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EARLY DEFENCE RESPONSES IN PLANTS INFECTED WITH PATHOGENIC ORGANISMS

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Abstract: Plant organisms possess a complex set of defence mechanisms that are responsible for preventing unfavourable interactions with other living organisms in their natural environment or for reducing negative effects of such interactions. They can be classified into two groups: early responses that occur immediately or shortly after contact with a pathogenic organism, usually in the proximity of the infection site, and late, usually transcription- and translation-dependent responses that take part in minimizing the long-term effects of the infection and in preventing further infections. Early responses are a mixture of distinct biochemical processes, leading to quick activation of enzymes, structural changes in components of the living cell, alteration of biochemical pathways and synthesis of intra- and intercellular signals. An important part of early responses are redox processes, especially the synthesis of large amounts of reactive oxygen species.

Key Words: Plant-Pathogen Interactions, Reactive Oxygen Species, Hydrogen Peroxide, Oxidative Burst, Hypersensitive Response

THE PHENOMENON OF PLANT RESISTANCE TO PATHOGENS

Compatibility and incompatibility between the pathogen and its host

Successful pathogen infection and disease in plants can occur if there is compatibility between the pathogen and its host. Other interactions are referred to as incompatible since they do not lead to successful infection. There are three possible reasons for genetic incompatibility between the pathogen and its host:

1. *nonhost interaction* – the plant species is unable to fulfil the pathogen's requirements, so the latter is unable to grow in the conditions it is faced with;

2. *nonhost resistance* – the host possesses sufficient preformed defence systems, such as structural barriers or toxic compounds that limit the growth and/or development of the pathogen;
3. *resistance* – the inducible defence mechanisms of the host are sufficient to restrict the pathogen's growth and/or development.

A phenomenon also exists where a genetically compatible host organism is able to limit the symptoms of and damage caused by pathogenic infection. Plants of this type are said to be disease-tolerant.

Temporal and spatial coordination of defence processes is required for successful resistance

A successful resistance response is dependent on the timely detection of the invading pathogen and the rapid and effective activation of defence mechanisms by the host plant. In the case of compatible interaction, one can often observe processes similar to those occurring in the resistant host during infection (e.g. the induction of certain genes, biochemical changes in the infected tissue), but they are weaker and take place too late to successfully restrict pathogen growth and to prevent disease.

Plant resistance responses are tightly coordinated by a network of various regulatory mechanisms. The complexity of this regulation has currently become one of the most fascinating research areas in plant biology.

LOCAL RESISTANCE PROCESSES ARE ACTIVATED AT THE SITE OF INFECTION

Immediate responses in invaded tissues

The pathogen is detected and the first responses occur at the site of infection within minutes of invasion. These responses are rapid events dependent on allosteric changes of several enzymes and fast chemical reactions. These include the massive synthesis of reactive oxygen species, including hydrogen peroxide (H₂O₂), named oxidative burst after a similar process occurring in the mammalian immune system, ion fluxes, cytoskeletal rearrangements, protein phosphorylation/dephosphorylation, NO synthesis, transcriptional and post-translational activation of transcription factors, and the induction of programmed cell death, often referred to as the hypersensitive response. The primary roles of these events are to act as the first line of defence, slowing down the pathogen's spread and initiating a signalling mechanism that leads to more fundamental changes in the metabolism of the infected plant.

Local responses change the metabolism and lead to the induction of defence genes

The detected presence of the pathogenic organism leads to metabolic changes in the infected tissue. Various pathogen elicitors can be detected via different

receptor mechanisms, but these signals later merge into a few common transduction pathways which control defence responses that are common for many different pathogen types. Secondary metabolic pathways are altered, most notably the phenylpropanoid pathway, which results in the accumulation of phenolic compounds. Phenolic compounds may exert a direct antimicrobial effect (phytoalexins), they may be deposited to form a physical barrier against pathogen spread (lignins), or they may participate in numerous plant defence responses as signalling compounds (salicylic acid, benzoic acid). A number of pathogenesis-related (PR) genes are activated, cell walls are structurally reinforced by oxidative cross-linking of hydroxyproline and proline-rich glycoproteins to the polysaccharide matrix and their *de novo* synthesis, and other defence-related proteins are synthesized, such as polygalacturonase-inhibiting proteins (PGIPs), which inhibit polygalacturonases produced by certain fungal pathogens. Other signal transduction compounds, such as jasmonic acid and ethylene, are synthesized and the respective signalling pathways may be activated, although the exact pattern of the response may be dependent on the pathogen type, the physiological state of the plant and the cross-talk between the various signal transduction pathways within the plant organism.

