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Light and developmental regulation of the *Anp*-controlled anthocyanin phenotype of bean pods

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Abstract. In the presence of the dominant allele of the *Anp* gene, bean pods present a purple-mottled phenotype. The purple pigmentation is variable from cell to cell in the pod epidermal layer and develops as a random mosaic. Three anthocyanidins, delphinidin, petunidin and malvidin, are involved in this purple pigmentation. Anthocyanins accumulated in vacuoles; anthocyanoplasts and cristal bodies were also observed occasionally. A developmental switch is a prerequisite for anthocyanin accumulation in the pods. This does not occur before day 4 after pollination and is controlled by light in competent pods. mRNAs for PAL, CHS, CHI, DFR and UFGT are induced in the pods, indicating that the general anthocyanin biosynthetic pathway is well conserved at both the biochemical and molecular levels in this species. mRNA steady-state level studies of PAL and CHS suggest that the light regulation occurs at the transcriptional level.

Key words: *Phaseolus vulgaris* – Anthocyanin biosynthesis – Genetic, light and developmental control – PAL and CHS mRNA steady state level

Introduction

Anthocyanins are widespread secondary metabolites involved in the generation of a diversity of bright red and blue colours in the plant kingdom (van Tunen and Mol 1990; Dooner et al. 1991). Because they are easily visible and because mutations affecting their accumulation are not lethal, these compounds have been extensively characterized at the biochemical and physiological levels in a wide range of plant species (Harborne 1988), but only in a few species, including maize, snapdragon, petunia and pea, at the genetic and molecular levels (Dooner et al. 1991). The various colours of the anthocyanins result from modifications (hydroxylation, methoxylation, acylation and glycosylation) at various positions of the flavonoid skeleton. Combinations of anthocyanins, the presence of co-pigments and pH differences may also influence the extent and quality of the colours within tissues (Harborne 1988).

It has been shown that environmental and developmental factors affect the accumulation and the spatio-temporal distribution of anthocyanins. The quality and intensity of light are certainly the most important of these environmental factors, but other parameters such as cold stress and pathogen infection have also been shown to stimulate anthocyanin biosynthesis (Mancinelli 1990).

Well-documented genetic and molecular analyses have demonstrated that several regulatory loci are involved in the spatio-temporal pattern of anthocyanin accumulation in maize, petunia and snapdragon (Dooner et al. 1991). Interestingly, these regulatory loci appear to be phylogenetically related. They belong to well-described transcriptional regulatory protooncogene families such as *myb* for *C1* or *myc* for *R* in maize (Taylor and Briggs 1990; Dooner et al. 1991; Goodrich et al. 1992). Similar regulatory proteins exist in unrelated plant fami-

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Abbreviations: PAL, Phenylalanine ammonia lyase; CHS, chalcone synthase; CHI, chalcone isomerase; DFR, dihydroflavonol-4-reductase; UFGT, UDP-glucose: flavonoid 3-*O*-glucosyl transferase

lies, in which they appear to control anthocyanin accumulation in a very similar manner (Taylor and Briggs 1990; Dooner et al. 1991; Jackson et al. 1991a, b; Goodrich et al. 1992). These different gene products appear to differentially regulate at least a number of the anthocyanin structural genes at the transcription level (Taylor and Briggs 1990). For example, in maize, *C1* appears to be involved in anthocyanin accumulation in the aleurone and scutellum of the kernel (Coe et al. 1988; Dooner et al. 1991). The *R* locus, which is represented by a multigene family in maize, regulates *C2* (Chalcone synthase) in the aleurone, while the transcription of *C2* is completely independent of the *R* locus in the seedling (Taylor and Briggs 1990). Anthocyanin regulation is thus a very complex network that may serve as a model for regulatory studies of other important plant functions.

In legumes, anthocyanin and other flavonoid-related compounds are very important metabolites. They are involved in a wide variety of important functions, such as plant defense against pathogens (isoflavonoids), nodulation signalling (flavones) and UV protection (anthocyanins) (Peters et al. 1986; Lamb et al. 1989). In bean, the anthocyanin distribution pattern is very complex in seeds, cotyledons, hypocotyls or pods, and differs considerably between different genotypes (Leaky 1988).

