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Expression of *CHS, CHI,* and *DFR* Genes in Response to Light in Small Radish Seedlings

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The expression patterns of the genes involved in flavonoid biosynthesis and the changes in anthocyanin content were investigated in small radish (*Raphanus sativus* L. var *sativus*) seedlings during light treatment. Anthocyanin content increased until day 4, reaching about 100-fold greater than the control plants, then decreased. *CHS* (chalcone synthase) mRNA reached a maximum level at 4 h, remained at relatively high levels until day 3, and then decreased rapidly. The *CHI* (chalcone isomerase) and *DFR* (dihydrofolate reductase) mRNA levels reached maximum at 6 h and day 2, respectively, but were decreased rapidly thereafter. All the genes were expressed strongly in hypocotyls, but were either expressed weakly in roots or not expressed at all in cotyledons. Genomic hybridization showed that the *CHS* gene belonged to a small multigene family, while the *CHI* and *DFR* genes were present in one copy per haploid genome.

Keywords: anthocyanin *CHS, CHI, DFR*, light treatment, small radish (*Raphanus sativus* L. var *sativus*)

INTRODUCTION

Anthocyanins are the most conspicuous class of flavonoids, widespread plant secondary metabolites, as they are the main pigments in flowers and fruits. They are important to many plant functions, such as insect attraction (Taiz and Zeiger, 1991) and protection against damage from UV irradiation (Hahlbrock and Griesbach, 1979). For example, anthocyanins are thought to protect plants from high intensity light and UV irradiation either by reducing the amount of light that reaches photosynthetic cells (Beggs *et al.*, 1987) or preventing polymerization or decomposition of DNA (Li *et al.*, 1993).

Genes involved in anthocyanin biosynthesis have been extensively studied in many plants such as maize, petunia, and snapdragon. Their studies have revealed that two classes of genes are involved. The first class includes the structural genes encoding phenylalanine ammonialyase (*PAL*), the first enzyme in the general phenylpropanoid pathway; chalcone synthase (*CHS*) and chalcone isomerase (*CHI*), the first and the second enzymes in the flavonoid pathway, respectively; and dihydroflavonol 4-reductase (*DFR*), the first enzyme leading to the anthocyanin production. The second class of genes, like the *C1* and *R* gene families in maize, regulates the activity of the structural genes, coordinating the spatial and temporal accumulation of the pigments (Holton and Cornish, 1995).

Structural genes for anthocyanin biosynthesis are regulated developmentally in a tissue specific manner and are induced by a variety of environmental stimuli, including visible light and UV irradiation (Lois, 1994), fungal elicitors (Lamb *et al.*, 1989), and cold treatment (Christie *et al.*, 1994; Leyva *et al.*, 1995).

Although the small radish accumulates a large amount of anthcyanin in its hypocotyl, molecular biological studies on the pigment biosynthesis of this plant have not been carried out so far. Accordingly, we report on the expression patterns of genes for anthocyanin synthesis and changes in anthocyanin content in young seedlings of the small radish plant in response to white light treatment.

MATERIALS AND METHODS

Plant Material

Seeds of small radish (*Raphanus sativus* L. var *sativus*) were purchased from TAKII Seed Company (Japan) and stored at 4°C until used. Seeds were sterilized with 30% H_2O_2 for 20 min, and washied and

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incubatied for 1 h in distilled water at room temperature. Seeds were planted in a plastic box wrapped with black vinyl on 3 MM filter paper (Whatman) soaked with Hoagland solution (Hoagland and Arnon, 1950) and left at 26°C in the dark for three days. Light treatment consists of exposure to continuous white light (150-160 mol/m^2/s) followed by the method of Leyva *et al.* (1995).

Anthocyanin Determination

Anthocyanins were extracted from seedlings by boiling them in a propanol : HCl : H_2O = 18 : 1 : 81 solution for 2 min followed by incubation at 26°C in the dark for 24 h (modified from Lange *et al.*, 1971). Extracts were centrifuged at 15,000 rpm for 10 min, and the supernatant was used to determine the absorbance at 535 nm and 650 nm. Anthocyanin concentrations expressed as absorbance at 530 nm per gram of fresh weight was calculated using the following equation.

