Notes

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Isolation and Characterization of a cDNA for Chalcone Synthase from Cultured Cells of the Strawberry *Fragaria ananassa*, cv Shikinari

Miei SAKURAI*, Yoshihiro OZEKI** and Tsukasa MORI***

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CHS (chalcone synthase, EC 2.3.1.74) is the first step enzyme involved in the flavonoid biosynthetic pathway. CHS cDNA was first obtained from cultured parsley cells [1], subsequently cDNAs of CHS have been obtained from various plant species. Furthermore, the mechanism of CHS gene expression regulated by cis-elements and transacting factors has been thoroughly examined in response to stresses, such as ultraviolet illumination [2] and elicitor [3].

Synthesized anthocyanin in strawberry fruit, has allowed the identification of the primary pigment, pelargonidin-3-glucoside (88%), and the secondary pigment, cyanidin-3-glucoside (12 %) [4]. It has been reported that the mechanism of ripening for strawberry fruit is not related to increased ethylene but a decline in the production of auxin in the achenes as the fruit matures [5].

We prepared a suspension culture of *F. ananassa*, cv Shikinari. The suspension-cultured cells were subcultured under the condition of darkness without anthocyanin accumulation. When the cells were illuminated, large quantities of anthocyanin rapidly accumulated [6]. In contrast with strawberry fruit ripening, auxin did not affect the anthocyanin accumulation in the suspension-cultured cells [7]. Furthermore, the anthocyanin components in cultured cells are different from those in strawberry fruit. The primary anthocyanin accumulated in cultured cells was peonidin-3-glucoside and the secondary pigment was cyanidine-3-glucoside [6].

The discrepancy between fruit and suspension-cultured cells suggests that the regulatory mechanisms for the expression of enzymes involved in anthocyanin biosynthetic pathway might be different under differentiated (*e. g.*, fruit ripening) and undifferentiated (*e. g.*, suspension-cultured cells) states. Recently, a partial CHS cDNA was identified in the ripening fruit of *F. ananassa*, cv Pajaro, using polymerase chain reaction differential display [8]. The first step in studying the regulatory mechanisms of anthocyanin biosynthesis in *F. ananassa* should be to obtain full-length CHS cDNA and examine the expression pattern during anthocyanin synthesis.

Suspended cell cultures of the strawberry *F. ananassa*, cv Shikinari, were established in 1993 [6] and subcultured in 100 m*l* of liquid Linsmaier and Skoog (LS) [9] medium supplemented with 3 % sucrose, 2,4-D (1 mg/*l*) and BA (0.1 mg/*l*) in 500 m*l* flasks. Cells were incubated on a gyratory shaker (80 rpm) under 800 lux (white fluorescent light: FL40SSW/37, Toshiba) at 25°C [6]. The cells were subcultured every week. Under these conditions, little anthocyanin accumulated. To induce anthocyanin production, seven-day-old subcultured cells (2 g of fresh weight) were transferred to fresh LS medium and cultured in darkness for four days, and thereafter cultured under 8,000 lux to produce anthocyanin. Cells were harvested at 0, 6, 12, 24, 36, 48, 60, 72, 84 and 102 hr after light illumination (8,000 lux).

For the preparation of cDNA libraries, total mRNA was extracted from 100 g fresh weight of cultured strawberry cells accumulating anthocyanin in an LS medium under 8,000 lux for one week using the SDS-phenol method. Total RNA was dissolved in TE-HPRI (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, and five units ml^{-1} ribonuclease inhibitor from human placenta) and layered on a 5.7 M CsCl cushion and precipitated by centrifugation at 110,000×g for 20 hr. Poly(A)$^+$ RNA was isolated from total RNA using oligo-d(T) latex (Roche, Tokyo) as described in the manual from the supplier. A cDNA library was constructed in λgt22A using the Super Script Lambda System for cDNA synthesis and cloning (BRL). The phage clones containing cDNAs for CHS mRNA were screened using a CHS cDNA probe derived from carrot [10]. A cDNA insert was digested separately with restriction enzymes, *Not*I and *Sal*I. The resulting DNA fragments were ligated into pSPORT I and transferred to competent cells (*E. coli* JM109). The DNA sequences were determined using an automated DNA sequencer (Applied Biosystems).