We have genetically characterized a bean locus, *Anp*, that controls a purple-mottled phenotype of the pods (Gantet et al. 1991). In this paper, we present the results of biochemical, physiological and molecular analyses of anthocyanin biosynthesis in the pods of a bean genotype harbouring *Anp* relative to pod development and light.

Materials and methods

Plant material

The AFN inbred line of *Phaseolus vulgaris* L. carries the dominant *Anp* locus. Seeds were a generous gift of H. Bannerot (INRA, Versailles, France). The plants were grown under controlled conditions in a greenhouse (Gantet et al. 1991) and different developmental stages were defined according to pod length after fertilization (Table 1). To study the light induction of anthocyanin biosynthesis, we wrapped pods (stage 7–8) or plants in the greenhouse with aluminum foil 3 days before harvest. Detached pods were immediately unwrapped, except for the controls, which were maintained in the dark. The detached pods were laid on Whatman paper no. 3 moistened with sterile distilled water, in petri dishes, and submitted to continuous fluorescent light (Philips PL 24W 183; 350 $\mu E\, m^{-2}\, s^{-1}$) at 20 °C. Two different pods were pooled for each sample. The light-exposed side of the pod was cut off and used for further analysis. For RNA extractions, the samples were directly dipped in liquid nitrogen and stored at −80 °C.

Anthocyanin characterization

For cytological studies, hand cross-sections and epidermal peels were mounted in water before observation under a Reichert Polyvar microscope.

For chemical analysis, total anthocyanins were extracted by macerating fresh tissue in methanol:HCl (99:1, v/v). For analysis of the anthocyanidins, the extract was strained through glass wool and dry-evaporated, *in vacuo*, at 37 °C. After dissolution in 2 *N* HCl, the anthocyanins were hydrolysed at 100 °C for 30 min in a water-bath. The hydrolysate was strained through glass wool, and the anthocyanidins were extracted by a minimal volume of isoamyl alcohol. The anthocyanidins were identified by two-dimensional thin-layer chromatography (TLC) analysis on cellulose plates (Merck 5716). The procedure was carried out as described elsewhere (Mullick 1969) using formic acid, HCl and water (100:2.5:37.5, v/v/v) for the first dimension and amyl alcohol, acetic acid and water (2:1:1, v/v/v) for the second dimension. Reference samples of anthocyanins were kindly provided by J.-P. Renou (Université d'Angers, France). Anthocyanidins were detected on plates by their pink-purple colour. Their R_F values were measured and compared to reference samples.

RNA analysis

The preparation of total RNA, Northern blots, hybridizations and the autoradiography were carried out as described previously (Mahé et al. 1992). Plasmids pSPP2 (containing a specific sequence for the *gpal2* gene transcript) (Liang et al. 1989), pCHS4 (*chs*) (Ryder et al. 1987) and pCHI1 (*chi*) (Mehdy and Lamb 1987) DNA bean probes were kindly provided by C. J. Lamb (Salk Institute, San Diego, Calif.). Plasmids pJAM 212 (*dfr*) (Martin et al. 1985) and pJ2AM 338 (*ufgt*) (Martin et al. 1991) snapdragon probes were gifts of C. Martin (John Innes Institute, Norwich, UK). In addition, a specific probe coding for the bean protein elongation factor EF 1-α (Axelos et al. 1989) was used as a constitutive control. This probe was kindly provided by B. Lescure (CNRS-INRA, Toulouse, France).

Quantification of the transcript levels was achieved by densitometric scanning of the autoradiograms (Biocom analyzer, Lecphor software) of RNA dot-blots. Each value obtained for GPAL2 or CHS mRNA steady-state levels were normalized with respect to the value obtained on the same filters for the EF 1-α probe and expressed in arbitrary units: A.U. = {[(PAL or CHS value)/(EF 1-α value)] × (EF 1-α mean value for one experimental condition)} Six independent repetitions were conducted for each experimental condition. The mean values from each experimental condition were compared using the Student's *t*-test.

