Responses of Living Hyphae Associated with Self and Non-self Fusions in the Basidiomycete Phanerochaete velutina

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Before fusion, hyphae of the basidiomycete Phanerochaete velutina responded similarly to one another when grown on a cellophane membrane, regardless of whether they were genetically the same or different. Long-range (up to 250 μm) curvature (homing) to specific sites in the lateral wall of recipient compartments often occurred in fusions involving main hyphal apices. Induction of tip outgrowth from lateral walls was most evident before short-range, tip-to-tip fusions resulting in H-connections between main hyphae. Spitzenkörper (apical bodies) became aligned with receptive sites before directed growth. A period (about 5–20 min) of expansion of the contact region preceded formation of a fusion pore. Fusions were abundant in the vicinity of septa, but never observed between tips of main hyphae which repelled one another. In fusions involving hyphae from the same thallus or of mating-incompatible homokaryons, the fusion pore usually enlarged until it occupied virtually the entire contact area. Except in the case of clamp-connexions, nuclear interchange was followed by aggregation and division in the pore region before septum formation. Between different heterokaryons, the fusion pore never expanded fully, nuclei were rarely exchanged, and rapid cytoplasmic lysis and vacuolation occurred. Lysis also occurred sooner or later between sexually compatible homokaryons; only in a few cases was dolipore dissolution and nuclear migration observed.

INTRODUCTION

The formation of hyphal fusions (anastomoses) is fundamental in both the vegetative and reproductive development of higher fungi. Fusions within the same thallus (self fusions) convert what was a radiate communication system into a network, and are also important in binding hyphae together in plectenchymatous aggregations. Fusions between genetically different thalli (non-self fusions) allow the possibility of genetic exchange, with the eventual outcome dependent on whether or not mechanisms of non-self rejection (somatic/vegetative incompatibility) or acceptance (sexual compatibility) are brought into operation (Rayner et al., 1984). Non-self acceptance mechanisms are essential for outcrossing in the somatogamous Basidiomycotina where they may be said to 'override' the rejection response (Rayner et al., 1984; Coates et al., 1985).

The cytological events associated with hyphal fusion are therefore of basic biological significance, and it is surprising that, after the pioneering efforts of Buller (1931, 1933), further real progress was long delayed (Gregory, 1984). However, recent light and electron microscope studies with Schizophyllum commune and Coriolus versicolor have demonstrated the formation of temporarily multikaryotic compartments before dolipore dissolution, nuclear migration and emergence of the dikaryon in sexual exchanges, as well as a remarkable nuclear replacement reaction in vegetative exchanges (Niederpruem, 1980a, b, 1984; Aylmore & Todd, 1984a; Todd & Aylmore, 1985). The latter reaction involves migration, via the fusion bridge, of a donor nucleus, or pair of conjugate nuclei, towards a recipient hypha in which the resident nuclei round up and degenerate. Division of the donor nuclei, and associated septum formation, then re-establishes the uninucleate or binucleate condition.
Both *C. versicolor* and *S. commune* exhibit what has been described as ‘normal’ nuclear behaviour (Boidin, 1971), having monokaryotic homokaryons (monokaryons) and dikaryotic mating-type heterokaryons (dikaryons) respectively with regularly uninucleate and binucleate compartments. The dikaryons are further characterized by possession of clamp-connexions. A major departure from this behaviour is found in members of the genera *Stereum*, *Phanerochaete* and *Coniophora* which have multinucleate hyphal compartments and verticillate (‘whorled’) clamp-connexions which can occur, in the case of outcrossing (heterothallic) forms, on both homokaryons and heterokaryons, i.e. ‘holocoenocytic’ behaviour (Coates et al., 1981; Boddy & Rayner, 1982; Rayner & Turton, 1982; Rayner et al., 1984). It is therefore of interest to compare the behaviour of these fungi at hyphal fusion with that of *C. versicolor* and *S. commune*. *Phanerochaete velutina* is particularly suitable for study since it possesses an unusually large proportion of wide diameter (about 10 μm) hyphae, has a unifactorial (bipolar) mating system (unpublished observations), anastomoses abundantly and, particularly significantly, exhibits a very rapid somatic incompatibility reaction.