Corrected A_{535} / g FW = (A_{535}-2.2 A_{650}) / g FW

Labelling of Probe DNAs

The probe for *CHS* was the 747 bp *Hin*dIII fragment of pSCHS1, which contains a full length *CHS* cDNA of mustard (*Sinapsis alba* L.) (Batschauer *et al.*, 1991). Those for *CHI* and *DFR* were the 724 bp and 1153 bp *Eco*RI/*Sal*I fragments of pCHI.CR and pDFR.CR (Shirley *et al.*, 1992), which contain *Arabidopsis CHI* and *DFR* cDNA, respectively. Probe DNAs were labelled with ^{32}P using the Prime-a-gene random labeling kit from Promega.

Extraction and Hybridization of Nucleic Acids

Total DNA or RNA was extracted from the seedlings using the method of Doyle and Doyle (1990) and Chomczynski and Sacchi (1987), respectively. A blot for DNA or RNA was prepared following the method of Sambrook *et al.* (1989) using 20 × SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) and Hybond N membrane (Amersham). Hybridization was carried out in a modified Church solution (1 mM EDTA, 0.25 M sodium phosphate, 1% BSA, 7% SDS) at 59°C for 20 h, after which the blot was washed with 0.1 × SSC/0.1% SDS and autoradiographed on an X-ray film at −25°C. Intensity of the hybridization was measured using a microdensitometer. The blot was deprobed by boiling the blot in 0.1% SDS solution and left until cooling and reused for another hybridization.

RESULTS AND DISCUSSION

Accumulation of Anthocyanins

Small radish plants, which were germinated in the dark for three days and then exposed to continuous white light, accumulated anthocyanin pigments in their hypocotyls (Fig. 1). Accumulation of anthocyanin reached a maximum rate at day 4 (0.9 A_{535} per gram fresh weight) and decreased thereafter. The rate of accumulation per day was 0.25 A_{535} per gram fresh weight for the first three days. A similar pattern of anthocyanin accumulation was reported for seedlings of *Arabidopsis* (Kubasek *et al.*, 1992), suggesting a general nature of anthocyanin accumulation in response to light. However, three varieties of grape produced anthocyanin even in darkness, in which the transcripts of the pigment biosynthesis genes were also found at a low level (Sparvoli *et al.*, 1994).

Genomic Complexity of the Structural Genes

In order to examine the complexity of anthocyanin genes in the genome of small radish, genomic hybridizations were carried out using heterologous probes. By probing with the *CHS* probe, a complex pattern with multiple hybridizing bands was obtained-10.5 kb, 5 kb, 3.8 kb, and 2.6 kb *Eco*RI fragments, and 5.1 kb, 4.8 kb, 3.9 kb, and 2.5 kb *Hin*dIII fragments, suggesting that the *CHS* gene in small radish is a member of a small gene family (Fig. 2A). Multiple copies of the *CHS* gene have been previously reported; 8-10 copies for *Petunia* (Koes *et al.*, 1989), 3-4 copies for the grape (Sparvoli *et al.*, 1994), and one for both *Arabidopsis* and parsley (Feibaumand

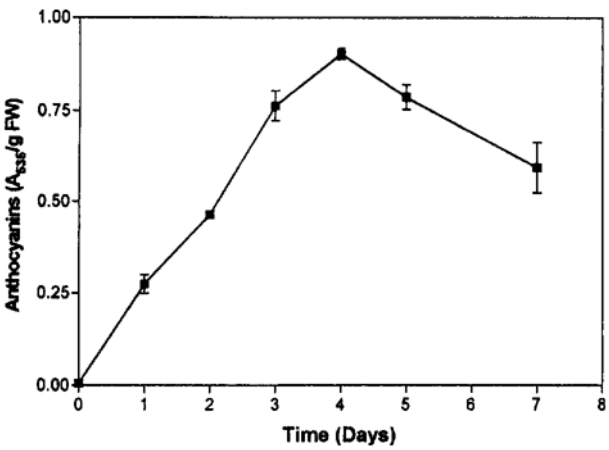

Fig. 1. Anthocyanin levels in small radish seedlings exposed to continuous white light up to seven days.

