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Vegetative regeneration on sexual organs in *Phycomyces blakesleeanus*

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Phycomyces blakesleeanus **produced an abundance of sexual organs when two mating types met on solid medium, but only about 14.7% of the sexual organs developed to the final stages. On the sexual organs showing arrested development, vegetative hyphae or dwarf sporangiophores (microphores) often regenerated. This vegetative regeneration was accelerated when the paired and looped progametangia were isolated from mycelia, when the counterparts of the progametangial cells constructing the loop were surgically incised, and when *Phycomyces* was mated at high temperature (25–27°C). A leaky-carotenogenic mutant, whose sexual reaction was imperfect and arrested at an intermediate stage even when mated with the wild type, also regenerated hyphae with a high frequency on these arrested intermediate organs. The vegetative regeneration seems to result from interruption of a cell-to-cell recognition system between cells of different mating type, which is believed to be essential for the mating process of this fungus in addition to the pheromonal actions.**

Key Words——carotenoid mutants; cell-to-cell recognition; fungus; mating; *Phycomyces;* regeneration.

The strengths of the fungus *Phycomyces* as an experimental material are its high sensitivity and sharp response to external stimuli such as light and gravity, its high capability for regeneration, and its remarkable mating response. These characteristics have allowed intensive studies on its behavioral physiology, genetics, and morphogenesis (reviewed by Galland and Lipson, 1987; Galland and Ootaki, 1987; Sutter, 1987). In particular, regeneration studies are of practical value for physiological exploration of organs, tissues, and cells, since regeneration patterns reflect potential physiological conditions.

In *Phycomyces*, regeneration can be achieved from any stage of the vegetative cycle: hyphae, small segments of sporangiophores, and even naked protoplasm (Gruen and Ootaki, 1970, 1972; Ootaki and Gruen, 1970; Weide, 1939). Sexual organs also regenerate hyphae or sporangiophores if the sexual process is interrupted (Burgeff, 1924; Orban, 1919). Such a remarkable capability for regeneration opens the way for the genetic study of this fungus by readily allowing heterokaryons to be formed between two or more genetically different strains; as regenerants from decapitated and grafted sporangiophores (Burgeff, 1912, 1924; Ootaki 1973, 1987), from naked and mixed protoplasm (Heisenberg and Cerdá-Olmedo, 1968; Weide, 1939), and from isolated and macerated sexual organs (Bergman et al., 1969; Gauger et al., 1980).

Sexual organs are formed when two heterothallic strains meet on solid medium, starting from formation of swollen and irregularly branched sexual hyphae (zygophores) and ending in production of zygospores after the successive development of intermediate sexual organs (Blakeslee, 1904; Sutter, 1975). This mating process is mediated by collaborative biosynthesis and action of mating pheromones, trisporoids (reviewed by Gooday, 1974; Kochert, 1978; Lemke, 1990; Sutter, 1987; van den Ende, 1976), but involvement of a cell-to-cell recognition system between two mating cells is also suggested (Satina and Blakeslee, 1930; Yamazaki and Ootaki, 1996).

In the present study, we describe the accelerated regeneration of vegetative hyphae or sporangiophores on the sexual organs resulting from exposure to high temperature, isolation from mycelia, mating with a sexually imperfect mutant, and surgical incision in sexual organs. Such acceleration of vegetative regeneration seems to be due to interruption of the recognition system between two mating cells.

Materials and Methods

Strain NRRL1555(−) of *Phycomyces blakesleeanus* Burgeff is the standard wild-type obtained from Northern Regional Research Laboratory, Peoria, Ill., and A56(+) is its isogenic strain obtained by Alvarez and Eslava (1983). C13(−) is leaky *carR*-mutant accumulating lycopene and a considerable amount of β-carotene (about 40% of the total carotenes; Hsu et al., 1974; Ootaki et al., 1973), which was obtained at California Institute of Technology by one-step mutation of NRRL1555 with nitrosoguanidine.

