INTRODUCTION

1. Canker stain disease

The canker stain disease is the most important plane fungal disease. The casual agent is the Ascomycete *Ceratocystis fimbriata* f. sp. *platani*, that is able to attack only species of *Platanus* genus (*P. acerifolia*, *P. orientalis* e *P. occidentalis*).

Other populations of *C. fimbriata* isolated from different hosts are able to attack only the natural host, like: potato (*Hypomaea batatas*), coffee (*Coffea arabica*), cocoa (*Theobroma cacao*) and aspen (*Hevea brasiliensis*) (Baker et al. 2003; Webster and Butler, 1976).

The disease was described for the first time in the USA (Jacson and Sleeth, 1935), where around 1920-1940 caused severe injuries in the plane trees of the most important cities of the North America (Walter and Schreiber, 1952). During the second world war the disease was introduced in Europe by infected stuff used to covered war materials. In fact the first centres of infection were: Napoli, Livorno, Siracusa, Marsilia and Barcellona; important harbour cities during the second war (Panconesi, 1973). The disease have assumed an epidemic way causing the death of many plants in streets and parks of many Italian and European cites (Panconesi, 1973; Cristinzio et al., 1973).

1.1 The plant host: *Platanus acerifolia*

Plane trees, genus *Platanus* L., form the only genus in the dicotyledon family *Platanaceae*, in the order *Hamamelidales*.

There are about 6-7 species, all trees from the northern hemisphere, mostly in temperate regions.
Platanus kerrii is found in Indochina, Platanus orientalis in West Asia and South Europe and Platanus occidentalis, P. racemosa, P. wrightii, P. lindeniana are from North America.

All plane trees are large, generally 20-50 m high. Flaking bark that peels away in thin sheets, often leaving a dappled trunk is a common characteristic. However forms occur in which the bark is retained.

Leaves are borne alternately on the stem. They are always simple (not split into leaflets). In most species they are palmate lobed and veined, (in P. kerrii leaves are unlobed, pinnately serrate and pinnately veined) (Fig.1).

The axillary bud on the shoot is covered completely during the growing season by the base of the petiole, which may be swollen to accommodate it.

The shoots and young leaves are covered by hairs or a fine down when young, this is probably to protect the young tissue from sunlight and water loss.

The hairs are usually shed as the leaves mature, but sometimes they are partially retained on the underside of the leaves.

Stipules are often present, part of each stipule forming a tube around the shoot, the rest of it forming a leafy extension.

Male and female flowers are borne on separate inflorescences and they are wind pollinated. The flowers form dense spherical heads, sometimes separately stalked, 1 to 12 on a stem (Fig.1B). The flowers may be covered by hairs as in the leafy shoot. Each female flower becomes an achene, united with the others on the flower-head to form the spherical fruit. Often the styles persist to form prickles on the fruit. The fruits persist through the winter on the temperate species.

Platanus acerifolia belongs to Magnoliopsida class, Hamamelidales order, Platanaceae Dumortier family.

Platanus acerifolia is a majestic plant obtained at the end of the 1600 from the inbreeding of P. orientalis and P. occidentalis (introduced from North America in the 1600 and today disappeared from Europe).

It is difficult to define the precise origin, but there are many evidences that it was present in Great Britain from the 1663 and before of the 1670 it was cultured in the Oxford botanic garden.
**Fig. 1A** Leaves of different plane trees.

**Fig. 1B** Flowers of different plane trees.
The plant is used like ornamental tree in urban areas because it is resistant to environmental pollution and decisive pruning.

There are variation within *P. acerifolia*. The different clones of *P. acerifolia* can vary in most of the visible characteristics. These include the leaf shape and colour, glossiness, colour and density of the hairs covering expanding leaves, bud shape and colour, stipules and their persistence, shoots, fruit size, bark colour, persistence of bark, branching habit, and the straightness of branches. In addition some of these characteristics can vary with the age and vigour of a tree, the season, and according to what part of the tree is being considered. One usually has to look at a combination of these factors to be have any idea which clone a tree belongs to.

1.2 The causal agent: *Ceratocystis fimbriata f.sp.platani*

Name: *Ceratocystis fimbriata* Ellis & Halsted f.sp. *platani* Walter.


Taxonomic position: Fungi; Ascomycetes (Bulletin OEPP/EPPO, 2003)

![Fig.2](image)

**Fig.2**  A) *C. fimbriata* colony and B) *C. fimbriata* conidia.

*Ceratocystis fimbriata* Ell. & Halst., causes serious wilt and canker-stain diseases on a wide range of plants world-wide. Some of the economically important agricultural and tree crops damaged by this pathogen include sweet
potato, cacao, stone fruit trees, poplar, rubber coffee and Eucalyptus spp. (Kile, 1993; Olsen and Martin, 1949; Pontis, 1951; Roux et al. 2000).

Although *C. fimbriata* has predominantly been reported from Central and South America (McCracken and Burkhardt, 1977), its occurrence is geographically widespread in temperate and tropical regions of the world.

The identification of *C. fimbriata* in disease reports and general taxonomic treatments has been based, for the most part, on morphological and cultural characteristics (Upadhyay, 1981).

Isolates from different hosts and geographical areas, however, have been shown to differ not only in colony morphology but also in growth rate and conidial state (Webster and Butler, 1967).

The name, *C. fimbriata f.sp. platani* (*Cfp*), has been assigned to *C. fimbriata* isolates that specifically infect plane trees (*Platanus* spp.). Although morphologically indistinguishable from the type species of *C. fimbriata* from sweet potato, the plane fungus is specifically pathogenic to its hosts. Furthermore, Webster and Butler (1967), in hybridization studies, showed that differences exist in *C. fimbriata* isolates from different hosts and origins.

*Cfp* attacks *Platanus occidentalis*, *P. orientalis* and their hybrid, *Platanus acerifolia*; the hybrid being the most susceptible.

This disease, indigenous to North America (Walter et al., 1952; Sinclair et al., 1987) and reported for the first time in Europe in 1972, has had a dramatic impact on plane trees growing in many southern European cities (Panconesi, 1972; Anselmi et al., 1994).

Recently, Britton et al. (1998) also found *Cfp* in some declining sycamore plantations of North Carolina, USA.

Canker stain can kill a large, vigorous, adult tree in 4±7 years.
1.3 Infective process

*C. fimbriata* f. sp. *platani* is able to attack plane tree only through wounds and roots anastomosis.

The fungus can invade the tree on principal and secondary branches subjected to frequency pruning, on the trunk where there are present many wounds from different origin and on the roots that emerge from the soil.

In the infected area the pathogen reproduce itself in asexual way in the first steps (after 2-3 days from infection) and then in sexual way (after 6-8 days from infection). In the forward days if a advanced to cambium causing the death and then continuing to medulla.

The mycelium penetrates also in lymphatic vessels causing chromatic alterations and cells necrosis.

