PLANT CELL RESPONSES TO INFECTION BY RUST FUNGI

by

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A thesis submitted in conformity with the requirements for the degree of
Doctor of Philosophy
Graduate Department of Botany
University of Toronto

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0-612-41313-6
PLANT CELL RESPONSES TO INFECTION BY RUST FUNGI,

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ABSTRACT

The cowpea rust fungus (*Uromyces vignae* Barclay)-cowpea (*Vigna unguiculata* [L.] Walp.) interaction was examined by cytological and molecular techniques to determine cellular responses during the monokaryotic stage of infection and changes in gene expression during the dikaryotic stage. Chapter one describes callose deposition around intracellular fungal structures, the main resistance response in cultivar Queen Anne. Ultrastructural examination revealed that the callose encasement was developed from the site where the fungus encountered the inside of the plant cell wall and was separated from the fungus by the extrahyphal membrane and an extension of plant plasma membrane. Treatment of plants with chemical inhibitors indicated that, similar to what is observed during the dikaryotic stage of infection, callose deposition in response to the monokaryotic stage is dependent on transcription, protein synthesis and glycosylation and microfilament polymerization. Experiments in which the fungus was killed by heat treatment indicated that callose deposition is normally suppressed in susceptible
plants and that, unlike the extrahaustorial membrane surrounding the dikaryotic haustoria of this fungus, the extrahyphal membrane is capable of generating callose. In chapter two, the role of the plant cytoskeleton in infection-induced nuclear movements and resistance-associated cell death, the hypersensitive response (HR) was investigated by fluorescent microscopy and treatments with anticytoskeletal drugs. The nuclear movements, which differ between the susceptible cultivar California Blackeye (CB) and the resistant cultivars Dixie Cream and Calico Crowder, were dependent on actin microfilaments. Observations of microtubule organization during the HR revealed that the sequence of events leading to protoplast collapse differed between the two resistant cultivars, suggesting a possibility of multiple pathways for cellular degradation during the HR. The microfilament network remained visible even at later stages of the HR and was necessary for this process to occur. Chapter three describes the use of mRNA differential display to identify two genes that are upregulated by infection in the susceptible cultivar CB. Both genes may code for proteins that were previously uncharacterized in plants. One is induced during early stages of infection and may encode a reverse transcriptase and the other is turned on only during haustorium formation and may encode a lipase-like protein.
ACKNOWLEDGEMENTS

I would like to thank the following people for their advice, support, inspiration and generous help:

M.C. Heath, (supervisor)

J.R.C Coleman, V.J. Higgins (supervisory commitee)

P.L. Tam, K. Sault, T. Ngyuyen (technicians)

D. Bonetta,

F. Ferreira,

M. Ghassemian,

J. Jebanathirajah,

M.J.R. Mould

P. McCourt

M. Škalamera

M. Popović

J. Kus

Without the help of these people this thesis would have never materialized.

This is for Polly.
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INTRODUCTION

Rust fungi are plant pathogens that can cause disease on a number of economically important crop species. They infect all major cereals except for rice (Saari and Prescott, 1985) and have been known to cause yield losses as high as 50% in some crops like peanuts (Subrahmanyam et al, 1985), sugarcane (Comstock et al, 1992) and coffee (Mendgen, 1997). Rust fungi infect a wide range of plant taxa including both monocot and dicot species of angiosperms as well as conifers and ferns. However, individual species of rust fungi have a limited host range which is most often restricted to a few plant species (Scott and Chakravorty, 1982). The limited host range is thought to be due to host-specific plant signals required for the development of rust fungi which are obligate biotrophic pathogens. Although certain races of rust fungi have been cultured in relatively simple media containing only sugars, amino acids, vitamins and minerals, they grew slowly and did not fully differentiate (Fasters et al, 1993; Maclean, 1982; Williams 1985). In nature, rust fungi require living plant tissue for growth.

Most rust fungi have complex life cycles consisting of up to five different spore stages. Species that go through all five spore stages are called macrocyclic and have two infectious stages that are completed on either one, for autoaecious, or two host species for heteroecious rust fungi. The two infectious stages are morphologically distinct. The basidiospore-derived monokaryotic stage usually penetrates plant epidermal cells directly, producing an intracellular hypha which subsequently branches out into an intercellular mycelium which develops intracellular M-haustoria (Littlefield and Heath, 1979). The
urediospore-derived dikaryotic stage penetrates plant tissue through stomatal openings, grows between plant cells and subsequently produces highly specialized D-haustoria inside mesophyll cells (Littlefield and Heath, 1979). Therefore, intracellular structures are produced during both stages of growth. Whether these structures are truly intracellular is debatable, since they remain enveloped by the invaginated plant plasma membrane so that the only plant structure penetrated is the cell wall. Nevertheless, fungal structures are in close contact with the plant protoplast in which they can cause considerable organelle rearrangements (reviewed by Heath and Škalamera, 1997). This interaction is an interesting example of a phenomenon in nature where walled cells from two different eukaryotic taxa are in close proximity and can influence each other’s behaviour. Therefore, investigation of rust fungi is important not only because it could lead to development of control measures for some major crop diseases, but also because it could elucidate basic mechanisms of certain types of cell-to-cell communication.

How plant cells respond to the development of intracellular rust fungal structures is the main focus of this thesis. I used the macrocyclic, autoaecious cowpea rust fungus (*Uromyces vignae* Barclay race 1) as the model system for investigation of cellular and molecular responses in the cowpea plant (*Vigna unguiculata* (L.) Walp.). For cytological investigations I used the monokaryotic stage since the first penetrated epidermal cells are easily accessible to microscopical observations. The dikaryotic stage was used for molecular experiments since infection is more synchronous, and substantial amounts of tissue could be obtained where the mycelium has reached a particular stage of growth (i.e. before or after the formation of fungal haustoria). Four cowpea cultivars were used in this
study which have similar responses to infection by either stage of the cowpea rust fungus (Heath, 1989). Cultivar California Blackeye (CB) is fully susceptible to the fungus, cultivars Queen Anne (QA), Dixie Cream (DC) and Calico Crowder (CC) are fully resistant so that fungal growth is arrested after only one or a few plant cells have been infected (Heath, 1989). At the cellular level, in the majority of infection sites in cultivar QA, resistance is manifested by encasement of fungal structures in callose containing deposits, while cultivars DC and CC demonstrate the typical hypersensitive reaction (HR) in which the infected cells die rapidly (Heath, 1989). In this thesis, I investigated certain aspects of callose deposition and the hypersensitive response as well as infection-induced plant organelle movements and gene expression. The work presented in chapters one and two is reproduced unmodified, with permission from two previously published papers (Škalamera et al, 1997; Škalamera and Heath, 1998) which I have written. I also performed all of the experiments described except for those used to obtain data for Figure 12, which were performed by Stefan Jibodh. Editorial decisions and decisions regarding experimental design were made in collaboration with my supervisor Michèle C. Heath.
LITERATURE REVIEW

Plant responses to rust fungal invasion most closely resemble those observed during attack by other fungal and oomycete biotrophs such as powdery and downy mildew pathogens. There are also some similarities between compatible plant-rust interactions and plant responses to infection by extensively studied mutualistic arbuscular mycorrhizal fungi. Therefore, this review will focus on comparing the plant-rust fungus interaction to these systems. The emphasis will be on changes in plant organelle distribution, deposition of callose, the HR and infection-induced gene expression. How these responses relate to others that are typically observed during plant-rust fungal interactions will be discussed in the Discussion part of this thesis. It should be kept in mind that many defensive responses are not specific to plant interactions with biotrophic fungi but represent ubiquitous plant responses to pathogen attack.

Plant organelle distribution

Changes in organelle distribution during invasion of plant cells by biotrophic pathogens is a well documented but poorly understood phenomenon (reviewed by Heath and Škalamera, 1997). During penetration of plant cells and subsequent development of intracellular hyphae or haustoria, the plant plasma membrane is invaginated and synthesized to envelop the invading structure. Although continuous with the plasma membrane this extrahaustorial (or extrahyphal) membrane (EHM) has a number of distinguishing characteristics that are particularly striking surrounding rust D-haustoria or
haustoria of downy and powdery mildew fungi (reviewed by Harder and Chong 1991). The EHM has been shown to lack cytochemically detectable ATP-ase activity (Chong et al, 1981, 1986; Coffey, 1983; Hickey and Coffey 1978; Spencer-Phillips and Gay, 1981; Woods et al, 1988; Woods and Gay, 1987), as well as differ from the plasma membrane in thickness, periodate-chromate-phosphotungstate (PACP) staining or by absence of intramembrane particles in freeze-etch replicas (reviewed by Harder and Chong, 1991; and Heath and Škalamera, 1997). Cytoplasm aggregates around intracellular fungal (and oomycete) structures and commonly contains various types of membranous structures most likely derived from the endoplasmic reticulum (ER). Accumulation of simple tubules and vesicles as well as complex lattices has been observed (Harder and Chong, 1991; Hickey and Coffey, 1977; Leckie et al, 1995; Littlefield and Heath, 1979). Depending on the fungal species or stage of growth, the type of structures may vary even in the same plant indicating that their formation is controlled by the pathogen. Although there has been a lot of speculation about the role of both EHM alterations and accumulation of endomembrane vesicles in fungal nutrition (Gay et al, 1987) and control of plant cell metabolism (Harder and Chong, 1991, Škalamera and Heath, 1997), there is little definitive evidence which can help elucidate the function of infection-induced membrane changes.

Even less is known about the significance of the infection-induced plant nuclear movements. Biotroph-induced nuclear movements have been most extensively studied during infection of cowpea by the monokaryotic stage of the cowpea rust fungus (Heath et al, 1997). In this system, the nucleus moves towards the fungus during the penetration
of the plant cell wall. This may be a non-specific plant response to damage of the cell wall since it has also been observed during various types of mechanical and chemical wounding (Aist, 1976; Gus-Mayer et al, 1998; Heath et al, 1997; Nagai, 1993). When the fungal penetration peg reaches the plant plasma membrane the nucleus moves away, a feature unique to rust infection. This happens in both resistant and susceptible cultivars as well as during aborted penetrations after a papilla has formed. Subsequently the nucleus becomes associated with the hyphal tip only in the susceptible cultivar (Heath et al, 1997). Similar associations have been observed with both M (monokaryotic)- and D(dikaryotic)-haustoria of this and other rust fungi (Bushnell, 1984; Littlefield and Heath, 1979) and develop earlier during the formation of downy mildew haustoria during both compatible and incompatible interactions (Coffey and Wilson, 1983; Freytag et al, 1994; Gross et al, 1993). The plant nucleus does not normally associate with powdery mildew haustoria (Aist and Bushnell, 1991), although there are exceptional cases in which the association does occur, such as in the incompatible interaction between cowpea and Erysiphe cichoracearum (Meyer and Heath, 1988b). The nucleus is also associated with fungal arbuscules, but not intracellular hyphae, during mutualistic interactions between plant roots and mycorrhizal fungi (Balestrini et al, 1992). The association in this system as well as during plant-rust fungal interactions is accompanied by changes in nuclear size and appearance (Balestrini et al, 1992; Bushnell, 1984), which may be, depending on the particular situation, indicative of upregulation or downregulation of gene transcription (Misteli and Spector, 1998). This suggests that one of the functions of the almost ubiquitous association between the plant nucleus and intracellular structures of biotrophs
may be to allow the fungus to exert control over plant gene expression. However, experimental evidence is needed to evaluate this and other proposed explanations for this phenomenon (reviewed by Heath and Škalamera, 1997).

The role of the plant cytoskeleton in directing and executing these cytoplasmic rearrangements is discussed in chapter two of this thesis.

_Calloose deposition_

Callose containing deposits in the form of papillae, encasements and plugs are commonly formed on the outside of the plant plasma membrane in response to wounding and pathogen invasion (Aist, 1976; Heitefuss, 1997). During plant-pathogen interactions they are believed to form barriers against chemical exchange and pathogen ingress, which may not always be effective (Aist, 1976). During plant infection with powdery and downy mildews they are often seen in both compatible and incompatible interactions, exhibiting only slight differences in frequency, size, composition or timing of deposition between the two types of interactions (Aist and Bushnell, 1991; Hächler and Hohl, 1984 and references therein). Although the differences between resistant and susceptible plants in amount of callose deposited in response to rust fungi are much more pronounced (Littlefield and Heath, 1979), it is often difficult to determine their role in defence. However, there are certain plant genotypes in which papilla formation is clearly associated with expression of resistance to biotrophic fungi.

One of the most thoroughly investigated examples of this is the _ml-o_ resistance of barley to _Erysiphe graminis_. This resistance is governed by recessive, naturally occurring
or induced mutations in a gene coding for a membrane-anchored protein unique to plants (Büsschges et al, 1997). Plants carrying the mlo allele exhibit durable resistance to all races of *E. graminis*, a resistance which is characterised by reduced pathogen penetration efficiency accompanied by rapid formation of oversize, callose-containing papillae in the plant (Jørgensen, 1992). Papillae formation was found to be dependent on the presence of calcium ions (Aist and Gold 1987), and treatments that reduced papilla formation increased penetration efficiency of the fungus (Stolzenburg et al, 1984 a,b). However, other mechanisms of resistance cannot be excluded in this system since mlo plants spontaneously form papillae and lesions mimicking the HR response in the absence of pathogens (Wolter et al, 1993). This "lesion mimic" phenotype has been found in a number of plant species, and is often accompanied by increased resistance to some pathogens that may be due to the expression of a number of defence-related responses (Dietrich et al, 1994 and references therein).

Race-specific resistance to the cowpea rust fungus in the cowpea cultivar QA is controlled by a single gene (Heath, 1993) and is manifested by deposition of callose around the intracellular fungal structures (Heath, 1971,1989; Heath and Heath 1989). Mechanisms of callose deposition in this interaction will be discussed in chapter one.

**HR**

The term "hypersensitivity " was first used by Stakman in 1915 to "indicate the abnormally rapid cell death of the host plant cells when attacked by rust hyphae". Since then, the HR has been recognized as a ubiquitous plant resistance response to pathogens
and has been extensively investigated particularly in resistance gene-dependent or parasite specific resistance. This is illustrated by the multitude of both early and recent reviews of the subject (e.g. Aist and Bushnell, 1991; Bushnell, 1982; Dangl, 1996; Doke et al., 1987; Goodman and Novacky 1994; Greenberg, 1997; Hammond-Kosack and Jones, 1996; He, 1996; Heath 1998b; Heath and Škalamera, 1997; Moerschbacher and Reisener, 1997). The main focus of more recent reviews is on two questions: 1) Is the HR a form of programmed cell death and 2) what is the role of the HR in defence? The idea that the HR is a form of programmed cell death is based on the observations that it is a genetically controlled process not only at the level of induction but also perhaps at the level of execution. It has long been recognized that triggering of the HR is dependent on the interaction between products of what are now called avirulence (Avr) genes from the pathogen and resistance (R) genes from the host plant (Flor, 1971), as has recently been confirmed by cloning of some of these genes (Bent, 1996). There are two lines of evidence suggesting that HR may be a programmed response. Firstly, there are additional genes that are not involved in determining specificity but are required for expression of this type of resistance (Bent, 1996), and secondly, the HR has been shown to require metabolic activity including gene transcription and active protein synthesis (Heath, 1998b). Some cytological similarities between HR and some other forms of programmed cell death in plants and animals have also been used as evidence for the programmed nature of this response (Heath, 1998b). The role of the HR in defence is difficult to assess because it often occurs concomitantly with a number of other defence responses that could all contribute to pathogen arrest (Hammond-Kosack and Jones 1996; Heath and
Nevertheless, the HR is likely to be a crucial component of plant resistance against obligate fungal biotrophs since these pathogens require living plant cells to survive.

Cytological changes accompanying the HR have been investigated in some detail during plant infection by biotrophic fungi and oomycetes. *In vivo* observations of infected cells by computer-enhanced video-microscopy have revealed some common features in the sequence of events during cell death caused by infection by incompatible downy mildew (Freytag *et al.*, 1994), powdery mildew (Aist and Bushnell 1991) and rust (Heath *et al.*, 1997) fungi. In all of these situations, pathogen penetration causes changes in cytoplasmic streaming which eventually stops. Subsequently, the cytoplasm becomes granular in appearance, the plant protoplast collapses and eventually accumulates autofluorescent and brown phenolic compounds (Aist and Bushnell, 1991, Freytag *et al.*, 1994; Heath *et al.*, 1997), which are often used as a marker for hypersensitive cell death (Goodman and Novacky, 1994). However, there are also considerable differences between these three plant-pathogen systems as well as between different genotypes within a system. One of the most obvious differences is the timing of cell collapse with respect to pathogen penetration which can take as little as a few minutes to a few hours. Differences are also observed in the sequence of other cytologically detectable responses such as changes in membrane permeability, pathogen deterioration, and occurrence of reactive oxygen species and cell wall-specific autofluorescence (discussed by Heath, 1998b). It is not clear whether these differences are due to different cell death pathways possibly involved in each situation, or differences in plant responses independent of the
Another common feature of the hypersensitive cell death during plant infections by rust, powdery mildew and downy mildew fungi is the dependence of this process on the microfilament component of the plant cytoskeleton, as will be discussed in chapter two.

