Short Communication

One Type of Chalcone Synthase Gene Expressed during Embryogenesis Regulates the Flavonoid Accumulation in Citrus Cell Cultures

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To elucidate the relationship between the expression of chalcone synthase (CHS) genes and the production of flavonoid in citrus cell cultures, two cDNA clones encoding CHS were isolated (CitCHS1 and CitCHS2) from the citrus. The accumulation of CitCHS2 mRNA was notably induced by embryogenesis but CitCHS1 mRNA was not. There was no detectable accumulation of flavonoid in the undifferentiated calli, but flavonoid accumulated after the morphological changes to embryos. These results indicate that two CHS genes differentially expressed during citrus somatic embryogenesis and CitCHS2 may regulate the accumulation of flavonoid in citrus cell cultures.

Key words: Cell culture — Chalcone synthase — *Citrus* — Flavonoid biosynthesis — Somatic embryogenesis.

Substantial progress has been made in elucidating the biosynthesis of plant phenolics, one major class of which is the flavonoids. Flavonoids are involved in many plant functions such as UV protection, defense against pathogen attack, legume nodulation, pigmentation and pollen viability (Firmin et al. 1986, Hahlbrock and Scheel 1989, Dixon and Harrison 1990). Due to such important functions, studies on the flavonoids have been extensively carried out and the genes involved in this pathway such as chalcone synthase (CHS) and chalcone isomerase (CHI) have been isolated (e.g. Hahlbrock 1981, Hahlbrock and Scheel 1989). Special attention is given to the regulation of CHS because of a key enzyme in the biosynthesis of all classes of flavonoids in plants. This enzyme catalyzes the condensation of 4-coumaroyl-CoA with three molecules of malonyl-CoA in order to form naringenin-chalcone (Kreuzaler and Hahlbrock 1975). It has been known that stress including UV light (Kreuzaler et al. 1983), elicitors (Junghans et al. 1993) and wounding (Junghans et al. 1993) induces the expression of CHS genes. Moreover, in some cases there are more than one CHS gene in the haploid genome (Ryder et al. 1987).

Citrus species are much of interest because they accumulate large amount of flavonoids, especially flavanone glycoside (Horowitz 1964). Some flavanone glycosides such as flavanone neohesperidosides, which accumulate in citrus species related to pummelo, such as grapefruit, sour orange and natsudaidai, lead to a bitter taste in the citrus fruits (Horowitz 1964). In addition to such bitter property of citrus flavonoids, Middleton and Kandaswami (1994) reported that some flavonoids from citrus have potential health-promoting properties including antiviral, anticancer and anticarcinogenic. Bracke et al. (1994) showed that the citrus tangeretin, which is one of the highly methylated flavones, inhibited the processes that shorten the life expectancy of tumor-bearing patients. Thus, citrus flavonoids are important agriculturally as well as pharmacologically.

Our interest lies in identifying the genes expressed with the induction of somatic embryogenesis and finally the regulation of flavonoid production in citrus cell cultures. It has been generally reported that grapefruit (*Citrus paradisi* Macf.) suspension cell (Lewinsohn et al. 1986) and undifferentiated calli of grapefruit and orange (*C. sinensis* Osbeck) (Barthe et al. 1987) lose or reduce the ability to produce flavonoids with one exception in lime (*C. aurantifolia* [Christm.] Swing.) cultures (Berhow et al. 1994). In the relationship between enzyme activities and anthocyanin production in suspension cells, Glietz and Seitz (1989) reported on two CHS forms with different molecular subunit weights and different isoelectric points from carrot suspension cultures, and showed the differential accumulation of these proteins in the anthocyanin synthesis. Ozeki et al. (1990) showed that CHS mRNA in carrot cells was in-
duced when the anthocyanin synthesis was initiated. In contrast, the amount of enzyme protein and mRNA for CHS was below the detectable levels, when anthocyanin synthesis was repressed (Ozeki et al. 1990). These reports imply that the production of anthocyanin in carrot cultured cells is regulated by CHS genes. This may also be applied to citrus, that is, low levels of flavonoid in citrus cell cultures are partially due to the lack of CHS protein. There had been no available information, however, based on the molecular aspect which occurred during citrus somatic embryogenesis. In this study, we report the relationship between the expression of CHS genes and the flavonoid accumulation in citrus cell cultures.

