Plant Mol Biol Rep (2011) 29:43–50
DOI 10.1007/s11105-010-0207-z

Regulation of Pigment-Related Genes During Flower and Fruit Development of *Bixa orellana*

Norma L. Rodríguez-Ávila · José A. Narváez-Zapata · Margarita Aguilar-Espinosa · Renata Rivera-Madrid

Published online: 20 April 2010

Abstract Annatto (*Bixa orellana*) is the sole source of the commercially important pigment bixin. The broad diversity of annatto phenotypes and the wide variation in pigment contents have restricted its use. Therefore, genetic improvement focusing on this variation is important in order to achieve optimal exploitation of annatto. To characterize and understand some of the genetic basis governing carotenoid production, two annatto cultivars with either pink or white flowers were selected on the basis of their contrasting pigment levels. Several complementary DNA (cDNA)-probes corresponding to genes involved in carotenoid biosynthesis (*dxs*, *psy*, *pds*, *β-lcy*, and *ε-lcy*) were cloned and analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR) in different tissues of these two annatto cultivars during flower and fruit development. In general, expression of most selected genes could be associated with pigment overaccumulation in these cultivars. The pink cultivar exhibited higher carotenoid contents and higher pigment-related gene expression, and the two variants analyzed showed notable differences in gene expression in the highest pigment producing tissues.

Keywords Annatto · *Bixa orellana* · Bixin · Carotenoids · Gene expression

N. L. Rodríguez-Ávila · M. Aguilar-Espinosa · R. Rivera-Madrid (✉)
Centro de Investigación Científica de Yucatán, A. C.,
Calle 43 No. 130, Col. Chuburná de Hidalgo,
97200 Mérida, Yucatán, México
e-mail: renata@cicy.mx

J. A. Narváez-Zapata
Centro de Biotecnología Genómica—Instituto Politécnico Nacional,
Boulevard del Maestro S/N esq. Elías Piña,
Col. Narciso Mendoza,
88710 Reynosa, Tamaulipas, México

Introduction

Carotenoids are derived from the isoprenoid biosynthetic pathway and are responsible for many of the red, orange, and yellow colors of plant flowers, fruits, and leaves (Wagner et al. 2002). In plants, the carotenoid pathway begins with the synthesis of the isoprenoid isopentenyl pyrophosphate (IPP; Fig. 1). The biosynthesis of IPP is catalyzed by 1-deoxy-D-xylulose-5-phosphate synthase (DXS) coded by the *dxs* gene (Lichtenthaler 1999). Subsequent biosynthesis of carotenoids from IPP involves a common route including their conversion into geranylgeranyl diphosphate (GGPP), which is the substrate for formation of the phytol (chlorophyll side chain) and the C_{40} molecule phytoene. Phytoene formation is catalyzed by phytoene synthase (PSY) coded by the *psy* gene and is considered the first regulatory step in carotenogenesis. Subsequently, through desaturation, catalyzed by two desaturase enzymes, phytoene desaturase (PDS) and ζ -carotene desaturase (ZDS), the colorless phytoene is transformed to the pink–red lycopene (Isaacson et al. 2002). Consequently, lycopene can be transformed into different lineal apocarotenoids such as crocetin and bixin, which are commercially valuable (Pfander and Schurtenberger 1982; Bouvier et al. 2003). In plants, two enzymes lead the cyclization of lycopene: lycopene-ε-cyclase (E-LCY) coded by *ε-lcy* and involved with the production of α-carotene and lycopene-*β*-cyclase (B-LCY) coded by *β-lcy*, which converts lycopene into β-carotene (Cunningham et al. 1996). This last cyclic molecule can be modified by the action of dioxigenases, hydroxylases, and epoxidases to produce physiologically important *β*-apocarotene such as abscisic acid (ABA; Cunningham 2002).

Annatto (*Bixa orellana*) is a tree–shrub native to the neotropics with seeds that contain high quantities of

