SECTION 10

Host Reactions – Plants

Pole 5

10.1 Host Reactions – Plants

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INTRODUCTION

Essential features of the various interactions occurring between bacteria and plants are summarized in Table 10.1. It should be noted that there are important differences in terminology applied to plant pathogenic bacteria and to animal pathogens. 'Pathogenicity' refers to the fundamental capacity of bacteria to invade plant tissues causing disease. 'Virulence' describes ability to invade a susceptible cultivar of the host plant, whereas 'avirulence' means failure to cause disease on resistant but not susceptible cultivars. In the plant pathology literature we therefore find strains described as 'pathogenic but avirulent', for example among pathovars of *Pseudomonas syringae* such as *P. s.* pv. *phaseolicola*, in which ability to cause disease in bean is based on the interaction between resistance genes in the host and avirulence genes in the pathogen (Mansfield *et al.*, 1994).

Perhaps the most common interaction for a plant pathogen is that occurring following dispersal to a non-host plant. If bacteria are able to enter plant tissues, a process which is normally dependent upon rain or mechanical damage, they will fail to multiply due to the activation of non-host resistance. In most, but not all cases, the expression of non-host resistance involves a hypersensitive reaction (HR) by the challenged tissue.

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Table 10.1. Summary of plant–bacterium interactions

Level of interaction and bacterial strain	Plants in general (non-hosts)	Host species	
General interactions		*Susceptible cultivar*	*Resistant cultivar*
Saprophyte	N[a]	–[b]	–
Animal pathogen	N	–	–
Plant pathogen	HR[c]	D[d]	HR
hrp mutant of plant pathogen	N	N	N
Varietal specificity			
Plant pathogen[e]		*Cultivar 1*	*Cultivar 2*
Race 1	HR	HR	D
Race 2	HR	D	HR

[a] N, null reaction; no symptoms of infection.

[b] Not applicable.

[c] HR, hypersensitive reaction; note that a few cases of non-host resistance are not expressed by the HR. [d] D, disease development.

[e] The races would be described as virulent or avirulent depending upon their respective success or failure to colonize susceptible or resistant cultivars.

The HR, which can be defined as the rapid necrosis of challenged plant cells leading to restricted multiplication or growth of an invading microorganism, also occurs during the expression of varietal resistance (Alfano and Collmer, 1996; Mansfield *et al.*, 1997). Cell death during the HR is associated with several biochemical changes within infected tissue including alterations to the plant cell wall and accumulation of phytoalexins, which generate antimicrobial conditions (Hahlbrock *et al.*, 1995). Major determinants of pathogenicity are the clusters of *hrp* genes, which control the ability of bacteria to cause the HR in resistant plants (whether host or non-host) and also the ability to cause disease in susceptible host cultivars (Bonas, 1994).

Recent studies comparing the development of wild-type and *hrp*$^-$ strains have drawn attention to the highly localized nature of responses occurring within challenged plant cells at sites of contact with bacteria; for example the deposition of phenolics, callose, and hydroxyproline-rich glycoproteins (HRGPs), and the production of active oxygen species (AOS). The development of our understanding of the elegance and structural complexity of the plant's resistance response has been achieved through the application of immunocytochemical and histochemical techniques at the level of electron microscopy (Bestwick *et al.*, 1995, 1997; Brown *et al.*, 1995).

Similar ultrastructural studies have also allowed the invasion of susceptible plants to be examined and mechanisms of pathogenicity dissected. The majority of bacterial pathogens are intercellular, multiplying in the spaces between plant cells (e.g. in the mesophyll tissue of leaves). However, several economically important pathogens, such as

Xanthomonas campestris pv. *manihotis*, are adapted to invade vascular tissues, particularly xylem vessels, within which bacterial multiplication may disrupt water flow and cause wilting. The lignin lined microenvironment of the vascular system provides particular challenges for the pathogen and the plant. In addition to variations in the tissue of choice for the bacterial pathogen, there are striking differences in the mechanisms of pathogenicity used. Soft-rotting pathogens are characterized by the production of a battery of plant cell wall degrading enzymes leading to the development of a lesion within which the bacterium feeds from killed tissues (Dow and Daniels, 1994). By contrast, many bacteria have a temporarily biotrophic phase of colonization, multiplying within the intercellular spaces, but initially causing no major changes to the invaded tissue. A good example of such a biotroph, which does not degrade its host's cell walls, is the pepper and tomato pathogen *X.c.* pv. *vesicatoria* (Brown *et al.*, 1993; Van den Ackerveken *et al.*, 1996).

The aim of this chapter is to illustrate how microscopy has been and may be applied to examine plant–bacterium interactions; emphasis is on the localization of responses in plant cells. A fascinating area of study concerns activation of the oxidative burst leading to AOS accumulation and subsequent intracellular signalling (Sutherland, 1991; Tenhaken *et al.*, 1995; Low and Merida, 1996). At present, few microscopical techniques have been developed to examine these topics in plants, but they provide good targets for which the close integration of ultrastructural and biochemical studies will be needed to develop a full understanding of bacterial pathogenesis and the subtle coordination of the plant's response occurring at the subcellular level. Such analyses will be greatly improved by the recent cloning of genes for resistance to bacterial infection and the emerging pattern that the encoded plant proteins may react directly with avirulence gene proteins to activate the HR (Staskawicz *et al.*, 1995; Scofield *et al.*, 1996; Van den Ackerveken *et al.*, 1996). As the receptors and ligands that determine the plant's response become more fully characterized, the signaling cascades leading to localized reactions will require detailed biochemical and structural clarification.

In the following sections some important components of the plant's response are introduced with reference to well-studied examples. The general methods described for examination of interactions by electron microscopy are applicable to most plant–pathogen combinations. The section 'Cellular Responses in Lettuce Challenged by *P. syringae* pv. *phaseolicola*: wild-type strains and *hrp* mutants' focuses on work which has allowed good definition of cell wall alterations occurring during resistance responses to non-pathogenic *hrp* mutants and demonstrated that wall alteration does not always require activation of the HR (Bestwick *et al.*, 1995). Mechanisms of pathogenicity and, in particular, cell wall degradation have been investigated thoroughly in work on the vascular pathogen *X. c.* pv. *manihotis* described in 'Invasion of Cassava by *X. campestris* pv. *manihotis*'. Analysis of degradative changes, which occur in the plant cell wall adjacent to invading bacteria has become an important model for the application of new methods in electron microscopy to plant tissues (Boher *et al.*, 1996; Vian *et al.*, 1996). Localization of secondary

metabolites has often been pursued by histochemistry, but more specific analyses require immunocytochemical methods. Recent successes in the detection of glucosinolates are described using approaches which should be adopted for work on other potentially antimicrobial compounds, in particular, phytoalexins. Alternative approaches using fluorescence microscopy to examine certain phytoalexins, notably those from cotton, are also considered in 'Localization of Phytoalexins'. This chapter concludes with comments on recent developments in our understanding of the mechanisms of signal exchange between bacteria and plants. The role of the plant cell wall as a barrier to the establishment of successful parasitism is emphasized.

◆◆◆◆◆◆ CELLULAR RESPONSES IN LETTUCE CHALLENGED BY *P. syringae* PV. *phaseolicola*: WILD-TYPE STRAINS AND *hrp* MUTANTS

The interaction between lettuce and *Pseudomonas* has provided a useful model for the application of electron microscopy to examine bacterial development and the plant's response. Microscopical studies have been combined with physiological and biochemical analyses. Inoculation of wild-type *P.s.* pv. *phaseolicola* into lettuce leaves induces a rapid HR. Tissues at inoculation sites collapse within 15 h, becoming brown and papery 24 h after infiltration. No macroscopic symptoms develop following challenge with *hrp* mutants. Key features of the plant's response, membrane damage (assessed by electrolyte leakage or failure to plasmolyse), and accumulation of the phytoalexin lettucenin A are summarized in Fig. 10. 1. Clearly, major changes in challenged tissues are associated with the macroscopic HR. However, when cellular reactions are analyzed, a more complex pattern emerges in which localized responses are observed in cells next to *hrp* mutants.

Conventional Electron Microscopy

Conventional ultrastructural studies, using transmission electron microscopy (TEM), enable the nature of the plant–pathogen interface to be observed, often providing clues to the biochemical basis for resistance or susceptibility. As with mammalian cell biology, ultrastructural analysis is now being increasingly adopted to provide a descriptive nomenclature for plant cell death events (Brown and Mansfield, 1988; Bestwick *et al.*, 1995; Levine *et al.*, 1996).

Unfortunately, most conventional fixation protocols offer degrees of fixation that may cause structural alterations that prevent sensitive cytochemical or immunocytochemical analyses. However, the greater degree of preservation and resolution of cellular structure usually afforded by conventional EM is useful in placing subsequent cyto- and

immunocytochemical studies within the context of the structural framework of the cell. Although cryofixation offers considerable benefits in the preservation of cell ultrastructure (Hoch, 1991; Rodriguez-Galvez and Mendgen, 1995), the relatively high cost of equipment and poor depth of tissue preservation often observed ensures that conventional fixation

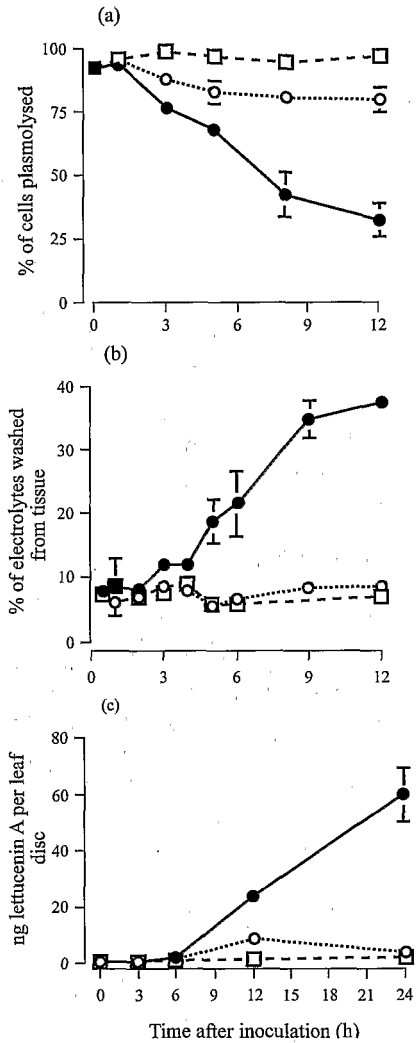

Figure 10.1. Characterization of responses of lettuce cells to wild-type and *hrp*⁻ strains of *Pseudomonas syringae* pv. *phaseolicola* (from Bestwick *et al.*, 1995). (a and b) Detection of membrane damage. (a) Numbers of cells retaining the ability to plasmolyze within inoculated tissues. Tissues were plasmolyzed in 0.8 M KNO_3 before fixation and embedding for conventional electron microscopy. Data are presented as means ± SE from eight separate experiments. (b) Electrolyte leakage from inoculated tissues. Data are the means ± SE from three experiments. (c) Phytoalexin accumulation. Levels of lettucenin A accumulation within inoculated tissues are presented as means ± SE of three independent extractions of leaf disks cut from inoculation sites. (Closed circles, wild-type; open circles, *hrp* mutant; open squares, inoculation with water alone)

protocols remain the most common method in the study of plant–pathogen interactions. A typical protocol for preparation of lettuce leaf material for examination by TEM is outlined in Table 10.2.

