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Genetics of Carotene Biosynthesis in *Phycomyces*

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Summary. Genetic analysis of carotenogenesis in *Phycomyces* is hampered by the inability of most mutants to complete the sexual cycle. Heterokaryons between complementing mutants or between a mutant and a helper strain are, however, fertile. Using this method crosses have been carried out between mutants representing the different known genes and phenotypes.

The gene for phytoene dehydrogenase, *carB*, is closely linked to *carRA*, a bifunctional gene specifying both lycopene cyclase and a product involved in substrate transfer. The regulatory gene *carS* lies on the same chromosome, but not close to the structural genes. The situation is thus reminiscent of an operon.

Two chemoinsensitive mutants, showing little or no response to the effects of retinol and 2-(4-chlorophenyl)-thiotriethylamine · HCl on carotenogenesis, carry mutations in separate genes. One of these is probably identical with *carRA*; the other is a new gene termed *carI*.

Key words: Phycomyces – Carotene biosynthesis

Introduction

The fungus *Phycomyces blakesleeanus* has often been used in biochemical and genetical studies of carotene biosynthesis (review by Cerdá-Olmedo and Torres-Martínez 1979). The characteristic yellow colour of the wild-type is due to the accumulation of β-carotene. White, red, light yellow and deep yellow mutants are easily spotted in cultures of mutagenized spores. Mutations are assigned to specific genes by complementation in heterokaryons.

Gene *carB* (Eslava and Cerdá-Olmedo 1974; Ootaki et al. 1973) determines phytoene dehydrogenase. Mutants lacking this enzyme are white and accumulate phytoene ("B phenotype").

Gene *carRA* (Torres-Martínez et al. 1980; Murillo et al. 1981) determines two separate functions, lycopene cyclase and substrate transfer. Mutants lacking lycopene cyclase are red and accumulate lycopene ("R phenotype"). Defective substrate transfer ("A phenotype") results in white mutants which, when grown in the presence of retinol, become yellow and accumulate appreciable amounts of β-carotene (Eslava et al. 1974). Mutants lacking both functions are white and do not respond to retinol ("RA phenotype").

Four copies of phytoene dehydrogenase and two copies of lycopene cyclase, integrated in an enzyme complex, carry out consecutively the four reactions needed to convert phytoene into lycopene and the two reactions needed to convert lycopene into β-carotene (De la Guardia et al. 1971; Aragón et al. 1976).

Gene *carS* controls the concentration of the final product (Murillo and Cerdá-Olmedo 1976); *carS* mutants are very deep yellow and accumulate large amounts of β-carotene ("S phenotype").

Retinol and other chemicals stimulate carotenogenesis (Eslava et al. 1974) and turn the wild-type deep yellow. 2-(4-Chlorophenyl)thiotriethylamine · HCl (CPTA) and other chemicals block lycopene cyclase (Coggins et al. 1970; Murillo 1980) and turn the wild-type red. There are mutants insensitive to both effects: they make β-carotene fairly normally, but do not become deep yellow in the presence of retinol or red in the presence of CPTA (chemoinsensitive mutants). These mutants were originally isolated by F. J. Murillo in our laboratory.

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Table 1. Strains of *Phycomyces* used in this work

Strain	Genotype	Phenotype
B36	*nicA101* (+)	Auxotroph for nicotinic acid; largely isogenic with the standard wild-type
C2	*carA5* (−)	White coulour
C9	*carR21* (−)	Red colour
C115	*carS42 mad-107* (−)	Intense yellow colour
C242	*carA5 nicA101* (+)	White colour; auxotroph for nicotinic acid
S102	*nicA101* (−)	Auxotroph for nicotinic acid
S119	*carA113* (−)	Chemoinsensitive
S144	*carI131* (−)	Chemoinsensitive
S240	*carR21 nicA101* (−)	Red colour, auxotroph for nicotinic acid
S241	*carR21* (+)	Red colour
S265	*carB10 dar-54* (−)	White colour; resistant to 5-deazariboflavin
S283	*carA5 carS42 nicA101* (−)	Yellow colour; auxotroph for nicotinic acid

The sexual cycle of *Phycomyces* allows genetic analysis by recombination (Cerdá-Olmedo 1975; Eslava et al. 1975a, b). After a long dormancy, the mating structure or zygospore germinates and produces a germsporangium containing thousands of germspores. The germspores in a germsporangium are usually the haploid products of a single meiosis. A major difficulty with the genetic analysis of carotenogenesis is the inability of the mutants to complete the sexual cycle: only mutants with the A phenotype make zygospores, although fewer than the wild-type. Complementing heterokaryons between the mutants are, however, fertile, and may be used to overcome this difficulty (Cerdá-Olmedo 1975). An appropriate cross would be $(a * b) \times c$, where * separates the components of a heterokaryon and x, the two opposite mating types; *a* carries a *car* mutation; *b* and *c* share an allele resulting in auxotrophy. Offspring results from $a \times c$ and $b \times c$ crosses. By requiring prototrophy, all offspring of the second cross and half of the first cross would be eliminated; genetic analysis is then limited to half the offspring of the $a \times c$ cross.