The processes described here are known as local acquired resistance and lead to a greater immunity of the infected tissue to subsequent infections.

THE FIRST LINE OF DEFENCE – DETECTION AND IMMEDIATE RESPONSE

Resistant plants are capable of detecting the infection almost immediately, and the first defensive responses can be observed within minutes after the infection. These rapid events are transcription-independent and they cause morphological and physiological changes in the infected cells and their surroundings. They are often a part of the hypersensitive response (HR) [1], which is a general term for a set of events leading to programmed cell death and the confinement of the attacking pathogen to the site of infection. Appearance of the HR is preceded by the intensive synthesis of reactive oxygen species (ROS) (so-called "oxidative burst" [2]), changes in cell membrane polarity and ion permeability, ROS-mediated cell wall reinforcement and protein phosphorylation/de-phosphorylation. Some of these events contribute to the limitation of pathogen spread within the infected tissue, while other events, e.g. ion fluxes or changes in protein phosphorylation state, additionally serve as a signal initiating transcription-dependent part of the local response, as well as the starting point for the transduction of systemic signals to distant parts of the plant (see Fig. 1). It is important to realise that proper defence responses in incompatible interaction must be tightly regulated in terms of their intensity and spatial appearance. Metabolic changes responsible for stopping the pathogen also have

a negative influence on the host cells, and this effect on the healthy parts of the plant must be minimized. On the other hand, the defensive reaction must be strong enough to be effective. These are the reasons for the existence of a complex signalling network involving several secondary messengers and providing fine control over the defensive processes on their various levels. Reactive oxygen species are thought to be an important element of this regulation scheme, together with other secondary messengers like salicylic acid, ethylene, jasmonic acid and nitric oxide.

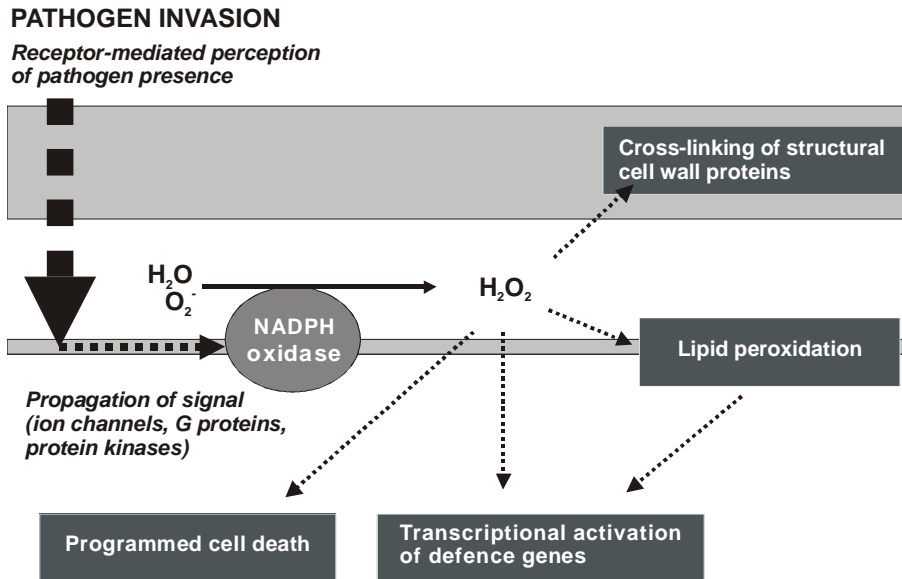


Fig. 1. Reactive oxygen species in the early defence responses of the plant cell.

Allosteric changes

The initial pathogen recognition by a specific receptor results in the activation of ion fluxes and oxidative burst. These early defence events are thought to be mediated through the regulation of plasma membrane-bound enzymes such as Ca²⁺-ATPase [3] and H⁺-ATPase [4], the activation of ion channels [5,6] and the induction of membrane-bound NADPH oxidase [7,8]. These processes induce changes in the electrical potential across the plasma membrane and in the pH gradient, which can affect the NADPH oxidase electron transport chain activating rapid production of H₂O₂. It is currently believed that the pathways that mediate such changes involve G proteins, changes in cytosolic Ca²⁺ concentration and protein kinases/phosphatases.