Here we describe a phase-contrast study of responses of living hyphae of *P. velutina* associated with both self and non-self fusions. A parallel study using electron microscopy will be reported by Aylmore & Todd (1986a, b).

**METHODS**

*Strains and culture procedures.* Homokaryotic strains of *P. velutina* were derived from single basidiospores deposited by basidiocarps (collected from woodlands near Bath) onto 2% (w/v) malt extract agar (MA) (20 g Munton & Fison spray malt A; 20 g Lab M agar no. 2 per litre) containing 100 μg novobiocin ml−1. Heterokaryotic strains were obtained from compatible matings between homokaryons or from isolations onto MA plus novobiocin and 2 μg benomyl ml−1 from wood or mycelium collected near Bath. Before the cytological studies, mycelia were grown on cellophane membranes [pre-soaked in 50% (v/v) glacial acetic acid and absolute ethanol for 20 min, washed, then autoclaved separately in distilled water at 69 kPa for 20 min] overlying 0.02% (w/v) MA.

*Microscopy.* A piece of cellophane (≤ 10 mm2) was cut from the periphery of a single colony (self fusion studies) or to include growing margins from two adjacent colonies (non-self fusion studies) and loaded into a microculture chamber (for details see Aylmore & Todd, 1984b). After overnight incubation at 22 °C in darkness the chambers were transferred to a Wild M20 microscope with camera attachment and observed at 22–25 °C by phase-contrast optics over a period of 48 h. Photomicrographs were taken on Kodak Technical Pan 2415 film. A total of 30 cases of self fusion, 20 of non-self fusion between heterokaryons, 20 of fusion between mating-compatible homokaryons and 11 of fusion between mating-incompatible homokaryons were followed.

**RESULTS**

*Responses and Spitzenkörper behaviour before fusion.*

Both self and non-self fusions were preceded by similar hyphal responses. Long-range curvatures, over distances as much as 250 μm often occurred before fusions involving main hyphal apices. These involved re-directed growth in a smooth arc towards an apparent specific receptive site in the lateral wall of an adjacent main hypha (Fig. 1b–e, j–l): occasionally curvature was initiated slightly ahead of the receptive site, so that the hypha swung through an arc > 90° (Fig. 1d). Apices of main hyphae repelled one another if their paths were convergent (Fig. 1f) and receptive sites generally only occurred in a region extending backwards from the rear of the apical compartment. Older compartments of main hyphae containing less dense cytoplasmic contents and hence more prominent nuclei were not receptive to fusion. These main hyphal fusions commonly occurred without induction of a growing point from the receptive site – that is, they were truly tip-to-side fusions, or there was only a minimal distortion of the lateral wall immediately before, or just after contact (Fig. 1j–l).

Short-range fusions, resulting in the formation of H-bridges between main hyphae, were also common (Fig. 1a), but usually resulted in less marked curvature and were preceded by development of a growing point by the receptive hypha (Fig. 1g–i). ’Appression’ fusions sometimes formed between hyphae which had been lying side-by-side for an extended period. These involved the direct opening of a fusion pore at a localized contact site.
Both long- and short-range fusions, but more particularly the latter, were frequently directed towards septal regions. In many such cases fusions were formed to the rear of the septum (Fig. 2a, b) but in others fusion occurred on both sides of the septum (Fig. 2c) or to the front of the septum (Fig. 2d–f). The latter situation almost certainly involved sites of potential hook cell formation. Figs 2(d)–2(f) also show the initiation and homing of two different branch hyphae towards the same receptive site. Successful fusion by one of these hyphae was associated with cessation of extension by the other. The successful hypha had also initiated a temporary response in an adjacent region of the wall of the recipient hypha.