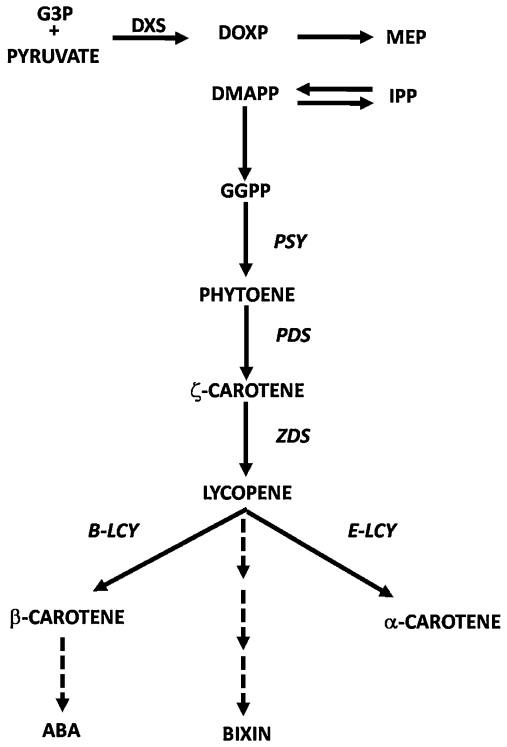

Fig. 1 Simplified carotenoid biosynthetic pathway. Lycopene can be directed to the production of lineal carotenoids such as bixin or cyclic carotenoids through the action of alpha and beta lycopene cyclase enzymes. *G3P* glyceraldehyde-3-phosphate, *DXS* 1-deoxy-D- xylulose-5-phosphate synthase, *DOXP* 1-deoxy-D-xylulose-5-phosphate, *MEP* 2-C-methyl-D-erythritol-2,4-cyclodiphosphate

pigments, the apocarotenoid bixin being the most abundant (Bittencourt et al. 2005a). Bixin is the second most commonly used natural industrial colorant (Giuliano et al. 2003). Nevertheless, the demand for bixin is not met by its supply because of the heterogeneity of bixin concentrations present in different commercial variants of annatto (Rivera-Madrid et al. 2006). The genetic regulation of the main pigment-related genes during the annatto plant's development and the accumulation of pigments still remain unknown. This has limited the effort made to classify the annatto cultivars based on bixin content and morphological traits.

In the present study, the transcriptional expression of five key genes, *dxs*, *psy*, *pds*, *ε-lyc*, and *β-lyc*, encoding enzymes related to carotenoid biosynthesis were investigated in two cultivars of *B. orellana* with white or pink flowers and contrasting bixin accumulation in their mature seeds (Rivera-Madrid et al. 2006). The analysis was complemented by total carotenoids and specific carotenoid-related molecule recorders. The expression response of these genes in both annatto plants could provide insights into the understanding of the molecular basis of carotenoid biosynthesis and accumulation during the genetic improvement process in this plant.

Materials and Methods

Plant Material

Samples of leaf, floral bud, open flower, immature fruit, immature seed, and mature seed tissues (from mature and dehiscent fruit) were taken from two *B. orellana* cultivars. These samples were collected from March to April of 2008 at a commercial plantation in Chicxulub, Yucatán, México. These variants were characterized as pink variant and white variant based on morphological and reproductive characteristics (Rivera-Madrid et al. 2006). Fresh tissues were photographically documented (Fig. 2) and then immediately frozen in liquid nitrogen and kept at −80°C for use in gene expression and pigment analysis.

Metabolite Analysis

Total carotenoids were measured using a spectrophotometric analysis by extracting pigments from 10 mg of freeze-dried tissues with 10 ml of chloroform in semi-darkness in an ice water bath. Each mixture was shaken vigorously for 5 min, filtered through a 0.45-μm nylon membrane and transferred to a 15-ml tube. Then, 100 μl of each extract was adjusted to 3 ml of chloroform and then measured with a spectrophotometer (resolution range, 1–4 nm from 400–700 nm; DU 650 Beckman Coulter). Extracts were measured at A_{404} to A_{480} nm, and the content was calculated using the equation reported by Wellburn (1994). Both high-performance liquid chromatographic (HPLC) and spectrophotometric results were the mean of three replicates; each was measured three times ± the standard error.

Specific carotenoid-related molecules, such as bixin, β-carotene, chlorophyll *a* and ABA were extracted from the different powdered tissues (0.1 g of tissues) with 1 ml of acetonitrile/methanol/isopropanol 75:10:15 (*v*/*v*/*v*) in a 1.5 ml microcentrifuge tube. Tubes were vortexed and centrifuged at 14,000 rpm for 10 min (Eppendorf Model 5414; Brinkmann Instruments, Inc., Westbury, NY), and the supernatant was filtered using Millex-GV13 (Durapore PVDF, 13-mm diameter and 0.22-μm pore size, Millipore, Billerica, MA). From this mixture, 25 μl was applied to a Hypersil ODS C-18 reverse phase column (25 cm×4.6 mm; 5-μm diameter beads) with a flow rate of 1 ml min^{-1}. The mobile phase consisted of solvent A (acetonitrile/methanol/isopropanol [75:10:15 by volume]) and solvent B (tetrahydrofuran), and the column was developed as follows: step 1, 100% solvent A at injection, for 15 min; step 2, linear increase to 100% solvent B in 4 min; step 3, 100% solvent B for 16 min; step 4, return to 100% solvent A for 1 min; step 5, 100% solvent A for 25 min. Calibration curves were constructed using pigment standards (Sigma) and were identified based on their column retention time relative to

