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EXPRESSION PATTERNS OF ETHYLENE AND POLYAMINE BIOSYNTHETIC GENES DURING FRUIT RIPENING IN STRAWBERRY

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Abstract: The expression pattern of genes related to ethylene and polyamine biosynthesis were analyzed in octoploid strawberry fruits in different ripening phases. RNA was isolated from *Fragaria x ananassa* Duch cv. Asia and used to synthesize cDNA by reverse transcriptase enzyme, which was applied in qPCR with primers designed for S-adenosyl-L-methionine synthases (*FaSAMS1-3*), S-adenosyl-L-methionine decarboxylases (*FaSAMDC1-3*), arginine decarboxylase (*FaADC*), spermidine synthase (*FaSPDS*), 1-aminocyclopropane-1-carboxylate synthases (*FaACS1-4*) and 1-aminocyclopropane-1-carboxylate oxidase (*FaACO1-4*) gene sequences. Based on the comparison made between the expression patterns of ethylene- and polyamine-related genes and those of the common precursor- and pathway shift-related genes, it appeared that with the exception of *FaSAMS2*, *FaSAMDC1* and *FaSAMDC2*, the genes involved in polyamine biosynthesis showed decreasing level of expression as ripening proceeded.

Keywords: ACC oxidase, ACC synthase, S-adenosyl-L-methionine, SAM decarboxylase, spermidine synthase

INTRODUCTION

The molecular background of ripening is a less well understood mechanism in non-climacteric fruits such as strawberry than in the ethylene inducible climacteric ones. Aharoni et al. (2002) carried out a high throughput microarray-based transcript analysis of strawberry ripening. Altogether 1100 strawberry ESTs from different ripening stages demonstrated that most of the comestible secondary metabolites were produced at the red stage of ripening. Based on this assay, Aharoni and O'Connell (2002) found that, in contrast to the achene, the ripening-related genes showed higher expression levels in the receptacle tissue. Investigating the RNA fingerprint of strawberry receptacle tissue Balogh et al. (2005) identified several genes which altered their expression pattern during the different ripening stages (green, white, pink and red stages). 130 transcript-derived fragments and partial cDNAs were isolated and sequenced. The ethylene and polyamine biosynthetic genes (1-aminocyclopropane carboxylate synthase and oxidase - *ACS* and *ACO*; spermidine synthase - *SPDS*) transcripts were identified in achenes and green flesh (Balogh et al., 2005).

Steps of ethylene biosynthesis in higher plants were described by Yang and Hoffmann (1984). The genes *ACS* and *ACO* encode the key enzymes in this pathway. The ACC synthase enzymes are encoded by a gene family, members of which encode isoenzymes with different regulatory functions in various cellular environments (Liang et al., 1992; Prescott and John, 1996). The amount of ethylene is relatively high in the green fruit of non-climacteric strawberry, it decreases at the white ripening stage and then it slightly rises until the end of ripening process (Perkins-Veazie et al., 1996). The moderate growth of ethylene starting from the white ripening stage is accompanied by an increase in the rate of respiration, similarly to the onset of climacteric ripening (Iannetta et al., 2006). In addition to ethylene, polyamines also play a major role in the ripening processes of fruits sharing a common precursor molecule, the S-adenosyl-L-methionine (SAM).

Polyamines are polycations with small molecular weight present in all living organisms (Cohen, 1998). Diamine putrescine (Put), triamine spermidine (Spd) and tetraamine spermine (Spm) are the most common polyamine forms in plants (Kaur-Shawhney et al., 2003). Similarly, to hormones, they play a role in replication, transcription, translation, membrane stabilization, regulation of enzyme-activity, cell division and elongation, as well as in the growth and development of plants (Galston et al., 1997; Walden et al., 1997) contributing thereby to the process of fruit ripening (Gil-Amado and Gomez-Jimenez, 2012). Promoting cell division and growth, influencing cell membrane structure and defense against the oxidative stresses are the main functions of polyamines during fruit development. The biosynthesis of putrescine can commence from ornithine or agmatine, which are synthetized from arginine by arginase and arginine-decarboxylase.