These strains were precultured on enriched PDA

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medium, consisting of potato broth (1 L), glucose (20 g), yeast extract (1 g; Difco), casitone (1 g; Difco), agar (15 g) and thiamine hydrochloride (1 ml of 0.05%), under continuous illumination with white fluorescent light (0.1 W/m^2; cool white FL40SD/38, Toshiba Elec., Tokyo, Japan) at 20°C.

For mating, two small pieces of mycelia isolated from precultures of different mating types were inoculated about 4 cm apart at confronting position on dialysis-membrane discs layered on 10 ml of synthetic glucose-glutamate-agar medium (SI medium; Sutter, 1975) in 60-mm Petri plates. 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). The cultures were aseptically kept at 20°C in the dark. To see the effects of high temperature, *Phycomyces* was also mated at 27°C, or transferred from 20°C to 27°C after surgical treatment.

Surgical incision was carried out in the looped progametangia at stage 4 (S4; see below), because of the technical advantage of their comparatively large size. Counterparts of the paired S4-progametangia, attached to mycelia or after isolation on plain agar plates, were incised with alcohol-sterilized iris scissors (Vannas, German Surgicals, Toronto, Canada) under a dissecting microscope and then kept in the dark.

The viability of spores was inspected by collecting spores in a vial containing distilled water and by streaking them on a 60-mm plate (about 10^4 spores/plate) containing solid glucose-asparagine-agar medium (SIV medium; Sutter, 1975) after activation at 48°C for 10 min to break dormancy. The spores were allowed to germinate at 20°C for 20 h in the dark.

Mitochondria were stained with 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$), dissolved in 99.5% ethylalcohol (5 μmol/L) and diluted 1/100 with distilled water, for 20 min in the dark (Matzke and Matzke, 1986). They were observed with a Nikon YF-EFD2 epifluorescent microscope equipped with a high-pressure mercury vapour lamp (HBO-100W/2), a 450-490 nm excitation filter and a 510-540 nm emission filter.

The χ^2 test at $P=0.05$ level was used for statistical comparisons.

Results

Regeneration on sexual organs attached to mycelium and after isolation About 2 d after inoculation, the mycelial fronts of two mating types met and started to develop intertwined zygophores, which reached the final stages after a further 1-2 d. In the mating process, eight developmental stages (S1-S8), based on morphological characteristics of the sexual organs (Sutter, 1975), were clearly distinguishable (Fig. 1): formation of zygophores (S1), intertwining of zygophores (S2), development of paired and club-shaped progametangia after enlargement of zygophores (S3), formation of looped progametangia owing to splitting at the middle region of the enlarged pillars (S4), development of gametangia and suspensor cells by delimitation of progametangia by septa (S5),

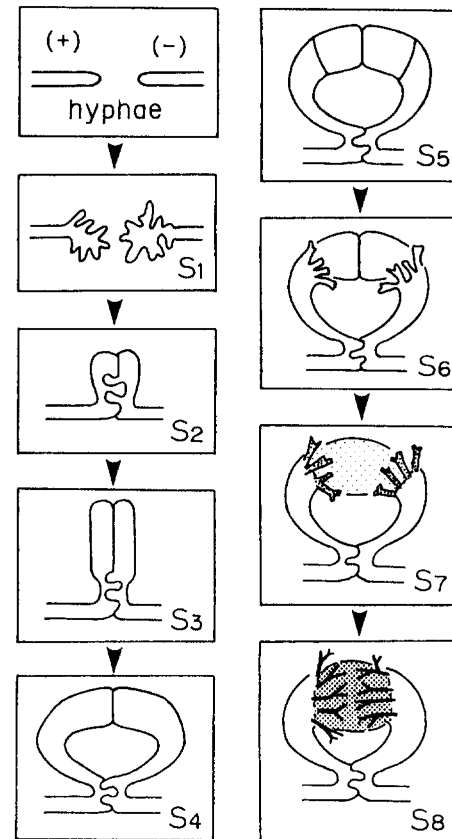

Fig. 1. Schematic diagram of mating response of *Phycomyces*.
Classification of developmental stages (S1-S8) after Sutter (1975).

emergence of thorn-like appendages on suspensors (S6), fusion of two gametangial apices to form a zygote (S7), and maturation of the zygote as a zygospore with a black coat (S8). Figure 2 shows the time course of development of the sexual organs and distribution of each developmental stage. The most intensive development of sexual organs occurred during the first 10 d, after which it practically ceased, resulting in accumulation of an abundance of the arrested S2 zygophores and S3-4 progametangia. Of the total of about 750 sexual organs developed, only about 110 organs (14.7%) reached the final S7-8 stages.

