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Excess carotenoids disturb prospective cell-to-cell recognition system in mating responses of *Phycomyces blakesleeanus*

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Carotenogenic mutants of *Phycomyces*, which accumulate excess β-carotene or its intermediates, always failed in zygospore development. No improvement occurred when such mutants were mated together with a helper wild type of the same mating type against the wild type of the opposite mating type. Addition of excess synthesized pheromone, trisporin B, also failed to improve the zygospore development, though the mating response was significantly activated in the early stages and abundant zygophores were formed. Exceptional acceleration of the zygospore development under these experimental conditions occurred in a regulatory albino mutant (*carA*), which does not accumulate excess intermediate carotenoids. Chemically- or genetically-induced overproduction of β-carotene or lycopene also inhibited the zygospore development. These results imply that the zygospore development of *Phycomyces* is maximal when the intracellular amount of β-carotene is optimal (=wild type), and that pheromones act mainly in the early stages of mating, while other factors such as the cell-to-cell recognition system may also be involved in the later stages. Intracellular accumulation of excess β-carotene or its intermediates probably disturb such later-stage factors.

Key Words——carotenogenic mutants; cell-to-cell recognition; heterokaryons; mating response; *Phycomyces*; trisporoids.

The heterothallic fungus *Phycomyces blakesleeanus* has two mating types, (+) and (−), and develops zygospores as the final product when their hyphae meet on solid medium (Blakeslee, 1904; reviewed by Sutter, 1987). The mating process is classified into eight developmental stages (S1–S8; Sutter, 1975): formation of zygophores as swollen and irregularly-branched hyphae (S1), intertwisting of two zygophores of different mating types (S2), progametangium formation after enlargement of the paired zygophores (S3), looping of progametangia owing to splitting at the middle region of the enlarged pillars (S4), formation of gametangia and suspensors on both progametangial cells by delimitation with septa (S5), appearance of thorn-like appendages on the suspensors (S6), zygote formation by fusion of two gametangia (S7), and maturation of zygote as zygospore (S8).

This mating process is mediated by pheromones, trisporoids, which are metabolites of β-carotene (reviewed by Gooday, 1974; van den Ende, 1976; Kochert, 1978; Sutter, 1987; Lemke, 1990). Carotenogenic mutants (genotype, *car*) are incomplete in their mating capabilities (Sutter, 1975). Not only β-carotene-deficient mutants such as albinos accumulating phytoene (*carB*) and red mutants accumulating lycopene (*carR*) but also leaky *carR* mutants containing 40–60% of β-carotene in addition to a large amount of lycopene (Ootaki et al., 1973; Hsu et al., 1974) and bright-yellow mutants accumulating 10–30 times more β-carotene (*carS*; Murillo et al., 1978) always fail to develop zygospores even when mated with the wild types. The only exceptions are *carA* albino mutants, which produce 1/4–1/100 of the number of zygospores of the wild types (Sutter, 1975). The mating development of these *car* mutants is always arrested at an early stage (S2-3). Thus, there is no clear-cut correspondence in this fungus between zygospore production and the amount of intracellular β-carotene.

Intergeneric or interspecific matings among some Mucorales fungi develop progametangia or gametangia but never produce the zygospores (Satina and Blakeslee, 1930). These results, together with the Sutter's findings, suggest that pheromonal actions may not govern the whole mating process and that other factors may be involved.

The pheromonal action-unitary theory, however, still cannot be ruled out, because the amounts and/or kinds of pheromonal products, or fungal sensitivity to pheromones may differ among different genera, species, and even strains of the same species (Sutter, 1975, 1977; Sutter and Whitaker, 1981; Drinkard et al., 1982). In addition, higher quantitative levels of pheromones may

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be required for the later mating stages. If these possibilities are valid, the mating behaviors of the *car* mutants may be interpretable in terms of pheromones.

In the present study, we demonstrate that intracellular accumulation of excess carotenoids disturb the mating of *Phycomyces*, probably by affecting the cell-to-cell recognition system, which appears to be involved in the later stages of mating, by analyzing the mating behaviors of heterokaryons of the *car* mutants and the effects of additional synthesized mating pheromones or inhibitors of β-carotene synthesis.

Materials and Methods

Strains The strains of *Phycomyces blakesleeanus* used in this work are listed in Table 1. NRRL 1555(−) and A56 (+) were obtained from Northern Regional Research Laboratory, Peoria, IL. and from Alvarez and Eslava (1983), respectively. All the C mutants were isolated from NRRL 1555 by NTG-mutagenesis at California Institute of Technology, CA (Heisenberg and Cerdá-Olmedo, 1968; Meissner and Delbrück, 1968; Ootaki et al., 1973). The *carA* mutants are deficient in substrate transfer system among carotenogenic enzymes and in negative regulation by β-carotene (Murillo et al., 1981). The *carB* and *carR* mutants carry mutations of the dehydrogenase gene and cyclase gene, respectively (Meissner and Delbrück, 1968). The leaky *carR* mutants contain a considerable amount of β-carotene in addition to predominant lycopene (Ootaki et al., 1973; Hsu et al., 1974). The *carS* mutant (C115) is probably a failure in the operation of end-product control in the carotenogenic pathway (Meissner and Delbrück, 1968; Murillo and Cerdá-Olmedo, 1976; Murillo et al., 1978, 1981). Both C158 and C166 slightly overproduce β-carotene, but the causal mechanisms are still unknown.

