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Genetics of lycopene cyclization and substrate transfer in β-carotene biosynthesis in *Phycomyces*

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SUMMARY

The mutations which block lycopene cyclization and those which stop substrate transfer along the carotene pathway are very closely linked in *Phycomyces*. Simultaneous blocking of both processes commonly results from single exposures to mutagens; and both blockings may be simultaneously removed after a second exposure. The frequencies of different kinds of mutants after treatments with the mutagens *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and ICR-170, their reversion patterns, and recombination analyses indicate that lycopene cyclization and substrate transfer are governed by separate segments of a single bifunctional gene.

1. INTRODUCTION

The fungus *Phycomyces blakesleeanus* owes its yellow colour to β-carotene. Colour mutants, either unable to synthesize β-carotene or accumulating it in atypical amounts, are readily found after exposure of the spores to different mutagens. The genetics and regulation of carotene biosynthesis in *Phycomyces* have been studied in considerable detail (review by Cerdá-Olmedo & Torres-Martínez, 1979).

Some colour mutants are red because they accumulate lycopene, a precursor of β-carotene (Meissner & Delbrück, 1968). They will be called R mutants in this report. They do not complement each other, thus defining gene *carR* (Ootaki *et al.* 1973), responsible for lycopene cyclase. Each carotenogenic enzyme complex contains two copies of this enzyme, which carry out the last two steps of β-carotene biosynthesis (De la Guardia *et al.* 1971).

Most colour mutants are white. Some of them, defective in gene *carB* (Ootaki *et al.* 1973; Aragón *et al.* 1976), complement all other mutants, but not each other. They are readily recognized by their high phytoene content, and were excluded from this report. Another group, which we shall call A mutants, are normally devoid of carotenes (except for trace amounts of β-carotene and others). However, in the presence of retinol and other chemicals, A mutants make

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considerable amounts of β-carotene (Eslava, Alvarez & Cerdá-Olmedo, 1974; Murillo, 1980). The A mutants do not complement each other, although they readily complement R and B mutants, thus defining gene *carA* (Ootaki *et al.* 1973). The product of gene *carA* is responsible for substrate transfer in carotenogenic enzyme complexes (Murillo *et al.* in preparation).

White mutants lacking all carotenes (except for trace amounts of lycopene), even in the presence of retinol (Eslava *et al.* 1974) are often isolated from the wild type. They complement neither *carR* nor *carA* mutants and have thus been considered double mutants, presumed to carry mutations in both the *carA* and *carR* genes (Ootaki *et al.* 1973). They will be called AR mutants in this paper, without any preconception as to their genetic nature.

The same AR phenotype may be obtained in two steps, as white mutants isolated from a red strain (Ootaki *et al.* 1973). They will be called 'double-step AR mutants' in this report. They must contain two separate mutations.

We have investigated the linkage relationships of these mutations and the relative frequencies at which they are induced and reverted by two mutagens: MNNG,* which causes mostly base substitutions (Whitfield *et al.* 1966; Prakash & Sherman, 1973; Coulondre & Miller, 1977), but also frameshift mutations (Yourno & Heath, 1969) and large deletions (Ishii & Kondo, 1975), and ICR-170,† which causes frameshift mutations (Roth, 1974; Culbertson *et al.* 1977).

2. MATERIALS AND METHODS

Table 1 lists the *Phycomyces blakesleeanus* strains used, together with their genotypes, phenotypes, and origins.

For media and culture conditions see Heisenberg & Cerdá-Olmedo (1968). Heterokaryons were obtained by surgical grafting of sporangiophores (Ootaki, 1973). For genetic analysis, random samples were taken from germspore pools combining the products of hundreds of zygospores (Cerdá-Olmedo, 1975).

Treatments with MNNG (Sigma Chemical Co., St Louis, Missouri, U.S.A.) followed Cerdá-Olmedo & Reau (1970). ICR-170 was the gift of the Institute for Cancer Research (Philadelphia, U.S.A.); a water suspension of spores (about 10^6 spores/ml) was heat-activated (15 min at 48 °C) and immediately exposed for 4 hours at room temperature and total darkness to a freshly-prepared aqueous solution of ICR-170 at a final concentration of 20 μg/ml. The safety precautions recommended by Ehrenberg & Wachtmeister (1977) were taken in working with mutagens. After repeatedly washing the mutagens away, the spores were plated on acid medium to look for colour mutants. Appropriate reconstruction experiments showed that colour mutants are readily detectable in plates containing more than 30000 colonies per plate. The mutants were classified according to their carotene content (De la Guardia *et al.* 1971) and response to retinol (Eslava *et al.* 1974).