1.4 Disease symptoms

The most important symptoms are: branches desiccation or whole crown.

On the infected trunks and branches there are the presence of necrotic areas (Fig.3). The tissues attacked from fungus present a characteristic dark and intense colour, sometimes blue, from which derives the disease name. The chromatic alteration can be derived from the wounds presence or fungus migration along the midollari rays, because of conidia dimension the fungus is unable to cross the vessels punctuations.

1.4 Pathogen diffusion and fight methods

Datum that *C. fimbriata* f. sp. *platani* can enter in the plants for contamination of a wound or through the radical anastomoses, the principal responsible for his diffusion is the man. In fact, the prunings, the cuts to apparato root them for the handmade article installation in the ground and above all during the
interventions aiming to the care and to the elimination of the sick plants, remarkable amounts of infected sawdust, in which the parasite can also keep him vital for a long time are produced.

![Fig.3 Disease symptoms. Transversal section of a trunk in which lenticular chromatic alteration are visible in radial sense along the medullary rays.](image)

The sawdust can be transported to work of the wind, of the vehicles in transit along the streets or through the streams, therefore allowing spreading it of the illness.

Of smaller importance instead it turns out the illness diffusion by insects and birds. Also, the infected or dead plants which remain in-place allow micelium to pass to the radical equipment of the next plants.

The elimination of the sick plants turns out, therefore, very important even though contaminated sawdust is produced (EPPO, 2003).
Unfortunately the various types of struggle tested against this illness (surgical balancing, chemical struggle, biological struggle, genetic improvement) have all, for reasons several, a poor effectiveness. In particular, the genetic improvement turns out very difficult because of the homogeneity of the guest. In fact, the totality of the plants cultivated in our plantations is almost formed by *P. acerifolia*, kind to quick increasing and fitted well to our environment, which shows a high genetic uniformity, probably deriving from the agamic diffusion of a narrow number of individuals multiplied prevalently on the road, factor which makes difficult the work of selection and improvement for the resistance to the canker.

Only it has been obtained recently in France, by crossing between plants susceptible of *P. orientalis* and resistant plants of *P. occidentalis*, the first clone of *P. acerifolia* which shows resistance to the coloured canker (Vigouroux and Olivier, 2004).

The only type of struggle which he has supplied with the positive results remains at all today the prevention, based on a few fundamental points: identifying and immediate pulling down of the sick plants; collection and diffusion of the infected material and disinfestations of the pulling down and tools area of work (EPPO, 2003).

In this regard when coloured cancer resistant *P. acerifolia* plants will be commercially available, they will be used to replace with the felled plants, after careful zone disinfestations, seen that the replacement with not resistant plane-trees always has supported the return to the illness after few years.

One understands, therefore, that a more in-depth knowledge of the illness, of the plant-pathogen interaction and the involved fungus factors turn out very important to hope to put point new strategies able to fight the coloured plane-tree canker.

For a bigger comprehension of the plant-pathogen interactions, both necessary having more information than they can show than also a special biochemical answer to an infection is criticizes in the phenomenon resistance (Hammerschmidt, 2003).
1.5 Cerato-platanin

Cerato-platanin (CP) is a small protein produced from the Ascomycete Ceratocystis fimbriata f. sp. platani (Cfp), the causal agent of plane canker stain, in the first growth steps (Walter et al., 1952; Sinclair et al., 1987; Scala et al., 2004) (Fig. 5).

CP is located in the Cfp cell wall of hyphae, conidia and ascospores (Ebbole, 1997; Wessels, 1997) (Fig. 4).

In host and non host plants, Cfp elicits defence-related responses, such as phytoalexin synthesis and cell death, suggesting it is one of the first fungal proteins involved in the recognition process and the elicitation of plant defence mechanisms (Pazzagli et al., 1999; Boddi et al., 2004).

Secreted CP is 120 amino acids in length, has a high percentage (40%) of hydrophobic residues, and contains four cysteines forming two S-S bridges: Cys20-57 and Cys60-115 (Pazzagli et al., 1999) (Fig. 6).

The protein primary structure is very similar to that of other secreted proteins from Ascomycetes, such as the product of the snodprot1 gene from Phaeosphaeria nodorum (accession no. O74238) and from Neurospora crassa (accession no. Q9C2Q5), the allergen Asp f13 from Aspergillus fumigatus (Kurup et al., 2000), the 19 kDa and CS antigens from Coccidioides immittis (Pan and Cole, 1995), and the product of gene sp1 from Leptosphaeria maculans (Wilson et al., 2002). All these proteins have been grouped by the European Molecular Biology Laboratory (EMBL) data bank into a new protein family, the CP family (EBI-InterPro IPR010829).
**Fig. 4** CP localization on the fungus cellular wall (A) Conidia and ascospores give positive result in immuno-gold labelling experiments. (B) Hyphae, conidia and ascospores appear strongly fluorescent in immunofluorescence experiments.

**Fig. 5** Dynamics of the production of CP depending on the time.
They are not always characterized by clear functional similarities, although in some cases they seem to be involved in phytopathological phenomena or immunological reactions: the product of the *snodprot1* gene from *P. nodorum* is produced during infection of wheat leaves (Sharen and Krupinski, 1970); the allergen Asp f13 causes an allergic reaction in humans (Kurup *et al.*, 2000); the CS antigen, a serine proteinase, is produced by the parasitic phase of *C. immitis*, the causative agent of a human respiratory disease (Pan and Cole, 1995); and the sp1 protein by *L. maculans*, the blackleg pathogen of *Brassica napus* (Wilson *et al.*, 2002). Moreover, the N-terminal region of CP is highly homologous with that of cerato-ulmin, a phytotoxic protein belonging to the hydrophobin family and produced by the *Ophiostoma* species responsible for Dutch elm disease (Yaguchi *et al.*, 1993; Pazzagli *et al.*, 1999; Del Sorbo *et al.*, 2000).

**Fig.6. CP amino acidic sequence.** The four residuals of cysteyne involved in the formation of two disolfur bridges and consequent characterized structure from two loops are highlighted.
Hydrophobins are small-to medium sized (75 to 120-amino acid) hydrophobic proteins produced by many saprophytic or pathogenic fungi (Wösten and de Vocht, 2000). The sequence similarity of hydrophobins is generally weak; however, they are characterized by a unique pattern of eight cysteine residues and by the consensus sequence CCN (Wösten, 2001). Hydropathy pattern scan was used to divide these proteins in two classes: class I proteins are highly insoluble, whereas class II proteins, which include cerato-ulmin, dissolve more easily in solvents such as ethanol or sodium dodecyl sulphate (Wessels, 1997). Hydrophobins on aerial hyphae form a water-repellent outer coating with a typical rod let pattern that, by lowering the surface tension, allows the fungus to escape the liquid and grow out into the air (Wösten et al., 1994). In vitro studies show that hydrophobins self-assemble into an amphipathic monolayer at the hydrophobic–hydrophilic interfaces. Some class I members have protein aggregates that resemble those of human amyloid proteins (Torkkeli et al., 2002; Stroud et al., 2003).

