Cytology of Non-self Hyphal Fusions and Somatic Incompatibility in
Phanerochaete velutina

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The somatic incompatibility reaction occurring at sites of fusion between hyphae of genetically
different secondary mycelia of Phanerochaete velutina has been examined using combined light
and electron microscopy. Hyphal compartments affected by incompatibility rapidly showed
increased vacuolation and the development of autophagic bodies throughout the cytoplasm.
Dense osmiophilic spherical bodies that developed within the vacuole lumen characterized the
early and highly regulated phase of the reaction. Eventually, as expansion of the vacuolar system
proceeded, more widespread degeneration began in the remaining cytoplasm, nuclei and
mitochondria. The large microtubule bundles present within this species showed variable
behaviour during degeneration of the cell compartments, either breaking down early on in the
process or persisting intact during breakdown of the other cell components. The incompatibility
finally caused extensive disruption and death of the compartments engaged in fusion and often
contiguous cells. Plugging of dolipore septa apparently restricted spread of the incompatibility
response along the fused hyphae.

INTRODUCTION

Somatic incompatibility is a common phenomenon between genetically different mycelia of
higher fungi (reviewed by Lane, 1981; Rayner et al., 1984). The incompatibility response is, in
the main, a post-fusion event which is often obvious as a reaction zone between non-self mycelia
in plate culture and natural substrata (Rayner & Todd, 1977, 1979). Whilst the reaction is well
recognized at the macroscopic level, little is known about the cytological details of the process.
In Neurospora crassa, fusion between heterokaryon incompatible strains causes the cytoplasm to
become granular and finely vacuolated, several compartments adjacent to the fusion often
showing the reaction. Plugging of the septal pores in the affected area was common.
Enlargement of the vacuolar system resulted in effective destruction of the fusion site (see
Garnjobst & Wilson, 1956). Further work on this species suggested that a membrane-associated
protein factor may be involved in triggering the incompatibility response (Wilson et al., 1961;
Williams & Wilson, 1966, 1968). In Podospora anserina, lysis occurs at the site of fusion and is
due to the activity of specific proteolytic enzymes synthesized from pre-existing mRNA at the
onset of the incompatibility reaction (Labarère et al., 1974; Boucherie et al., 1981). Similarly,
lysis has been associated with non-self fusions in Thanatephorus cucumeris (Fletje & Stretton,
1964), Endothia parasitica (Anagnostakis, 1977), Polyporus schweinitzii (Barrett & Usculpic,
1971) and Athelia (Sclerotium) rolfsii (Punja & Grogan, 1983). However, these observations are
based predominantly on light microscopy and there appears to be little information on the
ultrastructural aspects of these reactions.

In the basidiomycete Coriolus versicolor, which shows an obvious incompatibility response in
plate culture (Rayner & Todd, 1977, 1979), cytological analysis of fusing hyphae failed to reveal
any significant differences between self and non-self pairings. We noted, however, the presence
of abnormally shaped hyphae (so-called spindle cells) in the reaction zone between non-self
mycelia (Aylmore & Todd, 1984a). Here we record the ultrastructural features accompanying the somatic incompatibility occurring between different mycelia of *Phanerochaete velutina*. A similar study using light microscopy has recently been reported by Ainsworth & Rayner (1986). The rapid expression of the incompatibility reaction at the sites of fusion makes this fungus particularly suitable for these studies.

**METHODS**

*Strains and culture conditions.* Heterokaryotic secondary mycelia, synthesized from wild homokaryotic isolates (supplied by A. M. Ainsworth, University of Bath), were maintained on 2.0% (w/v) malt agar, as described in the accompanying paper (Aylmore & Todd, 1986). Somatic incompatibility between different mycelia was confirmed by pairing different isolates using this same medium. For cytological work, strips of each mycelium were cut from stock cultures and arranged in a 'V' shape, spaced between 2 and 4 cm apart on cellophane overlying 0.2% (w/v) malt agar. These were incubated at ambient temperature (18–21 °C) until the two isolates met.

*Light microscopy.* An area in which the leading hyphae, clearly traceable to either of the original inocula, were beginning to intermingle, was selected using a stereo dissecting microscope. Surrounding mycelium was scraped away to leave these groups of interacting hyphae attached to a small part of the parent colony in an area of cellophane about 10 mm². Care was taken to ensure hyphal fragments were not transferred between the isolates during this operation. This region of cellophane was cut free and loaded into the microculture chamber (see Aylmore & Todd, 1984b). After overnight incubation, non-self fusions were examined using a Leitz Dialux microscope fitted with Heine phase optics. Photomicrographs were taken on Kodak Technical Pan 2415 film.