Conversion of agmatine into putrescine is catalyzed by enzymes N-carbamoylputrescine-amidohydrolase and agmatine-iminohydrolase. The ornithine is transformed into putrescine by the ornithine decarboxylase (Gill and Tuteja, 2010). An aminopropyl group is bound to putrescine by the spermidine synthase enzyme generating triamine spermidine. Henceforward another aminopropyl

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group is attached to the spermidine by the enzyme spermine synthase resulting in a tetraamine spermine. The aminopropyl groups are ensured by decarboxylating SAM, which is catalyzed by the SAM-decarboxylase (Kuznetsov and Shevyakova, 2007). Earlier studies discussed the antagonistic effects of ethylene and polyamine metabolisms during fruit ripening in avocado and tomato. While the amount of polyamines decreases as the ripening process proceeds, that of the ethylene increases (Winer and Apelbaum, 1986; Saftner and Baldi, 1990).

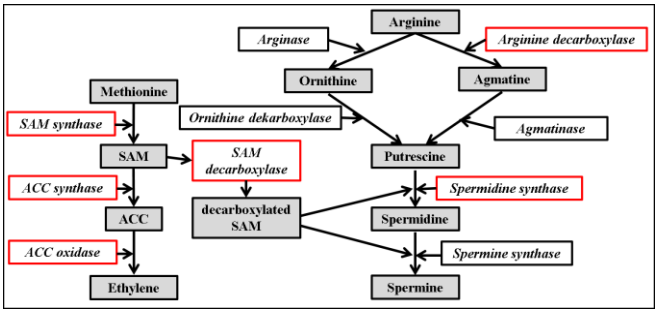

Fig. 1. Ethylene and polyamine metabolism. Expression levels of genes highlighted with red were measured in strawberry fruit at four different ripening stages.

By revealing the expression pattern of genes involved in the ethylene and polyamine metabolic pathways our primary aim was to better understand the mechanisms underlying non-climacteric fruit ripening, which still have elements to be clarified. Figure 1. shows the ethylene and polyamine metabolism. We measured the relative expression levels of the genes highlighted with red at four different ripening stages (green, white, pink and red).

MATERIALS AND METHODS

Plant material and RNA isolation

Fragaria x ananassa Duch. cv. Asia plants were grown in a greenhouse in Gödöllő, Hungary, 2015. Receptacle tissues were collected at four well distinguishable ripening stages (green, white, pink and red). The samples were frozen in liquid nitrogen and stored at -80˚C until processing. Total RNA was isolated from the samples as described by Salzman et al. (1999).

cDNA synthesis and qPCR analysis

The total RNA was converted into cDNA using ThermoFisher Scientific RevertAid First Strand cDNA Synthesis Kit and oligo-dT primer (Biocenter Ltd., Szeged, Hungary). The concentration and purity of the cDNA was determined by a NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, USA). The final concentration was adjusted to 20 ng/µl. For qPCR a Corbett RG-6000 real time thermal cycler (Qiagen, Hilden, Germany) was used. Reaction mix (20 µl) contained: 10 µl 2xABsolute qPCR SYBR Green Mix (ThermoFisher Scientific, Waltham, USA), 1.75 µl/primer (70 nM), 1 µl cDNA. PCR conditions were as follows: 95ºC 15 min (1 cycle), 95ºC 20 sec, 58ºC 20 sec, 72ºC 30 sec (40 cycle). Four technical replicates of qRT-PCR reactions were performed per primer pair. The relative expression levels were analyzed with the „Comparative quantification" application of the built-in software (Rotor-Gene Q Series Software) (Warton et al., 2004; McCurdy et al., 2008). We applied the actin housekeeping gene as reference. *Fragaria x ananassa* Duch. cv. Elsanta *FaACS1* and genes *FaACO1* (AY661301 and Y706156) were isolated and sequenced by Kiss et al. (2007). *FaACS2-4* and *FaACO2-4* were identified by BLAST analysis of *FaACS1* and *FaACO1*. The primers were designed based on the available whole genome sequence of *Fragaria vesca* L. (Shulaev et al., 2011) and tested both on genomic and cDNA of *Fragaria x ananassa* Duch. cv. Asia (Table 1.).

