PHENOL OXIDASES AND MORPHOGENESIS IN *PODOSPORA ANSERINA*[1]

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THE question of the relation between melanin synthesis and morphogenesis has been raised previously. HIRSCH 1954 and WESTERGAARD and HIRSCH 1954 concluded from their experiments with a wild-type strain of *Neurospora crassa* that the melanin pigments or the enzyme tyrosinase, a phenol oxidase which is responsible for the first steps in melanin synthesis, play a role in the formation of the protoperithecia. Further investigation of this question demonstrated that female sterile mutants of *N. crassa* have lost to a considerable degree the ability to form tyrosinase (BARBESGAARD and WAGNER 1959). The fact that the mutants do not form protoperithecia even when tyrosinase formation is triggered by induction (HOROWITZ *et al.* 1960), however, does not suggest a direct correlation between tyrosinase activity or melanin synthesis and morphogenesis.

In insects the phenol oxidases (particularly tyrosinase) show a strong catalytic activity during morphogenesis. From the extensive studies on Drosophila (HOROWITZ and FLING 1955, LEWIS 1960; LEWIS and LEWIS 1961; SEKERIS and MERGENHAGEN 1964) and Calliphora (KARLSON *et al.* 1964; SEKERIS and MERGENHAGEN 1964) it is, however, not clear whether the tyrosinase function intervenes in differentiation processes or whether it accompanies morphogenetic processes.

The observation that both allelic *minor* mutants of *Podospora anserina* show reduced melanin formation in addition to developmental defects, which manifest themselves as a reduction in the size of the perithecia, induced us to reexamine the question of the relation between melanogenesis and morphogenesis. In this paper we report experiments which demonstrate that in addition to macroscopically recognizable characteristics the *minor* mutation causes a qualitative change of the phenol oxidase laccase. This pleiotropic effect of the mutation is abolished by the action of a suppressor gene.

MATERIALS AND METHODS

The two strains S and s of *Podospora anserina* were used as wild-type strains. They differ from each other not only in the pair of *S/s* alleles which is responsible for barrage formation, but also in some extrachromosomal factors (RIZET 1952). The *S/s* locus belongs to linkage group III and is situated 5.6 corrected map units (limits 4.2–6.9) to the left of the centromere (KUENEN 1962b). Details concerning the development and genetics of the wild-type stocks can be found in ESSER 1956, 1959.

The two allelic mutants *minor-1 (m-1)* and *minor-2 (m-2)* were obtained by X-ray treatment of the wild-type stock s. The *minor* strains are morphogenetic mutants. They demonstrate a

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developmental defect in the formation of the perithecia. Their fruiting bodies reach only approximately one fourth of the volume of wild-type perithecia and contain correspondingly less asci. The mutants do not form aerial hyphae on corn-agar and appear less darkly pigmented than the green-black wild-type mycelium which is colored due to the presence of melanin pigments. The *m*-locus is situated to the left of the centromere in linkage group IV. The centromere distance in corrected map units is 8.5 (limits 7.1–9.9) for *m-1* and 9.8 (limits 6.6–12.9) for *m-2* (KUENEN 1962b).

The genetic methods used for crossing and tetrad analysis and for gene localization can be found in: ESSER 1956, 1959; KUENEN 1962a, b; ESSER and KUENEN 1965, p. 151 ff.

Biochemical methods: Culture methods for the production of mycelia for extraction, assay methods, partial purification, and serological characterization of the phenol oxidases have been described elsewhere (ESSER 1963a, b, 1966; ESSER, DICK and GIELEN 1964). Only those methods necessary for an understanding of this paper will be presented here.

We used partially purified mycelial extracts for enzyme assays and serological analysis, specifically the protein fraction obtained from the crude extract by 85% ammonium sulfate saturation. The nucleic acids had been removed previously from the crude extract.

One enzyme unit corresponds to a Δ E of 0.2 at 436 nm, 1 cm pathway at a temperature of 30°C. As substrate DL-3,4-dihydroxyphenylalanine (Dopa) is used for laccase and tyrosinase, potassium ferrocyanide for laccase, and tyrosine for tyrosinase. The specific activity (SPA) is defined as the number of enzyme units per mg protein.

For the serological studies an antiserum was used which we obtained from rabbits after injection of pure laccase. The antiserum is specific for laccase and does not react with tyrosinase. One unit of anti-enzyme is the amount of serum which inactivates 1 unit of laccase. In studies on the determination of the cross-reacting material (CRM-test) the enzyme activity was determined using the laccase substrate potassium ferrocyanide. The so-called CRM-value is equal to the quotient of enzyme units and anti-enzyme units. By definition it is 1 for the wild-type stock. Significant deviations from this value indicate a structural change of the enzyme. Corresponding values are presented with their standard errors.