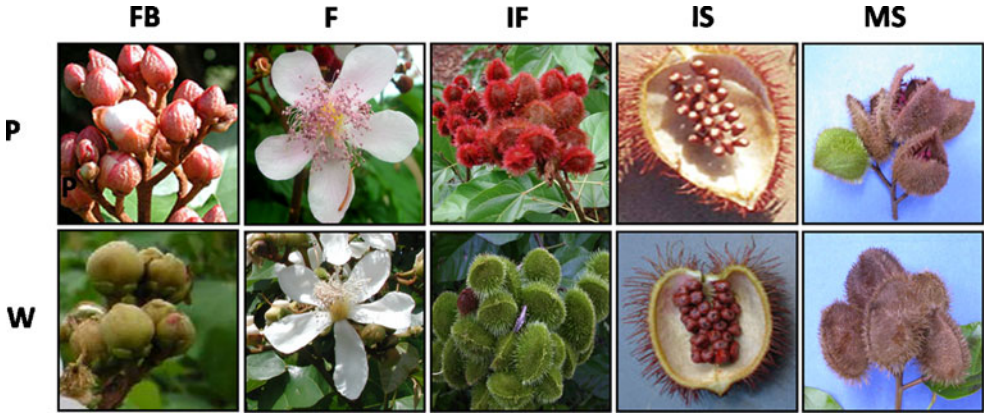

Fig. 2 Morphological characteristics and bixin production for floral and fructification organs in two annatto cultivars. *P* pink cultivar with pink flowers, red floral buds, and red fruit capsules; *W* white cultivar with white flowers, green floral buds, and green fruit capsules. *L* leaves, *FB*, floral buds, *F*, flower, *IF* capsules of mature fruit, *IS* immature seeds, and *MS* mature seeds

known standards and the absorption spectra of individual peaks.

Isolation of Pigment-Related Probes

Five genes encoding for key carotenoid biosynthesis enzymes were isolated from cDNA using PCR amplification with different sets of degenerate primers (Table 1). These primers were designed by aligning conserved regions of orthologous genes of other plants. Although unspecific amplifications were obtained in most cases, it was possible to obtain PCR products with high identities to the genes analyzed in this study. Sequence information of these PCR fragments was used to design a new set of specific primers to carry out the RT-PCR expression analysis (see Table 1). The cDNA was synthesized from messenger RNA (mRNA) isolated from 30-day-old *Bixa orellana* seedlings. All the amplifications were performed with 50 μl of PCR reaction mix containing 5 μl of reaction buffer, 5 units of *Taq* polymerase (Gibco-BRL, Rockville, MD), 1.5-mM $MgCl_2$, 50 pmol of each primer, 120-μM deoxyribonucleotide triphosphates (dNTPs) and 200 ng of template cDNA. The PCR reaction conditions (except for annealing temperature; see Table 1) were similar for all amplifications: initial denaturation at 94°C for 3 min, 35 cycles at 94°C (1.5 min) for denaturation, different annealing temperatures according to primer combination (1.5 min), 72°C (1.5 min) as extension temperature, and a final extension at 72°C for

Table 1 Gene probes of pigment-related genes isolated from *Bixa orellana* L. and PCR conditions

Gene probe	Accession numbers	Closest orthologous gene	Identity (%)	Primer set used for	Primer sequence	Temperature (°C)
dxs	AF479589	*DXS-Arabidopsis thaliana* (U27099)[a]	99	Cloning	5′TGGGCNATHTAYGTNTGCTG3′ 5′AARTTRTTRTARTCRTTNGCYTC3′	55
				Expression analysis	5′GGGAAGGATATGACGGGATG3′ 5′ATCATCTACTTCAGCTTCTCT3′	60
psy	AF196963	*PSY-Arabidopsis thaliana* (BT000450)	99	Cloning	5′TGGGCNATHTAYGTNTGCTG3′ 5′AARTTRTTRTARTCRTTNGCYTC3′	56
				Expression analysis	5′GGACCAGCATATGAGGCCATG3′ 5′ATCATCTACTTCAGCTTCTCT3′	55
pds	DQ924534	*PDS-Carica papaya* (DQ779922)	86	Cloning	5′TGGAARGAYGAYGGNGAYTGG3′ 5′AAYAAYGARATGYTNACNTGGCC3′	
				Expression analysis	5′GGAAAGATGATGATGGAGATTG3′ 5′TGTTCTTCAGTTTTCTGTC3′	58
β-lcy	AY836587	*β-LCY-Citrus sinensis* (DQ496223)	86	Cloning	5′TGGCCNAAYAAYTAYGGNGTNTGG3′ 5′ATGGTDCAYCCHGCMACWGG3′	57
				Expression analysis	5′TGAGGCTATGGATTTGCTTG3′ 5′TTCGATTTCTCTCCCTCAGC3′	58
ε-lcy	DQ145558	*ε-LCY-Daucus carota* (DQ192192)	88	Cloning	5′TGGAARGAYGAYGGNGAYTGG3′ 5′ATSAARATRCAYTGCAT3′	55
				Expression analysis	5′GGTTTAATTGGCCCAGATCTTC3′ 5′TTCTCCACCTCAACCTCAATG3′	55

[a] Accession number

10 min. Different PCR products (see Table 1) were purified using the Promega Wizard PCR Preps Purification System (Promega, Madison, WI), cloned in a TA cloning kit (Invitrogen, San Diego, CA), and then sequenced in an automatic sequencer (Davis Sequencing, LLC). The resulting sequences were blasted manually against the current database (National Center for Biotechnology Information), and any homologies were confirmed with the CLUSTALW program (http://dot.imgen.bcm.tmc.edu:9331/multialign). Phylogenetic analysis was performed following the unweighted pair group method with arithmetic mean (UPGMA) method using the free CLC Sequence Viewer software (http://www.clcbio.com/).