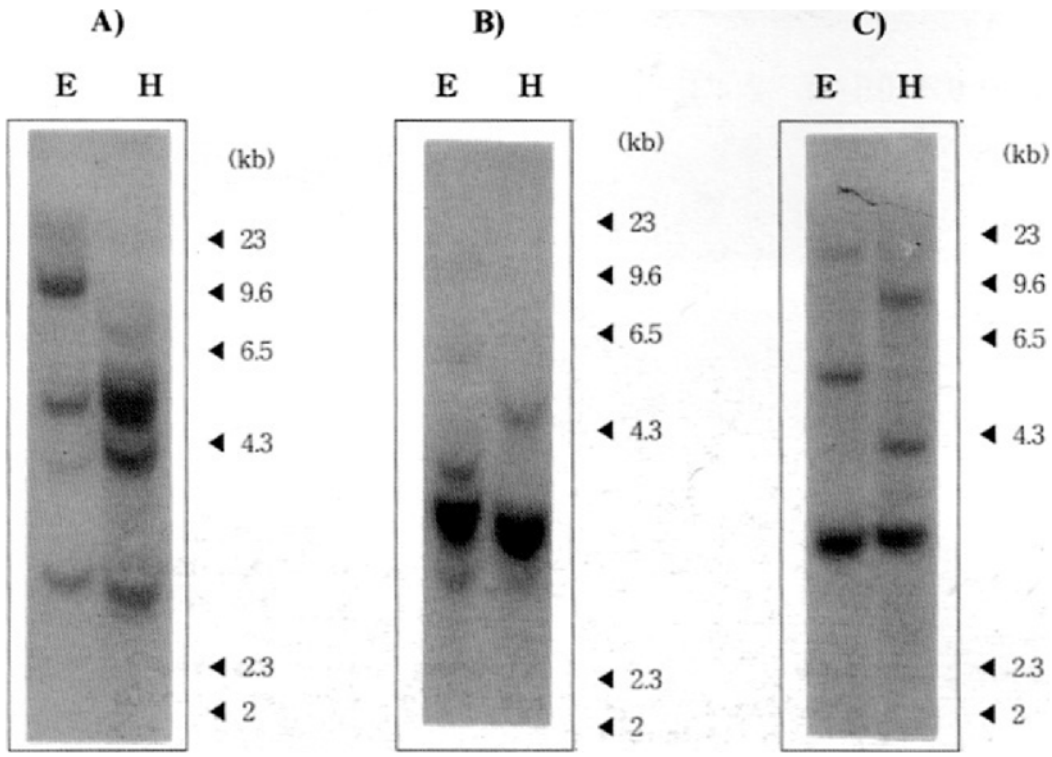

Fig. 2. Southern blot analysis of small radish genomic DNA for the genes involved in anthocyanin biosynthesis. Genomic DNAs digested with *Eco*RI (E) and *Hin*dIII (H) were eletrophoresed in a 0.7% agarose gel. After transfer to a nylon membrane, the blot was hybridized with the heterologous probes of *CHS* (A), *CHI* (B), and *DFR* (C), respectively.

and Ausubel, 1988). The hybridization pattern with other probes, however, was much simpler, and only a single band was detected. With the *CHI* probe, a 3.2 kb *Eco*RI fragment and a 3 kb *Hin*dIII fragment showed strong signals, implying that only a single copy of *CHI* exists in the small radish genome (Fig. 2B). One copy of *CHI* has been reported for grape (Sparvoli *et al.*, 1994) and *Arabidopsis* (Shirley *et al.*, 1992), while one or two for alfalfa (Mckhann and Hirsh, 1994), and two for *Petunia* (van Tunen *et al.*, 1988).

With the *DFR* probe, a 2.7 kb *Eco*RI fragment and a 2.8 kb *Hin*dIII fragment hybridized strongly, also suggesting a single copy of *DFR* in the small radish (Fig. 2C). Three copies of the *DFR* gene were reported for *Petunia* (Beld *et al.*, 1989), but only one for *Arabidopsis* (Shirley *et al.*, 1992) and grape (Sparvoli *et al.*, 1994). Based on the number of hybridization bands, it can be concluded that the complexity of anthocyanin biosynthetic genes in the small radish is similar to that of the grape.