Total RNA was extracted from 2 g fresh weight of cells and fractionated by electrophoresis in a 1.2 % formaldehyde-agarose gel, and then blotted onto a nylon membrane (HybondN$^+$, Amersham Japan). Hybridization was performed overnight at 65°C in 5×

* *Environmental Process Development Department, Research Institute, Ishikawajima Harima Heavy Industries, Co., Ltd., 1 Shinnakahara, Isogo-ku, Yokohama 235, Japan*
** *Department of Biotechnology, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184, Japan*
*** *Faculty of Fisheries, Hokkaido University, 3-1-1 Minato, Hakodate 041, Japan*

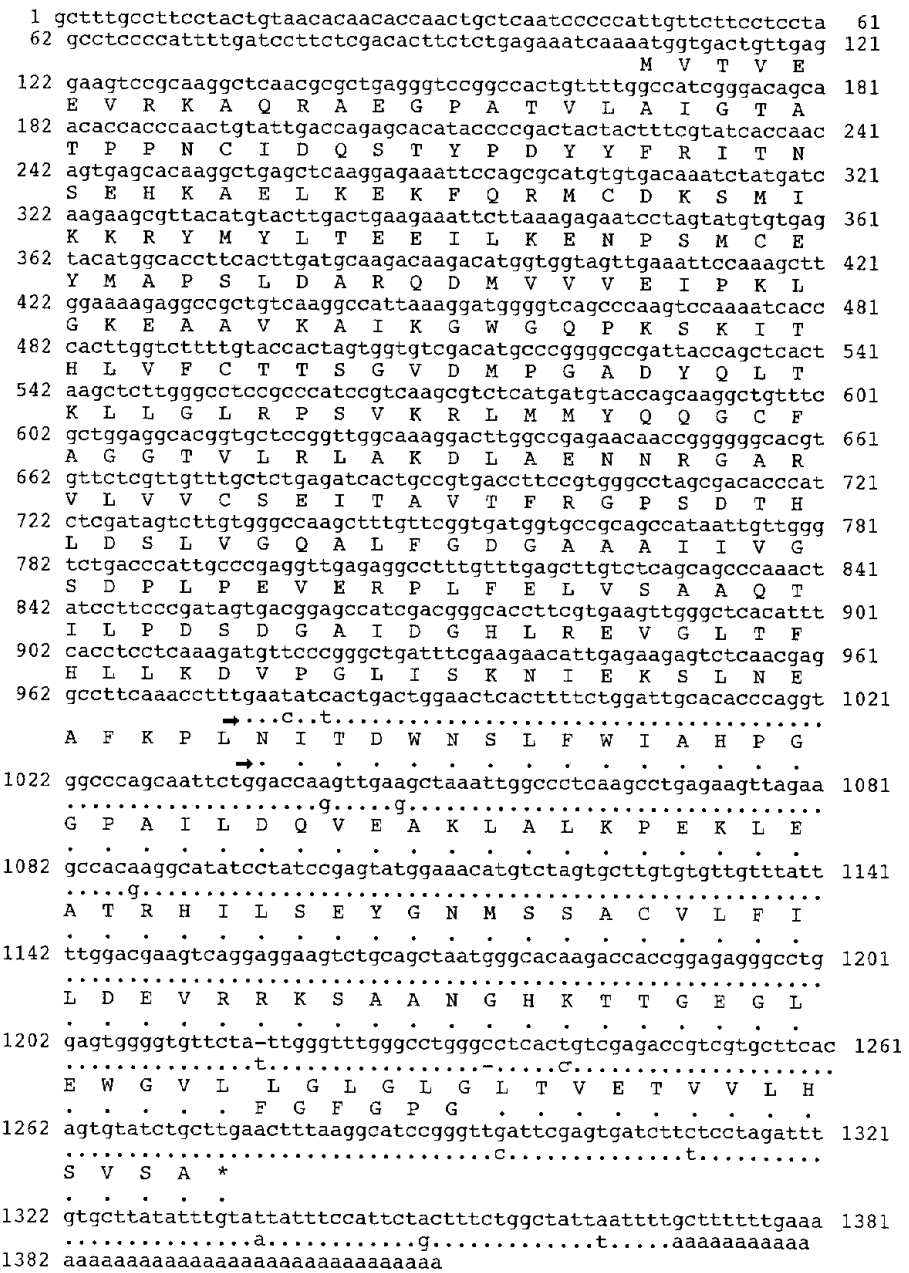

Fig. 1 The nucleotide and deduced amino acid sequences of CHS cDNA obtained from cultured strawberry cells.
The nucleotide and deduced amino acid sequences of CHS cDNA from cultured strawberry cells are shown by small letters and capital letters, respectively. Nucleotide and deduced amino acid sequences of a partial fragment of CHS cDNA from strawberry fruit reported by Wilkinson *et al.* (DDBJ/EMBL/Gen-Bank Database under the accession number U19942) are shown on the lane started by arrows. Same nucleotides and amino acids are shown by dots. The sequence data will appear in the DDBJ/EMBL/Gen-Bank Databases under the accession number AB003394.

SSC, 10×Denhard's solution, 10 mM of sodium phosphate (pH 6.5), 0.5 % SDS, 50% formamide, 100 μg ml^{-1} sonicated salmon sperm DNA and an RNA probe of antisense CHS, which was prepared from the cDNA clone obtained here using a DIG luminescent detection kit (Boehringer Mannheim). Washes were carried out using 0.2×SSC, 0.1 % SDS at 65°C. The membrane was exposed to X-ray film (FUJI-RX).

