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Anthocyanin accumulation and expression pattern of anthocyanin biosynthesis genes in developing wheat coleoptiles

N. AHMED*, M. MAEKAWA and K. NODA

Research Institute for Bioresources, Okayama University, Chuo 2-20-1, Kurashiki, Okayama, 710-0046, Japan

Abstract

Anthocyanin accumulation and expression pattern of anthocyanin biosynthesis genes were investigated in developing coleoptiles of wheat (*Triticum aestivum* L.). In epidermal cell layers of the growing coleoptiles of cv. Hope, anthocyanins started to accumulate between day 2 and 3 after germination, reached their maximum on day 6 and then decreased while another cultivar, Chinese Spring (CS) did not accumulate anthocyanin pigments. None of the six anthocyanin biosynthesis genes was upregulated in coleoptiles of both cvs. grown in the dark, whereas all genes were activated by light in coleoptiles of cv. Hope. Transcript levels of all the six genes were relatively low on day 2, increased from days 3 to 5 and then declined to almost non-detectable levels on day 6. In coleoptiles of CS grown in the light, the early biosynthesis genes (EBGs) were expressed, but the three late biosynthesis genes (LBGs) were not.

Additional key words: comparison of cultivars, pigments, transcript accumulation.

Introduction

Anthocyanins have been shown to accumulate in the epidermal tissues of plant organ where they play roles as antioxidants and in protecting DNA and the photosynthetic apparatus from high radiation fluxes and to attract animals for pollination. Genes required for anthocyanin biosynthesis are regulated in a tissue-specific manner during plant development as well as by a variety of environmental signals including UV radiation, temperature, wounding, fungal elicitors and infection by pathogenic fungi, phytohormone, sugar, bacteria and the presence of ions (Koes *et al.* 1994). The genes in the anthocyanin biosynthesis pathway have been divided into two groups (early biosynthesis genes, EBGs, and late biosynthesis genes, LBGs) due to their order in the biosynthesis pathway and activation by different regulatory genes in dicotyledonous plants (Fig. 1; Nesi *et al.* 2001).

Anthocyanin pigmentation is reported to occur in the allopolyploid *Triticum aestivum*, in coleoptile, first leaf or its sheath, culm, ring under node, auricle, ligule, rachis, glume, awn, anther, pericarp and aleurone layers (Gale and Flavell 1971, Zeven 1973). The presence or absence of pigmentation of plant parts, especially the purple coleoptile, is used to describe cultivars. Anthocyanins protect the developing seedlings from UV radiation, temperature fluctuations, drought and animal feeding.

The existence of such a diverse range of functions raises questions about how these compounds are synthesized and how their biosynthesis is regulated.

We report the accumulation of anthocyanins, expression pattern of anthocyanin biosynthesis genes *CHS*, *CHI*, *F3H*, *DFR*, *ANS* and *UFGT* in pigmented and non-pigmented wheat coleoptiles under light and dark conditions.

Materials and methods

Plants and growth conditions: Wheat (*Triticum aestivum* L.) cvs. Hope and Chinese Spring (CS) were used for this study. The developing coleoptiles of Hope exhibit red coloration whereas CS does not.

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Abbreviations: ANS - anthocyanidin synthase; CHI - chalcone-flavanone isomerase; CHS - chalcone synthase; CS - Chinese spring; EBGs - early biosynthesis genes; F3H - flavanone 3-hydroxylase; DFR - dihydroflavonol 4-reductase; LBGs - late biosynthesis genes; UFGT - UDP-glucose flavonoid 3-oxy-glucosyltransferase.

* Corresponding author; fax: (+81) 86-434-1249; e-mail: drmiannisar@yahoo.com

Grains of these lines were sterilized with 70 % ethanol for 1 min, then with 3 % sodium hypochlorite solution for 20 min, and finally washed five times with sterile water. Grains (20 grains per dish) were incubated on two layers of filter paper moistened with 6 cm^3 distilled water in a Petri-dish at 4 °C in the dark for 24 h to synchronize germination. The seedlings were grown in a growth chamber (*KM, Kiya Co.*, Osaka, Japan) at temperature of 23 °C and either in continuous white light (60 μmol m^{-2} s^{-1}) or darkness, for 7 d.

