CHAPTER 5

STRUCTURAL ASPECTS OF DEFENSE

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Summary

Plants can defend themselves very efficiently against phytopathogens. This resistance can be based on preformed resistance factors or it may be the result of infection-induced resistance reactions. Preformed and induced resistance mechanisms can be structural or chemical in nature. Clearly, the resistance of many plants to attack by potentially pathogenic micro-organisms is due to preformed structural properties such as an inappropriate surface hydrophobicity or topography which fails to supply the signals required for microbial ingress in addition to cell walls resilient to physical and chemical attack. Should a micro-organism overcome these preformed barriers, plants will almost invariably fall back on their second line of defense, i.e., active resistance reactions will be induced. Again, these may have structural aspects, such as local cell wall thickening and reinforcement, encapsulation of the penetrating pathogen in dead and often lignified or suberized cells, and even the formation of new meristems forming new layers of cells around the site of attempted microbial ingress. In this chapter, we will learn how plant cells build a strong cell wall as a protection against phytopathogens, and we will get to know the strategies that micro-organisms have adopted to circumvent or breach these cell walls. Finally, we shall explore the many ways plants have evolved to counteract microbial attacks - including preformed structural resistance factors and infection-induced structural resistance reactions. We will close the chapter with a discussion of the ways open to plant pathologists to unravel cause/consequence-relationships between plant defense mechanisms and disease.

Abbreviations

AGP	arabinogalactan protein
CAD	cinnamyl alcohol dehydrogenase
CCR	cinnamoyl-CoA reductase

CWDE	cell wall degrading enzyme
GAX	glucuronoarabinoxylan
GRP	glycine-rich protein
HRGP	hydroxyproline-rich glycoprotein
PAL	phenylalanine ammonia-lyase
PGIP	polygalacturonase inhibiting protein
PRP	proline-rich protein
THRGP	threonine hydroxyproline-rich glycoprotein

I. Introduction

Plants are completely resistant to the vast majority of the myriad of potentially pathogenic micro-organisms. Only a handful of microbes are able to overcome the multiple resistance barriers - preformed or induced - that protect a given plant species from pathogen attack. This so-called 'non-host resistance' of plants is often due to preformed resistance factors, which can be structural barriers or preformed chemical resistance factors. Only those microbes capable of breaching this line of non-specific resistance can colonize the plant successfully, i.e., the plant becomes a host species for this pathogen and so-called 'basic compatibility' is achieved. The ensuing selection pressure can lead to coevolution between host and pathogen and to the occurrence of race/cultivar-specific resistance phenomena: some cultivars of the host plant species develop resistance to some races or strains of the pathogen. This race/cultivar-specific resistance is always based on infection-induced active resistance mechanisms which may be structural or chemical in nature.

Clearly then, there are two different structural aspects of defense in plants against microbial pathogens: structural barriers as **preformed resistance factors** and structural barriers as **induced resistance mechanisms**. The former usually act at the level of host recognition or primary penetration of the pathogen, while the latter usually interfere either with primary penetration or with the establishment of a functional host/pathogen-relationship and the spread of the pathogen in the host tissue. A look at the recognition and penetration strategies of fungal and bacterial plant pathogens pinpoints the crucial steps where structural resistance factors or mechanisms may interfere with pathogen ingress and growth.

After having passively reached, or actively found, a suitable host plant, the pathogen has to recognize it, possibly orient itself on the surface, and initiate the penetration process in order to be able to use the plant as a source of nutrients. These processes are all vitally necessary components of infection, and failure of any of these invariably leads to resistance of the plant to the micro-organism.

Except for the rather few cases where the pathogen is motile and, thus, capable of actively searching out a suitable host plant (e.g., by the directional movement of oomycete zoospores, attracted by leaking root exudates, towards plant roots), or the inoculation of host plants by mobile vectors (e.g., viral inoculation via insects feeding on plants), contact with a host plant is accidental. Attachment of a fungal spore to a potential host plant and ensuing germination are usually thought to be

rather non-specific processes. Little is known at present concerning the specificity of surface attachment and whether chemical or structural surface properties - such as hydrophobicity - influence the process. Spore germination is usually triggered by the presence of moisture in the form of free water, or at least high humidity. In some cases, germination seems to be stimulated chemically by the presence of nutrients or even more specific host plant metabolites. Recently, surface hydrophobicity has been shown to be a possible structural trigger of spore germination (Shaw *et al.*, 1998).

In order to penetrate into the host tissue, the emerging germ tube has to breach the host plant's surface (Fig. 1). Penetration can be *via* natural openings such as stomata or lenticels or *via* self-inflicted wounds (e.g., during the emergence of lateral roots) or after traumatic damage (e.g., by agricultural practices or animal feeding). Alternatively, some pathogens penetrate directly through the outer periclinal epidermal cell wall into an epidermal cell or *via* the middle lamella between anticlinal epidermal cell walls into the underlying intercellular spaces between the mesophyll cells. In most, and possibly all, of these cases, penetration does not occur at random but only after recognition of an appropriate site such as a stoma or an underlying anticlinal cell wall. Recognition most likely occurs at the tip of the growing germ tube, and the triggering signals may be chemical or physical in nature. Looking for possible structural defense factors of plants against microbial infection, it can be concluded that an inappropriate surface hydrophobicity or topography leading to failure of attachment, germination, or correct recognition may present effective structural defense factors.

Figure 1. Penetration strategies of microbial plant pathogens

In contrast to bacterial pathogens, which, due to a lack of cutinases, can only penetrate into host plant tissues via natural openings, fungal pathogens have evolved a number of different ways to gain access to the nutrient pool of their host plants. Fungal penetration may be via wounds (1), via the cuticle and the middle lamella of an anticlinal epidermal cell wall (2), directly via the cuticle and the outer periclinal epidermal cell wall (3), or via stomata (4).

If the pathogen penetrates via the epidermal cell walls, then first the cuticle and later the middle lamella or the entire cell wall must be breached. If the initial penetration allows the pathogen access to the intercellular spaces of the host tissue, a secondary penetration through the cell walls of mesophyll cells, or the dissolution of the host cell walls, is often required in order for the pathogen to reach the intracellular nutrients of its host plant. Both cuticular and cell wall penetration may be either by chemical means (i.e., by the action of hydrolytic enzymes secreted by the pathogen) or by physical means (i.e., by the exertion of a highly localized pressure). Both the chemical and the physical structure of the cell wall, thus determine its resistance to microbial penetration, and **resilient cell walls** are therefore the major line of structural defense of plants against microbial pathogens.

If the host plant's cell wall in its native, pre-infection state is not completely resistant to penetration, the host cell may attempt to reinforce it during the actual process of penetration. In contrast to the above described preformed or passive structural barriers to resistance, this is an active process induced by the infection of the plant by the pathogen. The process does not necessarily involve the production of new cell wall material. Instead, re-arrangement or **cross-linking of pre-existing cell wall components** may render them more resistant to enzymatic hydrolysis or physical pressure. Usually, though, the **incorporation of new components into the existing wall** is concomitantly induced, and these are typically components that are easily cross-linked or polymerized, such as phenolics, offering additional strength to the wall. In many cases, the cell wall is further strengthened by the **local apposition of new wall material onto the existing cell wall**, forming a so-called 'papilla' which may later also become encrusted with polymerizing molecules. Lastly, the **production of new cell walls or even new tissue** may be attempted to isolate the infecting pathogenic micro-organism.

In all of the above cases of both preformed structural resistance factors and infection-induced structural defense mechanisms, it is the host plant's cell wall, its chemical composition or its physical form, that represents the crucial structural barrier to microbial attack. We will thus have to look firstly at the highly sophisticated, complex three-dimensional 'extracellular organelle' that is the cell wall. We will then get to know the tools phytopathogenic micro-organisms have developed to overcome the structural barriers of their host plants before we proceed to discuss the defense strategies adopted by the plants to counter the microbial attack. We close the chapter with a few thoughts about how to prove a presumed causal role of any one structural feature of a plant in resistance against a given pathogen.

II. The Potential Host Plant: How to Build a Strong Cell Wall

Cell walls were the first biological structures seen under a microscope, and they have continued to be the focus of detailed microscopic work. At the beginning of this century, plant cell walls were described as being composed of a middle lamella, primary and, sometimes, secondary and even tertiary cell wall layers in many forms. This century has seen an explosion of knowledge on the biochemistry of the diverse cell wall components that interact to elaborate a plant cell wall. Plant cells are surrounded

and shaped by a cell wall which physically and chemically protects individual cells and separates and connects neighboring cells. In plant cell walls, **cellulose** fibers take up the tensile forces while **hemicelluloses** and **pectins** make up the pressure-bearing matrix. Apart from these components, which appear to be present in every plant cell wall, additional molecules have long been known to be incorporated into, or layered onto, some cell walls in order to provide specialized functions: **lignin** to give strength to dead, turgor-less cells, e.g., in the xylem; **suberin** to impregnate cell walls and to make them impermeable to water, e.g., in cork cells and in the Casparian band of the endodermis; **cutin** and the epicuticular **waxes** of the cuticle to protect the above ground parts of land plants from desiccation. In addition, it is becoming increasingly apparent that cell walls also contain many different proteins and **glycoproteins** which fulfill both structural and enzymatic functions. All of these components have been investigated in great detail for a long time, but they still continue to yield new and surprising features. Based on our knowledge of the biochemistry of cell wall components, forged mainly from studies on plant cells growing in liquid suspension culture, several models have been proposed for their interactions to form a primary cell wall (Keegstra *et al.*, 1973; Lamport, 1986; Carpita and Gibeaut, 1993; Iiyama *et al.*, 1994). The advent of new and sophisticated microscopic methods in the past few years has kindled a new interest to look at the extremely more complex cell walls of intact plant tissues (Hoson, 1991, McCann *et al.*, 1995). In spite of this long standing interest, our current models of plant cell wall architecture remain approximate sketches at best (Fig. 2).

A. PRIMARY CELL WALLS OF SUSPENSION-CULTURED PLANT CELLS

The first models of plant cell wall architecture resulted from data obtained by investigating the primary walls of *Acer* cells rapidly growing in liquid culture. These can be regarded as a prototype for the primary cell wall of dicot plants. In contrast, the primary walls of some of the monocot plants, most notably the grasses, are made up of somewhat different components and, consequently, their architecture differs to some extent from that of dicots. Since cereals are monocots and play a major role in human nutrition, this necessitates an equally detailed interest in monocot cell walls.

1. *Dicot Plants*

Plant cell walls are made up of a fibrillar component - cellulose microfibrils (Fig. 3) - embedded in a complex matrix. Cellulose accounts for about one quarter of the dry weight of primary cell walls. Each cellulose microfibril, with a diameter of about 10 nm, is thought to be completely wrapped in an envelope of xyloglucan chains (Fig. 3) which mediate the surface interaction between the fibrillar component of the cell wall and the surrounding matrix. Neighboring microfibrils are about 20 to 40 nm apart, and individual xyloglucan molecules of about 200 nm length are thought to span the interfibrillar space, their ends hydrogen-bonding to different cellulose microfibrils. The cellulose and xyloglucan molecules thus form a three dimensional network in the cell wall, accounting for about one half of its dry weight (Fig. 2).

Figure 2. Architecture of a dicot plant cell wall

The typical primary cell wall of a dicot plant cell is thought to be composed of three independent networks. Cellulose microfibrils are covered by xyloglucans - individual xyloglucan molecules can hydrogen-bond to different microfibrils, thus cross-linking the fibrils to a cellulose-xyloglucan network. Pectic polymers built from linear (smooth) homogalacturonan and branched (hairy) rhamnogalacturonan domains are oriented perpendicular to the cellulose fibers - Ca⁺⁺-mediated egg box structures crosslink pectic molecules to the second, independent network in the cell wall. When cell growth has ceased, the final shape of the cell is fixed by the knitting of a third, proteinaceous network from hydroxyproline and proline rich glycoproteins through peroxidative formation of intermolecular isodityrosine bridges. In some cells fulfilling specialized functions, additional cell wall layers are laid down onto the inner surface of the primary cell wall, and this secondary wall can be modified, e.g., by the accretion of suberin or the incrustation of lignin. Upon wounding, breaches in the cell wall may be sealed by the rapid apposition of callose.

