Heterothallism and Mating Type Mutation in *Sclerotinia trifoliorum*

J. Y. Uhm and H. Fujii

Former research assistant and professor, respectively, Plant Pathology, Tokyo University of Agriculture, Tokyo, 156, Japan. Present address of the first author: Assistant professor, Department of Agricultural Biology, College of Agriculture, Kyungpook National University, Daegu, 635, Korea.

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ABSTRACT

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Each ascus of *Sclerotinia trifoliorum* contains four large and four small ascospores. The small-spore strains (strains derived from single small spores) are self-sterile, but cross-fertile only with those from large-spore strains (strains derived from large spores). On the other hand, the large-spore strains are always self-fertile, but their asci again show 4:4 segregation by spore size. From the mating behavior of small-spore strains, this fungus was determined to have bipolor heterothallism. Spore size difference is the pleiotropic expression of mating type. The mating types of large- and small-spore strains were designated as L and S, respectively. Formation of apothecia by large-spore strains is due to mutation for mating type and subsequent pairing of parental type and mutated nuclei in the same thallus; therefore, the resulting asci show segregation for spore size as well as mating type. However, the mating-type mutation occurs in one direction only, from L to S, since the S cultures are always self-sterile. The sexual role of microconidia was also demonstrated by successful mating in which spermatization of the sclerotia of small-spore strains was accomplished with microconidia of large-spore strains.

In the authors' previous work (23), two contradictory facts were found in the genetic system of ascospore dimorphism of *Sclerotinia trifoliorum*. Judging from the segregation patterns of large and small spores in the asci, the spore size difference was definitely controlled by a pair of alleles. However, such segregation was invariably observed, even in the asci of single-ascosporic strains.

If the large-spore strains (strains derived from single large spores) are homothallic and the perfect stages are formed by autogamy, no segregation in their asci would be expected. Therefore, in order to explain segregation in the asci of large-spore strains, self-fertility must be interpreted by a mechanism other than homothallism. This self-fertility of large-spore strains could be explained in two ways. One is the dikaryotic nuclear status as seen in *Neurospora tetrasperma* (4) and *Podospora anserina* (8), and the other is mating-type mutation similar to that found in *Saccharomyces cerevisiae* (1,10,11,14,20) and *Chromocrea spinulosa* (19). The present study tested the possibility of both mechanisms. For the former, the course of ascosporogenesis was cytologically examined. For the latter, however, the range of consideration is restricted, since mating-type mutation can be discerned only if the fungus is heterothallic.

This fungus was reported to be homothallic by Henson (13) and Keay (15), and this finding was supported by other researchers (2,3,9,17). Even authors who classified it homothallic noticed the presence of sterile strains. Sterility, however, either was not given adequate attention (13,15) or was attributed to an aberration (2).

In our previous work (23), it was demonstrated that small-spore strains (strains derived from single small spores) were sterile without exception and that those strains were similar to the large-spore strains in every other respect, but lacked the formation of apothecia. Moreover, it was discovered that such strains occurred under genetic control. From these two facts, the sterility of small-spore strains was postulated to be due to self-incompatibility rather than to other degenerative causes. Therefore, the approach to testing the occurrence of sterile small spores in which mating-type mutation was postulated was initiated by mating experiments to test the self-incompatibility of small-spore strains.

MATERIALS AND METHODS

Cytology. Young apothecia were divided into four pieces and fixed in Lu's fixative (18) for 24 hr at 25 C, hydrolyzed in 3N HCl at 70 C for 3 min, and stained with propionic iron haematoxylin (12). For better expansion of the chromosomes on the slide glass, snail gastric juice was usually applied after hydrolysis (7).

Mating. Matings were conducted by spermatizing the sclerotia with microconidia. To obtain a quantity of microconidia, each strain was cultured on an autoclaved potato slice; microconidia formed abundantly after 20 days. The sclerotia to be spermatized, 30 sclerotia per strain, were placed on water-soaked polyurethane. A few drops of microconidial suspension (~10^6/ml) were applied to each sclerotium with a capillary pipette. Fertilized sclerotia were incubated at 15 C. Insofar as possible, these processes were conducted aseptically.

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RESULTS

Ascosporogenesis. The course of ascosporogenesis from crozier formation to ascospore maturity was examined. In this paper, however, only that part directly relating to the present subject is described.