Anthocyanin extraction and quantification: Anthocyanins were extracted from the coleoptiles with 1 % HCl/methanol (v/v), as described previously by Christie *et al*. (1994) with some modifications. Day at rupture of seed coat was counted as day 1. Seedlings were harvested on each day from days 2 to 7 after germination. Anthocyanins were extracted from whole plants on day 2 and from coleoptiles on subsequent days. Fresh mass (1 g) was ground to a fine powder in liquid N_2 with a mortar and pestle, dissolved in extraction solution (1 % HCl/methanol, v/v) at room temperature and incubated at 4 °C for 4 h. The mixture was centrifuged at 10 000 *g* for 30 min and the supernatant filtered through a 0.22 μm filter (*Millex-GS*, *Millipore*, Bedford, USA). Absorbance, measured by spectrophotometer (*CE2021*, *CECIL Instruments*, Cambridge, England) of the cleared supernatant at 530 nm, served as a measure of anthocyanin content. Experiments were performed in triplicate.

Microscopic analysis: Digital images of the epidermal cell layers were captured with *Keyence VB-7010* CCD camera (Osaka, Japan). Coleoptiles grown for 3 d under continuous white light were used. Coleoptiles were separated and transverse sections were cut with a sharp razor. Microscopic images of the epidermal cell layers were visualized with *Nikon Eclipse E600* microscope (Tokyo, Japan).

Total RNA isolation and RT-PCR: Total RNA was extracted from whole seedlings at day 2 and from coleoptiles at subsequent days grown in light or dark using *Sepasol-RNA I* extraction solution (*Nacalai Tesque*, Osaka, Japan) according to the manufacturer's protocol.

Total RNA was treated with DNaseI (*Promega*, Wisconsin, USA) and reverse-transcribed with an anchored oligo (dt) primer (5'-GGCCACGCGTCG-ACTAGTTTTTTTTTTTTTTTT-3') and Superscript II reverse transcriptase (*Gibcobrl*, Rockville, USA), according to the manufacturer's instructions.

Primers for *CHS*, *CHI*, *F3H* and *DFR* were designed on the basis of the wheat genomic sequences reported to GenBank (*CHS*, AB187025; *CHI*, AB187026; *F3H*, 187027; *DFR*, AB 187028). Oligonucleotide primers for *ANS* and *UFGT* were designed from reported sequences of rice *ANS* (accession No. Y07955) and barley *UFGT* (accession No. X15694). Partial sequences were amplified effectively with cDNA of Hope or genomic DNA of CS as a template and sequenced. The deduced amino acid sequences of these fragments showed high similarity to sequences of corresponding genes listed in GenBank. The partial sequences of *CHS* (394 bp), *CHI* (434 bp), *F3H* (501 bp), *DFR* (231 bp), *ANS* (150 bp) and *UFGT* (399 bp) were amplified with specific primers for genes of the anthocyanin synthesis pathway (Table 1). The reaction was carried out under the following conditions: 5 min denaturation at 94 °C; then 35 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, in a *Gene Amp PCR system 9700* cycler (*Perkin Elmer*, New Jersey, USA). Nucleotide sequences of amplified genes were determined using an *Applied Biosystems 310* DNA sequencer and showed high similarity (80 - 90 %) to sequences of corresponding genes listed in GenBank (http://www.ncbi.nlm.nih.gov).

For quantitative RT-PCR assays, the total amount of cDNA in samples was standardized after the amount of

Table 1. Oligonucleotide primers used for to determine the transcript levels of anthocyanin biosynthesis genes in developing coleoptiles of wheat.

Primer	Gene	Sequence
TaCHS-L	*CHS*	5'-ATCACCCACCTCGTCTTCTG-3'
TaCHS-R	*CHS*	5'-AGGAGGTGGAAGGTGAGTCC-3'
TaCHI1-L	*CHI*	5'-GCAGTACTCGGACAAGGTGA-3'
TaCHI2-R	*CHI*	5'-GTTCGTTCACACCGAAACC-3'
TaF3H1-L	*F3H*	5'-CCTACTTCTCGTACCCGGTG-3'
TaF3H2-R	*F3H*	5'-GAACGTCGCGATCGACAG-3'
TaA1-L	*DFR*	5'-CTCATGGCTCGTCAGGAAG-3'
TaDFR1-R	*DFR*	5'-TCTTGGGAGTCGAAGTCCAT-3'
TaANS-S	*ANS*	5'-GTCTCCGCGCTCTCCTTC-3'
TaANS-A	*ANS*	5'-GCCCGTTGCTGAGGATCT-3'
TaUFGT-S1	*UFGT*	5'-TCCCCGGGGGTTCCTGGAGC-3'
TaUFGT-A1	*UFGT*	5'-ACAAACTATCTCGACAAACT-3'
TaUbi-L	*Ubiquitin*	5'-ACCGGCAAGACCATCACCC-3'
TaUbi-R	*Ubiquitin*	5'-GCTGCTCCACACCAGCAGA-3'