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

Group	Strain[a)]	Phenotype
wild type	NRRL 1555(−), A56(+)	Yellow, standard wild type, Isogenic (+) wild type
carA	C2(−), C3(+)	Albino, small amount of β-carotene
carB	C5(−)	Albino, phytoene accumulation
carR	C9(−)	Red color, lycopene accumulation
leaky *carR*	C13(−)	Orange color, lycopene accumulation, about 40–60% of β-carotene
carS	C115(−)	Bright yellow, about 15 times more β-carotene
β-carotene overproducing	C158(−)	Bright yellow, about 3 times more β-carotene;
	C166(−)	About 2 times more β-carotene
carAcarR	C171(−)	Albino, double *car* mutant
carBcarR	C172(−)	Albino, double *car* mutant

a) (+) and (−), mating types.

Culture conditions For vegetative subcultures, each strain was inoculated on glucose-asparagine-agar medium (SIV medium; Sutter, 1975) in Petri plates, supplemented with yeast extract (Difco, 1 g/L) and casitone (Difco, 1 g/L; SIVYC medium). The strains were cultured at 20°C under continuous white-fluorescent light (cool white FL40SD/38, Toshiba Electric., Tokyo, Japan) at about 0.1 W/m^2 at mycelial level.

For mating, small pieces (20 × 2 mm) of mycelial fronts, isolated from both mating-type subcultures, were inoculated about 4 cm apart at confronting positions on dialysis-membrane discs layered on solid media (glucose-glutamate-agar medium; SI) in 6-cm Petri plates, or on oblong strips of SI-agar medium when synthesized pheromones or chemicals were added (see below). The dialysis-membrane discs were washed with 1 mM EDTA and distilled water, and autoclaved before use to remove fungicidal coatings (Sambrook et al., 1989). This membrane-culture method is convenient for counting the sexual organs developed, because of formation of nearly monolayered mycelia. The cultures were kept at 17°C for 10 d in the dark. Sexual organs were counted under a dissecting microscope after being stained with 0.01% methylene blue in two different regions (5 × 5 mm each) randomly selected along the mating mycelial front (Yamazaki and Ootaki, 1996 a, b). The numbers of sexual organs in the two regions were combined and represented as the number per unit area (10 × 5 mm).

Heterokaryon formation and determination of nuclear proportion Heterokaryons were constructed by grafting the decapitated young sporangiophores from two genetically different strains (Ootaki, 1973, 1987). A high proportion of sporangiophores singly regenerated at the graft union was heterokaryotic. The spores were collected from the regenerant in distilled water and streaked on plates containing glucose-asparagine-yeast extract medium (GAY) acidified with HCl to pH 3.1 (Cerdá-Olmedo and Reau, 1970) after heat activation (48°C, 10 min). This medium promoted both carotenogenesis and colonization of mycelia, and allowed individual colonies with different shades of color markers to be counted.

Since the spores are predominantly multinucleate, the heterokaryons containing two different types of nuclei produce spores of three different nuclear combinations due to nuclear segregation in the sporangium, which on germination produce colonies with various shades of color (Heisenberg and Cerdá-Olmedo, 1968). The nuclear proportions of heterokaryons were estimated by applying the colony numbers counted to theoretical equations (Heisenberg and Cerdá-Olmedo, 1968; Cerdá-Olmedo, 1975).

Chemical treatments β-Carotene inhibitors, CPTA (2-(4-chlorophenylthio)-triethylamine hydrochloride) and EPTA (*p*-ethyl phenoxy-triethyl amine), given by Dr. H. Yokoyama, U.S. Dept. of Agri., Fruit and Veg. Chem. Lab., Pasadena, CA, were dissolved in distilled water and ad-

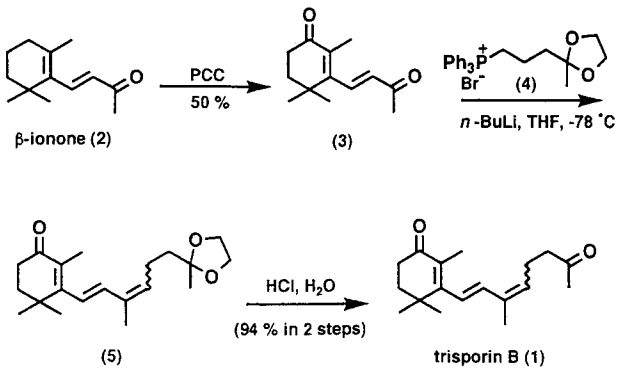

Fig. 1. Scheme of total synthesis of trisporin B.

ded to the melted SI-medium just before it was poured.