Cellulose

- Cellulose is a linear chain of glucose (β -D-glucopyranose) units connected in 1,4-glycosidic linkages.
- Around 4000-8000 cellobiose units make up a cellulose molecule of about 5-10 μ m length.
- Around 30-100 cellulose molecules tightly self-assemble *via* intermolecular hydrogen bonds to a linear cellulose microfibril of about 10 nm diameter. As the cellulose molecules in a fibril are staggered, the resulting microfibril is much longer than the individual molecules.
- The tensile strength of the microfibril is comparable to a steel wire of identical diameter.

Hemicelluloses

- Hemicelluloses are the non-pectic components of the cell wall matrix.

- Typical of dicot cell walls is *xyloglucan* which consists of a cellulosic backbone with three out of four glucose residues bearing an α -xylose residue which may be further substituted by a usually acetylated β -galactose which may in turn carry an α -fucose. These different side chains build regular heptasaccharide and nonasaccharide repeat units.
- The trisaccharide side chain of the nonasaccharide repeat folds back on the glucan backbone stabilizing a planar configuration of the molecule where all side chains are on one side of the backbone so that the opposite side can hydrogen-bond to the surface of a cellulose microfibril.
- Some stretches of the xyloglucan molecules are thought to completely wrap the cellulose microfibrils thus ensuring the individual identity of separate fibrils while other stretches may span the interfibrillar spaces thus cross-linking the microfibrils to a cellulose/xyloglucan-network.

Pectins

- Pectins, in concert with hemicelluloses, form the matrix of the cell wall.
- Pectins are defined as cell wall components containing galacturonic acid residues. The carboxyl groups convey a very hydrophilic character enabling pectins to attract large amounts of water which ensures the "apoplastic free space" for diffusion, the gelling properties and the pressure-bearing characteristics of the matrix.
- There are two basic types of pectic polysaccharides built on different backbones:
 - Homogalacturonan* consists of a linear unbranched chain of α -1,4-glycosidically linked galacturonic acid residues; 200 residues are thought to form a chain of around 100 nm length. Stretches of homogalacturonan molecules can dimerize either *via* divalent calcium cations cross-linking the carboxyl groups or *via* hydrophobic interactions dimerizing two highly methyl-esterified stretches of homogalacturonan. Partial dimerization leads to the formation of the pectic network.
 - Rhamnogalacturonan I* has a backbone of unknown length made of strictly alternating galacturonic acid and rhamnose residues and might be flanked on both sides by short consecutive stretches of highly methyl-esterified galacturonic acids.
- About every other rhamnose residue acts as a branching point by bearing an arabinan, galactan, or arabinogalactan side chain. Due to its strongly branched nature, rhamnogalacturonan I is described as the "hairy region" of pectin, as opposed to the "smooth region" of homogalacturonan.
- Most likely, homogalacturonan and rhamnogalacturonan I form different domains of the same pectic molecule.

Figure 3. Constitutive components of dicot plant cell walls

All primary and secondary cell walls of dicot plants are built from a set of major polysaccharide components: cellulose, xyloglucan, and pectin.

This cellulose/xyloglucan-network is embedded in the pectic matrix which makes up about one third of the dry weight of the cell wall. The pectins (Fig. 3) probably form a second, independent network in the cell wall which may not be covalently linked to the cellulose/xyloglucan network (Fig. 2). The intermolecular cross-links knitting pectic polysaccharides together are most likely Ca^{++} -egg box structures between non-esterified stretches of homogalacturonan, hydrophobic interactions between methyl-esters of homogalacturonan stretches, and, possibly, boron diesters between two rhamnogalacturonan II-stretches.

The structural glycoproteins (Fig. 4) - roughly 10 to 20% of the dry weight of the wall - may form a third independent network in the primary plant cell wall of dicot plants (Fig. 2). The major component appears to be the hydroxyproline-rich glycoprotein (HRGP) extensin, but proline-rich proteins may be involved as well. The only cross-links proposed so far to hold the protein network together are intermolecular isodityrosine bridges, but their existence still awaits experimental support.

2. Grasses

Primary cell walls of grasses are made up of somewhat different, though similar components compared to the typical primary cell walls of dicot plants (Table 1). The fibrillar component of both types of cell walls is made of cellulose, but the major hemicellulose of the grass cell wall is glucuronoarabinoxylan - which alone can account for about half of the dry weight of the walls - while only very small amounts of xyloglucan are present. In grasses, glucuronoarabinoxylan chains are believed to coat individual cellulose microfibrils and to span the interfibrillar spaces thus forming a cellulose/glucuronoarabinoxylan-network functionally equivalent to the cellulose/xyloglucan-network of the dicot cell wall.

The grass cell wall is rather poor in pectin - around 10% compared to about 30% in dicot walls - and the negatively charged glucuronoarabinoxylans are thought to partly substitute for the missing pectins. It can be expected that the same types of interpectic cross-links exist in the grass cell wall as discussed above for the dicot wall. Many of the arabinose residues of the glucuronoarabinoxylan carry ferulic acid or p-coumaric acid esters, and these may dimerize oxidatively to form diphenolic acid cross-links possibly substituting for missing interpectic links. Thus, a pectin and/or a glucuronoarabinoxylan/phenolic acid-network may exist in grass cell walls.

Grass cell walls are relatively poor in structural glycoproteins, which account for only about 2 to 10% of the cell wall dry weight. The relative importance of a presumed proteinaceous network in the grass cell wall is not known at present. However, grass cell walls may contain an additional polysaccharide component - a mixed linkage β -1,3- β -1,4-glucan - which may hydrogen-bond to cellulose and which may be covalently cross-linked to proteins. It has been speculated that mixed linkage glucan chains form an important intermediary network during phases of elongation growth of the cell.

Proteins, Glycoproteins, and Proteoglycans

- HRGPs, PRPs, GRPs, and AGPs are structural proteins.
- *Hydroxyproline-rich glycoproteins* (HRGPs, extensins) are characterized by post-translationally hydroxylated proline (hydroxyproline, hyp), which makes up about 40 % of the amino acid residues and often occurs in the repeated sequence ser-hyp-hyp-hyp-hyp. Some serins are substituted with a galactose residue while most hydroxyprolines carry side chains containing one to four arabinoses stabilizing the protein's helical secondary structure and giving it a stiff rod-like conformation. HRGPs might form a defined scaffold which determines the spacing of the cellulose microfibrils; intermolecular diphenylether isodityrosine bridges may cross-link HRGPs creating a three dimensional proteinaceous network.
- *Proline-rich proteins* (PRPs) have been implicated to be involved in the termination of cell growth when they may form a heteroprotein polymer with HRGPs.
- The synthesis of *glycine-rich proteins* (GRPs) appears to be correlated with the process of lignification of cell walls. GRPs most probably adopt a β -pleated sheet secondary structure with one side orientated toward the plasma membrane and the other side - which may contain aromatic amino acids serving as anchors for the phenolic lignin polymer - facing the cell wall.
- Some *arabinogalactan proteins* (AGPs) seem to be embedded in the plasma membrane, others are located in the middle lamella. They are thought to play important roles in the signal exchange between the cytoplasm and the extraplasmatic wall of a cell, or between adjacent cells, respectively. The protein part of the AGPs accounts for only around 2 % of their molecular weight; the rest is made up of highly branched arabinogalactans. AGPs, thus, are proteoglycans rather than glycoproteins.

Lignin

- *Lignin* is a polymer of phenylpropanols consisting of three slightly different monomeric units which are connected by a variety of covalent linkages thought to be produced in a random polymerization process. Lignin, as a random polymer, is extremely difficult to degrade enzymatically.
- Lignin of gymnosperm plants is made up of coniferyl (= ferulyl) alcohol, dicot angiosperm lignin contains in addition sinapyl alcohol, while monocot plants incorporate also p-coumaryl alcohol and phenolic acids.

● = major points of cross-linking
○ = minor points of cross-linking

Suberin, Cutin, and Waxes

- *Suberin*, a lignin-like phenolic acid polymer, and *cutin*, a polyester and polyether of hydroxy-, epoxy-, and oxo-acids and -alcohols, are laid down in layers alternating with layers of waxes. Both are extremely difficult to degrade enzymatically.
- *Waxes*, complex mixtures of esters of long chain fatty acids and alcohols with other components such as long chain hydrocarbons and fatty aldehydes, form water-insoluble barriers.

Callose

- Callose is a linear chain of β -1,3-linked glucose residues which can be produced extremely fast to reinforce an existing cell wall.
- Of equal importance may be the ease with which callose can be degraded by plant enzymes should the need arise.

Figure 4. Induced components of dicot plant cell walls

Both primary and secondary walls of individual plant cells contain components that are specific to a certain cell type or to a certain developmental stage of a cell, and that help the cell to fulfill its specific function: e.g., proteins, glycoproteins, and proteoglycans; lignin, suberin, cutin, and waxes; and callose.

Table 1. Typical primary cell walls of grasses as compared to typical primary cell walls of dicot plants.

	grass cell walls	dicot cell walls
microfibrils		
cellulose	~ 20%	~ 20%
matrix		
major hemicellulose	glucuronoarabinoxylan ~ 50%	xyloglucan ~ 20%
The backbone of glucuronoarabinoxylan (β -1,4-xylose) and of xyloglucan (β -1,4-glucose) closely resembles or is identical to cellulose, respectively. Both can, therefore, hydrogen-bind to the surface of cellulose microfibrils.		
pectins	homo- and rhamnogalacturonan ~ 10%	homo- and rhamnogalacturonan ~ 30%
The rather small amount of pectins present in monocot plants may lead to either a higher or a lower importance of the individual pectic cross-links in the grass wall compared to the dicot wall. A decision will have to await further experimentation.		
structural proteins	HRGP, PRP, GRP, and AGP ~ 2-10%	THRGP, PRP, GRP, and AGP ~ 10-20%
low molecular weight phenolics	ferulic and p-coumaric acid esterified and etherified to GAX and lignin ~ 2-10%	ferulic and p-coumaric acid esterified to pectins ~ 0-5%
lignin	sinapyl, coniferyl, and p-coumaryl monolignols; ferulate and p-coumarate esters and ethers	sinapyl and coniferyl monolignols

Primary cell walls of grasses - such as our most important crop plants, the cereals, e.g., wheat and rice - are built of somewhat different components compared to the typical primary cell wall of a dicot plant. While cellulose is the fibrous material in both cases, the major hemicellulose of grass cell walls is glucuronoarabinoxylan, while only very little xyloglucan is present. The negatively charged glucuronoarabinoxylan also at least partially takes over the role that pectins play in dicot plants. While the same pectic molecules appear to be present in both types of cell walls, they are clearly less abundant in grasses. Also, grass cell walls appear to be rather poor in glycoproteins. The fixation of shape once cell growth has ceased is most likely brought about by a peroxidative formation of diferulate crosslinking glucuronoarabinoxylan chains to which the monomeric ferulic acids are esterified. Lastly, the lignin found in grass cell walls is more complex in

3. *Complex Cell Walls of Plant Tissues*

In contrast to the artificial situation of plant cells growing in liquid suspension where all cells are permanently in the same state of differentiation, namely rapid spherical growth, cells of many different types, and in many different stages of differentiation, interact with each other to form a highly complex tissue in the whole plant. Cultured plant cells are surrounded only by a primary cell wall. In a plant tissue, all cells still in the process of growing and even many fully differentiated cells, such as leaf mesophyll cells, also contain only a primary and no secondary cell wall. However, all the different cells that make up a tissue are 'glued' together by the middle lamella, a structure which may not be present in cultured cells, at least if they grow as a very fine suspension of individual cells. Furthermore, it appears that the primary wall of a cell in a tissue is much less uniform than that of a suspension cultured cell.