During the two meiotic divisions spindles were situated longitudinally, but in postmeiotic mitosis the spindles were oriented transversely so that at telophase III four pairs of nuclei were arranged transversely (Fig. 1A). The eight nuclei gradually became realigned single file, and the intermediate stage between biseriate and uniseriate arrangements was frequently observed (Fig. 1B). Just before spore delimitation, when the nuclei were in interphase III, with spindle plaque at the same side, the eight nuclei were usually aligned in a single row (Fig. 1C).

The spore displacement that has frequently been observed in mature asci (23) is most likely to occur during this process of single-file alignment. This is because spore displacement is certainly a postmeiotic event, and through the spindle formation at the postmeiotic mitosis no spindle overlap is likely to occur.

When spore delimitation occurred, the nuclei were still in interphase III (Fig. 1D). After spore delimitation, two further mitotic divisions took place within the ascospores. Consequently, each ascospore contained four nuclei at maturity, regardless of spore size (Fig. 1E).

In the prometaphase I, nine bivalents were seen with fair certainty (Fig. 1F). This observation differed from that of Björling (2), who reported the haploid chromosome number to be six.

Self-incompatibility of small-spore strains and the sexual role of microconidia. Five small-spore strains, one from each tetrad isolated in the previous study (23), were selected to be sclerotial parents, and five pairs of large- and small-spore strains from the same tetrads were employed as microconidial parents. Matings were made in all possible combinations of the five isolates as sclerotial parents and 10 isolates as microconidial parents. In each mating, 30 sclerotia were spermatized.

When sclerotia of the small-spore strains were spermatized with microconidia of the large-spore strains, normal apothecia were produced. When spermatized with the small-spore strains, however, no apothecia were obtained. All the asci in the apothecia showed 4:4 segregation in spore size.

Production of apothecia on the small-spore strains may be interpreted as a result of fertilization between the two components. But to demonstrate fertilization fully, further evidence based on inheritance of specific traits is needed. In this study, however, no auxotrophic marker genes were available. Therefore, the difference in cultural morphology between the parents was used to demonstrate fertilization. A number of asci from the hybridized apothecia were dissected, and single ascospores were isolated in serial order with a micromanipulator. When grown on PDA plates, usually four pairs of morphologically distinct cultures were obtained from each ascus set. These showed wide variation in general morphology, especially in size, number, and distribution of the sclerotia on the agar plate.

The variability in morphology of the ascospore progeny was correlated with the morphological difference between the parent cultures; the greater the difference between the parent cultures, the more variable were the progeny. Among the single-ascosporic strains used in this experiment, one pair of large- and small-spore strains (1-2L, 1-4S) originating from isolate A22 were morphologically distinct from the rest (producing a relatively large number of small sclerotia), but the rest were rather similar to one another (Fig. 2). In every cross in which the two strains originating from A22 were used as one parental component, whether sclerotial or microconidial, morphology of the progeny was extremely variable (Fig. 3A and B). However, when two morphologically similar cultures were paired, their progeny showed a relatively narrow range of variation (Fig. 3C). When two isolates that originated from morphologically identical parents from the same ascus were paired, the progeny showed no variation at all (Fig. 3D).

The correspondence of the range of morphological variation of ascospore progeny to the morphological difference of parents provided further evidence for microconidial fertilization.