Synthesis and bioassay of pheromones A (−) mating type-specific pheromone, trisporin B (Fig. 1 (1)), was synthesized in three steps starting from β-ionone (2). β-ionone was first converted to diketone (3) by treatment with pyridinium chlorochromate (PCC) according to Müller et al. (1978). The Wittig reaction of this diketone (3) with the ylide generated from phosphonium salt (4) and *n*-butyl lithium (Isoe et al., 1971; Takahashi et al., 1988) and deprotection of compound (5) furnished a mixture of 9-*cis* and 9-*trans* trisporin B (1) (47% yield in three steps).

To investigate the effects of trisporin B, a small piece (20 × 2 mm) of each mating-type mycelium was inoculated on each edge of an SI-agar strip (25 × 50 × 1 mm), placed on a microscope glass slide. The slide was transferred on a U-shaped glass-rod support in a 9-cm Petri plate with a few ml of H_2O to maintain high humidity. After 2 d of culture in the dark, before the two mycelial fronts made physical contact, the central region of the agar strip (about 1 cm in width) was removed with a scalpel, and new SI-agar containing trisporin B (1×10^{-4}–3.7×10^{-3} M) was added into the gap between the two mycelial fronts. Solution of trisporin B in ethyl alcohol (0.2 ml) was emulsified with Tween 20 and liquid paraffin (0.1 ml each) and diluted with 10 ml of SI-agar medium.

Table 2. Zygospore development of *car* mutants of *Phycomyces*, accumulating two to three times more β-carotene than the wild type.

Strains[a]	Number of zygospores/unit area[b]
wild types	
NRRL 1555	136.1 ± 12.1
β-carotene-overproducing mutants	
C158 (about 3 times)	2.6 ± 0.9
C166 (about 2 times)	12.6 ± 1.4

a) The strains were mated against A56 (+).
b) The sexual organs at S7 and S8 were combined and represented as the number of zygospores per unit area (5 × 10 mm). Each datum shows the mean of four measurements with SE.

Trisporin B (1×10^{-4} M) was also added directly on both mycelial fronts just when they made contact, by dropping a drop of SI-liquid medium containing trisporin B and a small amount of Tween 20. These treatments were carried out under dim light to prevent disturbance of the mating response (Yamazaki et al., 1996) and from photo-decomposition of trisporin B. The mating was continued for further 4 d in the dark.

Results

Mating responses of *car* mutants and their heterokaryons Two β-carotene-overproducing mutants, C166 and C158, accumulating about 2 and 3 times more β-carotene, respectively, than the wild types, maintained the capability of zygospore development when mated with the wild type (Table 2), in sharp contrast to *carS* mutant (C115), which accumulates 15 times more β-carotene and is absolutely infertile (Sutter, 1975). These two mutants, however, developed only approximately 1/10 and 1/60 of the numbers of zygospores of the wild type, respectively (Table 2). The leaky *carR* mutant (C13) mated with the wild type produced an abundance of S2-zygophores and some early S3-progametangia but never developed the S8-zygospores (data not shown), confirming Sutter's results (1975).

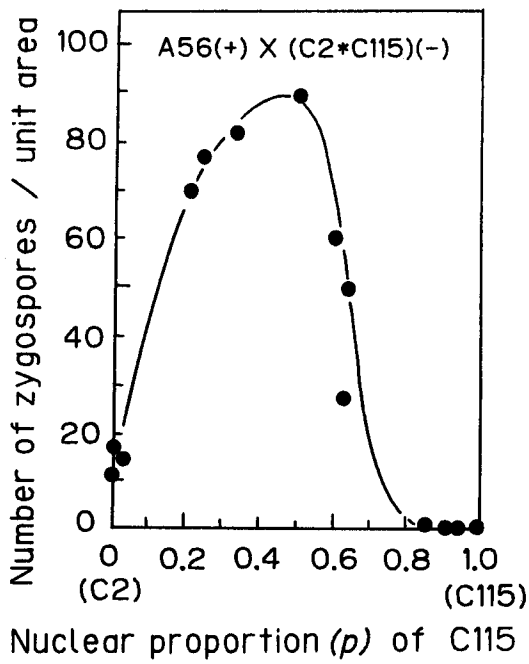

Fig. 2. Zygospore development of *Phycomyces* heterokaryons between two regulatory carotenogenic mutants, an albino mutant C2 and a β-carotene-overproducing mutant C115, as a function of nuclear proportion (*p*) of C115 in the heterokaryon.

The heterokaryons were mated against the wild type A56(+). Each point represents the result for one heterokaryon as the number of S7–8 zygospores developed in a unit area (5 × 10 mm). An asterisk (*) shows a heterokaryon between two genetically different strains.