All manipulations involved in enzyme purification and the serological studies were carried out at 0–4°C.

EXPERIMENTAL RESULTS

In addition to the morphological defect of strongly reduced size of the perithecia the *minor* mutants demonstrate on agar medium less melanin formation than the wild-type strain. As both *minor* characteristics are abolished under the influence of a suppressor gene, an analysis of the phenol oxidases responsible for the first steps in melanogenesis appeared desirable in view of the possible correlation of melanin formation and morphogenesis. Before the biochemical experiments are presented, experiments will be described concerning demonstration and mapping of the suppressor gene.

Identification and localization of the suppressor gene: In the course of mapping Podospora mutants by KUENEN (1962b) it was observed that the *minor* mutants of the wild-type strain s did not appear in the expected frequencies in F_1 from crosses with the wild-type strain S; indicating that the manifestation of the *minor* mutants was extensively suppressed in the S strain. Two possibilities might explain this observation: 1) An extra-chromosomal mechanism, since the strains s and S are not only different in their genomes but also in their plasmones. 2) The action of a suppressor gene. Preliminary tetrad analyses of the cross *s m-1* × *S m-1*, indicated the presence of a suppressor gene in the genome of *S*.

This gene which completely abolishes the macroscopically recognizable *minor*

defect was designated *su-m*. The respective wild-type allele which is present in strain s, was designated *su-m*$^+$. Thus the *minor* mutant should have the genotype *s m-1 su-m*$^+$; the wild-type strain s the genotype *s m-1*$^+$ *su-m*$^+$; the wild-type strain S the genotype *S m-1*$^+$*su-m*. This statement, made first as a working hypothesis, formed the basis for an extensive tetrad analysis with the mutant *m-1*; the important crosses are summarized in Table 1.

From the data of Table 1 it can be concluded: (1) The manifestation of the genotype *minor* is suppressed by a suppressor gene (*su-m*) and not by extrachromosomal factors. This statement is based on the following evidence: a) Segregation of the suppressor gene is independent of the gene *S* and its allele *s* (compare rows 1–3 with rows 4–6). b) This segregation is also not affected if instead of s the strain s^s (see RIZET 1952) is used which is characterized by an altered plasmone (rows 7 and 8). c) Also, in reciprocal crosses the segregation behavior of the suppressor gene is independent of the plasmone of the maternal parent (rows 2, 3, 5–8).

(2) The suppressor gene is closely linked to the *S/s* locus, i.e., both genes are situated on the same chromosome arm in linkage group III to the left of the centromere. This conclusion is derived from the single crosses as well as from the aggregate of the data from all the crosses (row 9): There is a significant deviation from a 1:1 ratio between parental and recombinant types in the prereduction class (column 8 and 9) (more information about linkage criteria will be found in ESSER and KUENEN, 1965, p. 166).

(3) The corrected gene-centromere distance for the suppressor gene *su-m* is 2.1 map units (limits 0.8–3.4). This value was calculated using the mapping function developed by KUENEN (1962a). As a basis for the calculation the post-reduction frequency of 4.2 (see table 1, column 11) was used. When determined in the same manner, the gene-centromere distance of 6.8 map units for the allele pair *S/s* (limits 4.9–9.1) is well within the limits of the already established value of 5.6 (see MATERIALS AND METHODS). The two genes are therefore approximately 4 map units distant from each other.

On the basis of these experimental results it can be understood that in the first preliminary crosses between the *minor* mutant of wild-type strain s and wild-type strain S the manifestation of the mutant character in the *S*-genome was suppressed to a considerable extent, because two factors contributed to the result: the existence of a suppressor gene in strain S and its close linkage to the *S* locus.

Other experiments which are not further described here have demonstrated that the *minor-2* gene is also suppressed by the gene *su-m*.

Analysis of the phenol oxidases: The wild-type strains of *Podospora anserina* form, besides larger amounts of laccase (E.C. 1.10.3.2) small amounts of tyrosinase (E.C. 1.10.3.1.) (ESSER 1963a). The laccase could be isolated in pure form and characterized by a series of physico-chemical parameters (ESSER *et al.* 1964). The tyrosinase could not yet be obtained in sufficient amounts for purification. However, also for this enzyme a number of properties are known from partially purified extracts which suffice for its differentiation from laccase.