Tab.1.

Primers used for qPCR

Primer name	Sequence	T_m (C°)
FvSAMS1_RT_F	CAACAAACGATTCTTGAAGACAGCTG	56.4
FvSAMS1_RT_R	AGACTGAGGCTTCTCCCACTT	54.4
FvSAMS2_RT_F	GTTTTTGAAGACTGCTGCTTATGGG	56
FvSAMS2_RT_R	AGCTTGAACCTTATCCCACTTGA	53.5
FvSAMS3_RT_F	CTCCTGAGCTTATGCCTCTCAGC	58.8

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FvSAMS3_RT_R	ATGGTCATTGTAGTACTCCACAGTGACTT	58.7
FvSAMDC1_RT_F	TGTTCATTGGATTTGAAGGGATACTGT	55.2
FvSAMDC1_RT_R	AGAGGATTCGTAGTCCTCATCTTC	55.7
FvSAMDC2_RT_F	GATCAACAAGCTATGAAGAGCTGG	55.7
FvSAMDC2_RT_R	GTTCTTCATGCTCAAACTCTCTTCAACTTC	58.9
FvSAMDC3_RT_F	GTAGCCAATCACTACTTCGACGCCT	59.3
FvSAMDC3_RT_R	TGAGAGTGAGGCCGAGAAGCGA	58.6
FvADC_RT_F	CTTCCACAACATGCCGTATCTG	54.8
FvADC_RT_R	TCAACCACTGCAGTATGACCACT	55.3
FvSPDS_RT_F	CAGAGAGTATATGGCTTCACATGCACAT	58.5
FvSPDS_RT_R	GGTCCCTCAGTAGAACAGAGCAT	57.1
FvACS1_RT_F	GAAGTGATGATCATCTCCATTTAGTTATGCAG	59.3
FvACS1_RT_R	GACTAGCTTCTAATTAGGTGGCATACCTA	58.7
FvACS2_RT_F	CTCATCCATGCACTCAACCTAGCCT	59.3
FvACS2_RT_R	CTTTGAGGTAGCAGATTGCAACAATCAG	58.5
FvACS3_RT_F	GCAATGAAAAGGATACGTGATTTCATGGG	58.7
FvACS3_RT_R	GCAACACATCTAATTCTTGAAGACCAAGATTTG	59.4
FvACS4_RT_F	CATTGCAACGCTTGAAGGCCTTTATAAC	58.5
FvACS4_RT_R	GATAGGGCTGCGATCGTCGAAAGA	59.1
FvACO1_RT_F	GCAGTGGAACTAGAGAAGCTGGCT	59.1
FvACO1_RT_R	GCATGGAGGGTAGTTGCTCACCT	58.8
FvACO2_RT_F	GGTGAATCATGGGATAGCCACTGAG	59.3
FvACO2_RT_R	CCTCAGTGTTAACTGCGTTAAGGCC	59.3
FvACO3_RT_F	AGTTGGTGACAGGTCTCCGAGCT	58.8
FvACO3_RT_R	GACAATCGAATTGCGCAGTGGCTG	59.1
FvACO4_RT_F	CGGGTGCTTTCAGTTAGTGAACCATG	59.5
FvACO4_RT_R	ACCGTGAACTTCCTCGAACCCATATG	59.5
FvACT_RT_F	GGACTCTGGAGATGGTGTCAGTCA	57.9
FvACT_RT_R	TCCCTGACTATTTCTCGCTCAGCAGT	59.7

Determination of total monomeric anthocyanins

The measurements of total monomeric anthocyanins were carried out according to a method described earlier (Lee et.al., 2005). The lyophilized samples (20 mg) were extracted by a solution of methanol:water:formic acid (60:39:1) for the measurements. The extracts were centrifuged with 8000 g at 4ºC for 10 minutes. The supernatant was investigated at two pH values (pH 1.0 and pH 4.5) and at two different wavelengths (at 520 and 700 nm). The results were expressed in cyanidin-3-glucoside equivalent based on the following equation.