The mature primary cell wall of about 100 nm thickness consists of only about three layers of cellulose microfibrils. Each layer, thus, resides in a different micro-environment, the outer layer facing the pectic middle lamella, the middle layer surrounded by the other two, and the inner layer facing the plasma membrane or, later, the secondary cell wall layers. Virtually nothing is known on the interaction of the primary cell wall with these neighboring layers. Immunocytological electron microscopic studies using antibodies to different cell wall components, however, revealed striking differences in the composition of the different parts of the primary cell wall. Antibodies cross-reacting with non-methyl-esterified epitopes of pectin stained the middle lamella, especially in cell corners, but also lined the inner surface of the primary cell walls along the plasma membrane. Antibodies cross-reacting with highly methyl-esterified pectic components, in contrast, evenly showed up across the whole width of the primary cell wall. Cell wall angles where three or more different cells meet are distinct from cell wall stretches that contact a single neighboring cell and these again differ from areas that face the intercellular space. These and related approaches will certainly shed new light on our understanding of the plant cell wall, but they can be expected to pose more questions initially than will be answered - e.g., how is this enormous complexity outside the cytoplasm regulated?

The wall of an individual cell is a highly differentiated organelle with domains differing in structure and - most likely - also in function. Cells are formed in meristematic zones where cell division occurs, they undergo elongation, differentiation, and eventually may senesce. Clearly, the primary wall of a cell constantly changes during these processes, but even today little is known of what these changes entail. Moreover, many cells that fulfill special functions within a plant tissue produce unusually, and often unevenly, thick primary cell walls or develop highly sophisticated secondary cell wall layers upon differentiation after elongation growth has stopped - e.g., thickening and cuticle excretion of the outer epidermal cell walls, and localized thickenings and lignification in tracheary elements.

An example of unevenly thickened primary cell walls is given by the outer walls of epidermal cells. In addition, cutin monomers and cuticular and epicuticular waxes (Fig. 4) are produced in epidermal cells and transported to the outer surface of the outer periclinal cell wall to reach the surface where they are laid down in layers and where the cutin is polymerized *in situ*. Besides protecting from transpiration loss of water,

the cuticle is also of paramount importance for warding off potentially phytopathogenic micro-organisms (Kerstiens, 1996).

One of the most prominent examples of secondary cell walls is certainly the intricate elaboration of cell wall thickenings in tracheary elements of the xylem where additional cellulose fibers and embedding matrix polymers are laid down only on certain parts of the cell walls. Eventually, lignin is incorporated into the walls of these cells (Fig. 4). The polymerization process of the monolignols taking place *in muro* leads to a growing lignin polymer infiltrating the primary cell wall, displacing the water as it does so. Eventually, when the polymerization process is complete, the lignin polymer serves to cement in place all the other components of the cell wall. The wall is then no longer permeable to water, and the mature tracheid dies. The ensuing loss of turgor pressure would result in a shrinking of the dead cell and most likely a collapse when the negative pressure (tension) of the transpiration stream is taken into account, were it not for the lignin impregnating and stiffening the cell wall of the tracheid.

III. The Pathogen: How to Breach a Plant Cell Wall

Almost any potential pathogen of above ground plant organs will first meet the waxes and the cutin of the cuticle as an obstacle in front of the nutrient pool within the plant cells (Fig. 1). If this barrier is crossed, most pathogens will then be confronted with the middle lamella of the plant cell walls, and many pathogens appear to prefer the middle lamella portion of their host cell walls for further ingress; penetrating into the leaf at the anticlinal junction between two adjacent cells. Most pathogens will eventually have to deal with the primary cell wall enveloping every host cell. Clearly, there are two ways of breaching through a cell wall. The more 'biological' way appears to be enzymatic hydrolysis of some or all of the cell wall components. But mere physical pressure is another way apparently adopted by some fungal pathogens.

A. PENETRATION WITH THE AID OF CELL WALL HYDROLYZING ENZYMES

Plant pathogenic micro-organisms are typically able to produce a wide array of plant cell wall degrading enzymes (Walton, 1994). This is certainly true for necrotrophic pathogens such as plant pathogenic bacteria, e.g., *Erwinia carotovora*, and many fungi, e.g., *Botrytis cinerea*. It is less true for biotrophic pathogens such as the obligately biotrophic rust and mildew fungi. But even these have to degrade the walls of their host cells very locally in order to come in intimate contact with the protoplast within.

Cell wall degrading enzymes are usually not produced constitutively in great amounts but they are induced by the appropriate substrates or small amounts of the breakdown products. As the pathogen finds its way into the plant tissue, different components of the plant cell wall are met consecutively, and the degrading enzymes are thus induced sequentially. We will organize our description of the relevant cell wall hydrolyzing enzymes according to this natural sequence.

1. *Cutinase*

Pathogens of above ground plant parts will first encounter the epicuticular wax layer covering the surface of land plants. Little is known on interactions between a potential pathogen and these waxes although an influence of a pathogen landing or growing on the outermost layer of a plant surface is clearly visible as an imprint when the pathogen is removed. Whether or not an enzymatic interaction or rather a physical process is causing the imprint, is mostly unknown. As the components of the epicuticular wax layer are rather low molecular weight compounds and are not polymerized, the wax layer appears to be easily breached, and no enzyme is thought to be necessary or known to be produced by plant pathogenic fungi.

Unlike the wax layer, the underlying cutin layers are complex polyesters and polyethers of different monomers. In order for a pathogen to penetrate this layer, either enzymatic hydrolysis or a considerable physical pressure can be expected to be necessary. Cutinases are esterases with a greater or lesser degree of specificity able to saponify the ester linkages of cutin. No enzyme is known that would be capable of cleaving the ether linkages of cutin but it would appear as if hydrolysis of the ester linkages suffices to weaken the cutin to a point where microbial ingress is possible. Cutinases are produced by many phytopathogenic fungi but they appear to be absent in bacteria. This may explain why bacteria always require pre-existing openings such as wounds or stomata to enter plant tissues.

The induction process of fungal cutinases has been studied in some detail (Fig. 5) (Podila *et al.*, 1988). Some fungi appear to produce low amounts of cutinase constitutively. When a fungal spore lands on a plant surface, the enzyme starts to degrade the cutin, producing cutin monomers. These in turn are taken up by the fungus and act as inducing signals to activate transcription of the cutinase gene. More enzyme is produced, and this autocatalytic cycle leads to rapid degradation of the plant cutin. Most likely this induction strategy is typical of many other cell wall hydrolyzing enzymes of microbial plant pathogens.

Figure 5. Induction process of fungal cutinases

Cutinases, similar to other cell wall degrading enzymes, are often induced by low molecular weight products of their own enzymatic action. The cutinase gene of some fungi is leaky so that low amounts of cutinase are produced constitutively. These begin to degrade the cuticle of a potential host plant as soon as the fungal spore has landed on a plant surface. The cutin monomers produced are taken up by the fungus and strongly induce the cutinase gene. More cutinase is produced so that the fungus can eventually degrade and penetrate the plant cuticle (Podila *et al.*, 1988).

It is important to note that some pathogens never penetrate the cuticle. The large group of root pathogens is not likely to ever encounter a cuticle proper, and some leaf or shoot pathogens avoid the cuticle by penetrating through wounds or through natural openings - as mentioned above for bacteria. Such a penetration mechanism, however, requires the successful location and recognition of stomata, a process that we will investigate in more detail later in this chapter.

2. Pectic Enzymes

Whether a pathogen has penetrated the cuticle to gain access to the plant tissue or whether it has entered the intercellular space of the host tissue via one of the alternative routes, it will invariably then be confronted with pectic components of the host cell wall. Some pathogenic fungi, e.g., *Claviceps purpurea*, initially penetrate only the cuticular membrane and then grow just below the cuticle, within the outermost, pectin-rich layer of the outer periclinal epidermal cell wall (Fig. 6). Other fungi penetrate deeper into the host tissue within the middle lamella of an anticlinal epidermal cell wall. The middle lamella, which is also the outermost layer of all 'internal' plant cells, is composed chiefly of pectins, at least in the case of dicot plants, and consequently, pectin degrading enzymes are produced early during the penetration process by many plant pathogenic organisms (Fig. 6). The complexity of the pectic polysaccharide fraction in cell walls is mirrored by the broad spectrum of the enzymes pathogens produce to degrade it (Fig. 7), although to date, only enzymes capable of depolymerizing homogalacturonan have been identified from pathogenic organisms (Alghisi and Favaron, 1995).

Any one plant pathogenic micro-organism usually produces several of these possible enzymes. Moreover, the enzymes are usually produced as families of isoenzymes so that the pectic polysaccharide component of a plant cell wall is attacked by a wide array of different enzymes. The reason for this immense complexity is not yet fully understood. It might be that we understand only too little of the complexity of the substrate. Owing to their exposed position in the plant cell wall, many specialized features may have evolved that help to protect the pectic molecules from enzymatic degradation, necessitating equally specialized enzyme activities. Moreover, it has become apparent that the pectins of the cell wall are a rich source of biologically active signal molecules. It should not be too surprising that surface molecules of plant cells act as signals recognized by neighboring cells in order to build a functionally intact tissue. But apparently, plant cells have also learned to interpret some of the degradation products of their pectins as a sign of alarm. Medium length oligomers of galacturonic acid, as produced by the action of endo-pectate hydrolase (also termed endopolygalacturonase), act as endogenous elicitors signaling to the plant cell the potential presence of a pathogen and triggering induced resistance mechanisms. Some micro-organisms appear to have counteracted this signaling mechanism by developing extremely active pectic enzymes which rapidly degrade pectic polymers down to very small oligomers, galacturonic acid dimer or trimer, which do not act as endogenous elicitors. Then again, plants contain polygalacturonase inhibiting proteins (PGIP) in their cell walls which slow down the activity of the enzymes so that medium length oligomers might be present long enough to be recognized by the plant cell. Another way around the problem of recognition might be for the pathogen to produce exo-cleaving enzymes, as these would only produce

Figure 6. Cutin and pectin degradation by a fungal parasite

Many phytopathogenic fungi are able to enzymatically degrade cutin and they can thus penetrate intact cuticles of potential host plants. Some fungi immediately continue to breach the underlying pectin rich region of the outer periclinal epidermal cell wall or they digest their way down the pectin rich-middle lamella of an anticlinal epidermal cell wall. Some fungi, however, after having penetrated the plant cuticle, exhibit a phase of subcuticular growth, as seen here with *Claviceps purpurea*, the causal agent of ergot, on rye: the fungal hypha (F) grows just below the cuticular membrane (cm) of the host cell (H) (a). A monoclonal antibody reacts with non-methylesterified regions of pectin in the host cell wall (hcw) only just in advance of the fungal cell wall (fcw), presumably due to the action of a fungal pectin methyl-esterase (b) (courtesy of K. Tenberge, Münster, Germany).

Figure 7. Pectin and pectate degrading enzymes

Pectin is a complex mixture of linear homogalacturonans and branched rhamnogalacturonans. The structure of both molecules may be further complicated by the modification with methyl and acetyl esters. Accordingly, microbial enzyme systems aimed at degrading plant pectins are fairly complex. Today, only enzymes degrading homogalacturonans have been studied with respect to plant-microbe interactions. These can first be separated into two groups, according to the substrate they prefer: pectate-degrading enzymes cleave non-methylesterified regions of homogalacturonan, while pectin-degrading enzymes act on highly methylesterified regions of the same molecules. Secondly, the enzymes can be grouped according to their mode of cleavage: hydrolases perform a typical hydrolytic cleavage where a molecule of water is added to the glycosidic linkage, while lyases cleave the glycosidic bond without the addition of water thus forming a double bond at the new non-reducing end. Theoretically, this makes already for four different enzymes: pectate hydrolase (usually named polygalacturonase), pectin hydrolase, pectate lyase, and pectin lyase. However, the second one, pectin hydrolase, has not been found so far. Polygalacturonase and pectin lyase are typical fungal enzymes, while bacteria are more likely to produce pectate lyases. However, this is not the end of the story. In addition, the enzymes can either cleave internal glycosidic bonds in which case they are named endo-cleaving enzymes, or they can act on the ultimate or pen-ultimate glycosidic bond near the non-reducing end of the polymer which makes them exo-cleaving enzymes. A pectin lyase can, thus, be an endo-pectin lyase or an exo-pectin lyase, etc. Pathogens with only pectate-degrading enzymes but devoid of pectin-cleaving activities would have problems to degrade highly methylesterified pectins. However, such organisms usually also produce a pectin methyl-esterase converting pectin to pectate which can then easily be degraded.

dimers or monomers of galacturonic acid. However, this is a tedious way of degrading a polymer (a bite at the end does not greatly influence the property of a polymer, while a bite in the middle cuts the molecule in half!) and it may give the host plant too much time to organize its defense. We seem to be witnesses to an ongoing, interactive process, where depending on the host/pathogen-interaction under investigation, either the pathogen or the host plant comes out as the winner.