In five similarly designed experimental series we have determined quantita-

TABLE 1

Summary of data from tetrad analyses used for the identification and localization of the suppressor gene su-m

1		2	3		4	5	6	7	8	9	10	11
				Analysis of F_1 according to the reduction of the genes *S/s* and *su-m⁺/su-m*								
	Cross	Asci analyzed		Type of reduction: gene 1	Post		Post	Pre	Pre		Percent postreduction	
				gene 2	Post		Pre	Post	Pre			
No.	genotype / phenotype	Number	Percent germination	Type of tetrad	Parental/ tetra	Recombinant/ tetra	Tetra	Tetra	Parental	Recombinant	*S* / *s*	*su-m⁺* / *su-m*
1	*s su-m⁺* o × *S su-m* O	68	93.2	.. ..	2	0	4	0	62	0	8.8	2.9
2	♀ *s su-m⁺* o × ♂ *S su-m* O	39	84.8	.. ..	3	0	1	0	35	0	10.3	7.7
3	♀ *S su-m* O × ♂ *s su-m⁺* o	46	92.0	.. ..	0	0	3	0	43	0	6.5	0
4	*s su-m* O × *S su-m⁺* o	203	87.5	.. ..	3	2	19	0	175	4	11.8	2.5
5	♀ *s su-m* O × ♂ *S su-m⁺* o	11	100.0	.. ..	1	0	1	0	9	0	18	9
6	♀ *S su-m⁺* o × ♂ *s su-m* O	19	79.2	.. ..	1	0	3	0	15	0	21	5
7	♀ s^S *su-m* O × ♂ *S su-m⁺* o	73	89.0	.. ..	4	0	7	0	62	0	15	5.5
8	♀ *S su-m⁺* o × ♂ s^S *su-m* O	91	89.2	.. ..	7	0	13	0	70	1	22	7.7
9	Sum:	550	88.6	.. ..	21	2	51	0	471	5	13.5±4.4	4.2±2.6

All crosses are two factor crosses in which the partners have different alleles for the *S*-locus and for the suppressor locus; at the *m*-locus all strains have the mutant allele *minor-1* (column 1). The phenotype is marked below each crossing partner (O = normal perithecia, o = small perithecia). Since the strains of *P. anserina* used are self-incompatible hermaphrodites, the perithecia of one cross come either from the female sex organs of one or from those of the other parent. We are dealing, therefore, in some crosses (rows 1 and 4) with two reciprocal crosses. For the establishment of extrachromosomal inheritance non-reciprocal crosses are essential. These are produced by so-called spermatization, placing the male gametes (spermatia) which contain practically no cytoplasm onto the mycelium of the partner. In this case only the latter transmits the plasmone through the female sex organs (rows 2, 3, 5–8). The actual and relative numbers of the analyzed asci are shown in columns 2 and 3. The method of identification of the asci according to the different patterns of meiotic reduction can be found in: ESSER and KUENEN 1965, p. 154 ff. Because of the fact that the spores of *P. anserina* contain two non-sister nuclei derived from postmeiotic mitosis, no clear distinction is possible between parental types and tetratypes on one hand and recombinant types and tetratypes on the other hand (columns 4 and 5), when both genes undergo post-reduction. This is true even if so-called abnormal five-spore asci are used, unless a time consuming post-analysis for each individual spore is carried out. We did not carry out post-analyses because in the present case unmistakable evidence for linkage could be established (details in ESSER and KUENEN 1965, p. 155 ff. and p. 19), based on the high pre-reduction values (columns 8 and 9).

tively the content of phenoloxidases of the wild-type strain, of the mutant *minor-1*, of the suppressed mutant *minor-1*, and of the wild-type strain with active suppressor gene. In addition, the laccase of the four strains was examined for qualitative alterations by use of the CRM-test. With exception of the genetic differences at the *minor* and the suppressor gene loci, all strains have the genome of the wild-type strain s. The results are presented in Table 2.

From the data of Table 2 the following results are obtained: (1) The *minor* mutant shows about half the phenol oxidase activity of the wild-type strain. This quantitative enzyme change is abolished by the action of the suppressor gene. In the wild-type strain the suppressor gene has, as expected, no measurable influence on the enzyme activity.

(2) A comparison of row 2 with row 3 shows that only the activity of laccase is diminished by the action of the *minor* gene. In contrast, the activity of the tyrosinase has increased. A further analysis of the tyrosinase was not possible because this enzyme is formed in too small amounts for purification which is necessary for the production of an antigen.

(3) The reduction of laccase activity in the *minor* strain is not caused by reduced synthesis of the enzyme but by a qualitative structural alteration of the laccase molecule. This is demonstrated by the CRM-values, since the mutant enzyme particles bound by the antiserum have only approximately one third the enzyme activity as compared to those of the other strains.

Similarly studies with *m-2* mutants have demonstrated that the laccase production is altered qualitatively by this mutant allele and can be restored by the suppressor gene.

Thus, a mutation at the *minor* locus does not only cause a defect in morphogenesis but also in the structure of the laccase. Both defects are completely abolished, as far as can be seen, by the suppressor gene.