Changes in gene expression

The overwhelming majority of studies concerning changes in gene expression during infection of plants by biotrophic fungi and oomycetes have concentrated on defence-related gene induction in resistant plants. Existence of these changes was indicated by early studies which did focus on susceptible as well as resistant plants and explored general and mostly quantitative changes in mRNA and protein metabolism (reviewed by Bushnell, 1984; Chakravorty and Scott, 1982; Manners and Scott, 1985). Subsequently, changes in expression of specific genes were characterized by differential screens of DNA clones between inoculated and uninoculated resistant plants, or by investigating expression of genes shown to be induced during defence response in other plant-pathogen systems.

The interaction between Phytophthora infestans and potato, parsley, soybean and tobacco is one of the best characterized plant-pathogen systems in terms of plant gene expression. A growing list of genes induced by this pathogen has been studied in terms of timing of gene expression, localization of transcripts in tissue, structure of promoters and effects of altered expression in transgenic plants (Freytag et al., 1994; Hahlbrock et al,
The majority of the genes induced in this system code for pathogenesis related (PR) proteins, a large group of proteins that have both known and unknown enzymatic functions and are associated with defence in plant pathogen interactions. They can also be induced in response to other stresses, as well as in response to certain developmental cues. As in many other systems, the expression of these genes is often induced in both resistant and susceptible plants infected with *P. infestans*. Some, but not all of them are induced earlier and to a greater level during incompatible interactions. Some are localized to the infection sites and some are induced systemically, so that it is hard to come to general conclusions about the role of these proteins in infection. However, a number of PR proteins have been shown to have antifungal activity *in vitro*. In particular, osmotin-like members of the PR-5 group of proteins such as AP24 from tobacco and P23 from tomato, were inhibitory against *P. infestans* and *P. citrophthora*, respectively (Rodrigo et al, 1993; Woloshuk et al, 1991). However, another tobacco osmotin was ineffective against *P. infestans* (Woloshuk et al, 1991), indicating a high level of specificity between different PR-proteins even within a group. A defensive role of some PR proteins is confirmed by studies of the *in vivo* effects of constitutive expression in transgenic plants. Expression of osmotin in potato plants delayed development of disease after inoculation with *P. infestans* (Liu et al, 1994), while the expression of PR1-a reduced susceptibility of transgenic tobacco plants to infection by *P. parasitica* var. *nicotianae* and *Peronospora tabacina* (Alexander et al, 1993). Increased PR-1 expression had no effect on some other pathogens, while the oomycete pathogens were unaffected by high levels of expression of other PR-proteins,
including a 1,3-β-glucanase and, not surprisingly, a chitinase (Alexander et al, 1993). This suggests that different PR proteins may have different functions in specific plant-pathogen interactions.

PR-proteins are also induced during infection of plants by the powdery mildew fungus *E. graminis*. Increase in levels of transcripts coding for other enzymes associated with defence such as peroxidases, phenylalanine ammonia lyase (PAL), as well as putative novel PR-proteins like oxalate oxidase, 14-3-3 protein kinase regulator-like protein, heat shock protein HSP90 and proteins of novel function have been observed (reviewed by Giese et al, 1997). Several of these transcripts showed two peaks of induction during the early stages of infection in barley corresponding to the timing of two penetration events initiated by the primary germ tube and appressorium of the fungus in both compatible and incompatible interactions. They included chitinase, HSP90, the 14-3-3-like protein and two enzymes that may contribute to the deposition of phenolics in cell walls and papillae, peroxidases and PAL (Brandt et al, 1992; Boyd et al, 1994; Clark et al, 1993; 1994; Thordal-Christensen et al, 1992; Walter-Larsen et al, 1993). Most of the transcripts induced in this interaction showed similar patterns of expression in both compatible and incompatible interaction at least during the first 24 h. At the later stages of infection when differences in growth between virulent and avirulent pathogens can be detected, a continued increase in expression of chitinase, peroxidase, 1,3-β-glucanase and several unknown proteins was observed in the resistant plants only (Boyd et al, 1994; Clark et al 1993; Davidson et al, 1987). This suggests that the fungus is capable of suppressing expression of these genes in susceptible plants after initially inducing them.
Similar observations have been made during plant interactions with mutualistic arbuscular mycorrhizal (AM) fungi in which plant defence genes are either not induced or are induced at lower level than observed during plant-pathogen interactions (reviewed by Gianinazzi-Pearson et al., 1996). Two lines of evidence suggest that this is due in part to active suppression of defence responses by the fungus. Firstly, AM fungi do trigger defence responses in myc− mutants of normally susceptible plants which are incapable of forming mycorrhizal and rhizobial associations (Harrison and Dixon, 1993); and secondly, mycorrhizal infection has been shown to suppress chemically induced PR-gene expression in host roots (David et al., 1998; reviewed by Hirsch and Kapulnik, 1998). Induction of a number of genes that may be involved in the establishment of mycorrhizal symbiosis has also been detected. They include genes with some similarities to nodulins which are expressed during rhizobial infections and may therefore be involved in plant root symbiotic associations in general (reviewed by Hirsch and Kapulnik, 1998), genes coding for cell wall proteins and tubulin that may be involved in altering cell development in the host, and genes coding for proteins that might be involved in the altered metabolism of mycorrhizal roots such as an ATPase (reviewed by Bonfante and Perotto, 1995).

Very little is known about specific changes in gene expression that may be involved in establishment of compatibility between plants and fungal or oomycete biotrophic pathogens, although such changes have been predicted by documented changes in protein and mRNA metabolism in susceptible plants infected by rust and powdery mildew fungi. In most cases these changes include the lack of an increase and sometimes a suppression of host mRNA synthesis during very early stages of infection that
may be followed by an increase in total host mRNA production days later (reviewed by Bushnell, 1984; Chakravorty and Scott, 1982; Manners and Scott, 1985). Changes in gene expression in plants infected by rust fungi will be discussed in chapter three.
CHAPTER ONE:

Callose deposition during the interaction between cowpea (\textit{Vigna unguiculata}) and the monokaryotic stage of the cowpea rust fungus (\textit{Uromyces vignae})

INTRODUCTION

Biotrophic fungal pathogens are distinguished by their common ability to form structures inside living plant cells. The intracellular fungal structures usually penetrate only the plant cell wall and remain enveloped by the invaginated plant plasma membrane, commonly referred to as the extrahaustorial or the extrahyphal membrane (Heath and Škalamera, 1997). Plant cells can react to this invasion with a variety of responses depending on the type of the interaction between plant and fungus. Typically, these responses can be very localised, so that they involve only the invaded cell or one of its components. Therefore, available methods for their investigation are often restricted to microscopical techniques. Understanding these localised responses is important for understanding plant-pathogen interactions since it is often at the level of an invaded plant cell that the outcome of infection is determined (reviewed by Heath and Škalamera, 1997).

One common localised response of plants to cell invasion is the deposition of callose around the invading structure (Aist 1976). Callose deposition can be triggered by chemical, physical or biotic stress and is thought to involve callose synthase, an enzyme localized to the plasma membrane (Delmer et al., 1993; Kauss 1987). What is particularly interesting about callose deposition triggered by fungal invasion is the diversity in the morphology and the manner of formation of the deposits. They have been observed in the
shape of papillae (eg. Sherwood and Vance, 1976), collars localized to the point of penetration (eg. Škalamera and Heath, 1996), or complete encasements of the invading structures (eg. Heath and Heath 1971). They can be deposited before (eg. McKeen and Rimmer, 1973) or after (eg. Heath, 1971) complete penetration of the plant cell wall. In the latter case they are separated from the fungal structure by the extrahaustorial membrane and the plasma membrane pushed inward by the growing deposit (Littlefield and Heath, 1979). Despite this variability, cellular events preceding and accompanying callose deposition are remarkably similar in all described situations and usually involve aggregation of cytoplasm (Kunoh et al 1985; Zeyen and Bushnell, 1979), endomembrane vesicles (Heath and Heath, 1971; Škalamera and Heath, 1995) and sometimes the nucleus at the damage site (Aist 1976). This suggests that although callose synthesis always occurs by a single process, initiation of callose deposition may involve various cellular mechanisms depending on the type of triggering stimulus.

This conclusion is supported by the results from the investigation of callose deposition during the cowpea (Vigna unguiculata [L.] Walp.) - cowpea rust fungus (Uromyces vignae Barclay) interaction. As demonstrated in previous studies (Heath, 1971; Heath and Heath, 1971; Heath, 1989), the interaction between the race 1 of the fungus and the resistant cowpea cultivar Queen Anne (QA) is characterized by formation of callose encasements around intracellular fungal structures. This occurs during infections initiated by either urediospores to give the dikaryotic- (Heath, 1971; Heath and Heath, 1971) or by the basidiospores to give the monokaryotic-parasitic stage of the fungus (Heath, 1989). In contrast, in the susceptible cultivar California Blackeye (CB), callose
deposits have been detected only associated with the D-(Littlefield and Heath, 1979) haustoria formed during the dikaryotic stage of the infection (Škalamera and Heath, 1996). These latter deposits occur infrequently in the form of collars around the haustorial necks (Škalamera and Heath, 1996). While the incidence of callose encasements in the resistant cultivar in urediospore-derived infections was reduced by inhibitors of transcription, protein synthesis, protein glycosylation and antimicrofilament agents, the incidence of collars in the susceptible cultivar was affected only by inhibitors of transcription and protein synthesis (Škalamera and Heath, 1996). This suggested that resistance-associated callose deposition in cultivar QA may require additional cellular mechanisms that are not necessarily involved in callose synthesis in general.

In this study we further investigated mechanisms of resistance-associated callose deposition by examining this phenomenon during the monokaryotic stage of infection. Our aim was to determine how this response compares with that observed during the dikaryotic stage of infection, since invasion hyphae produced during the monokaryotic stage differ considerably from D-haustoria in the degree of structural and presumed functional specialization (reviewed by Littlefield and Heath, 1979), and yet seem to trigger similar responses from the plant cells (Heath, 1989). We also examined callose deposition that is not associated with genetically-determined resistance, but is triggered by externally caused fungal death, to determine whether these two situations could be distinguished during monokaryotic infections as they can during the dikaryotic stage (Škalamera and Heath, 1996). We report that the location of callose deposits with respect to the intracellular penetration site differs from that observed during the encasement of D-
haustoria, although similar cellular processes seemed to be required for their deposition. The cellular response to fungal death also differed between invasion hyphae and D-haustoria in that in the former, callose deposition was initiated at cellular locations that were not sites of deposition during the resistance response.
MATERIALS AND METHODS

Plants

Cowpea (Vigna unguiculata) cultivars Queen Anne (QA) and California Blackeye (CB), were grown in Pro-Mix (Premier Brands Inc, New Rochelle, NY) in growth chambers maintained at 20-24°C, with a 16 hour photoperiod at a light intensity of 250μmol m² s⁻¹ as described previously (Perumalla and Heath, 1989).

Inhibitor treatments

Unless otherwise mentioned all inhibitors were purchased from Sigma Chemical Co., St Louis, Missouri. Actinomycin D (Calbiochem, San Diego, California), blasticidin S (gift from Dr. T. Tani, Kagawa University, Japan), cycloheximide, monensin and colchicine were dissolved in double distilled water. The remaining inhibitors were dissolved in an appropriate solvent and diluted with double distilled water to the required concentration. Final solvent concentrations were (v/v): 0.01% methanol for brefeldin A, 0.005% dimethyl sulfoxide (DMSO) for tunicamycin and cytochalasin E, and 0.5% DMSO for oryzalin. Inhibitor solutions were injected into the leaves of 10 day old plants using a 3 ml syringe and a 30 gauge needle as described previously (Perumalla and Heath, 1989). Control plants were injected with double distilled water or the appropriate solvent solution. Four infected plants were observed per treatment and 25 infection sites in each plant. Student’s T-tests were performed to compare inhibitor-treated plants to their respective controls. All experiments were repeated at least twice. Because values, but not trends, differed between experiments, data shown in the results represent one typical
Fungal material and inoculation

Teliospores of the cowpea rust fungus \(Uromyces \) \textit{vignae} race 1 (CPR-1) were produced on primary leaves of a susceptible cultivar and stored on dried leaves at room temperature. Sterilized teliospores (Heath, 1989) were inoculated on 2\% water agar in Petri dishes and incubated in a growth chamber at 22°C. At about 48 hr after inoculation (when the first basidiospores had been produced), agar blocks were cut from the plates and placed on the surface of primary leaves of the 10 day old plants. For the plants treated with inhibitors, the blocks were placed over the injected areas after the watersoaking of the tissue had disappeared, usually 30 min after injection. Plants were sprayed with water, and incubated in a dark moist chamber at 22°C until harvesting.

Heat-shock treatments

Inoculated leaves were heat-shocked by submerging in double distilled water at 50°C for 30 s.

Electron microscopy

Tissue pieces were prepared for electron microscopy using glutaraldehyde-osmium tetroxide fixation as described previously (Perumalla & Heath, 1991) and examined using a Phillips 201C transmission electron microscope operated at 60kV. The periodic acid-thiocarbohydrazide-silver proteinate (PA-TCH-SP) technique (Thiery, 1967) was used to
detect polysaccharides according to a previously reported method (Heath, 1988b).

**Light microscopy**

Tissue pieces were fixed and decolorized in boiling 95% ethanol. Tissue was subsequently stained in aniline blue (Sigma) (0.005% w/v in 0.07M K$_2$HPO$_4$) as described previously (Tighe & Heath, 1982). Tissue pieces were mounted in the aniline blue solution and observed under differential interference contrast (DIC) optics and epifluorescence irradiation using a Reichert-Jung Polyvar microscope. Callose was observed using UV irradiation filter cube U1 (excitation filter BP 330-380, barrier filter LP 418 and dichroic mirror DS 420).

In heat-shocked tissue, three plants were observed per cultivar, and two leaf pieces per plant. At least 20 infection sites were observed per leaf piece. All experiments were repeated at least twice.
RESULTS

Infection in the susceptible cultivar, CB and the resistant cultivar, QA

Development of infection structures from basidiospores in both the resistant and the susceptible cultivar corresponded to the reported observations from a previous light microscope study (Heath, 1989). Briefly, after the differentiation of germ tubes and appressoria, the epidermal cells were penetrated directly and intracellular invasion hyphae were formed (as shown for the cultivar CB in Figs. 1, 2, and 4). After the translocation of the fungal cytoplasm from the appressorium into the hypha, a plug of electron lucent material formed on the inside of the fungal wall near the penetration site (Figs. 1, 2, and 3). Unlike the fungal wall which was heavily stained, the plug material was only lightly stained after the PA-TCH-SP treatment involving incubation in TCH for 24 h (Fig. 3). The fact that such staining was similar after a 3, 18 or 24 h TCH incubation, but was absent if the periodic acid treatment was omitted, indicates that the plug contains low levels of simple polysaccharides (Thiéry, 1967) in addition to the previously reported β-1,3-glucans (Xu and Mendgen, 1994), which do not react with PA-TCH-SP. The hyphae grew through the plant cells by tip growth, and had reached or grown close to (Fig. 4) the inside of the plant cell wall in some sites at the time of harvesting. All intracellular fungal structures were surrounded by the invaginated plant plasma membrane which will be referred to as the extrahyphal membrane. The ultrastructure of the fungal cytoplasm and organelles was similar to that described for the bean rust fungus (Uromyces appendiculatus) in a compatible bean cultivar (Gold & Mendgen 1984). Therefore we will
not discuss it in detail in this paper. The examined fungal structures also did not differ from those observed for the cowpea rust fungus in the non-host bean plant (Xu and Mendgen, 1994).

In the susceptible cultivar CB, there were no visible signs of defence responses in the 10 sites examined at the early stages of infection (17-23 hr after inoculation). A possible exception was a small collar of material observed in some sites surrounding the region of the fungal plug near the penetration site. The material had same appearance as the plant cell wall except for the absence of the more fibrillar component of the wall. The collar stained almost as heavily as the plant cell wall after the PA-TCH-SP treatment (Fig. 3), indicating that polysaccharides with linkages other than β-1,3 glucan linkages were present in both structures. The cytoplasm surrounding the collar contained "vesicles" with PA-TCH-SP-staining contents and membranes that stained similarly to the plasma membrane and the extrahyphal membrane (Fig. 3); these "vesicles" were shown by serial sectioning to be evaginations of the latter membrane (similar to those shown in Fig. 28). In one site where the invasion hypha had grown close to the inside of the plant cell wall, no additional layers were observed on the plant cell wall close to the hypha (Fig. 4).

