Regulation of Anthocyanin Biosynthesis during Fruit Development in ‘Nyoho’ Strawberry

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Summary

The expression of anthocyanin biosynthesis pathway genes was investigated in ‘Nyoho’ strawberry fruits during fruit development. Fruit color changed from pale – green to white about 3 weeks after anthesis (white – mature stage). At this stage, anthocyanins rapidly accumulated until the full ripe. Total soluble sugar content also increased after white – mature stage. This includes sucrose but not glucose and fructose levels that remained constant during ripening. The transcript level of phenylalanine ammonialyase (PAL) and chalcone synthase (CHS) genes did not change markedly throughout the fruit development. The transcript level of chalcone isomerase (CHI) and dihydroflavonol 4-reductase (DFR) gene was high in the young fruit, decreased to an almost undetectable level at the white – mature stage, and then increased again until the fully ripe stage paralleling mostly the accumulation of anthocyanin. The latter corretion suggests the involvement of CHI and DFR in the regulation of anthocyanin biosynthesis during fruit ripening.

Key Words: anthocyanin, chalcone synthase, chalcone isomerase, dihydroflavonol 4-reductase, phenylalanine ammonialyase, strawberry.

Introduction

Fruit pigmentation is one of the significant aspects of fruit quality. In strawberry fruit, the accumulation of anthocyanins, the principal pigment, is developmentally regulated. Young fruit contains chlorophyll and phenolic compounds but undetectable levels of anthocyanins (Cheng et al., 1991; Montero et al., 1996). Chlorophyll, the predominant pigment in immature fruit, disappears at 3 weeks after anthesis (WAA) resulting in a white fruit surface, then anthocyanin accumulates until full maturity (Cheng et al., 1991; Montero et al., 1996).

Anthocyanin biosynthetic pathway involves several enzymes such as PAL (phenylalanine ammonialyase), CHS (chalcone synthase), CHI (chalcone isomerase), F3H (flavanone 3-hydroxylase), DFR (dihydroflavonol 4-reductase), LDOX (leucocyanidin dioxygenase), and UFGT (UDP Glc-flavonoid 3-O-glucosyl transferase). The genes encoding the above enzymes have been isolated and their developmental and tissue specific expression has been investigated in other plants (Holton and Cornish, 1995; Koes et al., 1994). In most cases, several of these genes are coordinately regulated. In grape berries, structural genes for PAL, CHS, CHI, F3H, DFR, LDOX, and UFGT were coordinately expressed during berry pigmentation. In Antirrhinum flowers genes encoding enzymes downstream from CHS in the pathway were expressed during flower color development (Jackson et al., 1992), whereas in lisianthus flowers genes downstream from DFR were expressed (Davies et al., 1993). DFR gene has been isolated from strawberry fruit and it was found to be coordinately expressed during fruit pigmentation (Moyano et al., 1998).

The purpose of this study was to examine the biochemical basis of anthocyanin accumulation and regulation in ‘Nyoho’ strawberry. In most strawberry cultivars, light conditions around the fruit surface affect fruit pigmentation (Urata et al., 1991; Miura et al., 1993). This increases labor costs, as fruit need to be manipulated in order to make them uniformly colored during commercial production. In contrast ‘Nyoho’ produces uniformly colored fruits without any consideration of incident sunlight or shading (Fushihara, 1995; Mochizuki, 1995). It was therefore of interest to examine the light independent characteristic of pigmentation in this cultivar. In this paper we analyzed the expression of the genes for PAL, CHS, CHI, and DFR involved in pigmentation in ‘Nyoho’ strawberries during fruit development.

Materials and Methods

Plant material

Strawberry plants (Fragaria × ananassa ‘Nyoho’) were transplanted in a mixture of Engei– Baido (Kureha) and Soil Mix (Sakata) (2:1 v/v) in 15cm pots and grown in a
greenhouse maintained between 17°C and 28°C with natural light condition. Experiments were carried out in February and March 1998. Samples were collected at 6 different developmental stages (Fig. 1): one WAA (stage 1); two WAA (Stage 2); about 3 WAA, when fruit surface became white with green achenes (stage 3); 2 to 3 days after stage 3, when pigmentation started from the distal end of the fruits (Stage 4); 4 to 6 days after stage 3, when distal half of a fruit was pigmented (Stage 5); and 7 to 10 days after stage 3, when whole the fruit was pigmented (Stage 6). Each sample consisted of a pair of the first lateral fruits from a terminal inflorescence: one fruit of the pair was used for anthocyanin analysis and RNA extraction; the other was used for sugar analysis.

**Analysis of anthocyanins and sugars**

One fruit of each pair was divided into 8 sections through the central axis. A pair of sections at opposite positions were soaked in 1% HCl–methanol at 4°C for 12 to 24 hrs. The rest of the sections were used for RNA extraction. Anthocyanin content was calculated from the absorbance at 510 nm. The other fruit of the pair was freeze-dried and analyzed of soluble sugars using HPLC as described previously (Kawabata et al., 1999).