A cDNA library (2×10^5 independent plaques) was constructed from mRNA of strawberry cells. More than 50 clones with positive signals was screened using a carrot CHS probe, and 7 clones were purified. One clone having 1410 bp length was sequenced. **Figure 1** shows the complete nucleotide sequence of the cDNA. The clone contained a 1170-bp open reading frame that encoded a 390-amino acid polypeptide with a molecular weight of 42,488 daltons.

The nucleotide sequence of a partial fragment of CHS cDNA (394 bp) obtained from ripening strawberry fruit [8] has 96.7 % similarity to that of cultured cells (**Fig. 1**). Three amino acids were different because of frameshift (**Fig. 1**). This

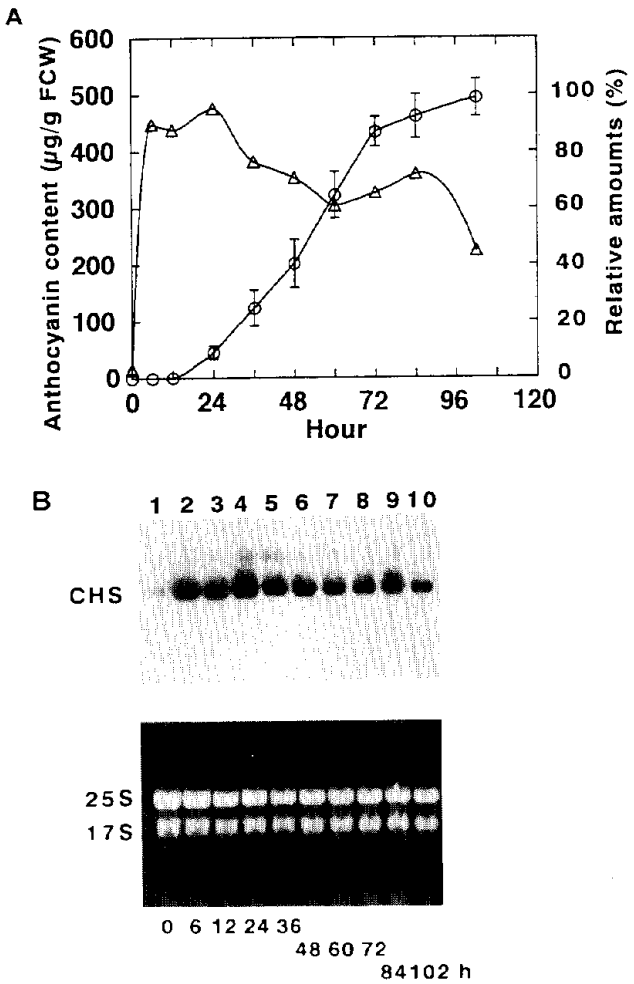

Fig. 2 (A) Changes of anthocyanin content (○) and relative amounts (%) of CHS mRNA (△) under a light condition.

Seven-day-old subcultured cells (2 g of fresh weight) were transferred to fresh LS medium and cultured under darkness for four days, and thereafter cultured under 8,000 lux. Cells were harvested at 0, 6, 12, 24, 36, 48, 60, 72, 84 and 102 hr after light illumination (8,000 lux), anthocyanin content was calculated (μg/g fresh cell weight) and mRNA was extracted.

(B) Northern blot analysis on CHS mRNA accumulation under 8,000 lux.

Ten μg RNA was loaded onto the gel and hybridization was carried out using a full length cDNA obtained in this work as a probe. Culturing time after light irradiation; lane 1: 0 hr, lane 2: 6 hr, lane 3: 12 hr, lane 4: 24 hr, lane 5: 36 hr, lane 6: 48 hr, lane 7: 60 hr, lane 8: 72 hr, lane 9: 84 hr, lane 10: 102 hr. 25S and 17S ribosomal RNAs were used as internal controls of RNA contents (bottom).

difference is thought to be caused by the difference of cultivar between Pajaro and Shikinari.

To examine the relationship between anthocyanin synthesis and the CHS gene expression of cells cultured under light, the anthocyanin content was monitored (**Fig. 2-A**) and northern hybridization was performed (**Fig. 2-B**).

When the cells were cultured under the condition of darkness, no anthocyanin accumulated. However, anthocyanin synthesis began in the cells 12 hr after transfer from darkness to a light condition of 8,000 lux (**Fig. 2-A**). Strong expression of the CHS gene was first observed in the cultured cells after 6 hr cultivation under 8,000 lux, and the high level expression was kept throughout the cultivation period until 96 hr (**Fig. 2-B**). The similar result was reported by Takeda *et al.* [11]. They investigated changes in CHS mRNA using carrot suspension cells induced by light. They suggested that anthocyanin synthesis was induced by the activation of the CHS gene and that CHS mRNA remained at a high level during anthocyanin synthesis under light illumination. However, the expression of the CHS gene was gradually stagnated after 24 hr (**Fig. 2-A**). This result was consistent with the anthocyanin content obtained after 72 hr, because it showed an equilibrium state.

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