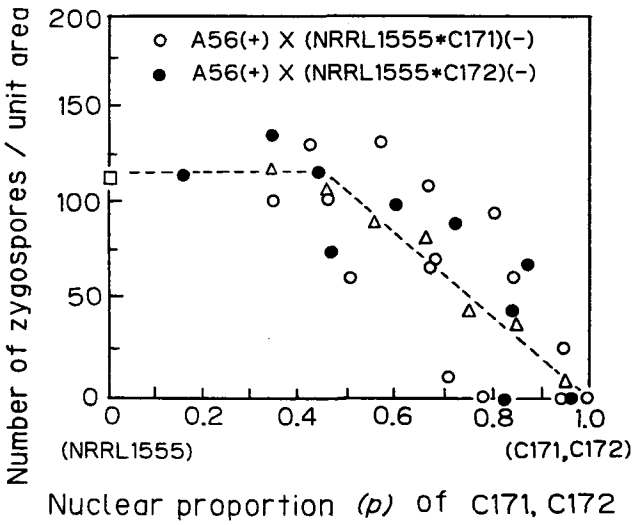

Fig. 3. Zygospore development of heterokaryons between the wild type (NRRL 1555) and double-*car*-mutants (C171, C172), as a function of the nuclear proportion (p) of double-*car* mutants.

The heterokaryons were mated against the wild type (A56). Open square, mating between A56(+) and NRRL 1555(−) as a control; open circles, heterokaryons with *carAcarR*; solid circles, heterokaryons with *carBcarR*. Open triangles represent the means of the zygospores of the indicated nuclear proportion±0.05. Broken lines were drawn along the mean values. See also the legend to Fig. 2.

Figure 2 shows the capability of zygospore development of the heterokaryons (C2*C115) between two regulatory mutants, C2 and C115, as a function of the nuclear proportion. When mated with the wild type (A56), production of zygospores was maximal at the best balance of nuclear proportion ($p=0.5$).

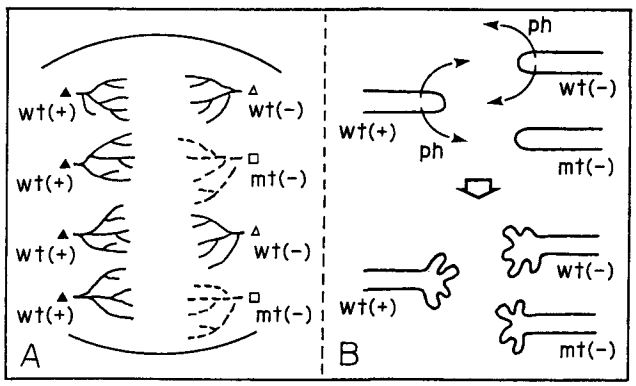

Fig. 4. A, Schematic diagrams of prospective mating responses of *Phycomyces* when the *car*(−) mutant (mt, open squares) was mated together with a helper (−) wild type (wt, open triangles) against the (+) wild type (solid triangles). B, Hyphae of all three strains are expected to develop zygophores equally, because of equal stimulation by pheromones (ph) secreted from the counterpart wild types.

Heterokaryons of diverse nuclear proportions were constructed between each of the double-*car* mutants and the wild type (NRRL 1555), and examined for their mating capabilities against A56(+). Normal zygospore production, as high as the wild-type control (A56×NRRL 1555), occurred whenever the heterokaryons contained more than a half of the wild-type nuclei (Fig. 3). The zygospore production, however, gradually decreased with an increase in the nuclear proportion of the double-*car* mutants. No significant difference was found in the mating responses between C171 and C172 as a component of heterokaryons.

Mating responses of *car* mutants with helper wild type

Since sexual pheromones are derived from β-carotene, pheromone production of β-carotene-deficient mutants may not be sufficient for stimulation of the counterpart hyphae (Sutter, 1975). To examine whether this is only the reason for infertility of these mutants, the *car* mutants were inoculated together with the wild type of the same mating type as a helper and mated against the wild type of the opposite mating type (Fig. 4A). In this system, all the strains inoculated, the *car* mutants and two kinds of the wild types, are assumed to be stimulated