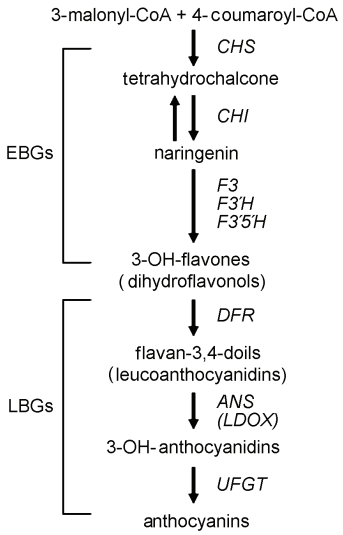

Fig. 1. Simplified schematic presentation of the anthocyanin biosynthesis pathway.

Ubiquitin mRNA was evaluated with two primers, TaUbi-L and TaUbi-R. PCR was also performed with 25, 30 and 35 cycles. Since all gene products increased linearly within the range of these cycles, 35 cycles were chosen to evaluate the expression level of anthocyanin biosynthesis genes. Quantification of the cDNA from the gel images was determined using *Scion Image* software (*Scion Corporation*, Maryland, USA).

Results

Accumulation of anthocyanins in developing wheat coleoptiles: Anthocyanin biosynthesis is limited in wheat to certain tissues, and it occurs during specific stages of development. The visible accumulation of these compounds usually reflects the activity of biosynthetic enzymes functioning in the pathway.

The coleoptiles of Hope during the first few days after germination exhibited a characteristic pattern of red pigmentation. This coloration started between days 2 and 3, reached a maximum level around day 5 and 6 (Fig. 2), and then faded to be almost non-pigmented a few days later. The anthocyanin contents were also determined in the developing coleoptiles of Hope. The amount of anthocyanins also started to increase between days 2 and 3, reached a maximum level on day 6, and then decreased on day 7 (Fig. 2). By contrast, neither red colour nor increase in anthocyanins was observed in developing coleoptile of CS.

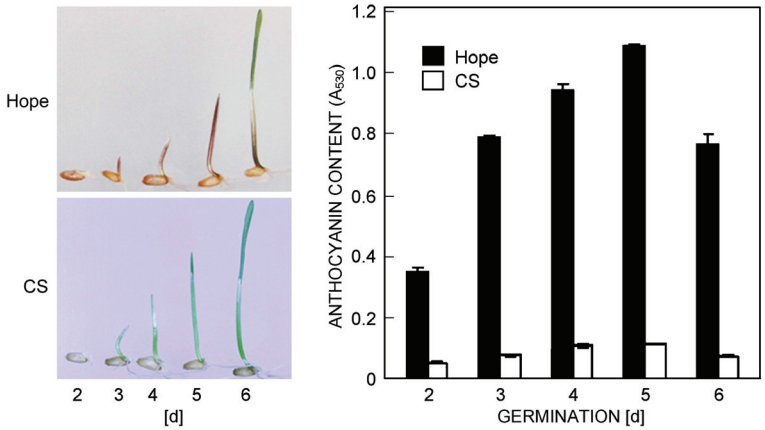

Fig. 2. Pigmentation and anthocyanin accumulation in developing coleoptiles of the wheat lines Hope and CS grown in light at 23 °C (2 - 6 d after germination). Means ± SE.

Localization of anthocyanins in developing wheat coleoptiles: To determine the localization of anthocyanin pigments transverse sections of hope and CS coleoptiles grown for 3 d under continuous white light were visualized under microscope. The pigments were accumulated in the epidermal layer cells of Hope coleoptiles whereas no pigments were detected in CS under same conditions (Fig. 3).