The sexual organs arrested at early stages often regenerated vegetative hyphae or dwarf sporangiophores (microphores) (Figs. 3, 4A). The microphores appear predominantly in the dark and are characterized by thin and short stalks (about 20 μm in diam and 1-3 mm in length), by septum formation (Fig. 4E), and by rather round spores (Fig. 4C), in contrast with gigantic sporangiophores (macrophores), which are about 100 μm in diam and more than 10 cm in length (López-Diaz and Cerdá-Olmedo, 1981). Since the sporangiophores regenerated on the sexual organs were predominantly microphores and since even the macrophores developed were quite thin and short, we use the term 'microphores' for all the regenerated sporangiophores in this study.

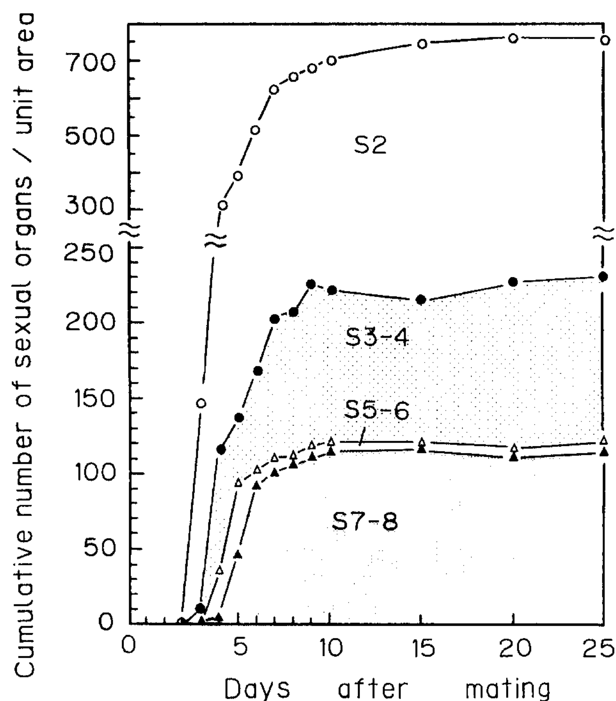

Fig. 2. Time course of successive development of sexual organs of *Phycomyces*.

The number of sexual organs of each developmental stage was cumulated and, therefore, the uppermost curve (open circles) represents the total number of sexual organs developed (S2-S8). The number of sexual organs was counted in a unit area (5×20 mm) along the mating line where the mycelial fronts of two mating types met. For simplicity, S5 and S6, and S7 and S8 were respectively combined. Note that population and distribution of each developmental stage remained invariable after around 10 d and that the development of many young sexual organs (S2-S4) was arrested. Mated at 20°C in the dark.