*Electron microscopy.* Non-self fusions showing various stages of the somatic incompatibility reaction were examined under phase optics before the whole mycelium was processed for electron microscopy. The protocol for this and details of the longitudinal sectioning of target cells are described elsewhere (Aylmore & Todd, 1984b). Sections were stained with lead citrate (Reynolds, 1963) and examined at 60–80 kV with a JEM 100S electron microscope.

**RESULTS**

The development of non-self fusions was followed. Our findings are based on at least 20 sets of observations.

*Light microscopy*

The general features of non-self fusion, in terms of pre-contact behaviour and fusion pore opening, appeared to be identical to those described for self fusions (Aylmore & Todd, 1986). However, after initial streaming and exchange of cytoplasm between compartments, events followed an entirely different course. Unlike self fusions, the point of contact remained open and was not re-partitioned at any stage by septum formation across the fusion channel. Within 30–40 min, small refractile bodies formed in many of the vacuoles present and intense cytoplasmic streaming was often evident. Growing apices or lateral branches rapidly ceased extension. Within 1–5 h the refractile structures had developed into large spherical bodies and this was accompanied by a general increase in the level of vacuolation throughout. The hyphal walls

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Fig. 1. Non-self fusions in *P. velutina*. *(a–h)* Hyphal compartments fixed at various stages of the incompatibility reaction. *(a)* The region just behind a hyphal apex fixed 20 min after fusion. Note the many small vacuoles (V). Points of appression between the vacuoles are arrowed. Smaller vesicles (VS) derived from dispersal of the apical apparatus are also evident. *(b)* Specimen fixed 40 min after fusion. Larger vacuolar structures with diffuse contents and regions of highly electron-dense material (arrowed) have formed. Numerous smaller vacuoles are also present. Note the swelling (SW) formed on the inner surface of the cell wall (CW). *(c, d)* Fixed 75 min after fusion, larger electron-dense structures formed within the majority of the vacuoles are present. Note the typical appearance of the cytoplasm and presence of intact mitochondria (M). *(e)* Aggregation of the electron-dense material within the vacuoles (V) resulted in the formation of large spherical bodies (arrowed) visible under phase optics. *(f)* A compartment fixed 8 h after fusion. Nuclei (N) persist in the degenerating cytoplasm. Note the diffuse chromatin (arrowed) and intact nuclear envelope (NE). *(g)* Cytoplasm in an advanced state of degeneration. Note the membranous fragments (arrowed) and nucleus (N) with separated nuclear envelope (NE). Numerous microtubules (MT) are dispersed throughout the region. *(h)* A compartment fixed 12 h after fusion showing completion of the incompatibility reaction. The cell wall (CW) contains mostly membranous debris. The plasmalemma has disintegrated. Bars, 1 µm.
showed increased refractility with the inner surface developing an irregular outline. These properties characterized the earlier stages of the incompatibility reaction. Nuclei and filamentous mitochondria remained visible in regions of intact cytoplasm. Between 1.5 and 6 h after fusion the vacuolar system expanded. The refractile structures within the vacuoles gradually dispersed while nuclei present in the remaining cytoplasm became visibly swollen. Progressive enlargement and coalescence of the vacuolar system continued until, between 10 and 15 h after fusion, the hyphal contents appeared to be plasmolysed and collapsed away from the cell wall.

The above sequence occurred uniformly throughout the fused segment and it was not possible to detect any centres of enhanced activity. Contiguous compartments commonly showed the same incompatibility reaction, often developing the symptoms simultaneously, but in other instances appearing normal for several hours before onset of vacuolation. In this way often one or two, and up to four compartments either side of a fusion site could show the reaction. All cross-walls remained present, non-self fusion failed to trigger septal dissolution.

The onset of incompatibility disrupted any further mitosis, clamp connection formation and septation. The zone of interaction between two mycelia contained many centres of die-back, forming within incompatible hyphae connected by fusion. However, transmission of the reaction was strictly intracellular; those hyphae growing in this region without having fused, even when in close physical contact with points of die-back, showed typical patterns of growth, lateral branch formation and mitosis. The high frequency of non-self fusion and the rapid onset and spread of the incompatibility reaction ensured that penetration of hyphae into another mycelium was minimized. The abnormally shaped hyphae found in mycelial interactions of *Coriolus versicolor* were not evident in this study.