Results

The purple-mottled phenotype of AFN pods: localization of anthocyanin

The mature pods of the bean with the AFN genotype present a typical purple-mottled phenotype. The intensity of the purple colour is directly related to high light exposure, while the mottled phenotype appears to be independent of light intensity. The AFN genotype harbours *Anp*, a single dominant genetic locus that is directly associated with both aspects of the AFN pod phenotype (Gantet et al. 1991). In contrast, other bean genotypes which do not contain the *Anp* locus (e.g. 'La Victoire') always have green pods under the same cultural conditions (Gantet et al. 1991). Cytological observations performed directly on hand cross-sections showed that purple pigments accumulated in the epidermal cell layer on-

Table 1. Definition of pod developmental stages relative to pod length and time after pollination in AFN bean pods

Developmental stage	Pod length (cm)	Days after pollination[a]
1	≤0.5	–
2	0.5; 2	2
3	2; 4	3
4	4; 6	4
5	6; 8	5–7
6	8; 10	7–8
7	10; 12	8–9
8	12; 13	10–11
9	>13	>11

[a] Approximated from Fig. 2

ly. The observation of epidermal peels revealed variations in the intensity of the purple colour from cell to cell: some cells presented a very strong purple colour, while neighbouring cells were unpigmented or pinkish (Fig. 1 A). In some cells, anthocyanoplast vesicles (Fig. 1 B) or cristal bodies (Fig. 1 C) were visible. Preliminary observations (P. Gantet, unpublished data) had shown that although all of the mature pods exhibited a mottled phenotype, the observed pattern of pigmentation was not strictly identical among the different pods. This suggests that the mottled phenotype could involve a random cell-autonomous anthocyanin synthesis.

Three different anthocyanidins are synthesized in the AFN pod epidermal cell layer

Because of their colour, we assumed that the purple pigments responsible for the AFN pod phenotype were anthocyanins. Peels of mature pods were macerated with methanol-HCl, and the extracts were then processed for anthocyanidins identification.

After TLC analysis, three different anthocyanidins (A, B, C) were detected in the AFN epidermal cell layer. Their R_F values were very close to the R_F values of the reference standards of delphinidin, petunidin and malvidin, respectively (Table 2). This identification was confirmed by co-analysis by TLC of the AFN pods extracts with the reference samples (unshown data). Delphinidin tri-hydroxylated on carbon residues 3′, 4′ and 5′, petunidin methoxylated on 3′ and hydroxylated on 5′, while malvidin methoxylated at both positions. This demonstrated that pod epidermal cells were able to carry out hydroxylation and methylation reactions both at 3′ and 5′ positions and that these cells were able to undergo all of the steps of the anthocyanin pathway.