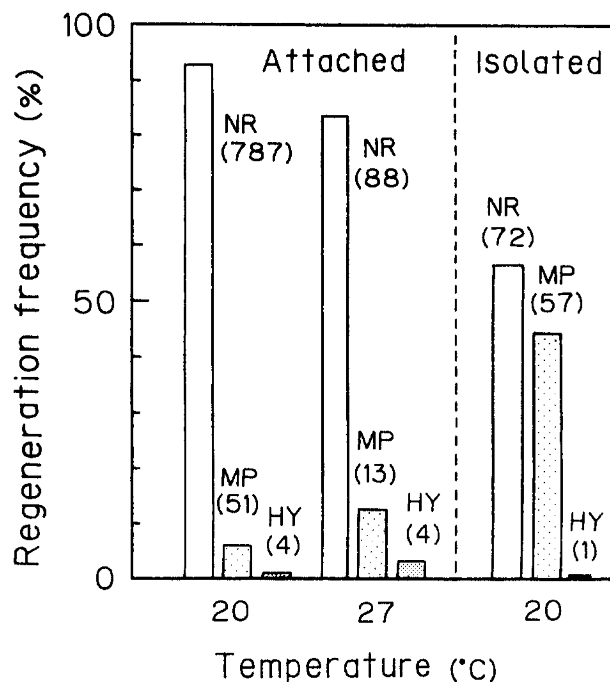

Fig. 3. Regeneration frequency of vegetative hyphae or microphores on S4 progametangia attached to mycelia or after isolation from mycelia.

NR, no regeneration; HY, hyphal regeneration; MP, microphore regeneration. Data are the sum of several measurements and the numbers in parentheses are the numbers of progametangia observed. The cultures mated at 20°C for 10 d were remained at 20°C or transferred to 27°C.

Regeneration frequencies of hyphae and microphores on the arrested S4-progametangia were about 0.5% (4/842) and 6.1% (51/842), respectively (Fig. 3).

The regeneration frequency increased sharply to 44.6% (58/130) when the S4 progametangia were isolated from mycelia and transferred to plain agar (Fig. 3). The predominant regenerates were also microphores.

Prior to emergence of regenerates or in the protuberances of regenerates, accumulation of cytoplasm and mitochondria was seen (Fig. 5). Such cytoplasmic accumulation often remained after the regenerates had emerged (Fig. 4E, white arrowhead).

Regeneration at high temperature Figure 6 represents the cumulative number of the sexual organs at different stages regenerating on a unit area (5×20 mm) of the plate as a function of temperature. The total sexual-reaction activity, particularly the activity at early steps (S2-4), was sharply accelerated by an increase in temperature to 27°C, but S7-8 zygospore development was totally depressed at above 25°C, agreeing with Hocking's description (1967). At above 28°C no mycelial growth was seen. The development of all sexual organs developed at 25-27°C was, therefore, arrested and proceeded no further than S4 at the maximum. With an increase in temperature, abnormally deformed or coiled progametangia appeared, and their frequency increased to almost 50% of the total S3-4 progametangia developed when mated at 27°C (Figs. 4B, 6 inset).

Frequencies of both hyphal (3.8%, 4/105) and microphore (12.4%, 13/105) regeneration on these arrested S4 progametangia at 27°C were significantly higher than those at 20°C (Fig. 3). The deformed or coiled progametangia were also capable of regeneration (Figs. 4B, C).

Regeneration on incised progametangia When the counterparts of S4 progametangial cells constructing the loops were surgically incised, the vegetative regeneration was evidently promoted (Figs. 4D, 4E, 7). Total regeneration frequency of about 30.3% (180/594) for the attached and incised sexual organs at 20°C (Fig. 7) was significantly higher than that of 6.5% (55/842) on the attached and intact organs (Fig. 3).

On the isolated progametangia, the incision synergistically accelerated the vegetative regeneration to 58.9% (76/129), 1.6% for hyphae (2/129) and 57.4% for microphores (74/129) (Fig. 7). The difference from the isolated but intact progametangia (44.6%, Table 3) was statistically significant.

Transfer of the incised progametangia from 20°C to 27°C slightly accelerated the regeneration frequency