Absorbance(A) = $(A_{520nm}-A_{700nm})_{pH1.0}$ - $(A_{520nm}-A_{700nm})_{pH4.5}$

Total anthocyanin content (TA) (mg/L) = A× Mw × dilution × 1000) / (ε/1), where in the case of cyanidin-3-glucoside standard Mw = 449.2 g/mol and ε = 26900 L/(mol×cm), 1 = length the light travels in cm.

Total anthocyanin content TA (mg/g DW) = [TA (mg/liter)/100]/extract (g)

Statistical analysis

The results represent the mean values of at least 3 measurements and were statistically evaluated using the standard deviation and ANOVA methods.

RESULTS AND DISCUSSION

The biosynthesis of ethylene proceeds through the following steps: SAM→ACC→ethylene catalyzed by enzymes ACS and ACO. The polyamines are produced by transfering an aminopropyl group from decarboxylated SAM (synthetized by SAMDC) to putrescine by the enzyme SPDS and to spermidine by the enzyme SPMS. The common precursor of ethylene and polyamine metabolism is provided by the genes *SAMS*.

First, the genes *SAMS* are responsible for S-adenosyl-L-methionine (SAM) synthesis, which serves as the initial molecule in both pathways.

As we can see in Figure 2a, *FaSAMS1* shows its highest activity in the green and its lowest activity in the pink phase, while the expression level of *FaSAMS2* is the highest at the white stage, followed by decline in its transcription level. The relative expression level of *FaSAMS3* is high in the green fruit and later it gradually decreases. The expression level of SAM synthase decreases as the ripening process proceeds in tomato (Kok et al., 2008) although SAM provides the methyl group for O-, N- and C-methyltransferases participating in the production of secondary metabolites like anthocyanins, various aroma and flavor substances (Roje, 2006). The accumulation of these metabolites is common in both the climacteric and non-climacteric fruits as ripening proceeds. Strawberry fruits infiltrated with

FaSAMS1-RNAi (RNA interference – in this biological process RNA molecules inhibit gene expression or translation) construct remained white at the infiltration sites, but anthocyanin synthesis was induced by ethephon (ethylene analogue) in these fruits (Sun et al., 2013). SAM synthase is unlikely to be the rate-limiting enzyme in the ethylene-biosynthesis (Yang and Hoffman, 1984), because it was observed in carnation that the enhanced ethylene biosynthesis was not accompanied by increased *SAMS* mRNA synthesis (Woodson et al., 1992).

Decarboxylated SAM is synthetized by the product of the *SAMDC* genes. These enzymes detour the flux from ethylene to polyamines, therefore *SAMDC*s are the key elements in balancing between these two products. Competition between the two pathways arises only if the quantity of SAM is limited but polyamine and ethylene biosynthesis use only 10% of the SAM present in the cell (Ravanel et al., 1998) and SAM supply is rich in the cell (Bregoli et al., 2002). The expression level of *FaSAMDC1* increases gradually during the process of fruit ripening. *FaSAMDC2* has a low rate of activity in the green phase and it increases at the white stage followed by a slight decrease in the red fruit. High level of expression of *FaSAMDC3* is found in the green phase then its activity declines (Figure 2a)