Induction of the Structural Genes by Light

In order to determine whether or not the accumulation of anthocyanin was accompanied by an increase in the mRNA levels of the genes, steady state levels of the *CHS*, *CHI* and *DFR* mRNAs were measured in seedlings exposed to white light up to day 6. All the genes were not expressed in the dark, but the expression was induced by light treatment (Fig. 3A). The *CHS* transcript was most abundant after 6 h of exposure, remained relatively unchanged until day 3, then started to decrease to about a half of the maximum level at day 6. The *CHI* mRNA also reached its peak at 6 h, however, thereafter decreased rapidly to 1/10 of the peak level at day 3. Transcripts of *DFR*, on the other hand, reached a peak at day 2, thereafter decreasing rapidly to about a half of the maximum level at day 3. Since the transcripts of *CHS* and *CHI* reached a peak at 6 h, expression patterns of three genes were examined at 1, 2, 4 and 6 h (Fig. 3B). The *CHS* transcripts started to appear at 1 h reaching a peak at 4 h and decreased a little bit at 6 h. Those of *CHI* and *DFR* increased continuously up to 6 h treatment.

Induction of the genes for anthocyanin biosynthesis by light has been reported in many plants. The transcripts were not found in dark-adapted *Arabidopsis*,

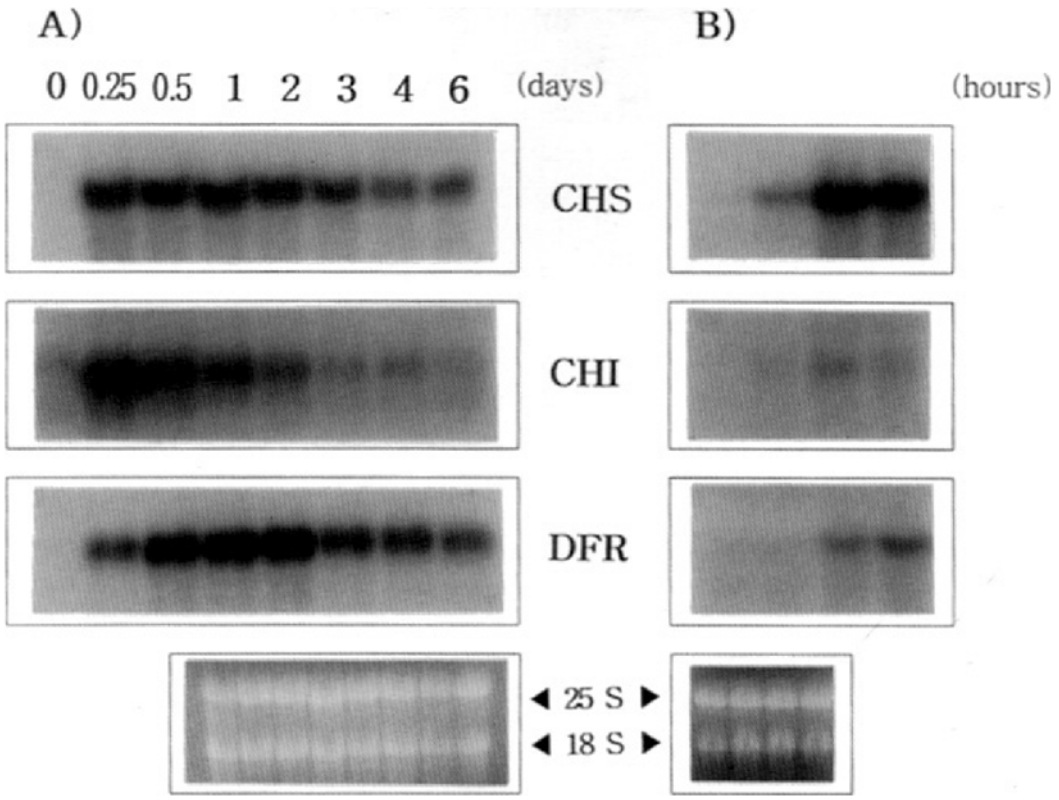

Fig. 3. Induction of the genes involved in anthocyanin biosynthesis by white light. Steady state transcript levels of *CHS, CHI,* and *DFR* were determined by RNA gel blot analysis in seedlings exposed to white light for 6 h to six days (A) and for 1 h to 6 h (B).

mustard, or cultured parsley cells, but were induced when exposed to light (Chappell and Hahbrock, 1984; Jahnen and Hahlbrock, 1998; Batschauer *et al.*, 1991; Kubasek *et al.*, 1992). Expression patterns of the *CHS, CHI* and *DFR* genes in the small radish plant were similar to those of *Arabidopsis* seedlings, in which all transcripts reached maximum level at 6 h (Kubasek *et al.*, 1992). But mRNA of *DFR* continued to increase up to day 3 in small radish. We do not know the significance of this difference, which may be the result of difference in either mRNA stability or *de novo* synthesis.