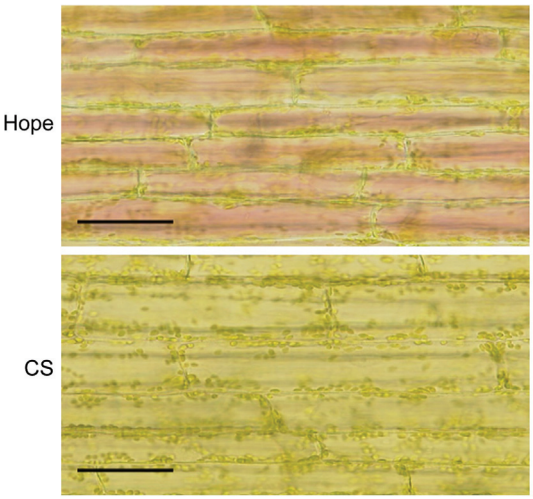

Fig. 3. Anthocyanin accumulation in epidermal cells of coleoptiles of cvs. Hope and CS. Coleoptiles grown in light at 23 °C for 3 d were used. *Bars* = 50 µm

Transcript accumulation of anthocyanin biosynthesis genes in developing coleoptiles: To determine whether the accumulation of anthocyanin pigments in wheat coleoptiles was accompanied by an increase in the level of mRNA corresponding to flavonoid biosynthesis genes, using RT-PCR we investigated the response of the six genes. Transcript levels of all genes were relatively low on day 2, increased from day 3 to day 5, and then declined to almost non-detectable on day 6 (Fig. 4). Three to six fold increases in transcript levels for different genes were observed. In CS the expression level of EBGs and

LBGs were also measured in developing coleoptiles from day 2 to day 7 (Fig. 4). There was no expression of LBGs (*DFR, ANS, UFGT*); although EBGs (*CHS, CHI, F3H*) were expressed similar to Hope and a 3 to 5 fold increase in transcript levels was observed. This expression profile of genes in the anthocyanin synthesis pathway correlated well with the accumulation pattern of anthocyanin in coleoptiles.

Induction of anthocyanin biosynthesis genes in white light: To determine whether the flavonoid biosynthesis genes are also light dependent in developing coleoptiles, we investigated the response of the genes expression by RT-PCR. Three day-old coleoptiles of Hope and CS were used to determine the transcript levels because the transcripts of EBGs and LBGs were peaked at this stage (Figs. 4, 5). All the transcripts were induced in the light. Coleoptiles grown in darkness did not show anthocyanin pigmentation. Expression of anthocyanin biosynthesis genes were also quite low when the two lines were grown in darkness and 4 to 5 fold decreases in transcript levels for different genes were observed.

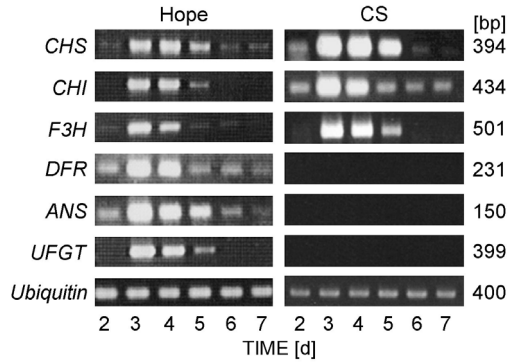

Fig. 4. Transcript accumulation pattern of anthocyanin biosynthesis pathway genes (*CHS, CHI, F3H, DFR, ANS* and *UFGT*). Coleoptiles of Hope and CS grown under light were harvested 2 - 7 d after germination.

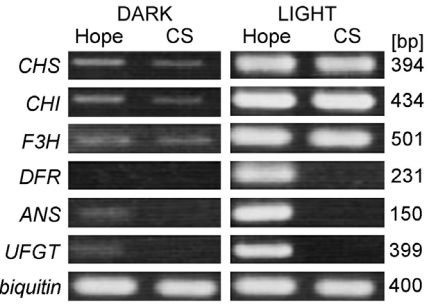

Fig. 5. Transcript accumulation of anthocyanin biosynthesis genes (*CHS, CHI, F3H, DFR, ANS* and *UFGT*). Coleoptiles of Hope and CS grown under light or darkness were harvested 3 d after germination.