During the ripening process of strawberry, the enzyme activity and expression level of SAMDC increased, which coincided with changes in physiological parameters such as anthocyanin, sugar, polyamine, auxin, abscisic acid and ethylene contents, indicating that SAMDC plays an important role in the ripening process of strawberry (Guo et al., 2018). Furthermore, ethylene-release curve in tomato showed high similarity to the expression patterns of genes *SlSAMDC*, with the amount of ethylene being at its highest at the beginning of the ripening at the orange stage (Van de Poel et al., 2012). The relative expression levels of *FaSAMDC1* and *FaSAMDC2* increased from the green to the red stages of fruit ripening, suggesting their major role in ripening process. The product of the gene *ADC* decarboxylates an arginine, initiating the metabolism of polyamines. The expression level of this gene is very high in the green phase then it continuously decreases (Figure 2a). Consequently, most of the decarboxylated arginine is produced at the beginning of ripening process and there is no need to produce more of it in the later phases. Previous studies also reported that the highest rates of transcription of the gene *ADC* were detected in the non-dividing elongating cells in peach (Ziosi et al., 2003).

The enzyme SPDS binds a decarboxylated SAM to putrescine, generating spermidine, which is a more complex form of polyamine. The expression level of *FaSPDS* in the green ripening stage is very high, followed by a decline in the white phase and then by a slight increase later on. Free polyamine levels are at their highest in the early phase of ripening, with their amount decreasing at later stages in tomato, peach, mango and grapevine (Saftner and Baldi, 1990, Bregoli et al., 2002; Malik and Singh, 2004; Agudelo-Romero et al., 2013), which, in our case, is well demonstrated by the relative expression curves of genes *FaADC* and *FaSPDS*.

The enzyme ACS converts the SAM to ACC, which is the direct precursor of ethylene, then ACO catalyzes the ACC→ethylene step. The transcription levels of *FaACS1*, *FaACS2*, *FaACS3*, *FaACO1*, *FaACO2*, *FaACO3* and *FaACO4* were found to be high in the green and pink fruits, the *FaACS4* transcription rate was almost zero in the green fruits and it was at its highest at the pink stage (Figure 2a). These findings are in accordance with our results showing that ethylene concentration is at its highest in the green strawberry, significantly declines when the fruit reaches the white stage, then it slightly rises till the red stage is reached (Perkins-Veazie et al., 1996; Sun et al., 2013). The expression rates of ACC synthase and ACC oxidase as well as the enzyme activity of ACC synthase are at their highest at the vérasion stage of the ripening process, followed by a decline at later stages of ripening in grapevine (Chervin et al., 2004; Terrier et al., 2005; Pilati et al., 2007).

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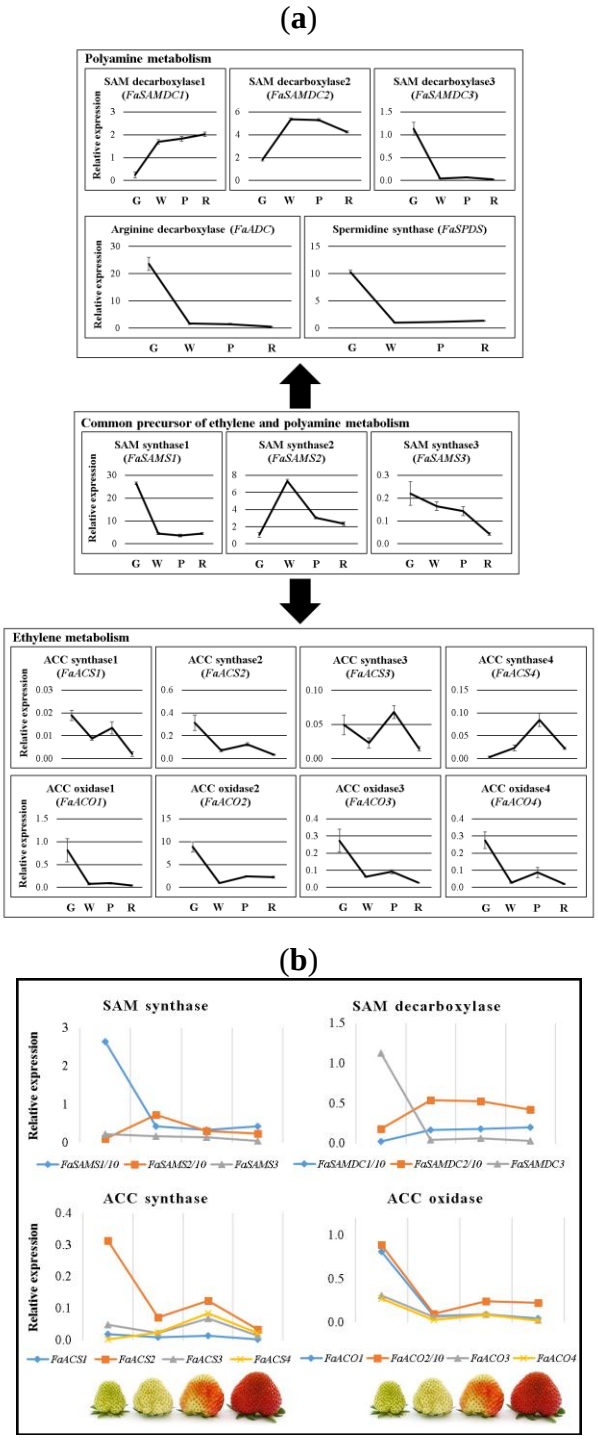