Infection in the resistant cultivar QA was not completely synchronous so that at the observed time (21 hr after inoculation), hyphae ranged in size and stage of development. The earliest stage observed was one site where the fungus just penetrated the plant cell wall, and the fungal cytoplasm had not completed its migration from the appressorium on the leaf surface (Fig. 5). In this site, no drastic changes in the plant cytoplasm and no new wall material could be detected at the penetration site (Fig. 5). In the remaining four sites
examined the intracellular fungal cytoplasm was separated from the basidiospore by an electron lucent plug of fungal material deposited inside the fungal wall (Figs. 6 and 7). In all four sites, a collar of plant cell wall-like material could be seen surrounding the plug region of the fungus (Figs. 6 and 7). Like the collars in the susceptible cultivar, those in cultivar QA were continuous with the plant cell wall (Fig. 6), but lacked its more obvious fibrils (Fig. 6). It appeared that the collar material was deposited directly against the fungal cell wall since no membrane profiles could be detected between the two structures (Fig. 7). A small plant cytoplasmic aggregate containing endomembrane vesicles surrounded the collar and in all four sites, the collar terminated at the point of junction between the plug and the intracellular hypha. In the region just distal to the plug, the invasion hypha contained a large vacuole (Fig.8) and the extrahyphal membrane was in close contact with the hyphal wall. No additional layers of material could be detected between the two structures. The surrounding plant cytoplasm was very thin and contained no organelles other than ribosomes (Fig. 8). In two of the observed sites, the intracellular hyphae were septate and had reached the inside of the plant cell wall (Fig. 9). In these sites hyphae were partially encased in a layer of material continuous with the plant cell wall that seemed to develop from the point where the extrahyphal membrane was in contact with the plant cell wall-associated plasma membrane (Fig. 9). In all regions the encasement was separated from the fungal wall by the two layers of the plant plasma membrane, the extrahyphal membrane and the plasma membrane surrounding the encasement (Fig. 10). The plant cytoplasm aggregated around the forming encasement and contained the nucleus and numerous endomembrane vesicles and dictyosomes (Fig. 11). The vesicles
varied in size and appearance so that some were smooth and some were associated with ribosomes (Fig 11).

In tissue stained with aniline blue and examined under the UV irradiation, the encasements were fluorescent (indicating presence of callose) while the collar and plug region was not.

*Effect of the inhibitors on fungus-triggered callose deposition in QA*

To determine whether the deposition of callose during the basidiospore-derived infection involved similar cellular processes as did callose encasement of haustoria during the urediospore-derived infection (Škalamera and Heath, 1996), QA plants were treated with specific chemical inhibitors. The concentrations of inhibitors used were at least equal to those that were shown to affect plant cells in previously reported studies (Heath, 1979; Perumalla and Heath, 1991; Škalamera and Heath, 1996). Colchicine and oryzalin were shown by immunocytochemistry to disrupt plant microtubules, while fluorescein-phalloidin staining demonstrated the lack of a microfilament network in cytochalasin E treated plants. At concentrations used in the current study, no adverse effects of the inhibitors on the fungus could be detected with the light microscope.

The incidence of callose deposits in response to hyphal presence in the plant cell was not affected by inhibitors of Golgi-associated vesicle transfer (monensin, brefeldin A), or microtubule polymerization (colchicine, oryzalin) (Fig. 12). In contrast, callose deposition was significantly reduced in the plants treated with inhibitors of transcription (actinomycin D), protein synthesis (cycloheximide), protein glycosylation in the ER
(tunicamycin) and an anti-microfilament agent (cytochalasin E) (Fig. 12).

Callose deposition triggered by heat-killed fungus

To determine whether initiation of callose deposition near the tip of the invasion hypha and not at the penetration site, was due to active suppression by the fungus during the initial penetration, we investigated the location of callose deposits in QA and CB plants in which fungus had been killed by heat-shock treatment, 18 h after inoculation. Heat treating infected leaves for 30 s at 50°C kills the fungus, which results in callose encasement of intracellular fungal structures in both resistant and susceptible cultivars (Heath, 1984). In tissue harvested 12 h after treatment, callose deposits detected by aniline blue fluorescence under UV irradiation appeared at a variety of cellular locations associated with the invasion hypha. Both completely and partially encased hyphae were observed, and among partial encasements, the most commonly observed type was encasement of the hyphal tip only (Fig. 13). Collars around the penetration site and deposits at other sites around the hypha were also seen (Figs. 14 and 15). Some sites contained both a callose collar and tip encasement (Fig. 16). The proportion of infection sites that contained a particular type of deposit did not differ between the susceptible and the resistant cultivar (Table 1).

In some infection sites, callose appeared to be deposited between the extrahyphal membrane and the hyphal wall (Fig. 15). To confirm this observation, plants were heat-shocked 15 h after inoculation and harvested for electron microscopy 12 h after heat shock. Tissue was heat-shocked earlier than in the previous experiments to maximize the
number of sites at which the invasion hyphae had not reached the inside of the plant cell wall. Six infection sites were observed in the resistant cultivar QA and five in the susceptible cultivar CB.

In all observed sites, fungal cytoplasm appeared disorganized and contained variously shaped electron opaque-granules (Figs. 17, and 21) which were absent in untreated hyphae. The organelles were difficult to distinguish (Figs. 17, 21 and 25) and often lacked membranes indicating that the fungus was damaged by the heat shock treatment. In the resistant cultivar, sections through the penetration site were obtained in four of the observed infection sites. In all four sites, electron-lucent material continuous with the plant cell wall was observed between the extrahyphal membrane and the hyphal wall (Figs. 17, 18, and 19). In two sites, this layer was similar to the collar observed in the untreated tissue in that it resembled the plant cell wall except for lacking fibrillar material (Figs. 17 and 18). In one of these sites the collar terminated in the region of the fungal plug (Fig. 18). In the other it was continuous with a more electron lucent, callose-like deposit that encased the hypha completely (Fig. 17). In the remaining two sites the layer surrounding the penetration site was continuous with and indistinguishable from material that encased the hypha (Fig. 19). The material was mostly electron-lucent and contained various amounts of electron opaque-granules as is typical of callose (Figs. 20 and 21). The thickness of encasements varied between the sites and between different regions within a site. In three of the observed infection sites the encasements were thin and incomplete (Figs. 20 and 22), so that it was possible to determine that the plant plasma membrane surrounding the encasement was continuous with the extrahyphal
membrane and that there were no membrane profiles between the hyphal wall and the
encasement. Therefore, the callose appeared to have been directly deposited in the
extrahyphal matrix. In the remaining sites, the encasements were complete. Both partial
and complete encasements tended to thicken towards the hyphal tip (Fig. 21). In this
region, trapped plant cytoplasm (Fig. 21), electron opaque granules (Figs. 21 and 26) and
membrane profiles (Figs. 23 and 24) could be detected trapped in the encasement near
the fungal wall. These profiles were sometimes quite long and closely appressed to the
fungal wall (Fig. 24), and were not seen to be connected to the extrahyphal membrane.
Serial sectioning confirmed that all hyphae terminated before they reached the inside of
the plant cell wall, so that the encasements surrounding the hyphal tip were not in contact
with the latter. The plant cytoplasm surrounding the encasements contained the plant
nucleus only in two sites where the encasements were not complete. Endomembrane
vesicles, endoplasmic reticulum and dictyosomes were observed in some regions
surrounding both complete and partial encasements in three of the six examined sites
(Figs. 25 and 26). However, in most sections the layer of cytoplasm surrounding the
encased hyphae was thin and contained ribosomes only (Figs. 20 and 22). Invaginations
of the extrahyphal membrane, containing mostly electron-lucent and sometimes also some
electron-opaque material were often seen in regions where an encasement had not formed
(Fig. 25 and also seen in the susceptible cultivar Fig. 27).

The ultrastructure of the infection sites observed in the susceptible cultivar was
similar to that described for the resistant cultivar. The only differences were observed in
the plant cytoplasm. In three of the five examined sites the plant cytoplasm was very
electron lucent and contained few if any ribosomes (Figs. 27 and 28), possibly as a response to the heat-shock treatment, since it has been shown to reversibly inhibit protein synthesis (Nagao et al., 1986). In all three sites, encasement material was seen between fungal wall and the extrahyphal membrane, and two sites had plant wall-like collars. In the site without a collar, elaborate membrane profiles were seen in the plant cytoplasm surrounding the fungal plug (Fig. 28). The membranes generally did not bear ribosomes and some were connected to the extrahyphal membrane. Although smooth membrane vesicles and whorls were observed in the cytoplasm of infected cells in the resistant cultivar, they were not as elaborate as in this site in the susceptible cultivar.
Figures 1-4. Electron micrographs of infection structures of *U. vignae* in the susceptible cultivar CB. Fig. 1. Longitudinal section of an invasion hypha (h). An electron lucent plug (p) has formed inside the fungal wall separating the hypha from the penetration hole (arrow) in the plant cell wall (W). Fig. 2. Fungal plug (p) is separating the invasion hypha (h) from the appressorium (a) outside the plant cell wall (W). A collar (arrow) of plant cell wall-like material has formed near the penetration site. Fig. 3. PA-TCH-SP treated section of the plug (p) region; TCH was applied for 24 h. The plant cell wall and the collar (arrow) are stained, and so are evaginations of the extrahyphal membrane, seen as vesicles (arrowhead). Dictyosomes (D) are only lightly stained. W-plant cell wall. Fig. 4. Invasion hypha (h) that has grown close to the inside of the plant cell wall (W) without causing any detectable response. (N-plant nucleus; n-fungal nucleus; V-plant vacuole; v-fungal vacuole). Figs. 1-3 bars = 1μm. Fig. 4 bar = 10μm.
Figures 5-8. Electron micrographs of infection structures of \( U. \) \( vignae \) in the resistant cowpea cultivar Queen Anne. Fig. 5. Invasion hypha (h) before complete translocation of cytoplasm from the appressorium. There is no collar material deposited at the penetration site (arrow). V-plant vacuole; W-plant cell wall; m-fungal mitochondrion. Fig. 6. Penetration hole (Ph) in the plant cell wall (W) after complete translocation of cytoplasm into the intracellular hypha. A collar (C) of plant cell wall-like material has formed around the region of the fungal plug (p) that separates the intracellular invasion hypha (h) from the penetration pore. Arrows-fungal cell wall. Fig. 7. Region of the fungal plug (p) surrounded by the collar (C). The collar does not extend around the invasion hypha (h) and is surrounded by the plant plasma membrane (large arrow). No membrane profiles can be detected between the fungal cell wall (small arrow) and the collar. The surrounding plant cytoplasm contains numerous endomembrane vesicles (Ve). Fig. 8. Invasion hypha sectioned between the fungal plug and the first septum. A very thin layer of plant cytoplasm (arrow) surrounds the hypha. V-plant vacuole; v-fungal vacuole; n-fungal nucleus. All bars = 1\( \mu \)m.
**Figures 9-11.** A plant cell in QA containing a partially encased invasion hyphae (h). Fig. 9. The callose encasement (E) is continuous with the plant cell wall (W) and covers the adjacent middle part of the hypha. N-plant nucleus; n-fungal nucleus; V-plant vacuole; v-fungal vacuole. Bar = 10μm. Fig. 10. Edge of encasement (E) showing that it is separated from the fungus by the extrahyphal membrane (large arrow) and the plant plasma membrane (small arrow). h-hypha. Fig. 11. Plant cytoplasm surrounding the hypha (top) and the encasement (E). Numerous endomembrane vesicles (Ve) and dictyosomes (D) are present. Figs. 10 and 11 bars = 1μm.
Figure 12. Effect of inhibitors on incidence of hyphae with callose encasements in the resistant cultivar QA, 24 hr after inoculation. Inhibitors marked "*" represent results of treatments significantly different from the control at P = 0.05. Inhibitors were injected 30 min-1 hr prior to inoculation. Control plants were injected with distilled water or the appropriate solvent solution. Error bars represent standard deviation. Inhibitor concentrations (μg ml⁻¹): actinomycin (10), cycloheximide (5), tunicamycin (1), monensin (0.7), brefeldin A (1), colchicine (40), oryzalin (60), cytochalasin E (0.5).
**Figures 13-16.** Location of callose deposits in heat-shocked, aniline blue-stained tissue. All shown types of deposits were observed in both the resistant (Figs. 13-14) and the susceptible (Figs. 15-16) cultivar. Deposits were identified by fluorescence under UV irradiation (a) and fungal structures were observed using DIC optics (b,c). Fig. 13. Most frequently observed type of deposit in heat-shocked tissue and the only type observed in the untreated resistant cultivar QA. Encasement is formed around the hyphal tip (13a, 13b-arrow). The fungal plug (p) at the penetration site can be visualised in another optical section (13c). h-hypha; v-fungal vacuole; W-plant cell wall. Fig. 14. Location of callose deposit around the point of penetration (14a, 14b,c-arrows, different optical sections). h-hypha; N-plant nucleus; p-fungal plug. Fig. 15. Callose deposit not in association with the plant cell wall. A small deposit (arrow) is seen in the extrahyphal matrix. h-hypha; p-fungal plug; W-plant cell wall. Fig. 16. Callose deposits formed at both the penetration site and fungal tip. h-hypha; p-fungal plug; v fungal vacuole; W-plant cell wall. All bars = 10 μm.
Figures 17-24. Ultrastructure of infection sites in the heat-shocked resistant cultivar QA. Fig 17. The fungal plug (p) region where a collar (C) of wall-like material has formed and is continuous with the plant cell wall (W) and the more electron-lucent parts of encasement (large arrow). Electron opaque granules (small arrow) are seen in the fungal cytoplasm. Fig. 18. A wall-like collar (C) that ends in the region of the fungal plug (p). Fig. 19. A collar (C) of electron lucent, callose-like material continuous with plant cell wall (W). Fig 20. Electron lucent, callose-like encasement (E) around the invasion hypha (h). There are no detectable membrane layers between the encasement and the hyphal wall (arrow). Fig. 21. Thicker encasement (E) near the fungal tip that is separated from the fungus in places (large arrow) by a thin layer of plant cytoplasm. Electron opaque granules (small arrow) are trapped in the encasement. The fungal cytoplasm is disorganized. h-hypha. Fig 22. Thin region of an encasement (E), showing continuity of the extrahyphal membrane (arrow) surrounding the encasements. h-hypha. Figs 23. and 24. Membrane profiles (arrows) trapped in some wider encasements. E-encasement; h-hypha; W-plant cell wall. All bars = 0.5 μm.
Figures 25-28. Ultrastructure of infection sites in heat-shocked resistant (25,26) and susceptible (27,28) cultivars. Fig 25. Large electron lucent vesicles (Ve) seen near a partially encased hypha (h). Vesicles were sometimes fused (arrow) with the extrahyphal membrane. Fig. 26. Cytoplasm surrounding an encasement (E) contains numerous profiles of endoplasmic reticulum (small arrow) and dictyosomes (D). Electron-opaque granules are trapped in the encasement (large arrow). Fig. 27. Invaginations of the extrahyphal membrane (arrow), containing electron lucent material that were seen in both cultivars in regions where there was very little cytoplasmic activity near the hypha (h). M-plant mitochondrion. Fig. 28. Large smooth membrane vesicles continuous (arrow) with the extrahyphal membrane in the fungal plug (p) region in a site where a collar was not formed. The plant cytoplasm is more electron lucent and contains fewer ribosomes than in other sites. All bars = 0.5 μm.
Table 1. Distribution of callose deposits in infected, heat-shocked resistant (QA) or susceptible (CB) cultivars.

<table>
<thead>
<tr>
<th>location of callose deposit with respect to the hypha†</th>
<th>average proportion (%) of infection sites per leaf piece (± standard deviation)*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>QA</td>
</tr>
<tr>
<td>tip</td>
<td>50 ± 15</td>
</tr>
<tr>
<td>complete</td>
<td>38 ± 17</td>
</tr>
<tr>
<td>penetration site</td>
<td>7 ± 6</td>
</tr>
<tr>
<td>tip and penetration site</td>
<td>6 ± 5</td>
</tr>
<tr>
<td>other</td>
<td>4 ± 4</td>
</tr>
</tbody>
</table>

† all locations include deposits outside and inside the extrahyphal matrix, which could not always be distinguished at the light microscope level.

