Theor Appl Genet (2004) 109:23–29
DOI 10.1007/s00122-004-1625-9

ORIGINAL PAPER

Yelena Borovsky · Michal Oren-Shamir · Rinat Ovadia · Walter De Jong · Ilan Paran

The *A* locus that controls anthocyanin accumulation in pepper encodes a *MYB* transcription factor homologous to *Anthocyanin2* of *Petunia*

Received: 3 December 2003 / Accepted: 2 February 2004 / Published online: 3 March 2004

Abstract Pepper plants containing the dominant *A* gene accumulate anthocyanin pigments in the foliage, flower and immature fruit. We previously mapped *A* to pepper chromosome 10 in the F_2 progeny of a cross between 5226 (purple-fruited) and PI 159234 (green-fruited) to a region that corresponds, in tomato, to the location of *Petunia anthocyanin 2* (*An2*), a regulator of anthocyanin biosynthesis. This suggested that *A* encodes a homologue of *Petunia An2*. Using the sequences of *An2* and a corresponding tomato expressed sequence tag, we isolated a pepper cDNA orthologous to *An2* that cosegregated with *A*. We subsequently determined the expression of *A* by Northern analysis, using RNA extracted from fruits, flowers and leaves of 5226 and PI 159234. In 5226, expression was detected in all stages of fruit development and in both flower and leaf. In contrast, *A* was not expressed in the sampled tissues in PI 159234. Genomic sequence comparison of *A* between green- and purple-fruited genotypes revealed no differences in the coding region, indicating that the lack of expression of *A* in the green genotypes can be attributed to variation in the promoter region. By analyzing the expression of the structural genes in the anthocyanin biosynthetic pathway in 5226 and PI 159234, it was determined that, similar to *Petunia*, the early genes in the pathway are regulated independently of *A*, while expression of the late genes is *A*-dependent.

Communicated by R. Hagemann

Y. Borovsky · I. Paran (✉)
Department of Plant Genetics and Breeding,
Agricultural Research Organization,
The Volcani Center, P.O. Box 6, 50250 Bet Dagan, Israel
e-mail: iparan@volcani.agri.gov.il
Fax: +972-3-9669642

M. Oren-Shamir · R. Ovadia
Department of Ornamental Horticulture,
Agricultural Research Organization,
The Volcani Center, P.O. Box 6, 50250 Bet Dagan, Israel

W. De Jong
Department of Plant Breeding,
Cornell University,
252 Emerson Hall, Ithaca, NY 14853, USA

Introduction

Anthocyanin pigments produce a range of colors in leaves, flowers, seeds and fruits. The biological functions of anthocyanins are diverse and include insect and bird attraction for pollination and seed dispersal and protection against photo-oxidative damage (Field et al. 2001; Winkel-Shirley 2001a). Anthocyanins comprise a major branch of flavonoid metabolism and their synthesis has been very well studied in *Petunia*, snapdragon and maize (Holton and Cornish 1995) and more recently in *Arabidopsis* (Winkel-Shirley 2001b). Mutations that alter pigmentation in these species allowed the identification of many structural and regulatory genes of the biosynthetic pathway.

The anthocyanin biosynthetic pathway branches off from the general phenylpropanoid pathway. The first enzyme of the pathway is chalcone synthase (CHS) that uses malonyl-CoA and 4-coumaroyl-CoA as substrates to form tetrahydroxychalcone. This chalcone is isomerized by chalcone isomerase (CHI) to form the flavanone naringenin, which is converted to dihydrokaempferol by flavanone 3-hydroxylase (F3H). This is subsequently hydroxylated by flavonoid 3′-hydroxylase (F3′H) or flavonoid 3′,5′-hydroxylase (F3′5′H) to form the dihydroflavonols DHQ and DHM. These colorless molecules are converted to colored anthocyanins by at least three steps involving the enzymes dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) and UDP glucose flavonoid 3-*O*-glucosyltransferase. Additional enzymes, such as anthocyanin rhamnosyltransferase (RT) and anthocyanin methyltransferase, further modify the anthocyanins.

Regulation of anthocyanin biosynthesis in *Petunia* involves a complex interaction among transcription factors and structural genes. *AN1* encodes a basic helix-loop-helix protein that directly activates DFR and a *MYB*

domain protein with unknown function (Spelt et al. 2000). *AN1* expression is dependent on other *R2R3 MYB*-domain genes (*AN2*, *AN4*) which activate transcription within the petal limb and anther, respectively. The activity of the latter transcription factors is regulated by a WD40 repeat protein AN11 (De Vetten et al. 1997). Additional factors such as JAF13 have been identified, although their exact function is not known. In maize, expression of the regulatory genes *r* and *c1* is required for transcriptional activation of all the structural genes in the pathway. In contrast, in snapdragon and *Petunia*, the pathway is controlled in two units. Mutations in the regulatory genes in *Petunia* indicate that the early genes in the pathway, i.e. CHS, CHI and F3H are expressed independently of the anthocyanin regulatory genes, while the expression of the late genes in the pathway, such as DFR, ANS and RT, are dependent on expression of the regulatory genes (Quattrocchio et al. 1993).