**Dot blot analysis of PAL, CHS, CHI, and DFR transcripts**

Fresh fruit tissue was frozen in liquid nitrogen and ground into fine powder in a mortar and pestle. Total RNA was extracted according to Manning (1991). Six total RNA samples were pooled to make a mixture representing each developmental stage. Five μg of total RNA for each stage was blotted onto nylon membrane (MagnaGraph, Funakoshi) and hybridized with cDNA probes for PAL (unpublished), CHS, CHI, and DFR (Kawabata et al., 1999). PAL cDNA clone was isolated from lisianthus petals as described previously for the other probes (Kawabata et al. 1999), except that specific primers (Forward: 5’-aaywsgnnaagayaaycc, Reverse: 5’-garcartnsgnccatgc; y: c, t; w: a, t; s: g, c; r: a, g; n: g, a, t, c) were used to amplify PAL cDNA fragments. The sequence of the amplified cDNA fragment has 76% homology with *Arabidopsis* PAL cDNA (Wanner et al., 1995). The probes were labeled with digoxigenin using DIG PCR Labeling Kit (Boehringer Mannheim). Signals were detected colorimetrically using DIG Nucleic Acid
Detection Kit (Boehringer Mannheim). Hybridization and signal detection were performed according to the manufacturer's instruction.

Results and Discussion

Anthocyanin content

Anthocyanin content (Fig. 2) was very low from stage 1 to 3. Although slight absorbance was observed at stage 1, the spectrum did not show a peak near 510nm, hence the absorbance was probably due to impurities such as chlorophyll rather than anthocyanins. Alternatively, a small amount of leucoanthocyanidins was possibly converted to anthocyanidins during the HCl extraction. Anthocyanins began to accumulate at stage 4 and increased rapidly thereafter, reaching a maximum at stage 6.

Sugar content

Sucrose was the main sugar in mature 'Nyoho' strawberry fruits (Fig. 3). Sucrose content increased continuously during fruit development, however, glucose and fructose contents remained almost constant from stages 3 to 6. Changes in sugar composition and sucrose accumulation coordinated with anthocyanin accumulation.

Gene expressions

The four genes analyzed did not show a similar pattern of expression (Fig. 4). Transcript level of PAL and CHS did not change markedly during fruit development. It has been reported that enzymatic activity of PAL has a peak during fruit ripening, which coincides with the rapid accumulation of anthocyanin (Given et al., 1988; Cheng et al., 1991). In that particular study, the activity was expressed on a protein basis; however the activity when expressed on a fresh weight basis remained constant throughout the ripening process (Cheng et al., 1991). Furthermore, PAL activity was found to be too high considering the production rate of anthocyanins in strawberry fruits (Cheng et al., 1991). Therefore, PAL is unlikely to play an important role in the regulation of anthocyanin synthesis during these stages of fruit development.

The expression of CHI and DFR genes showed a peak at stage 2, but decreased markedly at stage 3, and then increased consistently until stage 5. The latter increase coincided with the accumulation of anthocyanin. At stage 6 the expression of these genes decreased slightly, when anthocyanin ceased to accumulate. Coordinate expression of DFR with pigmentation has been found in 'Chandler' strawberry (Moyano et al., 1998). Our results show that CHI gene is another member of the pathway that is expressed during anthocyanin biosynthesis.

During the early stages of fruit development,

![Graph showing anthocyanin content with stages 1 to 6](image)

![Graph showing sugar content with stages 1 to 6](image)

![Graph showing gene expressions with stages 1 to 6](image)
transcript level of CHI and DFR genes was high although no anthocyanin accumulation was observed. This has also been reported for DFR (Moyano et al., 1998). The expression of CHS gene also showed a small peak at stage 2. It should be noted that PAL, CHS, CHI, and DFR are involved in not only the biosynthesis of anthocyanins but also those of other phenolic compounds, which are known to accumulate during early stage of development (Cheng et al., 1991; Montero et al., 1996). High level of the transcripts may be related to phenolic biosynthesis in the young fruit. During these stages, anthocyanin biosynthesis may be blocked at later steps of the pathway such as at the UFGT step as was proposed in grape berries (Boss et al., 1996).

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イチゴ‘女峰’の果実発達に伴うアントシアニン生成とその制御

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摘 要

イチゴ‘女峰’における果実の発達にともなうアントシアニン合成経路遺伝子の発現を調べた。果実の色は開花3週間後に薄い緑色から白色に変わり、その後成熟期までアントシアニンの蓄積は続けた。また、この時期から全糖含量が増加を始めた。スクロース含量が成熟期まで上昇し続けたのに対し、グルコースとフルクトース含量は成熟に伴ってほとんど変化しなかった。フェニルアラニンアミノアラーゼ(PAL)とカルコンシンターゼ(CHS)遺伝子の発現は果実の発育を通じて大きな変化を示さなかった。カルコンイソメラーゼ(CHI)とジヒドロフラボノール4-還元酵素(DFR)遺伝子の発現は、若い果実では高く、白熟期に著しく低下したのち着色伴って再び増加した。2回目のCHIとDFRの発現の上昇はこれらの酵素の発現が成熟期のアントシアニンの合成調節に関与することを示唆した。

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