Spitzenkörper were prominent in growing, hyphal tips, and in several cases it was possible to observe their displacement and alignment with a receptive site before curvature towards and fusion with such sites. A striking example is shown in Figs 3 and 4.
Fig. 2. (a, b) Homing and non-self fusion between a heterokaryotic apical compartment and the rear of a septum. The adjacent hook cell (H) had induced a growing point (arrowed) but fusion to form a clamp connexion had not occurred. (c) Non-self fusion between heterokaryons simultaneously involving one apical and two recipient intercalary compartments. (d-f) Self fusion within a homokaryon establishing an H-bridge between a hook cell (H) and lateral bulge (arrowed) at the front of a septum. Note the slight homing curvature of the tip (T) just before it ceased extension and the constricted vacuole (V) passing through the fusion pore. Slight transient tip induction (T) occurred to the rear of the septum but development ceased before (e). Bar marker represents 10 μm. Time (min) is indicated in the lower left-hand corner of (a), (b) and (d)-(j).

Fig. 3. Self fusion in a heterokaryon showing displacement of the Spitzenkorper (S) and homing of the hyphal apex (A) to induced tip (arrowed) in lateral wall of recipient compartment. Opening of the fusion pore (arrowed) preceded nuclear division (f) and dolipore septum synthesis (g), both occurring at the site of fusion. See Fig. 4 for interpretive diagrams of nuclear behaviour. Bar marker represents 10 μm. Time (min) is indicated in the lower left-hand corners.
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Fig. 4. Diagram illustrating the sequence of events during the self fusion shown in Fig. 3. T1–T3 and S1–S3 are nuclei derived from the apical and recipient compartments respectively. (a) Pore (P) visibly open. (b, c) Aggregation of apical and recipient compartment nuclei in pore region. (d–f) Arrowed nuclei in early stages of division. (g) Septum forming across pore (P); note daughter nuclei (D). Bar marker represents 10 µm. Time (min) is indicated in the lower left-hand corners such that time 0 corresponds to that of Fig. 3(a).

Events at and after self fusions

These are illustrated in Figs 3 and 4. Similar behaviour was exhibited by both homokaryons and heterokaryons. After contact a period (usually 5–20 min) of expansion of the interface region preceded the formation of a fusion pore. This pore then usually enlarged over a period of about 15 min until it occupied virtually the entire contact zone, although sometimes a fairly considerable interfacial rim remained. Fusion was followed by movement of cytoplasmic granules and vacuoles usually at first into the recipient hyphae, but thereafter in both directions. The behaviour of nuclei was complex, with initial movement of one or more into or out of the recipient hypha being followed by a general aggregation of four to six within the vicinity of the fusion pore. When the pore had been open for about 40–45 min, some or all of the aggregate entered a division cycle. Starting within a 5 min period there was dilation of the nuclear membrane accompanied by contraction and pronounced Brownian motion of the nucleolus. The nuclei then faded from view for up to 10 min, after which smaller, globular nuclei – presumably division products – became readily visible. These exhibited pronounced jerky movements, and
Fig. 5. (a, b) Development of hook cell during clamp-connexion formation in a heterokaryotic apical compartment. Hook initiation (arrowed) occurred adjacent to an aggregation of nuclei. Division of members of this aggregate was followed by migration of a daughter nucleus into the hook cell (b). (c) Hook cell of heterokaryon at onset of septation with a pair of daughter nuclei (arrowed). (d) Septate hook cell of a heterokaryon containing an undivided nucleus (N) and a daughter nucleus (D). Hook cell fusion failed to occur in this pseudoclamp-connexion. (e) Paired pseudoclamp-connexion in which neither hook cell fused with the subtending homokaryotic hypha. Bar marker represents 10 μm. Time (min) is indicated in the lower left-hand corner of (a) and (b).