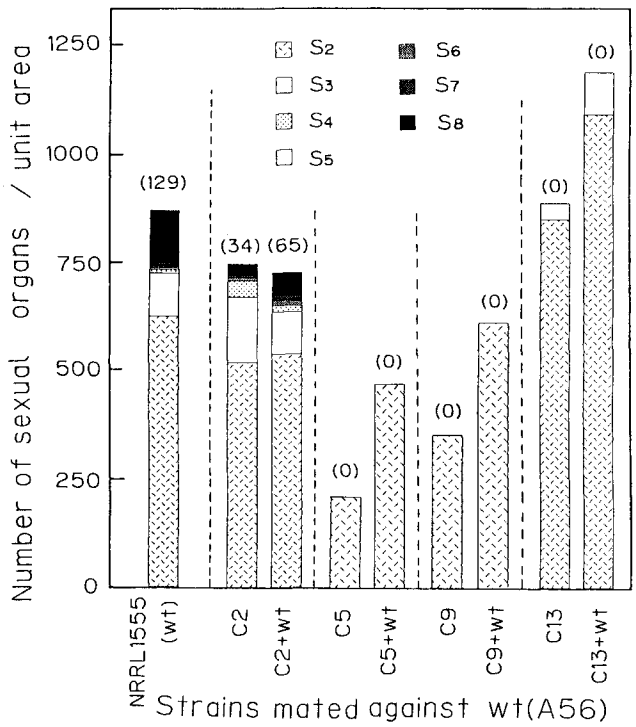

Fig. 5. Mating responses of carotenogenic mutants mated singly or together with a helper wild type (NRRL 1555) against the wild type (A56) of the opposite mating type.

The stages (S2-8) represent the developmental stages of sexual organs (see Introduction). The numbers of sexual organs at each stage were counted for a unit area (5×10 mm). The number of S7-8 zygospores developed are in parentheses. Note no recovery of zygospore development in the *car* mutants accumulating phytoene (C5) or lycopene (C9, C13).

Table 3. Effects of β-carotene inhibitors, CPTA and EPTA, on mating response of *Phycomyces*.

Concentrations (μg/ml)	Inhibitors	
	CPTA	EPTA
0	S8 (127.5±13.4)[a] yellow[b]	S8 (140.2±8.9) yellow
6.2	S8 (6.1±1.0) orange	S8 (56.2±4.2) orangish yellow
12.5	S2 (0) orangish red	S8 (10.3±1.1) orange
25	S2 (0) orangish red	S2 (0) orangish red
50	S1-2 (0) red	S2 (0) orangish red
100	S1-2 (0) red	S1-2 (0) red

a) The final stages of sexual organs developed (S1-S8) and average numbers of zygospores (parentheses) are shown. The sexual organs at S7 and S8 were combined and represented as the number of zygospores per unit area (5×10 mm). Each datum shows the mean of three measurements with SE.
b) Color of mycelia.

by the mating type-specific pheromones secreted from the opponent wild types (Fig. 4B). Recovery of normal zygospore development on the *car* mutants can be expected.

When C5, C9 and C13 were inoculated together with NRRL 1555(−) and mated against A56(+), the S4-8 sexual organs were formed only between the two wild types and not between the *car* mutants and A56 (Fig. 5). In the *car* mutants, the mating responses against A56 were always arrested at S2 (C5, C9) or, occasionally, early in S3 (C13), though their zygophore development was much more accelerated. The capability of zygospore development of C2 was significantly improved by the mixed culture with the wild-type helper (Fig. 5). In these observations, the mutants were distinguishable from the wild types by the color of hyphae and suspensor cells.

Effects of carotenogenic inhibitors Both CPTA and EPTA specifically block the cyclizations needed to convert lycopene into β-carotene and result in the accumulation of lycopene in a wide array of carotenogenic tissues, including *Phycomyces* mycelium (Coggins et al., 1970; H. Yokoyama, personal communication). With an increase in the concentrations of both inhibitors, the mycelia became red and deepened in color because of increasing lycopene-accumulation, but the zygospore development sharply decreased (Table 3).

Effects of synthesized trisporin B To determine the pheromonal activity, various concentrations of synthesized trisporin B were added to singly-cultured wild types, and zygophore development was investigated (Fig. 6). The first symptom of sexual response to pheromones was an arrest of hyphal growth prior to zygophore development (Drinkard et al., 1982). Since trisporin B is a (−) mating type-specific pheromone (Sutter and Whitaker, 1981; Sutter and Zawodny, 1984), it predominantly affected the (+) mating type (A56). At 1×10^{-4}-3.7×10^{-3} M of trisporin B, hyphal growth of A56(+) was arrested and zygophore formation started (Fig. 7 A, C). No such zygophore development, however, occurred on NRRL 1555(−) (Fig. 7 B, C).

Direct addition of trisporin B (1×10^{-4} M) in the mating regions caused significant acceleration of mating response only when the mating type of *car* mutants was minus (Fig. 7F, G; Fig. 8). The increases in the total numbers of sexual organs developed (ΣSO in Fig. 8) were about 138 and 145%, respectively, when C2(−) and C13(−) were mated against the wild types. Zygospore development was about twice when C2 was mated in the presence of trisporin B (Fig. 7F, G; Fig. 8). When C3(+) was mated, however, zygospore development was not improved (Fig. 7D, E; Fig. 8). In the leaky-*carR* mutant, addition of synthesized trisporin B also did not result in a recovery of zygospore development (Fig. 7H; Fig. 8).