The preservation of cellular detail obtained with lettuce using this conventional procedure is shown in Figs 10.2 and 10.3. Use of the standard EM approach has revealed that *hrp* mutants, although failing to cause the HR, do induce localized changes in the plant cell wall and cytoplasm. Particularly striking is the formation of paramural deposits or papillae adjacent to attached bacteria (see Fig. 10.2). The localized reactions to *hrp* mutants do not include the cytoplasmic disorganization characteristic of

Table 10.2. Routine method for the preparation of leaf tissue for electron microscopy

1. Quickly excise small pieces of leaf tissue (10 mm × 5 mm) and place under a drop of cold fixative (4°C). Fixative comprises 2.5% (v/v) glutaraldehyde in 50 mM piperazine-*N'N'*-bis (2-ethane sulfonic acid) (PIPES) buffer at pH 7.2 .
2. Cut sample into approximately 2 mm^2 pieces using a new razor blade and place in fixative for 12 h at 4°C or 4 h at room temperature (RT).
3. Wash in PIPES (10 min × 3).
4. Fix in 2% (v/v) osmium tetroxide in PIPES buffer for 2 h.
5. Wash in PIPES (10 min × 3).
6. Dehydrate in a graded series of increasing acetone concentrations – 50%, 70%, 80%, and 90% (v/v) acetone – 10 min each incubation, followed by three changes of 100% acetone of 20 min duration each.
7. Progressively embed in epon–araldite at 3:1 acetone:resin, 15 min; 2:1 acetone:resin, 12 h or overnight; 1:1 acetone:resin, 15 min; 1:2 acetone:resin, 15 min; 1:3 acetone:resin, 15 min; fresh resin, 24 h followed by a further change of fresh resin for 4 h.
8. Transfer to fresh resin in block molds and polymerize at 60ºC for 48 h.
9. Cut ultra-thin sections (70–90 nm) using a diamond knife (e.g. Diatome, Bienne, Switzerland) on a suitable ultramicrotome (Reichert Ultracut E, Milton Keynes, UK) and mount on 300 mesh uncoated copper grids (Agar Aids, Bishop's Stortford, UK).
10. Grid-mounted sections may be stained with uranyl acetate and lead citrate, the method being a minor modification to the procedure described by Roland and Vian (1991). The grids are immersed for 50 min, in the dark, in a solution of 4.5% (w/v) uranyl acetate, in 1% (v/v) acetic acid, at RT. After treatment, grids are washed in distilled water and transferred to individual drops of lead citrate, the lead citrate being prepared as described by Reynolds (1963). Before staining, drops of lead citrate are pipetted onto dental wax in a Petri dish, the base of which should contain filter paper (Whatman No.1) soaked in 1 M sodium hydroxide to serve as a trap for CO_2. Staining in lead citrate is usually undertaken for 2–4 min and grids are then washed in distilled water and allowed to dry.

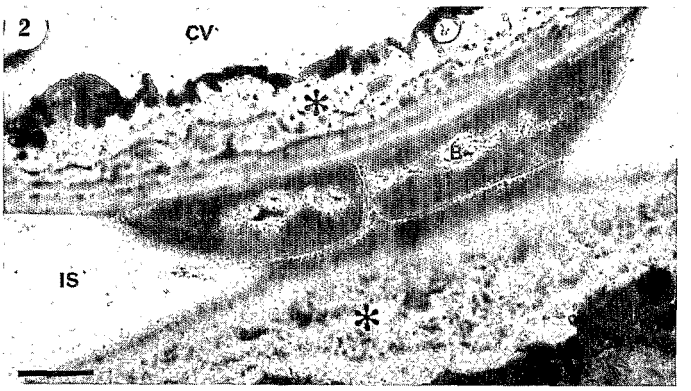

Figure 10.2. Development of paramural papillae. Cells of the non-pathogenic *hrpD* mutant of *P. s.* pv. *phaseolicola* (B), located between mesophyll cells of lettuce 24 h after inoculation. Layered, paramural deposits (asterisks) have developed in both mesophyll cells. (Bar = 0.5 μm; IS, intercellular space; CV, central vacuole; B, bacterium)

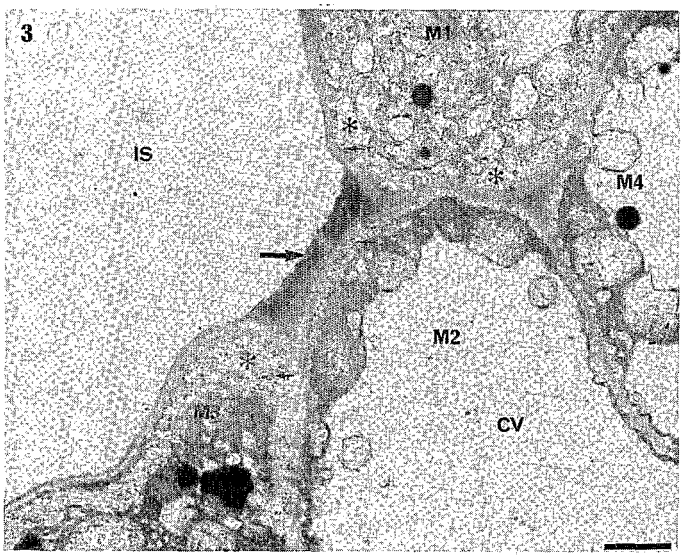

Figure 10.3. Onset of cytoplasmic disorganization during the HR of lettuce mesophyll cells in response to wild-type *P. s.* pv. *phaseolicola*. Bacteria (large arrow) are bordered by three mesophyll cells (M1, M2, and M3) in a specimen fixed 12 h after inoculation. Large and extensive paramural deposits are present within the cells immediately adjacent to the bacteria. The deposits comprise osmiophilic particles (small arrows) within a lightly stained matrix (asterisk) and have a layered appearance, which is most clearly defined in M2 and M3. The cytoplasm of M1 and M3 is extensively vacuolated and mitochondria are disrupted, although the tonoplast appears to be intact. Mesophyll cell M4 also shows evidence of paramural deposition, invaginations of the plasma membrane, and mitochondrial disruption. The bacteria are darkly stained and embedded in an electron-dense matrix. (Bar = 1 μm; IS, intercellular space; CV, central vacuole)

the HR in lettuce, which involves cytoplasmic vesiculation and localized membrane damage (see Fig. 10.3). The application of immunocytochemical and histochemical methods has provided further insights into the changes occurring in challenged lettuce leaf cells. The methods used are equally applicable to other plant tissues.

Cytochemical and Immunocytochemical Localization of Papilla Constituents

Phenolics

Many methods are available for the analysis of phenolic compounds in plants. A simple technique for their detection at the EM level based on reaction with $FeCl_3$ has been described by Brisson *et al.* (1977) as outlined in Table 10.3. Iron(III) chloride is electron dense, therefore allowing identification of sites of iron–phenol complexes, and has been used in light microscopy and EM of a number of plant–pathogen interactions. The method is of less use with naturally electron-dense structures such as the lignified cell wall. It may, however, be combined with subsequent X-ray microanalysis to identify sites of iron accumulation (Brisson *et al.*, 1977).

Table 10.3. Detection of phenolic compounds

1. Trim tissues to about $2mm^2$ pieces under a drop of cold (4°C) 2.5% (v/v) glutaraldehyde and fix for 5 h in a solution of 2.5% (v/v) glutaraldehyde containing 3% (w/v) freshly dissolved $FeCl_3$.
2. Wash in 50 mM PIPES buffer at pH 7.0, then in distilled water.
3. Dehydrate in a graded acetone series, embed in epon–araldite, polymerize, section, and view under TEM without further staining.
4. As a control, tissue should be fixed in the absence of $FeCl_3$.

Immunocytochemistry of hydroxyproline-rich glycoproteins (HRGPs)

A number of antisera have been raised for the detection of HRGPs. As with any immunocytochemical protocol, tissue should be fixed and embedded under conditions designed for maximum retention of antigenicity. Fixation with the dialdehyde, glutaraldehyde may achieve excellent ultrastructural preservation, but the higher degree of crosslinking that occurs relative to the monoaldehyde, formaldehyde, may cause reduction in, or even an elimination of, antibody recognition of epitopes within proteins (Roland and Vian, 1991). Furthermore, osmium tetroxide is a strong oxidant and can crosslink proteins as well as lipids, potentially leading to a reduction of available antigenic sites (Van den Bosch, 1991).

Epoxy resins are hydrophobic and this may have a deleterious effect on antigen preservation and antibody recognition. Polar resins, such as LR-white (London Resin Co., Basingstoke, Hants, UK), facilitate antigen

retention and antibody recognition with low background labeling, but they tend to result in poor ultrastructural preservation and/or resolution of intracellular structure. Treatment with conventional TEM stains after immunogold labeling may increase contrast within the sections. Cryofixation techniques provide high levels of antigen retention and preservation, but will not be covered here. In the absence of cryofixation, samples fixed at RT may be embedded at low temperature. Suitable resins for low temperature embedding include LR-white, Lowicryl K4M, and LR Gold. It is worth noting that, depending upon the antigen, adequate labeling can sometimes be achieved using glutaraldehyde and/or osmium tetroxide fixation followed by embedding in epoxy resins.

In order to detect nonspecific binding of the secondary antibody, sections should be incubated in the absence of the primary antibody. To determine the specificity of the immune serum it should be replaced by pre-immune serum at the same concentration. A further control should include the use of antisera pre-adsorbed with the target antigen. In all cases, labeling should be eliminated or at least severely reduced. A comprehensive review of immunocytochemical labeling techniques for use with plant tissues was given by Van den Bosch (1991).