To isolate cDNA clones for CHS, a cDNA library derived from Valencia orange (C. sinensis Osbeck) seeds, which was kindly supplied by Dr. Tetsushi Hidaka, was used for this purpose. The cDNA library was constructed using the Lambda ZAP XR directional vector cDNA synthesis kit (Stratagene, La Jolla, CA). An expressed sequence tag (EST) clone (accession no. C81661) that had high homology to CHS was used as a probe to isolate cDNAs with complete coding regions from the Valencia orange small seed cDNA library. EST clones, which were ligated into EcoRI/XhoI sites of pBluescript (Stratagene), were digested, and the fragment (approximately 200 bp) was recovered from an agarose gel using a NucleoTrap (Macherey-Nagel, Germany). The fragment was labeled using Dig-11-dUTP and a random primer DNA labeling kit (Boehringer Mannheim, Germany), and used as a probe for the isolation of CHS containing complete coding regions. Using an EST for the putative CHS gene as a probe, 14 positive clones were obtained. Among them, two clones with longer inserts were chosen for further analysis. These clones had a high degree of similarity to the sequences of two CHSs from tea (Camellia sinensis) (Takeuchi et al. 1994), namely, 66.8% to 69.4% identity at the nucleotide level, and 86.7% to 90.8% identity at the amino acid level. There were no other gene candidates with such high homology scores, including closely related genes such as stilbene synthase and acridone synthase. Therefore, two clones (CitCHS1 and CitCHS2) were identified as cDNA clones encoding CHS from citrus. To estimate the copy number of CHS clones in the citrus genome, Southern blot hybridization was carried out. Genomic DNA was isolated from mature Valencia orange leaves according to Del-laporta et al. (1983). Total DNA (10 μg) was digested with DraI, EcoRI or XhoI, electrophoresed on 1.0% agarose gels, and blotted onto nylon membranes (Hybond N, Amersham, U.K.). For the probes, the entire EcoRI/XhoI inserts of two CHS clones were used. The membranes were hybridized with CHS insert DNAs labeled using Dig-11-dUTP and a random primer DNA labeling kit (Boehringer Mannheim). Hybridization was carried out at 67°C, the membranes were washed twice for 15 min with 0.1× SSC and 0.1% SDS at 67°C, and then exposed to X-ray film (Hyperfilm, Amersham). Southern blot analysis indicated that only one strong signal with a faint band was revealed by the CitCHS1 as a probe, whereas a few clear bands with some additional weak signals were detected in the case of CitCHS2 (Fig. 1). This result suggested that CitCHS1 was present in single or a few copies, but some sequences related to CitCHS2 were present in the citrus genome. Furthermore, since hybridization patterns of CitCHS1 and

### Table 1 Flavonoid contents in the undifferentiated calli, greenish embryos and leaf

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>Undifferentiated calli</th>
<th>Greenish embryos</th>
<th>Leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg per gram dry weight)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neodiosmin</td>
<td>ND*</td>
<td>19.3±0.4</td>
<td>ND</td>
</tr>
<tr>
<td>Taxifolin</td>
<td>ND</td>
<td>3.2±0.8</td>
<td>ND</td>
</tr>
<tr>
<td>Diosmin</td>
<td>ND</td>
<td>ND</td>
<td>10.1±0.1</td>
</tr>
<tr>
<td>Isoberhoidin</td>
<td>ND</td>
<td>ND</td>
<td>7.8±0.1</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>ND</td>
<td>1.4±0.3</td>
<td>62.0±0.3</td>
</tr>
<tr>
<td>Total flavonoid</td>
<td>ND</td>
<td>30.4±2.3</td>
<td>103.2±1.3</td>
</tr>
</tbody>
</table>

* ND: Not detected.
CitCHS2 were different from each other, these cDNA clones originated from different loci.