Similar to cutinase, pectic enzymes are not usually produced constitutively in large amounts. Again, small amounts may be present to start the degradation of the substrate, and the degradation products - usually galacturonic acid oligomers which may or may not bear a double bond at their non-reducing end - are taken up by the micro-organism. Subsequent cytoplasmic degradation products then act as signals inducing transcription

of the appropriate genes for the pectic enzymes (Hugouvieux-Cotte-Pattat *et al.*, 1996). This autocatalytic, 'substrate induction' cycle guarantees rapid production of enough enzyme to degrade the pectic component of the middle lamella and of the primary cell wall of the host plant.

3. *Hemicellulases*

Pectic enzymes are by far the best studied cell wall degrading enzymes of phytopathogenic organisms but degradation of pectin appears not to be enough to allow cell wall penetration or to lead to bursting of the host cells. This is not surprising regarding the architecture of the plant primary cell wall which appears to consist of three independent networks (Fig. 2). When the pectic network is degraded and, thus, the matrix is partly removed from the cell wall, the load-bearing cellulose/xyloglucan-network is still in place. This network can rather easily be weakened significantly by cleavage of only a few xyloglucan bonds. If a (xylo)glucanase snips the interfibrillar domains of xyloglucan, this might weaken the network to the point where the cell wall can no longer withstand the internal turgor pressure. The bursting of the host cell not only releases its cytoplasmic content to the pathogen, but also renders any attempt at an induced, active resistance mechanism of this cell impossible (this, of course, would not be a good strategy for a biotrophic pathogen!). It may well be that one of the main reasons for the primary degradation of pectins is to expose the xyloglucan chains to enzymatic attack.

Xyloglucanases are typically endo-acting β -1,4-glucanases which can degrade the xyloglucan backbone only next to a glucose residue that is not substituted by a xylose. Owing to the rather regular organization of xyloglucan, this mode of cleavage gives rise to predominantly heptamers and nonamers of xyloglucan. These are presumed to be biologically active oligomers involved in developmental processes of the plant, but their participation in host/pathogen-interactions, either as pathogenicity factors or as endogenous elicitors of induced plant defense responses, has not been described.

In the case of grasses as potential hosts, the situation is a little different. Firstly, there are less pectins and, consequently, pathogens of grasses usually do not produce significant amounts of pectic enzymes early in the infection process. Also, xyloglucanases would probably be of limited value. It has been shown in some cases that grass pathogens produce a set of enzymes capable of degrading glucuronoarabinoxylan. This involves an acetyl esterase, an α -arabinosidase, and an endo- β -1,4-xylanase. However, little is known on the action of these enzymes on native glucuronoarabinoxylan and on the products formed.

4. *Cellulase*

Degradation of the cellulose microfibrils appears not to be a prerequisite for cell wall penetration, and we have seen why this is so. Moreover, the crystalline, water-insoluble nature of cellulose makes for a very difficult substrate. Nevertheless, many pathogens have evolved an enzymatic machinery for cellulose breakdown which is probably less involved in breaching structural barriers of plants but rather opens up the glucose reservoir of cellulose as a nutrient source for the pathogen. In the context of this chapter, it might

therefore suffice to summarize that the cellulase complex usually consists of at least three different enzymatic activities, an endo- β -1,4-glucanase, a cellobiohydrolase, and a β -glucosidase. Acting in concert, these three enzymes are able to bind to crystalline cellulose and to degrade it to cellobiose units and, eventually, to glucose residues.

5. *Proteases*

At this point, the careful reader should expect a section on microbial proteases able to degrade the third, proteinaceous network of the primary plant cell wall. However, virtually nothing is known about such proteases. Many phytopathogens are, of course, known producers of proteases, but these are usually enzymes able to degrade soluble proteins, and they can be envisaged as being involved in protein breakdown to produce amino acids as nutrients. The highly insoluble, structural cell wall glycoproteins, in contrast, are highly resistant to proteolytic attack. Even the soluble precursors of extensin are resistant to proteases unless the arabinose side chains are first removed. One might expect a specialized family of proteases adapted to the degradation of the insoluble cell wall proteins - similar to collagenolytic proteases - but these have received very little attention so far. One of the few reports comes from an obligately biotrophic rust fungus (Rauscher *et al.*, 1995), often falsely considered an unlikely candidate for cell wall degrading enzyme activities.

6. *Ligno-Peroxidases*

Very few organisms are capable of degrading lignified cell walls. For most pathogens, it is rather easy to avoid the few lignified cells in a host tissue - unless the host plant counters the pathogenic attack by induced lignification! The situation is clearly different for pathogens living in heavily lignified substrates such as wood. To date, the only known pathogenic micro-organisms capable of lignin degradation are the white rot fungi (Kurek, 1992).

Lignin, as a random polymer consisting of up to three different monomers connected via at least ten different covalent linkages (Ralph and Helm, 1993) and on top of that being completely water-insoluble, makes a very difficult substrate. On the one hand, the substrate is so varied that it is virtually impossible to produce an enzyme with an active center fitting to a significant portion of the molecule so that more than just a very few linkages can be broken. In addition, the C-O-C ether linkages and, especially, the direct C-C bonds are extremely stable and difficult to break. The strategy for lignin breakdown is, therefore, an unusual one: a peroxidase called ligno-peroxidase appears to oxidize a small, readily diffusible substrate, possibly veratryl alcohol (Piontek *et al.*, 1993). The oxidized product, a veratryl alcohol radical cation, apparently diffuses into the lignin polymer and is able to oxidize and cleave the phenolic ring. This will eventually give rise to a vast array of different lignin fragments the further metabolism of which is unknown.

B. PENETRATION BY PHYSICAL PRESSURE

While mechanical pressure is routinely cited as a possibility to breach the structural defense barriers of plants, there is little experimental evidence for this kind of host

cell wall penetration. One notable and long known example is the penetration behavior of zoospores of the obligately biotrophic fungal pathogen *Plasmodiophora* into root hairs of their cruciferous host plants where they cause the clubroot disease (Williams *et al.*, 1973). Inside the zoospore encysted on the surface of a root hair, a bullet-like 'Stachel' is visible which is virtually shot into the root hair cell, most likely by turgor pressure developing in the enlarging vacuole - the actual penetration process taking only seconds. The cytoplasmic content of the spore is then injected through this fine puncture into the host cell.

Another more recently investigated example of epidermal cell wall penetration by the action of pressure is the penetration of the rice blast fungus, *Magnaporthe grisea*, a necrotrophic pathogen of rice leaves (Howard and Valent, 1996). When the fungal spore has germinated on the surface of a rice leaf, a short germ tube develops which tightly adheres to the rice cuticle. An appressorium is formed at the tip of the germ tube which is separated from the rest of the germ tube by a septum. The cell wall of the appressorium becomes heavily melanized, a process somewhat reminiscent of lignification of plant cell walls. Incorporation of melanin renders the appressorial cell wall completely impermeable to larger solutes while its permeability for water is retained. The subsequent intracellular production of large amounts of glycerol leads to the production of an enormous turgor pressure within the appressorium which has been measured to almost 80 bar! This pressure must then apparently be released at a preformed breaking point facing the outer periclinal epidermal cell wall. The mere pressure is thought to be responsible for cuticular and cell wall penetration. The question of how this pressure release is regulated, i.e., how the fungus can penetrate the tough cell wall without also penetrating the directly underlying soft plasma membrane, has not even been addressed today. That pressure alone suffices for surface penetration can be shown when the fungus is allowed to germinate on a plastic surface of suitable hydrophobicity. Provided the appressorium can attach firmly to the surface, the fungus will penetrate any artificial substrate that is not too tough. Conversely, albino mutants of the fungus not able to produce melanin any more are incapable of cuticular penetration even though their enzymatic machinery is unchanged. Of course, while these observations allow the conclusion that physical pressure is involved in cuticular penetration, it may still be that *in planta*, a combination of pressure and enzymatic hydrolysis is active.

IV. Resistant Host Plants: How to Prevent Breaching of the Cell Wall

We have seen the intricate ways in which plants build their cell walls, and the equally sophisticated means by which phytopathogens have learned to breach these structural barriers. However, as we have stated early on, most plants are resistant to the attack by most potentially pathogenic organisms. In these cases, the tools of the pathogen are obviously inadequate to deal with the barriers put up by the plant. In the following sections, we will highlight some aspects of the possible interactions between host plants and microbial pathogens. We will first present the most crucial preformed resistance factors which plants may possess in order to ward off potential

pathogens passively, and we will then continue with a description of induced resistance reactions which may be triggered in an infected plant in order to actively prevent further ingress of the pathogen.

A. PREFORMED RESISTANCE FACTORS

The successful infection of a plant tissue by a pathogenic micro-organism is a complex process involving a number of critical steps. Each one of a number of requirements has to be met in order for the pathogen to be able to recognize the host, attach to it, move and orient itself on its surface, and penetrate into its tissues. Both chemical and physical - i.e., structural - signals appear to be involved in these processes (Hardham, 1992; Read *et al.*, 1992; Romantschuk, 1992; Gow, 1993; Schäfer, 1994; Mendgen *et al.*, 1996). Clearly, a plant not presenting required signals 'appropriately' may consequently be resistant to the attack by a pathogen relying on that signal.

1. Surface Hydrophobicity

Attachment and germination are *conditiones sine quibus non* for a successful infection of a plant by many phytopathogens. Already these very early events preceding host tissue penetration and colonization can fail due to structural features of the potential host plant. Perhaps the simplest of such features is the surface hydrophobicity. Generally, the contact angle of a water droplet, which is a measure of hydrophobicity, is extremely large on above ground plant surfaces (Holloway, 1969). Spores or germ tubes that can not adhere to such a hydrophobic surface cannot initiate a successful infection and the fungus will usually not be able to parasitize the plant. Pathogens, therefore, have adopted means to adjust their own surface hydrophobicity to that of the host plant, e.g., by the secretion of hydrophobins (Talbot *et al.*, 1993; Wessels, 1996), or vice versa, e.g., by enzymatic erosion of the cuticle.

Phytopathogenic bacteria which are flagellate, enter host plants via stomata or wounds and require a film of water on the leaf surface in order to be able to move. The hydrophobicity of the cuticle, however, favors the formation of little droplets of water rather than of a continuous film, greatly reducing the chances of bacterial ingress into host leaves.

Little experimental evidence is available to judge the importance of attachment as a presumably critical surface interaction. It has been shown that some fungal pathogens require a certain surface hydrophobicity or hydrophilicity in order to attach, and attachment has sometimes been proven to be essential for subsequent penetration. This appears evident in cases of appressorial penetration, especially when physical pressure is involved in breaching the epidermal cell wall. Here, the fungal structure has to tightly adhere to the cell wall lest the pressure exerted should only lift the appressorium off the plant surface. Attachment is most likely reached by the excretion of a sticky matrix (Nicholson and Epstein, 1991) and may be aided by the presence of cutinases and other esterases (Kunoh *et al.*, 1990; Deising *et al.*, 1992).

Similarly, it has been shown that oriented growth of a fungal germ tube on the host surface requires tight adhesion of the germ tube to the surface (Braun and Howard, 1994). Unlike many other fungal hyphae, a germ tube typically grows tightly appressed to

a surface, exhibiting a somewhat flattened profile in cross section and a 'nose down' profile in longitudinal section. An appropriate surface hydrophobicity appears to be a prerequisite for this type of growth behavior which in turn can be required to allow the fungal germling an orientation on the host surface guided by topographical features.