Protocols for immunogold labeling of HRGPs in lettuce leaf material using polyclonal antisera raised to melon and tomato HRGPs are described in Table 10.4, which illustrates how methods may have to be adapted to ensure specificity. The basic method for tissue preparation is applicable to most immunocytochemistry.

The accumulation of phenolics and HRGPs at reaction sites in lettuce is illustrated in Figs 10.4 and 10.5. Note that the material encapsulating bacteria on the plant cell wall contains secreted HRGPs.

Cytochemical Localization of Peroxidase, Catalase, and H_2O_2

Key players in the plant's response are peroxidase (POX) isoenzymes and AOS, such as H_2O_2 (Mehdy, 1994; Baker and Orlandi, 1995). Before describing techniques to detect enzyme activities and H_2O_2, it is important to explain why we have focused on these components of the resistance of lettuce to *P.s.* pv. *phaseolicola* . The production of H_2O_2 has been observed as a common feature of plant–pathogen interactions. H_2O_2 may have multiple roles; one important function is the oxidative crosslinking of proteins in the cell wall, which is catalyzed by peroxidases (Graham and Graham, 1991; Bradley *et al.*, 1992; Brisson *et al.*, 1994; Wojtaszek *et al.*, 1995). Plant POXs are monomeric heme containing proteins that are usually glycosylated. Isoperoxidases, arising from the transcription of different POX genes or via post-translational modification, are widely distributed in plant cells. In addition to their role in the H_2O_2 dependent crosslinking of cell wall proteins, such as the cell wall HRGPs or extensins (Fry, 1986), POXs are also involved in the final stages of lignin biosynthesis (Monties, 1989). Interestingly, POXs may themselves generate the H_2O_2 required for such crosslinking reactions via 'oxidase'-type activity. Although the *in vivo* reductant for POX-based H_2O_2 generation is unknown, NADH,

Table 10.4. Immunogold labeling of HRGPs

a. Fixation

1. Fix pieces of leaf tissue in 2.5% formaldehyde (freshly prepared) in 50 mM PIPES buffer, pH 7.2, for 16 h at 4°C.
2. Wash samples in PIPES (3 × 5 min) and distilled water (2 × 5 min).
3. Dehydrate in a graded ethanol series – 30%, 50%, 70%, 90% (v/v) ethanol – 10 min each change, followed by three changes of 100% ethanol, 10 min each change.
4. Progressively embed in LR-white resin medium grade (1:1 resin:ethanol, 60 min; 3:1 resin:ethanol, 60 min; fresh resin 12 h followed by a further change of fresh resin for 8 h).
5. Place samples in embedding capsules, add fresh resin and exclude air. Polymerize at 60°C for 24 h. Section and mount on uncoated gold grids (300 mesh).

b. Immunogold labeling of ultra-thin sections

1. For immunogold labeling prepare solutions in 20 mM Tris-buffered saline (pH 7.4) (TBS) containing 0.1% bovine serum albumin (BSA) and 0.05% (w/v) Tween-20 (TBST).
2. Sections to be labeled with anti-melon $HRGP_{2b}$ are blocked in 2% BSA (w/v) in TBST for 30 min at RT and then transferred to a 20 µl droplet of the primary antibody (Bestwick *et al.*, 1995). Sections to be labeled with polyclonal antisera raised to glycosylated tomato HRGP (Brownleader and Dey, 1993) are blocked for 30 min in 1% (w/v) BSA, 5% (v/v) non-immune goat serum (Sigma, Poole, Dorset, UK) and 0.5% (w/v) non-fat milk powder in TBST before being transferred to the primary antibody.
3. Sections are treated with anti-$HRGP_{2b}$ diluted 1:100 and 1:500 or anti-tomato HRGP diluted 1:100, 1:250, 1:500, 1:1000, and 1:10000 and incubated for 16 h at 4°C.
4. Sections are then washed in a stream of TBST from a wash bottle, and incubated in goat anti-rabbit antibody conjugated with IgG 10 nm Gold (Amersham International, Slough, Bucks, UK, or Sigma), diluted 1:25 in TBST, for 30 min at RT.
5. Grids are subsequently washed in a stream of TBST followed by washing in a stream of distilled water. Sections are either dried and examined without further treatment or are subjected to post-labeling staining.

c. Staining of sections after labeling

1. Rinse labeled grids in distilled water.
2. Place grids in a 20 µl droplet of 2.5% (v/v) aqueous glutaraldehyde for 5 min at RT in order to stabilize antibody complexes.
3. Rinse grids in a stream of distilled water and then stain for 20 min in 1% (w/v) aqueous osmium tetroxide at RT (minor modification to Berryman and Rodewald, 1990).
4. Wash in a stream of distilled water and dry.
5. Alternatively, following glutaraldehyde stabilization of antibody complexes, sections may be counterstained with uranyl acetate and lead citrate.

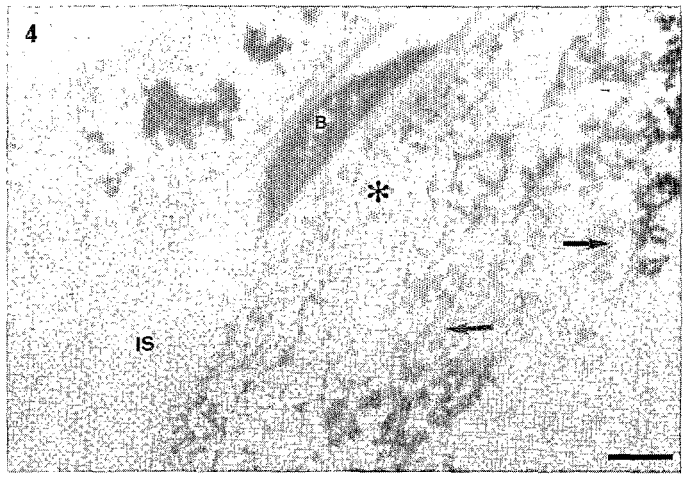

Figure 10.4. Detection of phenolics within papillae (asterisk) and endoplasmic reticulum (arrowed) in lettuce cells 8 h after inoculation with wild-type *P. s.* pv. *phaseolicola*. Note the layers of $FeCl_3$ stained material opposite the bacterial cell. The electron-dense materials seen in the plant cells in this micrograph are all positively stained for phenolics; without $FeCl_3$, only the bacterial cell has a similar degree of electron density. (Bar = 0.5 μm; IS, intercellular space; B, bacterium)

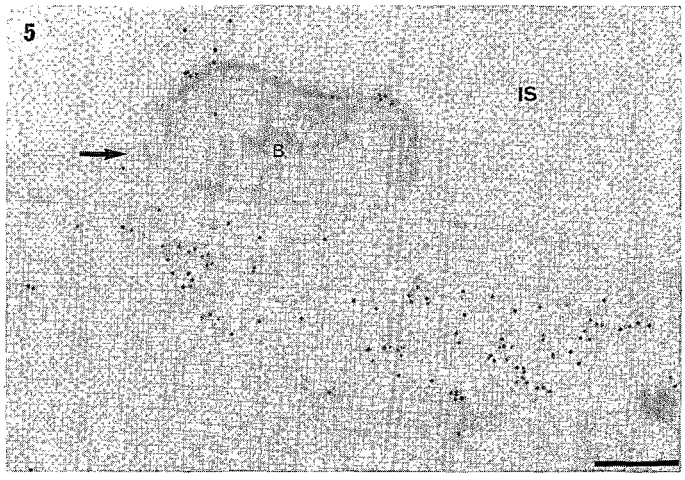

Figure 10.5. Immunocytochemical localization of HRGPs within a papilla, 5 h after inoculation of a lettuce leaf with wild-type *P. s.* pv. *phaseolicola*. Note that the papilla, located between the plant cell membrane and cell wall, is densely labeled and that labeling extends into the encapsulating material (arrowed) surrounding the bacterium (B). This specimen was embedded in LR-white resin and the section was not treated with conventional stains. In consequence, although immunolocalization is very successful, ultrastructural detail is not well resolved. (Bar = 0.25 μm; IS, intercellular space)

NADPH, thiols, phenolic compounds, and indole acetic acid (IAA) have been proposed as potential substrates (Halliwell, 1978; Vianello and Macri, 1991; Bolwell *et al.*, 1995).

In addition to an involvement in plant defense via modification of cell wall structure, it has been argued that AOS may act directly as antimicrobial agents within the apoplast. Peng and Kuc (1992) have demonstrated that H_2O_2 derived from peroxidase-based NADPH oxidation has the potential to prevent pathogen growth in tobacco. H_2O_2 may also influence gene expression, probably being involved in the induction of genes for antioxidant defense and phytoalexin biosynthesis (Devlin and Gustine, 1992; Levine *et al.*, 1994; Mehdy, 1994) and has also been implicated in the development of systemic acquired resistance (Chen *et al.*, 1993).

Importantly, increased AOS formation by plant cells may form a critical step in the progression towards hypersensitive cell death (Mehdy, 1994; Levine *et al.*, 1994, 1996). This may be achieved in two, not necessarily mutually exclusive, ways. H_2O_2 may participate in the iron/copper catalyzed Fenton and Haber–Weiss reactions to produce the highly reactive hydroxyl radical which may initiate lipid peroxidation, a reaction that has been observed in many examples of the HR (Halliwell and Gutteridge, 1989; Adám *et al.*, 1989, 1995). AOS may also serve as signals for the induction of a more complex programmed cell death (PCD) pathway in plants, possibly resembling the process of mammalian cell apoptosis (Levine *et al.*, 1994, 1996). Thus, AOS such as H_2O_2, and the enzymes that regulate their production are vitally important to plant defense reactions.

Enzyme activities

The compartmentation and redistribution of isoperoxidase is an important aspect in the physiological function of these enzymes. In order to identify changes in POX occurring at microsites within the plant cell, 3′,3′-diaminobenzidine (DAB) has been employed as the hydrogen donor for cytochemical localization. The oxidation of DAB yields a brown insoluble polymer, which forms an extremely electron-dense product (osmium black) with osmium tetroxide. The insolubility of the DAB oxide ensures that it does not diffuse from its site of formation during fixation or embedding. The use of DAB may also be manipulated to detect the activity of catalase (CAT), which normally catalyzes the conversion of H_2O_2 to molecular oxygen and water, but after glutaraldehyde fixation may show some POX activity (Frederick, 1991). Fixation conditions for cytochemical localization of enzymatic activity always represent a compromise between the need to provide ultrastructural preservation and maintenance of enzyme function. Usually, short fixation times with low concentrations of paraformaldehyde and glutaraldehyde mixtures followed by repeated buffer washing are employed. Despite the short periods of fixation excellent ultrastructural preservation is often possible.