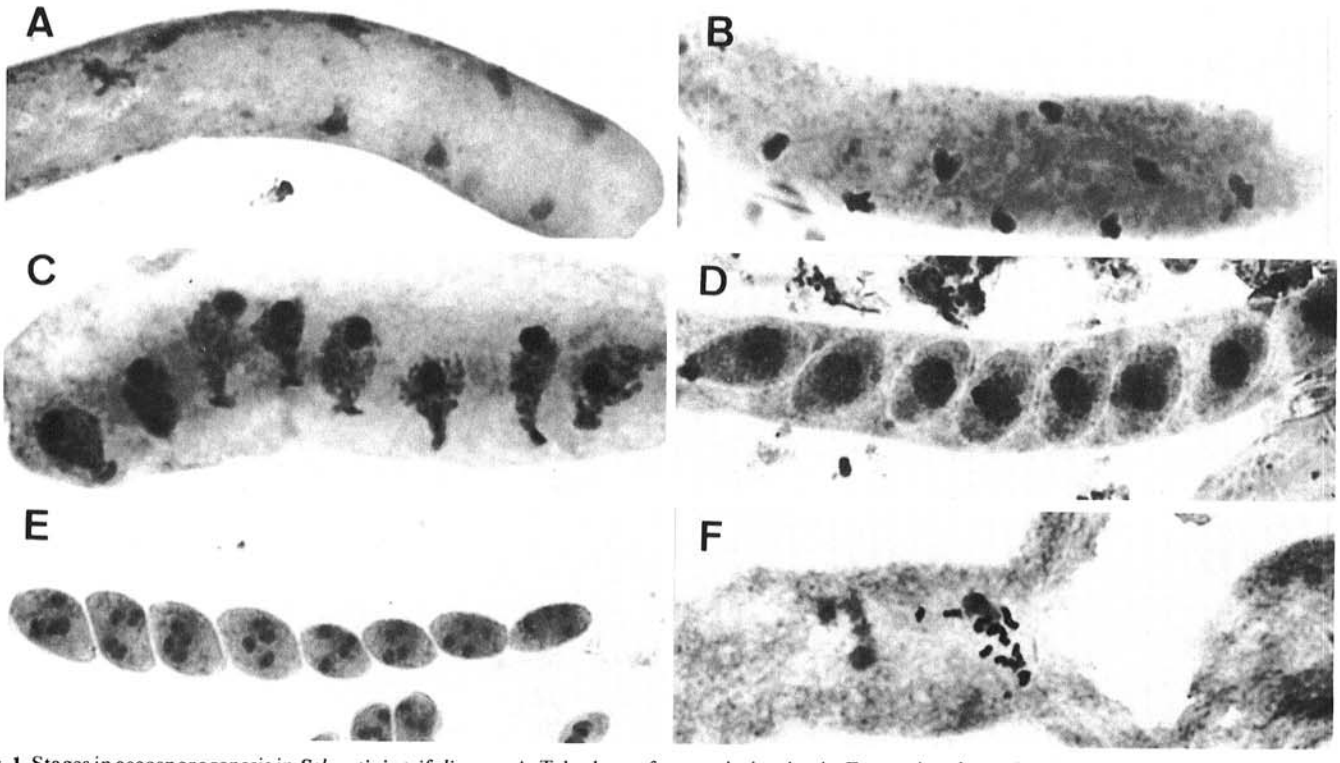

Fig. 1. Stages in ascosporogenesis in *Sclerotinia trifoliorum*. **A,** Telophase of postmeiotic mitosis. Four pairs of nuclei are initially arranged transversely. **B,** Intermediate stage of aligning from biseriate to uniseriate arrangement. **C,** Just before spore delimitation. Eight nuclei are aligned in a single row with spindle plaque at the same side. **D,** Newly formed uninucleate ascospores. **E,** Mature ascus showing first-division segregation for ascospore size. Each ascospore contains four nuclei regardless of spore size. **F,** First meiotic prometaphase (×3,200). Nine bivalents are visible.

Therefore, it is now certain that the sterile strains derived from small spores were self-incompatible, and that their microconidia were functional.

DISCUSSION

Ascosporogenesis of this fungus was that of the typical ordered tetrads, which is represented by *Neurospora crassa* (21,22), and no sign was found of dikaryotic nuclear status or diploidy (full or partial) in the large ascospores. Thus, fertility of the large-spore cultures could not be explained on the basis of their nuclear status.

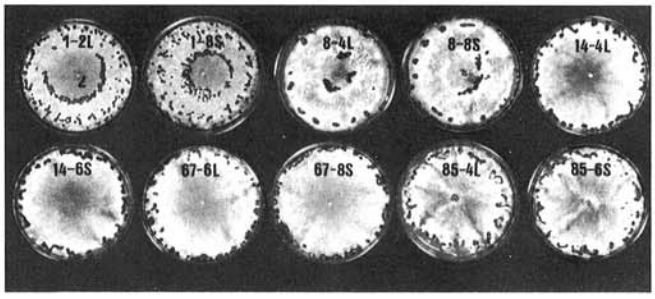

Fig. 2. Five pairs of single-ascosporic strains of *Sclerotinia trifoliorum* used in the mating experiment. Each pair was derived from different-sized spores of the same ascus.

However, in the second approach, evidence for regarding the fungus as heterothallic was obtained. From the facts that small-spore strains produced fertile apothecia by microconidial spermatization with large-spore strains and that the ascospore progeny showed variation in morphology when morphologically different cultures were mated, it was concluded that the small-spore strains are undoubtedly heterothallic. The fact that the small-spore strains are compatible only with the large-spore strains and incompatible with any strain derived from a small-sized spore suggests that this fungus is bipolar heterothallic, and that the large- and small-spore strains are of different mating types.