DISCUSSION

The question of a relation between melanin formation and morphogenesis can

TABLE 2

Phenol oxidase content and CRM values of four strains with different combinations of minor-1 *and* suppressor *alleles*

No.	Strain genotype	Wild type m_1^+ *su-m*$^+$	Mutant m_1 *su-m*$^+$	Suppressed mutant m_1 *su-m*	Wild type with suppressor m_1^+ *su-m*
	Relative enzyme content				
1	laccase and tyrosinase	1.00 (SPA 18.6)	0.51 ± 0.07	1.13 ± 0.07	0.93 ± 0.11
2	laccase	1.00 (SPA 28.5)	0.47 ± 0.15	1.15 ± 0.06	0.84 ± 0.23
3	tyrosinase	1.00 (SPA 0.5)	4.35 ± 1.15	0.74 ± 0.24	1.08 ± 0.18
4	CRM-value	1.00	0.34 ± 0.07	0.87 ± 0.08	0.81 ± 0.11

In rows 1–3 the relative amounts of enzyme relative to the enzyme content of the wild-type strains are listed. The conversion into absolute values can be carried out by using the specific activities (SPA) listed in the column "wild type".

be answered positively. In contrast to the findings with Neurospora described in the introduction where in spite of induction of tyrosinase no restoration of the morphogenetic defect occurs in Podospora the morphological as well as the melanin defect are repaired by the product of the suppressor gene. A series of additional data published elsewhere (ESSER 1966) also suggest a physiological connection of both processes.

Out of at least 14 known loci for melanin defective mutants in *P. anserina* 8 loci also control morphogenesis of the female sex organs *viz.* the perithecia. All 8 mutants have a phenol oxidase spectrum different from the wild-type strain. In the biochemically better analyzed sterile mutant *zonata*, for instance, two low molecular laccases are formed instead of the high molecular laccase of the wild-type strain which may be considered as subunits of the high molecular laccase (ESSER and MINUTH, in preparation).

Since all morphogenetic mutants of Podospora are distinguished from the wild-type strain by a macroscopically recognizable altered formation of melanin pigments, it may be asked whether the melanin itself or only the phenol oxidases are related to morphogenesis. For these are responsible only for the first steps of melanin formation up to the synthesis of indol-5,6-orthoquinone. The polymerization of this substance to melanin is not controlled by enzymes.

This question can be decided in favor of the phenol oxidases since the completely melanin-free mutant *albospora* forms normal fruiting bodies like the wild-type strain, although with white periodia and white spores. With respect to its phenol oxidase spectrum, however, no differences from the wild-type strain are found (ESSER 1966). A comparable observation was made by LEWIS (1960) in Drosophila. Neither the gene *sable* (black body color) nor a suppressor gene abolishing the sable effect have an influence on phenol oxidase activity.

When the qualitative alteration caused by the *minor* mutant is considered, it seems possible that morphogenesis is related not to the phenol oxidases in general but only to laccase. For the mechanism of this correlation two interpretations may be considered: In analogy to the enzymatically regulated syntheses in prototroph-auxotroph systems laccase could intervene directly in the physiological processes of morphogenesis. This would mean that in the *minor* mutants the normal development of the perithecia is inhibited due to an altered laccase. There exists also the possibility of an indirect correlation. This would occur if the *minor* mutation would cause an alteration in the physiological condition of the cell such that the synthesis of laccase as well as the course of morphogenesis are disturbed.

A decision between these two possibilities will have to wait for new experimental studies. Our data only suggest the existence of such a correlation. They can be used as a starting point for further genetic and biochemical investigations of morphogenesis and thus contribute to the understanding of the suppressor function; because two interpretations exist also for the mechanism of the suppressor function. On the one hand, the intracellular environment may be changed by a product of the suppressor gene such that e.g. due to a pH-shift in the mutants the wild-type conformation of the laccase can be reconstituted. On

the other hand, the suppressor effect may act at the level of protein synthesis by a repair of missense coding.

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SUMMARY

In the Ascomycete *Podospora anserina* the two allelic genes *m-1* and *m-2*, located in linkage group IV, have pleiotropic effects. They cause a drastic reduction in perithecial size and a reduction in the formation of melanin pigments of the mycelia. The expression of the mutant phenotype is abolished by the action of a suppressor gene (*su-m*) which could be localized by tetrad analysis in linkage group III.—Appropriate enzyme analyses have revealed that the alteration in pigmentation of the mutants depends on the formation of a qualitatively altered laccase (polyphenol oxidase). Since the action of the suppressor gene not only repairs the morphogenetic defect but also restores this enzyme alteration to the wild-type level, it may be concluded that formation of laccase and morphogenesis of perithecia are correlated. These findings may be used as a basis for further studies of the genetical and biochemical control of morphogenesis.

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