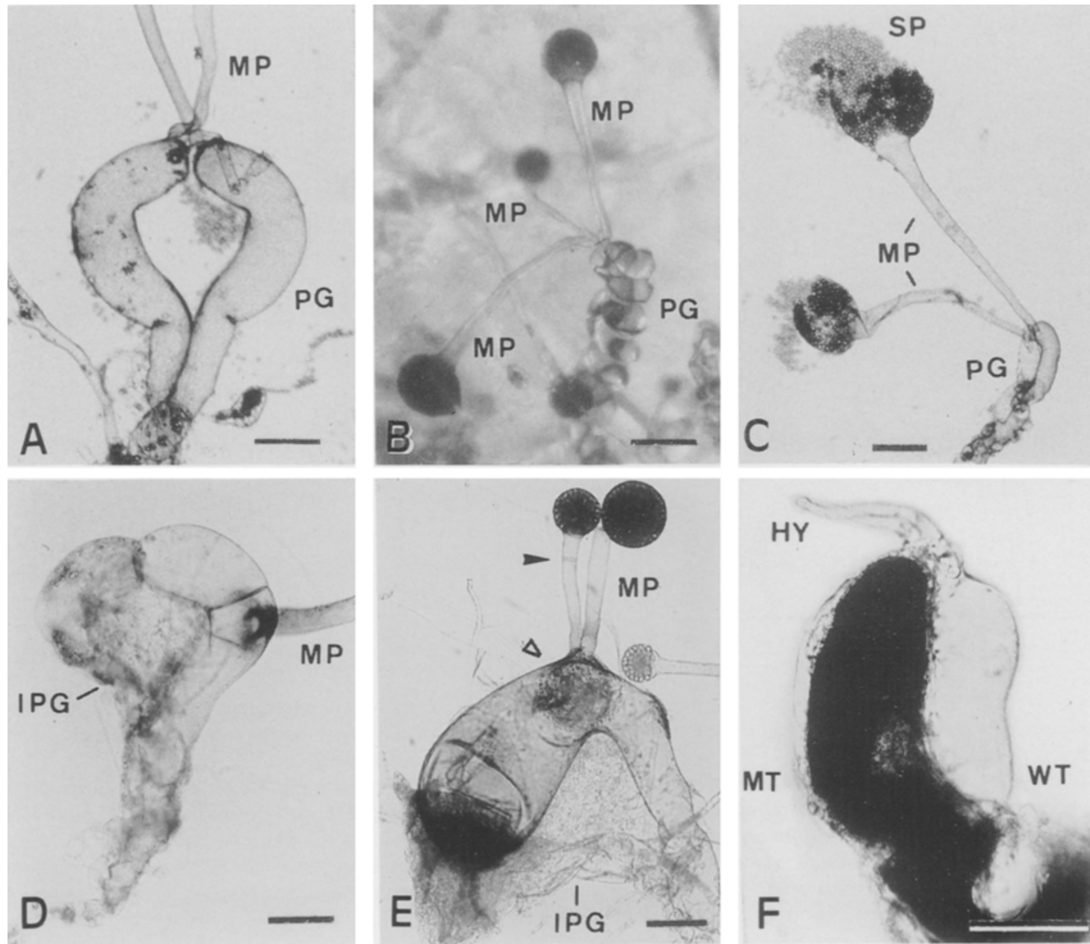

Fig. 4. Regeneration of vegetative hyphae or microphores on sexual organs.
A, a looped S4 progametangium (PG) with two regenerated microphores (MP). The present progametangium was formed between two wild types of opposite mating type, but further development was arrested. Small particles around the progametangium are spores dispersed from the regenerated microphores. Photographed 20 d after mating. B, An abnormally coiled progametangium at 27°C regenerating three microphores. C, Two microphores regenerated on S3 progametangium at 27°C. Sporangia produced rather round and viable spores (SP). D, Regeneration on a looped S4 progametangium whose one counterpart was surgically incised (IPG). Observed 14 d after incision. E, Incised progametangium regenerating two microphores. Note a septum (black arrowhead), which is one of the characteristics of microphores, and the accumulation of protoplasm in the region where microphore emerged (white arrowhead). F, Hyphal regeneration on early S3 progametangium formed between a wild type (WT), A56 (+), and a leaky carotenoid mutant, C13 (−), accumulating lycopene (MT). Bars 100 μm for A–D, and 50 μm for E and F.

from 30.3% (180/594) to 36.4% (44/121), but the difference was not statistically significant.

