The Molecular Mechanism of Protoplasmic Incompatibility and its Relationship to the Formation of Protoperithecia in *Podospira anserina*

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SUMMARY

In *Podospira anserina*, protoplasmic incompatibility due to interactions between non-allelic genes was suppressed by the effect of mutations in two modifier genes, *mod-1* and *mod-2*. It is shown that *mod-1* and *mod-2* are involved in the production of three specific proteins, a phenoloxidase and two previously identified proteases (Bégueret & Bernet, 1973*a*) which are associated with the phenomenon of protoplasmic disintegration. These enzymes, whose messengers are normally latent during vegetative growth, appear at this stage of the life cycle only as a consequence of incompatible gene interactions.

The *mod-1* and *mod-2* genes and each of the five incompatibility loci involved in non-allelic incompatibility systems also participate in the formation of the protoperithecia. This pleiotropic effect suggests that protoplasmic incompatibility is a deviation in the normal physiological processes of protoperithecial formation.

INTRODUCTION

Studies on protoplasmic incompatibility, a phenomenon of cytoplasmic lysis induced by the fusion of cells of unlike genotypes, have been reported in many fungi, including *Neurospora crassa* (Garnjobst & Wilson, 1956), *Didymium iridis* (Collins & Clark, 1968), *Physarum polycephalum* (Carlile & Dee, 1967) and *Podospira anserina*. The first extensive genetic studies (Rizet & Esser, 1952; Bernet, 1967) and biochemical investigations (Blaich & Esser, 1971; Bégueret, 1972) of this phenomenon were on *P. anserina*.

In *P. anserina*, incompatibility genes occur at nine loci. The five loci, *c*, *d*, *e*, *r* and *v*, are involved in the three non-allelic incompatibility systems *C/D*, *C/E*, *R/V*, and the *v* locus is also concerned in the allelic *V/vI* system. Most of the genetic investigations have been performed on the non-allelic systems, and allelic series were found at the *c*, *d*, and *e* loci giving rise to several *C/D* and *C/E* systems (Bernet, 1967). Physiological suppression of the protoplasmic incompatibility reaction between two strains that carry antagonistic genes, i.e. between strains *C* and *D*, *C* and *E* or *R* and *V*, requires the presence in both strains of a mutation in each of the modifier genes *mod-1* and *mod-2* (Bernet, 1971).

Biochemical investigations have been performed on the *R/V* system because of its temperature dependence. At 32 °C, the *RV* strain, which combines the non-allelic incompatible *R* and *V* alleles, exhibits the wild-type phenotype, but at 26 °C growth stops early in development and the cells lyse, as in the homologous *CD* or *CE* strains. When self-lysis was induced in *RV* cells by a temperature shift from 32 to 26 °C, proteases III and IV (enzymes which are absent from normal cells) were found (Bégueret & Bernet, 1973*a*). Furthermore, the addition of protease inhibitors such as phenyl methyl sulphonyl fluoride or β -phenyl pyruvic acid either reduced or completely abolished cell disintegration (Bégueret, 1973). The lytic

reaction was also prevented by cycloheximide but not by actinomycin D or 5-fluorouracil (Bégueret & Bernet, 1973*b*). Thus, it was postulated that protoplasmic incompatibility in *P. anserina* is due to proteolytic enzymes formed from messengers existing in the cells before the interaction between incompatibility factors (Bégueret, 1973; Bégueret & Bernet, 1973*a, b*).

Additional evidence for the involvement of proteases III and IV in cell disintegration is provided by investigations of strains carrying mutations at the *mod-1* and *mod-2* genes which suppress protoplasmic incompatibility. We here show that the production of proteases III and IV and of a novel enzyme, a phenoloxidase which like the proteases appears only in lysing cells, is affected by mutations in the *mod-1* and *mod-2* genes.