Table 10.5. Histochemical detection of CAT and POX activities

1. Cut leaf panels under fixative into 1–3 mm^2 sample pieces. Fixative comprises a mixture of 1% (v/v) glutaraldehyde/1% (v/v) paraformaldehyde at pH 7.0 in buffer A, 50 mM sodium cacodylate.
2. Fix for 45 min at RT.
3. Wash twice for 10 min in buffer A.
4. Transfer for a period of 30 min into either 50 mM potassium phosphate buffer pH 7.8 (buffer B) or 50 mM phosphate buffer pH 6.0 (buffer C).
5. Transfer to the cytochemical staining solution, comprising 0.5 mg ml^{-1} DAB and 5 mM H_2O_2 dissolved in either buffer B or C. To prevent auto-oxidation of the DAB, the staining medium should be prepared within 20 min of the incubation and maintained in the dark and with staining being undertaken in dim light. Optimal times for staining are determined by incubation for 10, 15, 30, 60, and 90 min.
6. Wash samples twice for 10 min in buffer B or C, and post-fix in 1% (v/v) osmium tetroxide in buffer A for 45 min, wash twice for 10 min in buffer A, and twice for 10 min in sterile distilled water (SDW).
7. Dehydrate in a graded ethanol or acetone series. If ethanol dehydration is employed, following the 100% ethanol wash samples should transferred to propylene oxide for two washes of 10 min duration each.
8. Embed samples in epon–araldite resin following progressive incubation in propylene oxide–resin mixtures or acetone–resin mixtures (depending upon the mode of dehydration). The ratio of solvent to resin decreases as follows; solvent:resin 3:1, 15 min; 2:1, 12 h or overnight; 1:1, 30 min; 1:2, 30 min; 1:3, 30 min.
9. Incubate in fresh resin for 12 h, transfer to fresh resin for 4 h, place in moulds containing fresh resin and polymerize at 60°C for 48 h. Section and mount on uncoated copper grids (300 mesh) and examine without further treatment or alternatively stain with uranyl acetate and lead citrate as previously described.
10. Controls should be conducted for non-inoculated and inoculated tissues. To inhibit all enzyme activity, following washing in buffer B or C leaf samples should be heated at 95°C for 15 min. This also allows sites of non-oxidized DAB binding to be detected. In order to determine the H_2O_2 dependency of staining, H_2O_2 should be omitted from the staining medium and, as H_2O_2 may be generated *in planta*, it may be necessary to pre-incubate tissues in buffer B or C containing 20 μg ml^{-1} CAT (commercial preparation from bovine liver, Sigma), then place samples in a staining medium containing CAT and from which H_2O_2 had been omitted. To inhibit endogenous CAT activity, samples may be incubated for 30 min in 20 μM 3-amino-1,2,4-triazole (ATZ) in buffer B or C before staining and during staining 20 μM ATZ should also be included in the staining medium. To inhibit POX, CAT and cytochrome oxidase activity, samples should be incubated in 5 mM potassium cyanide for 30 min before staining and again during staining cyanide should be included in the staining medium. As a total control, samples may be fixed and embedded in the absence of the staining medium.

CAT and POX may be differentially detected by alteration of pH and fixation times. Higher pH values, in the range pH 7.8–10, are used to detect CAT, while POX activity is usually detected in the range pH 5.0–7.0. The pH optima for CAT and POX will vary between tissues and the activities observed will probably represent the reactions of several isoforms. Before analysis, tissues should be extracted and pH optima for the reactions determined by spectrophotometric assay. Suitable CAT and POX assays are described by Aebi (1984) and Fielding and Hall (1978), respectively.

Protocols for CAT and POX detection in lettuce leaf tissue are detailed in Table 10.5. Samples of plant material should be cut as small as is practically possible. It should be appreciated that penetration of the DAB solution throughout the tissue is unlikely to be achieved unless either prolonged staining times, which enhance the possibility of staining artefacts arising from DAB auto-oxidation, or vacuum infiltration, which may dislodge bacteria from cell wall attachment sites, are used. Optimal times for incubation in DAB solutions should be determined in preliminary experiments. Controls are essential to define the specificity of the reactions observed. Tissues may be heated to abolish enzyme activity. Endogenous CAT activity is inhibited by incubation with ATZ, and sodium azide and potassium cyanide are used to inhibit POX. The requirement for H_2O_2 is assessed by omitting it from the reaction mixture.

Typical results from POX localization experiments are shown in Fig. 10.6. Cell wall alterations occurring in response to both wild-type and *hrp*$^-$ strains are associated with high levels of POX activity in the cell wall itself and also in the ER and associated vesicles in the adjacent cytoplasm. In contrast to the striking increase in activity associated with cell wall alterations, the onset of tissue collapse during the HR does not seem to be linked with any additional changes in POX activity.

The active oxygen species, H_2O_2

Direct attempts to localize AOS by light microscopy have included the use of nitroblue tetrazolium salt to detect O_2^- (Adám *et al.*, 1989), potassium iodide/starch to detect H_2O_2 (Olson and Varner 1993), and compounds that either fluoresce or have their fluorescence quenched on oxidation by H_2O_2 (Yahraus *et al.*, 1995). Detection of H_2O_2 as a product of enzyme catalysis has formed the basis of the localization, at the electron microscope level, of enzyme activities that generate the AOS in animals and plants (e.g. NADH oxidase and glycolate oxidase, Briggs *et al.*, 1975; Kausch, 1987). The histochemical assays used are based on the reaction of H_2O_2 with $CeCl_3$ to produce electron-dense insoluble precipitates of cerium perhydroxides, $Ce(OH)_2OOH$ and $Ce(OH)_3OOH$. This ultrastructural technique allows the precise localization of sites of H_2O_2 accumulation or production and has been used by Czaninski *et al.* (1993) in a study of endogenous H_2O_2 production in lignifying tissues. The following protocol (Table 10.6) has been applied to the identification of H_2O_2 at reaction sites in lettuce (Bestwick *et al.*, 1997) and also in tobacco, Arabidopsis and pepper leaves (J. W. Mansfield, I. R. Brown, S. Soylu and C. S. Bestwick, unpublished observations).

The distribution of cerium perhydroxide formation in lettuce reveals

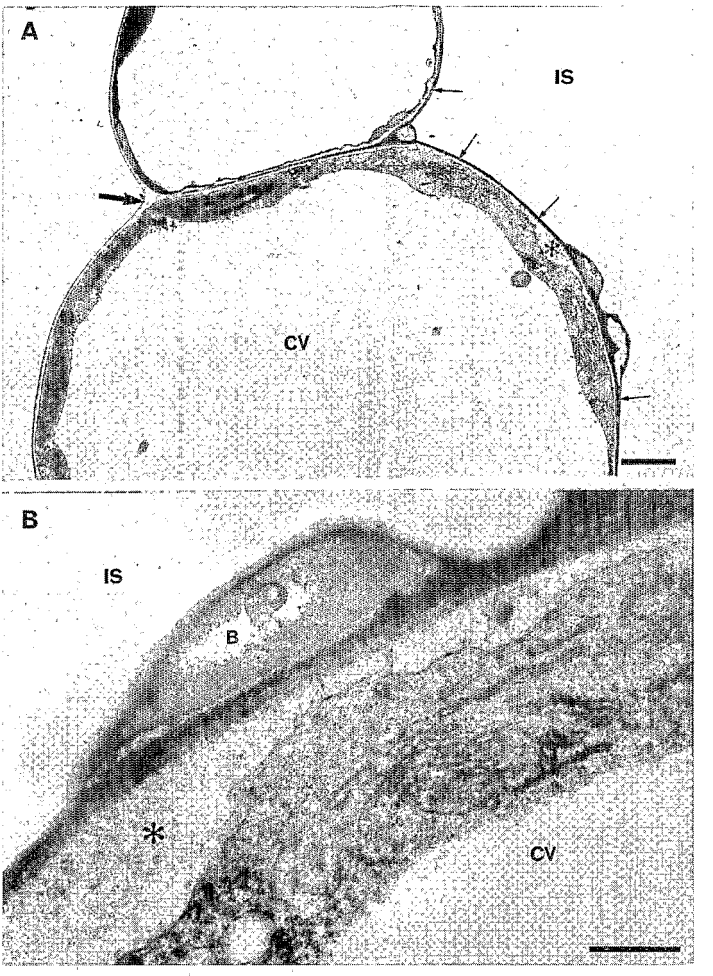

Figure 10.6. Peroxidase activity in the cell wall and cytoplasm of lettuce mesophyll cells 5 h after inoculation with wild-type *P.s.* pv.*phaseolicola*. (A) Low magnification (bar = 2 μm), showing the extension of electron-dense staining (small arrows) from sites of bacterial attachment into the plant cell wall. Note that the cell wall away from the bacteria is unstained (large arrow). The large papilla (asterisk) has patchy staining which is more distinct at higher magnification. (B) In this higher magnification (bar = 0.5 μm), staining for POX can be seen to extend into the material surrounding bacteria and activity is also associated with the ER and small vesicles in the dense plant cell cytoplasm at reaction sites. (B, bacterium; CV, central vacuole; IS, intercellular space)

that H_2O_2 production is concentrated at sites of bacterial attachment, and that the AOS is mainly associated with the plant cell wall (Fig. 10.7). Levels of H_2O_2 may be quantified based on the confluency and intensity of staining. Such quantification reveals that H_2O_2 formation is dramatically greater during the HR than in the response to the *hrp* mutant, which only causes localized cell wall alterations (Table 10.7). There is a clear association between hypersensitive cell death and elevated levels of H_2O_2. The results of our localization experiments raise the following important points about oxidative reactions and the plant's defense responses.