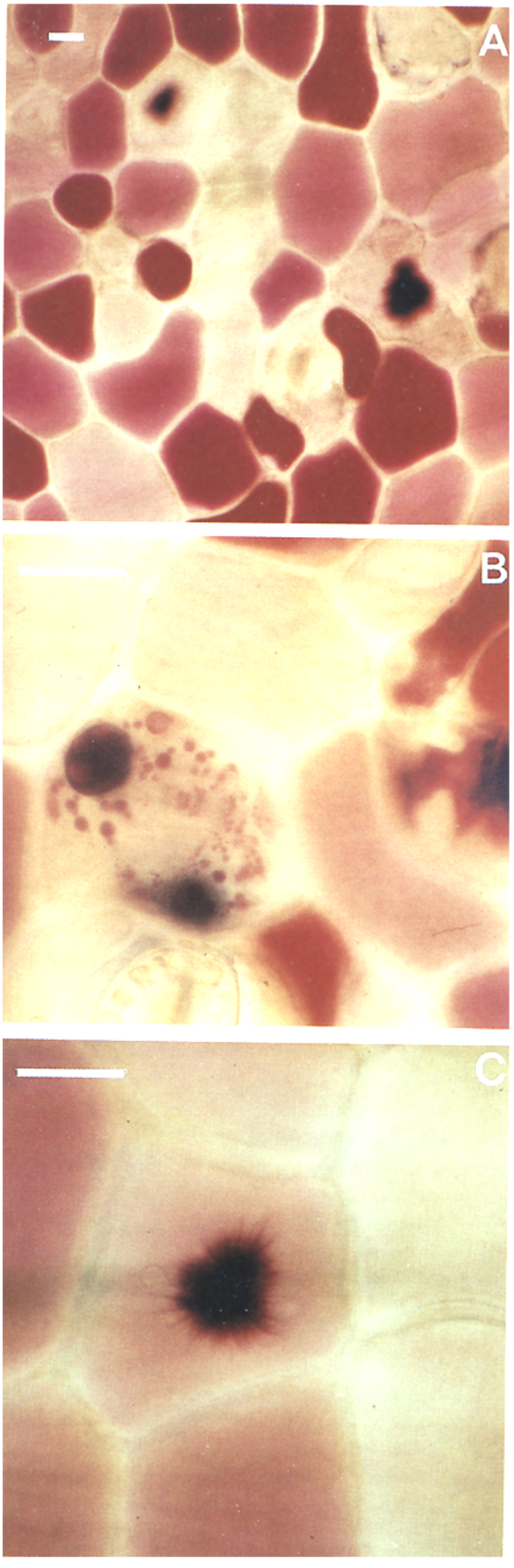

Fig. 1 A–C. Cytological observations of pod epidermal peels of the bean with the AFN genotype. **A** Direct observation of a peel of epidermal cells; **B** observation of anthocyanoplasts in an epidermal cell; **C** a cristal body in an epidermal cell. *Bars*: 10 μm

Table 2. Anthocyanidin TLC R_F values

	Control samples						Pod extracts		
	Dp	Pt	Cy	Mv	Pn	Pg	A	B	C
1st a	0.41	0.51	0.51	0.70	0.70	0.70	0.43	0.53	0.71
2nd	0.24	0.37	0.46	0.50	0.65	0.78	0.27	0.39	0.52

Dp, Delphinidin; Pt, petunidin; Cy, cyanidin; Mv, malvidin; Pn, peonidin; Pg, pelargonidin

[a] 1st, First dimension; 2nd, second dimension

A developmental switch is required for anthocyanin accumulation in AFN pods

The development of bean pods extends from the fertilization of the ovules until the release of the seeds. In order to know whether the accumulation of the anthocyanin pigments was related to pod development, a study was carried out under controlled growth conditions.

After fertilization, 30 pods on ten different individual plants were randomly sampled and their development followed, every 24 h for 20 days. The length of these pods was measured. During the first 2 days after fertilization, the length of the pods was too short to be estimated accurately. The relationship between the size of the pods and the number of days after fertilization is presented in Fig. 2. Pods elongated linearly during the first 11 days, then reached their maximal length (about 14 cm). The plateau phase corresponded to a radial growth followed by a yellowing of pods and a senescence phase. Pod developmental stages have been defined according to pod length (Table 1). The first eight developmental stages correspond to the linear growth phase of the pods, while the last developmental stage corresponds to full length pods.

The relative ratio of purple pigmented pods versus green pods was evaluated during the time course of pod development with the same sample of 30 pods. (Fig. 3 A). The overall data suggest the existence of a switch for the development of a purple anthocyanin pigmentation in the pods because the colour did not appear before day 5. Afterwards, the number of pods harbouring random pigmentation increased until day 10 when all of the pods turned to the red-mottled phenotype. The development of the pigmentation was concomitant with the linear phase of the pod elongation. Both pod elongation and pigmentation were complete by day 12, which may suggest a coordinated regulation between these two physiological processes.

