Journal of Experimental Botany, Vol. 52, No. 354, pp. 161–166, January 2001

Journal of Experimental Botany

SHORT COMMUNICATION

A simple approach to identify the first rice mutants blocked in carotenoid biosynthesis

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Received 22 May 2000; Accepted 29 August 2000

Abstract

Mutations blocking carotenoid biosynthesis, never before described for rice, are valuable for pathway manipulation and study. Similar to defects in ABA biosynthesis, mutations blocking the carotenoid pathway confer vivipary, but in addition also confer an albino seedling phenotype. Pigments extracted from rice mutants exhibiting the double mutant phenotype were analysed by HPLC (high pressure liquid chromatography); these results led to the identification of the first rice mutant accumulating an intermediate of the carotenoid biosynthetic pathway, phytoene, and a second mutant with almost no detectable carotenoids.

Key words: Carotenoid biosynthesis, mutations, rice, HPLC.

Introduction

Carotenoids are a structurally diverse group of pigments needed in plants for growth and development; they function as accessory pigments in photosynthesis, as photoprotectors preventing photo-oxidative damage, and as precursors to the plant hormone, abscisic acid (ABA) (Cunningham and Gantt, 1998; Robertson *et al*., 1966). Carotenoids are synthesized in plastids by nuclear-encoded enzymes and are derived from isoprenoid precursors made via a plastid-specific biosynthetic route (Lichtenthaler, 1999). In humans and animals, dietary carotenoids are essential precursors to Vitamin A and to retinoid compounds needed in animal morphogenesis (Bendich and Olson, 1989; Lee *et al*., 1981). Endosperms of food crops, such as maize and wheat, are low in provitamin A (1–10%) as compared with non-provitamin A carotenoids. Rice, an important food staple worldwide, accumulates no carotenoids in its endosperm and is therefore associated with Vitamin A deficiency in developing countries (Underwood and Arthur, 1996). For this reason, rice has been the subject of study and manipulation for improvement of endosperm carotenoids (Wurtzel *et al*., 1996; Ye *et al*., 2000).

With little known of the control of carotenoid accumulation in endosperm tissue, future research efforts to improve the carotenoid content of rice and other cereals would benefit from the availability of mutations affecting the rice carotenoid biosynthetic pathway. Such a collection of mapped and biochemically analysed mutants blocking or interfering with the pathway are available for maize since the white mutant endosperm phenotype is easily identified when compared to the normal yellow counterpart (Li *et al*., 1996; Robertson, 1975; Wurtzel, 1992). In maize, genetic loci are associated with particular biosynthetic steps, because of intermediates accumulating in mutant tissues (Neill *et al*., 1986; Robertson *et al*., 1966; Robertson, 1975; Treharne *et al*., 1966). Genetic and biochemical information regarding these loci is useful in identification of putative structural and regulatory genes involved in the plastid-localized carotenoid biosynthetic pathway, as in Li *et al*. (Li *et al*., 1996).

Mutations blocking the pathway lead to the accumulation of intermediates with pleiotropic phenotypic effects. Vivipary or precocious embryo germination is a consequence of a carotenoid-derived ABA deficiency and an albino seedling phenotype results from chlorophyll photo-oxidation occurring in the absence of photoprotective carotenoids. However, not all albino mutants are caused by carotenoid deficiencies; defects in chlorophyll

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biosynthesis would also lead to an albino phenotype, though, not in combination with vivipary. Similarly, blocks in ABA biosynthesis or perception may cause vivipary, but seedlings will remain green. Only mutations that specifically block the carotenoid biosynthetic pathway can manifest as a combination of vivipary with albino phenotype.

In an effort to study the regulation of carotenoid accumulation in rice, a search was made for putative carotenoid mutants which would be expected to exhibit both vivipary and albino seedling phenotypes. Among some viviparous mutants described by Yatou and Iida (Yatou and Iida, 1994), some also exhibited an albino seedling phenotype, suggesting that they were not simply mutations affecting biosynthesis or perception of ABA or chloroplast biogenesis. Therefore these unique viviparous mutants were analysed for the accumulation of intermediates of the carotenoid biosynthetic pathway, which are generally not present in non-mutant tissue (Neill *et al*., 1986).

Materials and methods

Plant materials

EMS (ethyl methane sulphonate) derived mutants of *Oryza sativa* L., 84NMEMdr2 and 90KHEMdr1 (Yatou and Iida, 1994), that segregated viviparous seeds which germinated into albino seedlings, were grown in the greenhouse with supplemental lighting at Lehman College, CUNY. Similarly, maize homozygous *vp5* albino mutant seedlings were germinated and grown under greenhouse conditions. Albino seedlings were collected and stored at −80 °C until used. Endosperms and embryos were dissected from mature rice seeds.