Measurement of mRNA by Reverse Transcriptase-Mediated PCR

Total RNA for the RT-PCR analysis was isolated following the protocol reported by Rodríguez-Ávila et al (2009). The SuperScript III First-Strand Synthesis System for the RT-PCR kit (Invitrogen, San Diego, CA) was used to synthesize cDNA according to kit instructions. RNA concentration was measured spectrophotometrically, and 2 μg was added to the cDNA synthesis reaction. After reverse transcription, the cDNAs of *dxs psy*, *pds*, and *β*- and *ε*-lycopene cyclases were amplified by PCR. One-tenth (2 μl per reaction) of the final cDNA product volume was added to PCR reactions specific to each gene analyzed. PCR tubes specifically for amplification of individual carotenoid-related genes and containing all the reaction components except the template were prepared in advance and frozen at −80°C until use. Specific primer combination and melting temperature conditions are summarized in Table 1. A parallel reaction with 25 cycles and specific primers (5′-CGGCTACCACATCCAAGGAA-3′ and 5′-GCTGGAATTACCGCGGCT-3′) for 18 S rRNA (GenBank accession no. AF206868) was run as a constitutive control for each PCR reaction. Replicates of each PCR reaction were carried out to confirm the results. The PCR amplification products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. The relative amounts of mRNA were confirmed using a Bio-Image Analyzer GS25 (BioRad, Hercules, CA). Preliminary PCR products were sequenced to verify identity in all the cases.

Results

Metabolites Analysis

The concentration of total carotenoids varied in all the stages of development for the plants studied, with the highest concentration generally found in mature seeds (Fig. 3). In addition, some specific carotenoid-related molecules were also evaluated. In this way, chlorophyll *a* contents were measured as an indirect indicator of the photosynthetic process. The white cultivar exhibited greater accumulation of chlorophyll *a* than the pink cultivar. Green tissues of this annatto cultivar such as immature fruits and flower buds also accumulated high chlorophyll *a* contents. The pink cultivar only accumulated chlorophyll *a* in its

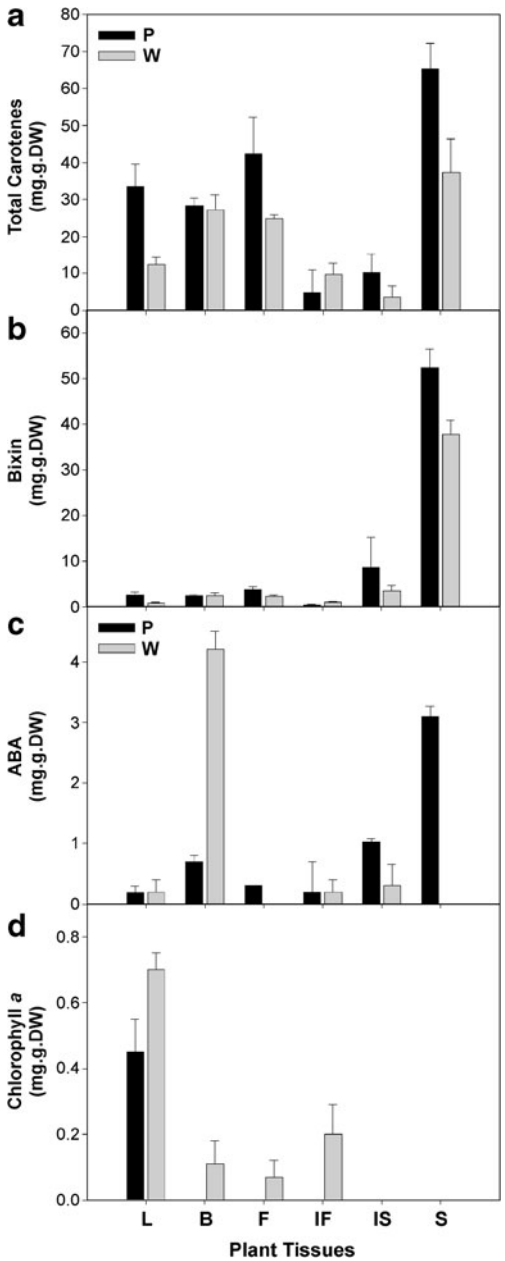

Fig. 3 Total carotenoids and specific carotenoid-related molecules in different plant tissues. **a** Total carotenoid contents, **b** bixin contents, **c** ABA contents, **d** chlorophyll *a* contents. Each value is the mean ± SD, n=3