The *mod* mutations have an additional effect. In strains carrying mutations at *mod-1* and *mod-2* loci, protoperithelial formation is blocked prior to early steps in their morphological differentiation (Boucherie, 1973). This pleiotropic effect of the *mod* mutations suggests a connexion between the incompatibility process and the formation of the female organs, and has led us to investigate whether the incompatibility genes might themselves be involved, like the *mod* genes, in protoperithelial development. Preliminary results (Boucherie & Bernet, 1974) revealed such a role for the incompatibility genes at the *d* and *e* loci. We show here that each of the five incompatibility loci involved in the non-allelic interactions also plays a role in protoperithecium formation.

METHODS

Origin of RV strains. The cross ♀ *Vr* × ♂ *v₁R* was the source of the conditional lethal self-lysing *RV* strains. For *RV* strains carrying mutations in the modifier genes *mod-1* and/or *mod-2*, these mutations were introduced from the male parent *v₁R*. The particular *mod* mutations used, *mod-1(1)* and *mod-2(1)*, are the most efficient for the suppression of protoplasmic incompatibility and protoperithecium formation. In standard conditions (26 °C), double mutant *mod-1(1) mod-2(1)* strains are nearly female sterile.

Origin of the recombinant c d e r v strains. These strains were derived from crosses between the three geographical races (A), (F) and (s). Progeny from the initial cross ♀ (A) × ♂ (F) were crossed with the (s) race and the appropriate *c d e r v* combinations selected. Mutations *mod-1(1)*, *mod-2(1)*, or *mod-1(1) + mod-2(1)* were added to these genotypes by a further cross with an (s) strain that carried both mutations. For each of the various *c d e r v* genotypes, four strains of each mating type were examined for the production of protoperithecia.

Preparation of cell-free extracts. Mycelia were grown in 100 ml liquid synthetic medium (Esser, 1969) in 1000 ml Roux bottles incubated at the desired temperature for 4 days. The mycelium was filtered through cheese cloth on a Buchner funnel, rinsed twice with cold distilled water and ground in a glass bead homogenizer in phosphate buffer (0.05 M, pH 7.0, plus 10⁻³ M-dithiothreitol). About 160 g wet wt mycelium was used in each experiment. The homogenate was centrifuged for 30 min at 20000 g to remove glass beads and cell debris. Solid (NH₄)₂SO₄ was added to the crude extracts up to 90 % saturation at 4 °C. The precipitate was collected by centrifugation and dissolved in a minimum volume of phosphate buffer (0.01 M, pH 7, plus 10⁻⁴ M-dithiothreitol). After dialysis the insoluble material was discarded and the extract mixed with 2 vol. cold acetone and kept for 7 h at -15 °C. The precipitate was recovered and suspended in a minimum volume of tris-HCl buffer (0.01 M, pH 8, plus 10⁻⁴ M-dithiothreitol) and exhaustively dialysed against this buffer. After dialysis the insoluble material was removed by centrifugation.

Chromatographic procedures. The soluble fraction was loaded on a TEAE-cellulose column (400 × 25 mm) previously equilibrated with the tris buffer. The column was washed

overnight and the proteins were eluted by increasing NaCl concentrations (0 → 0.5 M linear gradient). The flow rate was adjusted to 10 ml/h and 6 ml fractions were collected.

Proteolytic activity measurements. Extract (0.25 ml) was incubated for 30 min at 37 °C with 0.25 ml of a 1 % (w/v) casein solution (phosphate buffer 0.01 M, pH 6.7). The reaction was stopped by adding 1 ml of 20 % (w/v) trichloroacetic acid. The E_{280} of the supernatant was measured against a blank in which the reaction was stopped at zero time. One unit of proteolytic activity refers to the amount of enzyme that produces an extinction change equivalent to 10^{-4} M-tyrosine.

Phenoloxidase measurements. Phenoloxidases were analysed in crude extracts by polyacrilamide gel electrophoresis. Crude extracts (10 μ l) were applied at the top of 7.5 % (w/v) polyacrilamide gels (0.15 M-tris-glycine buffer, pH 8.5). Constant current (2.5 mA/gel) was applied until the bromophenol blue used as marker reached the bottom of the tubes. Gels were then incubated for 20 min at 30 °C in a 0.02 M DL-3,4-dihydroxyphenylalanine solution (in 0.05 M-phosphate buffer pH 6). The activity of the phenoloxidases is revealed by the appearance of a reddish pigment, the intensity of which was measured by spectrophotometric scanning.