Table 10.6. Detection of H_2O_2 in plant cells

1. Excise small tissue pieces (approximately 1–2 mm^2) from inoculated leaf panels and incubate at room temperature in freshly prepared 5 mM $CeCl_3$ in 50 mM 3-[*N*-morpholino]propane sulfonic acid (MOPS) (pH 7.2) for 1 h.
2. Fix in 1.25% (v/v) glutaraldehyde/1.25% (v/v) paraformaldehyde in 50 mM sodium cacodylate (CAB) buffer (pH 7.2) for 1h.
3. Wash in CAB buffer (2 × 10 min) and fix for 45 min in 1% (v/v) osmium tetroxide in CAB buffer.
4. Wash (2 × 10 min) in CAB buffer.
5. Dehydrate in a graded ethanol series – 30%, 50%, 70%, 80%, 90% (v/v) ethanol – 15 min each change and then three changes of 100% ethanol of 20 min duration each. Transfer to two changes of propylene oxide each for 20 min and progressively embed in Epon–araldite. Alternatively, dehydrate in a graded acetone series, then transfer directly into acetone-resin mixtures and progressively embed.
6. Place tissues for 12 h in pure resin followed by a change of fresh resin for 4 h and then place in blocks and polymerize at 60°C for 48 h.
7. Controls: As a total control, tissues are incubated in MOPS devoid of further additions. To confirm the presence of H_2O_2, the inoculated and non-inoculated tissue segments should be incubated for 30 min in 50 mM MOPS, pH 7.2, containing 25 μg ml^{-1} bovine liver catalase. The effects of endogenous catalase may be assessed by incubation in 20 μM ATZ.

Other inhibitors/effectors may be added to the incubation medium to examine the source of H_2O_2, for example, NADH/NADPH as substrates for POX or neutrophil-like NADPH oxidases. Potassium cyanide or sodium azide inhibit heme proteins such as POXs, and diphenylene iodonium chloride (DPI) inhibits neutrophil-like NADPH oxidase (Bestwick *et al.*, 1997).

Table 10.7. Quantitative assessment of staining with $CeCl_3$ to reveal H_2O_2 accumulation within the cell wall of lettuce mesophyll cells adjacent to bacteria (*Pseudomonas syringae* pv. *phaseolicola*)

Strain and time after inoculation (h)	Percentage of sites in each category of staining[a]							
	0	1	2	3	0	1	2	3
	Without aminotriazole				With aminotriazole			
Wild-type								
3	70	20	10	0	70	30	0	0
5	10	10	2	78	7	12	12	69
8	40	10	5	45	30	3	14	53
16[b]	66	18	16	0	19	50	31	0
***hrpD* mutant**								
3	80	20	0	0	83	14	3	0
5	52	24	8	8	63	19	3	16
8	58	14	14	14	64	6	19	11
16	77	3	17	3	80	9	0	11

[a] At least 30 sites were examined at each time from three replicate leaf samples. The minimum degrees of deposit assigned to each category of staining were: 0, none; 1, faint and patchy; 2, dense but patchy; 3, confluent and dense staining.
[b] By 16 h most cells had collapsed.

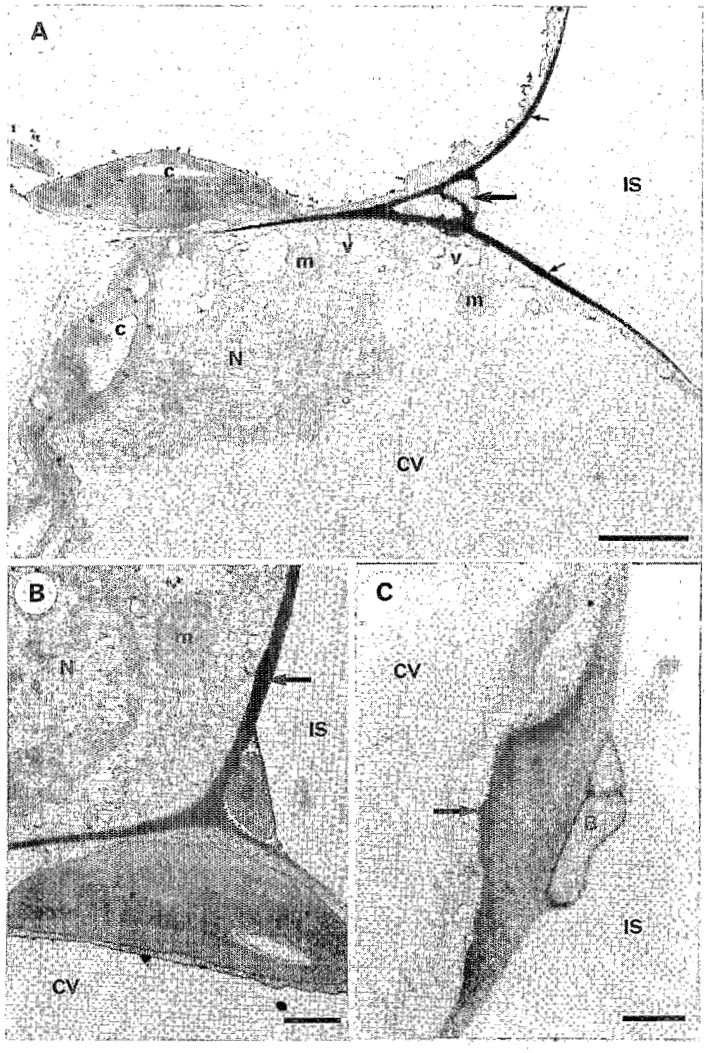

Figure 10.7. Localization of H_2O_2 accumulation in lettuce cells adjacent to wild-type *P. s.* pv *phaseolicola*. (A) Electron-dense deposits of cerium perhydroxides extending from the site of attachment of wild-type bacteria (large arrow) into the surrounding plant cell wall (small arrows). The small papillae formed are also intensely stained, 8 h after inoculation. Note that the cytoplasm of responding cells is already vesiculated near the bacteria but the nucleus (N), close to the reaction site, shows no signs of chromatin condensation or nuclear fragmentation (i.e. apoptic features); bar = 2 µm. (B) Restriction of H_2O_2 to one of a pair of adjacent mesophyll cells 5 h after inoculation. Note that the one shared wall of two cells is intensely stained, but staining only extends into the individual wall of one cell (arrow); bar = 1 µm. (C) Staining of a large papilla produced 8 h after inoculation with wild-type bacteria showing presence of H_2O_2 (arrow) at the papilla–plant cell membrane interface; bar = 0.5 µm. (B, bacterium; IS, intercellular space; CV, central vacuole; v, vesiculation; N, nucleus; c, chloroplast; m, mitochondrion)

Sources of H_2O_2

Although H_2O_2 is the principal AOS detected during plant–microbe interactions in soybean and lettuce, in other systems the superoxide anion (O_2^-) is readily detectable (Doke, 1983; Adám *et al.*, 1989; Levine *et al.*, 1994). H_2O_2 is produced following the enzymatic or non-enzymatic dismutation of O_2^- and may be able to diffuse freely into the apoplast or extracellular milieu. In the lettuce HR, the detection of H_2O_2 within the cell wall and its general absence from the cytoplasm implies that its site of production is either via extracellular enzymes or via oxidases bound at the membrane surface (Fig. 10.8). Membrane-bound neutrophil-like NADPH oxidase complexes and extracellular POXs have both been proposed as the agents of H_2O_2 generation. Studies in tobacco, soybean, potato, rose, and Arabidopsis support the involvement of a neutrophil-like NADPH oxidase (Low and Merida, 1996; Murphy and Auh, 1996). In bean and lettuce, however, H_2O_2 production in response to elicitors or bacteria may be linked to POX activities, since their inhibition virtually eliminates staining after treatment with $CeCl_3$ (Bolwell *et al.*, 1995; Bestwick *et al.*, 1997).

H_2O_2 and wall alterations

Whether a papilla is formed next to a wild-type bacterium or *hrp* mutant, in lettuce it contains both HRGPs and phenolic compounds (see Figs 10.4 and 10.5; see also Bestwick *et al.*, 1995). Thus, all the components required for crosslinking reactions are present at sites of bacterial attachment. Time course analysis reveals that the detection of HRGPs coincides with the appearance of peroxidase activity and H_2O_2, and is followed by phenolic deposition. However, perhaps surprisingly, major accumulation of H_2O_2 is only observed during the HR. Given the lower levels of H_2O_2, it seems possible that papillae and altered cell walls are actually less crosslinked in the interaction with the *hrp* mutant, although their appearance under the microscope is identical. Alternatively, and perhaps more plausibly, $CeCl_3$ may only be able to react with excess H_2O_2 not being used in crosslinking reactions in lettuce.

Progression of hypersensitive cell collapse and its relationship to apoptosis – a key role for AOS?

The response by both animal and plant cells to AOS production may be varied, leading to cell proliferation, adaptation, programmed cell death (PCD) or necrosis (Dypbukt *et al.*, 1994; Levine *et al.*, 1994; de Marco and Roubelakis-Angelakis, 1996). PCD describes the induction of a genetically programmed cell suicide mechanism; the main form of PCD recognized in animal cells is apoptosis. While PCD is a mechanistic term, apoptosis is descriptive, defining cell death events that share common morphological features and to which certain biochemical events have been linked (Arends and Wyllie, 1991). A second passive form of cell death is necrosis, which is observed in numerous pathological processes in animals following catastrophic environmental insult (Buja *et al.*, 1993; Kerr *et al.*, 1994).

A comparative summary of reactions observed during the HR in plants and in animal cells undergoing necrosis and apoptosis is given in Table 10.8. Detailed studies of the bacteria-induced HR in lettuce as outlined here, and in work on bean, soybean, and Arabidopsis, describe an apparently ordered collapse. Apoptotic features such as membrane blebbing and condensation of the cytoplasm are common to these HRs, although the timing of their appearance varies. There are, however, several changes that are not easily reconciled with those of apoptosis, in particular the absence of early nuclear fragmentation, which is a diagnostic feature of apoptosis in animal cells (Kerr *et al.*, 1994). In lettuce, one of the earliest

Table 10.8. Comparison of plant cell death events with the classical features associated with necrosis and apoptosis in mammalian cells

	Classical features of animal cell		Plant cell death events in plants – hypersensitive reactions in:			
Morphology	Apoptosis	Necrosis	Lettuce[a]	Bean[b]	Arabidopsis[c]	Soybean[c]
Nucleus						
Shrinkage	+	–	–	–	NR	+
Chromatin condensation	+	–	–	–	NR	+
Fragmentation	+	–	–	–	NR	NR
Cytoplasm and plasma membranes						
Plasma membrane blebbing	+[d]	+[d]	+	+	+	+
Vacuolation of cytoplasm	+	+	+	+	NR	NR
Pronounced early protoplast shrinkage	+	–	–	–	+	+
Organelles						
Mitochondrial swelling and rupture	–	+	+/–	+	NR	–
Peroxisome damage	–	+	+/–	+	NR	NR
Chloroplast damage	NA	NA	–	+	NR	+
Corpse morphology						
Cytoplasmic condensation	+	–	+/–	+	+	+
Apoptotic bodies[e]	+	–	+/–	–	+	+

NR, Not reported, NA not applicable.