Discussion

Sutter (1975, 1987) revealed that β-carotene-deficient mutants such as *carB*, *carR* and double *car* mutants were infertile in terms of zygospore development when mated against the wild type, probably because of their poor secretion of pheromones. Indeed, β-carotene-deficient mutants induced few zygophores on the counterpart hyphae of the opposite mating type (Sut-

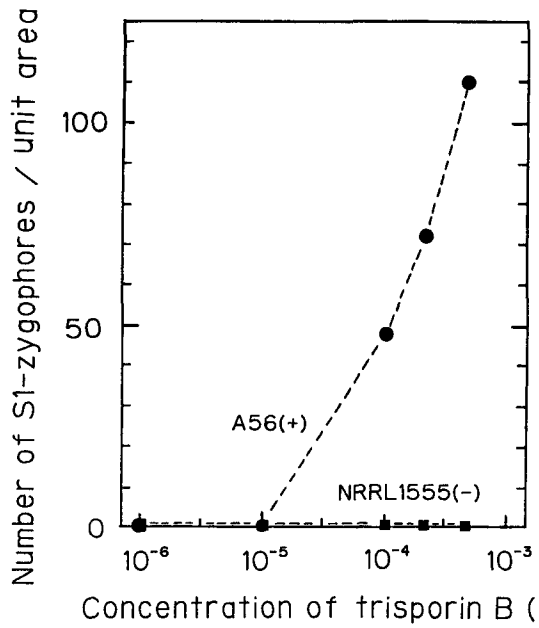

Fig. 6. Number of zygophores developed in a unit area (5×5 mm) of the wild types, A56(+) (solid circles) and NRRL 1555(−) (solid squares), in the presence of synthesized trisporin B at various concentrations.

Note the larger effects on the (+) wild type. Both strains were separately cultured on independent plates in the dark.