Petunia is a member of the Solanaceae family, which includes important crop species such as tomato, pepper, potato, eggplant and tobacco. Anthocyanin pigmentation is an important component of the natural variation in these species and affects traits such as immature fruit color in pepper and eggplant, tuber skin color in potato and flower color in potato, eggplant and pepper. Anthocyanin-related color mutations have been described in these Solanaceae species, e.g. the *F* and *I* loci that control flower and skin color in potato (Van Eck et al. 1993, 1994), the anthocyanin gainer (*ag*) locus in tomato (Rick and Butler 1956) and the *A* locus that controls anthocyanin accumulation in various parts of the pepper plant (Daskalov and Poulos 1994). Nevertheless, the genes controlling this variation have not yet been identified. We recently mapped *A* to pepper chromosome 10 in a region that corresponds to the chromosomal regions containing *ag* in tomato, *F* and *I* in potato and a major gene for the presence of anthocyanin in eggplant fruit (Ben Chaim et al. 2003; Doganlar et al. 2002; Pillen et al. 1996), indicating that these loci are likely to be orthologous. The mapping of structural and regulatory genes of the anthocyanin biosynthetic pathway in tomato (De Jong et al. 2003) indicated that *Petunia An2* mapped to chromosome 10, to the same region where the *A* locus mapped in pepper (Ben Chaim et al. 2003). This suggested *An2* as a candidate for *A*.

The objectives of the present study were: (1) to identify and clone the *A* locus, using mapping information of structural and regulatory genes of the anthocyanin pathway, (2) to determine the expression pattern of *A* and the structural genes in wild-type and mutant lines and (3) to characterize the molecular basis of variation at this locus in *Capsicum*.

Materials and methods

Cloning of *A*

To clone the pepper homologue of *An2*, we first identified a tomato expressed sequence tag (EST; cTOA12C2; http://www.sgn.cornell.edu) with high homology to *Petunia An2* (82% nucleotide identity) and then designed primers [5′-GGAGT*N*AGGAAAG-GT*N*CATGG-3′ (*N*=A+C+G+T), 5′-GTCATCTTTGTCTAATGT-GTTTGTG-3′] against two conserved regions in the *Petunia* and tomato sequences. These primers were used in RT-PCR with pepper RNA obtained from young purple fruit of the *C. annuum* inbred line 5226 (obtained from Dr. C. Shifriss, The Volcani Institute, Israel). For first strand cDNA synthesis, we used total RNA extracted with an RNeasy plant mini kit (Qiagen). The RNA was reverse-transcribed with avian myeloblastosis virus reverse transcriptase (CHIMERx), using random primers. The 460-bp amplified product of 5226 was cloned and sequenced. Cloning of all PCR products was done with the pDrive vector (Qiagen) and all sequences were determined at The Center for Genomic Technologies, The Hebrew University of Jerusalem. The sequence from the partial cDNA was used to design primers for rapid amplification of cDNA ends (RACE) analysis in order to clone a full-length pepper cDNA. RACE experiments were performed with the Smart RACE system of BD Bioscience (Clontech) according to the manufacturer's protocol. For 5′ and 3′ first-strand cDNA synthesis, we used the gene-specific primers 5′-CGTTTGCTGTTCTTCCCGGCAATC-TCCC-3′ and 5′-GCCGCATATAAAGAGAGGTGACTTTGG-3′, respectively. The PCR products from the RACE experiments were cloned and sequenced. The sequence of the full-length cDNA of *A* was deposited in the GenBank as accession AJ608992. In order to determine the genomic sequence of *A*, we used a forward primer that contained the start codon of the open reading frame (ORF; 5′-GATATCATGAATACTGCTATT-3′) and a reverse primer from the 3′ untranslated region (UTR; 5′-CTTACATTGAAGATG-CGTGGA-3′). These primers amplified a 1,690-bp fragment, using genomic DNA of 5226 as a template for PCR.

Genetic mapping

Two F_2 mapping populations that segregated for anthocyanin accumulation and were used to position *A* in the pepper genetic map were described by Ben Chaim et al. (2003). Briefly, the first population was derived from an inter-specific cross of *C. chinense* PI 159234 that does not accumulate anthocyanin and the *C. annuum* inbred line 5226 that has purple foliage, stem, flower and immature fruit. The second population was derived from the cross of *C. annuum* inbred line 100/63 that does not accumulate anthocyanin and the *C. annuum* line IL-579 that contains an introgression in chromosome 10 from *C. chinense* PI 152225 and has a purple anther filament. We showed that the genes that control flower and fruit color in the 5226 × PI 159234 cross and filament color in the 100/63 × IL 579 cross map to the same chromosome region and are likely to be allelic. A total of 295 F_2 plants from both populations were scored for anthocyanin accumulation and used to map the candidate gene for *A* by RFLP analysis, as described by Ben Chaim et al. (2001).