Carotenoid extraction and HPLC analysis

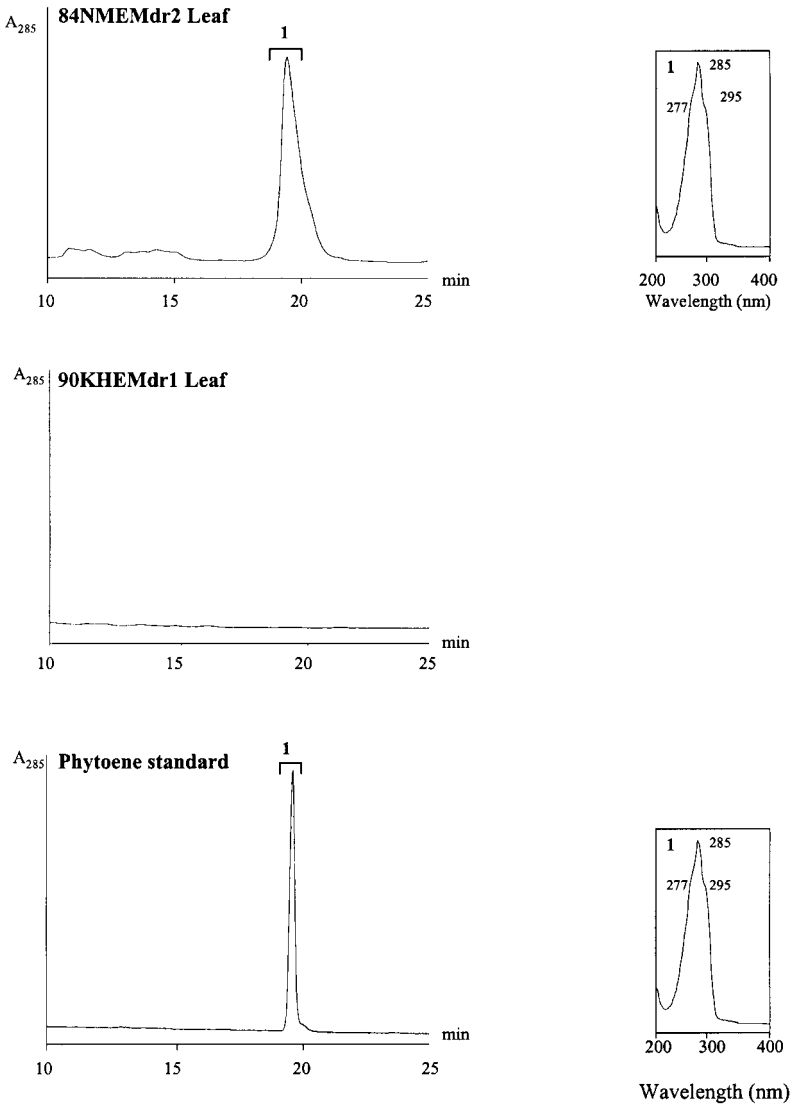
Tissues from rice or maize mutants, derived from mutant seeds segregating from self-pollination of a plant heterozygous for the mutation, were used for extraction of carotenoids under low light using a modified method (Britton, 1985; Fraser *et al*., 1994). In the case of the rice mutants, because of the easily discerned albino plant phenotype, all leaves were homozygous for the mutation, whereas embryos and endosperm were from a mixture of seeds, approximately 25% of which were homozygous for the mutation. One gram of frozen tissue (stored at −80 °C) was ground in liquid N_2, suspended in 10 ml methanol and centrifuged at 9000 *g*, for 10 min at 4 °C. After the addition of 1 ml 60% (w/v) KOH, the supernatant was heated at 65 °C for 20 min. The mixture was extracted three times in 10% (v/v) diethyl ether in hexane, the organic phase evaporated under N_2, dissolved in 700 µl methanol and stored in amber vials at −20 °C. A Waters HPLC system with 600 controller and pump, 996 Photodiode Array Detector, and WISP 717 autosampler were used to separate samples by reverse phase chromatography on a Spherisorb-ODS (5 µ, 250×4.6 mm) column, eluted with acetonitrile:methanol:isopropanol (85:10:5, by vol.) (Sandmann *et al*., 1993) at a flow rate of 1 ml min^{-1}. Peaks were identified by spectrophotometric profiles (as characterized by three absorption maxima and peak II/III ratios), and retention times which matched those of the authentic phytoene standard. The phytoene standard was extracted from albino maize seedlings of the homozygous *vp5* mutant which accumulates phytoene (Neill *et al*., 1986; Wurtzel, 1992), a carotenoid intermediate that has also been possible to extract and identify from a genetically engineered bacterium (Li *et al*., 1996).