**Electron microscopy**

Ultrastructural observations confirmed that self and non-self fusions occurred by a common mechanism. However, in non-self situations, subsequent re-partitioning was inhibited and the fusion channels remained open, allowing mixing of cytoplasm and spread of the incompatibility reaction between the compartments. The apical vesicles present in any growing points became dispersed soon after fusion. A notable feature of the incompatibility reaction was the increased level of vacuolation. This first became evident as small vacuoles arising in clusters in specimens fixed within 20 min of fusion (Fig. 1 a). Close appression of the vacuole membranes (arrowed in Fig. 1 a) suggests the possible coalescence of these into larger structures. Vacuoles formed initially were mostly devoid of contents although some enclosed small osmiophilic bodies and membranous structures. Vacuolation became increasingly obvious in specimens fixed between 20 and 90 min after fusion although these were characterized by diffuse amorphous contents and the formation of large, highly osmiophilic bodies within the vacuole lumen (Figs 1 c and 1 d). Autophagic structures were common in these hyphae (Fig. 2 d) and many of the larger vacuoles contained membranous debris (Fig. 2 b). Condensation of the vacuolar material gave rise to the formation of large, highly osmiophilic bodies, circular in section, equivalent to the refractile spheres readily observed under phase optics (Fig. 1 e). These structures were characteristic of the

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**Fig. 2.** Non-self fusion in *P. velutina.* (a) Photomicrograph showing two points of fusion (F) between hyphae of different mycelia. Note their vacuolated appearance. (b) Composite showing median longitudinal section through the bracketed area (i) shown in (a), fixed 60 min after opening of the fusion. The large vacuoles (V) contain amorphous material and membranous structures (arrowed). Numerous lightly stained swellings (SW) occurred on the inner surface of the cell wall. A large bundle of microtubules (MT), nuclei (N) with single nucleoli (NU) and mitochondria (M) occurred intact within the cytoplasm. (c) A section through point (ii) arrowed in (a). Note the lightly stained swelling (SW) arising from the inner surface of the cell wall (CW). The individual microtubules (MT) of the bundle are clearly defined. (d) An autophagic structure present in a hypha showing the incompatibility reaction fixed 90 min after fusion. A fragment of cytoplasm (CY) containing a densely stained mitochondrion (M) is present within the vacuole lumen (V). (e) Nuclei (N) and mitochondria (M) within a pocket of intact cytoplasm separating two vacuoles (V). Note the single nucleolus (NU) and region of condensed chromatin associated with the nuclear envelope (NE).
incompatibility reaction. Apart from the development of the vacuolar system, the other cell compartments in the earlier stages of the reaction showed no obvious modification and the nuclei and mitochondria had typical structure (see Fig. 2e).

Specimens fixed between 2.5 and 8 h after fusion showed further enlargement of the vacuolar system but persistence of cytoplasmic pockets containing intact nuclei and mitochondria. The dense osmiophilic spheres present in the larger vacuoles degenerated. After 6 h any remaining cytoplasm began to degenerate and appeared progressively less dense with a reduction in the numbers of ribosomal particles. Chromatin began to break down, but remained within the nuclear envelope (Fig. 1f). Filamentous mitochondria fragmented into smaller, more spherical, forms. Hyphae in advanced stages of the incompatibility reaction (Fig. 1g) contained remnants of nuclei and mitochondria. Separation and degeneration of the nuclear envelope was a feature of nuclei in this condition. Eventually, collapse of the plasmalemma and disruption of any remaining organelles resulted in virtually empty hyphal shells containing only membranous debris (Figs 3e and 1h). Pockets of cytoplasm occasionally remained, often in association with microtubular bundles (see below).