Only circumstantial evidence exists so far for a role of G proteins in the signal transduction from the receptor. There are reports that the recombinant α -subunit of a G protein can directly activate a plasma membrane Ca^{2+} channel [9], and that heterotrimeric G protein activates a membrane-bound phosphatase to induce the dephosphorylation of the proton pump (H^+ -ATPase) [10,11]. It has been proposed that the C-terminal LRR domains of some R proteins may interact, directly or via an as yet unidentified protein intermediate, with a G protein coupled receptor [12]. Cholera toxin (CTX) can activate signalling pathways dependent on heterotrimeric G proteins. It has been shown that expression of the CTX gene in transgenic tobacco leads to elevated resistance against *Pseudomonas tabaci* and to constant induction of the set of PR genes known to be activated during pathogenesis [13]. It is possible that in the early response phase the G protein-dependent signalling pathway involves inositol 1,4,5-triphosphate (IP3) and phospholipase C (but not phospholipase D) activation [14,15]. Recently, plant homologues of the animal *rac* gene, encoding a small G protein, have been isolated from tobacco, *Arabidopsis*, maize and alfalfa. In tobacco, antibodies raised against the human protein Rac2 detected a strong immunoreactive cytosolic protein in elicitor-treated cells. The appearance of this protein was related to the production of ROS by the elicited cells [16]. Moreover, transgenic tobacco plants expressing the antisense *Ms-rac1* gene from alfalfa fail to develop necrotic lesions during the hypersensitive response [17]. In mammalian cells, Rac induces the activation of NADPH oxidase leading to superoxide production. It has been demonstrated that activated *rac* genes from maize can induce superoxide production when expressed in a mammalian system [18]. This strongly supports the hypothesis that Rac-like proteins in plants serve as signal transducers from the receptor to NADPH oxidase and possibly to other branches of the regulatory network during early defensive responses. It is also an indication of the remarkable structural and functional conservation of Rac proteins between the plant and animal kingdoms during evolution.

Ion channels and ion fluxes

Plasma membrane permeability through the activation of membrane-located ion channels changes immediately after pathogen recognition. This results in ion fluxes, mainly Ca^{2+} and H^+ influx, and K^+ , H_2PO_4^- and Cl^- efflux. Increases in intracellular calcium content have been described for various types of stress conditions including heat shock, chilling, excessive salt concentration, drought and anaerobic stress [19]. Calcium is also required for the action of ethylene [20]. Cytosolic Ca^{2+} concentration increases in response to race-specific elicitors as well as in response to some non-race-specific signals [21].

Certain types of incompatible interactions are strictly dependent on Ca^{2+} influx. In such cases, the blocking of calcium signalling prevents oxidative burst and the hypersensitive response [22,23]. On the other hand, oxidative stress, such as

the administration of H_2O_2 , can stimulate increases in cytosolic calcium concentration [24]. Most likely, Ca^{2+} ions induce NADPH oxidase, either directly by interacting with a Ca^{2+} -binding motif of NADPH oxidase [25,26], or indirectly via a homologue of the mammalian p47^{phox} protein [25]. In neutrophils, p47^{phox} is phosphorylated by protein kinase C and helps activate the NADPH oxidase [27]. Ca^{2+} may also act in favour of NADPH oxidase activation by calmodulin through a calmodulin-dependent NAD kinase that supplies NADPH during the assembly and activation of the oxidase [28]. The resulting oxidative burst further induces Ca^{2+} influx, causing induction of massive electrolyte leakage. This induction occurs because increased cytosolic calcium concentration causes the opening of outward-bound anion (Cl^- and H_2PO_4^-) channels and closing of inward-bound K^+ channels. Such anion efflux promotes cell membrane depolarisation, which might be responsible for opening outward-bound K^+ channels and K^+ efflux. Apart from its direct effect on plasma membrane permeability, Ca^{2+} can activate protein kinases that pass the signal down to the oxidative burst or to the other parts of the signalling network [29].