leaves. Another important photosynthetic carotenoid pigment, β-carotene, was also measured. However, β-carotene contents were only detected in the leaves of both cultivars (data not shown). Specific carotenoid derivatives, such as apocarotenoids, were also evaluated. Significant differences in the bixin contents were observed in the two annatto cultivars (Fig. 3b). Pink cultivars exhibited higher bixin content in mature seeds even when bixin accumulation began at the immature seed stage. The contents of the β-apocarotenoid ABA were also evaluated in order to find out the role of the *β-lcy* gene in regulating the proportion of carotenoids directed to each branch of the carotenoid pathway and driving the carotenoid diversification observed in annatto plants (Fig. 3c). Thus, ABA presented higher concentrations in the tissues of the white variant and, in general, in those tissues of both annatto cultivars where the lowest bixin accumulation was observed.

Isolation of Pigment-Related Probes

Partial probes of the carotenoid-related genes *dxs*, *psy*, *ε-lcy*, and *β-lcy* from *B. orellana* L. were isolated using degenerate primers designed from conserved regions in orthologous genes of other plants. GenBank accession numbers are summarized in Table 1. All the resulting sequences were suitable probes due to their reliable size, high similarity when blasted with other orthologous genes, and high conservation levels when aligned with other carotenoid-related genes. When compared with sequences reported in the public database, the probes of this study were shown to have a percentage identity from 86% to 99%. *dxs* and *psy* probes exhibited the highest identity (99%) with respect to *dxs* and *psy* orthologous genes from *Arabidopsis thaliana*. *pds*, *β-lcy*, and *ε-lcy* also presented high levels of identity (86% to 88%) with other orthologous genes from plants (Table 1). The identity of probes was reflected in the UPGMA tree showing a short distance of 0.9 to 0.6 with reference to other plant orthologous genes (Fig. 4).

Pigment-Related Gene Expression

The *dxs* gene involved in the initial steps of the carotenoid pathway showed a constitutive expression in all tissues of different plant development stages (Fig. 5). Linear carotenoid-related genes such as *psy* and *pds* showed a differential expression during development stages in both annatto cultivars. The accumulation of *psy* transcripts was highest in immature seeds of the pink annatto cultivar. The white cultivar only showed a slight accumulation of this *psy* transcript. Likewise, *pds* transcripts were up-regulated during all development stages in the pink cultivar and presented their peak in expression in the immature seed stage. The white annatto cultivar also presented a *pds* peak in expression in immature seed tissues, but its expression profile was less prominent in other tissues than in its annatto counterpart.

Cyclic carotenoid-related genes such as *ε-* and *β-lcy* presented a very active expression in leaves and immature fruits in both annatto cultivars. Expression of *β-lcy* mRNA differed from that of *ε-lcy* mRNA among the different tissues analyzed and its expression profile was also notably different between both cultivars. Transcripts of this gene (*pds*) were detected in leaves, immature fruits, and immature seeds of the pink cultivar, whereas in the white cultivar, they were only detected in leaves, floral buds, and flowers, the more photosynthetic tissues (Fig. 5).

In general, all carotenoid-related genes analyzed, with the exception of the *ε-lcy* gene, showed a peak in expression in immature seeds, the tissue where the accumulation of pigments began. Moreover, these expression profiles seem to be more pronounced in the more pigment productive cultivar: the pink annatto cultivar.

Discussion

Pigment Measurement

Carotenoids and chlorophylls usually present a very close balance in photosynthetic plant tissues such as leaves and immature fruits (Kennis et al. 2001). Thus, chlorophyll *a* content was evaluated to investigate the possible role of total carotenoids in relation to photosynthetic process in the different tissues analyzed. The white cultivar exhibited differential chlorophyll *a* contents more related to all green tissues, displaying a similar profile with respect to total carotenoid accumulation. In contrast, chlorophyll *a* was only detected in leaves of the pink cultivar. Variable chlorophyll *a* content has been reported in the leaves of the closely related "Peruana CPATU" *B. orellana* L. variant (Matsumoto et al. 2002). β-carotene, an important photosynthetic pigment, was only detected in the leaves of both variants, suggesting a rapid turnover of this molecule into other carotenoid-related compounds. A difference in the chlorophyll *a* to ß-carotene ratio during developmental stages in this annatto cultivar suggests a different physiological role of these carotenoids to the photosynthetic process, as has previously been observed in green algae (Sanchez-Estudillo et al. 2006). It is also known in plants that carotenoid accumulation is linked to plant development stage (Merzlyak and Solovchenko 2002).