* *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

† 2-methoxy-6-chloro-9[3-(ethyl-2-chloroethyl)amino propylamino]acridine.2HCl.

Table 1. Phycomyces *strains*

Strain	Genotype	Origin	Notes
NRRL1555	(−)	Nature	Standard wild type
B141	(+)	Backcrosses of UBC21 into NRRL1555	Largely isogenic with the standard wild type
B142	(+)	Backcrosses of UBC21 into NRRL1555	Largely isogenic with the standard wild type
C2	*carA5* (−)	From NRRL1555 via MNNG	White, phenotype A
C6	*carRA12 madF48* (−)	From NRRL1555 via MNNG	White, phenotype AR
C9	*carR21* (−)	From NRRL1555 via MNNG	Red, phenotype R
C115	*carS42 mad-107* (−)	From NRRL1555 via MNNG	Intense yellow colour
C171	*carA30 carR21* (−)	From C9 via MNNG	White, double-step AR phenotype
C242	*carA5 nicA101* (−)	From C2, UBC21, and S102, via crosses	White, phenotype A auxotroph
S102	*nicA101* (−)	From NRRL1555 via MNNG	Auxotroph
S104	*carS42 carRA115 mad-107* (−)	From C115 via MNNG	White, phenotype AR
S124	*carS42 car-119 mad-107* (−)	From C115 via ICR-170	White, phenotype AR
S125	*carS42 car-120 mad-107* (−)	From C115 via ICR-170	White, phenotype AR
S160	*carS42 carRA115 car-136 mad-107* (−)	From S104 via MNNG	Yellow
S161	*carS42 carRA115 car-137 mad-107* (−)	From S104 via MNNG	Yellow
S165	*carS42 carRA115 car-141 mad-107*	From S104 via MNNG	Red

3. RESULTS

(i) *A and R phenotypes are due to closely-linked mutations*

R mutants are unable to complete the sexual cycle (Heisenberg & Cerdá-Olmedo, 1968; Sutter, 1975), but heterokaryons containing an R component are fertile (Cerdá-Olmedo, 1975). Recombination with R mutants can be consequently studied in crosses in which one of the parents is a heterokaryon containing both the desired R mutant and a helper strain. We have studied the cross C242 × (C9*S102), where C9 is an R mutant and C242 an A mutant. Both C242 and S102 carry the marker *nicA101*, which is very stable (spontaneous revertant frequency, less than 10^{-7} in a vegetative cycle) and segregates independently of both the A (Eslava, Alvarez & Delbrück, 1975*b*) and R (Cerdá-Olmedo, unpublished) characters. All the progeny from C242 × S102 must be auxotrophic for nicotinic acid and are readily excluded by plating on minimal medium. The prototrophic half of the progeny from C242 × C9 is used to study recombination between the A and R phenotypes.

Zygospore germination started about 100 days after setting up the cross. Table 2 gives the results of random germspore analyses. The A and R phenotypes could yield two kinds of recombinants: wild type (yellow) and double mutant (presumably white, AR phenotype). Neither was found. In fact, about 0·5%

Table 2. *Cross C242 × (C9*S102). Genotypes* carA5 nicA101 (+) × [carR21 (−)* nicA101 (−)]. *Heterokaryons with different nuclear proportions were used for crosses no. 1 and no. 2*

	Cross no. 1	Cross no. 2
Pooled germsporangia	660	343
Viable germspores:		
On nutrient medium	$7{\cdot}7 \times 10^4$	$1{\cdot}37 \times 10^5$
On minimal medium plus nicotinic acid	$2{\cdot}16 \times 10^4$	$6{\cdot}37 \times 10^4$
On minimal medium	$2{\cdot}1 \times 10^3$	$2{\cdot}33 \times 10^4$
Progeny studied*	1518	642
Phenotype proportions:		
Yellow	0·46	0·24
White A	0·38	0·47
White AR	0·00	0·00
Red	0·16	0·29
Prototrophic progeny studied†	951	714
Phenotype proportions:		
Yellow	0·00	0·00
White A	0·53	0·485
White AR	0·00	0·00
Red	0·47	0·515
Proportion of germsporangia carrying C9 progeny‡	0·19	0·73
Estimated number of meioses involving C9	125	250

* Colonies obtained by plating germspores on minimal medium plus nicotinic acid.
† Colonies obtained by plating germspores on minimal medium.
‡ Estimated as double the proportion of prototrophs among colonies grown on minimal medium plus nicotinic acid.

of the prototrophic progeny was yellow (not included in Table 2), but analysis of their vegetative spores showed that they were not recombinants, but heterokaryons containing white and red, or white and auxotrophic yellow components. The heterokaryosis of some germspores is not surprising (Cerdá-Olmedo, 1975).