Oxidative burst

Both plant and animal cells possess the ability to produce and detoxify reactive oxygen species. In plants, under normal physiological conditions, ROS are produced during the process of molecular oxygen assimilation. Under stress conditions, protective mechanisms are overridden and massive ROS production can be observed – this process is called oxidative burst. This bears striking similarity to a defence mechanism of mammalian immunological system, in which a similar oxidative burst occurs in phagocytes. Rapid and intensive production of ROS is a common phenomenon in many stress responses including photoinhibition, hypoosmotic stress, drought, cold shock and pathogen infection [30,31]. Excessive ROS concentration leads to cellular damage and ultimately to cell death, primarily through damage to the photosystem II reaction centre and to membrane lipids. This is why plant cells possess the following detoxifying mechanisms:

1. Superoxide dismutases (SOD) catalyse the dismutation of O_2^- and $\text{HO}_2\cdot$ to H_2O_2 . They are metal-containing enzymes (Mn, Fe or Cu/Zn) found in the cytosol, mitochondria, chloroplasts, peroxisomes and glyoxysomes.
2. Ascorbate peroxidases (APX) play a role in the scavenging of H_2O_2 . Ascorbate also directly scavenges $\cdot\text{O}_2^-$ and H_2O_2 . It is regenerated by dehydroascorbate reductase and at the expense of glutathione, which is in turn regenerated by glutathione reductase at the expense of NADPH. Glutathione can also react directly with $\text{OH}\cdot$. In addition to its role in ascorbate regeneration, this ascorbate/glutathione cycle is important for the regeneration of α -tocopherol, which is an important antioxidant that scavenges lipid peroxides.

- Catalase is a commonly occurring enzyme converting H_2O_2 to H_2O and O_2 . It plays a significant role in reducing H_2O_2 levels in peroxisomes. Most of the catalases are located in peroxisomes but some may be found in glyoxysomes. A mitochondrial localisation of catalases is also suggested in some cases [32].
- In *Nicotiana plumbaginifolia*, as well as in tobacco, three catalase classes have been described but only two of them, Class I (*Cat1*) and Class II (*Cat2*), are expressed in mature leaves – Class III catalases is found in seeds) [33]. There are striking differences in the expression of these genes during the HR: while *Cat1* mRNA levels drop to undetectable levels in the infected area, *Cat2* is strongly induced around the site of infection [34].

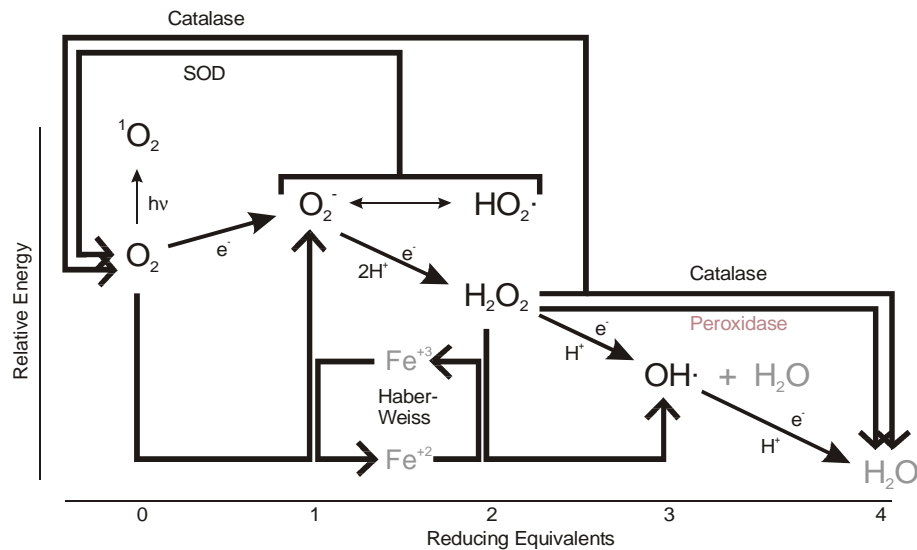


Fig. 2. Possible chemical reactions involving ROS in the plant cell.

Oxidative burst, together with calcium concentration increase, is one of the first responses observed in plant tissue during incompatible reaction. In suspension cultures, two distinct peaks of ROS production can be observed: the first within 30 minutes after pathogen challenge, and the second occurring 1.5-6 hours after elicitation. In some cases, compatible interactions are also able to induce ROS production, but only the first peak is observed – this is considered a non-specific response [31]. H_2O_2 seems to be a major stable ROS compound generated during oxidative burst. The nature of the chemical interaction of H_2O_2 with O_2^- makes it difficult to establish which of the two particles is synthesized first (see Figure 2).