To ascertain whether or not the kinetics of pod pigmentation observed *in planta* was dependent upon development and not due to the young pods receiving a limited light exposure, *in vitro* experiments were carried out. Forty to one hundred and fifty pods corresponding to the

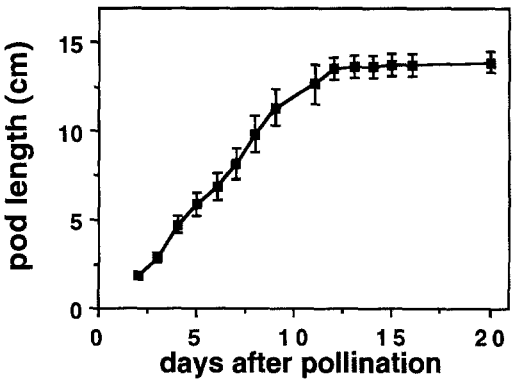

Fig. 2. Time course of pod elongation after fertilization in the bean with the AFN genotype. For each time point, a sample of 30 pods was analysed. *Bars* represent the 95% confidence interval

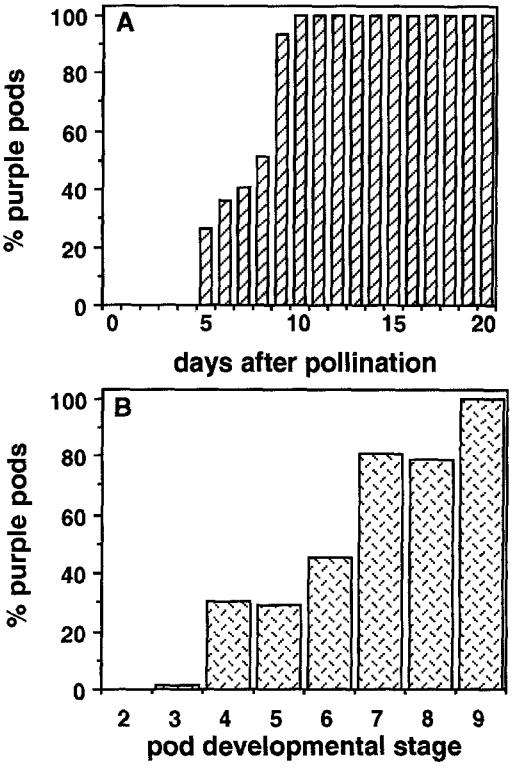

Fig. 3 A, B. Appearance of the purple phenotype during pod development. **A** In planta. For each time point, the same sample of 30 pods was followed, the proportion of purple pods versus green pods was calculated after visual classification. **B** *In vitro*. For each developmental stage, samples of 40–150 pods were exposed to light for 110 h before the proportion of purple pods versus green pods was calculated after visual classification

different development stages (defined in Table 1) were collected from different plants and submitted to a continuous illumination with high fluence-rate white light. The collected pods were maintained in a humid environment and remained alive, but they did not elongate further. The ratio of purple pods versus green pods was calculat-

ed for each stage after 110 h of illumination *in vitro* (Fig. 3 B). After detachment from the plant, not a single young pod (stage 2) turned purple after strong illumination. This was not the case of more mature pods (stages 4–9), which were able, in a significant proportion to accumulate anthocyanin under the same experimental conditions. This suggests that the initial developmental switch necessary for the induction of anthocyanin accumulation is independent of light. In pod developmental stages 3, 4 and 5, the pigmentation was mainly restricted to the ventral and dorsal veins of the pods, whereas it extended throughout the pod in later stages. This experiment provides a strong argument in favour of a developmental switch for anthocyanin biosynthesis.

Light is necesssary for anthocyanin accumulation in AFN pods

Green pods at developmental stages 7–8 (Table 1) were wrapped with aluminium foil *in planta* 3 days before harvesting. They were submitted to the same illumination conditions as in the previous experiment. It became clear that at these stages anthocyanin pigmentation is related directly to light stimulation since the pods maintained in aluminium foil did not produce an increase in measurable absorbance at 530 nm (Fig. 4). The low level of absorbance at 530 nm of Methanol-HCl extracts of green pods maintained in the dark could be typically ascribed to the absorbance of the degradation product of chlorophyll in this acidic solvent (Mancinelli 1990). Thus, once pod epidermal cells become competent to synthesize anthocyanins, the accumulation of these pigments is positively regulated by light.