Expression analyses

Total RNA (10 μg) extracted from five stages of fruit development, flower petals and young leaves was used for Northern blot experiments. Hybridization probes from the anthocyanin biosynthetic pathway included: the full ORF of pepper *A*, *Petunia CHS* obtained from Dr. David Weiss (the Hebrew University of Jerusalem), tomato *DFR* (Z18277), *Petunia ANS* (X70786), *Petunia F3′5′H* (AF081575) and tomato putative *CHI* (EST cTOB11G7).

Expression of *A* and *CHI* was also determined by RT-PCR. For *A*, the primers 5′-CGTTTGCTGTTCTTCCCGGCAATCTCCC-3′ and 5′-GCCGCATATAAAGAGAGGTGACTTTGG-3′ were used for amplification. For *CHI*, we used the *Petunia* gene (X14589) and the tomato EST sequence to synthesize two primers from conserved sequences: 5′-GAGAGTGCTATTCC*W*TTTCT*R*G-3′ and 5′-CTGA*W*TCATCTT*Y*AG*M*GAAGC-3′ (*W*=A+T, *R*=A+G, *M*=A+C, *Y*=C+T). These primers amplified a 342-bp fragment from pepper fruit RNA. After cloning and sequencing this amplified

product, we synthesized two pepper-specific primers (5′-AAA-AGCTCCAAGGAGTTGACC-3′, 5′-ACCAGGTGGGAAGTTTT-CACT-3′) that amplify a 287-bp fragment from the *CHI* gene. Two primers from the pepper 18S ribosomal RNA gene (AA840641) were used as a positive control for RT-PCR: 5′-GTGCTCAAAG-CAAGCCTACGTC-3′ and 5′-GACTATGAAATACGAATGCCC-CC-3′.

Anthocyanin extraction and quantification

Samples of fresh pepper (0.1–0.3 g of skin with pericarp) were ground in liquid nitrogen and the pigments were extracted in the dark with 2 ml of cold methanol:water:acetic acid (11:5:1; Markham and Ofman 1993). Extracts were spun for 10 min at 22,000 *g*, leaving the anthocyanins in the supernatant. Further purifications were with equal volumes of hexane. Samples were then concentrated to 0.5 ml, hydrolyzed by boiling in 2 N HCl for 1 h and passed through a 0.45μm polyvinylidene difluoride filter (Nalgene).

Anthocyanidin composition was determined using a HPLC (Shimatzu, Japan) equipped with a LC-10AT pump, a SCL-10A controller and a SPD-M10AVP photodiode-array detector. Extracts were loaded onto a RP-18 column (Vydac 201TP54) and separated at 27 °C with the following solutions: (A) H_2O, pH 2.3 and (B) H_2O:MeCN:HOAc (107:50:40), pH 2.3. Solutions were applied as a linear gradient from a ratio of 4:1 (A:B) to 3:7 over 45 min, and held at a ratio of 3:7 for an additional 10 min at a flow rate of 0.5 ml/min. Anthocyanidins were identified by comparing both the retention time and the absorption spectrum at 250–650 nm with those of standard purified anthocyanidins.

Results

In order to determine the position of *Petunia An2* on the pepper genetic map, we used *An2* and a tomato EST (cTOA12C2) with high homology to *An2* as RFLP probes in two mapping populations. Southern blot analysis revealed a single-copy gene that completely co-segregated with *A* in 295 F_2 individuals (Fig. 1).

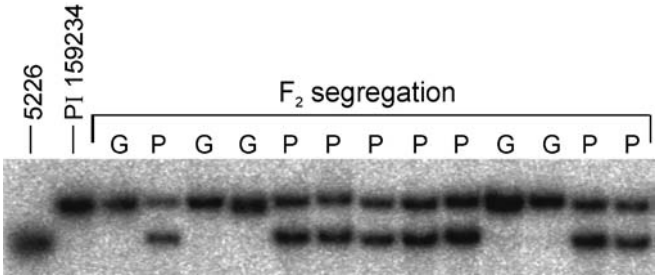

Fig. 1 Co-segregation of *Petunia An2* and pepper fruit color in an F_2 cross of 5226 × PI 159234. *G* DNA from green-fruited plant, *P* DNA from purple-fruited plant

Given the tight linkage between *An2* and *A* and the parallel nature of the phenotypes controlled by these loci in *Petunia* and pepper, it was strongly indicated that the two genes are orthologous. Therefore, we cloned the orthologue of *An2* from pepper. Using primers from a region conserved in *Petunia AN2* and a homologous tomato EST, we were able to amplify a partial cDNA from pepper fruit. Subsequently, by means of RACE analysis, we isolated a full-length pepper cDNA.