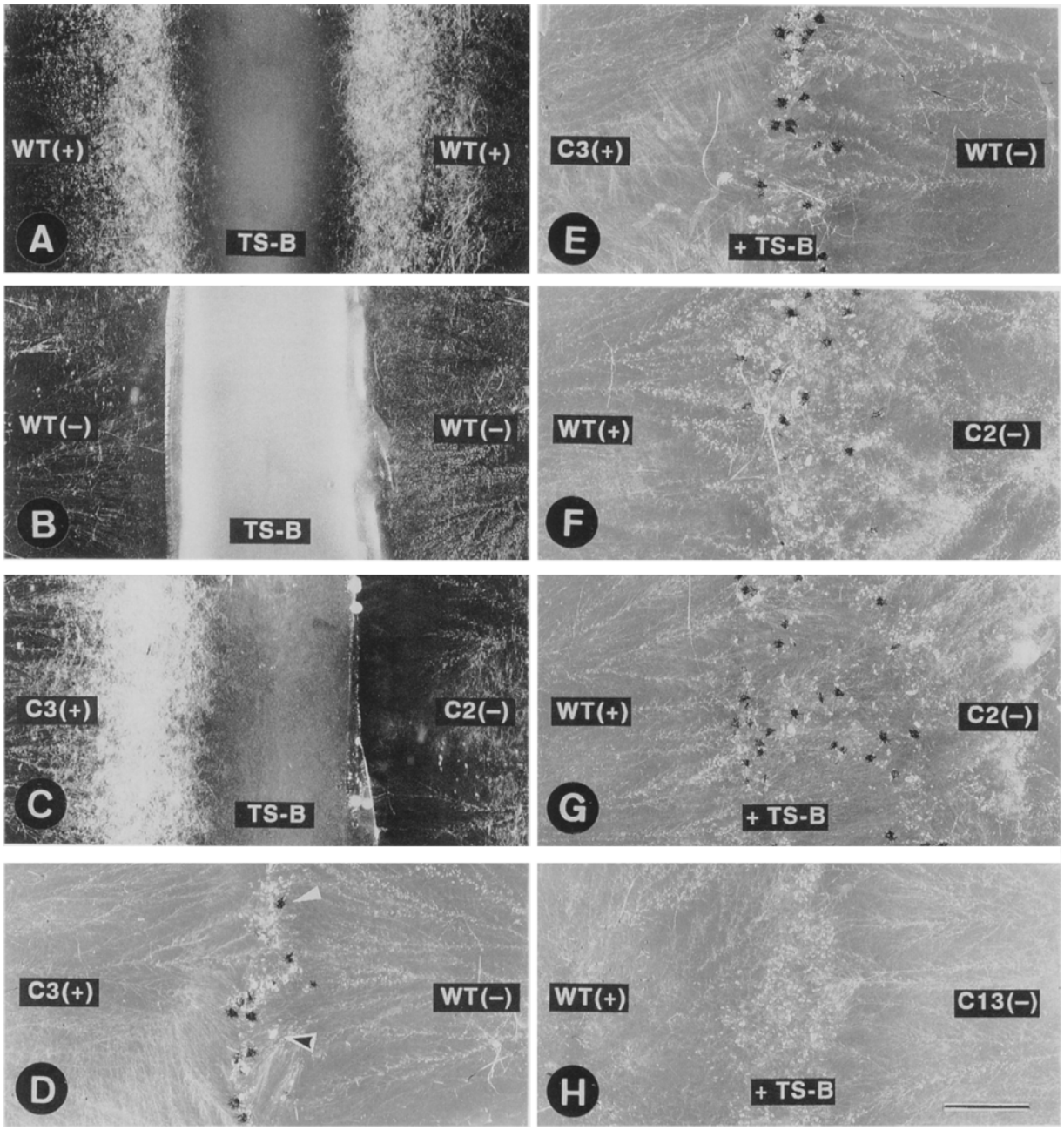

Fig. 7. Mating responses of *Phycomyces* in the presence of synthesized trisporin B (TS-B).

A–C: When the hyphae of (+) and (−) mating types reached the center zone of agar medium containing trisporin B, the growth of (+)-mating-type hyphae was arrested and abundant zygophores were formed. A, A56(+) against A56(+), as a control showing intensive induction of zygophores on both hyphal fronts; B, NRRL 1555(−) against NRRL 1555(−), as another control showing no zygophore-stimulation by trisporin B; C, mating between two carotenogenic mutants, C3(+) and C2(−), representing the development of abundant zygophores only on the C3 side (left). D–H: Trisporin B was directly added to the mating zone. D-E, mating responses between C3(+) (left) and NRRL 1555(−) (right) in the absence (D) or in the presence (E) of trisporin B, showing no significant induction of zygospore development by trisporin B. White and black arrow heads show S8-zygospore and S4-gametangium, respectively. F–G, mating responses between A56(+) (left) and C2(−) (right) in the absence (F) or in the presence (G) of trisporin B, showing zygophore stimulation on A56(+) hyphae and, as a result, acceleration of zygospore development. H, mating between A56(+) (left) and the leaky-*carR* mutant C13(−) (right) in the presence of trisporin B, showing development of many S2 and some S3 zygophores in the mating zone, due to mating activation of A56 hyphae. Note no zygospore development. Bar = 5 mm.

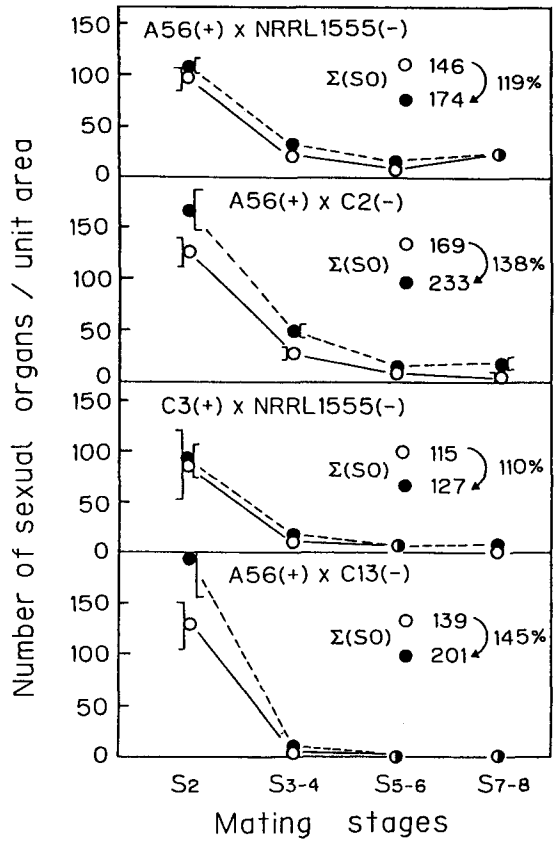

Fig. 8. Mating responses of *Phycomyces* in the absence (Tween 20 alone) or presence of synthesized trisporin B.

Pheromone was directly added to the mating zone. Sexual organs at each stage (S2-8) per unit area (5×10 mm) were counted and represented as a mean of three measurements with SE. The mating responses of *car* mutants of (−) mating type (C2, C13) were significantly accelerated by additional trisporin B when mated against A56(+), probably because of pheromonal stimulation of A56. Note a higher rate of increase in the total number of sexual organs (ΣSO) in the mating combinations of A56×C2 and A56×C13 than the others. In general, the presence of Tween-20 rather suppressed the zygospore development.

ter, 1975). Complementary heterokaryons for carotenogenesis such as *carA***carB*, *carA***carR*, and *carB***carR* recovered the capability of zygospore development when their nuclear proportions were well balanced (Heisenberg and Cerdá-Olmedo, 1968; Ootaki et al., 1973; Cerdá-Olmedo, 1975; Roncero and Cerdá-Olmedo, 1982), probably because of recovery of normal production of β-carotene and the resultant pheromones. The *carA* mutants containing a small amount of β-carotene maintain a partial capability of zygospore development. These results imply that the magnitude of zygospore-development capability corresponds to the amount of intracellular β-carotene.