For some pathogenic fungi, hydrophobins are essential for pathogenicity because they form an amphipathic membrane that modifies the hydrophobicity of the leaf surface, enabling the fungus to attach itself to the host surfaces (Whiteford and Spanu, 2002). Some hydrophobins also have a structural role in lining the air channels of fruiting bodies. In spite of having four cysteines and a different hydrophathy profile, CP shares some structural and functional characteristics with hydrophobins that associate this protein with the hydrophobin family: it is moderately hydrophobic, it has the CSN sequence aligned with the consensus motif CCN, its secondary structure prediction indicates the presence of mainly β-sheet, and it is an important component of the cell wall of hyphae, ameroconidia, and ascospores (Boddi et al., 2004). In vitro conditions CP shows a distinct tendency to form supramolecular aggregates (Carresi et al., 2006, Pazzagli et al., 2006).

Ten microlitres droplets of CP 100 µM applied on host and non host leaves seem to elicit defence-related events, such as phytoalexin and phenol synthesis, intercellular and intracellular disorganization of spongy parenchyma cells, cell plasmolysis and/or necrosis (Bennici et al., 2005) (Fig.7).
CP induced the synthesis of umbelliferone, the major reference phytoalexin from plane, and of glyceollin, a phytoalexin from soybean, a non-host plant for \textit{Cfp}. In tobacco leaves, cell necrosis and fluorescent phenolic compound synthesis have been demonstrated (Pazzagli \textit{et al.}, 1999)(Fig.8).

\textbf{Fig.7} Sections of leaves of \textit{P. acerifolia} observed to the optic microscope later 24 hours. A) Control B) and C) Leaves treated with CP.
Recently, Alami et al. (1998, 1999) isolated another protein from germinating Cfp spores, glycoprotein GP66, able to elicit the phenolic metabolism and the synthesis of umbelliferone, scopoletin and xanthoarnolin cell suspension cultures derived from the susceptible plane, P. acerifolia. It is known that in most plant-micro organism interactions, plant defence mechanisms are activated at the initial phase of the pathological process (Agrios, 1997; Jackson and Taylor, 1996). This activation needs perception by the host of an external, physical and/or chemical stimulus, and must be rapid for plant defences to be effective against microbial attack.

At the moment, CP seems to be a surface protein suitable for release by Cfp in the extracellular environment; this hypothesis is in agreement with the thesis
that CP is one of the first fungal substances able to interact with the host plane.

1.6 Other fungal secretion proteins without enzymatic activity

1.6.1 Elicitins

Elicitins are highly conserved small proteins secreted by species of the genus *Phytophthora*. Pure elicits can, alone, cause a hypersensitive response, including cell death, and induce systemic acquired resistance in tobacco. Historically, *Nicotiana tabacum* was the plant used to assess biological activity during the purification of elicits, and so their definition is primarily biochemical in nature, as is the understanding of their action. They are thought to be major determinants, or avirulence factors, mediating the basic resistance of tobacco against *Phytophthora* species. The role of elicits in *Phytophthora* biology is not yet clear, but they may function as do fungal hydrophobins, and so they might also act as pathogenicity factors in other plant-pathogen interactions. Several lines of evidence suggest that elicits' effects on responsive plants such as tobacco, may be mediated by a specific binding event which is transduced along signalling pathways to trigger defence responses (LLoyd, 1995).

Plants have the ability to recognize potential pathogens and resist them by inducing various defence mechanisms. Molecules derived from pathogens are targets for plant recognition and can elicit defence responses even in the absence of the pathogen. These elicitors include nonspecific molecules, such as conserved structural components of the fungal cell wall, the bacterial outer membrane or flagella, and specific molecules produced by particular strains of pathogens such as the avirulence proteins (Avr proteins) secreted by some fungi (e.g. Avr9 and AvrL567) and type III effectors produced by some bacteria (e.g. AvrB and PopP2) (Montesano et al., 2003; Dodds et al., 2004; Lahaye, 2004).
Elicitins are small elicitor proteins produced by the pathogenic *Oomycete* genera *Phytophthora* and *Pythium*, although not by all species of *Pythium*. *Phytophthora* species possess a family of elicitors and elicitor-related proteins divided into three classes (Ponchet *et al*., 1999; Tyler, 2002; Baillieu *et al*., 2003; Qutob *et al*., 2003), but the term elicitors generally refers to Class I elicitors, which are secreted abundantly in culture and are well conserved among *Phytophthora* species.

The primary structure of elicitors has been determined after sequencing of purified proteins and/or after sequencing of cloned genes and cDNAs. All known elicitors share a conserved elicitor domain from amino acids 1 to 98. Five different classes have been defined based on the primary structure. Class I-A and I-B enclose 10-kD elicitors that display only the elicitor domain and thus are 98-amino acid-long proteins. Some have an acidic pI, are called elicitors, and belong to class I-A.

Some have a basic pI, are called elicitors, and belong to class I-B. Class II contains highly acidic elicitors, which possess a short hydrophilic C-terminal tail (five to six amino acids long). Class III encloses elicitors with a long (65–101) amino acid C-terminal domain rich in Ser, Thr, Ala, and Pro, an amino acid composition and distribution that suggests potential O-glycosylation sites (Kamoun *et al*., 1997). Elicitors from *Pythium* spp. have been either classified into a distinct group called the *Pythium* spp. group (Kamoun *et al*., 1997) or as a subgroup of class I (Ponchet *et al*., 1999). Although several class I-A and I-B elicitors have been purified to homogeneity and investigated for their biological activity, there are no reports on the isolation and biological activities of class II and class III elicitor proteins. Biological activity of elicitors has been most studied on tobacco (*Nicotiana tabacum*) plants and tobacco cell cultures. Elicitors are usually applied through the vascular system, either by application to the stem of decapitated plants or to the petiole of detached leaves. This mode of treatment leads to the systemic movement of elicitors, with and elicitors being equally well translocated (Devergne *et al*., 1992; Zanetti *et al*., 1992). This property explains elicitor capacity to induce distal HR and systemic acquired resistance (SAR) against fungal
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Phytopathogens (Kamoun et al., 1993; Bonnet et al., 1996; Picard et al., 2000). The elicitin-induced HR is correlated with features of programmed cell death, production of ethylene, and expression of typical defense responses such as phytoalexins and PR proteins (Milat et al., 1991; Keller et al., 1996; Levine et al., 1996). Induction of HR and systemic acquired resistance by elicits has been observed in most species of Nicotiana and in some cultivars of Brassica rapa and Raphanus sativus, but not in Solanum species, Capsicum species, Lycopersicon esculentum, or Arabidopsis (Kamoun et al., 1993; Bonnet et al., 1996; Keizer et al., 1998). Although elicits from different species show various HR-inducing activities, elicits from different Phytophthora species usually elicit HR in the same range of plants, indicating that responsive plants recognize elicits as a conserved feature of Phytophthora species. Thus, elicits seem to be intermediate between general and specific elicitors.