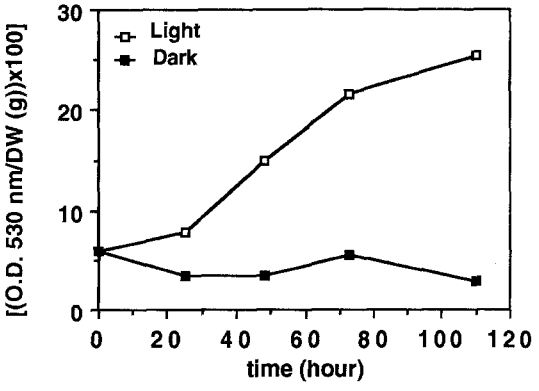

Fig. 4. In vitro kinetics of light-stimulated anthocyanin accumulation in fully developed pods (stages 7–8). Green pods harvested at developmental stages 7–8 were wrapped with aluminium foil 3 days before maturity and then exposed or not exposed to light. Anthocyanins were extracted as described in the Materials and methods. Each time point represents the average of ten independent experiments with 2 pods from different plants. *DW*, Dry weight

Anthocyanin genes are expressed in AFN pods:pal and chs mRNA steady-state levels increase in the presence of light

Northern analyses using *pal, chs*, and *chi* bean probes and *dfr* and *ufgt* snapdragon heterologous probes were performed. mRNAs with the expected sizes of 2.5 kb (*pal*), 1.4 kb (*chs*), 1 kb (*chi*) 1.4 kb (*dfr*) and 1.7 kb (*ufgt*) accumulated in illuminated AFN pods (developmental stage 7–8) *in vitro* (75 h of continuous illumination as previously described; see Fig. 5). This suggests that the different enzymatic steps for the anthocyanin biosynthetic pathway reported for other species (Martin et al. 1991 a, b) are well conserved in bean (Fig. 6).

In maize seedlings, it has been shown that anthocyanin accumulation is regulated at the transcriptional level (Taylor and Briggs 1990). In order to verify whether or not this is the case in bean pods, the steady-state levels of transcripts from the *pal* and *chs* genes were investigated after light exposure *in vitro*. PAL is the first enzyme of the phenylpropanoid pathway and CHS is the key enzyme of the flavonoid catalysing the formation of the first C15 compound (Fig. 6). Total RNAs were isolated from green pods belonging to developmental stage 7–8 (Table 1) and from pods exposed to light for 6 and 75 h *in vitro*. These time points were chosen on the basis of previous reports on flavonoid-related mRNA accumulation in bean and corn seedlings (Liang et al. 1989; Taylor and

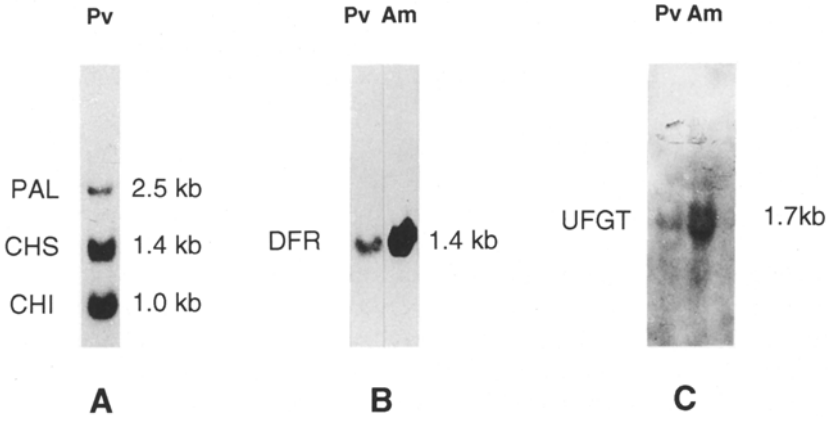

Fig. 5 A–C. Anthocyanin-related mRNA steady-state level studies. **A** GPAL 2, CHS and CHI; hybridizations were carried out with 50% formamide at 42 °C. **B** DFR; hybridization was carried out with 38% formamide at 47 °C. **C** UFGT; hybridization was carried out with 26% formamide at 47 °C. *Pv*, Total RNA of AFN bean pods (stage 8) illuminated 75 h *in vitro*; *Am*, total RNA of purple *Antirrhinium majus* flowers