Fig. 2. Relative expression levels of genes involved in ethylene and polyamine metabolism in four different ripening stages of *Fragaria x ananassa* Duch. cv. 'Asia'. (G=Green stage; W=White stage; P=Pink stage; R=Red stage). Data are the means of four replicates (±SD). Error bars represent significant differences at P< 0.001 (a).
The relative expression of FaSAMS1-FaSAMS3, FaSAMDC1-FaSAMDC3, FaACS1-FaACS4 and FaACO1-FaACO4 genes merged in one diagram. The expressions FaSAMS1/10, FaSAMS2/10, FaSAMDC1/10, FaSAMDC2/10 and FaACO2/10 refer to the relative expression level values divided by ten (b).

Anthocyanins accumulate in significant amounts from the vérasion/pink ripening stage in grapes and strawberries (Halbwirth et al., 2006; Pilati et al., 2007; Griesser et al., 2008; Carbone et al., 2009). In accordance with these observations, the anthocyanins in our experiments also showed increased rates of accumulation from the pink ripening stage. (Figure 3). In an earlier study while investigating the relative expression levels of

genes involved in anthocyanin biosynthesis such as chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*FHT*), dihydroflavonol 4-reductase (*DFR*), anthocyanidin synthase (*ANS*) and flavonoid 3-glucosyltransferase (*F3GT*) in strawberry the largest amounts of mRNA transcribed from these genes were found in the pink ripening phase (Thill at al., 2012). Therefore, the moderate increase of *FaACS1-FaACS4* and *FaACO1-FaACO4* transcript levels found in the pink fruits may suggest that ethylene is needed both for the completion of the ripening process and for the accumulation of anthocyanins. Furthermore, the ethylene peak coincides with the increased accumulation of pigments during the fruit ripening process in tomato (Van de Poel, 2012).

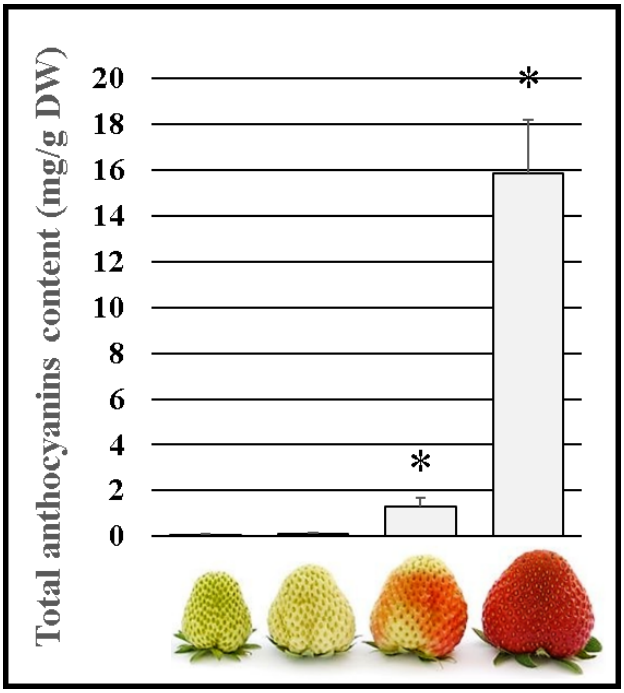

Fig. 3. The total amount of anthocyanin in the green, white, pink and red ripening phases in strawberry. Data are the means of three replicates (±SD). The asterisk represents significant differences at P< 0.001.