A contradiction of this scheme, however, is presented by the deficiency of zygospore development in the β-carotene-overproducing (C115) and β-carotene-leaky *carR* mutants (Sutter, 1975). Together with our findings that C166 and C158, which contain about 2 and 3 times more β-carotene, developed respectively only 1/10 and 1/60 of the numbers of zygospores of the wild type, these results imply that the zygospore-development capability does not correspond linearly to the amount of intracellular β-carotene, and that accumulation of β-carotene above a certain level inhibits zygospore development, though its mechanisms are still unknown (Salgado et al., 1989). This was also supported by the fact that mating capability of C2*C115 heterokaryons depended greatly on the nuclear proportion (Fig. 2): the zygospore production was maximal at the best balance of nuclear proportion ($p=0.5$) but sharply decreased with the increase in nuclear proportion of C115. The amount of β-carotene in the heterokaryons increased exponentially with the increase in *carS* nuclear proportion: at $p=0.5$ the heterokaryons produced almost the same amount of β-carotene as the wild type, and at $p=0.7$ β-carotene was almost twice that of the wild type (Murillo and Cerdá-Olmedo, 1976). These facts agree with the mating responses of C166 and C158.

In the heterokaryons between the wild type and the double *car* mutants, which contain an undetectable amount of β-carotene, the zygospore development was normal when the wild-type nuclei accounted for more than half of the nuclear population (Fig. 3). The capability of zygospore development and the yellow color of mycelia gradually decreased and paled with the decrease in the nuclear proportion of the wild type. These results imply that the amount of β-carotene in the wild types is the optimal for the zygospore development, and that in heterokaryons both mating response and β-carotene synthesis occur normally when at least half of the nuclei in the population are normal.

As well as excess β-carotene, accumulation of excess intermediate carotenes such as phytoene and lycopene also inhibits the zygospore development, in particular at the later stages of mating. The phytoene-accumulating C5 and lycopene-accumulating C9 and C13 always failed to produce zygospores (Sutter, 1975), even though C13 contains more β-carotene than *carA* mutants. The loss of capability of zygospore development in the lycopene-accumulating wild types induced by CPTA or EPTA (Table 3) supports the above results.

We assume here that in *Phycomyces* pheromones act predominantly in the early stages of the mating process, probably up to the S2-zygophore formation (Sutter, 1977), and that another factor such as the cell-to-cell recognition system (Manocha, 1990) acts predominantly in the later stages. Accumulation of excess β-carotene or intermediate carotenes inhibits this later recognition system.

The fact that the mixed culture of *car* mutants with a helper wild type produced an abundance of zygophores (Fig. 5) implies that zygophore-stimulating pheromones from the helper were supplied normally to the *car* mutants. If pheromones governed the entire mating process, zygospores would develop normally between the *car* mutants (C5, C9, and C13) and the wild type

(A56), as found between two wild types (A56×NRRL 1555). The failure of zygospore development in the *car* mutants may support our speculation. Acceleration of zygospore production in the *carA* mutant by the helper wild type, in a contrast to the other *car* mutants (Fig. 5), may be due to no accumulation of specific intermediates of carotenoids (Meissner and Delbrück, 1968; Ootaki et al., 1973).

Addition of synthesized trisporin B stimulated significantly the zygophore development on (+)-mating-type hyphae, supporting the results of Sutter and his collaborators that the mating type-specific precursors of trisporic acids are zygophore-stimulating pheromones in *Phycomyces* (Sutter, 1975, 1977; Sutter and Whitaker, 1981; Drinkard et al., 1982). Our finding of unrecoverable zygospore production in the leaky *car* mutant by addition of trisporin B (Figs. 7, 8), however, may provide evidence of involvement of multiple steps in the mating, ruling out the possibility that quantitative differences in pheromones regulate the whole mating processes.

In C13×A56, the mating response was always arrested at S2 or very early S3. Not only C13 but also A56 stopped at this stage, even though A56 genetically possesses the complete mating capability. Some information from the counterpart C13 that is essential for the further development of A56 may be blocked. Sassen (1962) also assumed that some chitinolytic enzyme-inducing signals came from the mating partner when two gametangial cells enzymatically fused at S6-7. The existence of such an intercellular recognition system is also supported by the fact that vegetative hyphae or sporangiophores regenerated on a progametangial cell when its counterpart cell was surgically incised (Yamazaki and Ootaki, 1996b). Deformed or coiled progametangia obtained at a high temperature (25-27°C) also induced regeneration of vegetative organs with a high proportion (Yamazaki and Ootaki, 1996b). These regenerations seem to result from a disturbance of the recognition system between two gametangial cells. The extracellular fibrils connecting two progametangial cells may contribute to the maintenance of close intercellular communication (Yamazaki and Ootaki, 1996a).

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