Viable counts on nutrient medium are notably higher than those on minimal medium plus nicotinic acid, presumably due to a non-specific increase of germination by the richer medium.

Each germsporangium usually contains the mitotically-amplified products of a single meiosis (Cerdá-Olmedo, 1975; Eslava *et al.* 1975*a*, 1975*b*), in this case either C242 × C9 or C242 × S102. The proportion of meioses involving C9 is best estimated as twice the proportion of prototrophs in the progeny. Thus we estimate having studied about 375 C242 × C9 meioses without finding any recom-

binants for the A and R phenotypes. There is a more than 95% probability that the recombination frequency between the mutations responsible for the A and R phenotypes is less than 1% (Spiegel, 1974).

(ii) *Genetic analysis of the AR phenotype*

The AR strains are also defective in sexual reproduction. The standard wild type NRRL1555 was used as a helper strain in crosses of strain C6 against the wild types B141 and B142. Zygospores germinated about 100 days after setting

Table 3. *Genetic analysis of the AR phenotype. Cross no. 1 B141 × (C6*NRRL1555). Cross no. 2 B142 × (C6*NRRL1555). Cross no. 3 B142 × (C6*NRRL1555). Heterokaryons with different nuclear proportions were used in the different crosses*

	Cross no. 1	Cross no. 2	Cross no. 3
Pooled germsporangia	50	50	617
Progeny studied	1584	815	2820
Phenotypes proportions:			
Yellow	0·696	0·54	0·868
White A	0·00	0·00	0·00
White AR	0·304	0·46	0·132
Red	0·00	0·00	0·00
Proportion of germsporangia carrying C6 progeny*	0·60	0·92	0·264
Estimated number of meioses involving C6	30	46	163

* Estimated as twice the proportion of white progeny.

up the cross. Analysis of individual germsporangia showed that practically all contained viable germspores. Neither R nor A recombinants were found in analyses of pooled germspores (Table 3). Additionally, a total of $2{\cdot}8 \times 10^6$ germspores from cross no. 3 were plated at a density of 3×10^4 germspores per plate, which should allow for the detection of rare red colonies, but these were not found.

The proportion of germsporangia carrying progeny of C6 may be estimated as twice the proportion of white progeny; we have analysed about 239 meioses involving C6 nuclei without finding any recombinants. The AR phenotype must be due to a single mutation, or to two closely-linked mutations. There is a more than 95% probability that the recombination frequency between two such mutations is less than 1·5% (Spiegel, 1974).

(iii) *Induction of colour mutants in yellow strains*

New A, AR, and R colour mutants have been looked for after treatment of strains NRRL1555 and C115 with MNNG and ICR-170 (Table 4). The marked colour difference between the two original strains might be expected to influence the detectability of different colour mutants, but in fact the same relative

numbers of each kind of mutant were obtained from both original strains. The two mutagens, on the other hand, induced very different mutant spectra.

(iv) *Induction of colour mutants in white strains*

Several white strains were treated with MNNG and ICR-170 to look for further mutation to either red or yellow phenotypes (Table 5). Since these phenotypes cannot be selected, the less frequent mutations would go undetected.

Table 4. *Search for new colour mutants in yellow strains*

Mutagen	Strain	Colonies examined	New colour mutants		
			A	AR	R
MNNG	NRRL1555	$4{\cdot}7 \times 10^6$	14	4	3
	C115	$1{\cdot}5 \times 10^5$	5	1	1
	Sum	$4{\cdot}9 \times 10^6$	19	5	4
ICR-170	NRRL1555	$8{\cdot}4 \times 10^5$	0	3	0
	C115	$3{\cdot}0 \times 10^5$	0	2	0
	Sum	$1{\cdot}1 \times 10^6$	0	5	0