The isolation of plant homologues of mammalian NADPH oxidase [25,26] led to the assumption that, as in animals, this enzyme is responsible for ROS generation during oxidative burst. The existence of Ca^{2+} -binding motifs in NADPH oxidase further supports the possibility of the involvement of this enzyme in the hypersensitive response. However, recent findings suggest that peroxidase H_2O_2 -generating systems may be at least partially responsible for ROS production [35]. Most probably, the oxidative burst occurs at the cell surface, although the appearance of ROS is not limited to the apoplast, since H_2O_2 is a small diffusible molecule that can freely cross lipid membranes [36].

Several roles have been proposed for oxidative burst (see Figure 1):

1. a direct toxic effect on micro-organisms [37,38];
2. promoting the oxidative cross-linking of cell wall hydroxyproline- and proline-rich glycoproteins, thus protecting them against enzymatic degradation [39];
3. increasing the rate of lignin polymer formation in the cell wall, which would create a physical barrier against the spread of some pathogens [2,40];
4. induction of phytoalexin synthesis [41];
5. a signalling role.

Substantial evidence indicates that H_2O_2 is the major factor causing cell death during the hypersensitive response, due to its cytotoxic effect. However, recent results for tobacco, induced with cryptogein, point to a 9-oxylipin pathway involving the lipoxygenase-dependent peroxydation of fatty acids as the main cause of cell death during the HR [42].

Reactive oxygen species generated during the oxidative burst, particularly H_2O_2 , are an indispensable factor in signalling pathways leading to the hypersensitive response on one hand, and to the activation of local and systemic resistance on the other. Direct application of H_2O_2 can induce several pathogenesis-related genes. A similar effect has been obtained in catalase-antisense plants [43]. Remarkably, enzymes that scavenge H_2O_2 are down-regulated during pathogenesis [44]. H_2O_2 can also act as a modulator of MAP kinase-dependent signalling pathways (see above).

Apart from the unquestionable role in the early response against pathogen infection, ROS and particularly H_2O_2 are critical factors taking part in the induction of transcription-dependent resistance responses, both local and systemic.

Hypersensitive response

The reaction of plants to pathogen invasion results in the activation of two distinct signalling pathways, one leading to the induction of programmed cell death at the site of infection, the other activating resistance mechanisms in the infected tissue, as well as in other parts of the organism. Incompatible interactions often manifest themselves in tissue necrotisation in and around the pathogen entry point. Such a response was historically referred to as the

hypersensitive reaction, but is now more frequently called hypersensitive cell death. However, it is not clear whether the HR is the cause or the result of the stoppage of pathogen growth.

In animals, programmed cell death is often realised as a set of defined steps leading to cell dismantling, termed apoptosis, and regulated by the caspase signalling cascade [45]. Some features of animal apoptosis are present in plants, including nuclear condensation and regular DNA cleavage into characteristic apoptotic "ladders". The oxidative burst and overall changes in the cell redox state are important factors in triggering cell death. Recent research indicates that death does not occur through a direct cytotoxic effect of ROS [42]; they are rather an element of a signalling network in the cell, regulating both the HR and resistance. The production of $\cdot\text{O}_2^-$ and H_2O_2 was shown to induce hypersensitive cell death [46,47,48], although the exact signalling pathway leading to it remains to be elucidated. Remarkably, transgenic plants with reduced catalase [44, 49] or ascorbate peroxidase [44] levels develop spontaneous necrotic changes and induce systemic resistance responses or are hyper-responsive to pathogen infection. Further underlining the importance of a high ROS level in the HR is the finding that ROS scavenging systems such as CAT and APX are suppressed during the HR [34,49,50]. Some reports indicate that the oxidative burst alone may not be sufficient (and sometimes not be required at all) to induce hypersensitive cell death [51,52]. It is likely that hypersensitive cell death is regulated by several partially overlapping signalling pathways leading to a similar phenotype.

The oxidative burst is accompanied by the rapid synthesis of nitric oxide in the infected tissue. In animals, NO can generate highly toxic peroxynitrite radicals when combined with $\cdot\text{O}_2^-$. Still, similarly to ROS, NO can have either a toxic or protective effect depending on its concentration and the physiological state of the tissue. NO, acting as an acceptor, can neutralize more toxic peroxides produced as a result of oxidative damage and it can also stop the propagation of radical-mediated lipid oxidation [53]. NO has the ability to potentiate the induction of hypersensitive cell death [54] and may be an indispensable component of the signalling pathway leading to the HR. It may also activate responses via specific signalling pathways involving G proteins and Ca^{2+} [21,55]. It is unclear at present to what extent NO is involved in Ca^{2+} - and MAP kinase-dependent regulation of NADPH oxidase.