Table 2. shows the fold-changes of gene expression between two successive ripening stages. If we compare the transcription levels of the genes in the green fruits with those in the red ones an increase can be observed in genes *FaSAMS2*, *FaSAMDC1*, *FaSAMDC2* and *FaACS4*, while the relative expression levels of all the other genes like *FaSAMS1*, *FaSAMS3*, *FaSAMDC3*, *FaSPDS*, *FaACS1*, *FaACS2*, *FaACS3*, *FaACO1*, *FaACO2*, *FaACO3* and *FaACO4* declined.

Tab. 2.
The fold-change of relative expression levels between two successive ripening stages. (+number x up regulated; -number x down regulated; - the difference is less than 0.25); (W/G=White stage/Green stage; P/W=Pink stage/White stage; R/P=Red stage/Pink stage; R/G=Red stage/Green stage)

Gene name	W/G	P/W	R/P	R/G
SAM synthase1 (FaSAMS1)	-5.97	-1.45	+1.46	-5.92
SAM synthase2 (FaSAMS2)	+7.05	-2.41	-1.28	+2.26
SAM synthase3 (FaSAMS3)	-1.37	-	-3.50	-5.50
SAM decarboxylase1 (FaSAMDC1)	+7.12	-	-	+8.41
SAM decarboxylase2 (FaSAMDC2)	+2.91	-	-1.25	+2.30
SAM decarboxylase3 (FaSAMDC3)	-28.25	+1.75	-2.33	-37.66
Arginine decarboxylase (FaADC)	-14.10	-	-2.46	-40.60
Spermidine synthase (FaSPDS)	-10.20	-	-	-7.75
ACC synthase1 (FaACS1)	-2.00	-	-	-2.00
ACC synthase2 (FaACS2)	-4.42	+1,71	-4.00	-10.33

ACC synthase3 (FaACS3)	-2.50	+3.50	-7.00	-5.00
ACC synthase4 (FaACS4)	+2.00	+4.00	-4.00	+2.00
ACC oxidase1 (FaACO1)	-11.57	+1.28	-2.25	-20,25
ACC oxidase2 (FaACO2)	-9.25	+2.52	-1.07	-3.92
ACC oxidase3 (FaACO3)	-4.50	+1.50	-3.00	-9.00
ACC oxidase4 (FaACO4)	-9.00	+3.00	-4.50	-13.50

The relative expression curves of the strawberry ethylene and polyamine biosynthetic genes show partial similarity to the results of several earlier studies (Aharoni and O'Connell 2002; Agudelo-Romero et al., 2013; Sun et al., 2013) and it can be concluded that ethylene plays a fundamental role in the fruit coloration processes of non-climacteric strawberry as well, similarly to the climacteric fruits.

CONCLUSION

According to our results, the genes involved in both the ethylene and polyamines biosynthesis show varying levels of expression during the ripening process of strawberry fruit. The relative expression levels of *FaSAMS2*, *FaSAMDC1*, *FaSAMDC2* and *FaACS4* increased as ripening proceeded indicating that these genes might play an important role in the ripening process of strawberry. Although there is no ethylene peak during the ripening of non-climacteric fruits, ethylene can still play a vital role in the ripening of strawberry fruit since the rising expression levels in the pink stage of *ACC synthases* and *ACC oxidases* may indicate that ethylene – as a signal molecule – modulates the production of anthocyanins in the late ripening phases.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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