Regeneration on sexual organs formed by mating between the wild type and a sexually-imperfect mutant The leaky *carR* mutant, C13, produced an abundance of S2 zygophores and early S3 progametangia but never developed the S7-8 zygospores (data not shown), confirming Sutter's results (1975). Regeneration frequency on these arrested sexual organs was about 8.0% at 20°C, significantly higher than the 2.0% on the sexual organs formed between two wild types (Table 1). Hyphae were the predominat regenerate (Fig. 4F), in a sharp contrast with the S4 progametangia, which regenerated predominantly microphores (Figs. 3, 4A).

Morphology and viability of spores The microphores regenerated on the sexual organs produced about 10^3–10^4 spores per sporangium, equivalent to about 1/10–1/100 of the macrophore spores (data not shown). In outward appearance, these regenerated microphore spores were rather round and small (about 5×5 μm), as were the ordinary microphore spores developed on mycelia in the dark, in contrast with the ellipsoidal spores of the macrophores ($4\text{-}6 \times 5\text{-}10$ μm; Eslava, 1987; Ootaki et al., 1991). The viability of the regenerated microphore spores was about 63.7% (290/455), as high as that of the ordinary microphore spores (62.9%, 418/665), but lower than that of the macrophore spores (86.0%, 416/484) (data not shown).

Discussion

Phycomyces has a remarkable capacity for regeneration, which can be achieved from any vegetative or sexual organ. Intensive and quantitative studies, however, have

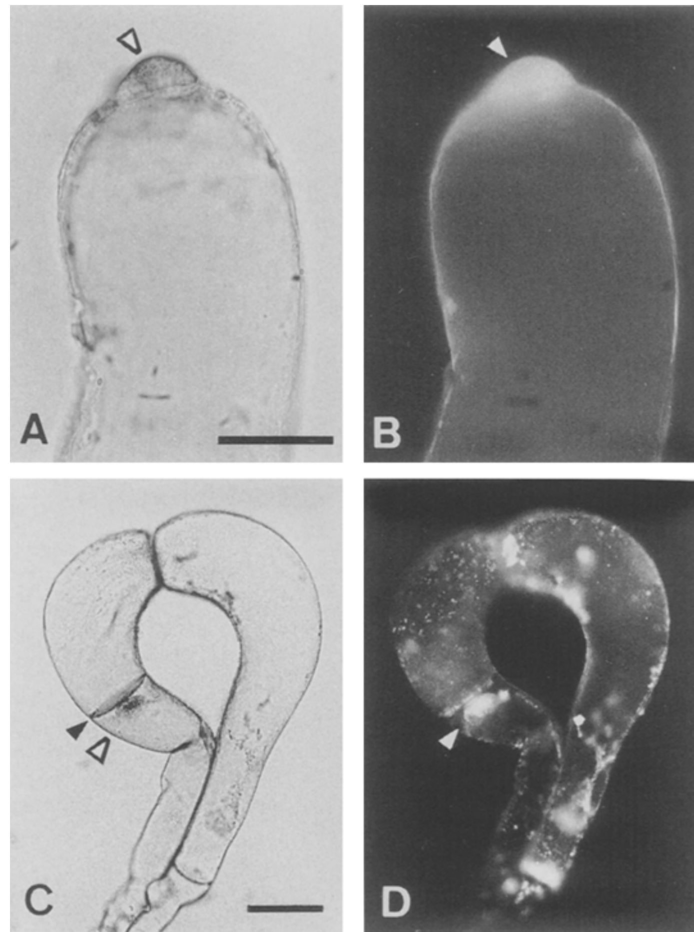

Fig. 5. Accumulation of cytoplasm and mitochondria in the regions where vegetative regeneration occurred.
A, B. A protuberance of a vegetative regenerate on the tip of a deformed progametangium accumulating mitochondria (white arrowheads). Only a counterpart of the progametangial cells is shown. C, D. A looped progametangium showing a septum (black arrowhead) and the initial protuberance of a regenerate. Mitochondrial accumulation is seen in the protuberance (white arrowhead). These deformed progametangia were obtained at 25°C. Bars 50 μm for A and B, and 100 μm for C and D.

been made only on regeneration from vegetative organs such as hyphae and macrophores (Galland and Ootaki, 1987; Gruen and Ootaki, 1970, 1972; Ootaki and Gruen, 1970), from their protoplasm (Heisenberg and Cerdá-Olmedo, 1968; Weide, 1939) and from protoplasts (Binding and Weber, 1974; Suárez et al., 1985). Although regeneration from sexual organs is known (Galland and Ootaki, 1987; Orban, 1919) and has been applied for heterokaryon formation (Bergman et al., 1969; Gauger et al., 1980), the present study is the first quantitative analysis of spontaneous or experimentally-induced regeneration on sexual organs from the viewpoint of demonstrating the existence of a cell-to-cell recognition system (Manocha, 1990).