Results

A number of viviparous mutants have been described, two of which also exhibited an albino seedling phenotype, characteristics expected for mutants blocked in carotenoid biosynthesis (Yatou and Iida, 1994). To test whether any carotenoid intermediates accumulated in albino tissues of these mutants, pigments were extracted from albino leaves of viviparous mutants 84NMEMdr2 and 90KHEMdr1 and subjected to HPLC analysis. In addition to retention time on reverse-phase columns, individual carotenoids and carotenoid intermediates are characterized by their spectral properties, consisting of three peak maxima with a unique spectral shape (ratio of peaks II/III), which are influenced by the solvent used. Therefore, the standard was analysed under identical separation and solvent conditions, and the HPLC system utilized a diode array detector to obtain spectral information for each eluted peak. As shown in Fig. 1, leaf tissue from mutant 84NMEMdr2 contained one spectral peak (peak 1) at 285 nm with a retention time of 19.45 min, which was absent in the albino tissue from mutant 90KHEMdr1; albino leaves from 90KHEMdr1 were found to contain only a trace amount of coloured carotenoid products absorbing at a maximum of 450 nm (data not shown). As shown in the top panel of Fig. 1A, the spectrum of peak 1 showed the characteristic three peak maxima and peak shape of the compound phytoene. Moreover, as shown in Fig. 1B, the peak shapes, characterized by the peak II/III ratio, matched between peak 1 of 84NMEMdr2 and the phytoene standard. The phytoene standard was obtained from an albino maize mutant, *vp5*, known to accumulate phytoene, a compound that does not accumulate in normal green tissues (Neill *et al*., 1986; Wurtzel, 1992) and a carotenoid intermediate that was also verified by extraction and identification from a genetically engineered bacterium (Li *et al*., 1996). The chromatogram and spectrum of the phytoene standard, which eluted at 19.65 min, is shown in the bottom panel of Fig. 1A.

To examine the tissue specificity of the mutation in 84NMEMdr2 further, pigments were extracted from embryo and endosperm dissected from mature seeds obtained from a self-pollinated plant heterozygous for the 84NMEMdr2 mutation. The extracted compounds were analysed by HPLC and compared with that of the mutant leaf tissue. For lack of a visible phenotype, the predicted 25% homozygous mutant seeds could not be separated from normal seeds in a segregating population. However,

A



B

Sample	Peak	Retention Time (min)	Absorbance maximum (nm)[a]	Ratio II/III[b]	Pigment accumulation
84NMEMdr2 leaf	1	19.45	277, **285**, 295	1.31	Phytoene
90KHEMdr1 leaf	---	---	---	---	---
Phytoene standard	1	19.65	277, **285**, 295	1.33	Phytoene

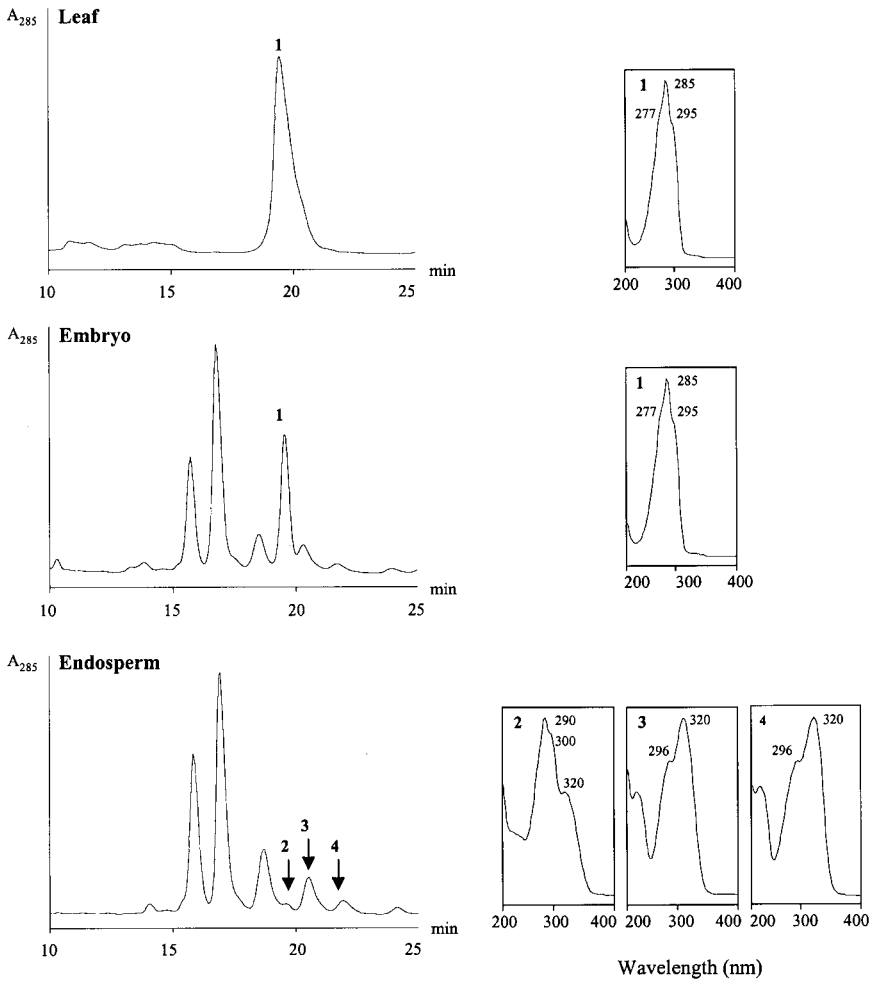
[a]Main absorbance maximum is shown in bold.