[a] Bestwick *et al.* (1995, 1997) and unpublished observations.

[b] Brown and Mansfield (1988).

[c] Levine *et al.* (1996).

[d] Blebbing is highly pronounced in classical apoptosis.

[e] Apoptotic bodies in plants refers to the presence of discrete membrane-bound cell remnants.

features of the non-host HR induced by *Pseudomonas syringae* pv. *phaseolicola* is swelling of mitochondria, a feature often seen in necrosis. During the early stages of the lettuce HR no protoplast shrinkage is observed, but this response is reported to be a prominent feature of the HR in soybean and Arabidopsis (see Table 10.8). Interestingly, in lettuce, two separate cell fates are observed following vacuolation of the cytoplasm. Most commonly, the tonoplast ruptures and disruption of the plasma membrane occurs (Bestwick *et al.*, 1995), but in some cases the tonoplast appears to remain intact while the cytoplasm condenses. These observations suggest further complexity within responding plant tissues as two types of cell death process seem to operate within a single HR lesion.

The widespread occurrence of HR-specific lipid peroxidation suggests that AOS production is involved in some aspect of the cell death program in plants. A toxic shock associated with excess and localized H_2O_2 production by the host may cause a self-induced necrosis, as illustrated by the rapid destruction of cells early on in the lettuce HR. The AOS may then serve either directly or indirectly to induce PCD in surrounding cells. The concentration of H_2O_2 at reaction sites may determine the cell death process activated within individual cells. Variation in AOS concentrations may therefore account for the variability in the descriptions of ultrastructural cell collapse both within an individual HR lesion within the same species responding to different avirulent pathogens, and between HRs in different species (Levine *et al.*, 1994; Ryerson and Heath, 1996; Bestwick *et al.*, 1997). Detailed ultrastructural studies of cell death events in different plants and tissues, including DAB and $CeCl_3$ based cytochemistry, may eventually characterize a common structural framework for 'plant apoptosis'.

◆◆◆◆◆◆ INVASION OF CASSAVA BY *X. campestris* PV. *manihotis*

Successful invasion of plant tissues by several pathovars of *X. campestris* is characterized by degradation of the plant cell wall. The interaction between *X.c.* pv. *manihotis* (*Xcm*) and its host plant cassava has been studied in detail at the cellular level. The cassava pathogen is of particular interest as it preferentially invades the vascular tissue, multiplying rapidly within xylem vessels (Boher *et al.*, 1996).

Nicole and colleagues have used a range of immunocytochemical and advanced histological techniques to examine changes occurring in the plant and the production of extracellular polysaccharides by *Xcm*. A particularly valuable approach has been to use gold-labeled high-affinity probes such as the purified enzymes β 1-3 glucanase, cellulase (β 1-4 glucanase), and laccase to localize callose, cellulose, and phenolics, respectively (Boher *et al.*, 1995, 1996). Binding between the labeled enzymes and their substrates is sufficiently strong to allow retention of the gold probe throughout the strict washing procedures applied to treated sections. A protocol for preparation and use of gold probes, including lectin conjugates, is given in Table 10.9. Benhamou (1996) has described variations of

Table 10.9. Preparation of gold probes for histochemical localization at the EM level

a. Preparation of gold particles

1. Prepare 0.01% tetrachloroauric acid (100 ml) in a siliconized Erlenmeyer flask and heat to boiling.
2. Rapidly add x ml of 1% sodium citrate (x varies according to gold size; 4, 6 or 8 ml gives 15, 10 or 8 nm particles) and keep boiling. The initially violet purple color turns red–orange, indicating stabilization of the gold solution.

b. Preparation of gold probes for laccase

1. Adjust the pH of the gold solution close to the isoelectric point of the protein.
2. Add 10 ml of the colloidal gold to 100 µg of protein; after 3 min add 10 µl of 1% polyethylene glycol 20 000 and centrifuge for 1 h at 4°C at 60 000 × g.
3. Collect the red mobile pellet, which contains the usable gold probe; resuspend in 1 ml buffer adjusted to the pH of activity of the protein.
4. The gold complex may be stored at 4°C for several weeks.

c. Procedure for labeling using enzyme- or lectin–gold complex

1. Immerse ultra-thin sections on grids for 15 min in 0.01 M phosphate-buffered saline (PBS) containing 0.05% Tween-20 adjusted to the pH of activity of the protein.
2. Transfer onto a drop of the gold probe for 30 min in a moist chamber.
3. Jet-wash sections with 0.01 M PBS pH 7.1, followed by distilled water.
4. Optionally silver intensify the gold label.
5. Stain sections on grids with uranyl acetate and lead citrate.

A two-step procedure should be used with some lectins; in this case, the sections are first incubated on grids in the unbound probe before being floated onto a drop of gold-conjugated glycoprotein.

this method and has used gold complexes to study several substrates occurring within plant cell walls.

Returning to cassava, Boher *et al.* (1995) described the invasion of leaves by *Xcm*. Following stomatal penetration, bacterial colonies developed in the intercellular spaces and were typically surrounded by a fibrillar matrix. Immunocytochemistry using antibodies to epitopes in the bacterial polymer xanthan (Fig. 10.8a) indicated that the matrix was predominantly of bacterial origin. Close to degraded plant cell walls, however, the matrix also contained fragments of pectin and cellulose as revealed using antibodies or exoglucanase–gold complex, respectively. The distribution of components of the polysaccharides surrounding bacterial cells is clearly illustrated in Figs 10.8b and 10.8c. During the infection process, host cell walls and middle lamellae (rich in pectin) displayed extensive disruption, regardless of how the tissue was colonized (i.e. intercellularly in the leaf parenchyma or finally intracellularly in xylem vessels and tracheids).

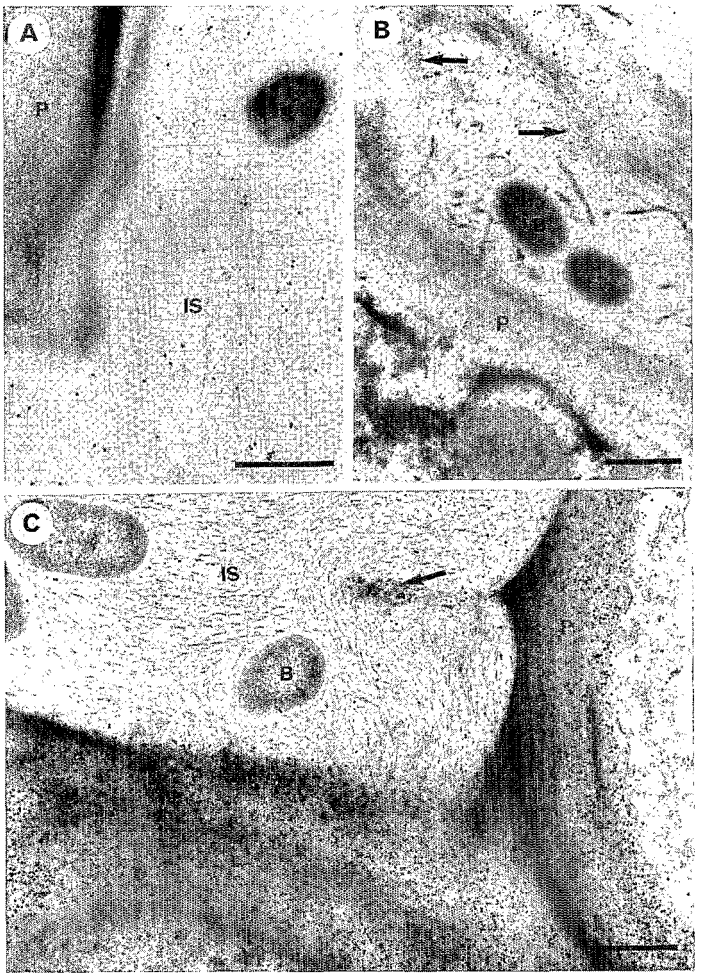

Figure 10.8. Invasion of of cassava leaf tissue by *Xanthomonas campestris* pv. *manihotis*. (A) Immunogold labeling of xanthan with the XB3 monoclonal antibody. Gold particles are seen over the fibrillar bacterial exopolysaccharides (EPS) within the intercellular space (IS), but not over the plant cell wall; bar = 0.5 μm. (B) Immunogold labeling of pectin with the JIM 5 monoclonal antibody. Gold particles decorate the degraded middle lamella between mesophyll cells; fragments of pectinaceous material (arrows), are also seen within the bacterial EPS; bar = 0.5 μm. (C) Gold labeling of β-1,4-glucans with an exoglucanase conjugated to colloidal gold. Labeling occurs over the primary cell wall and over wall fragments (arrow) located within the bacterial EPS; bar = 0.5 μm. (B, bacterium; IS, intercellular space; P, plant cell wall)

Multiplication of bacteria in xylem cells is accompanied by pronounced alteration of the plant cell wall. Modification of the labeling pattern of the middle lamella indicated that pectin was altered in pit areas over which no, or few, gold particles were seen. The fibrillar sheath surrounding the pathogen in the lumen of vessels was always associated with degraded areas of walls and middle lamella. Anti-pectin antibodies labeled electron-dense material found in the vessels, indicating the release of pectin gels, which may contribute to vascular occlusion and the wilting

symptom characteristic of infection. The secretion of plant cell wall degrading enzymes leading to disruption of the cellulose (β 1-4 glucan) and pectin (α 1-4 polygalacturonic acid) components, is clearly a major feature of successful infection by *Xcm*. Ultrastructural studies indicate the striking variability that may occur in the microenvironment within and around bacterial colonies. Individual bacterial cells may be bathed in pectic and cellulosic fragments or alternatively surrounded by their own EPS. The changes in growing conditions will regulate the expression of genes that determine pathogenicity.