Female fertility determinations. Protoperithecia do not occur before the mycelia have reached the edge of the Petri dish (9 cm diam). To investigate female fertility, strains were grown on Rizet's medium and were exposed to light for five days after the cessation of growth (Rizet & Engelman, 1949).

RESULTS

Effect of mutations in mod-1 and mod-2 genes on the proteolytic enzymes

Three proteases were found in cell extracts of normal strains (Bégueret & Bernet, 1973a). The first eluted, proteases A and B, were both sensitive to ovomucoid, *p*-chloromercuribenzoate and phenyl methyl sulphonyl fluoride but differed from each other in molecular weight and pH-dependence. The third, protease C, was an acid endopeptidase insensitive to these inhibitors. These three proteases were similarly found in the extracts from the *RV* strains cultured at 32 °C (Fig. 1a) whether these carried the *mod-1(r)* and *mod-2(r)* mutations or not, indicating that these genes are not involved in the formation of proteases A, B or C.

Release of the incompatibility reaction in the *RV* strain by lowering the temperature from 32 to 26 °C, was followed after 2 h by an increase in the protease level. When the extraction was performed after the strain had spent 12 h at 26 °C, proteolytic activity was distributed between four different fractions (Bégueret & Bernet, 1973a). These corresponded to proteases A and B, which eluted first and represented 10 % of the total proteolytic activity, and two novel acid endopeptidases, proteases III and IV, that were different from protease C. The latter was never found in lysing cells.

When the *RV* strain carried the *mod-2(r)* mutation, the elution profile of the proteolytic activity found in the lysing cells was unchanged (Fig. 1b). It can thus be concluded that the *mod-2(r)* mutation alone has no effect on any of the proteolytic enzymes. For *RV mod-1(r)*, strain in which the temperature shift also induces the protoplasmic self-lysis reaction, protease III was not detected (Fig. 1c), indicating that the *mod-1* gene is involved in the formation of protease III and that protease IV is sufficient for cell disintegration. In the double mutant *RV mod-1(r) mod-2(r)* strain where the temperature shift did not lead to cell lysis, protease III was still absent and the level of protease IV was reduced to about 5 % of that found with the *RV mod-1(r)* strain (Fig. 1d). Of the five proteolytic enzymes detected in the fungus, proteases A, B, C, III and IV, only III and IV, which are specific to lysing cells, are affected by mutations in the *mod-1* and *mod-2* genes.

Fig. 1. Elution profiles on a TEAE-cellulose column of the proteolytic activity from a *RV* strain cultured at 32 °C (a), and from strains *RV mod-2(r)* (b), *RV mod-1(r)* (c) and *RV mod-1(r) mod-2(r)* (d) cultured 4 days at 32 °C and shifted to 26 °C for 12 h. Underlined fractions were pooled and further analysed by chromatography on DEAE-Sephadex and then on G-100 Sephadex. Each enzyme was tested for pH dependence and sensitivity towards the inhibitors ovomucoid, phenyl methyl sulphonyl fluoride and *p*-chloromercuribenzoate to allow comparisons with proteases A, B, C, III and IV (Bégueret & Bernet, 1973a).

Effect of the mod mutations on the phenoloxidases

In crude extracts from a normal strain, or from a *RV* strain cultured at 32 °C, a single phenoloxidase was present, phenoloxidase A. This was temperature-sensitive and exhibited a R_T of 0.1. When the *RV* strain was shifted to 26 °C an additional enzyme was found, phenoloxidase B. This was temperature-resistant and exhibited a R_T of 0.3. These features resemble those previously described for laccases I and III (Esser, 1963).