2. *Inappropriate Surface Topography*

While surface hydrophobicity seems critical in the attachment of spores, subsequent growth of the emanating germ tube appears to be influenced both by surface hydrophobicity and topography. A fascinating example of oriented germ tube growth and recognition of a stoma on the surface of an adequate host plant is given by the germ tubes of most rust fungi on their way to a stoma needed for penetration into the host plant tissue (Read *et al.*, 1992; Mendgen *et al.*, 1996).

a. Oriented Germ Tube Growth. We have already mentioned above the necessity for the fungal germ tube to quickly reach a host stoma before the resources of the spore are depleted. Chemo-attraction to stomata - based on pH-gradients, changes in epicuticular wax composition, or volatiles emanating from the stomata - has been proposed as a possible mechanism for oriented germ tube growth, but experimental evidence is largely missing. In contrast, convincing evidence has now accumulated for a surface orientation of the fungal germ tube based on topographical features of the outer epidermal cell walls. Using artificial ridges on plastic surfaces, it was clearly shown that the germ tube tip can sense a depression in the surface - such as naturally brought about by the underlying anticlinal cell walls of the epidermis - and orient its further growth perpendicular to this depression.

On a typical grass leaf, it is readily understandable that this simple behavior will guide the germ tube at a relatively short path to a stoma: the epidermal cells of a grass leaf are long and narrow and organized in longitudinal rows, stomata are aligned in some of these rows, and stomata in one row are staggered compared to those in the next stoma-containing row. Thus, a fungal germ tube is highly likely to meet a depression brought about by a longitudinal anticlinal cell wall and this will lead to a transverse growth of the germ tube perpendicular to the long axis of the leaf. Chances are high then that the germ tube tip will meet a stoma at the first stoma-containing row it crosses. However, should it grow in between two stomata, it is rather likely to meet one in the next stoma-containing row.

The situation is less clear on a typical leaf of a dicot plant where the pattern of epidermal cells is less evident. However, computer simulations have shown that a perpendicular re-orientation of germ tube growth at each epidermal cell border encountered will usually lead to a 'homing in' on a stoma.

Rather simple orientation strategies, thus, lead growing germ tubes of different rust fungi to stomata - a necessary prerequisite for successful penetration of the pathogen into its host tissue. It would appear reasonable to assume that a changed pattern of epidermal cells might misguide the fungus, thus rendering the plant resistant. The fungus, not finding a stoma to penetrate, would rapidly exhaust the nutrient reservoir of the spore and starve. Evidence for such a structural resistance factor has been reported for several non-host plants inoculated with different rust fungi (Heath, 1977).

b. Recognition of a Stoma. Simply reaching a stoma, though, is not good enough for successful penetration. Upon contact with a stoma, the germ tube tip has to recognize that it has reached its destination, growth has to be stopped, and an appressorium as the first of a series of infection structures has to be differentiated (Read *et al.*, 1992; Mendgen *et al.*, 1996). Again, chemical signals have been implicated in this recognition process but experimental evidence is stronger for structural signals provided by the topography of the stoma.

It had long been known that germ tubes of some rust fungi, when growing on artificial substrates, may be induced to form appressoria by scratches in the surfaces. In an elegant series of experiments using polystyrene replicas of micro-manipulated silicon wafer templates, it was now possible to precisely identify the topographical signal that induces appressorium differentiation in the bean rust fungus (Hoch *et al.*, 1987): a single step up or down of 0.5 μm height is a necessary and sufficient signal. This is exactly the height of the stomatal lip surrounding bean stomata.

It is perhaps surprising that such a simple signal is a 'safe' enough trigger for such a crucial differentiation step of the fungus. Both failure to recognize a stoma reached and erroneous 'recognition' of a stoma not present, could be fatal events leading to fungal death. On the other hand, it would appear rather simple for a plant to slightly change the dimension of its stomatal lip and thus evade fungal recognition and become resistant. However, the observations known so far appear to indicate that recognition of a precisely dimensioned stomatal lip is a general mechanism rust fungi use to penetrate dicot plants.

Again, the situation is different on grass leaves. Rust fungi which infect grasses do not react to scratches on artificial surfaces, and variations of the dimensions of the ridges or grooves on the polystyrene surfaces did not immediately furnish a developmental signal for these fungi (Allen *et al.*, 1991). However, after overgrowing a series of narrowly spaced ridges, grass-infecting rust fungi may differentiate appressoria (Read *et al.*, 1997). Comparing this topography with the surface of a grass leaf, it was concluded that the series of narrowly spaced anticlinal walls of the cells forming a gramineaceous stomatal complex may be the signal recognized by the rust fungi of grasses. However, *in vitro*, only about half of the germ tubes developed an appressorium, and this only after growing over a dozen or so narrowly spaced ridges - far more than the number of narrowly spaced anticlinal walls in a grass stomatal complex. It has long been known that the wheat stem rust fungus can easily be chemically induced to form appressoria - a component of the epicuticular wax and a volatile substance emanating from the leaves appeared to act synergistically (Grambow and Riedel, 1977). These chemical signals may work in conjunction with the topographical signal. In fact, the combination of physical and chemical triggers led to the formation of an appressorium at the tip of almost every fungal germ tube (Moerschbacher and Read, unpublished observation).

Thus, it would appear that rust fungi which infect grasses use a combination of signals to identify with reasonable certainty that a stoma has been reached. Maybe the developmental program for building the topography of the gramineaceous stomatal complex is less fixed than in dicots and reliance on topographical features alone for stomatal recognition, therefore, is not certain enough. Interestingly, the resistance of

a certain barley species, *Hordeum chilense*, to cereal leaf rusts was shown to be based on failure of the germ tubes to recognize stomata (Rubiales and Niks, 1992). The epicuticular waxes of this barley variety form finger-like protrusions reaching over the stomata. Most likely, the fungus is thus no longer able to feel the real surface of the stoma, it fails to recognize having reached its destination, and keeps on growing - a fascinating case of structural resistance of a host plant to a fungal pathogen.

We have concentrated the description of surface topography as an aspect of structural resistance of plants to pathogens on the rust fungi not because the phenomenon is restricted to this group of pathogens but because these are by far the most intensively studied. However, surface topography can be expected to play a critical role during the process of infection by many pathogens, certainly by all those which prefer a specific site of their host's surface for penetration. A rather different example of stomatal morphology acting as a determinant of resistance or susceptibility is given by citrus fruits where the conformation of the cuticle around the stoma is thought to either prevent or allow the passage of water droplets containing bacterial pathogens. This is a wide and promising field for future research.

3. *Resilient Cell Walls*

The above sections have dealt with surface attachment and recognition leading to a correct positioning of the microbial infection structures for the subsequent attempt at penetrating into the host tissue. This site of attempted penetration may be an outer periclinal wall of an epidermal cell, an anticlinal wall between two epidermal cells, or a stoma. Even in the latter case, the pathogen has only reached the intercellular spaces of a host tissue, and the formidable barrier of the plant cell wall in all its complexity is still to be overcome. We have described the cell wall in detail above, and we have also elaborated on the machinery evolved by the pathogens to breach, or partially circumvent and then breach, this barrier. In this section, we shall summarize rather briefly the counter-mechanisms plant cells have developed to specifically make their cell walls resistant to pathogenic attack.

a. Cutin and Suberin. The first line of defense and possibly the single most important is the excretion and polymerization of the cuticle at the outside of the outer periclinal plant cell walls. The waxes incorporated into the cuticle proper, and the epicuticular waxes on the very surface, hinder the diffusion of cell wall degrading enzymes into the wall (and prevent the diffusion of nutrients from the leaf cells to the micro-organisms on the surface). The underlying cutin polymer, as a polyester and polyether, is obviously difficult to degrade enzymatically.

Clearly, an intact cuticle is a paramount obstacle that can only be breached by a very limited number of micro-organisms. In plant organs exhibiting secondary growth, the epidermis is replaced by layers of cork cells, and these are impregnated with suberin. Similar to cutin, suberin is a random polymeric material (chemically related to lignin and cutin) (Schmutz *et al.*, 1993; Bernards *et al.*, 1995), and it is equally laid down in alternate layers with waxes. Consequently, it can be assumed that suberin fulfills similar functions as cutin in the warding off of phytopathogenic micro-organism, albeit this has been much less studied. Nothing is known about possible 'suberinases' but

those can be expected to be produced by some fungi specialized on the infection of plant tissues protected by cork cells.

b. Substitution and Cross-Links. Once a pathogen has breached the cuticle or cork layers, the primary cell walls of the host cells are exposed to chemical and, possibly, physical attack. The best way to protect the wall from enzymatic degradation is to chemically change the substrates for the microbial enzymes. The best way to prevent the wall from being pierced by pressure is to increase its physical strength by strategically introducing inter-polymer cross-links.

Chemical modification is a way to protect pectins and hemicelluloses from enzymatic degradation. We have seen that pectic molecules may partially be methyl-esterified or they may bear acetyl groups. The degree of substitution by and the distribution of methyl-esters on homogalacturonan chains has been shown to determine their degradability by pectic enzymes (Forrest and Lyon, 1990; Mort and Chen, 1996). However, due mainly to a lack of adequate analytical techniques to compare the substitution patterns of pectins from different plants, it is not yet clear to what extent such a substitution really is involved in successfully protecting pectins from microbial degradation in disease. Addition of phenolic acid esters or ethers to pectins and hemicelluloses is also thought to protect the substituted molecules from enzymatic depolymerization, but experimental evidence is largely lacking (except maybe for the reduced biodegradability of phenolic acid-rich cell walls by rumen micro-organisms) (Ralph and Helm, 1993; Wallace and Fry, 1994). Also, little is known about the substitution of hemicelluloses and its influence on enzymatic degradation, e.g., acetyl groups on some xylose residues in the backbone of glucuronoarabinoxylans and on some galactose residues in the di- or trisaccharide side chains of xyloglucan.

Interpolymeric cross-links to physically strengthen the plant cell wall are an integral concept in the architecture of the primary cell wall to create the three independent networks. Cross-links may be defined as being linkages that are introduced *in muro* and not during synthesis of the polymers (Iiyama *et al.*, 1994). They can occur between identical molecules or between different types of molecules and they may or may not be mediated by low molecular weight cross-linkers. Examples are the crystallization of cellulose molecules to form insoluble microfibrils, the Ca^{++} -mediated cross-links between homogalacturonans, the postulated isodityrosine bridges between two hydroxyproline-rich glycoproteins and the phenolic acid bridges between glucuronoarabinoxylans. The only indisputable examples for mixed polymer cross-links are the hydrogen-bonding of xyloglucans or arabinoxylans to cellulose microfibrils. There can be no doubt that all of these cross-links are absolutely required to build a coherent cell wall structure, but the relative contribution of one type of cross-link versus another are not known. Consequently, their individual role in strengthening the cell wall against the physical impact of microbial penetration attempts, though certainly crucial, remains undetermined.

c. Lignin. Lignification clearly is a superb way of making a cell wall almost completely resistant to both chemical and physical attack. Moreover, lignin replaces the water in the cell wall thus making it perfectly impermeable to water and thus leakage

of nutrients from the plant cells to a potential pathogen and, in the reverse direction, to cell wall degrading enzymes or toxins from the pathogen to the plant cells. Of course, there must be a serious drawback: cells with lignified cell walls will die as they are shut off from water and nutrients supplied from the rest of the plant. Thus, whereas preformed lignification is a means of stabilizing certain cell types such as xylem elements or sclerenchyma fibers in performing a duty to the whole plant physiology and morphology, wound-induced lignification, in which a ring of cells with lignified cell walls encircles a wound, is an efficient protection of the surrounding tissue from secondary infection by opportunistic micro-organisms (see below).

d. Silicon. A further way of impregnating cell walls and making them resistant - probably to chemical degradation and possibly to physical offense - is given by the deposition of silicon in the plant cell walls. However, in spite of the near ubiquity of silicon, little is known about the chemical composition or the site of deposition of silicon in plants. Clearly, the leaves of some plants, notably grasses including some cereals, can be very rich in silicon making them unpalatable for grazing animals, but convincing evidence for protection against pathogenic infection is rare (Zeyen *et al.*, 1993), most likely due to a lack of research into this question.