Resistance to *Xcm* in cassava is expressed by restricted colonization of the xylem vessels. Limitation of bacterial growth and spread is

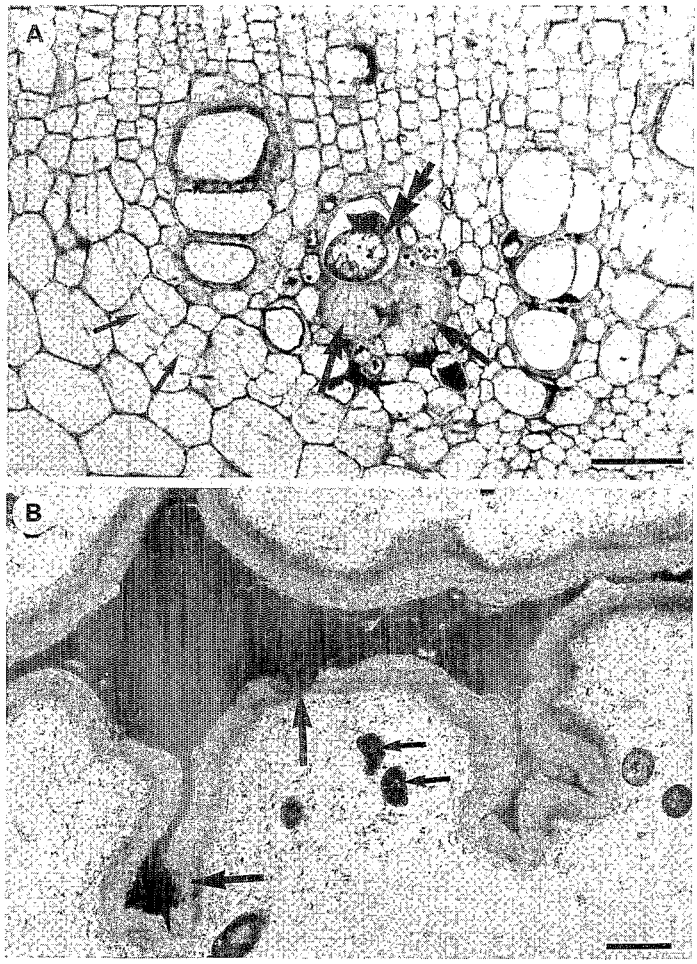

Figure 10.9. Microscopy of tyloses in vessels of resistant cassava plants infected with *Xcm*. (A) Semi-thin section stained with toluidine blue showing cambial cells (small arrows) close to an area of infected xylem. A large tylose (double arrow) and dense bacterial colonies (large arrows) are present in adjacent vessels. (bar = 50 μm). (B) Dead bacterial cells (small arrows) close to a digitate tylose that appears to secrete electron-dense compounds (large arrows) within a xylem vessel. (bar = 1 μm).

occasionally observed within generally susceptible cultivars, but is more frequent and common within resistant genotypes, for example TMS91934 (Kpémoua *et al.*, 1996). Sites of restricted multiplication were associated with lignification, suberization, and callose deposition within phloem and xylem parenchyma, as determined by labeling with histochemical stains, gold conjugates, and immunocytochemistry. A special feature of the more resistant tissues was the production of tyloses, which often blocked xylem vessels as illustrated in Fig. 10.9. The tyloses displayed autofluorescence under ultraviolet (UV) excitation and were found to be active in the secretion of osmiophilic phenolic compounds. Bacteria close to the tyloses often appeared greatly distorted, indicating the generation of locally toxic conditions within responding vessels.

◆◆◆◆◆◆ LOCALIZATION OF PHYTOALEXINS

The accumulation of phytoalexins (low molecular weight antimicrobial compounds) within challenged plant tissues is a particularly well-documented mechanism of disease resistance (Bailey and Mansfield, 1982; Harborne, 1987; Hahlbrock *et al.*, 1995). Biochemical analysis clearly demonstrates synthesis and accumulation of the secondary metabolites. The toxicity of compounds such as lanicilene C and lettucenin A to bacterial plant pathogens is also easily demonstrated *in vitro* (Essenberg *et al.*, 1982, Bennett *et al.*, 1994; Pierce *et al.*, 1996). Although considerable progress has been made using a variety of techniques, precise localization of the phytoalexins at infection sites has been difficult to determine.

Phytoalexins belong to a wide range of chemical families. They are structurally diverse and have different properties, which in some cases aid their analysis within infected tissues. At the light microscope level, localization has been achieved on the basis of inherent color as with the red deoxyanthocyanidin phytoalexins of sorghum (Snyder and Nicholson, 1990). The specificity of such analyses can be enhanced by microspectrophotometry (Snyder *et al.*, 1991), but estimation of concentration using this technique is complicated by the presence of other absorbing substances.

Autofluorescence has also been used with some success, notably with wyerone derivatives in *Vicia faba*, and lacinilenes in cotton (Mansfield, 1982, Essenberg *et al.*, 1992a,b). Specificity can again be enhanced by microspectrofluorometry, but this should be used with caution, particularly when attempting quantification, because of errors due to quenching at high fluorophore concentrations and/or by other solutes. Histochemistry is generally applicable to groups of related compounds and therefore lacks specificity (Mace *et al.*, 1978; O'Brien and McCully, 1981; Dai *et al.*, 1996). The approach has, however, been used with success in locating terpenoid aldehydes and flavanols, some of which are phytoalexins, in cotton (Fig. 10.10). Phenolic phytoalexins may also be localized at the EM level using $FeCl_3$ and laccase–gold complexes as outlined previously.

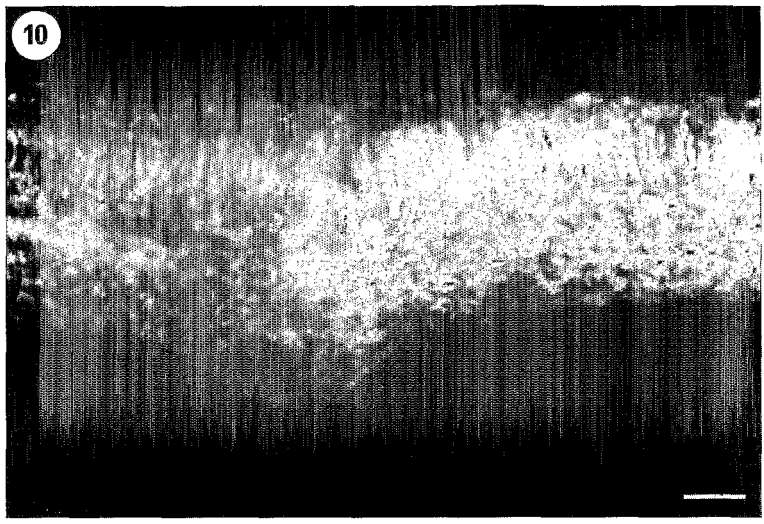

Figure 10.10. Detection of flavanoids by fluorescence after staining with Neu's reagent. The micrograph, taken using UV excitation, is of a section of a cotyledon of resistant cotton (*Gossypium hirsutum*) infected by *Xanthomonas campestris* pv. *malvacearum* race 18. The strong white fluorescence of the inoculated tissue (right side) indicates an accumulation of flavanoids; by contrast, the non-infected tissue (left side) does not fluoresce (bar = 50 μm).

A very specific method, but one that is not generally available, is laser microprobe mass analysis. Moesta *et al.* (1982) have used this technique to localize glyceollin to groups of cells in soybean. The fine beam of the laser is focused on sites within single cells and ionized and volatilized phytoalexin are detected and identified by mass spectrometry. This approach is substance specific, but does not produce quantitative data. The laser also tends to damage more tissue than would be ideal, limiting localization to at best the level of a single cell.

Fluorescence-activated cell sorting has been used to isolate cells containing high concentrations of the sesquiterpene phytoalexins found in yellow–green-fluorescent cells that have undergone the HR in cotton cotyledons inoculated with an avirulent strain of *X. c.* pv. *malvacearum* (Pierce and Essenberg, 1987). Responding mesophyll tissues were digested with macerating enzymes and suspensions containing mixtures of responding and unaffected cells sorted into two groups, either cells with bright fluorescence or those with at most a weak response (Fig. 10.11). High concentrations of lacinilene C and 2, 7-dihydroxycadalene were found in the highly fluorescent cells. This study is one of few in which an attempt has been made to correlate localization with quantitative analysis. The overall conclusion was that more than 90% of the most active phytoalexins recovered from whole cotyledons are concentrated in and around fluorescent cells at infection sites. Average phytoalexin concentrations in these cells were calculated by dividing the tissue's total phytoalexin content, determined by extraction and HPLC, by the water

content of the fluorescent cells (Essenberg *et al.*, 1992a). Results indicated cellular concentrations of lacinilenes much greater than those required to inhibit bacterial growth *in vitro* (Pierce *et al.*, 1996).

The rapid activation of *de novo* biosynthesis that leads to phytoalexin accumulation in plants has provided invaluable systems for studies of gene expression (Dixon and Paiva, 1995). At the tissue level, *in situ* hybridization has been used to determine the timing of the appearance of mRNA transcripts for certain key enzymes such as phenylalanine ammonia lyase and chalcone synthase (Schmelzer *et al.*, 1989). Immunocytochemistry has yielded corresponding information about the appearance of the enzymes themselves (Jahnen and Hahlbrock, 1988). Results obtained indicate that phytoalexin synthesis occurs predominantly in living cells around those undergoing the HR and that the phytoalexins are transported to and accumulate within the dead cell (Bennett *et al.*, 1996). The mechanisms underlying the transport of phytoalexins into cells undergoing the HR are not understood.

Subcellular localization of phytoalexins has rarely been attempted. Immunocytochemistry has considerable potential for detection of low molecular weight secondary metabolites and is increasingly being used with success in plant tissues. A recent example is work on the glucosinolate sinigrin, which is related to phytoalexins in *Brassica* spp. such as oilseed rape (Hassan *et al.*, 1988; Bones and Rossiter, 1996). Sinigrin is a substrate for the myrosinase enzyme, which generates antimicrobial isothiocyanates. In order to detect sinigrin, polyclonal antibodies were raised to a sinigrin–BSA conjugate prepared as summarized in Table 10.10, an adaptation of the method first used by Hassan *et al.* (1988). The key step is the synthesis of the activated sinigrin hemisuccinate, which reacts with free amino groups in proteins such as BSA. Sinigrin is particularly suitable

Table 10.10. Preparation of sinigrin–BSA conjugate

1. React sinigrin (31 mg) with succinic anhydride (38 mg) and anhydrous pyridine (6.5 µl) in anhydrous dimethylformamide (DMF, 250 µl) for five days at RT under argon.
2. Add excess diethyl ether to precipitate the sinigrin hemisuccinate (SHS) product and collect by centrifugation. Redissolve in methanol and repeat the ether precipitation three times.
3. Dry SHS over phosphorous pentoxide and dissolve 43.7 mg in 370 µl of DMF containing *N*-hydroxysuccinimide (22.1 mg) and *N,N'*-dicyclohexylcarbodiimide (20.5 mg); stir for 12 h at 4°C. Centrifuge to remove precipitate.
4. Add supernatant containing the active ester of sinigrin to BSA (16.5 mg) in 1.56 ml phosphate buffer (pH 7.6) and stir at 4°C for 24 h.
5. Desalt the resultant sinigrin–BSA conjugate using a BioRad 10DG column (Bio-Rad Laboratories, Hemel Hempstead, Herts, UK). Conjugation can be confirmed by infrared spectrophotometry and SDS–PAGE analysis.