Discussion

Anthocyanin pigmentation in developing coleoptiles of Hope coincided with expression of both EBGs and LBGs of the anthocyanin synthesis pathway (Fig. 2*A*, 4). By contrast, the EBGs but not the LBGs were upregulated in the cultivar, CS, which did not develop pigmented coleoptiles (Fig. 4). Anthocyanin pigmentation of coleoptiles is controlled by three genes, *Rc1*, *Rc2* and *Rc3* that are located on the short arm of chromosomes 7A, 7B and 7D, respectively (Zeven, 1973, Khlestkina *et al.* 2002). Kuspira and Unaru (1958) reported that CS carries a recessive allele of *R* gene. Gale and Flavell (1971), using chromosome substitution lines of Hope 7A, 7B and 7D in CS, suggested that *R* on chromosome 7 has a regulatory role in anthocyanin production. Nisar *et al.* (2003) showed that maize transcription factor genes *C1* and *B-Peru*, when transiently transfected using a microprojectile bombardment method, induced anthocyanin pigments in CS coleoptiles. Himi *et al.* (2005) reported that *Rc* gene for coleoptile colour is a transcription factor that regulates *DFR* in wheat coleoptile. Two transcription factors that regulate only the late genes in the flavonoid biosynthesis pathway have been previously reported. The *Del* gene of *Antirrhinum majus* activated the expression of *F3H*, *DFR*, *Candi* (mutant name), and *UFGT* in flowers (Martin and Gerats 1993). The *TT2* gene of *Arabidopsis* also regulated the expression of *DFR* and leucoanthocyanidin reductase (Nesi *et al.* 2001). Therefore it appears that the *R* gene encodes a transcription factor for LBGs that functions similarly to the *C1* and *B-Peru* gene products. This expression profile of genes in the anthocyanin synthesis pathway correlated well with the accumulation pattern of anthocyanin in coleoptiles. This result suggested that the expression level of anthocyanin biosynthesis genes may be closely related to the anthocyanin pigment accumulation in wheat coleoptiles. A similar finding has already been demonstrated by Lu and Qing (2006) in wild potato. Anthocyanins were accumulated in the epidermal cells of Hope coleoptiles. Anthocyanin accumulation has been described in a number of plant species. In *Petunia hybrida*, anthocyanins could be observed in the epidermal cells of most organs such as stems, leaves and flower limbs (Huits *et al.* 1994). In soybean, mature black seed coat contained anthocyanins that were accumulated in the epidermal layer of the seed coat (Todd and Vodkin 1993). Our findings are consistent with the previous results indicating that the anthocyanin accumulation is tissue specific and highly conserved between different plant species.

Anthocyanin biosynthesis is modulated by environmental stimuli such as light, temperature, and nutrient supply, as well as by internal stimuli such as growth regulators, metabolites, and the particular developmental stage of the competent tissue (Mol *et al.* 1998). Light is

one of the most important environmental stimuli regulating anthocyanin accumulation and acts both as an essential stimulus and as a factor that modulates the intensity of pigmentation. To determine whether light affects the accumulation of coleoptile anthocyanin pigmentation. We investigated the response of the six genes by RT-PCR analysis in developing wheat coleoptiles to white light. Light also upregulated these genes in developing coleoptiles and 3 to 5 fold increase in transcript levels was observed for different genes of the anthocyanin biosynthesis pathway (Fig 5). These results indicate anthocyanin pigmentation in wheat coleoptiles is induced by light.

Marrs and Kaufman (1991) suggested that anthocyanin mRNA accumulation in various plant tissues is dependent on light. Petroni *et al*. (2000) and Piazza *et al*. (2002) have reported that light regulation of anthocyanin biosynthesis is mediated through transcriptional activation of the biosynthetic genes. Two major classes of transcription factors have been reported to regulate the expression of genes in the anthocyanin synthesis pathway, namely the R/B family (basic helix-loop-helix type), and the C1/Pl family (MYB type) (Ludwig *et al.* 1989, Cone *et al.* 1986, Paz-Ares *et al.* 1987). The light signal transduction pathways promote rapid induction of the MYB-related factors and these factors mediate the expression of the anthocyanin biosynthesis genes. Over expression of the transcription factors leads to constitute expression of the pathway (Grotewold *et al*. 1994, Borevitz *et al.* 2000) even in the absence of light.

During these investigations, the steady-state levels of six anthocyanin biosynthetic genes were accumulated upon light treatment. The results obtained during these studies are also consistent with the previous findings that anthocyanin biosynthesis gene regulation is dependent on light. The molecular mechanism involved in the light regulation of anthocyanin biosynthesis genes in wheat coleoptiles is developmentally regulated or mediated by light induced transcription factors still remains to be identified.

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