Pepper *A* has an ORF of 786 bp, encoding a protein of 262 amino acids, that is preceded by a 5′UTR of 69 bp and followed by a 3′UTR of 238 bp (Fig. 2A). Comparison of the nucleotide and protein sequence of *A* with other sequences in GenBank indicated that it shares the highest degree of homology with *Petunia AN2* and the recently isolated tomato *ANT1* (61% and 56% amino acid identity, respectively; Mathews et al. 2003; Quattrocchio

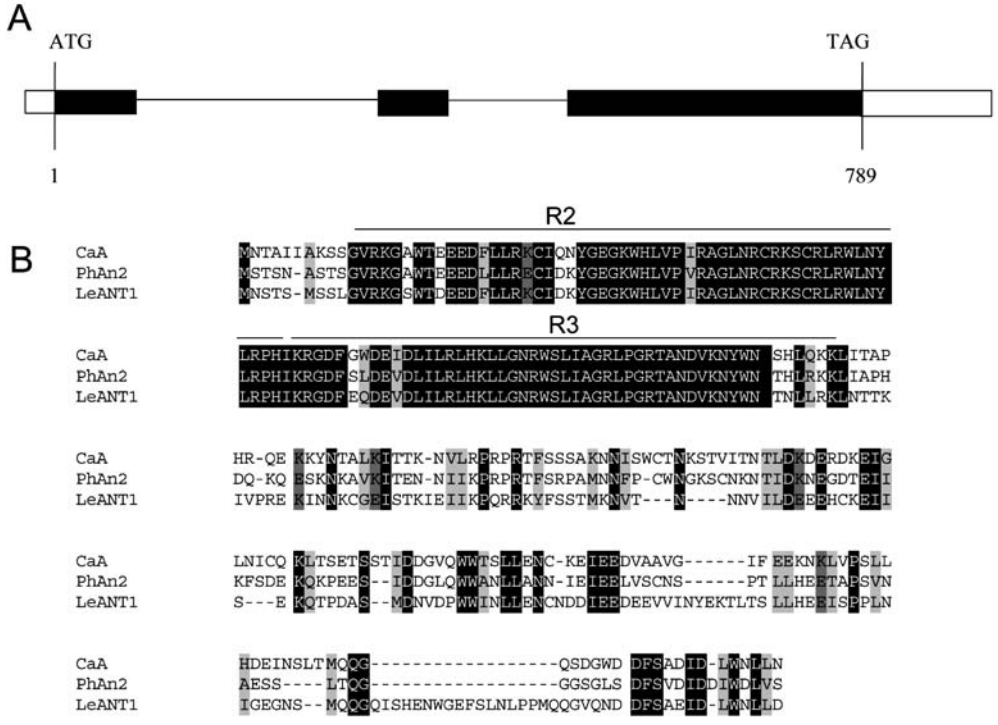

Fig. 2A, B Structure of *A* and its alignment to other Solanaceae *MYB* genes. **A** Schematic diagram of *A* showing the coding region (*black boxes*), 5′ and 3′ un-translated regions of the cDNA (*white boxes*) and introns (*solid lines*). **B** Comparison of deduced amino acid sequences of three *R2R3 MYB*-domain genes. *CaA Capsicum annuum A*, *PhAn2 P. hybrida An2*, *LeANT1 Lycopersicon esculentum ANT1*. *Black color* indicates identical amino acids, *gray color* indicates similar amino acids. *Lines* above the sequence indicate the position of the *R2* and *R3 MYB* domains

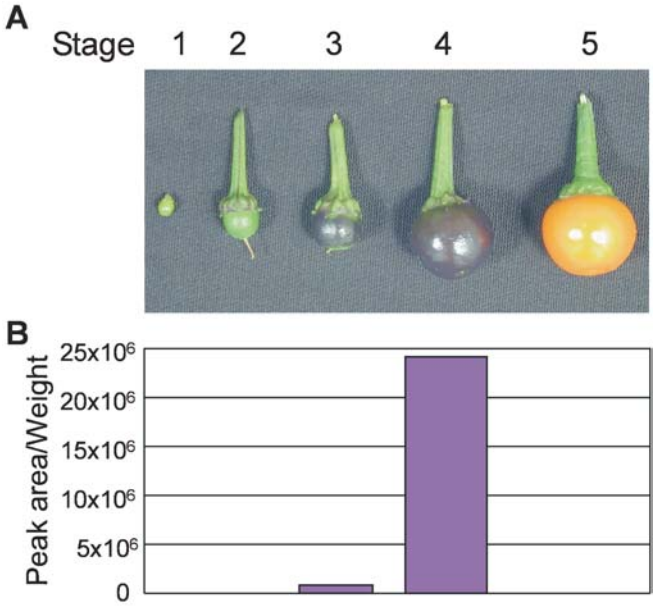

Fig. 3A, B Fruit color change and anthocyanin accumulation during fruit development of 5226. **A** Fruit color changes from anthesis to ripening. **B** Anthocyanin accumulation, measured by HPLC, from anthesis to ripening. Developmental stages: *stage 1* ovary at anthesis, *stage 2* young fruit at 5 days post-anthesis, *stage 3* young fruit at 10 days post-anthesis, *stage 4* mature unripe fruit, *stage 5* ripe fruit