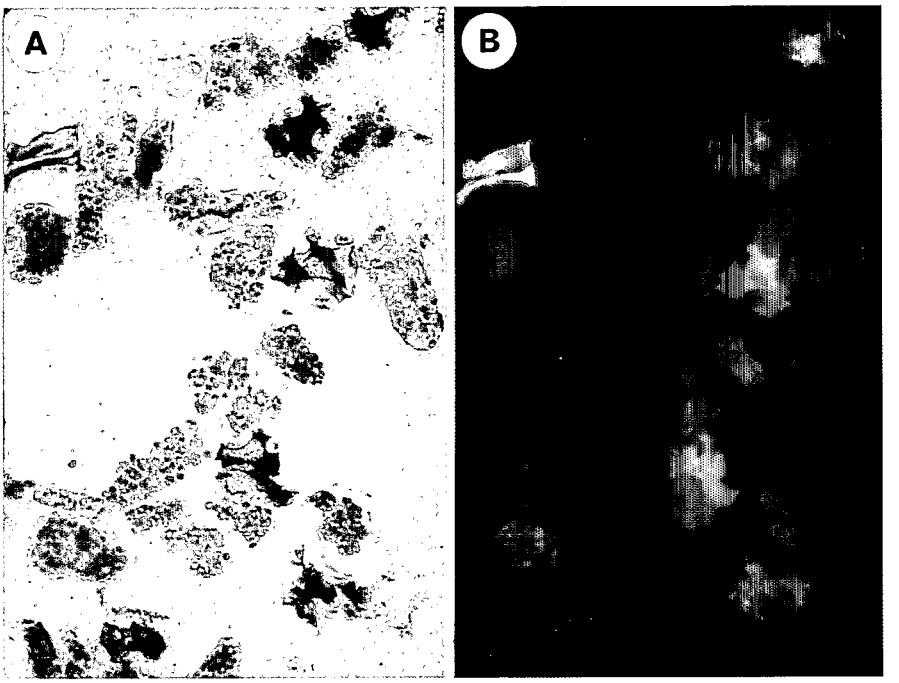

Figure 10.11 (A) Transmitted white light and (B) fluorescence (excitation, 460–485 nm) micrographs of mesophyll cells isolated from a cotyledon of the bacterial blight-resistant cotton line OK1.2 inoculated with *X.c.* pv. *malvacearum*, in preparation for fluorescence activated cell sorting. The yellow green autofluorescent cells which have undergone the HR, contain high concentrations of the lacinilene phytoalexins. Red autofluorescence in the undamaged cells is due to chlorophyll. (Micrographs kindly provided by Margaret Pierce.)

for conjugation via the hydroxy moiety of the glucoside. For other compounds of interest it may be necessary to generate such a functional group by chemical modification and it should be borne in mind that this might alter the antigenic properties of the molecule (Jung *et al.*, 1989).

Combined use of the antisinigrin antiserum with antibodies raised to the myrosinase protein has allowed precise subcellular localization of the two components of the glucosinolate generation system. The enzyme is found in myrosin grains and is also co-localized with its substrate, sinigrin, within protein bodies in aleurone-like cells found in the cotyledons of *B. juncea*, as shown in Fig. 10.12 (Kelly *et al.*, 1998). Retention of the small sinigrin molecule during dehydration and embedding implies crosslinking during fixation or the presence of sinigrin–protein complexes in the plant cell. Further application of immunocytochemistry to studies of phytoalexins, perhaps combined with cryofixation methods to enhance retention of the compounds, has considerable promise for unraveling the cellular defense reactions of plants.

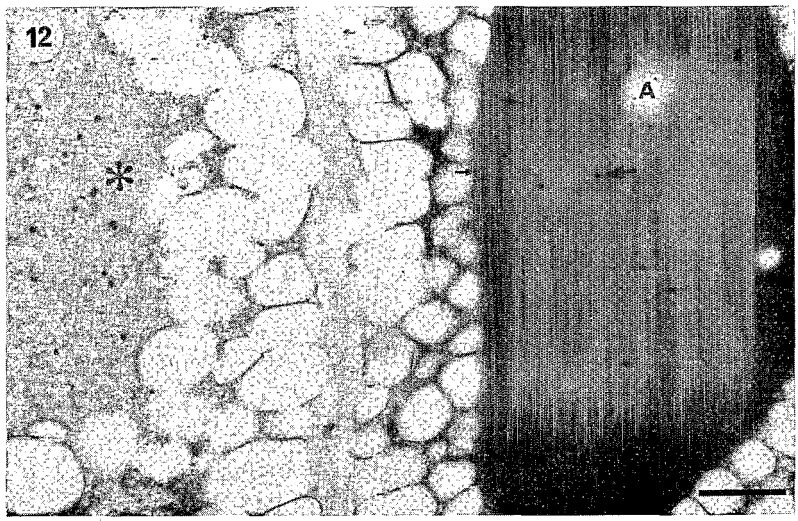

Figure 10.12. Immunocytochemical localization of sinigrin (small gold particles, small arrow), and myrosinase (large gold particles, large arrow), within cotyledon cells in a seed of *Brassica juncea*. Note that myrosinase and its substrate sinigrin are co-localized within the protein body of an aleurone-like cell (A); myrosinase is also located separately within a myrosin grain in an adjacent cell (asterisk) (bar = 0.5 μm). (Micrograph kindly provided by Peter Kelly and John Rossiter.)

◆◆◆◆◆◆ CONCLUDING REMARKS

The fundamental difference between the pathology of plants and animals is the presence in the plant of a complex cell wall, which acts as a barrier to contact between the plant cell membrane and the invading bacterium. An important determinant of pathogenicity in many phytopathogens is

their ability to degrade the plant cell wall (e.g. *Xcm* and subspecies of *Erwinia carotovora;* Alfano and Collmer, 1996). With soft rotting pathogens, enzymic digestion of the wall provides a direct source of carbohydrates. Destruction of the wall also leads to plant cell death and the release of cytosolic nutrients for necrotrophic growth from the killed cell. Ultrastructural studies reveal that the wall is also a dynamic matrix associated with various oxidative reactions including the generation of H_2O_2, phenolic deposition, and protein crosslinking. Although this section is primarily concerned with the plant's responses to infection, it is important to consider recent discoveries concerning the mechanisms by which plant pathogens circumvent the cell wall and deliver pathogenicity and virulence determinants.

Genetic evidence strongly supports the concept that certain bacterial proteins may be delivered or 'injected' into plant cells in a manner analogous to that described for the animal pathogens *Yersinia* and *Salmonella* (Bonas, 1994; Rosqvist *et al.*, 1994; Perrson *et al.*, 1995; Van den Ackerveken *et al.*, 1996). Transfer of the pathogenicity determinants, the Yops, from *Yersinia* into animal cells has been demonstrated using confocal laser microscopy to study movement of immunocytochemically labeled proteins (Rosqvist *et al.*, 1994). Similar approaches should be applied to the study of plant–bacterium interactions.

Although a source of nutrients for necrotrophs, the cell wall presents an intriguing barrier to the transfer of pathogenicity determinants from the more biotrophic pathogens. There is increasing evidence that specialized pili may be involved in the delivery of proteins from *P.s.* pv. *tomato*

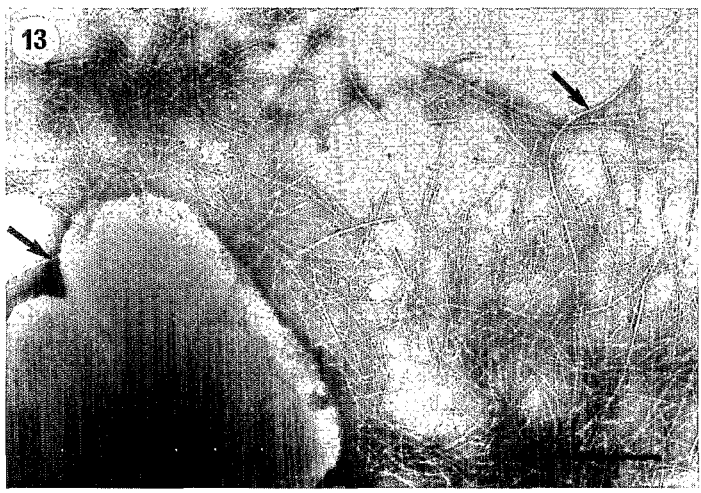

Figure 10.13. Production of filamentous *hrp* pili by *P.s.* pv. *tomato* after growth in *hrp* gene-inducing conditions. Note that the fine pili are clearly distinguished from flagella (arrows). The bacterium was negatively stained using 1% potassium phosphotungstate at pH 6.5 (bar = 0.5 μm). (Micrograph kindly provided by Elina Roine and Martin Romantschuk.)

and protein/DNA complexes from *Agrobacterium tumefaciens* (Fullner *et al.*, 1996; Roine *et al.*, 1997). For example, the *hrpA* gene from the *hrpZ* operon in *P.s.* pv. *tomato* has recently been found to encode a filamentous, pilus-like protein as illustrated in Fig. 10.13 . The pili produced by *hrpA* and the *virB* complex in *A. tumefaciens* (Fullner *et al.*, 1996) may allow a form of conjugation to take place with the plant cell, a possibility first proposed by Lichtenstein (1986). Fullner *et al.* (1996) suggest that pili first attach to the recipient plant cell to establish a stable mating pair and that the pili may then retract to create a channel for movement of proteins into the plant cell. How the pilus may allow transfer through the wall remains unknown. Possibly, pili may be able to penetrate through the loose matrix of polysaccharides that constitutes the wall and make contact with receptors on the plant cell membrane.

The 'pilus story' is a good example of the increasingly close links that are being forged between molecular genetics and cell biology. Use of the microscopical approaches outlined in this chapter with genetically well defined plant/bacterium interactions should allow further mysteries of signal exchange and 'life in intercellular space' to be revealed.

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