Patterns of temporal accumulation of anthocyanin and mRNAs of the genes involved in pigment biosynthesis did not coincide each other (Fig. 4). The mRNA of *CHS* reached a peak first at 4 h, followed by those of *CHI* at 6 h and *DFR* at day 3. However, anthocyanin levels reached a peak at day 4, probably reflecting stability of the enzymes involved. On the other hand, anthocyanin biosynthetic genes were induced in the order of biosynthetic steps. Their coordinated induction has been previously demonstrated in snapdragon flowers (Martin *et al.*, 1991), *Arabidopsis* seedlings (Kubasek *et al.*, 1992) and *Perilla* leaves (Gong *et al.*, 1997). This type of induction may suggest that different plants have a common regulation mechanism, in which some

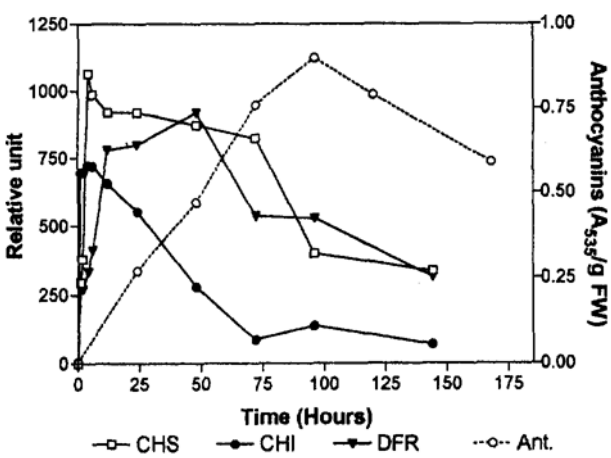

Fig. 4. Accumulation of anthocyanin and expression of anthocyanin biosynthetic genes in response to light. This figure was generated by combining the Fig. 1 with quantified data of Fig. 3 using a microdensitometer.

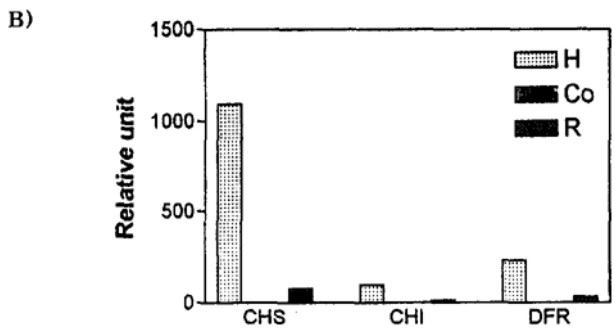

Fig. 5. Spatial expression of the anthocyanin biosynthetic genes. Seedlings exposed to white light for two days were used. H, hypocotyl; Co, cotyledon; R, root.

regulatory genes coordinately control the expression of groups or subgroups of the genes involved in the anthocyanin synthesis pathway (Gong *et al.*, 1997).

Spatial Expression of the Structural Genes

In order to examine whether the location of anthocyanin accumulation is the same to that of mRNA synthesis, the expression patterns of the anthocyanin biosynthesis genes were examined in different organs of seedlings, which were exposed to light for two days (Fig. 5). As expected, all the genes were expressed strongly in hypocotyls where anthocyanin accumulated, and they were not expressed in cotyledons where the pigment did not accumulate. However, they were expressed weakly in roots where the pigment did not accumulate, suggesting the synthesis of flavonoids other than anthocyanins in the root of the small radish.

In the cotyledons of mustard, the *CHS* mRNA was not only expressed strongly in the lower epidermis where anthocyanin accumulated, but also expressed weakly in the upper epidermis and cells in the lower epidermis where the pigment did not accumulate (Nick *et al.*, 1993). This result suggested that not all the cells synthesizing the *CHS* mRNA did accumulate the pigment.

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