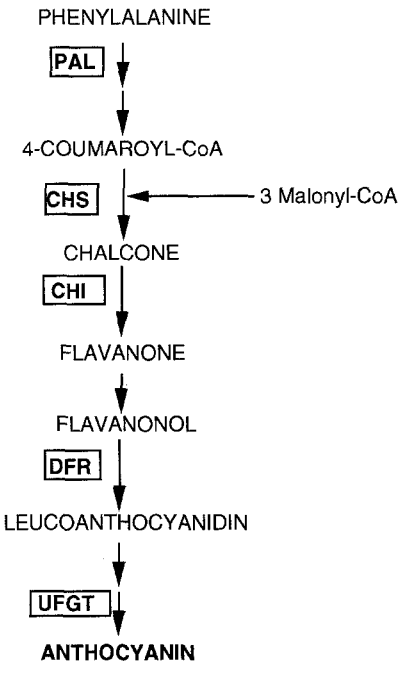

Fig. 6. Proposed anthocyanin biosynthetic pathway in AFN pods possessing the *Anp* gene

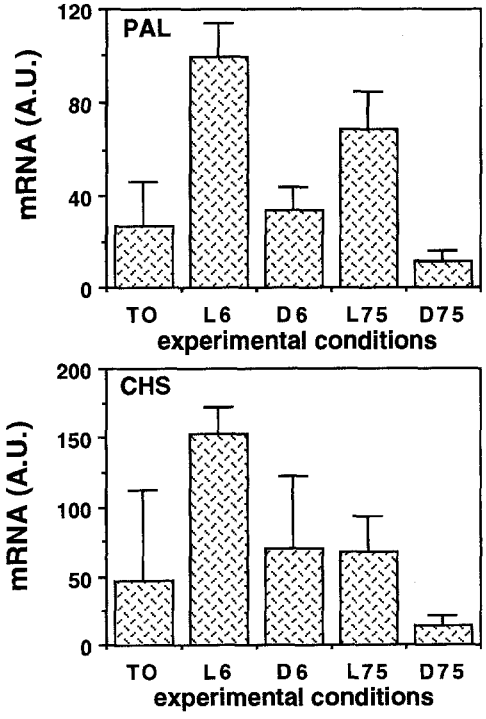

Fig. 7. Light induction of pal and chs mRNA accumulation in AFN pods (stage 7–8). *A.U.*, Arbitrary Unit (see Materials and methods); *T0*, start of the experiment; *L6* and *L75*, 6 and 75 h of light exposure; *D6* and *D75*, corresponding dark controls. Each value is the mean of six independent repetitions of the experiment. *Bars* indicate the upper limit of the 95% confidence interval

Briggs 1990). The quantification of *pal* and *chs* transcript levels was achieved after RNA dot-blot hybridization (Fig. 7). The variability, shown by the confidence interval bars, seems to be related to the heterogeneous physiological state of the pods, which were randomly sampled on different plants. A significant increase ($\alpha = 0.05$) in both *pal* and *chs* mRNAs levels occurred 6 h after light treatment (Fig. 7) compared to the levels of control pods maintained in the dark. Six hours after the onset of light, the pods maintained in the dark did not significantly accumulate *pal* or *chs* mRNAs. These data strongly suggest that light-dependent anthocyanin biosynthesis is regulated at the transcription level in AFN pods.

Discussion

In bean, almost all of the organs can be pigmented. The patterning and the variety of the colours can be very complex and present differential organ and spatio-temporal specificities (Leaky 1988). However, while this kind of phenotype has already been studied in some detail at the genetic and molecular level in a few species (Dooner et al. 1991), this is not the case in *Phaseolus vulgaris*. The reason certainly resides in a lack of well-documented specifics of the genetics of this species (Dooner et al. 1991; Leaky 1988). Because we were confident that studying such a diversity of colour patterns would contribute to a better understanding of the plant pigmentation regulatory network, a well-characterized bean genotype was studied. This genotype (AFN) accumulates purple pigments in various organs: seed coats, hypocotyls, cotyledons, flowers and pods. A gene, *Anp*, is involved in the accumulation of the purple pigments in the pods (Gantet et al. 1991). *Anp* mutants are affected in the mottled purple colouration of the pods but develop a normal pigmentation in other organs. In this report, we present the characteristics of the purple-mottled phenotype in a genotype harbouring *Anp* as a dominant allele in order to further identify the molecular nature of this locus.