Annatto pigment content in seeds varies from 2% to 6% among different cultivars (Rivera-Madrid et al. 2006); however, the possible role of the different carotenoid-related genes in the pigment variation observed has not been assessed to date. Quantification of carotenoid-related

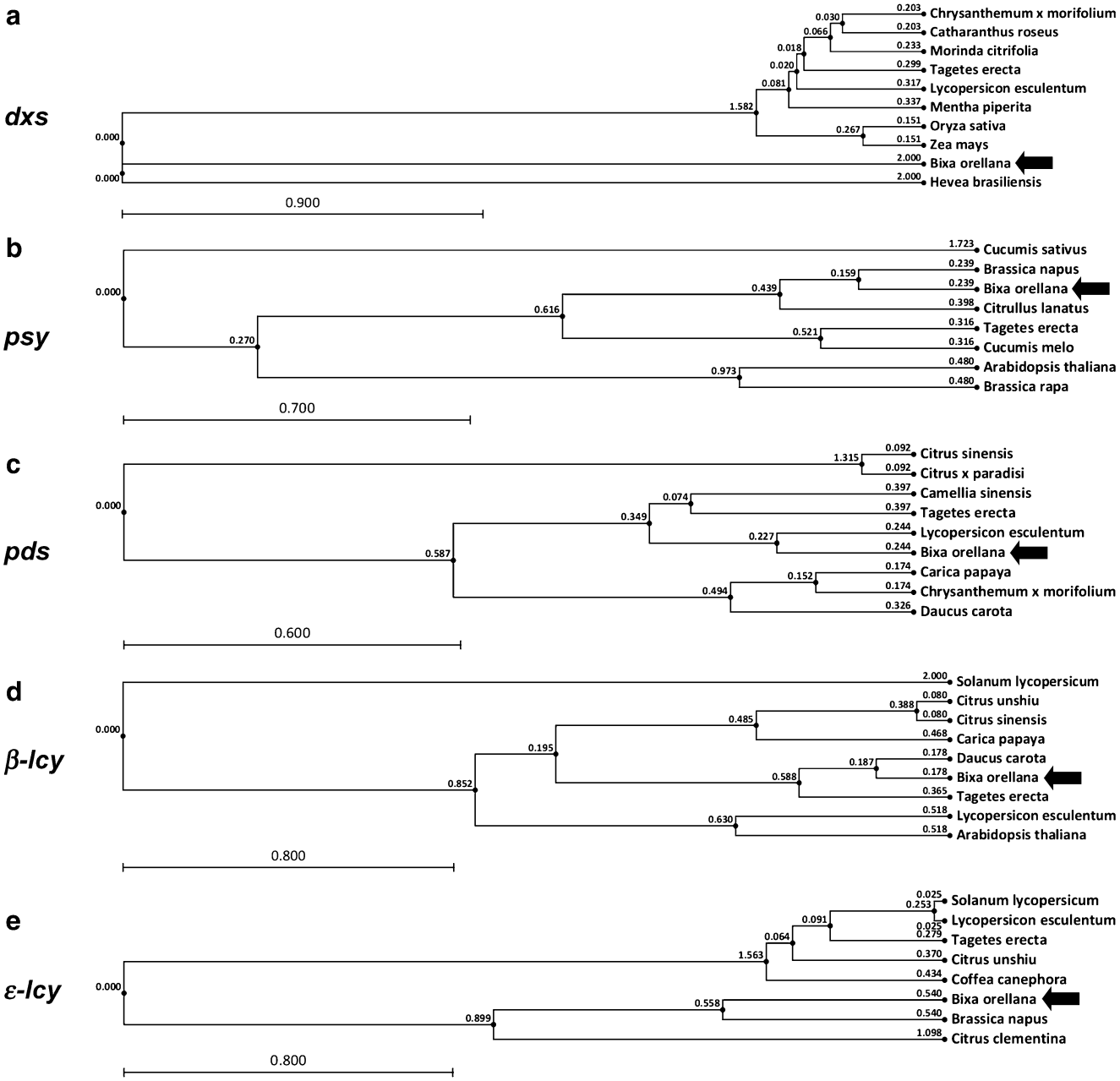

Fig. 4 Phylogenetic trees of obtained probes compared with related genes of different plants. The trees were constructed according to UPGMA analysis. **a** *B. orellana* DXS, **b** *B. orellana* PSY, **c** *B. orellana* PDS, **d** *B. orellana* LYCE, and **e** *B. orellana* LYCB. The arrows indicate the sequences reported in this study

molecules analyzed by HPLC shows their progressive accumulation during the different developmental stages in both annatto cultivars, showing a more drastic difference in their mature seeds. The highest contents of bixin were observed in the seeds of the pink cultivar that may explain the wide commercial use of this annatto cultivar. Vice versa, ABA contents were mainly present in the floral bud of the white cultivar; hence, it is possible that an enzymatic mechanism is leading to the accumulation of such a β-apocarotenoid product in this variant, instead of the lineal apocarotenoid bixin, as was observed in the pink cultivar.