Table 5. *Search for new colour mutants in white strains*

Strain	Phenotype	Mutagen	Colonies examined	New colour mutants	
				Yellow	R
C2	A	MNNG	$4{\cdot}2 \times 10^5$	12	0
		ICR-170	$8{\cdot}2 \times 10^5$	0	0
C171	AR, double-step	MNNG	$1{\cdot}6 \times 10^5$	0	65
C6	AR, single-step	MNNG	$1{\cdot}54 \times 10^5$	0	7
		ICR-170	$2{\cdot}46 \times 10^6$	0	0
S104	AR, single-step	MNNG	$2{\cdot}4 \times 10^5$	3	13
S124	AR, single-step	MNNG	$1{\cdot}8 \times 10^5$	0	0
		ICR-170	$5{\cdot}6 \times 10^5$	0	0
S125	AR, single-step	MNNG	$9{\cdot}3 \times 10^4$	0	0
		ICR-170	$7{\cdot}8 \times 10^5$	0	0

No colour changes were induced by ICR-170, suggesting its inability to revert or suppress mutations originally caused by MNNG or itself, but the data on this are relatively scarce.

MNNG often reverted the white A strain C2 to the wild-type yellow phenotype, but did not mutate it to red. The double-step AR mutant C171 was often mutated to red, but not to yellow; the vast majority of these red mutants were less pigmented than the original C9 strain, as if they had only partially recovered the A function. Thus, reversion and/or suppression of the A mutation is common, but need not be accompanied by change of the R function.

The single-step AR mutants C6 and S104 were mutated to red, indicating reversion and/or suppression of their defective A function. The three yellow mutants from S104 are particularly interesting because they show simultaneous

recovery of the A and R functions in one case in which they had been simultaneously lost. The mutants from C6 and S104 could not have been contaminants, since they show the peculiar phenotypes conferred by mutations *madF48* and *mad-107* on the sporangiophores of the original strains.

Finally, no colour changes were observed after MNNG treatment of the ICR-170-induced AR strains S124 and S125, but we did not examine sufficient colonies to make this a firm conclusion.

Table 6. *Genetic analysis of yellow revertants Cross no. 1 B142 × S160. Cross no. 2 B142 × S161*

	Cross no. 1	Cross no. 2
Pooled germsporangia	182	201
Progeny studied	$\sim 10^6$	$\sim 10^6$
Phenotype proportions:		
Yellow	1·00	1·00
White	0·00	0·00
Red	0·00	0·00

Table 7. *Genetic analysis of strain S165. Cross C242 × (S102*S165)*

Pooled germsporangia	589
Variable germspores	
On nutrient medium	$1{\cdot}7 \times 10^5$
On minimal medium plus nicotinic acid	$5{\cdot}0 \times 10^4$
On minimal medium	$1{\cdot}6 \times 10^4$
Progeny studied*	407
Phenotypes proportions:	
Yellow	0·28
White A	0·45
White AR	0·00
Red	0·27
Prototrophic progeny studied†	1317
Phenotype proportions:	
Yellow	0·00
White A	0·47
White AR	0·00
Red	0·53
Proportion of germsporangia carrying C165 progeny	0·64
Estimated number of meioses involving C9	377

* Colonies obtained by plating germspores on minimal medium plus nicotinic acid.
† Colonies obtained by plating germspores on minimal medium.

(v) *Genetic analysis of colour mutants from white strains*

Two yellow revertants from the single-step AR strain S104 were designated S160 and S161 and crossed with the wild type B142. Table 6 shows that only yellow progeny were found; so both S160 and S161 must be considered true revertants, and not the result of intergenic suppression.

The red mutants from S104 are sexually incompetent, like the other red mutants. Strain S102 was used as a helper strain to cross strain S165, one of the red mutants from S104, with the white A strain C242. Table 7 shows the results of the cross; the estimate of the number of meioses involving S165 nuclei assumes that the germsporangia were all fertile, but this was not independently checked. The lack of yellow or white prototrophic recombinants indicates that the mutation responsible for the red phenotype is closely linked to the mutation originally responsible for the white AR phenotype.