To determine whether phenoloxidase B is synthesized in *RV* strains as a consequence of the temperature shift, actinomycin D and cycloheximide were added just before the cultures were exposed to 26 °C. While actinomycin D had no effect, cycloheximide inhibited both phenoloxidase B and the increase in total proteolytic activity (Table 1). Thus it is suggested

Fig. 2. Incompatibility relationships between the geographical races (A), (F) and (s) of *Podospora anserina* determined by alleles at the *c*, *d*, *e*, *r* and *v* incompatibility loci (from Bernet, 1967). Incompatible gene combinations are connected by a line: *R* and *V* for the *r* and *v* loci, for the *c*, *d* and *e* loci, *c*(s) and *d*(A) in the *C/D* system, *c*(s) and *e*(A), *c*(A) and *e*(F) in the *C/E* system, and *V* and *v*₁ in the allelic *V/v*₁ system.

Table 1. *Effect of actinomycin D and cycloheximide on phenoloxidase B and on the level of proteolytic activity in RV strains shifted from 32 to 26 °C*

Inhibitors were added 2 to 3 h before the temperature shift at final concentrations of 33 µg/ml for actinomycin D and 6 µg/ml for cycloheximide. In each experiment the first number corresponds to the measure at 32 °C and the second to that after 12 h incubation at 26 °C.

	Inhibitor added		
	None	Actinomycin D	Cycloheximide
Proteolytic activity	0.5, 2.9	0.6, 2.3	0.7, 0.8
Relative activity of phenoloxidase B	0, 100	0, 100	0, 10

that phenoloxidase B, like proteases III and IV, arises from preformed RNA messengers. To investigate whether the synthesis of phenoloxidase B was induced simply as a consequence of the activity of proteases III and IV, i.e. by an increase in the lysing cells of the level of aromatic amino acids, the *RV* strains were cultured at 32 °C on a medium containing high concentrations (100 mg/ml) of the three aromatic amino acids. In these conditions phenoloxidase B was always absent, suggesting that this enzyme arises, like proteases III and IV, as a consequence of the interaction between incompatible factors rather than being a secondary effect.

Phenoloxidases A and B were investigated in *RV* strains that carry the *mod-1(r)*, *mod-2(r)* or *mod-1(r)* and *mod-2(r)* mutations, at 32 °C and after 12 h culture at 26 °C. Phenoloxidase A was always present. Phenoloxidase B, whose synthesis is triggered off by the temperature shift, was affected only when the *RV* strain carried both *mod* mutations, when the activity of this enzyme was reduced to less than 10 % of that found in the three other genotypes. It may be concluded that the formation of phenoloxidase B is under the same control as protease IV.

Effect of incompatibility genes on the female fertility of the double mutant mod-1(r) mod-2(r) strains

From the results reported above we saw that *mod-1* and *mod-2* mutations affect three enzymes, proteases III and IV and phenoloxidase B, which appear during vegetative growth only as a consequence of the non-allelic gene interactions *C/D*, *C/E* and *R/V*. Since mutations at *mod-1* and *mod-2* genes also prevent the formation of female organs (Boucherie & Bernet, 1974), the incompatibility genes at the *c*, *d*, *e*, *r* and *v* loci, might also play a role in the formation of the protoperithecium. To test this hypothesis, several recombinant strains

Fig. 3. Effect of alleles at the *c*, *d*, *e*, *r* and *v* loci on the distribution of protoperithecia in double mutant *mod-1(r) mod-2(r)* strains grown at 20 °C. Mycelia originate from a single inoculum (■) deposited off centre in order to observe the effect of the extent of radial growth.

were constructed by appropriate two-way and three-way crosses between the geographical races (A), (F) and (s) of the fungus. Incompatibility relationships between these races, involving loci *c*, *d*, *e*, *r* and *v*, are shown in Fig. 2. In the progeny of the crosses, *CD* [i.e. *c*(s) *d*(A)], *CE* [i.e. *c*(s) *e*(A) or *c*(A) *e*(F)] and *RV* were lethal self-lysing genotypes in which growth stopped early in development and were discarded. Most of the viable genotypes were examined for female fertility. All the strains exhibited wild-type fertility. Protoperithecia started to form when the mycelium reached the edge of the Petri dish and they eventually developed at a rather uniform density ($10^3/\text{cm}^2$) over the entire surface of the cultures.