Pigment-Related Gene Expression Profiles

Expression of *dxs* mRNA was active during all stages of development in both cultivars, probably because of its important function as a regulatory enzyme in the plastidial isoprene pathway in plants (Lichtenthaler 1999). The *psy*

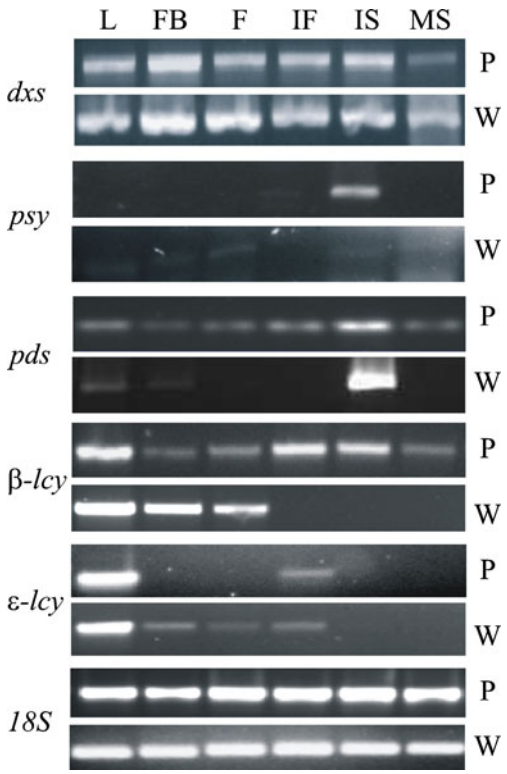

Fig. 5 Expression analysis of the *dxs*, *psy*, *pds*, *β-lcy*, and *ε-lcy* genes by RT-PCR during plant development. The presence of *dxs*, *psy*, *pds*, *β-lcy*, and *ε-lcy* mRNA was detected by PCR amplification with specific primers. Ribosomal 18 S cDNA was amplified as an internal control

mRNA expression coincided with the large carotenoid accumulation in the immature seeds of the pink cultivar. Expression of *psy* mRNA during pigment accumulation has been reported in other plants (Giuliano et al. 2003; Hugueney et al. 1996; Karvouni et al. 1995). However, *psy* mRNA was not detected in the mature seeds of neither annatto cultivars. This is in agreement with the results of Jako et al. (2002) who found that only one of 870 expressed sequences tags analyzed from *B. orellana* L. seeds corresponded to the *psy* gene. The combination of these results suggests an important role for phytoene synthase during carotenoid and bixin accumulation during seed development.

C_{40} linear carotenoid biosynthesis was analyzed by measuring the expression of *pds*. The *pds* mRNA expression during seed formation coincides with the increasing bixin recorded in mature seeds. This is probably due to the rapid conversion of C_{40} linear carotenoids into bixin. Therefore, our results suggest that phytoene desaturase probably plays an important role in the accumulation of carotenoids and bixin in *B. orellana*.

C_{40} cyclic carotenoid formation was analyzed by measuring the expression profiles of *ε-lcy* and *β-lcy* transcripts. These genes are generally regulated together during the photosynthetic process and direct the formation of α-carotene and β-carotene in an equimolar relation in the active antenna complex (Kennis et al. 2001; Cunningham and Gantt 1998). The *ε-lcy* mRNA expression peak was reached in leaf tissues and immature green fruits that also showed an overall up-regulation in the white cultivar. Similar results have been reported for leaves and developing fruit in *A. thaliana* and *L. esculentum* (Cunningham et al. 1996; Rosati et al. 2000), suggesting that ε-lycopene cyclase genes have an important role in photosynthetic pigment accumulation. The *β-lcy* mRNA expression coincided with the accumulation of photosynthetic pigments in the white cultivar. The expression of this gene was also detected in a number of tissues preceding a higher accumulation of the β-apocarotenoid ABA. Differently, *β-lcy* mRNA expression coincided with the accumulation of carotenoids in the pink cultivar. Thus, the expression of the *β-lcy* gene is probably related to the biosynthesis of β, β′-xanthophylls, which are accumulated in the colored petals of the pink variant and with the production of β-apocarotenoids such as ABA and other β-carotene derivates not analyzed here (i.e., methyl (all-E)-8′-apo-β-caroten-8′-oate), which have previously been reported in mature seeds of *B. orellana* (Bittencourt et al. 2005b; Tirimanna 1981).

Finally, the knowledge obtained from the present study supports the selection of candidate genes that could be used as markers for elite *B. orellana* cultivars with higher pigment contents and to identify different alleles related to pigment production. Further research is needed to clarify the balance of different important apocarotenoids such as ABA and bixin in different *B. orellana* cultivars.

Acknowledgments This work was supported by the International Foundation for Science (IFS) F/2932-2, the Consejo Nacional de Ciencia y Tecnología (CONACYT) 46541, and the UC Mexus-CONACYT. Rodríguez-Ávila was supported by a CONACYT PhD grant (no. 196432). We would like to acknowledge the technical support provided by R. Ku-Cauich in *dxs* cloning.