From these facts, the successive segregation by spore size associated with the correlated mating type in the asci of a large-spore strain may be appropriately interpreted by applying the concept of mating-type mutation. Although both large- and small-spore strains are certainly of different mating types, the genetic relationship between spore size and mating type requires further analysis; at this moment it could be said that mating type and spore size are the bilateral expression of a single gene. Therefore, we designate the mating type of the two strains according to the spore size from which those strains were derived: L to the large-spore strain and S to the small-spore strain. Consequently, every ascus, not only of crossed apothecia, but also of single-ascosporic strains, can be said to be heterozygous for mating type, since every ascus shows dimorphism in spore size.

In a heterothallic fungus, every ascus must be heterozygous for mating type, and mating type is usually controlled by a single pair

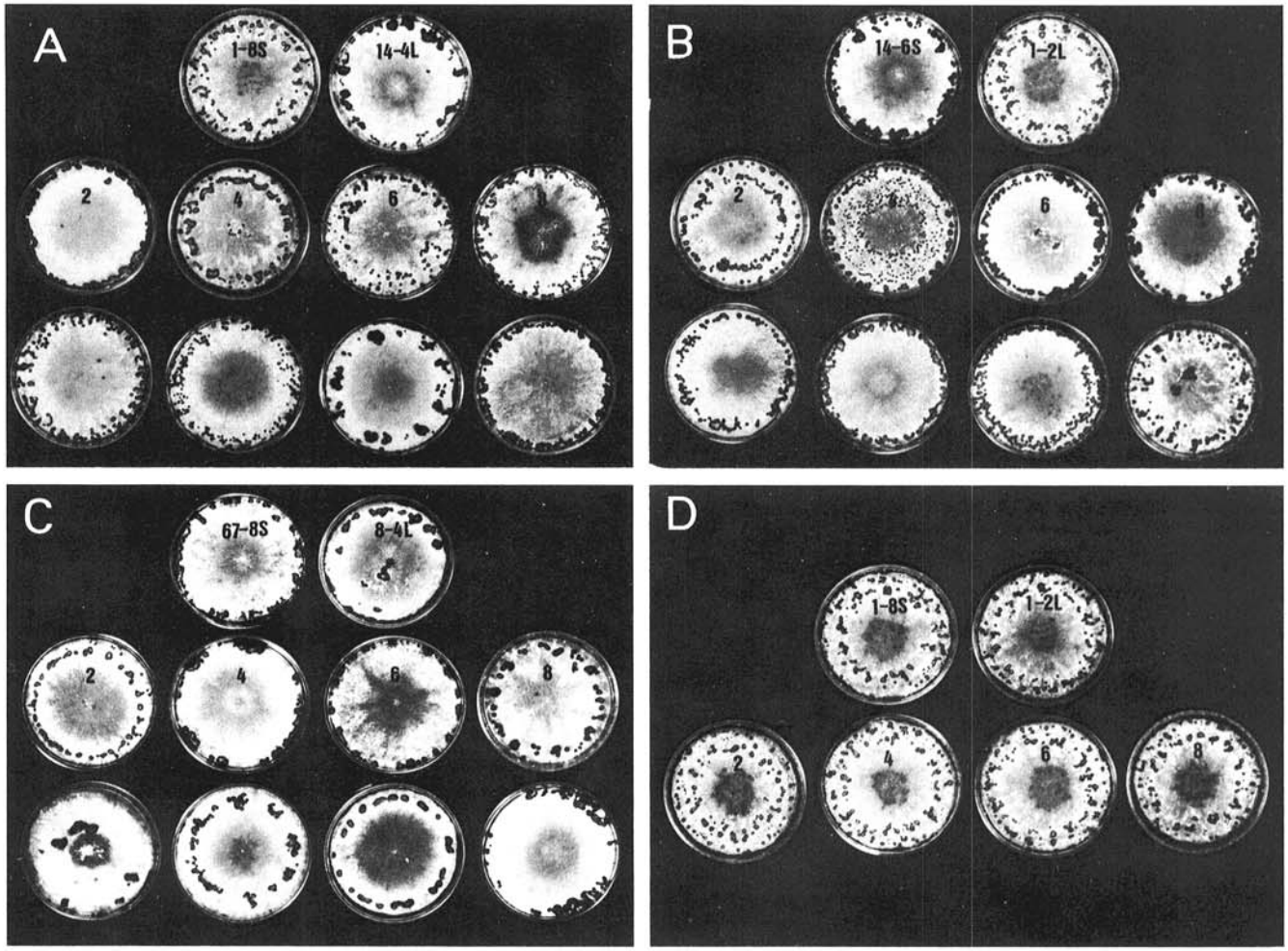

Fig. 3. Morphological variability of ascospore progeny of *Sclerotinia trifoliorum* related to the morphological difference between the parent cultures. **A** and **B,** The progeny show a wide range of morphological variation when two morphologically distinct cultures are crossed. **C,** The progeny are less variable when morphologically related cultures are crossed. **D,** The progeny show no variation and are quite similar to the parent cultures when two morphologically identical cultures, which were derived from the different-sized spores of the same ascus, are crossed.