The septa directly containing the fused compartments invariably showed an occlusion reaction, typically involving discrete plugs of amorphous electron-dense material completely blocking either end of the dolipore channel, leaving the central region clear (Fig. 3f). The precise time at which these formed was not established, although they were present in specimens fixed after 45 min of fusion. The parenthesomes and septal swellings remained intact, playing no visible part in the occlusion process. The plugged septa were stable structures, evident in a range of specimens fixed at various times up to 6 h after fusion. However, in hyphae more advanced in the vacuolation process, such septa showed a more dramatic change in structure with the septal swellings cleaving away from the cross-wall or deforming around the pore plug to give a hemispherical pad which completely sealed off that compartment (Fig. 3h). The plugging reaction was not confined to the septa positioned directly around the point of fusion; the second and third cross-walls, moving in either direction, commonly showed a similar response (Fig. 3g). Those in more distant positions still usually displayed only partial plugging. All cross-walls remained intact with no evidence that non-self fusions between secondary mycelia triggered septal dissolution. Cell walls in affected compartments developed ingrowths on their inner surface (Figs 2b, 2c and 3c) which were similar in structure and staining properties to the material of the septal swellings. These were widespread in both lateral walls and septal plates in all specimens fixed after 1 h of fusion.

In the majority of active hyphal compartments, cytoplasmic microtubules are closely associated to form large bundles which run longitudinally through the hyphae (Aylmore et al., 1985). For up to 3 h after fusion, during the earlier stages of vacuolation, these remained intact, with individual microtubules clearly visible (Figs 2b and 2c). However, in specimens fixed in more advanced states of degeneration, between 3 and 10 h after fusion, the behaviour of the

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Fig. 3. Non-self fusions in P. velutina. (a) Photomicrograph of a tip-to-side fusion (F) between somatically incompatible hyphae. (b) Median longitudinal section through the bracketed area in (a) fixed 60 min after fusion. Note the large vacuoles (V) and regions of intact cytoplasm (CY). (c) Enlargement of the fusion from (b) showing incomplete expansion of the open fusion pore (FP). Swellings (SW) have formed on the inner surface of the cell wall. (d) Photomicrograph showing three points of fusion (F) between somatically incompatible hyphae. (e) Enlargement of the bracketed area in (d) fixed 10 h after fusion. The fusion pore (FP) remained open. Only cell debris was present within the cell wall (CW). (f) Near median section through a dolipore septum of compartment forming a fusion with an incompatible hypha, fixed after 45 min, before extensive vacuolation had occurred. Note the discrete electron-dense pores (PP) obstructing both ends of the dolipore channel leaving the central region clear. The parenthesomes (P) and septal swellings (SS) remained intact and played no part in the occlusion reaction. (g) Median section through the second septum away from an incompatible fusion showing a plugging reaction similar to that shown in (f). (h) The septum (SP) of a fused compartment fixed after 10 h. The septal swellings on one side have cleaved away from the septal plate whilst on the other they have deformed around the electron-dense pore plug (PP). (i) The apical septum of an unfused compartment growing in the interaction zone. Note the typical dolipore structure with septal swellings (SS), parenthesomes (P) and unoccluded pore channel.
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microtubules was varied. In many instances the microtubular elements of the bundle became indistinguishable, the whole structure appearing progressively diffuse (Fig. 4a), eventually degenerating with the rest of the cytoplasm. Certainly, many fusions fixed after total collapse of the cell contents contained membranous debris only and were devoid of these structures (Figs 1f and 3e). In some specimens, however, the microtubules persisted. Fig. 1(g) shows an example in which numerous single microtubules occurred in a specimen well advanced in the incompatibility reaction. Persistence of the microtubules in such situations was not invariably associated with disaggregation of the bundle. Virtually empty compartments could contain regions in which the bundles remained intact, often within a small pocket of cytoplasm associated with the septa (Figs. 4d and 4e). In a single example, one end of a compartment contained innumerable microtubules packed tightly together into a complex three-dimensional structure rather than a discrete bundle (Figs 4b and 4c). The regular packing arrangement of the associated microtubules is evident in Figs 4c and 4e.

The septal plugging, distortion of cell wall structure and vacuolation associated with the incompatibility reaction were confined to the fused hyphae and not transmitted extracellularly to other cells growing nearby (Fig. 3i). Those self fusions occurring between genetically identical hyphae but within the interaction zone between mycelia behaved as described previously, becoming re-partitioned by septum formation across the fusion pore (see Aylmore & Todd, 1986).