B. INDUCED RESISTANCE REACTIONS

When a micro-organism has breached all the preformed barriers mentioned above, which a plant has put up as a passive, structural means of protection, and threatens to colonize the host tissue, plant cells usually exhibit typical morphological reactions as a second line of active structural defense. All of these infection-induced, active resistance mechanisms involve the formation of new cell walls or cell wall materials or a change in composition or interaction of existing cell walls or cell wall materials (Fig. 8). As early as 1926, Young described wall appositions (*callosities*) during penetration of leaf cells by numerous fungi. Similarly, root cells were shown to exhibit tuber-like wall appositions around the hyphae of pathogenic and mycorrhizal fungi (Burgeff, 1932). During the past few years, these wall appositions have been studied with renewed interest focusing on their role as a possible means of resistance. Their complex composition has been analyzed and was further characterized with the advent of new probes. It was shown that the normal plant wall, as well as the callose-appositions, could be modified by the infection-induced addition and cross-linking of phenolics, suberin, lignin, proteins, calcium, and silicon. In addition, walls may contain inhibitors of enzymes or toxins that deter a possible parasite. Numerous reviews have covered cell wall alterations such as modifications in thickness (Rice, 1945; Aist, 1976; 1983) or changes in composition after infection (Akai and Fukutomi, 1980; Vance *et al.*, 1980; Ride, 1983; Smart, 1991; Heitefuss, 1997).

1. Intracellular Events Before and During Penetration

Even before actual penetration of the cell wall has been attempted, the underlying host cells can very often be shown to be reacting to the presence of the pathogen.

Figure 8. Induced structural resistance mechanisms of a challenged host plant

When the preformed structural and biochemical resistance factors that protect a given plant from the attack by most potentially phytopathogenic microorganisms have failed, a second line of defense is activated, including induced structural and biochemical resistance reactions. In some cases, a newly developed meristem may produce a new layer of cells which may eventually become suberized or lignified in order to localize an ingrowing pathogen (1). In other cases, the cell walls of attacked plant cells may be fortified by the incorporation of new cell wall components (2), often accompanied by the apposition of new cell wall material in the form of a papilla (3). In yet other cases, the cytoplasm of a penetrated host cell may undergo rapid structural changes which may even lead to host cell death - the hypersensitive resistance reaction HR - e. g. by the intracellular polymerization of monolignols (4). The occurrence of none of these different types of induced structural resistance reactions (1-4) is specifically linked to any one of the penetration strategies shown in the figure.

a. Permeability Changes of the Plasmalemma and Gene Induction. Plant cells can respond rapidly to the presence of a potential pathogen. Early reactions include the depolarization of the plasma membrane (Kauss, 1990), ion fluxes and changes in the internal Ca^{++} -concentrations (Scheel, 1998) upon elicitor application, and the stimulation of an oxidative burst (Brisson *et al.*, 1994). It is not yet fully understood whether these early responses are causally related to rapid changes in gene expression that have been measured within minutes of contact with pathogens or pathogen derived signals (Lamb, 1994; Dangl *et al.*, 1996). Clearly though, induced resistance responses very often require the transcriptional activation of defense genes.

b. The Cytoplasmic Aggregate. Light microscopy of living cells revealed cytoplasmic changes within minutes after the first contact between host and parasite. Most impressive are changes in the cytoskeleton during these early interactions (Gross *et al.*, 1993; Kobayashi *et al.*, 1994). Other responses include acceleration of cytoplasmic streaming, increase in cytoplasmic strands, and migration of the nucleus towards the area of fungal

penetration. As a result, a cytoplasmic aggregate assembles near the infection site (Bushnell and Bergquist, 1975). In the interaction between the powdery mildew pathogen (*Erysiphe graminis*) and barley (*Hordeum vulgare*), the number of cytoplasmic strands increased dramatically by the time appressoria form on the epidermis, i.e., about four to five hours prior to actual host penetration by the fungus (Aist and Bushnell, 1991).

c. Endo- and Exocytosis at the Penetration Site. Conventional electron-microscopic studies exhibited an accumulation of vesicles in the cytoplasmic aggregate during infection of barley by *E. graminis* f. sp. *hordei* (Zeyen and Bushnell, 1979). After ultrarapid freezing, the endo- and exocytotic nature of such vesicles could be resolved. At the penetration site of the cowpea rust fungus (*Uromyces vignae*) into epidermal cells of broad bean (*Vicia faba*), numerous coated pits and coated vesicles were observed on the plant plasma membrane suggesting greatly enhanced endocytosis and secretion of material at the penetration site (Xu and Mendgen, 1994). Most coated vesicles were filled with β -1,3-glucans (Fig. 9). These glucans might have originated from the wall of the penetrating fungus due to the activity of host plant glucanases. β -1,3-Glucans from fungal walls, which can be elicitors of plant defense responses, are recognized by soybean cells through receptor-like binding sites associated with the plasma membrane. They bind elicitor molecules in a saturable and reversible manner (Hahn, 1996; Umemoto *et al.*, 1997). Endocytosis of fungal elicitors might serve as a system to clear fungal cell wall components from the plant surface after signal transduction and elicitation of a second messenger system inducing phytoalexin synthesis or other plant defence reactions. In addition, endocytosis could be involved in recycling the elicitor receptors.

Coated vesicles have also been observed around haustoria of *Uromyces vignae* in

Figure 9. Endocytosis of β -1,3-glucans by coated vesicles on the plasmalemma of epidermal cells from *Vicia faba* at the penetration site of *Uromyces vignae*

Different stages of endocytosis (a, b) and multivesicular body (c). x 125 000, adapted from Xu and Mendgen (1994).

the host *Vigna sinensis* (Stark-Urnau and Mendgen, 1995) and around haustoria of the smut fungus, *Ustilicystis waldsteiniae*, in the host plant *Waldsteinia geoides* (Bauer *et al.*, 1995). These results show that endo- and exocytotic events may also occur at later stages of fungal development within host cells, especially around symbiotic structures such as haustoria.

During infection of legumes by *Rhizobia*, the plasma membrane is invaginated and the bacteria produce an infection thread which grows through the root cortical cells. The plant membrane along this thread is studded with coated vesicles, and smooth vesicles are situated close by (Robertson, 1982). Obviously, the early contact zone between plants and invading micro-organisms in this symbiotic interaction is also characterized by endocytotic and exocytotic events which culminate in characteristic extensions of the plasma membrane, in the modification of the existing wall and the subsequent production of large wall appositions. Therefore, the infection site of bacterial and fungal pathogens is an interesting area to study vesicle-mediated transport of molecules, a field of research that has attracted much interest in the last years (Low and Chandra, 1994; Hawes *et al.*, 1995).

2. *Modification of Plant Cell Wall Composition after Infection*

A typical sign of plant cell wall penetration or even attempted penetration which is easily visible with a light microscope is the halo of stainable material around the penetration site. UV-light induces fluorescence of the halo and this is most likely due to the deposition of phenolic material, e.g., esterified phenolic acids or lignin. Quantitative interference microscopy on onion leaves attacked either by *Botrytis cinerea* or *Colletotrichum circinans* suggested that if the interaction was susceptible, mass was lost or, if resistant, mass was increased within the halo area (Russo and Pappelis, 1981). Cytochemical studies with antibodies raised against cell wall components and against hydrolytic enzymes produced by the pathogen suggest a very complex picture: At first, wall material is degraded at the penetration site of the fungus and around hyphae growing within host cell walls (Fig. 6) (Müller *et al.*, 1997; Podila *et al.*, 1995; Xu and Mendgen, 1997). As a result, the plant starts modifying pre-existing cell wall components and also may incorporate additional cell wall components into the cell wall halo at the site of attempted penetration. In barley, the area in the host wall halo was shown to accumulate lignin, silicon and phenolics (Kunoh, 1990; Zeyen, 1991). Also in many other host-parasite combinations, hydroxyproline-rich glycoproteins and thionins accumulated after infection (Bohlmann, 1994; Heitefuss, 1997; Showalter, 1993). Pre-existing and/or newly incorporated cell wall proteins and phenolics can be cross-linked by peroxidases. The hydrogen peroxide required for these reactions may be provided by an oxidative burst (Fig. 10) (Brisson *et al.*, 1994).

3. *Apposition of New Wall Material onto the Existing Cell Wall*

The above described modifications of the existing host cell wall at the site of attempted microbial ingress do not appear to be able to prevent the eventual breaching of the cell wall. Rather, they may slow down the penetrating micro-organism enough to give the host cell time to produce new cell wall material to locally thicken and reinforce the wall. Such wall appositions or papillae have been observed in leaves, stems and roots of plants after infection by many pathogens. A recent comprehensive review

Figure 10. H_2O_2 -production in the oxidative burst

Plant cells, similar to some animal cells such as human phagocytes, are able to rapidly produce active oxygen species by partially reducing molecular oxygen. A plasmamembrane-bound NADPH-oxidase (NADPH-OX) may transfer a single electron to O_2 producing the superoxide radical anion, O_2^- , which then spontaneously, or catalyzed by superoxide dismutase (SOD), converts to hydrogen peroxide, H_2O_2 . Alternatively, H_2O_2 may be produced directly from O_2 by the oxidase function of a peroxidase (POX) transferring two electrons and two protons to O_2 . The resulting hydrogen peroxide can act as a co-substrate in a number of peroxidase-catalyzed coupling reactions. If two tyrosine residues from different proteins are thus dimerized to isodityrosine, the two protein molecules become cross-linked in the process. Similarly, the dimerization of two ferulic acids to diferulate may cross-link two pectin or arabinoxylan molecules to which they are attached. Lastly, the polymerization of monolignols to the lignin polymer is dependent on a peroxidative radicalization of the precursors. All of these cross-links lead to the stabilization of the plant cell wall rendering it more resistant to enzymatic and physical attack.

on wall appositions by Heitefuss (1997) concludes that papillae or wall appositions may be a major factor preventing pathogen ingress into the host cell, but the speed of host reaction relative to fungal growth and the chemical composition of cell wall material seem to regulate the outcome of the interaction. Only very few examples can be cited here.

a. Leaf Pathogens. At the penetration site of *E. graminis* f. sp. *hordei*, distinct papillae or wall appositions arise in localized areas between the plasmalemma and the cell wall of epidermal cells of barley (*Hordeum vulgare*). This system has attracted considerable interest because this pathogen can infect epidermal strips from coleoptiles. Thus, the host/parasite-interaction is extremely suitable for microscopical and cytochemical studies. With this technique, it has been shown that papillae may vary considerably in their speed of formation and in composition. One major component is β -1,3-glucan. In addition, silicon, phenolic compounds, calcium, lignin and even the secretion of hydrolytic enzymes into this structure has been observed (Aist and Bushnell, 1991). The number of components of this wall apposition increased with the advent of new or better probes. Very often, an increase of one of these components has been correlated with increased resistance to penetration by the pathogen.

Carver *et al.* (1992) were able to induce 'super-susceptibility' in compatible barley/powdery mildew-interactions after the application of α -aminooxy- β -phenylpropionic acid, an inhibitor of phenylalanine ammonia-lyase (PAL). Therefore, phenolic compounds appear to play a major role in penetration efficiency. Russo and Bushnell (1989) compared papillae induced by *E. graminis* and wall appositions induced by wounding. Both structures were positive for carbohydrates, callose, and protein, and negative for lignin and suberin. Only wall appositions induced by wounding were positive for cellulose and pectin whereas papillae induced by the fungus were positive for phenolics and a basic staining material.

An exceptional case of inherited resistance is observed with the recessive alleles (*mlo*) of the *Mlo*-locus. Each resistance allele of the locus acts in a non-race-specific manner and confers resistance to almost all isolates of *E. graminis* f. sp. *hordei* (Jørgensen, 1987). The development of the fungus is arrested in the papilla. This wall apposition is very similar in morphology and composition in the susceptible and the resistant plant. Experiments with *mlo*-resistant plants have shown that micromolar concentrations of both 2-deoxy-D-glucose, an effective inhibitor of callose deposition in plants, and chlorotetracyclins, a Ca^{++} chelator, drastically decrease the size of the wall apposition in response to fungal attack. At the same time, penetration rates of the fungus increase with these smaller wall appositions. It is obvious that also in this case, the timing of papilla formation and its final size and composition are crucial for the outcome of the interaction (Gold *et al.*, 1986; Smart, 1991; Zeyen and Ahlstrand, 1993; von Röpenack *et al.*, 1998).