In the present work, we found a considerably high frequency of vegetative regeneration on the sexual organs when their sexual development was arrested by mating at high temperature (above 25°C), by isolation from mycelium, by destruction of the counterparts of the paired progametangial cells, and by mating with the sexually-imperfect mutant.

At high temperature, the vigorous development of S2 zygophores and S3 progametangia (Fig. 6) implies acceleration of pheromonal production or activity and the predominant involvement of pheromones in the early steps of the mating process. The arrest of further sexual development of these young organs, however, implies the involvement of factor(s) other than pheromonal actions in this mating process, particularly in the later steps. Total failure of sexual completion in the deformed or coiled progametangia, probably due to the disturbance of the development of extracellular fibrils connecting and tightening the paired progametangial cells (Yamazaki and Ootaki, 1996), implies that the cell-to-cell recognition system is a candidate for the second factor and that it acts predominantly in the later steps of the mating process.

If our hypothesis is valid, the intercellular recognition system may exist after S3, because the sexual arrest by high temperature occurred at S3-4 and the incision in the counterparts of S4 progametangial cells induced regeneration in a high proportion of the remaining cells (Fig. 7).

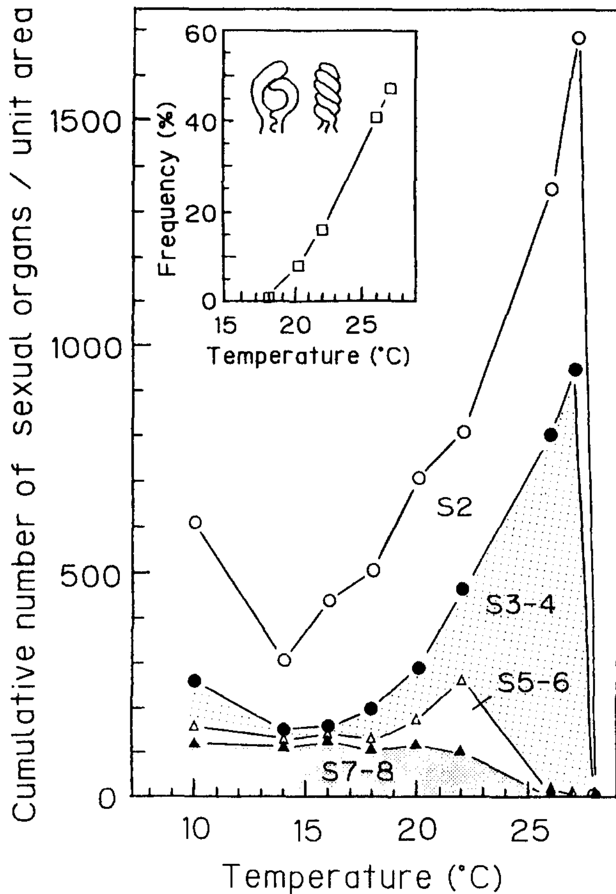

Fig. 6. Frequency of sexual organs at different developmental stages as a function of temperature.
Inset: frequency of abnormally deformed or coiled progametangia found in the S3-4 progametangia developed. See also legend to Fig. 2.