[b]Ratios of heights of the main absorbance maximum versus the longest wave maximum were measured from the base line.

Fig. 1. HPLC analysis of pigments accumulating in albino leaves of mutants 84NMEMdr2 and 90KHEMdr1 compared with phytoene standard. (A) Chromatograms on the left and spectra of numbered peaks on the right. Phytoene standard is extracted from albino leaves of homozygous *vp5* maize. (B) Peak retention times and spectral characteristics leading to compound identification.

as shown in Fig. 2A, phytoene could still be detected as peak 1 (retention time about 19.5 min) in extracts prepared from dissected embryos, but not from endosperms. The endosperm sample showed a number of small peaks with retention times (peaks 2–4) in the vicinity of that of the phytoene standard; however, examination of the peak spectra, shown on the right of Fig. 2A, revealed that the compounds did not match that of the phytoene standard. In Fig. 2B, a comparison of peak retention times, peak maxima, and peak ratios between

A



B

84NMEMdr2	Peak	Retention Time (min)	Absorbance maximum (nm)[a]	Ratio II/III[b]	Pigment accumulation
leaf	1	19.45	277, **285**, 295	1.31	phytoene
embryo	1	19.50	277, **285**, 295	1.31	phytoene
endosperm	2	19.58	**283**, 295, 301	1.49	---
endosperm	3	21.91	296, **300**	---	---
endosperm	4	24.11	296, **300**	---	---

[a]Main absorbance maximum is shown in bold.

[b]Ratios of heights of the main absorbance maximum versus the longest wave maximum were measured from the base line.

Fig. 2. HPLC separation of pigments extracted from leaf, embryo and endosperm of the rice mutant 84NMEMdr2. (A) Chromatograms on the left and spectra of numbered peaks on the right. (B) Peak retention times and spectral characteristics leading to compound identification.

the standard and mutant tissue samples, corroborated the presence or absence of phytoene. Therefore, phytoene appeared only to accumulate in the leaf and embryo, but was not detectable in the endosperm of 84NMEMdr2 plants.

Discussion

Evidence of a carotenoid mutant of rice was suggested by the phenotype of several mutants for which viviparous seeds germinated into albino seedlings (Yatou and Iida, 1994). Mutant tissues were analysed from two mutants,

and phytoene was detected in the albino leaves of 84NMEMdr2, while albino leaves of 90KHEMdr1 showed only trace amounts of coloured carotenoids and no carotenoid intermediates accumulated. Mutant 84NMEMdr2 also accumulated phytoene in embryo but not in endosperm tissue.

Mutant 90KHEMdr1 was similar in biochemical phenotype as some maize mutants, such as *vp12* (Maluf *et al*., 1997), that exhibit vivipary and albino phenotypes, but do not accumulate carotenoid intermediates; the absence of intermediates combined with reduced coloured carotenoids suggests that the products of those loci may function at steps upstream of the pathway such as in providing the isoprenoid precursors of carotenoids. This mutation represents a mutant class that could be useful in furthering study of the new alternative IPP biosynthetic pathway unique to plastids (Lichtenthaler, 1999).