References

Bittencourt C, Felicissimo MP, Pireaux JJ, Houssiau L (2005a) Study of annatto from *Bixa orellana* seeds: an application of time-of-flight secondary ion mass spectrometry. Spectrosc Europe 17:16–22

Bittencourt C, Felicissimo MP, Pireaux JJ, Houssiau L (2005b) ToF-SIMS characterization of thermal modifications of bixin from *Bixa orellana* fruit. J Agric Food Chem 53:16–19

Bouvier F, Dogbo O, Camara B (2003) Biosynthesis of the food and cosmetic plant pigment bixin (annatto). Science 300:2089–2091

Cunningham FX Jr (2002) Regulation of carotenoid synthesis and accumulation in plants. Pure Appl Chem 74:1409–1417

Cunningham FX, Gantt E (1998) Genes and enzymes of carotenoids biosynthesis in plants. Annu Rev Plant Physiol 49:557–577

Cunningham FX, Pogson B, Sun Z, McDonald KA, DellaPenna D, Gantt E (1996) Functional analysis of the β and ε lycopene cyclase enzymes of *Arabidopsis* reveals a mechanism for control of cyclic carotenoid formation. Plant Cell 8:1613–1626

Giuliano G, Rosati C, Bramley PM (2003) To dye or not to dye: biochemistry of annatto unveiled. Trends Biotech 21:513–516

Hugueney P, Bouvier F, Badillo A, Quennemet J, d'Harlingue A, Camara B (1996) Developmental and stress regulation of gene expression for plastid and cytosolic isoprenoid pathways in pepper fruits. Plant Physiol 111:619–626

Isaacson T, Roñen G, Zamir D, Hirschberg J (2002) Cloning of tangerine from tomato reveals a carotenoid isomerase essential for the production of carotenoid and xanthophylls in plants. Plant Cell 14:333–342

Jako C, Coutu C, Roewer I, Reed DW, Pelcher LE, Covello PS (2002) Probing carotenoid biosynthesis in developing seed coats of *Bixa orellana* (Bixaceae) through expressed sequence tag analysis. Plant Sci 163:141–145

Karvouni Z, John I, Taylor JE, Watson CF, Turner AJ, Grierson D (1995) Isolation and characterization of a melon cDNA clone encoding phytoene synthase. Plant Mol Biol 27:1153–1162

Kennis JT, Gobets MB, Ivo H, van Stokkum M, Dekker JP, van Grondelle R, Fleming GR (2001) Light harvesting by chlorophylls and carotenoids in the photosystem I core complex of *Synechococcus elongatus*: a fluorescence upconversion study. J Phys Chem 105:4485–4494

Lichtenthaler HK (1999) The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol 50:47–65

Matsumoto SN, Viana AES, São JAR, Cardoso AD, Aguilar MAG (2002) Growth and photosynthetic characteristics of annatto plants. In: Ming LC, Craker LE, Scheffer, MC (eds.) I Latin-American symposium on the production of medicinal, aromatic and condiments plants. ISHS Acta Hortic 569

Merzlyak MN, Solovchenko AE (2002) Photostability of pigments in ripening apple fruit: a possible photoprotective role of carotenoids during plant senescence. Plant Sci 163:881–888

Pfander H, Schurtenberger H (1982) Biosynthesis of C20-carotenoids in *Crocus sativus*. Phytochemistry 21:1039–1042

Rodríguez-Ávila NL, Narváez-Zapata JA, Aguilar-Espinosa M, Rivera-Madrid R (2009) Full-length genes enrichment by using an optimized RNA isolation protocol in *Bixa orellana* recalcitrant tissues. Mol Biotech 41:1–6

Rivera-Madrid R, Escobedo-Medrano RM, Balam-Galera E, Vera-Ku M, Huges H (2006) Preliminary studies toward genetic improvement of annatto (*Bixa orellana* L.). Sci Hort 109:165–172

Rosati C, Aquilani R, Dharmapuri S, Pallara P, Marusic C, Tavazza R, Bouvier F, Camara B, Giuliano G (2000) Metabolic engineering of beta-carotene and lycopene content in tomato fruit. Plant J 24:413–420

Sánchez-Estudillo L, Freile-Pelegrin Y, Rivera-Madrid R, Robledo D, Narváez-Zapata JA (2006) Regulation of two photosynthetic pigments-related genes during stress-induced pigments formation in the green alga *Dunaliella salina*. Biotech Letters 28:787–791

Tirimanna ASL (1981) Study of the carotenoid pigments of *Bixa orellana* L. seeds by thin layer chromatography. Microchim Acta 11:11–16

Wagner T, Windhövel U, Römer S (2002) Transformation of tobacco with a mutated cyanobacterial phytoene desaturase gene confers resistance to bleaching herbicides. Z Naturforsch 57c:671–679

Wellburn AR (1994) The spectral determination of chlorophylls *a* and *b*, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. J Plant Physiol 144:307–313