* based on average of 6 leaf pieces and at least 20 infection sites observed per leaf piece
Ultrastructural observations of the cells of the resistant cowpea cultivar QA infected with the basidiospores of the cowpea rust fungus have revealed two types of plant cell responses that may be associated with defence: 1) the formation of a collar of wall-like material around the fungal penetration site and 2) the deposition of callose encasements around the intracellular structures. In this study we identified callose using UV-light induced fluorescence in aniline blue treated tissue at the light microscope level, or as the electron-lucent material that does not stain with PA-TCH-SP in electron micrographs. Immunocytochemical studies have confirmed the presence of β-1,3-glucans in similar deposits that have previously been identified as callose in different plant tissues by aniline blue staining and ultrastructural morphology (Hussey et al., 1992; Meikle et al., 1991; Northcote et al., 1989). The plant cytoplasm surrounding both collars and callose encasements contained numerous endomembrane profiles, particularly the endoplasmic reticulum, supporting the previous suggestion that these organelles may be involved in their secretion (e.g. Škalamera and Heath, 1995). In contrast, the cytoplasm surrounding the intracellular hypha in regions away from the either type of deposit was remarkably devoid of organelles. The extrahyphal matrix in this region was also barely perceptible.

The lack of staining with aniline blue indicates that the collars do not contain callose. Similar, but smaller, collars were also observed in the susceptible cultivar CB, which stained for polysaccharides with linkages other than β-1,3 glucan linkages as indicated by PA-TCH-SP staining. Deposition of this wall-like, collar material may be a
common response of epidermal cells to rust fungal invasion hyphae, since it was also observed in cowpea rust-infected, non-host broad bean plants (Xu and Mendgen, 1994), and in the interaction between the bean rust fungus and French bean (Gold and Mendgen, 1984). These collars in our study did not extend beyond the region of fungal plug separating the appressorium from the intracellular invasion hyphae. A similar plug is also present in the primary hypha of the bean rust fungus (Gold and Mendgen, 1984) and has been shown to contain 1,3-β-glucans in the cowpea rust fungus (Xu and Mendgen, 1994). The slight staining of the plug after PA-TCH-SP treatment suggests that additional linkages may also be present.

Although complete callose encasements were previously observed in the cultivar QA infected with the monokaryon of the cowpea rust fungus (Heath, 1989), the fact that they develop from the cell wall next to the growing invasion hypha and not from the penetration site as they do around D-haustoria (Heath, 1971; Heath and Heath, 1971; appendix 1), is a novel finding. However, like the callose encasements that develop around D-haustoria in this cultivar (Škalamera and Heath, 1996), the incidence of those around invasion hyphae was reduced by inhibitors of transcription, protein synthesis and glycosylation and antimicrofilament agents. Therefore, the same processes seem to be involved in callose deposition and induction regardless of the parasitic stage of the fungus used to infect the plants or its original site of deposition. In a previous study (Škalamera and Heath 1996), small callose collars occasionally induced by the dikaryon in the susceptible cultivar CB, or complete encasements formed when D-haustoria were killed by polyoxin D, were not affected by inhibitors of glycosylation and antimicrofilament
agents. Similarly, these inhibitors did not reduce callose deposition induced by chemical wounding of uninfected plants, and antimicrofilament agents did not affect callose deposition induced by the cowpea rust fungus in non-host bean plants (Perumalla and Heath, 1991). These observations suggested that protein glycosylation and actin microfilaments may be involved specifically in the signalling between the plant and the fungus that results in callose deposition associated with resistance of cultivar QA (Škalamera and Heath, 1996). The results of the current study suggest that the same signalling occurs between the plant epidermal cell and the basidiospore-derived invasion hypha as occurs between a mesophyll cell and the D-haustorium.

The general lack of callose deposition in rust-infected tissue has been suggested to be the result of active suppression by the living fungus (Heath, 1984, 1988a). In support of this hypothesis, callose deposition occurred both in the susceptible cultivar, and around the penetration region in QA, when the invasion hypha was killed by heat shock. An interesting question is why the invasion hypha, unlike the D-haustorium, can prevent callose deposition around the penetration site in both cultivars while it is unable to prevent that triggered in QA by the close proximity of the hypha to the perimeter of the plant cell. Although this latter type of callose deposition was not seen in the susceptible cultivar in the present study, it has been reported to be occasionally induced in susceptible plants by invasion hyphae of other rust fungi (Gray et al, 1983; Kohno et al, 1977).

In untreated QA tissue, internally-triggered encasements around invasion hyphae, like penetration-triggered encasements around D-haustoria (Heath and Heath 1971), were
not deposited between the extrahyphal (or extrahaustorial) membrane and the fungal wall but were continuous with the plant wall and lined with extensions of the plant plasma membrane. It is possible, therefore, that the extrahyphal and the extrahaustorial membranes are not capable of generating callose, reflecting their differentiation from the normal plant plasma membrane (Heath and Škalamera, 1997). This hypothesis may be true for the extrahaustorial membrane surrounding D-haustoria since killing the haustorium in susceptible tissue almost invariably results in an encasement separate from the extrahaustorial matrix (Heath 1988a). However, it seems incorrect for invasion hyphae since, in the present study, their death also resulted in callose deposition in the extrahyphal matrix. The fact that callose also has been observed in the matrix region around monokaryotic (M-) haustoria (Hopkin and Reid, 1988; Littlefield and Heath, 1979; Stark-Urnau and Mendgen, 1995) and intracellular hyphae (Al-Khesraji and Lösel, 1981) during several compatible interactions between plants and rust fungi supports other evidence (Heath and Škalamera, 1997) that the plant membrane surrounding the fungus in the monokaryotic stage is not as highly modified as that surrounding D-haustoria. In the case of M-haustoria callose deposition is usually seen only around older haustoria (Littlefield and Heath 1979; Stark-Urnau and Mendgen 1995) that may be unable to suppress this process.

A puzzling observation from our ultrastructural study was that thick callose deposits near the tips of heat-killed invasion hyphae contained trapped cytoplasm and membranes as typically seen for callose encasements that grow up from the plant wall (rather than being formed within the extrahyphal matrix) despite the fact that these
encasements were not in contact with the plant wall. The simplest explanation is that these parts of the encasement were formed by the formation of large, membrane-bound, sheets of callose in the plant cytoplasm that eventually fused, in places, with the extrahyphal membrane.

Resistance to race 1 of the cowpea rust fungus in the cultivar QA is thought to be determined by a single dominant gene (Heath, 1994). It differs from the more commonly observed types of resistance in that the rapid death of invaded plant cells (i.e. the hypersensitive response) does not occur in the majority of infection sites (Heath, 1971; Heath, 1989). Our results suggest that the type of callose deposition observed in this cultivar is dependent on and specific to the expression of that resistance regardless of the parasitic stage of infection. However, its location is specific to the type of fungal structure that triggers it (i.e. the invasion hypha or the D-haustorium), and may differ from that triggered by fungal death.
CHAPTER TWO

Changes in the cytoskeleton accompanying infection-induced nuclear movements and the hypersensitive response in plant cells invaded by rust fungi.

INTRODUCTION

The cowpea rust fungus (*Uromyces vignae* Barclay, race 1) is an obligate biotrophic pathogen capable of forming infection structures inside living host-plant cells. A well documented but poorly understood feature of the intracellular invasion of plants by biotrophic fungi is the positioning of plant cell components in a precise location with respect to the fungus (reviewed by Heath and Škalamera, 1997). Most commonly observed responses are the creation of cytoplasmic aggregates surrounding the fungus, nuclear movements, and rearrangements of endomembranes, particularly the endoplasmic reticulum. The plant organelle positions may vary in different plant-pathogen systems, as well as between compatible and incompatible interactions within a system. However, certain organelle arrangements are often typical for a particular interaction, implying that the plant cells are capable of sensing the location of the incoming pathogen and moving their organelles accordingly (reviewed by Heath and Škalamera, 1997).

During the invasion of cowpea (*Vigna unguiculata* (L.) Walp) epidermal cells by the monokaryotic stage of the cowpea rust fungus, the plant nucleus moves to, and then migrates away from, the penetration site as the fungus enters the cell. The nucleus
subsequently moves towards and remains close to the tip of the growing invasion hypha in the susceptible cultivar, while it remains away in resistant cultivars in which the invaded cells subsequently die during the so called hypersensitive response (HR) (Heath et al., 1997). The lack of nuclear movement towards the hyphal tip is one of the first detectable signs of incompatibility in cells of the resistant cultivars and often precedes the cessation of cytoplasmic streaming, granulation of the plant cytoplasm and protoplast collapse. Although nuclear positioning with respect to the pathogen may differ in other systems, a sequence of distinct cellular events is also observed in a number of incompatible interactions between plants and biotrophic fungi in which infection results in a resistance-dependent plant cell death (reviewed by Heath and Škalamera, 1997). Although this type of cell death is a ubiquitous plant defense response that has attracted a lot of scientific attention because of some similarity to animal programmed cell death (apoptosis) (Heath, 1998b), a number of questions still remain unanswered. A particularly important question is whether there is a common sequence of cellular events that is uniquely characteristic of the HR.

Cytoplasmic changes and organelle movements in response to various environmental or developmental stimuli are dependent on, and sometimes controlled by the cytoskeleton (reviewed by Williamson, 1993). The common changes in organelle positioning during the resistance response against biotrophic fungi indicate a need for investigating how the plant cytoskeleton relates to the cytoplasmic changes observed prior to the HR. Although the changes in distribution of plant microtubules and microfilaments during fungal infection have been reported for a number of systems
(Baluška et al., 1995; Gross et al., 1993; Kobayashi et al., 1992, 1994), there has only been one such study directly related to the HR. That study (Kobayashi et al., 1994) investigated infection of flax by the dikaryotic stage of the flax rust fungus which invades mesophyll cells. In contrast, the monokaryotic stage of the cowpea rust fungus penetrates epidermal cells directly, allowing in vivo observation and detailed documentation of the relationship between resistance, organelle movements and cell death (Heath, 1997).

In this paper we use immunocytochemistry and anticytoskeletal agents on this monokaryotic rust system to determine the relationship between the cytoskeleton and cellular events preceding and during either the establishment of a successful biotrophic relationship, or the initiation of the HR. We use one susceptible and two different resistant cultivars to differentiate between changes that are specific to an interaction from those that may be a general feature of the HR.
**MATERIALS AND METHODS**

*Plants*

Cowpea (*Vigna unguiculata*) cultivars California Blackeye, Calico Crowder and Dixie Cream were grown and inoculated when 9 days old by placing basidiospores of the cowpea rust fungus (*Uromyces vignae*, race 1) on the lower leaf surface as described previously (Škalamera and Heath, 1997).

*Drug treatments*

Stock solutions of taxol (gift from F. Dicosmo, University of Toronto), oryzalin (gift from R. L. Peterson, University of Guelph) and cytochalasin E (Sigma Chemical Co., St. Louis, MO, USA) were made in DMSO (10 mg ml⁻¹) and diluted to the desired concentration with double distilled water. The drug or control (DMSO at the appropriate concentration) solutions were injected into the intercellular spaces of leaves of 9 day old plants prior to inoculation as described previously (Škalamera and Heath, 1997).

*Staining for microtubules*

Plants were injected (as above) with primary fixative (2.8% paraformaldehyde, 0.3% glutaraldehyde in microtubule stabilizing buffer (MSB), pH 7.2, containing 50 mM 1,4-piperazine-diethansulfonic acid (PIPS), 5 mM EGTA, and 1 mM MgSO₄). Injected areas were immediately excised from the plants and submerged into
secondary fixative (same as the primary but containing 3.7% paraformaldehyde) for 60 min on ice. Strips of lower leaf epidermis were peeled off using watchmakers forceps and floated cuticle side up on a drop of fixative on a coverslip at room temperature. All subsequent steps were performed by exchanging solutions in the drop of liquid supporting the epidermal strips. After several washes in MSB, epidermal cells were permealized by incubation in cell wall degrading enzymes (0.02% cellulyisin and 0.006% pectinase in MSB, for 20 min) and (after more MSB washes) in detergent (0.01% Triton X-100 in MSB, for 15 min). The strips were then washed in several changes of phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 7mM Na$_2$HPO$_4$, 1.5 mM KH$_2$PO$_4$, and 0.01% NaN$_3$, pH 7.3) and placed for 45 min in a blocking solution (2.5% bovine serum albumin(BSA) in PBS). Subsequent incubations in the primary and secondary antibodies took at least 3 h each. Incubation in the primary mouse monoclonal anti β-tubulin antibody (N357; Amersham International plc, Buckinghamshire, UK, 1:200 dilution in PBS containing 1% BSA) was followed by at least five, 10 minute washes in the blocking solution. Since tubulin-label intensity or pattern did not change if the incubations in either primary or the secondary antibody were longer than 3 h, the epidermal strips were left overnight in the secondary, FITC-conjugated sheep anti-mouse antibody (F-2883, Sigma, 1:25 dilution in the blocking solution). After several washes in the blocking solution the strips were drained and allowed to dry prior to mounting in 80% glycerol in PBS. Tissue was observed using a Reichert-Jung Polyvar microscope equipped with differential interference contrast (DIC) optics. Fluorescence of labelled tubulin was detected under blue light
epifluorescence irradiation using filter cube B1: exciter BP 450-495, barrier LP 520, dichroic DS 510.

Staining for microfilaments

Procedure 1 (filament cables): Plants were treated as above except for the following changes in solutions: 2% DMSO was added to MSB; and 0.2 mM 4(p-maleimidophenyl) butyric acid N-hydroxysuccinimide ester (MBS) (Sigma), triton-X 100, 0.005 % (v/v) and 2% (v/v) DMSO were added to the primary fixative. The permealization with cell-wall degrading enzymes was omitted and the epidermal strips were incubated in TRITC-labelled phalloidin (1.6μM in MSB) (Sigma) for at least 3 h, washed, dried and mounted in 80% glycerol in MSB. Tissue was observed using G1 filter combination (exciter HQ 535/50, dichroic Q565LP, emitter HQ 620/60sb).

Procedure 2 (filament networks): Plants were treated as in procedure 1 except that the concentration of glutaraldehyde in fixatives was lowered to 0.025% and that plants were first injected with MBS (0.2 mM) and TritonX-100 (0.05% in MSB) and the leaf pieces then incubated in the primary fixative (30 min) and transferred to the secondary fixative (45 min). Subsequent steps were as in procedure 1.

Effects of antimicrotubule agents on nuclear movement and plant responses

Tissue was fixed in hot ethanol (95%), stained overnight for DNA with DAPI (4′6-diamidino-2-phenylindole) (5 μg ml⁻¹ in double distilled water), and mounted in water. Tissue was observed as described above. Autofluorescence of cell components
was detected using blue light (B1 filter cube) and nuclear position using UV epifluorescence (U1 filter cube: exciter BP 330-380, barrier LP 418, dichroic DS 420).
RESULTS

Infection of cowpea by the monokaryon of *U. vignae* has been described previously (Heath et al., 1997 and references therein). Briefly, epidermal cells are penetrated directly and an intracellular invasion hypha forms. The hypha grows through epidermal cells by tip growth and in the absence of resistance responses in the susceptible cultivar California Blackeye (CB), branches and invades neighbouring cells, eventually establishing an intercellular mycelium bearing intracellular haustoria. Although infected cells in the susceptible cultivar remain alive throughout infection, those of the resistant cultivars Dixie Cream (DC) and Calico Crowder (CC) die and most hyphae do not grow beyond the length of 20 μm in DC and 30 μm in CC. Here, we describe the initial 16 h of infection by which time about 80-90% of infected cells have died in the resistant cultivars.

*Microtubule (MT) distribution*

In uninfected cells (Fig. 29a-c) of all cultivars, two major interconnected MT arrays were detected using indirectly labelled anti-tubulin antibody: the cortical array consisted of MTs close to the inside of the plant cell wall (Fig. 29b) and a perinuclear array surrounded the nucleus (Fig. 29c). Infected cells (Fig. 29d-l) contained an additional array that surrounded the invasion hypha, herein called the perihyphal array. The perinuclear and perihyphal MTs appeared to be particularly sensitive to fixation, since they could only be detected if the tissue was fixed on ice and the exposure of tissue to the cell wall degrading enzymes and detergent used to permeabilize the cells
was limited to short time intervals and dilute concentrations. Fungal MTs were not labelled since hyphal walls were not permealized by the enzymes used to allow the antibodies access to the plant cytoplasm. Since infection was not synchronous enough to allow direct time course experiments, investigated infection sites were grouped into categories (at least 100 sites per category) according to hyphal length: I - hyphae shorter than $12 \pm 2 \mu m$, II - hyphae between $16 \pm 2 \mu m$ and $20 \pm 2 \mu m$ and III - hyphae longer than $22 \pm 2 \mu m$. Data presented in Figure 30 represent the proportion of sites within a category exhibiting a particular MT arrangement. To ensure that the lack of antibody-fluorescence was due to the absence of MTs and not a labelling artefact, only sites that had at least some MTs were included. The position of the plant nucleus and evidence of any defence responses, such as cell wall autofluorescence, were also recorded for each site. Sites that contained more than one hypha per cell (less than 1 %) (Fig. 29g-i) were excluded.