For extraction and analysis of flavonoids, the method by Kanes et al. (1993) was applied with a slight modification. Lyophilized and grounded sample tissues (100 mg) were placed in a 1.5 ml plastic centrifuge tube and extracted with 1 ml of a 1:1 (v/v) mixture of dimethylsulfoxide (DMSO) and methanol per sample tube. The supernatant was collected after 3 min centrifugation, and passed through a 0.2 μm filter into the sampling bottle. After diluting with distilled water, an aliquot was injected on HPLC column. The chromatographic system consisted of Hewlett-Packard model 1100 pumps, automatic sampler, a Hypersil ODS reverse phase column (Hewlett-Packard, 125 × 4 mm) and a UV diode array detector. The gradient elution schedule consisted of an initial 2 min of 10 mM H₃PO₄ and 20% methanol followed by a linear gradient of 100% methanol in 55 min at a flow rate of 1 ml min⁻¹. The detector was set to measure spectra from 220 to 400 nm and monitor the eluent at 285 nm. Known standards of nine flavones: eriocitrin, neoeiocitrin, narirutin, naringin, hesperidin, neohesperidin, naringenin, poncirin and neoponcirin; those of ten flavones: isorhoifolin, rhoifolin, diosmin, neodiosmin, luteolin, apigenin, sinensetin, acacetin, nobiletin and tangeretin; and those of four flavonols: rutin, kaempferol, 3,3',4',5,6,7,8-heptamethoxyflavone and natsudaidain were used. Flavonoid contents in the undifferentiated calli, greenish embryos and leaf are shown in Table 1. Flavonoids were below the limits of detection in the undifferentiated citrus calli, and flavonoids were detected in greenish embryos. The detectable flavonoids were different depending on the tissues. The major flavonoids detected in the greenish embryos and in the leaf were neodiosmin and hesperidin, respectively. These results indicated the changes in flavonoid metabolic pathway during the morphogenesis.

Expression of CHS clones was analyzed during citrus somatic embryogenesis. Embryogenic calli of Valencia orange were maintained on callus growth medium of MS (Murashige and Skoog 1962) including 0.2 M sucrose and 5 × 10⁻⁵ M kinetin in a glass tube. Approximately 1-month-old calli on the above medium were then transferred to hormone-free MS medium supplemented with 0.1 M galactose and 0.1 M sorbitol (Gal-Sor) medium for inducing somatic embryogenesis (Hidaka and Omura 1989). Calli were transferred onto fresh Gal-Sor medium at 1-month-intervals and harvested at each transfer for total RNA extraction. For detailed expression analysis, calli

![Fig. 2 Northern blot analysis of CitCHS1 and CitCHS2 during citrus somatic embryogenesis. Stage 1, undifferentiated calli; stage 2, pale yellowish calli with globular shape; stage 3–4, greenish embryos; stage 5, small green plantlets. Bars = 5 mm.](image)
were harvested 3, 5 and 11 d after transferring onto fresh Gal-Sor medium. Total RNA was isolated from each callus as described by Ikoma et al. (1996). Total RNA (10 μg) was electrophoresed on 1.0% agarose gels containing formaldehyde and blotted onto nylon membranes (Hybond-N, Amersham). The loading of equal amounts of RNA was checked by gel staining with ethidium bromide. The hybridization was performed at 42°C, the membranes were washed twice for 15 min with 0.1 × SSC and 0.1% SDS at 67°C, and then exposed to X-ray film (Hyperfilm, Amersham). Both transcripts corresponding to CitCHS1 and CitCHS2 mRNAs were hardly detectable in the undifferentiated calli grown on MS medium containing 0.2 M sucrose and 5 × 10⁻⁵ M kinetin (Fig. 2). When the calli were transferred onto MS medium containing Gal-Sor medium for induction of somatic embryogenesis (Hidaka and Omura 1989), the accumulation of transcript corresponding to CitCHS2 mRNA was induced at high level one month after transfer and continued up to the formation of small green plantlets. In contrast to CitCHS2, the transcript corresponding to CitCHS1 mRNA was hardly accumulated in either proliferation or the somatic embryogenesis process. When the northern blot analyses were carried out using CHI, flavanone 3-hydroxylase (F3H) and flavonol synthase (FLS) cDNAs, which exist in the down-stream of flavonoid biosynthetic pathway, the mRNA accumulation for CHI was not detectable at stage 1 like CitCHS2, but for F3H and FLS, it was detectable even in the undifferentiated calli at stage 1 (unpublished data). This indicates that CitCHS2 may be a primary key enzyme of flavonoid biosynthetic pathway for the flavonoid accumulation in citrus cell cultures. Northern blot analysis using mRNAs at 3, 5 and 11 d after transferring the calli onto Gal-Sor medium showed that the transcript for CitCHS2 was detected even in the sample incubated for 3 d, indicating that the induction of accumulation of CitCHS2 mRNA occurred so quickly (Fig. 3). The transcripts for CitCHS1 mRNA were hardly detectable at any days (data not shown) as shown in Fig. 2. Thus, the genes of CitCHS1 and CitCHS2 were differentially regulated during citrus somatic embryogenesis and the expression of CitCHS2 correlated with flavonoid accumulation.