Elicitins have two visible effects on tobacco plants. First, they may cause tissue necrosis, at nanomolar concentrations, and second, they may protect the plant from subsequent pathogen infection. Initially, the stunting of the growth of tobacco plants and the appearance of necrotic lesions on their leaves after inoculation with some Phytophthora species led to the search for a causative factor (Bonnet, 1985).

A toxic activity was isolated from mycelia but was soon isolated in much greater amounts from mycelial culture filtrates. During this early period of characterization the different nature of tobacco's responses to Phytophthora species or their culture filtrates became apparent when necrosis was monitored on leaf tissue after application of active fractions to a distant location: roots, a freshly decapitated stem, or leaf petioles (LLoyd, 1995).

Elicitins have been reported to bind phytosterols with 1:1 stoichiometry, to transfer sterol between phospholipidic membranes, and to pick up sterols from plant plasma membranes (Mikes et al., 1998; Vauthrin et al., 1999). Given that Phytophthora and Pythium species are unable to produce sterols, which are essential for their reproduction (Hendrix, 1970), they must take up sterols from their host plants. Therefore, elicits could be essential factors for the
propagation of Phytophthora and Pythium species (Blein et al., 2002), but the importance of elicitors for pathogen viability or pathogenicity has not been confirmed experimentally.

1.6.2 AVR proteins

Host specificity in plant–pathogen interactions has been described by the gene-for-gene model (Flor, 1942; 1946). This model postulates that, for every dominant gene determining resistance in the host plant, there is a matching dominant gene conditioning avirulence in the pathogen. The simplest biochemical model for perception of an avirulence (AVR) protein by a resistant host plant involves direct interaction of the AVR protein with the matching resistance (R) gene product (Gabriela and Rolfe, 1990; Keen, 1990). Perception of an avirulence protein by the host plant elicits a hypersensitive response (HR), culminating in resistance. To date, a variety of R and Avr genes have been cloned (Takken and Joosten, 2000). However, the number of host–pathogen relationships for which a direct interaction between R and Avr gene products has been detected is still very limited (Luderer and Joosten, 2001). In fact, for most gene-for-gene relationships studied so far, experimental evidence is more consistent with indirect perception of an AVR protein by an R protein than with a direct physical interaction between these proteins (Luderer and Joosten, 2001). Indirect perception of an AVR protein by an R protein implies that at least a third component is required for specific recognition.

The Arabidopsis RPM1, RPS2, and RPS5 NBS-LRR-type R proteins confer resistance by detecting changes in host proteins that are modified by the effector function of their corresponding Avr proteins from the bacterial pathogen Pseudomonas syringae (Mackey et al., 2002; Shao et al., 2003). P. syringae isolates virulent toward these R genes lack the corresponding Avr genes, and population studies of the R gene loci in Arabidopsis have found little genetic diversity and suggest that simple balanced polymorphisms for
functional and non functional alleles have been maintained over long evolutionary time scales (Stahl et al., 1999; Mauricio et al., 2003). The Avr protein that abolish recognition must also result in loss of its effector function, which can have a fitness penalty for the pathogen. Thus there is no selective pressure for diversification of the corresponding \( R \) and \( Avr \) genes. In contrast, recognition by direct \( R–Avr \) protein interaction raises the possibility of a gene-specific arms race leading to diversification of both \( R \) and \( Avr \) genes (Stahl et al., 2000), because such an \( R \) protein in the host may be countered by alterations to the pathogen Avr protein that abolish recognition but retain effector function with little or no fitness penalty to the pathogen. However, there has been very little empirical evidence available to test this prediction. Although many plant \( R \) gene loci are highly polymorphic with diversifying selection playing an important role in the evolution of new recognition specificities (Michelmore and Meyers, 1998), and some pathogen \( Avr \) gene families also show significant variation and diversifying selection (Lahaye and Bonas, 2001), there are few examples where diversity in corresponding \( R \) and \( Avr \) genes has been studied. In one case, diversifying selection and high levels of polymorphism were observed in the corresponding \( ATR13 \) and \( RPP13 \) genes, from \textit{Hyaloperonospora parasitica} and \textit{Arabidopsis}, respectively (Allen et al., 2004; Rose et al., 2004). However, it is not known whether the variation in these genes is associated with differences in recognition specificity as expected if diversification results from \( R–Avr \) counter selection. Conversely, although the \textit{Arabidopsis} \( RRS1-R \) and rice \( Pita \) \( NBS-LRR \) class \( R \) proteins interact directly with their cognate Avr proteins PopP2 and Avr-Pita from \textit{Ralstonia solanacearum} and \textit{Magnaporthe grisea}, respectively (Jia et al., 2000; Deslandes et al., 2003).

### 1.6.3 Idrophobins

Filamentous fungi produce small proteins, hydrophobins, that are localized on the outer surface of their cell walls. These proteins confer water repellent properties to many conidia, hyphae and multicellular structures. They do this
because they form amphipathic membranes whose hydrophobic side is exposed to the exterior whilst the hydrophilic surface is bound to the cell wall polysaccharides. At the time of writing, over 50 hydrophobin sequences from a wide range of species have been deposited in the databases, these include hydrophobins from saprophytic moulds, edible mushrooms, plant pathogen sand mutualistic symbionts (fungi forming mycorrhizae or lichens).

Many of these species have several hydrophobins: for example six different hydrophobin genes have been isolated from both *Pleurotus ostreatus* (Asgeirsdottir *et al*., 1998; Penas *et al*., 1998) and the tomato pathogen *Cladosporium fulvum* (Nielsen *et al*., 2001; Segers *et al*., 1999), and four have been isolated from *Schizophyllum commune* (Wessels *et al*., 1995). Hydrophobins are now generally thought to be ubiquitous in filamentous fungi. There is usually a low level of similarity between the nucleotide sequences of the hydrophobins. The homologies are more apparent when hydrophobin amino acid sequences are compared. All hydrophobins have eight cysteines in a conserved array. The consensus pattern, Xn-CX5–10-C-C-X11–44-C-X8–23-C-X5–9-C-X6–18-C-Xm (Wessels, 1996), is now thought to be one of the distinguishing features of a hydrophobin.

Sequence comparisons show that the hydrophobins can be grouped into two distinct groups: Class I and Class II. This division into two classes based on sequence differences also appears to be reflected in their different solubility. Thus, although both classes of protein self-assemble to form aggregates, the aggregates of Class II hydrophobins can be dissolved in hot SDS or 60% ethanol, whereas the Class I hydrophobins are insoluble in aqueous solvents and can only be dissociated by harsh treatment with organic solvents such as concentrated tri-fluoroacetic or formic acids (Wessels, 1994). Class I hydrophobins were present in filamentous fungi before the separation of the *Basidiomycetes* and *Ascomycetes*.