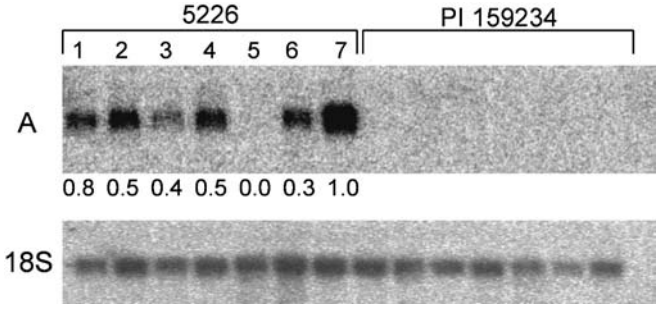

Fig. 4 Northern blot analysis of *A* in purple-fruited (5226) and green-fruited (PI 159234) genotypes. Stages of development and tissues: *lane 1* ovary at anthesis, *lane 2* young fruit at 5 days post-anthesis, *lane 3* young fruit at 10 days post-anthesis, *lane 4* mature unripe fruit, *lane 5* ripe fruit, *lane 6* flower petal, *lane 7* leaf. *Numbers* below each lane in the image of hybridization with *A* represent the relative transcript abundance of *A*, as quantified by densitometry of the images of *A* and 18S rRNA produced by a phosphor imager (Fuji FLA 5000) and normalized for RNA loading, using Image Gauge ver. 3.3 software

et al. 1999; Fig. 2B). As with other *MYB*-domain genes, the *R2R3 MYB* domain in the N-terminal half of the proteins was highly conserved among the three solanaceous genes, while little conservation existed in the C-terminal half of the proteins. Comparison of the genomic DNA and cDNA sequences of *A* revealed the presence of two introns in the gene. The first intron of 441 bp resides between amino acids 45 and 46 in the *R2* domain of the *MYB* gene and the second intron of 254 bp resides between amino acids 87 and 88 in the *R3* domain.

During fruit development of the purple-fruited parent 5226 after anthesis, the ovaries and young fruits initially remain green. Only after about 10 days post anthesis does anthocyanin become visible, peaking 20 days post-anthesis (Fig. 3A). At ripening, anthocyanin disappears and the fruit turns orange due to the accumulation of carotenoid pigments. In order to study the accumulation of anthocyanin and its relationship to the expression of *A* and structural genes in the anthocyanin biosynthetic pathway, we extracted anthocyanin from five stages of fruit development and analyzed it by HPLC. The predominant anthocyanidin in the pepper fruit was determined to be delphinidin. Its accumulation, as measured by HPLC, fit the visible developmental pattern, indicating that anthocyanin is not masked by carotenoids in ripe fruit but is rather degraded at this stage (Fig. 3B). No anthocyanin was detected either by eye or by HPLC in the green-fruited parent PI 159234 during any stage of fruit development.

By extracting total RNA from the five stages of fruit development of both parents, we were able to determine the mode of expression of *A* by Northern analysis (Fig. 4). In 5226, transcript accumulation was detected in all stages of fruit development, except in ripe fruit, and in flower petals and leaves. Due to the low level of expression of *A*, we did not detect a hybridization signal in ripe fruit by Northern analysis. However, using RT-PCR we were able to detect transcript also in this stage (data not shown). The highest expression level of *A* during fruit development was detected in ovaries, prior to the accumulation of anthocyanins. A high level of expression was also detected in leaf tissue.

In contrast to 5226, no transcript was detected in any stage of fruit development, flower or leaf in PI 159234 using either Northern or RT-PCR analyses. Similar results were obtained for another green-fruited genotype Cv. 'Maor' (data not shown). The lack of expression of *A* in PI 159234 and 'Maor' can explain the absence of anthocyanin accumulation in these genotypes. In order to determine whether changes in the coding region of *A* in the green-fruited parents could account for the lack of detectable expression, we PCR-amplified the *A* gene using genomic DNA from these genotypes. The length of the PCR product in PI 159234 and 'Maor' was indistinguishable from that in 5226, indicating that no major rearrangements occurred in the coding region of the recessive allele at *A*. The amplified product from 'Maor' was cloned and sequenced, but no sequence variation was detected, compared with 5226. A sequence comparison was performed between 5226 and 'Maor' in order to avoid potential confounding effects caused by interspecific differences. Both 'Maor' and 5226 belong to *C. annuum*, while PI 159234 belongs to *C. chinense*.