There were no statistical differences between cowpea cultivars in the arrangement of perihyphal MTs which were present in 70-80% of infection sites in all three cultivars. In most sites, MTs surrounded the whole hypha (Fig. 29d-f, j-l), while in 25-30% of category III, perihyphal MTs were present only around the hyphal tip.

The MT arrangement in the susceptible cultivar changed very little compared to the uninfected cells, even in the cells that contained two or more hyphae (Fig. 29g-i). The only exception was the absence of MTs around the fungal penetration site observed in 30-56% of categories II and III (Fig. 29g-i). The MT absence in this region of the cell was only rarely observed in the two resistant cultivars. The proportion
of sites in which both cortical and perinuclear arrays were present and intact was significantly lower in the resistant cultivars than in the susceptible cultivar in categories II and III (Fig. 30b). For these categories the plant nucleus was generally close to the hypha in the susceptible cultivar CB while it remained away in the resistant cultivars (Fig. 30a).

Surprisingly, the disappearance of perinuclear and cortical MT's during HR-cell death proceeded differently in the two resistant cultivars. In the cultivar DC, most cells in categories II and III had fragmented or absent cortical MTs, but the perinuclear MTs were still present while the reverse was true for cultivar CC (Figs. 30c-d and 31a-b). The proportion of sites in CC in which cortical MTs were fragmented or absent increased with increased hyphal length. However this proportion was significantly lower than that observed in cultivar DC (Fig. 30c). Cells lacking either MT array often showed no other signs of cell damage although, autofluorescence of cell walls was also observed but only in the cultivar CC (Fig. 31c) at 30-50% of the sites in categories II and III. Cells that contained undisrupted MTs and autofluorescent cell walls constituted about 5.8% of all the sites observed.

MTs and nuclei in the cells surrounding the infected cell sometimes aggregated towards the infected cell. This was particularly prominent around infected cells in the resistant cultivars that, unlike those reported in Figure 30, lacked MTs and were at the latest stages of death (eg. protoplast collapse, autofluorescence of cytoplasm) (Fig. 31d).
**Microfilament (MF) distribution**

We used two fixation methods which produced different labelling patterns of MFs. When the MF stabilizer MBS was included in the fixative with higher glutaraldehyde concentrations (0.3%) (procedure 1), rare thick MF cables which were often attached to the nucleus were observed in uninfected cells of all cultivars (Fig. 32a). In the infected cells (Fig. 32c-d) MF cables were also attached to the hypha in the majority of sites in the susceptible cultivar regardless of the position of the nucleus (Fig. 33). In the resistant cultivars significantly fewer sites contained cables attached to either the hypha or the plant nucleus. This difference was more pronounced in categories II and III, which were presumably closer to or at the early stages of the cell death process (Fig. 33).

In the fixation (procedure 2) where cells were treated with MBS prior to fixation in a lower glutaraldehyde concentration (0.025%), the MFs were arranged in a dense network of fine filaments with aggregates surrounding the nucleus and the chloroplasts of uninfected cells (Fig. 32b). The arrangement in the infected cells of both resistant and susceptible cultivars did not differ from that observed in uninfected cells except that filament aggregation was observed surrounding the hypha (Fig. 32e-f). In the resistant cultivars, the network was disrupted or absent at a higher proportion of sites in categories II and III so that the incidence of sites with an intact network was significantly lower than in the comparable categories in the susceptible cultivar (Fig. 33). In some (9.3% in DC; 12.7% in CC) resistant cells showing signs of cell death such as protoplast collapse, some microfilaments could also be seen (Fig. 32g-h). This was
never observed in cells labelled for MTs. There were no detectable differences in either network or cable filament arrangements between the two resistant cultivars.

**Effects of anticytoskeletal drugs**

To determine whether an intact cytoskeleton was required for plant responses to infection, plants were injected with anticytoskeletal drugs prior to inoculation and observed 14 h after inoculation. The concentrations of drugs were chosen that had visible effects on the cytoskeleton in labelling studies, but did not effect fungal growth or cause plant tissue damage. In tissue treated with taxol (inhibitor of MT depolymerization) plant MTs appeared thicker and brighter than in controls and dense MT aggregates which were not observed in the untreated tissue were visible around chloroplasts and nuclei. In tissue treated with oryzalin (inhibitor of MT polymerization) MTs were either absent or fragmented in the majority of cells. Cytochalasin E (inhibitor of MF polymerization) caused fragmentation or disappearance of MFs and cessation of cytoplasmic streaming up to 6-8 h after treatment; however, these effects were completely reversed by 12 h (data not shown).

In the susceptible cultivar, nuclear movement toward hyphae longer than 16 μm was inhibited by cytochalasin E, while taxol and oryzalin had no effect (Table 3).

In the resistant cultivars, proportions of sites showing signs (Heath et al, 1997) of HR cell death were significantly reduced in plants treated with cytochalasin E and were not affected by treatments with taxol or oryzalin (Table 2). However, taxol and oryzalin significantly reduced development of cell autofluorescence (which occurs after
other signs of cell death) in the resistant cultivar DC. Although a reduction in the proportion of autofluorescent, infected cells was repeatedly observed in the cultivar CC, this was not statistically significant due to the high variability observed in this cultivar (Table 2).

Interestingly, cytochalasin E repeatedly but not significantly reduced fungal growth in the susceptible cultivar (Table 3), but increased it in the resistant cultivars (Table 2), indicating that these effects are probably due to the drug’s effects on plant responses and not on the fungus.
Figure 29. Microtubule arrangements in uninfected (a-c) and infected (d-l) plant cells. Nuclei and/or fungal hyphae are shown by differential interference contrast (DIC) optics (a,d,g,j) and cortical (b,e,h,k) and perinuclear and/or perihyphal (c,f,i,l) MTs in corresponding optical sections taken under blue-light fluorescence. (a-c) MT arrangements representative of uninfected cells in all three cowpea cultivars. Perinuclear MTs can be distinguished from the gray background nuclear fluorescence as white lines (c, arrow); (d-f) Cell of the susceptible cultivar CB in a late stage of infection (hyphal length ~ 35 μm); (g-i) cell of the cultivar CB containing two hyphae. Cortical MTs are absent around one of the hyphal penetration points (g and h, arrows); (j-l), cell of the resistant cultivar DC prior to development of any visible signs of cell death or defence (hyphal length ~ 5 μm). All figures are similar magnification. Bar = 10 μm. c-chloroplast; n-nucleus; h-hypha; g-guard cell.
Figure 30. Analysis of incidence of nuclear association with hypha (a) and different microtubule responses (b, c, d) in plant cells grouped according to the length of the invading hypha (hyphal length category: I - less than 12 ± 2 μm, II - 16 ± 2 μm to 20 ± 2 μm, III more than 22 ± 2 μm). Black bars represent susceptible cultivar CB, while the white and grey bars represent resistant cultivars DC and CC respectively. The absence of white bars in category III is due to the lack of hyphae that grew to this length, absence of bars in any other category represents 0. Each bar represents % sites obtained by examining at least 100 infection sites combined from 3 labelling experiments (*significant difference between cultivars at P ≤ 0.03, chi square test using raw data, not %)
a) nucleus near the hypha

b) all MT arrays present

c) cortical MTs disrupted

d) perinuclear MTs absent
Figure 31. MT arrangements in infected resistant plants. DIC (a) and corresponding fluorescence (b) micrograph of two infection sites in the cultivar DC. In both cells, cortical MTs are absent while perinuclear MTs can still be seen (b, arrows); (c), Cell of the cultivar CC showing autofluorescence of the plant cell wall (arrow); (d) Infected cell in the cultivar DC without any MTs due to the HR. MTs and nuclei of the surrounding cells are oriented toward the infected cell. Bar = 10 μm. n-nucleus; h-hypha; g-guard cell.
Figure 32. MF arrangement in uninfected (a,b) and infected (c-h) cells. Position of organelles and fungal hyphae are shown under DIC optics (c,e,g) and rhodamine-phalloidin labelled MFs in corresponding micrographs taken using green-light fluorescence (d,f,h). MF arrangement using fixation procedure 1 is shown in a and d, and that using procedure 2 in b,f and h. (c,d) Susceptible cultivar CB MF cables are attached to the hyphae and to the nucleus (d). Subsidiary cells (s) often fluoresced more intensely than others (d); (e,f) MF network arrangement observed in all cultivars at early stages of infection; (g,h) Resistant cultivar DC, MF network is still visible (h) when (g) the protoplast is collapsing (small arrow) away from the plant cell wall (large arrow). Bar = 10 μm. c-chloroplast; n-nucleus; h-hypha; g-guard cell.
Figure 33. Analysis of incidence of microfilament arrangements in infected cells grouped according to the length of the invading hypha (hyphal length category: I - less than $12 \pm 2 \mu m$, II - $16 \pm 2 \mu m$ to $20 \pm 2 \mu m$, III more than $22 \pm 2 \mu m$) (a) Incidence of sites with filament cables (procedure 1) attached to nucleus and the hypha; (b) incidence of sites with intact filament network (procedure 2). Black bars represent susceptible cultivar CB, while the white and grey bars represent resistant cultivars DC and CC respectively. The absence of white bars in category III is due to the lack of hyphae that grew to this length, absence of bars in any other category represents 0. Each bar represents % sites obtained by examining at least 100 infection sites combined from 3 labelling experiments (*significant difference between cultivars at $P \leq 0.03$, chi square test using raw data, not %).
a) actin cables attached to the hypha and nucleus

b) intact filament network
TABLE 2. Effect of anticytoskeletal drugs on plant defense responses 14 h after inoculation in the resistant cowpea cultivars DC and CC. All values are from one of at least two separate experiments and represent mean (± standard error) of four plants (50 infection sites per plant).

<table>
<thead>
<tr>
<th>Inhibitor, concentration</th>
<th>cultivar</th>
<th>treatment</th>
<th>mean hyphal length (μm)</th>
<th>cell death* (% sites)</th>
<th>autofluorescent cell wall or cytoplasm (% sites)</th>
</tr>
</thead>
<tbody>
<tr>
<td>taxol, 85 μg ml⁻¹</td>
<td>DC</td>
<td>drug</td>
<td>15.1 ± 0.3</td>
<td>64 ± 12</td>
<td>0 ± 0*</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td></td>
<td>14.5 ± 0.4</td>
<td>68 ± 12</td>
<td>16 ± 4</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>drug</td>
<td>15.4 ± 0.3</td>
<td>53 ± 7</td>
<td>39 ± 18</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td></td>
<td>17 ± 1</td>
<td>70 ± 14</td>
<td>64 ± 17</td>
</tr>
<tr>
<td>oryzalin, 60 μg ml⁻¹</td>
<td>DC</td>
<td>drug</td>
<td>14.6 ± 0.9</td>
<td>76 ± 4</td>
<td>26 ± 10*</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td></td>
<td>15.2 ± 0.4</td>
<td>83 ± 3</td>
<td>52 ± 12</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>drug</td>
<td>18 ± 1</td>
<td>65 ± 20</td>
<td>41 ± 40</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td></td>
<td>18 ± 0.6</td>
<td>73 ± 23</td>
<td>73 ± 23</td>
</tr>
<tr>
<td>cytochalasin E, 0.5 μg ml⁻¹</td>
<td>DC</td>
<td>drug</td>
<td>17 ± 1</td>
<td>58 ± 9*</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td></td>
<td>16 ± 1</td>
<td>96 ± 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>drug</td>
<td>18 ± 2</td>
<td>53 ± 16*</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>control</td>
<td></td>
<td>17 ± 0.5</td>
<td>88 ± 7</td>
<td></td>
</tr>
</tbody>
</table>

* granulation of cytoplasm, protoplast collapse and fluorescence under blue light of cytoplasm or cell wall were considered signs of plant cell death
* significantly different from control at P ≤ 0.05 (t-test)
TABLE 3. Effect of anticytoskeletal drugs on the nuclear movement towards the hypha in the susceptible cowpea cultivar CB. All values are from one of at least two separate experiments and represent mean (± standard error) of four plants (50 infection sites per plant).

<table>
<thead>
<tr>
<th>Inhibitor, concentration</th>
<th>treatment</th>
<th>mean hyphal length (μm)</th>
<th>plant nuclei next to hypha (% sites)</th>
</tr>
</thead>
<tbody>
<tr>
<td>taxol, 85 μg ml⁻¹</td>
<td>drug</td>
<td>18 ± 2</td>
<td>80 ± 8</td>
</tr>
<tr>
<td>oryzalin, 60 μg ml⁻¹</td>
<td>drug</td>
<td>18 ± 1</td>
<td>76 ± 3</td>
</tr>
<tr>
<td>cytochalasin E, 0.5 μg ml⁻¹</td>
<td>control</td>
<td>18 ± 1</td>
<td>84 ± 6</td>
</tr>
<tr>
<td>cytochalasin E, 0.5 μg ml⁻¹</td>
<td>drug</td>
<td>15 ± 4</td>
<td>15 ± 11*</td>
</tr>
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</table>

* Only the sites in which the hypha was longer than 16 μm were included in the count, as plant nuclei were not consistently associated with shorter hyphae in CB.
* significantly different from control at P ≤ 0.05 (t-test)
DISCUSSION

We have shown that both MTs and MFs persist in epidermal cells of susceptible cowpea leaves infected with the cowpea rust fungus. This and similar observations from plants infected with other fungal pathogens (Baluška et al., 1995; Kobayashi et al., 1992, 1994) as well as nonpathogenic mycorrhizal fungi (Uetake et al., 1996, 1997), suggest that establishment of successful fungal biotrophy inside plant cells can occur without major disruption of the plant cytoskeleton. MTs in the infected cowpea cells formed a perihyphal array surrounding the fungus. In view of the close association between the plasma membrane and MTs (Lloyd et al., 1996) and since intracellular fungal structures are surrounded by a continuation of the plasma membrane (Heath and Škalamera, 1997), it is surprising that MT aggregates around intracellular hyphal masses were previously reported only for mycorrhizal infection of orchid protocorms (Uetake et al., 1996). This may be a reflection of the sensitivity of perihyphal MTs to fixation as demonstrated in the cowpea rust system.

The importance of fixation methods is also illustrated by our finding that varying glutaraldehyde concentration and timing of the addition of the MF stabilizing agent MBS (Sonobe and Shibaoka, 1989), produced different MF patterns in the cell. Higher glutaraldehyde concentration and MBS addition to the fixative resulted in a commonly seen (Reuzeau et al., 1997) pattern of thick actin cables in the cell, while the lower concentration and prefixation treatment with MBS resulted in a network of fine actin filaments resembling the one reported for in vivo observations of onion epidermal cells injected with rhodamine-phalloidin (Reuzeau et al., 1997). Although this latter result
may suggest that the cable pattern is an artefact, it is also possible that while the network of fine filaments is destroyed by the harsher fixation, the cables only become accessible to label after such treatments so that the two observed patterns represent different subsets of actin filaments created by different actin binding proteins or by the multiple isoforms of actin which coexist in plant tissues (Janßen et al., 1996).

In our study, both the actin network filaments and the cables were aggregated around the fungus in the infected cells of the susceptible cultivar. Actin aggregates were also observed around intracellular hyphae during mycorrhiza-formation (Uetake et al., 1996). This aggregation coincides with the accumulation of endoplasmic reticulum (ER) around the intracellular fungal structures observed in our system as well as during other plant-fungal interactions (Heath and Škalamera, 1997). The association between MFs and ER has been extensively documented both in fixed tissues (Staehelin, 1997) and more recently in living cells (Reuzeau et al., 1997) suggesting a role for MF in organizing ER distribution, and therefore perhaps in stabilizing ER structures surrounding fungal hyphae. The role of MFs in organizing cytoplasmic components during intracellular plant-fungal interactions is more clearly demonstrated by the inhibition by the antimicrofilament drug, cytochalasin E, of plant nuclear migration close to the longer hyphae normally observed in the susceptible cowpea cultivar. In our study, the nuclear position was not affected by the antimicrotubule drugs taxol and oryzalin. Similarly, in cultured parsley cells infected with the oomycete Phytophthora infestans, nuclear movements coincided with localized depolymerization of MT, and were disrupted by cytochalasin D but not by taxol and the MT-disrupting agent
amiprophosmethyl (Gross et al., 1993). Therefore, it seems that the fungus and oomycete-induced nuclear movements resemble wound-induced movements which are dependent on the actin cytoskeleton rather than MTs (Nagai, 1993). It is significant that in our study the MF cables were frequently not attached to the hypha or nucleus in the resistant cultivars in which the nucleus remains away from the longer hyphae.