No Class II hydrophobins have been found so far in the *Basidiomycetes*. Class I and Class II hydrophobins have very little sequence similarity over and above the conserved cysteine arrays. It is possible that Class II hydrophobins have evolved within *Ascomycetes*, independently of the Class I proteins and
after the separation of the *Basidiomycetes*. The similarities between the two classes therefore represent a phenomenon of convergent evolution (Fig. 9).

**Fig. 9.** An un-rooted phylogenetic tree that shows the degree of similarity between hydrophobins whose sequences are deposited in the database. Note that CFT1a, b and c represent the three separate core domains of the Claviceps fusiformis trihydrophobin.

It was demonstrated that all the cysteines in mature SC3 and SC4 of *S. commune* are involved in intra molecular cross-linkage sand that this is likely to be the case for many of the other hydrophobins (de Vries *et al.*, 1993). The topology of cerato-ulmin from *Ophiostoma ulmi* was elucidated. In this protein the cysteines form disulphide bonds with each other in the following configuration: Cys 1 and Cys 2, Cys 3 and Cys 4, Cys 5 and Cys 6, Cys 7 and
Cys 8; thus four loops are formed (Yaguchi et al., 1993). One of the roles for these intra molecular bonds in SC3 has recently been established. The disulphide bridges were reduced and the sulphhydryl groups blocked with iodo acetamide. The resulting proteins were unable to re-form disulphide linkages but self-assembled spontaneously in the absence of a hydrophilic/hydrophobic interface. The characteristics of the membrane formed were indistinguishable from that of unmodified SC3. This suggests that the cysteines oxidized to form intra molecular bonds assist in retaining SC3 in a monomeric, soluble state during passage through the secretory pathway. The disulphide bonds thus inhibit the premature assembly of the hydrophobins prior to secretion into the wall and the growth medium (de Vocht et al., 2000). The rather surprising fact that not all cysteines that might form disulphide bonds are necessary for biological activity has also recently been shown for other cysteine-rich fungal proteins, for example the extracellular elicitors produced by C. fulvum (Luderer et al., 2002). One of the main actions of SC3 is to reduce the surface tension of the growth medium and to allow the hyphae to emerge into the air (Wösten et al., 1999). Such a function may well be shared by other hydrophobins. (Lugones et al., 1998).

Other hydrophobin less mutants from different fungi, for example O. ulmi, also produce fewer aerial hyphae (Bowden et al., 1996; Brasier et al., 1995). Another function of SC3 is to aid maturation of the fungal cell wall: in wild-type S. commune, as the fungus develops, the cell wall glucans become linked to chitin and are thus insolubilized. In the ΔSC3 mutants this does not happen and the soluble glucan is secreted in the medium (van Wetter et al., 2000).

It is becoming apparent that hydrophobins are structural proteins that perform a variety of very different functions. The overall picture is rather complex because not only does each fungus often have more than one hydrophobin, but also some individual hydrophobins have more than one function. The fact that filamentous prokaryotes such as Streptomyces have functional analogues of hydrophobins suggests that these proteins are necessary for filamentous growth and dissemination by spores and conidia. This proposition is further
corroborated by the observation that fungi that do not form true hyphae (e.g. \textit{Saccharomyces cerevisiae}) do not have hydrophobins.

### 1.7. Plant-pathogen interactions

Plants are hosts to thousands of infectious diseases caused by a vast array of phytopathogenic fungi, bacteria, viruses, and nematodes. A relatively small proportion of pathogens successfully invade the plant host and cause disease. Plant–pathogen interactions, particularly those involving biotrophic parasites, are governed by specific interactions between pathogen \textit{avr} (avirulence) gene loci and alleles of the corresponding plant disease resistance (\textit{R}) locus. When corresponding \textit{R} and \textit{avr} genes are present in both host and pathogen, the result is disease resistance. If either is inactive or absent, disease results. The simplest model that accounts for this genetic interaction requires that \textit{R} products recognize \textit{avr}-dependent signals and trigger the chain of signal-transduction events that culminates in activation of defence mechanisms and an arrest of pathogen growth.

Plants recognize and resist many invading phytopathogens by inducing a rapid defence response, termed the hypersensitive response (HR). The HR results in localized cell and tissue death at the site of infection, which constrains further spread of the infection (Dixon \textit{et al.}, 1994) (Fig. 10). This local response often triggers non specific resistance throughout the plant, a phenomenon known as systemic acquired resistance (SAR) (Fig. 10) (Ryals \textit{et al.}, 1996). Once triggered, SAR provides resistance to a wide range of pathogens for days. The HR and SAR depend on interaction between a dominant or semidominant resistance (\textit{R}) gene product in the plant and a corresponding dominant phytopathogen avirulence (\textit{Avr}) gene product, as predicted by Flor (Flor, 1971). It has been predicted that phytopathogen \textit{Avr} products function as
ligands and host R products function as receptors in an interaction leading to plant resistance to disease (Gabriel and Rolfe, 1990; Jones, 1997). Over the past 3 years, numerous R genes were cloned from several plant species. Although these genes confer resistance to diverse bacterial, fungal, viral, and nematode pathogens, their products share striking structural similarities, which suggests that certain signalling events are held in common in plant defence (Dangl, 1995; Staskawicz et al., 1995). The largest class of R genes encodes a ‘nucleotide binding site plus leucine-rich repeat’ (NB-LRR) class of proteins (Fig.11). Plant R genes seem to encode receptors that interact directly or indirectly with elicitors (ligands) produced by pathogen Avr genes (Gabriel and Rolfe, 1990; Jones, 1997). The existence of cytoplasmic and transmembrane classes of R protein indicates that some are specialized to detect secreted ligands or surface components from the pathogen, and some are dedicated to recognize ligands that appear inside the cell. Because of the structural similarities among many cloned R genes, a likely candidate motif for ligand binding is the LRR domain. LRR domains are found in diverse proteins and function as sites of protein–protein interaction, peptide–ligand binding and protein–carbohydrate interaction (Jones and Jones, 1996; Kajava, 1998). In addition, each R protein contains a conserved nucleotide-binding (NB) site, which in other proteins is critical for ATP or GTP binding (Saraste et al., 1990); but it is not clear how or which of these nucleotides is bound.
The plant $R$-$Avr$ gene interaction triggers a signal transduction pathway leading to the HR and SAR. The primary local response in $R$-$Avr$ interaction is induction of the HR and limitation of pathogen growth and spread. ROIs may play a key signaling role in the induction of the HR. $R$-$Avr$ interaction–induced signal transduction events lead to rapid induction of gene expression and defense responses. SA is a molecule that is involved in local (1) and systemic (2) resistance responses (Baker et al., 1997).
Fig. 11 Representation of the location and structure of the five main classes of plant disease resistance proteins. The largest class of R proteins, the NB-LRR class, are presumably cytoplasmic (although they could be membrane associated) and carry distinct N-terminal domains (Dangl and Jones, 2001).