of alleles (24). Therefore, the large-spore strains, even though they are self-fertile, may well be regarded as heterothallic from the segregation for spore size or mating type in every ascus, and the occurrence of mating-type S in single-spore L cultures can only be interpreted as a result of mutation at mating-type locus L to the S allele. Apothecial formation in large-spore strains is thus due to mating-type mutation and subsequent pairing of dissimilar nuclei. The resulting asci show segregation for mating type and spore size. On the other hand, because single-spore S cultures are completely self-sterile, it can be concluded that mating-type mutation occurs in only one direction from L to S, but not from S to L.

Briefly combining the above elements of spore size, mating type, mating-type mutation, and heterothallism, the situation can be summarized as follows: spore size and mating type are tentatively considered as the bilateral expression of a single gene; small-spore strains are heterothallic, hence self-sterile; large-spore strains are heterothallic until mating-type mutation occurs, but after mutation show balanced homothallism and hence is self-fertile with 4:4 spore-size segregation.

Mating-type mutation is well established in *Saccharomyces cerevisiae* and in *Chromocrea spinulosa*. The situation in *C. spinulosa* is very similar to that of *S. trifoliorum* in every respect: 16 spores are produced in each ascus, eight large and eight small, arranged in typical first- and second-division segregation. Also similar to *S. trifoliorum*, the colonies grown from small ascospores are sterile, and those from large ones are fertile, but their asci show 8:8 segregation for spore size. Mathieson (19) explained that this successive segregation is due to mating-type mutation that occurs only in a colony of large-spore strains.

As for the genetic relationship between mating type and ascospore size, two possibilities are considered. Either the allele determining the mating type may be the same allele that controls spore size, or the two characters are controlled by separate alleles so closely linked that no crossing over occurred in the sample of more than 100 asci tested. However, considering the complete absence of all large-spored asci among hundreds observed, the former is the most likely explanation. If the latter is postulated, then because the asci from single L cultures display ascospore dimorphism, the mutation for mating type and for spore size would always have to occur simultaneously. This means an absolute co-mutation in the two loci; the occurrence of that in every ascus is difficult to conceive. The factors determining mating type and ascospore size are inseparable so far, and therefore should be regarded as a pleiotropic expression of one gene.

The frequency of mating-type mutation has not been determined. No methods to calculate the frequency of mating-type mutation have been established, since in this fungus the asexual spore stage is lacking, and individual mutation is almost undetectable. In *Saccharomyces*, the homothallism exhibited by a certain strain is due to the extremely frequent mutation of mating types rather than to stable self-compatibility (11). Mathieson also encountered difficulties in calculating the mutation ratio due to the lack of available methods for detecting individual mutations; however, he presented an approximate rate as less than 50 in 10^6, regarding each stroma as equal to one mutation and the number of nuclei present in a culture as more than 10^6.

Even though several problems remained unsolved, the evidence seems to be sufficient to regard *S. trifoliorum* as basically heterothallic, and to conclude that fertility exhibited by large-spore strains is due to mutation at the mating-type locus that occurs only in those thalli.

Apart from the mating-type problem, the function of microconidia in this species was discovered. In species of closely related genera (ie, *Botryotinia convoluta* [6] and *Stromatinia gladioli* [5]) the microconidia have been demonstrated to function as spermatia. However, in the genus *Sclerotinia* (including *S. trifoliorum*, the present material) no definite function of the microconidia has previously been demonstrated. According to available reports (2,3,9,16), the ruling opinion on the sexual role of microconidia is rather negative. Some researchers (2,9) have assumed it to be a functionless male organ, since they regarded this fungus as homothallic and did not recognize an ascogonium. In this study, however, spermatization was conducted by applying the microconidia to sclerotia, and the result was quite convincing. Therefore, it was concluded that, at least in this species, the microconidia are functional spermatia.

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