Fig. 6. (a) Unilateral non-self fusion between heterokaryons with faster lysis occurring in the apex after homing and opening of a very small pore. (b–i) Bilateral non-self tip-to-side fusion between heterokaryons showing progressive lysis and vacuolation (f–h) occurring after incomplete pore opening (e) resulting in virtually empty hyphal shells (i). This was accompanied by transient globular dark structures occurring within vacuoles (arrowed), and localized wall accretion (A). Bar marker represents 10 μm. Time (min) is indicated in the lower left-hand corner of (b)–(i).

Some continued to do so, and to remain small with a minute nucleolus for up to 60 min or more, suggesting that they might have been defective. About 15 min after the onset of division, a dolipore septum began to form across the fusion pore, closure being completed after about 10–15 min. However, this occasionally failed to occur.
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A similarly-timed sequence of nuclear aggregation and division, followed by septation, regularly occurred in extending apices and at sites of branch initiation from intercalary compartments. When there was concomitant formation of a clamp-connexion, hook cells contained one of the following: a single post-division nucleus; a pair of post-division nuclei; a single undivided nucleus, or one of each (Fig. 5).

Clamp-connexions were relatively rare on homokaryotic thalli and usually ceased development before hook cell fusion thereby permanently entrapping nuclei in pseudoclamps (Fig. 5).

Events at and after non-self fusions

Fusions between hyphae belonging to different, mating-incompatible homokaryons exhibited a similar behavioural sequence to that in self-fusions. However, those between different heterokaryons and between mating-compatible homokaryons departed radically from this pattern.

Fusions between heterokaryons. These were characterized by the rapid development, after incomplete opening of the fusion pore (see below), of a unilateral or bilateral lytic reaction. Unilateral reactions were predominantly confined to one of the participating compartments, that of the homing hypha (Fig. 6a). They occurred very rapidly, before the fusion pore was visible or when its presence could only be inferred by the movement of granules into the recipient hypha. Bilateral reactions affected both the participating compartments equally, and sometimes spread to two or three immediately neighbouring compartments (Fig. 6b–i). Enlargement of the fusion pore usually occurred until it was clearly visible, but expansion to the extent seen in self fusions was never observed.

The fusion pores were traversed by mitochondria, cytoplasmic granules and vacuoles but nuclei were not seen to migrate through them except, occasionally, as an apparent result of equilibration of cytoplasmic pressure. Within 5 min of opening of the fusion pore, nearby cytoplasmic activity in the recipient compartment, as manifested by the motion of organelles, temporarily subsided before initiation of a phase of progressive vacuolation (Fig. 6g). Dark globular structures containing oscillating darker fragments occurred transiently within the vacuoles (Fig. 6h), apparently originating by invagination of surrounding cytoplasm. As vacuolation proceeded, localized wall accretions (Fig. 6g, h) became visible throughout the lytic region, and were associated with a marked increase in refractility. After about 10 h the fusion compartments were reduced to virtually empty shells (Fig. 6i) and sometimes were invaded by intra-hyphal hyphae.

The example shown in Fig. 6(b–i) involved fusion with the rear of an apical compartment, and development of the lytic reaction was associated with disappearance of the Spitzenkörper from the recipient hypha before the onset of vacuolation.

Fusions between mating-compatible homokaryons. The majority of these fusions resulted in either a rapid lytic reaction of the type observed between heterokaryons, or in delayed lysis. Rapid lysis predominated in early encounters between interacting thalli whereas delayed lysis was characteristic of later encounters. The behaviour pattern preceding lysis paralleled that in self fusion and involved opening of the fusion pore, followed by almost synchronous nuclear division in the pore region and, in most cases, formation of a dolipore septum across the region. Vacuolation of the fusion compartments eventually followed, according to the pattern described previously, and was usually complete within 10 h of septal closure.