Three anthocyanidins, delphinidin, petunidin and malvidin, accumulate in the pigmented epidermal cells of pods. Previous reports have mentioned the presence of pelargonidin, cyanidin and delphinidin in the bean seed testa (Stanton and Francis 1966) and of malvidin in the seedlings (Nozzolilo 1971). An organ-specific differential regulation of the hydroxylation and methylation steps of the anthocyanin biosynthetic pathway might exist. This has already been shown in petunia where flower limbs accumulate 3′–5′ methoxylated anthocyanidin (malvidin) and flower tubes accumulate only 3′ methoxylated anthocyanidin (petunidin) (A.G.M. Gerats, personal communication). The anthocyanins were shown to accumulate in specialized subcellular structures of the epider-

mal cell layer of the pods. These structures have been reported in other plant species and are referred to as anthocyanoplasts, they may isolate the last enzymatic steps of anthocyanin biosynthesis from the unfavorable acidic environment of the vacuolar sap (Small and Pecket 1982; Yasuda and Shinoda 1989).

In AFN pods, the anthocyanin-dependent pigmentation is distributed as a mosaic in the epidermal cell layer that provides the basis for the mottled phenotype. It is not known whether the *Anp* locus is involved in this apparent random biosynthesis of the anthocyanins, since a mutation in this locus affects both aspects of the phenotype simultaneously (anthocyanin and mosaic). However, the purple-mottled phenotype has also been observed in the epidermal layer of AFN hypocotyls (P. Gantet, unpublished data) and this phenotype is unaffected by the *Anp* mutation. This suggests that the mottled patterning involves a locus other than from *Anp*. The analysis of crosses between genotypes harbouring either mottled or uniformly pigmented pods would be necessary to check this point. The mosaic distribution of anthocyanins observed in the pod epidermis favours a cell-autonomous process for the accumulation of these secondary metabolites in AFN. A cell-autonomous dependent accumulation of these pigments has already been reported in snapdragon (Jackson et al. 1991 a, b). The mechanism is not completely understood, but anthocyanin regulatory loci such as *del* or *R* appear also to be regulated at the transcriptional level in a cell-autonomous manner.

In AFN pods, anthocyanin accumulation is regulated to a certain extent by development and light. A developmental switch is necessary to allow anthocyanin biosynthesis under high fluence-rate white light. Such a switch for anthocyanin biosynthesis is also required in mustard seedlings (Steinitz et al. 1976). This switch seems to be independent of light because very young pods are unable to accumulate anthocyanins even if they are maintained under light for 5 days *in vitro*.

Once pods are competent to accumulate anthocyanin, the anthocyanin biosynthesis is dependent on light. In AFN pods, the genes coding for the different enzymes of the anthocyanin biosynthetic pathway are transcriptionally active. Steady-state level studies of *gpal2* and *chs* mRNA suggest that the transcription of these two genes could be regulated by high fluence-rate light, this is the case for the snapdragon corolla or maize kernel (Taylor and Briggs 1990; Dooner et al. 1991; Goodrich et al. 1992). In the *Anp*-deficient bean genotype 'La Victoire', no anthocyanin can be detected in the pod epidermis (Gantet et al. 1991). This suggests that the *Anp* locus could be a structural gene involved in the organ-specific anthocyanin accumulation. This case was already found in petunia, where the *po* mutant, affected on the CHIA gene promoter, is characterized by a lack of flavonoid production in the anthers but not in the petals (van Tunen et al. 1991). On the other hand, the *Anp* locus could encode a transcriptional regulatory protein similar to the *R* protein family. In maize, *R* is involved in the developmented light and spatio-temporal regulation of the anthocyanin biosynthesis (Taylor and Briggs 1990). Molecular tools are presently being used to identify the *Anp* locus in bean.

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