In order to determine whether the early and late genes in the anthocyanin biosynthetic pathway are co-regulated by *A* during pepper fruit development and in other anthocyanin-accumulating tissues, we analyzed the ex-

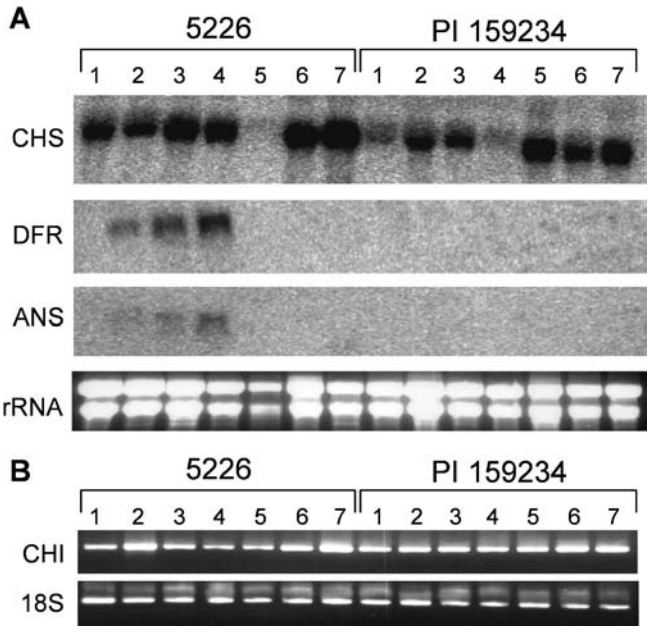

Fig. 5A, B Expression of structural genes of the anthocyanin biosynthesis pathway in purple-fruited (5226) and green-fruited (PI 159234) genotypes. **A** Northern blot analysis using probes of *CHS*, *DFR* and *ANS*. Ethidium bromide-stained gel is shown to indicate the approximately equal loading of RNA. **B** RT-PCR analysis for *CHI*. 18S rRNA primers were used as a control for amplification. Stages of development and tissues: *lane 1* ovary at anthesis, *lane 2* young fruit at 5 days post-anthesis, *lane 3* young fruit at 10 days post-anthesis, *lane 4* mature unripe fruit, *lane 5* ripe fruit, *lane 6* flower petal, *lane 7* leaf

pression of selected genes in the pathway in 5226 and PI 159234 by Northern or RT-PCR analysis. The early genes *CHS* (Fig. 5A) and *CHI* (Fig. 5B) were detected in all tissues in both parents. For both *F3′5′H* and *CHI*, we could not obtain a hybridization signal by Northern analysis. To overcome this for *CHI*, we cloned a partial *CHI* cDNA from pepper and used its sequence to design primers for RT-PCR analysis. Transcripts of the late genes *DFR* and *ANS* were detected in 5226 but not in PI 159234. Transcripts of both genes were first detected about 5 days after anthesis, reached a maximum level in mature unripe purple fruit and then declined in ripe fruit. These results indicate that, similar to *Petunia*, the early genes in the pathway are regulated independently of *A* expression, while expression of the late genes is *A*-dependent.

Discussion

The genetics and biochemistry of anthocyanin biosynthesis and regulation have been studied extensively in model species, such as *Petunia*, *Arabidopsis* and maize (Holton and Cornish 1995; Winkel-Shirley 2001b). However, the genes that control natural variation in anthocyanin accumulation are not known in most crop plants. In the vegetable crops of the Solanaceae family, several mutants impaired in anthocyanin biosynthesis have been known for many years, but few of the genes controlling this variation have been cloned. Recently, through genetic mapping in tomato and the use of comparative maps of other Solanaceae, several pigmentation genes were identified as potential candidates for known color mutants in tomato, potato, pepper and eggplant (De Jong et al. 2003). In addition, T-DNA activation-tagging experiments in tomato identified a transcriptional regulator of anthocyanin biosynthesis, termed *ANT1*, that has high homology with *Petunia An2* (Mathews et al. 2003).

Our linkage analysis, expression pattern and phenotypic similarity with *Petunia* anthocyanin mutants, collectively, indicate that the pepper *MYB* gene isolated by us is a likely candidate of the *A* mutation. However, we cannot exclude the possibility that, despite our strong evidence for such association, *A* could be controlled by another tightly linked gene. A final proof for the function of the putative *A* gene will require complementation of the mutant phenotype by the wild-type gene.

RFLP analysis indicated that *A* is present as a single gene in the pepper genome, similar to *An2* in *Petunia* (Quattrocchio et al. 1999). This result is in contrast to recent mapping data in tomato, in which *Petunia An2* was mapped to two linked loci in a region in which the *anthocyanin gainer* (*ag*) mutation is mapped (De Jong et al. 2003). Similarly, the *F* and *I* loci that control anothocyanin accumulation in flower and tuber skin of potato are also linked but considered as distinct loci (Dodds and Long 1956).

The mode of expression of the anthocyanin biosynthesis genes in purple and green pepper genotypes indicated that the transcription of only the late genes in the pathway are dependent on the expression of *A*, similar to the expression pattern observed in *Petunia* (Quattrocchio et al. 1999). In contrast, tomato *ANT1* was found to carry a broader regulatory function, as both early and late genes in the pathway and additional genes involved in modification and transport of anthocyanins were up-regulated when *ant1* was over-expressed (Mathews et al. 2003). A similar response was obtained with an activation-tagging mutant, *pap1-D*, isolated in *Arabidopsis*, in which over-expression of a *R2R3 MYB* regulator related to *Petunia An2* induced activation of both early and late genes in the phenylpropanoid pathway (Borevitz et al. 2000). This broad transcriptional activation of the pathway in activation-tagging mutants therefore appears to overcome the stringent control on anthocyanin structural gene expression during plant development.