In six cases the self fusion behaviour pattern was followed not by lysis, but by rounds of septal erosion and nuclear migration. Septal erosion began 2–3 h after nuclear division in the pore region, and with the pore septum if present. After erosion, which always left a prominent septal rim (Fig. 7a), nuclear migration occurred, but was mostly restricted to six to ten compartments away from the original fusion pore by intact septa. Aggregation of many nuclei behind an intact septum was followed by their individual disintegration over a period \( \geq 2 \) h (Figs. 7b–d). The disintegration process began with expansion of the nucleolus until the entire nucleus became spherical and appeared dark (Fig. 7c). Approximately 2 min later the contents faded completely, leaving behind a spherical membrane (Fig. 7d) which gradually disappeared.
Nuclear migration had ceased, in all cases examined, within 24 h of appearance of the first eroded septum. The hyphae in which migration had occurred contained sparsely distributed nuclei and both intact and eroded septa. Some of these septa had formed after the onset of migration.

DISCUSSION

The extensive observations of hyphal fusions made by Buller (1931, 1933) led him to propose that all anastomoses are essentially tip-to-tip, such that even where one hypha grows directly towards the side of another, fusion is always preceded by induction of a tip from the lateral wall of the recipient. This led to the suggestion that the sequence of fusion is initiated by action at a distance (telemorphosis) not exceeding 10–15 μm, followed by a phase of zygotropism in which the stimulated hyphae grow towards one another before fusing end-to-end (Burnett, 1976). Two theories were proposed to account for these observations. One postulated the production of a single diffusible chemical from a growing hyphal tip which initiated both the telemorphotic and zygotropic responses (Raper, 1952). The other suggested that fusion between tips was a result of overlap of haloes of low concentration of staling products (Park, 1961, 1963; Robinson & Park, 1965; Park & Robinson, 1966). The latter theory fails to account for initiation of hyphal tips in tip-to-side confrontations, and neither theory accounts for the mutual repulsion between main hyphal tips mentioned by Burnett (1976) and observed during the present study.
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More recent studies questioned these earlier ideas in that observations of fusions made by growing fungi on cellophane membranes and at low nutrient levels have shown that tip-to-side fusions do occur (Watkinson, 1978; Aylmore & Todd, 1984a; Todd & Aylmore, 1985). This was attested further by the present observations.

Apart from the existence of tip-to-side fusions, an extra dimension to the problem of hyphal anastomosis was provided by the observations of long-range curvatures. These implied a much earlier and more far-reaching response than that proposed for telemorphotic induction (see above), together with the probability of initiation by receptive sites in the lateral wall of recipient hyphae. This probability was further emphasized by the behaviour shown in Figs 2(d) and 2(e), which also showed that induction of a tip from a receptive site is likely when another tip is in the vicinity. The positions in which fusions occurred, particularly near septa and well behind the apices of recipient main hyphae but not in old wall regions, strongly suggest that the recipient sites are in fact incipient branch sites of limited duration. If two hyphae lie adjacent or parallel to one another, then it may further be expected that the respective appression or H-bridge fusions will only occur where the receptive sites are in spatiotemporal alignment. This does much to explain the observed patterns.

Also in favour of a site-directed response is the parallel between long-range curvatures during hyphal fusion and those in the homing reactions with arthroconidia (oidia) and basidiospores which have been observed in several Basidiomycotina (Bistis, 1970; Kemp, 1970, 1977, 1980; Fries, 1981, 1983). In the case of basidiospores, it may be significant that in Leccinum homing is only induced following germination to produce a spherical germ vesicle from which germ tubes would eventually emerge. Production of a germ vesicle is itself induced by the nearby presence of hyphae of the same or a closely related species: these hyphae then home towards, and attach to the germ vesicle (Fries, 1981, 1983).

The mechanism underlying curvatures remains obscure however. The behaviour of the Spitzentörkörper might suggest some role in detection of a stimulus from a receptive site, but since displacement of this body is a general feature before growth curvature (Girbardt, 1955, 1957; Grove, 1978), cause and effect are difficult to resolve. It may be significant that those fungi which possess Spitzentörkörper (Ascomycotina and Basidiomycotina) are just those in which hyphal anastomosis occurs most readily.