Recombinant strains carrying the *mod-1(r)* or the *mod-2(r)* mutations showed normal female fertility. Strains carrying both *mod-1(r)* and *mod-2(r)* mutations were practically female sterile at 26 °C, but showed a noticeable restoration of fertility at 20 °C. However, important differences in the distribution of protoperithecia over the surface of the mycelia were observed (Fig. 3). Investigations on eight strains of each genotype showed that the differences in female fertility depended solely on the *c d e r v* genotype. None of the recombinant strains showed wild-type fertility because of the absence of protoperithecia in an outer area of the mycelium, 5 to 6 mm in width. In general, the most fertile strains were those carrying the *v*₁ gene from the (F) geographical race associated with *R* from (F) or *r* from (A). For the third viable combination at the *r* and *v* loci, i.e. the *rV* genotype, derived from the (s) race, the female fertility depended on the nine *c d e* combinations investigated. The three most sterile genotypes were formed with alleles of independent origin, i.e. *c*(F) *e*(A) *d*(F), *c*(F) *e*(A) *d*(s), *c*(A) *e*(A) *d*(F). Furthermore, for the strains that carried the *d*(A) or *d*(F) genes at the *d* locus, the most fertile *c e* combinations were *c*(A) *e*(A) for *d*(A) and *c*(F) *e*(F) for *d*(F).

From these results it can be concluded that the five incompatibility loci *c*, *d*, *e*, *r* and *v* are involved in protoperithecium formation, since at 20 °C they suppress to various extents

the inhibitory effect of the *mod* mutations. Moreover, it seems likely that the *c*, *d* and *e* incompatibility loci operate through the effect of specific combinations of these three genes, rather than through addition of their individual gene effects.

DISCUSSION

The effect of mutations at the *mod-1* and *mod-2* genes on proteases A, B, C, III and IV and phenoloxidasases A and B has been investigated. Only proteases III and IV and phenoloxidasase B, whose messengers are normally latent during vegetative growth and which appear as novel proteins during the protoplasmic incompatibility reaction, depend on *mod-1* and *mod-2* genes. The *mod-1(r)* mutation abolishes the activity of protease III. Although this mutation is recessive, *mod-1* is not the structural gene for protease III, because the product of *mod-1*, but not protease III, is sensitive to streptomycin and magnesium (Bernet, Bégueret & Labarere, 1973). From these latter properties it was postulated that *mod-1* determined a ribosomal factor which, from the present results, appears essential for the translation of the messenger of protease III. The *mod-1* factor also plays a role in the formation of protease IV and phenoloxidasase B, since the *mod-2(r)* mutation suppresses these enzymes when it is in association with a mutation in *mod-1*. Because of this interaction with *mod-1* and the fact that *mod-2(r)* is dominant over its wild-type allele (Bernet *et al.* 1973), *mod-2* is not thought to be the structural gene for protease IV but rather a regulator gene for protease IV and phenoloxidasase B. Thus, the three non-allelic incompatibility interactions *C/D*, *C/E* and *R/V* have in common the induction from latent mRNA molecules of the synthesis of proteins that are also under the control of *mod-1* (protease III) or *mod-1* plus *mod-2* (protease IV and phenoloxidasase B).

The suppression of the formation of the female organs by the *mod* mutations also requires mutations in both *mod* genes (Boucherie & Bernet, 1974). The restoration of female fertility to *mod-1 mod-2* strains by different allele combinations at the *c*, *d*, *e*, *r* and *v* incompatibility loci suggests that the latter also have a role in the formation of protoperithecia. Thus, protoplasmic incompatibility and protoperithecium formation are related phenomena, since we have shown that they are under the control of seven common loci: the *c*, *d*, *e*, *r* and *v* incompatibility loci and *mod-1* and *mod-2*. We consider that protoplasmic incompatibility arises from the deviant expression in vegetative cells of a physiological process normally involved in the formation of the female organs. On this hypothesis, protease IV and phenoloxidasase B are essential for protoperithecial formation, and the function of the incompatibility genes is to induce the synthesis of these enzymes, following the cessation of vegetative growth.

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