In rust fungus infections, wall thickenings and papillae have been observed very often adjacent to the penetration hypha of the haustorial mother cell in incompatible or non-host combinations (Taylor and Mims, 1991; Harder and Chong, 1991). They can be quite variable in appearance, ranging from electron-translucent structures, with callose as a major constituent, to electron opaque, granular deposits with silicon as a major constituent. However, studies with silicon-depleted bean plants indicated that reduced silicon deposition increased the frequency of haustorium formation in mature leaves. Treatment of bean leaves with inhibitors that influence the deposition of callose, silicon, or autofluorescent compounds very often resulted in an increase of haustorium development in bean (*Phaseolus vulgaris*) (Heath and Stumpf, 1986).

One of the most striking features of susceptible plants infected by rust fungi is the superficial lack of response to infection (Heath, 1997). Double inoculation experiments with non-pathogenic fungi indicated that pathogenic rust fungi, or their haustoria, can suppress wall depositions (Fernandez and Heath, 1991). Haustoria of *Physopella zeae* prevented wall matrix formation around intercellular hyphae also formed by this fungus. In contrast, death of the dikaryotic haustorium of *U. appendiculatus* (Heath, 1988) or aging of monokaryotic haustoria of *U. vignae* (Stark-Urnau and Mendgen, 1995) (Fig. 11) resulted in rapid encasement of the intercellular fungus. These results suggest that a continued metabolism of the biotrophic pathogen is needed to prevent deposition of plant cell wall components, including callose, around fungal structures.

Bacterial pathogens may also induce morphologically very similar wall appositions. The *hrp*-genes of *Xanthomonads* are responsible for basic pathogenicity and the ability of the bacterium to induce the hypersensitive reaction of host tissue. Strains of *Xanthomonas*

Figure 11. Deposition of plant cell wall material around monokaryotic haustorium of *Uromyces vignae* in *Vigna sinensis*

Young haustorium, exhibiting profiles of coated pits (a) and old, inactive haustorium (b) ensheathed by wall layers composed of different components. fw - fungal wall, fpm - fungal plasma membrane, M-ha - monokaryotic haustorium, ehm - extrahaustorial membrane, ema - extrahaustorial matrix, HRGP - hydroxyproline-rich glycoprotein, AGP - arabinogalactan protein.

campestris pv. *vesicatoria* with mutations in *hrp*-loci fail to multiply in the plant tissue and induce localized reactions of pepper mesophyll cells. Large wall appositions containing phenolics, callose, and hydroxyproline-rich glycoproteins are found next to the bacteria encapsulated within an amorphous matrix. The rapid and localized responses to such mutants can be suppressed by the pathogenic strains of the same bacterium. Suppression of the wall reaction requires the clustered *hrp*-loci in *X. c.* pv. *vesicatoria*. The mechanism by which such wall appositions influence bacterial multiplication is unknown (Boher *et al.*, 1996).

b. Root Colonizing Micro-Organisms. Transmission electron-microscopy has helped considerably to study the development of wall thickenings and the extent of wall appositions in epidermal and cortical cells of the root tip. Bishop and Cooper (1983) compared tomato root invasion by *Fusarium oxysporum* f. sp. *lycopersici* or *Verticillium albo-atrum* and pea root invasion by *Fusarium oxysporum* f. sp. *psii*. Hyphae of the pathogens adhered to the host root cell walls by means of a matrix typical in morphology for each pathogen. Colonization of the root tissue occurred by intercellular hyphae. Host cells reacted with the development of wall appositions within epidermal and cortical cells. Penetration of host walls was by constricted hyphae and localized wall degradation. Continued deposition of material occurred around penetration hyphae

which resulted in elongated penetration papillae. These wall appositions were lignified and resistant to enzymatic and chemical degradation. Wall appositions did not seem to prevent infection. New fixation techniques such as high-pressure freezing followed by freeze substitution have improved this picture. They revealed that during infection of cotton root tips by *F. oxysporum* f. sp. *vasinfectum*, host cytoplasm including cell organelles remained intact after infection. Cell wall synthesis proceeded during fungal penetration and proliferation within host cells. Hyphae became completely encased by wall appositions. Callose was a major component of the appositions (Fig. 12). Other constituents such as pectic homogalacturonan (Fig. 13), xyloglucan (Fig. 14) and arabinogalactan (Fig. 15) were also present in the wall appositions and were secreted into this area by Golgi-derived vesicles. These results show that wall appositions consist of the normal components of the plant wall and additional components, produced in stress situations. Most hyphae of the pathogen, although encased by wall appositions, did not seem to be inhibited in their growth and developed further. During subsequent growth of the fungus between the cells, wall appositions were less pronounced. Hyphae within cells were surrounded by a matrix, a structure reminding of the extrahaustorial matrix observed around the haustoria of many rust fungi (Rodríguez-Gálvez and Mendgen, 1995a, b).

In potato tubers infected with *Phytophthora infestans*, cessation of fungal growth in incompatible reactions is very often accompanied by wall appositions at the penetration site and by the encasement of fungal haustoria with callose-containing material. A positive correlation between resistance and relative abundance of wall appositions was found (Hächler and Hohl, 1984). Also *P. cinnamomi* induced the development of such wall appositions in root tissue of resistant *Zea mays* (Hinch *et al.*, 1985). In *P. sojae*, the speed of callose deposition was always higher in the resistance response than in susceptible roots (Enkerli *et al.*, 1997). However, the possibility that callose deposition is a secondary effect after the halt of fungal growth could not be excluded.

Also the induction of defense reactions by other microorganisms or molecules secreted by them seem to enhance plant resistance against a root pathogen. Tomato plants treated with elicitors such as fungal β -1,3-glucans or chitosan showed increased resistance to *F. oxysporum* f. sp. *radicis-lycopersici* (Benhamou *et al.*, 1996). Root tissue from such plants exhibited restriction of fungal growth and the formation of numerous wall appositions at sites of attempted penetration. These papillae varied greatly in their appearance. Cytochemical tests detected callose and pectin throughout the wall apposition whereas cellulose was concentrated over the external portion.

Even arbuscular mycorrhizal fungi can induce wall appositions in symbiotically defective plants. Mutants isolated after chemical mutagenesis of pea and *Faba* bean exhibited impaired symbiotic abilities vis-à-vis both arbuscular mycorrhizal fungi and *Rhizobium*. Such mutants elicited normal appressorium formation at the root surface, but further development of the mycorrhizal fungus was arrested at this stage. These aborted infections are characterized by an abnormally thick re-enforcement of epidermal and hypodermal cell walls in contact with the symbiont. Within these secondary structures, phenolic compounds, callose, and PR1 proteins accumulate (Gollote *et al.*, 1993). In a tomato root system partly colonised by an arbuscular mycorrhizal fungus, wall appositions were produced also in non-mycorrhizal parts of

Figure 12. Callose distribution within wall appositions in cells of cotton root tips after contact with hypha of *Fusarium oxysporum* f. sp. *vasinfectum* detected with a polyclonal serum against β -1,3-glucan, labelled with colloidal gold. x 30 000, (courtesy of E. Rodriguez-Galvez, Piura, Peru).

Figure 13. Homogalacturonan distribution after penetration by *F. oxysporum* detected with the monoclonal antibody JIM 5, labelled with colloidal gold. x 20 000, from Rodriguez and Mendgen (1995b).

Figure 14. Xyloglucan distribution after penetration by *F. oxysporum* detected with the monoclonal antibody CCRC-M1, labelled with colloidal gold. x 17 000, from Rodriguez and Mendgen (1995b).

Figure 15. Arabinogalactans within wall appositions produced by cells of cotton roots after infection with *F. oxysporum* f. sp. *vasinfectum* detected with the monoclonal antibody CCRC-M8, labelled with colloidal gold. x 35 000 (courtesy of E. Rodriguez-Galvez, Piura, Peru).

Figure 16. Rhizodermal cells of cotton (elongation zone) infected with *F. oxysporum* f. sp. *vasinfectum*. Plant cell and fungal hypha appear intact. x 45 000, from Rodriguez-Galvez and Mendgen (1995a).

the root after infection with the pathogen *P. parasitica*. PR-1a protein detected within these wall appositions and in the hyphae of the pathogen correlated with induced resistance (Cordier *et al.*, 1998).

These results show that, from a morphological point of view, wall appositions in response to fungal or bacterial infections have a striking resemblance to one another. However, their composition and especially their speed of development varies considerably. Therefore, the event that initiates the production of a papilla and the process that regulates the composition of the wall apposition need to be elucidated before we can prove that wall appositions are primarily involved in plant resistance. Molecular methods are needed to solve this question. To date, it is only obvious that papillae can inhibit invading micro-organisms if speed of production and composition of the papilla adequately match the penetration strategy and machinery of the pathogen. On the other hand, the infection thread initiated by *Rhizobia* has some striking resemblance to wall appositions, but obviously supports bacterial invasion. Modification in its composition can inhibit further development of the symbiotic interaction.

4. Production of New Cells or New Tissue

The induced structural resistance mechanisms described above were highly localized and involved a single host cell only. In some cases, however, the reaction may encompass several cells, and additional cells may even be produced *via* induced mitosis in order to isolate the pathogen.

a. Tyloses. The vascular tissue of many plants can produce a morphologically very specific type of defense, the tyloses. Occlusion of vessels with tyloses and gels appears to be associated with resistance in banana, cotton, hop, tomato, elm, and many other plants to wilt diseases. The process of tylose formation is very often similar in different plants (Beckman and Talboys, 1981; Beckman *et al.*, 1982). After the development of protective layers within parenchyma cells, the pit membranes between the xylem parenchyma cells and xylem vessels are disrupted and the living cytoplasm of the parenchymatous cell expands into the vessel lumen, produces a new wall, and may fill the lumen of the vessel. The walls of tyloses apparently contain callose, pectin, phenols, and suberin (Rioux *et al.*, 1998; Robb *et al.*, 1979; Pearce and Holloway, 1984). These compounds may be involved in the protection of tylose walls against the penetration by wilt pathogens. Also vacuoles of both xylem parenchyma cells and tyloses accumulate electron opaque material and these substances, too, may contribute to the protection against further fungal growth. In addition to vessel occlusion, gel formation adds to the blocking of vessels. There is only circumstantial evidence to suggest that gel formation and tylose development are rapid enough to localize the pathogen. Pathogen propagules are only rarely associated with tyloses and gel - a surprising fact if the latter are entrapment points. Since gels and tyloses are also observed after wounding and plant stress (Bishop and Cooper, 1984), they may not represent a specific reaction against fungal pathogens.

b. Barrier Zone Formation after Infection with Ophiostoma Ulmi. The Dutch elm disease pathogen *Ophiostoma ulmi* causes serious wilting in elms, *Ulmus americana* and *U. europaeus*. A comparison of host and non-host trees inoculated with aggressive and non-aggressive isolates of the pathogen has shown that barrier zone formation is an important factor in defense of the disease (Rioux, 1996). A continuous barrier prevented further spread of the fungus completely (Rioux and Oullette, 1991). Barrier zone formation is a multi-component system. It consists of cells in phloem and xylem, mainly fiber cells, with lignified and suberized walls and vessel elements occluded by suberized tyloses. Thus, vessels in the xylem invaded by the pathogen may be completely surrounded by these barrier zone cells.

V. Towards a Causal Role of Structural Barriers in Plant Resistance

How can we tell whether a given structural feature of a plant, whether preformed or pathogen-induced, is causally involved in making this plant resistant to some potential pathogen? Though difficult to answer, this is a question of paramount importance to our understanding of plant resistance or susceptibility and, vice versa, of pathogenicity and virulence of a micro-organism. Different strategies have to be employed in trying to solve these problems as they relate to preformed resistance factors on the one hand and to infection-induced resistance reactions on the other hand.

A. PREFORMED RESISTANCE FACTORS

The simplest way of finding out whether a given preformed structure is essential in protecting a plant from a pathogen would be to compare the susceptibility of different plants which do or do not possess the structure under investigation. One fundamental problem with this approach is the fact that most structural features of a plant serve multiple purposes, probably none can be expected to be involved in the defense against pathogens alone, e.g., the cuticle protects not only from pathogens, but also from desiccation. Thus, plants lacking such a feature altogether will be difficult to obtain, and studies using such plants would almost certainly be prone to many artifacts. Another major problem with this approach is the genetic stability of most structural traits which are usually the indirect products of many genes, greatly compounding molecular genetic approaches. Nevertheless some such studies have been undertaken with some success.