The nature of the stimulus, whether it be physical or chemical, is also of concern. The involvement of diffusible chemicals in telemorphic and zygotropic responses during sexual development, as well as their implication in homing reactions (Fries, 1983), makes them the more obvious candidates. In an experiment with P. velutina grown on a cellophane membrane above a circulating low nutrient medium, similar responses to those described here were still observed (B. C. Phipps, unpublished, cited by Cooke & Rayner, 1984); however, the intended prevention of establishment of chemical gradients may have been ineffective due to boundary layer formation. Long-range curvatures are rarely seen on nutrient rich agar media.

Nuclear behaviour and septation during self fusion exhibited markedly different patterns from those reported previously for Coriolus versicolor and Schizophyllum commune (Aylmore & Todd, 1984a; Todd & Aylmore, 1985). Thus the nuclear replacement reaction was not observed, and, except in clamp-connexions, septation was always directly across the fusion pore, where numerous nuclei divided, rather than at the site of division of an individual nucleus or pair of nuclei. Nevertheless the capacity to undergo the same process of degeneration which precedes replacement in C. versicolor and S. commune was exhibited, after mating-compatible fusions, by nuclei whose migration was blocked by an intact septum. These differences in behaviour are probably associated with lack of strict control of numbers of nuclei (up to 80 have been observed) in the holocoenocytic compartments of P. velutina compared with the rigorously uni- or binucleate compartments of monokaryons and dikaryons in C. versicolor and S. commune. This probability is enhanced by recent observations with another basidiomycete, Chondrostereum purpureum, which may be more closely related to Phanerochaete, but in which typical nuclear replacement reactions have been observed, together with regularly binucleate compartments in the heterokaryon (A. M. Ainsworth, unpublished).
The other major departure from the behaviour of C. versicolor and S. commune was in the rapidity of onset of the lytic reaction. In these latter fungi, although somatic rejection is easily observed in plate culture, fusions between dikaryons grown under the same conditions as described in the current paper all resulted in the same behavioural sequence as that seen in self fusions (Aylmore & Todd, 1984a; Todd & Aylmore, 1985). This implies that the rejection responses are slow to develop and/or precluded by the nuclear replacement reaction.

The occurrence of rapid or delayed lytic interactions between mating-compatible but not between incompatible homokaryons correlated with behaviour during interactions in plate culture on 2% malt agar. Here migrating lytic zones precede emergence of a stable heterokaryon in between compatible homokaryons whereas interactions are weak or slow to develop between incompatible ones. Moreover, heterokaryon–heterokaryon and heterokaryon–homokaryon interactions result in a strong rejection response unless the strains are genetically similar. It is therefore probable that in P. velutina the presence of complementary mating alleles determines both rapid somatic rejection in heterokaryon and heterokaryon–homokaryon confrontations, and the overriding of such rejection between homokaryons. This parallels the behaviour which has been described for Neurospora crassa (see Perkins & Barry, 1977). However, in other basidiomycetes, such as Stereum hirsutum, somatic rejection can be expressed independently from the presence of different mating-compatibility alleles (Coates et al., 1981, 1985; Coates & Rayner, 1985a, b).

The lytic reactions after non-self fusions strongly resemble the 'lethal' cytoplasmic reactions which have been reported to follow homing to arthroconidia and basidiospores in Coprinus and Leccinum respectively (Kemp, 1977; Fries, 1981, 1983). However, in this case the reactions follow fusion with spores of closely related species, and not with the same species. Since at least the spores would be homokaryotic, it is likely that lethal reactions following non-self fusions with the same species would either be delayed, or overridden by mating compatibility.

A further parallel, particularly evident in the rapid reaction shown in Fig. 6(a), is with hyphal interference, whereby contact, but not fusion, between hyphae of different species results in lysis of one or both participant compartments. In Ascocholus cremalatus contacted by Coprinus heptemerus increased refractility and vacuolation occurs (Ikediugwu & Webster, 1970; Ikediugwu, 1976) which appears to be fundamentally similar to that in non-self interactions of P. velutina (see also Aylmore & Todd, 1986b).

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