1.8 Plant defence mechanisms

1.8.1 Hypersensitive response (HR)

The resistance of some plants to infection by certain pathogenic strains reflects the presence of disease resistance (R) genes, which are predicted to encode receptors for pathogen-derived molecules (Feys et al., 2000; Baker et al., 1997). The protein products of R genes act at, or near, the beginning of signalling pathways that invoke the defence responses. The absence of R genes renders plants susceptible to infection and in severe cases they may die. In other cases, the plant, although
infected, may outgrow the pathogen for long enough to complete its life cycle. Unlike animals, plants lack an immune system that produces specialized cells, that can attack and eliminate pathogens. Instead, plants have apparently opted for more general defence strategies. One such strategy, which has analogies in animals, is the induction of PCD. In plants, this phenomenon forms part of the hypersensitive response (HR) and its classification is based mainly on morphological criteria of the resultant cell-death lesions as well as the functional suppression of pathogen growth (Morel et al., 1997; Heath, 2000; Heath, 2000b). The HR occurs at the site of pathogen entry and involves PCD in and around the infection site. It is also accompanied by the induction of plant defence responses that serve to confine the pathogen and protect the plant. (Fig. 12 and 12A)

The HR can be triggered by a wide variety of pathogens and occurs within a few hours following pathogen contact. The HR is often conditioned by the presence in the pathogen of an avirulence (avr) gene, the direct or indirect product of which is recognized by a plant possessing the corresponding resistance (R) gene. An interaction leading to disease is termed compatible and, when resistance is effective, the interaction is called incompatible. This specific pathogen recognition accounts for many, but not all, plant disease resistances (Dangl, 1995; Staskawicz et al., 1995). The simplest mechanistic model is that the avr gene encodes a ligand that is recognized by the product of the matching R gene which then triggers the HR and disease resistance (Bent, 1996). In addition, molecules from the pathogen called elicitors are able to trigger HR (Ebel and Cosio, 1994). Plant receptors are also thought to be involved in recognition of these elicitors (Umemoto et al., 1997).

**Fig. 12.** Tobacco mosaic virus/N gene interaction: a classic hypersensitive response (HR) model system. Tobacco leaves (*Nicotiana tabacum* cv. Samsun NN) were either mock treated (left) or inoculated with TMV, U1 strain (right) (Lam et al., 2001).
Fig. 12A. Hypersensitive response in single lettuce mesophyll cells penetrated by haustoria of an incompatible isolate of the biotrophic fungus *Bremia lactucae*. The lettuce *R* gene is *Dm*? (Bennett et al., 1996).

### 1.8.2 Programmed cell death (PCD)

Plants employ PCD mechanisms for the expression of tissue and organ functions, and for efficient nutrition and reproduction (Pennell and Lamb, 1997). Plant PCD occurs in senescent leaves and petals, in germinating seed tissues (such as aleurone layers and embryonic suspensors), in the xylem of vascular bundles, in the tissues of reproductive organs (such as stomium, tapeta, and ovaries), in the reproductive organ primordia of dioecious plants, in root caps, and in cortex that is forming aerenchyma (Pennell and Lamb, 1997). The cells of these tissues or organs display characteristic features as their PCD progresses. The activation of cell-death-associated hydrolytic enzymes, protein degradation, and breakage of nuclear DNA strands are observed frequently (Fath *et al*., 2000; Fukuda, 2000; Sugiyama *et al*., 2000; Funk *et al*., 2002). However, little was known about the regulatory mechanisms that control these events until recent progress was made in
several areas. The induction of cell death in plants seems to be a common response to many different types of biotic and abiotic stress. Cell death associated with the HR may be only one of a larger set of cellular responses that are co-ordinately activated by different stress signals. In terms of combating pathogen invasion, it is most likely involved in restricting viral pathogens that have replicated beyond a certain titre within the plant cell. The timing of this cell-death induction relative to the rate of replication of the virus is apparently critical for the effective prevention of virus escape into the phloem of the plant host, after which point systemic infection will ensue. Cell death may also be involved in restricting the growth of obligate bacterial and fungal pathogens that infect living plant tissues.

Because many fundamental biological mechanisms are well conserved among organisms of different kingdoms, those related to developmental PCD are also expected to share common cytological and molecular biological aspects in different organism (Fig. 13). The developmental PCD is regulated by hormones or intrinsic biologically active molecules. Developmental PCD must be coordinated with other physiological processes, such as growth and differentiation, occurring in the tissue, organ, or individual. This type of regulation may be helpful in orchestrating different physiological processes. ‘Death signals’ are mediated by pleiotropic signal transduction machinery. These pathways also have roles in cell proliferation and specification, and to date, no pathways appear to transduce only developmental death signals. The details of such pleiotropic signal transduction pathways, which are ultimately reflected in different kinds of cellular responses, are largely unknown, but they may also have a role in the coordinated progression of cell proliferation, differentiation and death during development. PCD is accompanied by the activation of specific hydrolytic enzymes, the actions of which often result in apparently shared cytological phenotypes of dying cells (Kaufmann et al., 2001). Instead, plants have a unique PCD process that includes the large lytic vacuole as a main player, as typically observed in tracheary elements PCD (Fukuda, 2000). The degradation of cell contents in tracheary elements PCD starts at vacuolar collapse, which causes the release of hydrolytic enzymes and
allows them to attack organelles (Fig. 14). For example, degradation of nuclear and chloroplast DNA can be completed within 20 minutes of vacuole rupture, whereas chlorophyll is degraded much more slowly (Obara et al., 2001).

Fig. 13. (a) In metazoan developmental PCD, hormone, cytokine, and growth factor signals are transduced to invoke gene expression that results in the activation of apoptotic machinery. (b) Plant cells undergo developmental PCD in response to hormone regulated signalling and gene expression. These signals can also regulate growth and differentiation in certain specific cases. (Kuriyama and Fukuda, 2002).

Fig. 14. Brassinosteroids (BRs) induce PCD, as well as the formation of secondary walls. PCD-specific hydrolytic enzymes, such as an S1-nuclease, RNases and cysteine proteases, are synthesized and accumulate in the vacuole. The transport of organic anions into the vacuole is inhibited, in association with the enlargement of the vacuole. The enlarged vacuole bursts, then shrinks and fragments. The collapse of the vacuole causes hydrolytic enzymes to invade the cytoplasm and to attack various organelles, resulting in the degradation of cell contents and part of the cell walls.
1.8.3. Reactive oxygen species (ROS), Nitric oxide (NO) and salicylic acid (SA).