After carrying out such crosses, we now report on the linkage relationship of the *car* mutations.

Materials and Methods

Phycomyces strains used in this work are listed in Table 1, together with their phenotypes and genotypes. Strain B36 was obtained from A. P. Eslava (Universidad de Salamanca); C strains, from the California Institute of Technology; the others were developed in this laboratory. For media and culture conditions see Heisenberg and Cerdá-Olmedo (1968). Heterokaryons were prepared by surgical grafting of sporangiophores (Ootaki 1973). Nuclear proportions in the heterokaryons were calculated from spore segregations (Heisenberg and Cerdá-Olmedo 1968). Carotene content was determined according to De la Guardia et al. (1971).

Two kinds of genetical analysis were performed: mass spore analysis and tetrad analysis (Cerdá-Olmedo 1975). For mass spore analysis the germspores of many germsporangia were pooled; samples were plated on acid (pH 3.3) medium and the frequency of different phenotypes among the resulting colonies was determined.

For tetrad analysis, the germspores of each germsporangium were collected and studied separately. Each germsporangium is assumed to contain the products of a single meiosis. For any pair of markers each germsporangium may be classified as PD (parental ditype), NDP (non-parental, or recombinant ditype) and T (tetratype). Incomplete tetrads, missing one or more expected phenotypes, were also found. Zygospore germination started from 56 to 150 days after setting up the crosses.

Results

Close Linkage of R and A

A detailed tetrad analysis was carried out on the cross (C242 * S241) x (C2 * C9). The respective genotypes are [*carA5 nicA101* (+) * *carR21* (+)] x [*carA5* (−) * *carR21* (−)], and the carotene phenotypes [A * R] x [A * R]. Only white A mycelia came out of 61 germsporangia, implying A x A crosses. Only red R mycelia were produced by 21 germsporangia, implying R x R corsses. Mixed mycelia, products of the two possible and genetically equivalent A x R crosses, were found in 40 germsporangia. Of these, 39 produced only white A and red mycelia. This indicates that the genetic determinants of the A and R phenotypes are very closely linked and confirms the results of Torres-Martínez et al. (1980).

The one exceptional germsporangium gave rise to white and red parental mycelia, wild-type yellow recombinants, and to a special kind of yellow mycelium which could not be stimulated by retinol. This segregant may represent the phenotype of the *carRA* gene combining the two mutational alterations of the parents; in such a case, the exceptional germsporangium would be a tetratype.

Close Linkage of A and B

A detailed tetrad analysis was conducted on the cross C242 x (S240 * S265). The corresponding genotypes are *carA5 nicA101* (+) x [*carR21 nicA101* (−) * *carB10 dar-54* (−)], and the carotene phenotypes, A x (R * B).

Table 2. Mass spore analysis of the cross C242 x [C115 * S102]

Genotypes: *carA5 nicA101* (+) x [*carS42 mad-107* (−) * *nicA101* (−)]	
Carotene phenotypes:	A x[S * wild-type]
Pooled germsporangia	159
Proportion of germsporangia carrying C115 descendants[a]	0.35
Estimated number of meiosis involving C115	56
Prototrophic phenotypes[b], proportions:	
White A	0.34
Deep yellow S	0.30
Yellow wild type + yellow AS	0.36

[a] Estimated as proportion of C115 nuclei in the heterokaryon
[b] Of 637 colonies produced by plating germspores on minimal medium

Table 3. Tetrad analysis of the cross B36 x S 283

Genotypes:	*nicA101* (+) x *carA5 carS42 nicA101* (−)
Carotene phenotypes:	Wild type x AS
Complete tetrads	
Parental ditypes	26
Non-parental ditypes	4
Tetratypes	26
Incomplete tetrads	
Monotypes	
Parental	0
Non-parental	1
Tritypes	7

Descendants of the A x R cross were found in 44 germsporangia: 37 parental ditypes, 6 monotypes (all red, may be incomplete PD tetrads), and one incomplete tetratype (white A, red R, wild-type yellow; all auxotrophs). This result further confirms the close linkage of the genetic determinants for the A and R phenotypes.

Descendants of the A x B cross were found in 13 germsporangia: 8 parental ditypes, 4 monotypes (all white B), and one recombinant-containing germsporangium, which must have arisen from more than one meiosis (white B auxotroph, yellow auxotroph, yellow prototroph). Thus, the genetic determinants of the A and B phenotypes are closely linked. That is to say, genes *carB* and *carRA* are very close in the genome.