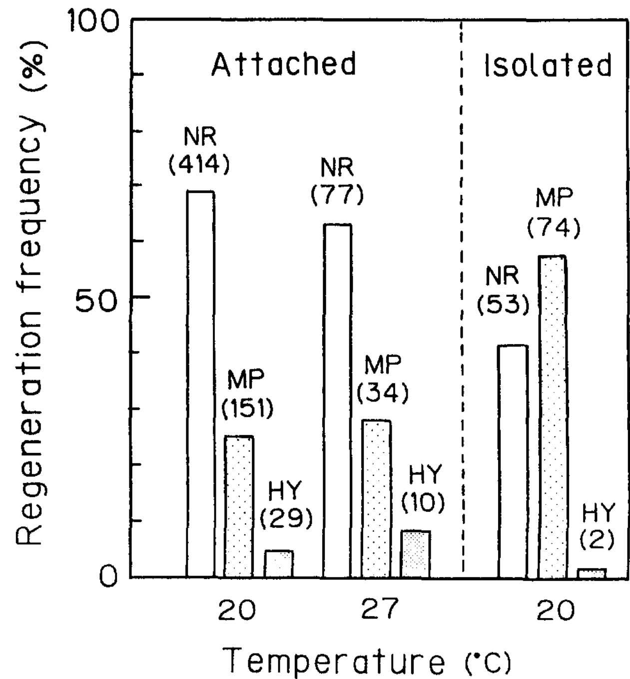

Fig. 7. Regeneration frequency of vegetative hyphae or microphores at an incision in S4 progametangia attached to mycelia or after isolation.
A counterpart of the looped S4 progametangium was surgically incised 10 d after mating. See also the legend to Fig. 3.

Sasen (1962) also assumed that some chitinolytic enzyme-inducing signals came from the mating partner when two gametangial cells enzymatically fused at S6-7.

Involvement of a second factor other than pheromonal action in the mating process is supported by the fact that there is no linear correlation between the zygospore production and the amount of intracellular β-carotene, which is the precursor of the sexual pheromones (Sutter, 1975); both superyellow mutants accumulating several times or more β-carotene and the mutants accumulating intermediate carotenoids, as seen in C13, always failed to complete the mating process at S2-3 (Sutter, 1975). Excess accumulation of β-carotene and its intermediates may interrupt the second factor, probably the recognition system.

Table 1. Regeneration of vegetative hyphae or microphores on sexual organs at early stages (S2-3).

Strains mated[a]	Regeneration		
	No regeneration	Microphores	Hyphae
WT (+)×WT (−)	2681 (98.0%)	14 (0.5%)	42 (1.5%)
WT (+)×C13 (−)	2682 (92.0%)	10 (0.3%)	224 (7.7%)

a) WT, wild types of mating type (+), A56, and (−), NRRL1555. C13, a leaky carotenogenic and sexually imperfect mutant. Mated at 20°C for 20 d in the dark.

Isolation of progametangia from mycelia greatly promoted the vegetative regeneration (Figs. 3, 7). Sudden loss of turgor pressure by isolation may disturb the cell-to-cell recognition system, and/or disconnection of signals from other organs through hyphal channels may stimulate the regeneration capability.

The promotional effect of high temperature on regeneration on the incised progametangia was insignificant (Fig. 7). This implies that high temperature acted more directly on the deformation of the progametangia, which disturbs the normal cell-to-cell recognition system, than on activation of vegetative regeneration itself.

The S2-3 sexual organs mostly regenerated hyphae and the S4 progametangia predominantly regenerated sporangiophores (micro- or macrophores), implying that the former are akin to hyphae and the latter to sporangiophores. The fact that a sporangiophore-deficient mutant (*imb*) also lacks the capability for progametangial development (Gutiérrez-Corona and Cerdá-Olmedo, 1985; Galland and Ootaki, 1987; Sutter, 1987) may support the above assumption though the progametangia showed neither phototropic nor gravitropic responses, in sharp contrast with the macrophores (data not shown).

Although at present the practical mechanisms of the cell-to-cell recognition system in *Phycomyces* are

unknown, such regeneration analysis can serve as a useful tool for elucidating not only sexual mechanisms but also the regulation systems between the vegetative and sexual cycles. Such a conversion from the sexual phase to a vegetative phase which produced viable spores seems to be strategically advantageous for the survival of this fungus when it encounters an adverse environmental situation.

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