The introduction of molecular oxygen (O2) into our atmosphere by O2-evolving photosynthetic organisms 2.7 billion years ago, reactive oxygen species (ROS) have been the unwelcome companions of aerobic life (Halliwell and Gutteridge, 1989). In contrast to O2, these partially reduced or activated derivatives of oxygen (singlet oxygen, superoxide anion, hydrogen peroxide and hydroxyl radical) are highly reactive and toxic, and can lead to the oxidative destruction of cells (Asada and Takahashi, 1987). Consequently, the evolution of all aerobic organisms has been dependent on the development of efficient ROS-scavenging mechanisms. In recent years, a new role for ROS has been identified: the control and regulation of biological processes, such as growth, cell cycle, programmed cell death, hormone signalling, biotic and abiotic stress responses and development (Kovtun et al., 2000; Foreman et al., 2003). The use of ROS as signalling molecules by plant cells suggests that, during the course of evolution, plants were able to achieve a high degree of control over ROS toxicity and are now using ROS as signalling molecules. Controlling ROS toxicity while enabling ROS such as H\textsubscript{2}O\textsubscript{2} or O\textsuperscript{2-} to act as signalling molecules appears to require a large gene network (Fig.15).

Most forms of biotic or abiotic stress disrupt the metabolic balance of cells, resulting in enhanced production of ROS. Simple organisms, such as bacteria or yeast, sense the enhanced production of ROS using redox-sensitive transcription factors and other molecular sensors, activate different ROS defence pathways, and regulate their metabolic pathways to lower the production rate of ROS (Costa and Moradas-Ferreira, 2001; Georgiou, 2002). This ‘basic cycle’ of ROS metabolism maintains a low steady-state level of ROS in cells. Variations on this pathway could have originated during evolution and contributed to the use of ROS as signalling molecules to control more specialized processes such as plant growth and defence, hormonal signalling and development (Fig.16).
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Fig. 15 Modulation of reactive oxygen species (ROS) signalling by the reactive oxygen gene network of plants. Different cellular signals (e.g. pathogen recognition or stress perception) result in the enhanced production of ROS in cells by the ROS-producing pathways of the network. ROS are perceived by different ROS sensors and activate cellular responses (e.g. pathogen or stress defence) (Mittler et al., 2004).

Fig. 16. Generalized model of the reactive oxygen species (ROS) signal transduction pathway. ROS can be detected by at least three mechanisms (ROS receptors, redox sensitive transcription factors and phosphatases). Detection of ROS by receptors results in the generation of Ca2+ signals and the activation of a phospholipase C/D (PLC/PLD) activity that generates phosphatidic acid (PA). PA and Ca2+ are thought to activate the protein kinase OXI1. Activation of OXI1 results in the activation of a mitogen-activated protein kinase (MAPK) cascade (MAPK3/6) and the induction or activation of different transcription factors that regulate the ROS-scavenging and ROS-producing pathways. The activation or inhibition of redox-sensitive transcription factors by ROS might also affect the expression of OXI1 or other kinases and/or the induction of ROS-specific transcription factors. Inhibition of phosphatases by ROS might result in the activation of kinases such as OXI1 or MAPK3/6. Two different loops are shown to be involved in the ROS signal transduction pathway. A localized or general defense response (a negative feedback loop; solid green line) can be activated to suppress ROS, whereas a localized amplification loop (positive feedback loop; red dashed line) can be activated to enhance ROS signals via the activity of NADPH oxidases. Salicylic acid (SA) and nitric oxide (NO) might be involved in this amplification loop as enhancers. Abbreviations: HSF, heat shock factor; PDK, phosphoinositide-dependent kinase; TF, transcription factor (Mittler et al., 2004).
Much effort has been devoted to identification of the earliest responses to pathogen invasion. The earliest detectable cellular events are ion fluxes across the plasma membrane and a burst of oxygen metabolism that produces reactive oxygen intermediates (ROIs), such as superoxide and hydrogen peroxide (Scheel, 1998). A clear causal link between these events and defence induction was demonstrated in cultured cells of parsley, in which elicitor-induced ion fluxes are required for induction of the oxidative burst, but not vice versa (Jabs et al., 1997) (Fig. 17). The oxidative burst is in turn required for activation of defence gene induction and production of antimicrobial metabolites.

Fig. 17. Regulation of local and systemic defence responses by ion fluxes, reactive oxygen intermediates (ROIs), nitric oxide (NO) and salicylic acid (SA). Receptor-mediated ion fluxes trigger localized production of NO and ROIs immediately after pathogen recognition. These second messengers synergistically induce cell death, defence gene expression, and production of SA and more ROIs, establishing a putative feedback loop in which the response is amplified.
The role of ROIs in the defence response is particularly interesting and controversial at the moment. ROIs have been associated with apoptosis of mammalian cells, indicating a role in cell death during the HR in plants (Lamb and Dixon, 1997).

Two putative accomplices have been identified in recent investigations of interactions between salicylic acid (SA), nitric oxide (NO) and ROIs. SA has long been associated with defence induction in plants: exogenous SA is sufficient to induce plant defence gene expression and systemic acquired resistance (but not cell death) (Ryals et al., 1996). NO collaborates with ROIs to trigger cell death in the mammalian immune response (Hausladen and Stamler, 1998) and many studies provide evidence that NO also interacts with ROIs and SA in plants to induce the HR and defence gene expression (Durner et al., 1998; Delledonne et al., 1998).

How might these three small molecules interact to promote defence induction? An important observation in the above studies is that the contributions of NO, SA and \( \text{H}_2\text{O}_2 \) appear to be synergistic rather than additive, implying that they interact directly and cooperatively in a signal amplification mechanism. ROIs and NO stimulate SA biosynthesis, and SA in turn potentates ROI–NO-dependent responses. These features suggest that receptor dependent pathogen perception triggers a positive feedback loop of ROI–NO production and SA accumulation, which rapidly amplifies the initial signal and guarantees timely defence activation (Fig. 11). Culmination of this cycle in HR cell death could release ROIs, NO and SA into intercellular spaces, and these compounds could directly inhibit pathogen growth or ‘warn’ neighbouring cells of an imminent invasion, or both.

### 1.8.4 Jasmonic acid and ethylene

The ROI–SA–cell-death response, described above, has received the majority of recent experimental attention. However, this response is not germane to every plant–pathogen interaction. For example, recent genetic studies in
Arabidopsis have revealed resistance responses that operate independently of SA accumulation and are mediated by jasmonic acid (JA) and the gaseous hormone ethylene (ET) (Dong, 1998). JA and ET are also plant growth regulators, suggesting overlap between the regulatory components of development and defence. ET–JA-dependent responses are utilized differentially against pathogens with contrasting modes of attack (Thomma et al., 1998) (Fig. 12). The ET–JA-dependent defence response is activated by pathogens that kill plant cells to obtain nutrients. In contrast, the SA-dependent response is triggered by a pathogen that obtains nutrients from living plant tissue. This observation raises the intriguing possibility (yet to be generalized) that plants can activate distinct defence responses tailored to specific types of parasites. Several studies have also suggested that the ET–JA and SA responses are mutually inhibitory (Dong, 1998). Such potential cross talk is again suggestive of a capacity for selective defence deployment.

