c	4.77±0.32c

Values are means ± SD values of six replicates. Different letters (a-d) within the same column indicate significant difference at $p < 0.05$ by Duncan's test

DISCUSSION

The study demonstrated that ALA and/or GNT application could improved the color of 'Qinyang' apple peel (Fig. 1), and promoted the accumulation of anthocyanin and total polyphenols in 'Qinyang' apples 20 days before commercial harvest (August 10, 2013) (Fig. 2, Fig. 4). Different studies proved in this regard that treating fruits with ALA and/or GNT before harvest induced higher synthesis rates of anthocyanin (WANG *et al*., 2004; XIE *et al*., 2013; ZHU *et al*., 2007a; ZHU *et al*., 2007b; ZHU *et al*., 2010). In addition, the study further observed that ALA and/or GNT took effect on polyphenol compounds in 'Qinyang' apples peel (Tables 2 and 3). The results indicated that flavonoids of DT were drastically decreased and significantly increased by ALA and/or GNT. ALA and/or GNT presented minimal effects on chlorogenic acid, becasuse chlorogenic acid was not a main polyphenol in apple peel (AWAD *et al*., 2000; MAYR *et al*., 1995).

Mechanisms, which ALA and/or GNT take effects on accumulations of polyphenol and anthocyanin in apple, are not elucidated. Phenylalanine ammonialyase (PAL), a light dependent enzyme catalyzing the first step of phenylalanine metabolic pathways, can promote to polyphenols production and its expression peaks after 24 h light exposure and then decreased (WANG *et al*., 2006a; XIE *et al*., 2013; ZHU *et al*., 2010). The results of the study indicated that *PAL* expression was not induced by AT, and inhibited by AGT and GT (Fig. 5A). This did not agree with Xie and Zhu's results (XIE *et al*., 2013; ZHU *et al*., 2010). Because the induction of *PAL* expression by ALA and/or GNT was time-dependent, there was not a chance to observe that the *PAL* expression was induced in the AGT, GT, and AT. Fig. 4 shows that ALA and/or GNT could up-regulate the expression of *PAL* and increase its activity. It is important to notice that *CHS* gene encodes the CHS enzyme, which in turn catalyzes the first committed step of flavonoid biosynthesis, and promotes the condensation of p-coumaroyl-CoA with malonyl-CoA molecules to produce chalcone. Chalcone considered as the precursor for a diverse set of flavonoids (KODURI *et al*., 2010). Fig. 5B shows the expression of *CHS*, which clearly indicated that CHS transcripts could accumulate to higher levels in apples treated with ALA plus GNT than those treated otherwise. The results of the study indicated that *CHS* was more strongly responsive to GNT than that of ALA in promoting polyphenols accumulation in apples. *DFR* expression in AGT and GT was induced, but not by ALA (Fig. 5C), which indicated that *DFR* was not a key gene for ALA-induced anthocyanin accumulation, but a key gene for GNT-induced anthocyanin accumulation. *F3H* expression was induced by ALA and/or GNT, and presented no difference between GT and AT (Fig. 5D), indicating that *F3H* was not a key gene for both ALA and/or GNT-induced anthocyanin accumulations. Furthermore, *LDOX* expression in AGT and AT was strongly up-regulated, but not in GT (Fig. 5E), which meant that *LDOX* was not a key gene for GNT-induced anthocyanin accumulation, but a key gene for ALA-induced anthocyanin accumulation. *MYB10* regulated anthocyanin synthesis by

controling of *UFGT* instead of other up-stream structural genes involved in anthocyanin synthesis (HONDA *et al*., 2002; ZHANG *et al*., 2014). Thus, the expression profile of *MYB10* was relatively similar to that of *UFGT* (Fig. 5F; Fig. 5G). It followed that ALA had synergistic promoting effects on anthocyanin accumulation in apples mostly by up-regulating of *CHS*, *F3H*, *UFGT*, and *MYB10* rather than *DFR*. And GNT promoted anthocyanin accumulation in apples mostly by up-regulating *CHS*, *DFR*, *F3H*, *UFGT* and *MYB10* rather than *LDOX*.

The study concluded that ALA and/or GNT had very evident effects on enhancing apple coloration; the concentrations of anthocyanins and flavonols increased significantly compared with CT, and the expressions up-regulation of anthocyanin-related genes were associated with red peel in 'Qinyang' apple. Accordingly, more investigation needed to be done to elucidate possible mechanisms by which ALA and/or GNT promoted anthocyanin accumulation in early-maturity apple. Besides, ALA and/or GNT applications were a new approach for bagging-saving and field management costs as well as reducing orchard pollution in apple production.

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