DISCUSSION

This work shows that somatic incompatibility in P. velutina results in the death of the participating cells, and confirms that it serves as a barrier to exchange of nuclei and cytoplasm. The destruction of the interface between mycelia effectively prevents translocation, thereby maintaining their individuality (Todd & Rayner, 1980). This reaction is confined to the fusion segment of one or a few contiguous compartments. That it spreads so rapidly within and between those compartments affected might indicate that the signal responsible is mobile and resides in the cytoplasm and is not associated with one of the larger organelles. Apparently the stimulus is able to traverse dolipore septa before they become occluded. However, the reaction is eventually confined and is prevented from spreading extensively by septal sealing. The mechanism of sealing is the same as that found when hyphae are damaged experimentally. This involves a two stage process; initially, the dolipore channel is occluded by the formation of electron-dense plugs and this is followed by re-modelling of the septal swellings over and around the pore plugs to give a permanent seal (see Fig. 3h). The parenthesomes and septal swellings play no part in the initial plugging process (for full discussion of this see Aylmore et al., 1984). The same occlusion reaction seems to be a general response to loss of viability due to both physical and metabolic damage. In N. crassa, the simple septal pores also plug as a result of cytoplasmic lysis at sites of incompatible fusions (Garnjobst & Wilson, 1956) although this is likely to be caused by an entirely different process involving the so-called Woronin bodies (Reichle & Alexander, 1965; Wergin, 1973). Limited spread of the incompatibility reaction reported in Sclerotium rolfsii (Punja & Grogan, 1983) and Thanatephorus cucumeris and T. pratolicus (Fletje & Stretton, 1964) suggests septal plugging may also occur in these species. This

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Fig. 4. Non-self fusions in P. velutina. (a) Specimen fixed 3 h after incompatible fusion. The individual microtubules of the bundle (in brackets) are ill-defined and appear to be partially dissolved. The diffuse material present in the large vacuoles (V) appears to be aggregating into discrete electron-opaque bodies (arrowed). Note the dense cytoplasm and intact mitochondria (M). (b) A complex aggregate of closely-packed microtubules (MT) present in an extensively vacuolated (V) compartment of a specimen fixed in the advanced stages of the incompatibility reaction. (c) An enlargement of the bracketed area in (b). Individual microtubules are evident, tightly packed at various orientations in a complex array. (d) A small pocket of cytoplasm surrounded by vacuoles (V) contains two areas of sectioned microtubule bundle (in brackets). Note the cytoplasm of the adjacent compartment is also in an advanced state of degeneration. (e) A serial section through the upper area bracketed in (d). The discrete bundle is shown in transverse section. The individual microtubules are closely packed in parallel array.
is consistent with Buller’s original suggestion that septa function in this way to contain damage of any sort (see Buller, 1933). It may be significant that the truly coenocytic lower fungi, which lack septa, also fail to form somatic fusions and there seems to be an association between the absence of septa and the inability to fuse somatically.

The reaction is clearly not simply a system of unregulated lysis of organelles. The ultrastructural features indicate enhanced activity of the vacuolar–lysosomal system of the hyphae and it seems that the demise of the compartments involves, at least in the initial stages, a regulated process of autophagic digestive vacuolation. However, this must be viewed as a complex system and it was not always possible to identify the heterogeneous components of the lysosomal system (see Pitt, 1975). Certainly in its earliest stages, the reaction involved the development of vacuoles, lysis apparently being confined within the membranes surrounding these organelles. The integrity of the cytoplasmic compartment was maintained until the reaction was well advanced. The dense osmiophilic spherical bodies that characteristically occurred within the vacuoles (Fig. 1 e) probably represent ‘residual bodies’ derived from compaction of partially digested material.

The features and time course of the reaction in Phanerochaete velutina, as seen under light microscopy, are similar to those described for other species (Garnjobst & Wilson, 1956; Anagnostakis, 1977; Punja & Grogan, 1983) and it is probable that the same basic mechanism is involved. This ultrastructural work has provided useful information which now needs to be complemented with a biochemical approach. Further insight may be gained using cytochemical techniques in attempts to localize specific hydrolytic enzymes during the development of the lysosomal system after the onset of the reaction. How the death of the fused hyphae described here relates to other processes of necrosis or apoptosis (programmed cell death; see Wyllie, 1985) or general ageing phenomena remains to be seen. Fungi clearly possess a recognition system which has a great capacity for specificity and is able to distinguish self from non-self. Detailed examination of interacting cells during this work failed to reveal the nature of the recognition system or target organelles involved. However, it is obvious that a form of digestive vacuolation characterizes the reaction but whether this represents the primary expression of the somatic incompatibility mechanism or is merely a consequence of more subtle cellular changes is not clear.

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REFERENCES


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