For the above reasons, it is often easier to approach the problem from the other side, i.e., to compare pathogens that have or do not have the ability to overcome the structural barrier under debate. This competence of the pathogen (e.g., the production of an enzyme degrading a certain cell wall component) is more likely to be controlled by a single gene or a manageable number of genes and, consequently, it may eventually become possible to compare wild type and mutant strains of a pathogen differing only in this or these gene/s.

1. *Plants with Enhanced or Suppressed Levels of a Presumed Resistance Factor*
In order to evaluate a structural feature's involvement in plant resistance to pathogenic attack, plants overexpressing or underexpressing a certain gene or a small set of genes thought to be involved in the building of a structural plant feature have been produced. Overexpression can be achieved under the influence of a suitable promoter, while underexpression may be reached by anti-sense or co-suppression technology. Two examples of such approaches shall be cited here.

Tomato plants were transformed with the gene for tobacco anionic peroxidase thought to be involved in lignin production in tobacco (Lagrimini *et al.*, 1993). The transgenic tomato plants exhibited higher levels of lignin in healthy plants and increased deposition of soluble phenolics and lignin upon wounding when compared to wild type tomato plants. In spite of these increases, resistance to different pathogens (*Fusarium oxysporum* f. sp. *radicis-lycopersici*, *F. oxysporum* f. sp. *lycopersici*, *Verticillium dahliae*, and TMV) was not increased in the transgenic plants. Disappointing as this result is, it certainly does not allow the general conclusion that lignin is not involved in resistance of tomato to microbial pathogens.

Using co-suppression as a strategy, tobacco plants with decreased levels of PAL and, consequently, phenylpropanoid products, were produced (Maher *et al.*, 1994). These transgenic plants showed increased susceptibility to a fungal pathogen, *Cercospora nicotianae*, indicating that PAL products are involved in the resistance of tobacco to this fungus. However, the major phenylpropanoid in these plants was soluble chlorogenic acid so that no conclusion can be drawn concerning an involvement of structural features in resistance.

2. *Pathogens with Enhanced or Lowered Activities of Cell Wall Degrading Enzymes*

The same question can be addressed from the opposite site, i.e., that of the pathogen. If deletion of a given cell wall degrading enzyme (CWDE, e.g., endopolygalacturonase) in a micro-organism results in loss of its pathogenicity (i.e., if the pathogen becomes a saprophyte and is no longer able to infect and cause disease) or loss of its virulence (i.e., if the pathogen becomes less virulent and causes less severe symptoms) then the cell wall component degraded by that CWDE (e.g., pectate) may tentatively be considered a structural feature involved in pathogen resistance of this plant. Classical studies in this field have recently been complemented by a molecular genetic approach, where the genes for CWDEs were inactivated by site-directed mutagenesis. However, some problems remain with this reasoning, as we will see below when we will discuss some recent examples of this approach.

a. Cutinase. Initial biochemical and enzymatic studies indicated that cutinase was a key factor in the infection of pea plants by *Fusarium solani* f. sp. *pisi*. But when cutinase-deficient mutants of the fungus were produced, the results obtained in infection experiments were contradictory. Some authors found that the cutinase-deficient mutant was equally virulent as the wild type and concluded that this particular cutinase is not crucial for penetration of the outer periclinal epidermal cell walls (Stahl and Schäfer, 1992). Other authors, using the same pathogen and host plant, found decreased virulence of the mutant and concluded that cutinase is a virulence factor causally involved in penetration (Rogers *et al.*, 1994). The first result would have no implication for our basic question - is cutin critical for resistance? - as the fungus may possess additional cutinase genes, and as we know that fungal pathogens may be able to breach the cuticle by sheer physical force. The second result, however, would imply that cutin and, thus, the cuticle, is a factor involved in resistance of pea plants to potential pathogens.

The reverse experiment has also been performed: transformation of an obligate wound pathogen (*Mycosphaerella*) with a cutinase gene from a different pathogen (*Fusarium*) allowed the former to penetrate the intact cuticle of its host plant (papaya) and to cause disease (Dickman *et al.*, 1989). Apparently, cutin is a decisive resistance factor effective against the wild type of this wound pathogen.

Clearly, the question is still open as to how important fungal cutinases and, thus, the plant cuticle really are in deciding on pathogenicity and resistance.

b. Pectic Enzymes. The same approach as detailed above has been used to investigate whether pectin degrading enzymes of a number of bacterial and fungal plant pathogens are causally involved in infection or pathogenesis. Pectin is a rather complex cell wall component, and we have seen that there is a great variety of enzymes capable of degrading just one component of pectin, namely homogalacturonan. Consequently, the molecular genetic work needed to produce null-mutants no longer able to degrade pectin is rather complex.

The phytopathogenic bacterium most intensely studied in this respect, *Erwinia chrysanthemi*, is known to produce a number of different pectic enzymes when grown in liquid culture on pectin as a carbon source (Barras *et al.*, 1994; Hugouvieux-Cotte-Pattat

et al., 1996). Single and multiple mutants deficient in one, several, or all of these enzyme activities exhibited decreased virulence, but all of these mutants were still pathogenic to some extent, possibly due to a 'spare' set of pectic enzymes induced *in planta*.

These results allow the tentative conclusion that pectins are likely to be involved in resistance of plants to pathogens, as a deletion of some pectic enzymes leads to decreased virulence. The main problem with this reasoning is the possibility that pectin degradation may be essential for the pathogen's nutrition rather than for penetration of the structural defense barrier of the host plant.

B. INDUCED RESISTANCE REACTIONS

Somewhat different strategies have to be employed if one is to find out whether a given infection-induced mechanism of structural defense is causally involved in making a plant resistant to the attack by a phytopathogen. Again, the simplest approach would be to find or make a plant that is no longer able to perform the resistance mechanism under investigation, and to compare the susceptibility of this plant with that of the wild type. However, such a mutant could principally be impaired either in the triggering or in the execution of the resistance reaction, and both cases have their drawbacks. A mutant impaired in the triggering of an induced structural resistance mechanism is almost certainly also impaired in the triggering of other resistance mechanisms, as it has become apparent that defense responses are usually complex syndromes consisting of a number of different resistance mechanisms, the triggering of which are certainly not independent (Moerschbacher and Reisener, 1997). A mutant impaired in the execution of an induced structural resistance mechanism is most likely also impaired in some fundamental aspect of plant growth or development, as the resistance mechanisms make use of biosynthesis machinery and cell wall components that are also involved in the normal building of a cell wall in a healthy plant.

1. Inhibitor Studies

The approach taken therefore involves the interference with the execution of the induced resistance mechanism at the time and at the site of pathogen attack. This has not yet been achieved using a molecular genetic approach, instead, such studies have relied on the use of specific inhibitors of the process under investigation. Usually, the compound used will inhibit an enzyme involved in the biosynthesis or deposition of a cell wall component. Ideally, these can be applied only just at the time when the resistance reaction is being executed and only just at the site of attempted pathogenic attack - reducing the unwanted side effects of the inhibition on general processes of growth and development of the plant. We are still far from this 'ideal' situation described above, but novel methods of microscopy allowing *in vivo* observations and micro-applications of inhibitors should make this scenario more likely in the near future.

Another important problem to be realized is the specificity of the inhibitors involved - and the specificity of the inhibited reaction. Ideally, the inhibitor used should only inhibit one reaction, and ideally, this reaction should only be involved in the formation of one product, i.e., the structural barrier investigated. Both criteria are difficult to meet, as we will see in the examples described below.

a. Inhibitors of Lignin Biosynthesis. The inhibitor approach has repeatedly been used to investigate the relative importance of induced lignification in a given resistance reaction of a plant against a pathogenic micro-organism. We will summarize these attempts as an example to discuss the limitations of this approach.

The first experiments aimed at inhibiting induced lignification used competitive inhibitors of phenylalanine ammonia-lyase (PAL). These inhibitors are not completely specific for PAL (some also inhibit ethylene biosynthesis) and PAL (being at the beginning of the general phenylpropanoid pathway) is not specific for lignin biosynthesis. Depending on the host/pathogen-combination investigated, resistance was either partly broken by the PAL-inhibitors or the inhibitors had no influence on the outcome of the interaction. With the restrictions indicated, these results may be interpreted as indicating that direct or, more likely, indirect PAL-products are causally involved in the former cases of induced resistance reactions. They can not, though, be taken as a reliable indication that the deposition of lignin is involved in these induced resistance reactions.

Later studies used highly specific suicide inhibitors of cinnamoyl-CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD). Both of these enzymes are involved only in the specific branch pathway of monolignol biosynthesis (Whetten and Sederoff, 1995). The results were similar as seen with the PAL-inhibitors: some resistance reactions were partly negated by the inhibitors so that the fungus was able to colonize rather larger areas of plant tissue, while in other host/pathogen-combinations, the CAD-inhibitors did not influence the resistance reaction. The conclusion now would be that lignin biosynthesis is causally involved in the former cases of induced resistance reactions.

Interestingly, the effect of PAL- and CCR/CAD-inhibitors have been compared directly in a few host/pathogen-combinations, and the different results obtained may shed light on the relative importance of induced lignification versus other PAL-dependent resistance reactions in these host/pathogen-interactions.

In wheat plants highly resistant to the wheat stem rust fungus, inhibition of PAL and CAD both partly broke hypersensitive resistance, i.e., penetrated host cells did not lignify and collapse, and the fungus was able to develop a larger colony within the host tissue (Moerschbacher *et al.*, 1990). The effects of the CAD-inhibitors were more marked than those of the PAL-inhibitors presumably hinting at induced cellular lignification as a major factor in the hypersensitive resistance reaction, where monolignol polymerization in the cytoplasm is thought to be the method of active programmed cell death (Moerschbacher and Reisener, 1997).

The same inhibitors were employed in a series of studies on mildew resistance of barley as conditioned either by the *Mla*-gene or by the *mlo*-gene (Zeyen *et al.*, 1995). In case of the *mlo*-gene, mildew development is stopped by the rapid production of papillae at the sites of attempted epidermal penetration. In contrast, in the case of the *Mla*-gene, papillae are produced but appear not to be responsible for resistance. Instead, the fungus can usually penetrate the papilla, but the penetrated epidermal cell then undergoes hypersensitive cell death, stopping further fungal development. Again, PAL- and CAD-inhibitors had basically the same effect, namely, partly negating the *Mla*-dependent hypersensitive resistance reaction but not influencing the *mlo*-dependent

papilla-involving resistance reaction. Apparently, the hypersensitive reaction requires the production of lignin while the papillae are resistant to fungal penetration even without the incrustation with lignin and other phenolics.

Recently, the PAL- and CAD-inhibitors have been used with yet another host/pathogen-system, namely *Arabidopsis* plants inoculated with the powdery mildew fungus (Mauch-Mani and Slusarenko, 1996). In this system, the fungus can make limited growth in the host leaf before being stopped by a hypersensitive type of resistance reaction also correlated with the deposition of lignin. In this system, too, did both inhibitors have similar effects, both reducing the efficiency of the resistance reaction allowing for increased fungal growth, but the effect of the PAL-inhibitors clearly outdid that of the CAD-inhibitors, indicating that lignin biosynthesis is one, but not the major factor in resistance here. The second indirect PAL-product probably crucially involved in resistance in this system has tentatively been identified as salicylic acid.

We should finish this description of inhibitor studies with another word of caution. Even in the cases where a major causal involvement of monolignol biosynthesis has been shown, the results do not allow the conclusion that the polymerization of the phenylpropanols to the lignin polymer itself, i.e., the production of a lignified, structural barrier, is causally involved in the induced resistance reaction. Alternatively, the monolignols produced may be fungitoxic - and the subsequent polymerization of the monolignols to the lignin polymer might merely be a detoxification reaction by the plant. In that case, the defense reaction induced would be a chemical rather than a structural resistance mechanism - a distinction relevant only for this chapter, but not for plant or fungus.

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