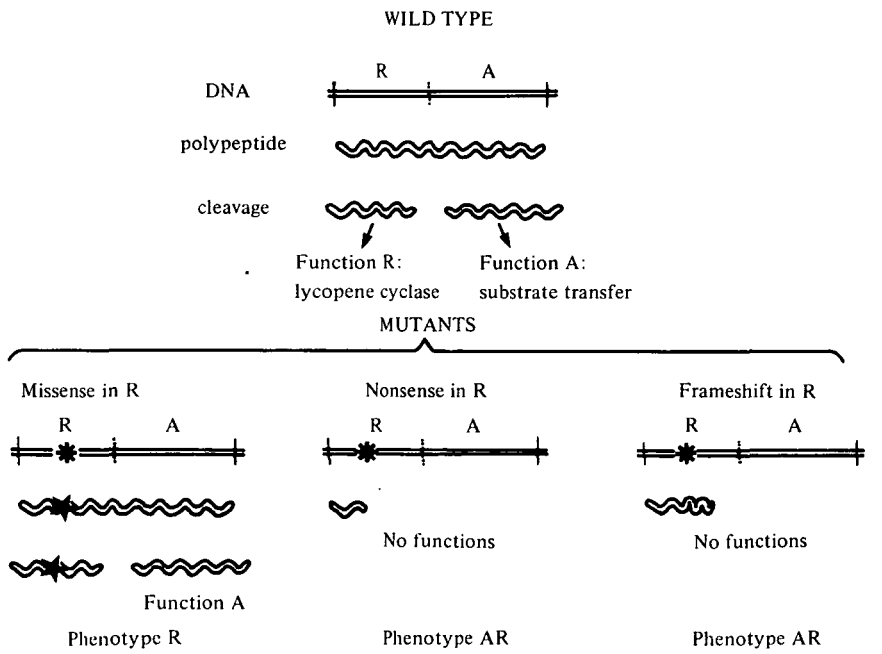

Fig. 1. Proposed structure of the *carRA* cluster-gene and expected phenotype of mutants in segment R.

4. DISCUSSION

The Results show that the genetic determinants for the related A and R functions are closely linked, a rather uncommon situation in eukaryotes. No recombinants have ever been found; from the number of meioses studied, the recombination frequency must be less than 1 %, which falls within the intragenic range in *Phycomyces*: recombinants were found between two alleles of gene *nicA* by studying only 175 meioses (Eslava *et al.* 1975*a*).

The mutant hunts indicate a special relationship between the genetic determinants for the A and R functions, beyond their physical proximity in the genome and their involvement in the same overall process of carotenogenesis. The frequency with which AR single-step mutants revert to wild type cannot be reconciled to the concept of two neighbouring, but separate, genes.

We propose that the A and R functions are determined by contiguous DNA segments, cotranscribed to a single mRNA, and cotranslated to a single polypeptide (Fig. 1). Several examples of such multifunctional genes or cluster-genes are known in the fungi (Giles, 1978). The results suggest that the R segment is proximal to the gene's beginning (5′ end of the mRNA and NH_2 end of the polypeptide). Phenotype R would arise from missense mutations in the R segment; phenotype AR, from nonsense or frameshift mutations in segment R; phenotype A, from any mutations in segment A. Thus, base substitutions induced by MNNG could give rise to each of the three phenotypes; some of the MNNG-induced A and AR mutants could also result from frameshift mutations. ICR-170 would have induced only frameshift mutations.

The R phenotype of strain C9 must be the result of a missense mutation, since its defective lycopene cyclase competes with the wild type enzyme for a place in the enzyme complexes (De la Guardia *et al.* 1971); the fact that heterokaryons C5*C6 do not accumulate lycopene or γ-carotene (Aragón *et al.* 1976) suggests that C6 has no lycopene cyclase able to compete with the wild type enzyme, as would be expected from a nonsense mutation.

Single-step AR mutants could revert to wild type through a single-step mutation at the original site or nearby, as witnessed by the yellow revertants of strain S104. Red mutants could also be obtained from single-step AR mutants through reversion or suppression in such a way that translation proceeds, but the R segment does not become functional. Strain S165 is most likely a revertant replacing a premature stop with an amino acid inappropriate for the R function. Alternatively, S165 could have a new initiating codon inside the R segment (Exinger & Lacroute, 1979).

All carotenogenic enzyme complexes, whatever their lycopene cyclase capability, are equally able to mobilize substrates for carotene production in C2*C9 heterokaryons (De la Guardia *et al.* 1971). If the two functions had to reside in the same polypeptide, one might expect that the enzyme complexes containing an A mutant product would be inactive in carotenogenesis.

This difficulty may be solved by assuming that the polypeptide is split into separate R and A proteins, as drawn in Fig. 1. There are well-documented cases of post-transcriptional cleavage of polypeptides, particularly in animal viruses (Hershko & Fry, 1975). We need not assume cleavage of the polypeptide if the A function is mediated by a diffusible product, able to promote substrate transfer in all carotenogenic enzyme complexes.

We propose designating *carRA* the bifunctional gene. The notations *carR* and *carA* could be used for each of the gene's segments, and the mutations affecting these segments. As examples, in Table 1, *carR21*, *carA5*, and *carRA12* indicate mutations affecting only segment A, only segment R, or both segments, respectively.

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