The existence of plant mutants exhibiting spontaneous tissue necrotisation in the absence of a pathogen [see Ref. 56,57] indicates that hypersensitive cell death is transcription-dependent and may be a tightly regulated phenomenon. It is likely that hypersensitive cell death occurs both in compatible and incompatible interactions, induced by extensive tissue damage by the invading pathogen in the former instance, and in the latter case triggered by resistance mechanisms and to help to limit the pathogen invasion.

LOCAL ACQUIRED RESISTANCE

Changes in metabolism

Apart from hypersensitive cell death, numerous changes take place in the tissue surrounding the infection site. These processes enhance the plant's defensive response against the attacking micro-organism as well as build up resistance against subsequent infections.

Initial responses triggered by the infection are further amplified in the subsequent stages of the response. The activity of defence-related signal-transduction pathways leads to the activation or repression of numerous genes. The expression pattern of some of these genes is common for various stress responses, others are specifically induced or repressed only during pathogenesis. Many elicitor-responsive genes are involved in various biochemical pathways from both the primary and secondary metabolism. The secondary metabolism, in particular, has been studied in detail, since many of its products exhibit defence-related properties either directly enhancing protection of the plant or taking part in the regulation of other defence-related genes.

Phenylpropanoid metabolism is heavily modified after pathogen infection in all the experimental systems analysed. Phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase, and 4-coumarate:CoA ligase are strongly activated during stress responses [58]. This leads to the activation of subsequent branches of the phenylpropanoid pathway, and to the production and accumulation of various soluble and cell wall-bound phenolics that have been shown to have direct antimicrobial effect (e.g. phytoalexins), or to function as secondary messengers (e.g. benzoic acid, salicylic acid and its derivatives, jasmonic acid, ethylene; see below). Large carbon fluxes into the secondary metabolism are possible due to increased supplies of substrates from primary metabolic pathways such as the shikimate pathway or the activated methyl cycle which are upregulated as a result of elicitation [59-61].

Various proteins are synthesized as a result of infection. A distinct group of proteins that is closely linked with the defensive response has been named "pathogenesis-related" (PR) (see below). Some of them play regulatory roles, others possess defined enzymatic activities that may directly influence the attacking pathogen, while the function of other PR proteins remains to be determined.

Pathogenic infection is a serious threat to the integrity of the organism and the whole metabolism must be reprogrammed to provide enough resources for efficient resistance. The drastic reduction of Rubisco levels [61] or arrest of the cell cycle and cell proliferation [62] are examples of such metabolic accommodation that have been described in cases of potato and parsley, respectively, but it is likely that many more similar cases exist.

Changes in physical properties of cell components

Several effective strategies of resistance against pathogen infection rely on changes in the physical properties of some cell components:

1. callose and lignin deposition – small papillae are often formed beneath sites infected with biotrophic fungi, probably serving as a physical barrier blocking fungal penetration into plant cells; callose depositions in the plasmodesmata are likely to restrict cell-to-cell virus movement;
2. extracellular hydroxyproline-rich glycoproteins (HRGPs) – H_2O_2 induces the process of rapid crosslinking of HRGPs to the wall matrix, increasing the density of the wall; later in the course of infection, *de novo* synthesis of HRGPs initiates additional lignin polymerisation;
3. polygalacturonase-inhibiting proteins – these extracellular proteins inhibit cell-degrading polygalacturonases present in some necrotrophic pathogens, resulting in an increased abundance of oligogalacturonides which may trigger additional defence responses;
4. microtubule depolymerisation – the microtubular network is rapidly depolymerised around the penetrating fungal hyphae; actin filament-mediated translocation of the nucleus and cytoplasm to such sites has also been observed.

CONCLUSIONS

An efficient defence response against pathogen infection relies upon correct pathogen recognition, the activation of immediate defence processes in the infected tissue and the triggering of various transcription- and translation-dependent processes in the infected organism via a tightly coordinated signalling network. Redox processes are an important element of early defence responses at and around the site of infection. The infection results in changes in plant metabolism, in the enhancement of defence processes, and in the activation of signalling transduction cascades leading to the development of systemic acquired resistance.

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