The organization of the cytoskeleton in the resistant cowpea cultivars was similar to that observed in the susceptible cultivar at the initial stages of infection, but the cytoskeletal arrays became fragmented and eventually undetectable as the cells underwent the HR. Despite the lack of effect of antimicrotubule drugs on nuclear positioning in the susceptible cultivar, disappearance of MTs coincided with the lack of nuclear movement towards the fungus in the resistant cultivars and occurred in cells in which no other signs of ensuing cell death could be detected. Interestingly, in the resistant cultivar DC, cortical MTs were degraded before perinuclear MTs while the sequence was reversed in cultivar CC, suggesting that in addition to multiple molecular signals that can initiate the HR (Heath, 1998b), plant cells may possess multiple pathways for the execution of the death process that can operate in different race-cultivar interactions within the same plant pathogen system. Disappearance of cortical MTs in DC occurs after the initiation of the sustained increase in the cytoplasmic calcium concentration at the time of plant cell wall penetration by the fungus (Xu and Heath, 1998). Since an increase in cytoplasmic calcium levels can both cause disintegration of MTs (Lloyd et al., 1996), and be caused by opening of plasma membrane calcium channels induced by MT depolymerization (Thion et al., 1996),
these processes may also be linked during the HR. Disruption of MTs was also observed in plant cells undergoing the HR during the interaction between flax and an incompatible race of the dikaryotic stage of flax rust fungus (Kobayashi et al., 1994) and between *Erysiphe cichoracearum* and the non-host cowpea plant (Meyer and Heath, 1988b), but not during early stages of cell death in cowpea triggered by heavy metal salts (Meyer and Heath, 1988a). This suggests that disruption of MTs might be a specific and early sign of the HR.

Treatment of cowpea plants with taxol or oryzalin reduced the incidence of cell autofluorescence in our study. Since autofluorescence develops in infected cells as one of the last stages of cell death (Heath, 1998a), this process is probably dependent on the MTs of the surrounding cells which became oriented towards the infected cell. Taxol and oryzalin also reduced the incidence of autofluorescent halos and papillae in non-host barley cells infected with *Erysiphe pisi* (Kobayashi et al., 1997b). Autofluorescence is thought to indicate the presence of phenolic compounds and cytoplasmic autofluorescence is frequently used to indicate cell death (Bennett et al., 1996). Since in our study neither taxol nor oryzalin had an effect on cell death, our data confirm that cell death precedes and can occur in at least temporary absence of autofluorescence as previously observed in lettuce infected with *Bremia lactucae* (Bennett et al., 1996) and in the cowpea-cowpea rust interaction (Heath, 1998a). This may explain the claimed inhibition of the HR by oryzalin and taxol in the flax-flax rust system (Kobayashi et al., 1997a), since indicators of phenolic deposition were included in the criteria for cell death determination.
In contrast to MTs, MFs could still be detected in some cells that were clearly collapsing in the resistant cultivars in our study. Protoplast collapse in this system does not occur by tonoplast burst but by the stepwise contraction-like movements (Heath, 1998a) that might involve MFs. The essential role of MFs in the HR is confirmed by the reduced incidence of dead cells in plants treated with cytochalasin E. Cytochalasins have also been shown to increase penetration efficiency of a number of normally non-pathogenic fungi in several non-host plants (Kobayashi et al, 1997b,c) and to inhibit the HR during the potato-Phytophthora infestans (Tomiyama et al, 1982) and barley-powdery mildew interactions (Hazen and Bushnell, 1983), as well as to reduce other resistance responses such as callose deposition during the cowpea rust fungus interaction with bean or cowpea (Perumalla and Heath 1991, Škalamera and Heath, 1996, 1997). Together these data demonstrate the importance of MFs in the expression of plant resistance. However it is not clear whether their role is through their function in cytoplasmic streaming or through their role in signal transduction (Lloyd et al., 1996). A signalling role has been indicated during resistance-induced callose deposition in the cowpea-cowpea rust fungus interaction, where cytochalasin E affects fungal-, but not chemical-induced callose deposition (Škalamera and Heath, 1996). Since in our study MF patterns in the two resistant cultivars did not differ from each other, our observations suggest that while the changes in MTs may differ during plant-pathogen interactions, MF changes may be a general feature of the HR cell death.

Recent evidence suggests that the HR in resistant host plants is triggered by a signal transduction cascade starting with the interaction between specific resistance
genes-encoded receptor-like proteins and specific pathogen-derived elicitors (Heath, 1998b). The cultivar DC used in this study has two resistance genes that may interact with two fungus derived elicitor peptides to trigger a cascade of events that eventually result in cell death-associated DNA cleavage and activation of cysteine proteases (Heath, 1997). Current data suggest that these events involve changes in the cytoskeleton, an increase in calcium concentration (Xu and Heath, 1998) and infection-associated organelle movements.
CHAPTER THREE:

Cowpea rust fungus-induced changes in plant gene expression identified by mRNA differential display

INTRODUCTION

The interaction between rust fungi and susceptible plants is characterized by formation of fungal structures both inside and between plant cells, often without the triggering of any detectable defence responses from the plant. As a requirement for the obligate biotrophy of rust fungi, there is very little plant tissue damage during the initial stages of infection and even the invaded plant cells can remain alive for days (reviewed by Heath and Škalamera, 1997). In contrast, comparable amount of invasion by nonbiotrophic pathogens, or even wounding by abiotic factors, normally triggers a battery of plant defence responses including wall appositions i.e deposits on the cell walls (reviewed by Aist, 1976), production of antimicrobial compounds (Kuč, 1995) and induction of pathogenesis related (PR) gene expression (reviewed by Bowels, 1990; and Kombrink and Somssich, 1997). These responses to non-biotrophs are observed both in the resistant and to a lesser degree in susceptible plants. The invasion of plant tissues by rust fungi could be expected to produce similar amount of both physical and perhaps chemical irritation to trigger plant defence responses and yet they are largely absent during compatible interactions. Therefore, it can be concluded that rust fungi are not simply avoiding the triggering of defence responses in susceptible plants, but have developed mechanisms to actively suppress them (Heath, 1995).
At the same time, rust fungal invasion of susceptible plant cells can cause changes in the distribution of cellular organelles, particularly the endomembranes and the nucleus (reviewed by Bushnell, 1984; Heath and Škalamera, 1997). These changes observed at the early stages of infection occur at the same time as changes in protein profiles specific to the compatible interaction of oat and *Puccinia coronata* f. sp. *avenae* (Yamamoto and Tani, 1982), and flax and *Melampsora lini* (Sutton and Shaw, 1986), as detected by one and two dimensional gel-electrophoresis. At the later stages of infection, changes were detected in whole plant metabolism, including hormone production, rates of photosynthesis and respiration, and nucleic acid and carbohydrate synthesis (reviewed by Bushnell, 1984; Mendgen, 1997). Since many of these responses are controlled at the level of gene expression, these observations indicate that rust fungi must be able to induce specific changes in plant gene expression at both early and late stages of infection. Analysis of these changes particularly during the compatible interactions is important, since it may significantly improve our understanding of the infection process and eventually provide means for disease control.

Despite their potential significance, there have been relatively few studies on changes in plant gene expression during plant-rust fungal interactions. Most of these studies concentrated on incompatible interactions and characterization of expression patterns of transcripts that have previously been identified as defence-related genes in other plant-pathogen systems. Increase in transcript levels of β-1,3-glucanase and chitinase has been reported in the incompatible interaction between wheat and the
wheat stem rust (Münch-Garthoff, et al, 1997), while the mRNA levels encoding thauamatin-like proteins, β-1,3-glucanase, phenylalanine ammonia-lyase (PAL), PR-1 and 3-phosphoglycerate kinase all increased during the interaction of oat and incompatible and non-pathogenic isolates of *Puccinia graminis*, (Lin et al, 1996,1998). Resistance response to rust fungi also resulted in an increase in enzyme activities and presumably mRNA levels of a phospholipase in wheat (Ocampo and Grambow, 1997) and β-1,3-glucanase, chitinase, PAL and peroxidase in cowpea (Fink et al, 1991; Deising and Mendgen 1992). In addition, specific screening for cDNA clones representing genes induced during the resistance response of oat to *Puccinia graminis* has revealed an increase in a putative glucose-regulating protein and a transcript for an unknown protein (Lin et al, 1998). In some of these studies compatible interactions were also investigated and it was revealed that chitinase transcript levels are increased slightly in wheat 6 days after inoculation with *P. graminis f.sp tritici* (Münch-Garthoff, et al, 1997) and that some defence gene transcripts were induced in oat 24 to 30 hr after inoculation with a compatible isolate of *P. graminis f. sp. avenae* (Lin et al, 1996, 1998). In the latter case the response was non-specific since it was also observed in mock-inoculated plants and was possibly due to the oil used to in the inoculation procedure (Lin et al, 1996,1998). So far there has only been one report of a gene that is induced by a rust fungus infection only in a susceptible plant. Roberts and Pryor (1995), used subtraction hybridization between uninoculated and inoculated plants to generate cDNA clones specific for the compatible interaction between flax and *Melampsora lini*. They were able to identify only one plant gene, possibly an aldehyde
dehydrogenase, that was induced 4 days after inoculation (Roberts and Pryor, 1995). Therefore, molecular events involved in the early stages of the establishment of compatibility between plant cells and rust fungi remain virtually uncharacterized.

This lack of information is due in part to the methods used to identify differentially expressed genes. These methods relied on subtractive hybridization procedures which require substantial amounts of tissue and only allow detection of relatively abundant mRNAs. Given the small amount of fungal growth and therefore the small percentage of plant cells that are initially exposed to the fungus during rust infection, it is to be expected that any changes in plant gene expression might be represented by only a small proportion of total plant transcripts. This type of change can only be detected by a more sensitive technique, such as mRNA differential display (Liang and Pardee, 1992; 1995). Differential display requires a small amount of tissue since it is based on amplifying selected mRNA species by using specially designed random primers during the polymerase chain reaction (PCR). The amplification products, representing different mRNA species are then displayed as bands of different mobility on a polyacrylamide gel. Additional advantages of this method are that multiple samples can be compared at the same time and that both upregulation and downregulation of genes can be analysed simultaneously (Liang and Pardee, 1992; 1995).

In this study, I used a modified differential display procedure to analyse changes in gene expression during the compatible interaction between cowpea (Vigna unguiculata) and the dikaryotic stage of the cowpea rust fungus (Uromyces vignae).
The dikaryotic stage was chosen for two reasons: 1) initially the fungus grows intercellularly and then starts producing intracellular haustoria, so that the effects of both intra- and inter-cellular growth can be compared and 2) the infection is relatively synchronous so that different growth stages can be separated by different sampling times. This is a report of the preliminary finding that shows that differential display is sensitive enough to detect changes in plant gene expression as early as 6 h after inoculation, and that these changes may involve plant genes that were not previously characterized or associated with plant responses to pathogens.
MATERIALS AND METHODS

Plants

Cowpea (Vigna unguiculata (L.) Walp.) cultivar California Blackeye was grown in Pro Mix (Premier Brands Inc. New Rochelle, NY) in growth chambers maintained at 20-24°C, with a 16 h photoperiod at light intensity 250 μmol m⁻² s⁻¹ as described previously (Perumalla and Heath 1989).

Fungal material and inoculation

Upper and lower leaf surface were inoculated by brushing with washed urediospores of the cowpea rust fungus (Uromyces vignae, Barclay, race 1) as described previously (Perumalla and Heath 1991). Inoculated plants were sprayed with water and placed in a dark moist chamber until harvesting.

Nucleic acid isolation

For all RNA isolation experiments a small leaf piece was cut from the plant at the time of harvesting and kept in 100% ethanol for microscopical evaluation of the infection levels. RNA was isolated by the phenol/sodium dodecyl sulfate (SDS) method (Ausabel et al, 1988). Briefly, plant leaves were ground in liquid nitrogen and immediately placed in phenol equilibrated with grinding buffer (0.1M LiCl, 0.1M Tris, 0.01M EDTA, 1% SDS, pH8) and incubated at 60°C for at least 20min. The aqueous phase was collected by centrifugation, extracted with phenol/chloroform and chloroform and the RNA was precipitated twice in 2M LiCl in 70% ethanol. RNA was
stored in 0.3M sodium acetate in 70% ethanol at -20°C until use when it was collected by centrifugation and redissolved in water. RNA was treated with DNase I (Pharmacia/Biotech, Baie d’Urfé, Quebec) according to manufacturers recommendations. The DNase was removed by phenol/chloroform extraction.

Plant DNA was isolated by grinding leaves in liquid nitrogen. The powder was thawed, incubated in 2X cethyl triethylammonium bromide (CTAB) (2%CTAB (W/v), 0.1M Tris-HCl, 20 mM EDTA, 1.6M NaCl, 1% polyvinylpyrrolidone (Mr 40 000, pH 8) at 65°C for 20 min and extracted with an equal volume of chloroform:isoamyl alcohol (24:1). After centrifugation, 1/10 volume of 10% CTAB containing 0.7M NaCl was added to the aqueous phase which was extracted with chloroform:isoamyl alcohol again. DNA was precipitated by adding one volume of CTAB precipitation buffer ((1% CTAB, 50 mM Tris-HCl, 10 mM EDTA pH 8) and incubated at 4°C overnight. DNA was collected by centrifugation, dissolved in TE buffer (10 mM Tris-HCl, 1mM EDTA, pH 8), treated with RNase (1mg/ml), extracted with an equal volume of phenol:chloroform (1:1), and precipitated using 0.3M sodium acetate in 70% ethanol.

Fungal DNA was isolated from urediospores pregerminated in water overnight and collected by filtration as described by Bhairi et al (1989).

Plasmid DNA was isolated by the alkali lysis method from bacteria grown in LB broth (Sambrook et al, 1989).

**Differential display**

Differential display was performed by combining and modifying the procedures
of Liang et al, (1995) and Doss (1996). Total mRNA from leaves was reverse transcribed using M-MuLV-reverse transcriptase (20U/15μg of RNA)(Boehringer-Mannheim-Canada (BMC), Laval, Quebec) in the supplied buffer (50mM Tris-HCl, 40mM KCl, 6mM MgCl₂, 10 mM dithioerythriol, pH 8.3) with addition of dNTP’s (1 mM) and one of the four anchored poly T primers (S'(dT)₁₂ VX, Operon Technologies, (OT) Alameda, CA) (0.8 μM). Total volume was 25μl. Prior to the addition of the enzyme, RNA was denatured for 15 min at 65°C, and cooled to 37°C, at which temperature the reaction was allowed to proceed for at least 1h. 2 μl of this reaction mixture was used in the following PCR reaction mixture (total volume 23μl): 10mM Tris-HCL pH 8.3, 1.5mM MgCl₂ 50 mM KCl, 0.2 mM dNTP’s, 1μM poly T primer, 0.4 μM random 10-mer primer (OP-26, OT), 5U of TaqDNA polymerase (BMC). The reaction was performed in the Robocycler (PerkinElmer, Norwalk, CT.) for 30 cycles of 30' at 95°C, 2 min at 40°C, 30' at 72°C, with the final extension step at 72°C for 10 min. 7 μl of each reaction mixture was run on a 7.2% non-denaturing polyacrylamide gel (Sambrook et al, 1989) at 400V for 6 hr.

Silver staining of the polyacrylamide gels

To avoid fixing the gel to glass plates, gel transfer to different solutions was aided by supporting it with a large piece of filter-paper. The gel was fixed in 10% (v/v) acetic acid overnight, rinsed three times in MiliQ-filtered water, and transferred to the staining solution (1% AgNO₃, 0.055% formaldehyde) for 3 h. The gel was developed in prechilled Na₂CO₃ (30%) to which formaldehyde (final 0.11%) and Na₂S₂O₃
(1.5 mg l\(^{-1}\)) were added. The developing reaction was stopped by the addition of 10% acetic acid, the gel was rinsed in water and dried on a gel drier (Bio-Rad, Hercules, CA).

\textit{Reamplification of differentially expressed bands}

Bands were excised from the dried gel and incubated in 30 \(\mu l\) of water at 70\(^\circ\)C for 1hr. Half of this volume was used in a PCR reaction (40 \(\mu l\)) which was similar to the original one except that it was allowed to proceed for 40 cycles. Bands were purified by agarose gel electrophoresis. DNA in agarose was either used for probes or as a template in another PCR reaction to obtain enough product for cloning.