S is Loosely Linked to A

Two crosses involving *carS* were studied. The first (Table 2) suggests linkage between the A and S determinants and estimates the recombination frequency at 0.36. The cross led to the isolation for the first time of *carA5 carS42* recombinants, whose "AS phenotype" has been described in detail elsewhere (López-Díaz and Cerdá-Olmedo 1980). They are yellow, contain a little more β-carotene than the wild-type and their carotene accumulation is not increased by light.

Mass spore analysis is notoriously unreliable in the study of weak linkages in *Phycomyces*. Tetrad analysis of another cross (Table 3) confirms the existence of linkage, since the number of parental ditypes, 26, clearly exceeds that of non-parental ditypes, 4. From Table 3 the recombination frequency is estimated at 0.34, in good agreement with the first cross.

Thus the A and S phenotypes depend on genes of the same chromosome, but not close to each other.

Table 4. Genetic analysis of the chemoinsensitive mutants

Cross No. 1	C242 x [S144 * S102]
Genotypes: *carA5 nicA101* (+) x [*car-131* (−) * *nicA101* (−)]	
Carotene phenotypes:	A x [I * wild-type]
Cross No. 2	C242 x [S119 * S102]
Genotypes: *carA5 nicA101* (+)	x [*car-113* (−) * *nicA101* (−)]
Carotene phenotypes:	A x [I * wild-type]

	Cross No. 1	Cross No. 2
Pooled germsporangia	187	185
Proportion of germsporangia carrying I descendants[a]	0.40	0.35
Estimated number of meiosis involving I nuclei	75	65
Viable germspores studied on minimal medium	1,092	1,126
Prototrophic phenotypes[b], proportions		
White A + yellow I	0.52	1.00
White AI	0.20	0.00
Yellow wild type	0.28	0.00

[a] Estimated from separate, partial tetrad analyses of the same crosses
[b] Of colonies produced by plating germspores on minimal medium

Genetic Analysis of the Chemoinsensitive Mutants

The chemoinsensitive mutants were unable to form zygospores. Table 4 gives the mass spore analyses of heterokaryon crosses involving two different mutants.

Mutation *car-113* is very close to mutation *carA5*, since no recombinants were obtained at all; in fact *car-113* may well lie in the A section of gene *carRA*.

Mutation *car-131* is unlinked to *carA5*, since the recombination frequency was estimated at 0.48; thus *car-131* defines a new gene, which we designate *carI*.

The complete allele designation, *carI131*, has been used in Table 1. The *carA5 carI131* recombinants show the expected "AI phenotype": white colour and no stimulation by retinol.

Discussion

The close linkage of genes *carB* and *carRA* means that the two enzymes carrying out the six last steps in carotenogenesis and the A gene product, responsible for substrate transfer, all depend on a small segment of the genome. This small segment is located about 10 map units from the centromere of one of the chromosomes (Eslava et al. 1975b). Few cases of close linkage between the genetic determinants of related biochemical functions are known in the Fungi Examples of this situation are, in *Saccharomyces cerevisiae*, the *GAL7, GAL10* and *GAL1* genes controlling galactose utilization (Bassel and Mortimer 1971), and, in *Neurospora crassa*, the *arom* genes controlling the biosynthesis of aromatic aminoacids (Giles 1978) and some *al* mutations affecting carotene biosynthesis (Subden and Trelkeld 1970). None was known before now in *Phycomyces*. However, mutations affecting carotenogenesis in the bacterium *Rhodopseudomonas capsulata* are all closely linked on a small chromosome segment (Yen and Marrs 1976).

One may wonder, by extension of the *carRA* situation, whether *carB* and *carRA* are only sections of a single gene. This is unlikely, since no known mutants have lost all three functions and since all B mutants complement with all R and A mutants. But one should keep in mind that the number of mutants characterized biochemically and genetically is still small.

Phytoene dehydrogenase and lycopene cyclase are needed in the fixed stoichiometric proportion of two of the former for each one of the latter. They must be well co-regulated to avoid imbalance when the rate of synthesis of the final product is drastically altered (Murillo and Cerdá-Olmedo 1976; Murillo et al. 1978). Imbalance between the two functions might have led, for example, to an oversupply and accumulation of lycopene, but this was never observed. The close linkage is thus likely to be put to use in the required co-regulation, as in the classical operon model. In fact, gene *carS* strongly reminds of a repressor gene (Murillo and Cerdá-Olmedo 1976). But we know of no operator mutants and generally lack information about the molecular details of carotene regulation.

The two chemoinsensitive mutants turned out to be genetically independent. Mutation *car-113* is probably in section A of the *carRA* gene, since we found no recombinants between *car-113* and *carA* and since the A substrate transfer function is clearly related to retinol action (Eslava et al. 1974). We have thus indicated in Table 1 mutation *carA113*.

The new gene *carI* is so far represented only by allele *carI131*, present in the other chemoinsensitive mutant. Additional extensive work on the chemoinsensitive mutants, carried out in Seville by several co-workers, will be published elsewhere.

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