Our examination of two unrelated non-pigmented mutants belonging to two different *Capsicum* species revealed that *A* is not expressed in either species. The lack of divergence in the gene sequence between the wild-type and mutant alleles implicated that differences in the promoter region are the likely cause of differences in *A* gene expression. Preliminary results of determining the level of expression of *A* by real time RT-PCR in purple genotypes with varying degrees of anthocyanin pigmentation showed a positive correlation between the level of

anthocyanin pigmentation and the expression level of *A*. Similarly, in genotypes (such as *C. frutescens* BG-2816) in which pigmentation is tissue-specific (e.g. the fruit is pigmented but the flower is not) the presence or absence of anthocyanin is correlated with the expression of *A*. This indicates that *A* is the major determinant of variation in anthocyanin accumulation in pepper. We previously reported that pigmentation of different tissues, such as fruit, flower, leaf and stem, in 5226 is controlled by the *A* locus. The only tissue in which anthocyanin pigmentation is not controlled by *A* is the anther. Odland (1960) concluded that anther pigmentation is controlled by an independent gene from *A*, designated *S*. This situation parallels *Petunia*, in which pigmentation of the corolla and tube is controlled by *An2* but pigmentation of the anther is controlled by an as yet un-cloned regulator, *An4* (Quattrocchio et al. 1998). Linkage data reported by the latter study indicated that *An4* and the transcription regulator *jaf13* are linked to each other in *Petunia*. Because *jaf13* was mapped to chromosome 8 in tomato (De Jong et al. 2003), it will be possible to test whether *S* is mapped in chromosome 8 of pepper, which may suggest a possible correspondence of pepper *S* and *Petunia An4*.

The A protein belongs to a family of *R2R3 MYB*-domain genes that act as regulators of diverse developmental processes. In *Arabidopsis*, 125 members of this family were detected and their function is starting to be unraveled, using knockout mutants (Stracke et al. 2001). Within the Solanaceae, the *blind* gene that controls the formation of lateral meristems was recently cloned and shown to be a *R2R3 MYB* gene (Schmitz et al. 2002). Searching GenBank with the sequence of *A* revealed no hits from pepper, indicating that *A* is the first *R2R3 MYB* gene isolated from this genus.

Most of the information on the synthesis and regulation of the anthocyanin biosynthesis pathway originated from studies on flower pigmentation in the model species *Antirrhinum* and *Petunia*. In contrast, very little is known about the expression and regulation of the pathway in fruit. In a few studies, such as in strawberry and blueberry, some of the structural genes were isolated and their expression was determined during fruit development (Jaakola et al. 2002; Moyano et al. 1998). A *MYB*-domain regulatory gene was isolated from strawberry that acts as a repressor of transcription of the anthocyanin pathway (Aharoni et al. 2001). *MYB*-domain genes were also isolated from grapes and shown to regulate anthocyanin biosynthesis (Kobayashi et al. 2002). Because of the natural variability in anthocyanin pigmentation, the *Capsicum* genus offers an excellent system to study the pathway in fruit. To date, except for *A*, no genes from the anthocyanin biosynthetic pathway or their regulators have been isolated in pepper. Because these genes are known in the solanaceous species *Petunia*, it will be a relatively simple task to isolate them in pepper and in other Solanaceae by exploiting the sequence similarity among these genomes. The isolation of additional regulatory genes from pepper will make it possible to determine their role in controlling the large amount of variation of anthocyanin pigmentation in pepper fruit.

Acknowledgements This research was supported by the United States–Israel Binational Agricultural Research and Development (BARD) Fund Grant No. IS-3225-01C and by a subcontract with Cornell University, Department of Plant Breeding, under Prime Agreement 2001-52101-113347 from the USDA Cooperative State Research Education and Extension Service.

References

Aharoni A, De Vos CH, Wein M, Sun Z, Greco R, Kroon A, Mol JN, O'Connell AP (2001) The strawberry FaMYB1 transcription factor suppresses anthocyanin and flavonol accumulation in transgenic tobacco. Plant J 28: 319–332

Ben Chaim A, Paran I, Grube R, Jahn M, Wijk R van, Peleman J (2001) QTL mapping of fruit related traits in pepper (*Capsicum annuum*). Theor Appl Genet 102:1016–1028

Ben Chaim A, Borovsky E, De Jong W, Paran I (2003) Linkage of the *A* locus for the presence of anthocyanin and *fs10.1*, a major fruit-shape QTL in pepper. Theor Appl Genet 106:889–894

Borevitz JO, Xia Y, Blount J, Dixon RA, Lamb C (2000) Activation tagging identifies a conserved *MYB* regulator of phenypropanoid biosynthesis. Plant Cell 12:2383–2393

Daskalov S, Poulos JM (1994) Updated *Capsicum* gene list. Capsicum Eggplant Newsl 13:15–26