\textit{Extraction of DNA from agarose gels}

All DNA used for cloning or sequencing was purified by agarose gel electrophoresis. DNA was extracted from the gel by heating (15min at 50\(^\circ\)C) in 2 volumes of 6M NaI, collected by binding to silica (suspension in 3M NaI, 5\(\mu\)l per 500\(\mu\)l of the gel solution) for 15 min on ice and centrifuging (10' at 12000xg). Silica bound DNA was washed 3 times in the wash buffer (50 mM NaCl, 40 mM Tris-HCl pH 7.5, 2.5 mM EDTA, 50% ethanol) by repeated cycles of centrifuging and resuspending in the wash buffer. The DNA was released from the silica by heating in water (15min at 50\(^\circ\)C) and centrifuging to remove the silica pellet.
Northern and Southern blot analysis

Agarose gel electrophoresis, and nucleic acid transfer to Nytran membranes (Schleicher & Schuell, Keene, NH) for both RNA and DNA were performed as described by Sambrook et al, (1989). Probe synthesis, prehybridization, hybridization and subsequent washes were as described by Glover and Hames (1995).

Cloning and sequencing of the amplified bands

Gel purified bands were blunted using Vent DNA polymerase (New England Biolabs, Beverly, MA) according to manufacturers recommendations, ligated into Bluescript SK+/- phagemid (Stratagene, La Jolla, CA) and transfected into E.coli DH5α cells (Stratagene) as described by Sambrook et al (1989). Plasmid DNA was recovered by alkaline lysis (Sambrook et al, 1989), gel purified and sequenced by The Core Molecular Biology Facility (York University, North York, Ont.)
RESULTS

During infection of cowpea by the dikaryotic stage of the cowpea rust fungus, fungal urediospores germinate on the leaf surface to form germ tubes that penetrate through stomatal openings. The fungus initially grows intercellularly and subsequently forms haustoria inside plant cells. The first haustoria can be observed between 12 and 14 h after inoculation (hai) and they are observed in almost all infection sites by 24 hai. Originally tissue was harvested for RNA isolation 6 and 18 hai, to compare effects of intra- and inter-cellular fungal growth. However, microscopical examination revealed that at these times infection levels varied greatly between experiments, so the time points were moved to 8 and 24 hai respectively. The results presented here include RNA samples where at least 45% of stomata on both upper and lower leaf surface were penetrated and at least 72% of the infection sites contained haustoria by 18 hai and at least 92% by 24 hai. Uninoculated plants were also harvested at these times to differentiate the changes caused by the day/night cycle from those caused by infection.

Optimizing the PCR reaction and silver staining of the gel for the differential display

The PCR cycle parameters used in this study were the same as those described previously for similar types of primers (Doss, 1996; Liang et al, 1995). Varying concentration of nucleotides (0.2 - 5 mM) or primers (0.02 - 0.2 µM) had no detectable effect on the number of bands or the amount of product. Increasing the concentration of MgCl₂ from that supplied in the buffer (1.5mM) had different effects on different
primer combinations, but in general higher concentrations resulted in more product but decreased the number of observed bands (Fig. 34), so that in subsequent experiments 1.5 mM was used. The maximum volume of the cDNA synthesis reaction mixture that could be added to the 23 μl of PCR reaction was 2 μl. Not surprisingly, the number of bands and the amount of product obtained was most dependent on the quality and the amount of RNA in the cDNA synthesis reaction. Even a slight degradation of RNA resulted in PCR products that produced smears rather than distinct bands after electrophoresis. It was determined that the optimum amount of RNA in the cDNA synthesis reaction was 15 μg. Either increasing or decreasing this amount by 25% resulted in significant reduction in the number of bands (Fig. 35). In all experiments, samples from inoculated tissue with both increased and decreased amount of RNA in the cDNA synthesis reaction were run as controls to eliminate false positives (bands that appear but actually are not differentially expressed between samples) due to slight variation in the amount of template. Other controls included no template in the PCR reaction mixture to detect contamination of solutions, and mock reverse transcribed sample (MRT) for which the cDNA synthesis reaction was performed without the enzyme. This was done to identify the PCR products that were due to the incomplete removal of DNA from the RNA sample. Only bands that were differentially expressed in at least two RNA samples from separate inoculation experiments were considered.

Samples were run on a polyacrylamide gel that was considerably smaller (15X30cm) than those normally used for sequencing and radioactively labelled differential display. The gel concentration was increased to 7.2% (instead of 6%). This
was done to allow easier handling and improve resistance to tearing during the staining procedure. This still provided adequate resolution to allow identification of single bands. The staining procedure was based on that described by Bassam et al, (1991), with variations in the concentrations of formaldehyde in the staining and developing solutions, and with an increase in the staining time. The procedure reported here allows for detection of as little as 10 pg of DNA in a single band. Supporting the gel with filter paper during transfer through solutions is preferable to fixing it to glass plates or a polyester backing film as previously described (Bassam et al, 1991), since it is considerably less expensive and gels can be stored for months (and possibly indefinitely) dried on the filter paper as opposed to being destroyed whenever the glass plates need to be reused. Even bands cut out from gels 6 months after they were dried, were successfully reamplified.

_Differentially expressed bands_

Four out of the possible 104 primer combinations were tested. At least four differentially expressed bands were detected, representing putative genes that were upregulated (band5) and downregulated (band6) (Fig. 36) during the formation of haustoria, as well as genes induced by intercellular infection (band3, band4). Here we describe characterization of two of these bands.

Band3 could be detected in samples from inoculated tissue 6, 8, 18 and 24 hai (Fig. 36) but not in comparable samples from uninoculated tissue, while band5 (Figs. 35 and 36) could only be detected in samples from 18 and 24 hai, i.e. after the
formation of intracellular haustoria. Both of these bands probably represent low copy number plant genes since each detected a low number of bands on Southern blots containing plant DNA cut with EcoRI, BamHI and HindIII even under non stringent conditions (Sambrook et al, 1989)(Figure 37B). Neither of these were of fungal origin, as verified by no hybridization to dot blots using at least 30 μg of fungal DNA per dot under stringent conditions (Sambrook et al, 1989). However, under non-stringent conditions the probe from band3 did produce a faint signal, suggesting some sequence homology to a fungal gene (Figure 37A). Probes from either band failed to detect an interpretable signal (faint bands or smears were observed) on Northern blots containing 15 μg of total RNA per lane even after prolonged exposure (up to 14 days).

After cloning into pBluescript, both PCR products were sequenced, and the sequences were subjected to a BLAST search (Altschul et al, 1997). Band3 (Figure 38, appendix 2) had 92% identity at the nucleotide level to a part of BCNT, a bovine brain protein with an unknown function that contains a retrotransposon-like element (Nobukuni et al, 1997). Band3 contained sequence homology to the portion of this protein that corresponded to a bovine reverse transcriptase and was also homologous to a number of animal and viral reverse transcriptases at 30-50% identity and 50-80% similarity at the predicted amino acid level. Among higher plants, only short sequence homology was detected to a number of Arabidopsis and rice ESTs or genomic BAC ends, and a portion of an EST from the salt-induced ice plant.

Band5 (Figure 39) showed only a short region (54 nucleotides) of homology to the human hepatic lipase gene (87% identity at the nucleotide level) and had a 20
nucleotide sequence that was identical to a portion of the variable region of the heavy chain of three mouse immunoglobulins G. Among higher plant sequences, BLAST detected only one uncharacterized genomic clone from Arabidopsis which had 84% identity with Band5 over a 45 nucleotide stretch.
Figure 34. Ethydium bromide-stained agarose gel (2%) showing the effects of changing MgCl₂ concentration on the number and amount of PCR products. Two different primer combinations are shown, both included anchored primer 5'(T)₁₂ VC and either 5'GATCAAGTCC (1) or 5'GATCTGACAC (2) as random-binding 10-mers.
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![Gel Image]

size (kb): -2, -1.5, -0.6
Figure 35. Silver-stained polyacrylamide (7.2%) differential display gel showing samples amplified using 5'(T)$_2$VC-5'GATCAAGTCC primer combination. RNA samples were obtained from two separate inoculation experiments (8, 24 hai for one and 6 and 18 hai for the other). For each lane except the controls, 15μg of total RNA was used in the cDNA synthesis reaction 10% (v) of which was used as template for the PCR. Control lanes: 1,6-mock-reverse transcribed template; 4,7- no template; 2,5- 25% more RNA in cDNA synthesis reaction; 3-25% less RNA in the cDNA synthesis reaction. Arrows point to the size of band5 present only in the inoculated samples 18 and 24 hai.
hai:

inoculated (+/-):

control lanes:

size (kb)

2

1.5

0.5
Figure 36. Enlarged portions of differential display gels showing difference in expression of band3, band5 and band6. Band3 was obtained from the 5'(T)$_{12}$VC-5'GATCATGGTC, band5 (whole gel shown in figure 2) from 5'(T)$_{12}$VC-5'GATCAAGTCC and band6 from 5'(T)$_{12}$VC-5'GATCTGACAC primer combinations. MRT-mock-reverse transcribed control.
hai: 8 8 24 24
inoculated (+/-):
    -  +  -  +
MRT

band3

band5

band6
Figure 37. DNA dot-blot (A) and Southern blot (B) analysis using band3 and band5 as probes under non-stringent (Sambrook et al., 1989) conditions. A: 30µg of fungal or plant DNA was used. Under stringent conditions, there was no signal observed from the fungal sample. B: 15µg of restriction enzyme-digested plant DNA was loaded per lane on an agarose gel (0.7%). Similar pattern was observed under stringent conditions. (Note: band3 results are from two separate blotting experiments as indicated by the division between the lanes.)
Figure 38. Sequence alignment between band3, and portions of the bovine reverse transcriptase (BRT, accession# Z25525) and bovine brain protein BCNT (accession# AB005652). Unmatched bases are indicated in bold letters.
Figure 39. Sequence of band5 (A). Primer sequence is indicated in bold letters, sequence matching portion of the mouse mRNA for IgG variable region (accession# Z22038) and IgM V(H)NZA4 (accession# X63805) are underlined. B: Sequence alignment between portions of band5 and exon1 of the human hepatic lipase gene (accession# M35425), with unmatched bases indicated in bold letters.
### A

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DISCUSSION

The results of this study have demonstrated that there are detectable and specific changes in plant gene transcription during early stages of infection of cowpea by a compatible race of the cowpea rust fungus. These changes have been suspected to occur during plant-rust-fungal interactions since the early reports of changes in general mRNA and protein metabolism in infected plants although most of these studies involved relatively late stages of infection (reviewed by Chakravorty and Scott, 1982; and Bushnell, 1984). Our experiments demonstrated that these changes can be systematically studied by mRNA differential display. In this study, the use of only four out of the possible 104 primer combinations has detected at least four differentially expressed genes, suggesting that other primer combinations may lead to identification of a substantial number of plant genes effected by rust fungus infection.

Differential display has been previously applied to study gene expression during a plant-fungus interaction by Benito et al (1996). They used this method to identify Botrytis cinerea genes expressed only during its infection of tomato plants and not during growth on media. In a control experiment they were able to detect bands originating from the fungus in a mixture of plant and fungal DNA when fungal DNA constituted only 1% of the total. In addition, some of the fungal genes that they identified could only be detected by Northern blot analysis at later stages of infection when fungal growth was more substantial than at the earlier stage used for differential display (Benito et al, 1996). These experiments demonstrate superior sensitivity of differential display compared to Northernms and may explain our failure to detect a
signal from differentially displayed bands on Northern blots using the same mRNA sample. Although DNA contamination of the RNA samples is a common source of bands that can not be detected by Northern.texts (Liang and Pardee, 1995), this possibility can be excluded since the bands described were absent from the RNA samples that were not reverse-transcribed, i.e., in which the only template for the PCR would be the contaminating DNA. The data also confirm previous reports that the sensitivity of differential display is not strictly dependent on the use of radioisotopes in the PCR mixture (eg. Doss, 1996) since I was able to detect rare messages using silver staining of the gel. Additional PCR based methods for differential screening of mRNA have been recently developed. Amplified fragment length polymorphisms (AFLP) is a technique similar to differential display except that primers include adaptor sequences, simplifying the subsequent cloning of the bands (Bachem et al, 1996). Suppression subtractive hybridization is a method for generating differentially regulated cDNA probes based on a PCR reaction in which differentially expressed sequences are selectively amplified from a complex mixture (Diathchenko et al, 1996). Both of these methods are more sensitive than the classical subtractive hybridization-based techniques and will lead to identification of other rare transcripts involved in control of cellular processes. In addition cDNA microarray analysis (Schena, 1996), a novel method for analyzing expression of large numbers of cloned sequences simultaneously could contribute to understanding of pathogen-induced gene expression.

Two putative genes that are differentially expressed during the interaction between the cowpea rust fungus and a susceptible cowpea cultivar were identified in
my study. Band3 was detected in samples from inoculated tissue 6, 8, 18 and 24 hai, corresponding to the stages both before and after the haustorial formation. Southern blot analysis confirmed that this is a plant gene which most likely exists as a single copy in the cowpea genome. Sequence analysis and data base searches indicated that it is similar to the reverse transcriptase sequence that forms a part of a LINE repetitive insert in the cDNA for BCNT, a bovine brain protein of an unknown function (Takahashi et al, 1998). LINEs are a type of retrotransposon that form a family of highly repetitive elements that are interspersed in eukaryotic genomes. They belong to a group called non-LTR type retrotransposons which are distinguished from the LTR-type by the absence of long terminal repeats and some other structural components (reviewed by Weiner et al, 1986). Both LTR and non-LTR-type retrotransposons are found in plants. The best characterized so far are the LTR-type Ty1-copia group which are ubiquitous in plants (Kumar, 1996). Copia-like retrotransposons are commonly not active during normal plant development but are induced by various types of biotic and abiotic stress (Wessler, 1996). The one most extensively studied is the tobacco Tnt1 retrotransposon expression which was studied both in tobacco and as reporter gene constructs in transgenic Arabidopsis and tomato plants. It was found that Tnt1 can be induced by pathogen attacks including compatible and incompatible interactions with Cladosporium fulvum and cucumber mosaic virus in tomato, Pseudomonas syringae in Arabidopsis, and TMV in tobacco. In addition, Tnt1 was induced by biotic elicitors and abiotic stresses such as wounding and treatments with salicylic acid, methylviologen and CuCl₂ (reviewed by Grandbastien et al, 1997). In all of these cases expression of
Tnt1 was restricted to the affected tissue areas suggesting that this is a stress-induced localized response possibly linked to plant defence responses (Grandbastien et al., 1997). It is possible that Band3 is part of a similar element in cowpea, since it is expressed early during infection and could represent a non-specific stress response comparable to the induction of defence gene transcripts by a compatible race of Puccinia graminis observed in barley (Lin et al., 1996, 1998). Low levels of expression, as indicated by the PCR product that could not be detected on conventional Northern blots, could be due to the transcripts being induced only in localized areas surrounding the fungus. However, Band3 shares no significant sequence homology to a family of interspersed Ty1-copia-like retrotransposons found throughout the cowpea genome (Galasso et al., 1997). This is confirmed by our failure to detect multiple bands on southern blots even when they were hybridized to Band3 under non-stringent conditions. This suggests that if Band3 is indeed part of a retrotransposon, it represents an element that has not yet been characterized in plants. This is likely since Band3 shares sequence homology with a number of uncharacterized plant EST's including one from the ice plant that may be induced by salt stress. It is also possible that Band3 is part of a plant retrovirus since it also showed sequence homology to a number of animal retroviruses. This could explain its observed binding to fungal DNA on dot blots under non-stringent conditions, since viral particles have been observed in the cowpea rust fungus throughout its life cycle (Littlefield and Heath, 1979). Alternatively Band3 could be part of a protein unrelated to retrotransposons into which a retroelement or a virus was inserted as is the case with BCNT (Takahashi et al., 1998). It will be possible
to distinguish between these possibilities once the corresponding full length cDNA and genomic clones have been obtained for Band3.

In contrast to Band3, Band5 was detected only in RNA samples from infected plants 18 and 24 hai i.e. after the formation of fungal haustoria, so that it possibly represents a plant gene involved in the establishment of intracellular fungal structures. It is likely that it is a single copy plant gene which shares no significant sequence homology to any fungal genes as evidenced by southern and dot blot analysis. It shares only short stretches of similarity with characterized sequences from the GenBank. One is to a human hepatic lipase gene, the other is to the variable region of the heavy chain of the three different mouse immunoglobulins G. This indicates that the protein may be involved in a metabolic function. Metabolic functions are likely to be altered by infection as suggested by the recent characterization of two haustorium-specific genes from the bean rust fungus coding for a putative amino acid transporter and a plasma membrane H⁺-ATPase, which are thought to aid the metabolite exchange between plant fungus haustoria (Hahn et al, 1997; Struck et al, 1998). Involvement of lipases in the response of plants to biotrophic fungal pathogens is also indicated by the observation of cytochemically detectable lipase activity induced in pea cells by the haustoria of a compatible Erysiphe graminis (Chard and Gay, 1986). However, any speculation on the function of Band5 will have to await characterization of the full length cDNA and genomic clones of the corresponding gene.