**Fig. 18.** Genetically defined relationships between salicylic acid (SA)-dependent and ethylene–jasmonic acid (ET–JA)-dependent defence responses in Arabidopsis. The SA-dependent response is deployed against a biotrophic pathogen that obtains nutrients from living cells, whereas the ET–JA response is activated by necrotrophic pathogens that kill plant tissue.
1.8.5 Pathogen related proteins (PR)

PRs are implicated in plant defence, they have not been identified because of their anti-pathogenic action, but solely because of their accumulation in infected plants. Eleven families of PRs have now been officially recognized (Van Loon et al., 1994), but additional pathogen-induced proteins with potential anti-pathogenic action keep being described (e.g. Broekaert et al., 1995). PRs have been identified in at least nine plant families, with those in tobacco and tomato characterized best. It is now known that they comprise four families of chitinases (PR-3, -4, -8 and -11), one of 1,3-glucanases (PR-2), one of proteinase inhibitors (PR-6), and one specific peroxidase (PR-9), as well as the PR-1 family with unknown biochemical properties, the thaumatin-like PR-5 family, and the birch allergen Betv1-related PR-10 family. Not all families are represented in any plant species, but each family may comprise several members. Together the PRs form a set of pathogen-induced proteins that may be considered as stress proteins. It has been suggested that in induced plants the accumulated intercellular proteins form the first line of defence to a challenging pathogen and, if this fails and the tissue is disrupted, the release of the vacuolar PRs functions as a second line, engulfing the pathogen with lytic enzymes (Mauch and Staehelin, 1989).

1.8.6 Signalling mechanisms

Signal transduction cascades link recognition and defence responses through second messengers conserved among most eukaryotes.

In plants, no major differences in signalling mechanisms have been observed upon perception of race cultivar-specific or general elicitors (PAMPs) (Nurnberger and Scheel, 2001). However, individual recognition events appear to dictate specific signalling routes that employ a distinct set of secondary messengers and activate a characteristic portion of the complex defence machinery. Changes in cytoplasmic Ca\(^{2+}\) levels, the production of
reactive oxygen species and nitric oxide (NO) as well as the post-translational activation of MAPK cascades are commonly reported to signal the activation of innate immune responses in plants (Jonak et al., 2002; Nurnberger and Scheel, 2001).

Plasma membrane-located plant Ca\textsuperscript{2+} channels were shown to be responsive to the oomycete elicitor Pep-13 (Zimmermann et al., 1997) and to race-specific elicitors from Cladosporium fulvum (Gelli et al., 1997). Moreover, PAMP induced influx of extracellular calcium causes transient elevation of cytosolic Ca\textsuperscript{2+} levels (Lecourieux et al., 2002). Elevated levels of cytoplasmic calcium are crucial signal transduction components in animal innate immunity as well. Pharmacological evidence is provided for the requirement of influx Ca\textsuperscript{2+} from the extracellular space in plant cells, the participation of internal stores in elevating cytosolic Ca\textsuperscript{2+} levels can not be ruled out (Lecourieux et al., 2002; Galione and Churchill, 2002).

Nitric oxide is an essential factor for the activation of innate immune responses in humans as well as in insects (Underhill and Ozinsky, 2002). The same molecule was found to be produced upon treatment of plants with PAMPs as well as upon pathogen infection, suggesting that it may be important for the activation of innate defence mechanisms (Clarke et al., 2000). Very recently, it has been reported the biochemical purification of a tobacco nitric oxide synthase (NOS) in which enzymatic activity is activated upon pathogen infection (Chandok et al., 2003).

Mitogen-activated protein kinases constitute central points of cross-talk in stress signalling in plants including the protection against microbial invasion (Barton and Medzhitov, 2003). Mitogen-activated protein kinase (MAPK) cascades are major components downstream of receptors or sensors that transduce extracellular stimuli into intracellular responses in yeast and animal cells (Widmann et al., 1999; Davis, 2000). The basic assembly of a MAPK cascade is a three kinase module conserved in all eukaryotes. MAPK, the last kinase in the cascade, is activated by dualphosphorylation of the Thr and Tyr residues in a tri peptide motif (Thr–Xaa–Tyr, where Xaa could be Glu, Gly, Pro or Asp) located in the activation loop (T-loop) between subdomains
VII and VIII of the kinase catalytic domain. This phosphorylation is mediated by a MAPK kinase (MAPKK or MEK), which, in turn, is activated by a MAPKK kinase (MAPKKK or MEKK). There are multiple members of each of the three tiers of kinases in a cell, which contribute to the specificity of the transmitted signal (Widmann et al., 1999).

Because plants are sessile, they must adapt to adverse environmental conditions by adjusting their metabolism. Thus, it is important to be induced by various elicitors and pathogens (Zhang et al., 2000; Romeis et al., 1999).

Other members in the same subfamily such as \textit{MMK4} from alfalfa (later called \textit{SAMK}), \textit{MPK3} from \textit{Arabidopsis} and \textit{ERMK} from parsley (\textit{Petroselinum crispum}) are induced likewise at the mRNA level (Jonak et al., 1996; Ligterink et al., 1997). Their counterpart in wheat, \textit{TaWCK1}, is also activated transcriptionally by a fungal elicitor (Takezawa, 1999). These results suggest that stress induced transcription of genes for MAPKs evolved early in plants, before the divergence of dicots and monocots, and might play an important role in plant defence responses (Fig. 13). The transcripts of a second subfamily of MAPKs represented by \textit{MsTDY1} and \textit{OsBWMK1}, are induced by wounding and pathogen infection as well (He et al., 1999).

Although members of MAPK subfamilies I, II, III and V have been implicated in plant stress responses, their exact functions remain elusive. An increasing body of evidence suggests that a subset of plant responses to biotic and abiotic stresses is shared, such as the generation of reactive oxygen species (ROS) and the activation of early defence genes (Somssich and Hahlbrock, 1998).

MAPKs are likely to be one of the converging points in the defence-signalling network (Fig. 18). In animal and yeast cells, most of the substrates for stress-activated MAPKs are transcription factors (Widmann et al., 1999; Davis, 2000). Activation of a MAPK leads to the phosphorylation of transcription factors, which, in turn, activate gene expression. In support of a similar role in plants, a MAPK was shown to translocate into the nucleus upon activation in parsley cells treated with the Pep25 elicitor (Ligterink et al., 1997). However, to date, no substrates have been identified for any plant MAPKs.
Fig. 18 Convergence of various stress stimuli onto mitogen-activated protein kinase (MAPK) pathways. MAPK cascades are implicated in signalling defence responses of plants that are under several biotic and abiotic stresses, including pathogen invasion, wounding, high salinity, high or low osmolarity, extreme temperature, drought, reactive oxygen species (ROS), ozone, and ultraviolet (UV) irradiation.