There have been a few reports dealing with the relationship between CHS and flavonoid biosynthesis using cell cultures. In carrot cell suspension cultures, two different forms of CHS with different molecular subunit weights and different isoelectric points were identified, where these two CHS forms were differentially induced depending on the anthocyanin accumulation (Gleitz and Seitz 1989). In the present study, expression of CitCHS1 and CitCHS2 in citrus was also regulated differently during the embryogenesis, which led to the flavonoid accumulation. This is the first evidence of these two different CHS genes differentially expressed in citrus cell cultures. In addition, Ozeki et al. (1990) reported another carrot suspension cultures in a medium lacking 2,4-dichlorophenoxyacetic acid (2,4-D), in which embryogenesis was induced at high cell density and anthocyanin synthesis at low density. They showed that CHS mRNA in cells was induced 5 d after transfer to medium lacking 2,4-D, when the anthocyanin synthesis was induced (Ozeki et al. 1990). The induction of CitCHS2 mRNA by the condition for embryogenesis was, thus, faster than that of CHS in carrot suspension cultures and preceded the apparent morphological differentiation of the callus. It has been reported that the expression of CHS genes is regulated through the response to sugars (Takeuchi et al. 1994), and sucrose is known to induce CHS expression (Tsukaya et al. 1991). However, sucrose in the callus growth medium did not induce the expression of either CitCHS1 or CitCHS2 in the citrus cell cultures as shown at stage 1 of Fig. 2. Moreover, the production of flavonoids is closely related to the citrus embryogenesis in this study, because it has been reported that grapefruit suspension cell (Lewinsohn et al. 1986) and undifferentiated calli of grapefruit and orange (Barthe et al. 1987) lose or reduce the ability to produce flavonoids. The repressed flavonoid biosynthesis was recovered by the induction of somatic embryogenesis as indicated by Galewsky and Nessler (1986) and Ozeki and Komamine (1981). Our results, that flavonoids were below the limits of detection in the undifferentiated citrus calli and flavonoid accumulation started in the greenish embryos, coincided with their reports. Thorpe et al. (1971) reported that phenylalanine ammonia-lyase (PAL) activity was detected in grapefruit callus cultures. They also showed that there was no detectable formation of naringenin and its glycosides unlike the intact grapefruit, although the callus possessed the ability to convert...
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phenylalanine to cinnamic acid (Thorpe et al. 1971). These results indicate that CitCHS2 rather than PAL may play an important role on the switching of flavonoid synthesis in citrus cell cultures.

In conclusion, we have shown the differential expression of CHS genes and the close correlation between flavonoid biosynthesis and expression of CitCHS2 in citrus cell cultures. The manipulation of CHS genes would provide us with effective synthesis of flavonoids in citrus cell cultures. Work on the expression analyses of genes existing in the phenylpropanoid pathway between PAL and CHS such as cinnamate 4-hydroxylase and 4-coumarate:CoA ligase is necessary for the elucidation of detailed regulation of flavonoid biosynthesis.

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References


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