In conclusion, I have demonstrated that a sensitive technique such as the differential display can lead to identification of previously unknown plant genes
associated with rust fungal infection of susceptible plants. Future experiments including other sensitive techniques for analysis of gene expression such as Northern blot analysis using purified mRNA, S1 nuclease protection assays and possibly quantitative RT-PCR, may lead to better understanding of the role of Band3 and Band5 in the establishment of compatibility between plant cells and fungal haustoria.
GENERAL DISCUSSION

The results of investigations presented in this thesis indicate that there are differences and similarities between race-cultivar-specific responses of cowpea plant cells to infection by the cowpea rust fungus. Furthermore, it was shown that plant cell responses to intracellular fungal invasion occur early during the interaction and that they can be localized to specific regions of the cytoplasm of the infected cell. This was particularly evident during the establishment of compatibility between the plant cell and the intracellular hypha in the susceptible cultivar CB. Once the fungus has penetrated into the cell, begun tip growth, and reached a hyphal length of 15 μm or more, four distinct regions of host cytoplasm could be recognized by ultrastructural studies and immunocytochemistry at the light microscope level. They include the region of cytoplasm away from the fungus, and the cytoplasm surrounding three parts of the hypha: the plug near the cell penetration point, the middle and tip. The region of plant cytoplasm away from the fungus does not seem to differ from the cytoplasm of uninfected cells in terms of distribution of cytoskeletal elements, endomembranes and other organelles. Cytoplasm surrounding the hyphal middle is thin and mostly devoid of organelles. Near the fungal penetration site and in the cytoplasm surrounding the fungal plug, elaborate membrane profiles, which are not normally seen in the uninfected cells, can be detected by electron microscopy. This region is often devoid of microtubules but contains microfilaments, as demonstrated by immunocytochemistry and histochemistry. It is tempting to assume that the
microtubules are absent in order to accommodate microfilament-based endomembrane vesicle traffic that may be involved in the deposition of the plant cell wall-like collars which were observed around the fungal penetration point in some sites. Notably, both the collars and the absence of microtubules were only observed in a portion of infection sites. However, both the structure and the location of collars in the periplasmic space suggest that they were deposited prior to fungal penetration as discussed by Littlefield and Heath (1979). It is possible that the absence of microtubules, and the membrane elaborations, are related to the formation of the extrahyphal membrane to which some of the elaborations are connected. In view of the close association of microtubules with the plasma membrane and their possible role in structural orientation of the cell wall components (reviewed by Williamson, 1991), the absence of microtubules in this region is likely to facilitate the transition between the original plasma membrane and the newly formed extrahyphal membrane. One of the changes associated with this transition may involve the temporary inhibition of the callose synthase enzyme as demonstrated by the experiments with the heat-killed fungus (Chapter 1). Since the lack of microtubules in the fungal penetration region was only observed in the susceptible cultivar, it is also possible that this phenomenon is a consequence of cytoplasmic changes associated with the suppression of plant defence responses. This may involve changes in cytoplasmic calcium concentration or pH which have been shown to affect microtubule stability (reviewed by Cyr and Palevitz, 1995), as well as the presence or absence of more complex molecules potentially capable of interacting with microtubules such as the suppressors
of defence responses secreted by the fungus. The existence of such suppressor activity has been demonstrated for a number of biotrophic fungi including a rust fungus (reviewed by Knogge, 1997; Baissmann and Kogel, 1992).

The cytoplasm surrounding the fungal tip contains endomembrane vesicles and aggregates of both microtubules and microfilaments which may serve to stabilize the position of the nucleus in this region. Association of the plant nucleus with the fungal tip was dependent on actin microfilaments. One of the possible explanations for this association is that it might be related to the metabolic activity required to accommodate fungal growth which only occurs in this region. Proximity of the nucleus may be required for the synthesis of the extrahyphal membrane which may originate from the nuclear envelope (Heath et al., 1997). Alternatively this association may shorten the distance for diffusion of molecules between the fungus and the plant nucleus and thereby facilitate the plant-fungus signal exchange that results in some of the changes in plant gene transcription including both the suppression and activation of specific genes described in chapter three. Although the cytological and molecular investigations were performed on different infectious stages of the fungus, the similarity of the cytologically detectable responses to the intracellular structures produced during the two infectious stages of the fungus (Heath, 1997) suggests that they may cause similar changes in gene transcription. Therefore, changes in plant gene transcription during the formation of dikaryotic haustoria may be related to phenomena that are common to both infectious stages, such as the suppression of callose synthesis, accumulation of endomembranes in cytoplasmic regions surrounding the fungus, and
the proximity of the nucleus. In particular, it is not surprising that one of the plant genes that is upregulated during the formation of haustoria may code for a lipase-like protein suggesting that it might be involved in lipid metabolism. Upregulation of such a gene may play a direct or indirect role in endomembrane redistribution and membrane synthesis observed during infection. Moreover, suppression of a transcript as detected in this study may be related to the suppression of a particular plant defence response, since the cytological evidence suggests that the fungus is turning off plant defence responses during the establishment of compatibility. Finally, the observed high sensitivity of techniques necessary to detect the changes in gene transcription may reflect the localized nature of these responses, reflecting the localized nature of the cytologically detectable responses. Localized responses and the suppression of plant defences, possibly through suppression of gene transcription, during the compatible interaction are not a unique feature of the cowpea-cowpea rust fungus system but are commonly observed or suspected in susceptible plants infected by other biotrophic fungi as discussed throughout this thesis.

During the hypersensitive cell death in cultivars DC and CC, cortical and perinuclear microtubular arrays seem to be degraded independently of each other and in a different sequence between the two cultivars. It is not clear whether this is a reflection of differences in cytoplasm surrounding each array which may differ during particular stages of the cell death process. In that case the cytoplasmic characteristics which affect stability of microtubules may differ considerably between the regions in the immediate proximity of the nuclear envelope and the cytoplasm opposing the cell
wall. This would suggest that the plant cell is capable of controlling the environment in the two regions independently of each other in response to infection. An alternative explanation to the independent timing of degradation of cortical and perinuclear microtubule arrays would be that either the microtubules or microtubule-associated proteins that stabilize each array are degraded by different proteases which are activated during the hypersensitive cell death in this system (D'Silva et al, 1999). Existence of activation cascades involving multiple proteases during programmed cell death has been documented in animal systems (Thornberry, 1998). Further studies involving the biochemistry of plant microtubules and associated proteins as well as cellular mechanisms of programmed cell death in plants are necessary before the phenomena described in this thesis can be fully understood.

It is interesting that although callose deposition is the main cytologically detectable resistance response in cultivar QA, the HR occurs in a portion of infection sites which may vary depending on experimental conditions from 20-50% so that in some sites both responses are observed. This suggests that these responses may exhibit some functional redundancy. The possibility that one of these defence responses can compensate for the absence of the other is supported by the observation that callose encasement of rust hyphae often occurs instead of the HR in detached leaves of cowpea cultivars which normally exhibit HR-type resistance but require exogenous kinetin to express this response when excised from the plant (Heath, unpublished). The observations that fungal growth increases in resistant plants in which cell death is suppressed by treatments with inhibitors of certain cellular processes (chapter 2; Heath
et al, 1997) suggest that the HR may be arresting fungal growth.

It is intriguing that most of the inhibitors which affected callose deposition in QA also inhibited hypersensitive cell death in other cowpea cultivars which do not respond to rust infection by depositing callose. This is true of inhibitors of transcription and protein synthesis (Heath et al, 1997) which inhibit callose synthesis in general, as well as of antimicrofilament agents (chapter 2) which inhibit only the resistant-associated callose in cultivar QA (Heath and Škalamera, 1996). This raises a question whether the inhibited cellular functions are involved only in the execution of these resistance responses or whether they have an additional role in signal recognition that leads to the expression of resistance. A signalling function seems particularly plausible for actin microfilaments since an antimicrofilament agent reduced the incidence of callose deposits in response to the incompatible race of the fungus but not in response to chemical wounding (Heath and Škalamera, 1996). In addition, hypersensitive cell death was still prevented in resistant plants treated with antimicrofilament agents hours after the microfilament arrays in the cell had recovered from the treatment (chapter 2). Interestingly the hypersensitive cell death in resistant cultivars QA and DC was not inhibited by protein glycosylation inhibitors which specifically inhibited resistance-associated callose deposition in QA (D. Mellersh and Heath, M.C., unpublished), suggesting that if similar molecular pathways are involved in triggering these two phenomena, they may diverge at the level of protein glycosylation.

The existence of different cellular pathways leading to the expression of resistance is strongly suggested by our observations of the cowpea-cowpea rust fungus
system as well as evidence from other plant-pathogens systems. The most obvious evidence is the differences in cellular phenotypes of defence responses expressed by the different resistant cultivars, i.e., callose deposition in QA, fast HR in DC, and rapid cell wall autofluorescence preceding the HR in cultivar CC. Differences in timing of disappearance of separate microtubule arrays during the HR in CC and DC are a less obvious example of this phenomenon. These cultivars are not isogenic lines so that they differ in both genetic background and the number and possibly the type of resistance genes that they carry (Chen and Heath, 1992; Heath, 1994; Ryerson and Heath, 1996). Variability in cytological phenotype caused by different resistance genes is commonly observed during plant-pathogen interactions. Among the better characterized examples of this is the HR response in barley-powdery mildew interactions which occurs after haustorial formation in cultivars carrying Mla resistance genes, but occurs during very early stages of, and possibly prior to, cell penetration in lines carrying Mlg resistance genes (Görg et al, 1993). Separate molecular pathways for these two forms of the HR are indicated by the fact that the expression of Mla-resistance depends on the presence of the Rar1 gene while the Mlg-dependent HR does not. Neither Mla nor Mlg-type resistance expression require Ror1 and Ror2 genes which are required for the expression of the race-nonspecific mlo-type resistance (Peterhänsel et al, 1997 and references therein) discussed in the literature review. In Arabidopsis, a number of loci controlling resistance towards the downy mildew pathogen, Peronospora parasitica, or towards the bacterium, Pseudomonas syringae, have been found to depend on either the presence of the EDS1 or NDR1 gene, but
never both, indicating the presence of at least two pathways leading to resistance (Aarts et al, 1998). Furthermore, *EDS1* and *NDR1*- requiring resistance was not correlated with the type of pathogen, indicating that specificity for either pathway is controlled by the resistance gene locus (Aarts et al, 1998). It is not clear whether these two pathways are diverging at the level of signal transmission as the authors suggest, or at the requirement for some process involved in the actual execution of defence responses.

In summary, the data presented in this thesis add to the growing amount of evidence that biotrophic fungal pathogens trigger numerous responses from the plant cells at the early stages of infection. Some of these responses which may be crucial for the outcome of infection, can only be detected by cytological and sensitive molecular techniques that only recently became available. Future characterization of differentially expressed genes should address the question what is the specific role of these genes during infection. This would involve analysis of full length clones of these genes and their expression patterns during different stages of infection in both resistant and susceptible plants. In addition, investigation of the relationship between the changes in gene expression and cytologically observed responses may help elucidate some parts of the complex signalling pathways between plants and rust fungi that are required either for the establishment of compatibility or for mounting a successful defence against the pathogen.
APPENDIX 1: Diagrams showing the location of callose deposits during the monokaryotic and the dikaryotic stages of infection in the resistant cultivar QA.

**MONOKARYOTIC STAGE INFECTING AN EPIDERMAL CELL**

**DIKARYOTIC STAGE INFECTING A MESOPHYLL CELL**
APPENDIX 2. Alignment of one of the 6 possible predicted amino acid sequences for band3 and the amino acid sequence of *Bos taurus* BCNT (accession# d1022577).

band3 2 IMVKRVRNAVGLGNYNLKNDMISVRFQGKPFNIRVIQVYAPTNAEAEAEVERFYEDLQDL ++VNKRVRNA++G NLKNDMISVRFQGKPFPN+ VIQVYAPT AEE EV RFYEDLQ L
BCNT 308 LIVNKRVRNAIIGCNLKNDMISVRFQGKPFLTVIQVYAPTTPYABEGEYRFYEDLQHL

band3 182 LELTPQKDVLFIIGDWNAKVES*ETPGITGKFGGLGIRNEAGQRLIEFQENALVITYNTL
LE+TP+ DVLFIIGDWNAKV S E PGITG+FGLG++NEAG+RLIEFC N LVITNTLF
BCNT 368 LEITPKIDVLFIIGDWNAKVGSQEIPG+GTRFGGLGMQNEAGRRLIEFCHHNRLVITYNTL

band3 362 QQHKRLYTWSTPGQHRRQIDYICRQRWRNCIQSAKTRPGADCG 499
QQ RRLYTWST P G++R+QIDYI+C QRWR+ +QSAKTRPGADCG
BCNT 428 QQPSRRLYTWSTPDGYRDQIDYIIICRQRWRSSVQSAKTRPGADCG 473
REFERENCES


powdery mildew. Protoplasma 185: 140-151


Chong J, Harder DE, and Rohringer R. (1986) Cytochemical studies on Puccinia graminis f.sp. tritici in a compatible wheat host. II. Haustorium mother cell walls at the host cell penetration site, haustorial walls and the extrahaustorial matrix. Can J Bot 64: 2561-2575


Davidson AD, Manners JM, Simpson RS, and Scott KJ. (1987) cDNA cloning of mRNAs induced in resistant barley during infection by *Erysiphe graminis* f.sp *hordei*. Plant Mol. Biol. 8:77-85


Doke N, Chai HB, and Kawaguchi A. (1987) Biochemical basis of triggering and


Gold RE, Mendgen K. (1984) Cytology of basidiospore germination, penetration and


Gross P, Julius C, Schmelzer E, and Hahlbrock K. (1993) Translocation of cytoplasm and nucleus to fungal penetration sites is associated with depolymerization of microtubules and defence gene activation in infected cultured parsley cells. EMBO. 12: 1735-1744


defense responses. Plant Cell 8: 1773-1791


**Hazen BE, and Bushnell W.R.** (1983) Inhibition of the hypersensitive reaction in barley to powdery mildew by heat shock and cytochalasin B. Physiol. Plant Pathol. 23: 421-438


**Heath MC.** (1971) Haustorial sheath formation in cowpea leaves immune to rust infection. Phytopathology. 61: 383-388

**Heath MC.** (1979) Partial characterization of the electron opaque deposits formed in the non-host plant, French Bean, after cowpea rust infection. Physiol. Plant Pathol. 15: 141-148


**Heath MC.** (1988a) Effect of fungal death or inhibition induced by oxycarboxin or polyoxin D on the interaction between resistant and susceptible bean cultivars and the bean rust fungus. Phytopathology. 78: 1454-1462


Heath MC. (1998a) Involvement of reactive oxygen species in the response of resistant (hypersensitive) or susceptible cowpeas to the cowpea rust fungus. New Phytol. 138: 251-263


Hickey EL, and Coffey MD. (1978) A cytochemical investigation of the host-parasite interface in Pisum sativum infected by the downy mildew fungus Peronospora pisi. Protoplasma 97:201-220


Manners JM, and Scott KJ. (1985) Reduced translatable messenger RNA activities in leaves of barley infected with Erysiphe graminis f.sp hordei. Physiol. Plant Pathol. 26: 297-308


McKeen WE, Rimmer SR. (1973) Initial penetration process in powdery mildew infection of susceptible barley leaves. Phytopathology 63: 1014-1053


Škalamera D, and Heath MC. (1995) Changes in the plant endomembrane system associated with callose synthesis during the interaction between cowpea (Vigna unguiculata) and the cowpea rust fungus (Uromyces vignae). Can. J. Bot. 73: 1731-1738


Škalamera D, and Heath MC. (1997) Callose deposition during the interaction between cowpea (Vigna unguiculata) and the monokaryotic stage of the cowpea rust fungus (Uromyces vignae). New Phytol. 136: 511-524


Staehelin LA. (1997) The plant ER: a dynamic organelle composed of a large number
of discrete functional domains. Plant J. 11: 1151-1165

Stakman EC. (1915) Relation between *Puccinia graminis* and plants highly resistant to its attack. J. Agric. Res. 4:193-199


*infestans.* Plant Cell 3: 619-628


**Xu H, Mendgen K.** (1994) Endocytosis of 1,3-β-glucans by broad bean cells at the penetration site of the cowpea rust fungus (haploid stage). Planta 195: 282-290