The mutant 84NMEMdr2 confers a block early in the carotenoid biosynthetic pathway. As shown in Fig. 3, the conversion of phytoene to zetacarotene is mediated by the enzyme phytoene desaturase (PDS); a block at this step would be predicted to cause phytoene accumulation, a biosynthetic intermediate not ordinarily found in green tissues (Neill *et al*., 1986). However, a block causing phytoene accumulation might be an indirect effect of blocking biosynthesis of plastoquinones, pigments that participate in the electron transfer reactions associated with phytoene desaturation (Beyer *et al*., 1989; Mayer *et al.,* 1990, 1992; Norris *et al*., 1995). Therefore, the block in carotenoid biosynthesis could represent a defect in a component required for function or expression of PDS or for plastoquinone biosynthesis. For example, the maize viviparous mutations *vp5* and *vp2* both confer phytoene accumulation in albino seedlings and endosperm (Neill *et al*., 1986); however, the *vp5* locus was found to encode PDS (Hable *et al*., 1998; Li *et al*., 1996), while the *vp2* locus was found to be involved with plastoquinone biosynthesis (R Luo and ET Wurtzel, unpublished results). Since a block leading to phytoene accumulation may be due to alterations related to PDS expression, the transcript level in the normal and mutant tissue was tested. No differences in PDS transcript level, as tested by RT-PCR, were found (data not shown). Therefore, the presence of phytoene is not due to a block in accumulation of PDS transcripts. It is possible that the presence of phytoene is due to a block in production of an active plastid-localized PDS protein, to a block in the plastoquinone pathway, or to some other explanation. It was not possible to use chemical complementation to test for blocks in the plastoquinone pathway, since the complemented phenotype would be green and therefore indistinguishable from the normal segregating counterparts.

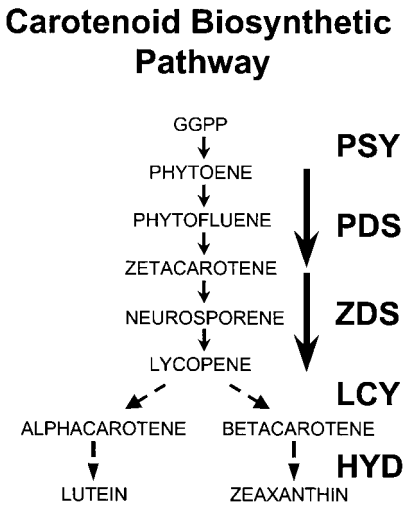


Fig. 3. Carotenoid biosynthetic pathway in higher plants. Enzymes are indicated on the right with arrows indicating the corresponding catalytic steps: PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, zetacarotene desaturase; LCY, alpha- and beta-lycopene cyclases; HYD, beta- and epsilon-hydroxylases.

The viviparous phenotype of both rice mutants was apparently caused by a defect in accumulation of the carotenoid precursor to ABA. It is surprising that the 84NMEMdr2 mutant phenotype was only evident in embryo and leaf tissue, and not in endosperm. This is unlike the case in maize where mutations conferring phytoene accumulation affect both the leaf and the endosperm tissue (Neill *et al*., 1986). Recent experiments in engineering of the rice endosperm carotenoid pathway with a gene encoding PSY, resulted in endosperm phytoene accumulation; if there was any endogenous PDS, it was insufficiently active since no further phytoene desaturation products were detected (Burkhardt *et al*., 1997). The absence of endosperm phytoene in the rice mutant 84NMEMdr2 also suggests that the endogenous endosperm pathway is not active, otherwise some phytoene might accumulate in the mutant. Furthermore, from the work of Ye *et al*., it is apparent that rice endosperm cannot produce carotenoids and requires transgenes in order to do so (Ye *et al*., 2000). Alternatively, the mutation may be tissue-specific.

The first rice mutants blocked in carotenoid biosynthesis were identified using a simple approach of screening for vivipary combined with an albino seedling phenotype, a strategy easily applied for identification of carotenoid mutants in other plants. Further analysis of the rice mutations described here will shed light on the functions of the associated gene products encoded by the normal alleles and to determine whether these mutations represent blocks in any new genes. This simple approach can be used to further expand the number of loci related to carotenoid and isoprenoid biosynthesis.

Acknowledgements

We thank Mr David Cain (Lehman College, NY) for his excellent care of rice plants grown in the greenhouse. We thank Dr Barbara Meurer-Grimes for help in the HPLC analysis. This research was funded in part by grants from The Rockefeller Foundation International Rice Biotechnology Program, The National Institutes of Health (No. 5 SO6 GM08225), and The City University of New York (CUNY-HEAT and PSCUNY research awards).

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