De Jong WS, Eannetta NT, De Jong DM, Bodis M (2003) Candidate gene analysis of anthocyanin pigmentation loci in the Solanaceae. Theor Appl Genet Vol 108:423–432

De Vetten N, Quattrocchio F, Mol J, Koes R (1997) The *an11* locus controlling flower pigmentation in petunia encodes a novel WD-repeat protein conserved in yeast, plants, and animals. Genes Dev 11:1422–1434

Dodds KS, Long DH (1956) The inheritance of colour in diploid potatoes. II. Three-factor linkage analysis. J Genet 54:27–41

Doganlar S, Frary A, Daunay MC, Lester RN, Tanksley SD (2002) Conservation of gene function in the Solanaceae as revealed by comparative mapping of domestication traits in eggplant. Genetics 161:1713–1726

Field TS, Lee DW, Holbrook NM (2001) Why leaves turn red in autumn. The role of anthocyanins in senescing leaves of red-osier dogwood. Plant Physiol 127:566–574

Holton TA, Cornish EC (1995) Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell 7:1071–1083

Jaakola L, Maatta K, Pirttila AM, Torronen R, Karenlampi S, Hohtola A (2002) Expression of genes involved in anthocyanin biosynthesis in relation to anthocyanin, proanthocyanidin, and flavonol levels during bilberry fruit development. Plant Physiol 130:729–739

Kobayashi S, Ishimaru M, Hiraoka K, Honda C (2002) *Myb*-related genes of the Kyoho grape (*Vitis labruscana*) regulate anthocyanin biosynthesis. Planta 215:924–933

Markham KR, Ofman DJ (1993) *Lisianthus* flavonoid pigments and factors influencing their expression in flower colour. Phytochemistry 34:679–685

Mathews H, Clendennen SK, Caldwell CG, Liu XL, Connors K, Matheis N, Schuster DK, Menasco DJ, Wagoner W, Lightner J, Wagner DRY (2003) Activation tagging in tomato identifies a transcriptional regulator of anthocyanin biosynthesis, modification, and transport. Plant Cell 15:1689–1703

Moyano E, Portero-Robles I, Medina-Escobar N, Valpuesta V, Munoz-Blanco J, Caballero JL (1998) A fruit-specific putative dihydroflavonol 4-reductase gene is differentially expressed in strawberry during the ripening process. Plant Physiol 117:711–716

Odland ML (1960) Inheritance of flower color in *Capsicum annuum* L. Proc Am Soc Hortic Sci 76:475–481

Pillen K, Pineda O, Lewis CB, Tanksley SD (1996) Status of genome mapping tools in the taxon Solanaceae. In: Paterson

AH (ed) Genome mapping in plants. Landes, Austin, pp 281–307

Quattrocchio F, Wing JF, Leppen TC, Mol JNM, Koes RE (1993) Regulatory genes controlling anthocyanin pigmentation are functionally conserved among plant species and have distinct sets of target genes. Plant Cell 5:1497–1512

Quattrocchio F, Wing JF, Woude K van der, Mol JNM, Koes R (1998) Analysis of bHLH and MYB domain proteins: species-specific regulatory differences are caused by divergent evolution of target anthocyanin genes. Plant J 13:475–488

Quattrocchio F, Wing J, Woude K van der, Souer E, Vetten N de, Mol J, Koes R (1999) Molecular analysis of the *anthocyanin 2* gene of petunia and its role in the evolution of flower color. Plant Cell 11:1433–1444

Rick CM, Butler L (1956) Cytogenetics of the tomato. Adv Genet 8:267–382

Schmitz G, Tilmann E, Carriero F, Fiore C, Cellini F, Theres K (2002) The tomato *blind* gene encodes a *MYB* transcription factor that controls the formation of lateral meristems. Proc Natl Acad Sci USA 99:1064–1069

Spelt C, Quattrocchio F, Mol JNM, Koes R (2000) *anthocyanin 1* of petunia encodes a basic helix-loop-helix protein that directly activates transcription of structural anthocyanin genes. Plant Cell 12:1619–1631

Stracke R, Werber M, Weisshaar B (2001) The *R2R3-MYB* gene family in *Arabidopsis thaliana*. Curr Opin Plant Biol 4:447–456

Van Eck HJ, Jacobs JME, Dijk J van, Stiekema WJ, Jacobsen E (1993) Identification and mapping of three flower colour loci of potato (*S. tuberosum* L.) by RFLP analysis. Theor Appl Genet 86:295–300

Van Eck HJ, Jacobs JME, Stam P, Ton J, Stiekema WJ, Jacobsen E (1994) Multiple alleles for tuber shape in diploid potato detected by qualitative and quantitative genetic analysis using RFLPs. Genetics 137:303–309

Winkel-Shirley B (2001a) Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol 126:485–493

Winkel-Shirley B (2001b) It takes a garden. How work on diverse plant species has contributed to an understanding of flavonoid metabolism. Plant Physiol 127:1399–1404