REVIEW ARTICLE

Oxidative burst: an early plant response to pathogen infection

Przemysław WOJTASZEK

Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowskiego 12/14, 61-704 Poznań, Poland

As plants are confined to the place where they grow, they have to develop a broad range of defence responses to cope with pathogenic infections. The oxidative burst, a rapid, transient, production of huge amounts of reactive oxygen species (ROS), is one of the earliest observable aspects of a plant's defence strategy. First this Review describes the chemistry of ROS (superoxide radical, hydrogen peroxide and hydroxyl radical). Secondly, the role of ROS in defence responses is demonstrated, and some important issues are considered, such as: (1) which of the ROS is a major building element of the oxidative burst; (2) the spatial and temporal regulation of the oxidative burst; and (3) differences in the plant's responses to biotic and abiotic elicitation. Thirdly, the relationships between the oxidative burst and other plant defence responses are indicated. These include: (1) an oxygen consumption, (2) the production of phytoalexins, (3) systemic acquired resistance, (4) immobilization of plant cell wall proteins, (5) changes in membrane permeability and ion fluxes and (6) a putative role in hypersensitive cell death. Wherever possible, the comparisons with models applicable to animal systems are presented. Finally, the question of the origin of ROS in the oxidative burst is considered, and two major hypotheses, (1) the action of NADPH oxidase system analogous to that of animal phagocytes, and (2) the pH-dependent generation of hydrogen peroxide by a cell wall peroxidase, are presented. On the basis of this material, a third 'unifying' hypothesis is presented, where transient changes in the pH of the cell wall compartment are indicated as a core phenomenon in evoking ROS production. Additionally, a germin/oxalate oxidase system which generates H_2O_2 in response to pathogenic infection is also described.

INTRODUCTION

Throughout their life cycle, plants have to react to various threats coming from the outside environment. As plants are sessile, they have developed a broad range of strategies, collectively known as 'defence' or 'stress' responses, to protect themselves against biotic and abiotic stresses. Infections by pathogenic fungi, bacteria, and viruses are among the more serious dangers plants have to cope with.

Upon sensing the invading micro-organism, plants can evoke, in a co-ordinate fashion, one, two or many defence mechanisms in an attempt to restrict pathogen growth and, finally, to destroy it [2,3]. General defence reactions, such as cell wall reinforcement, phytoalexin production and the accumulation of antimicrobial proteins, are employed, and their temporal and spatial regulation are the most decisive factors governing the outcome of the host–pathogen interactions [susceptibility (disease) or resistance (no disease) to a given micro-organism] [4,5]. In numerous incompatible interactions, these reactions are often associated with the death of a small number of cells at the site of infection, known as the 'hypersensitive response' (HR) [6–9]. Initiation of a resistance response requires perception of signal molecules, either synthesized by the invading organism or released from plant cell walls. These signal molecules have collectively been termed 'elicitors', but only a few have been defined at the molecular level as oligosaccharides, (glyco)proteins, and glycopeptides [10]. The elicitors may be specific for a particular plant host–microbe system or are very general molecules, e.g. components of cell walls of the invading microbe [10]. When mixtures of elicitor molecules or purified elicitors have been used to induce defence reactions in suspension-cultured plant cells, many novel aspects of the early defence response have been established. These include rapid and transient responses that occur mainly at the plant cell surface and are based on the activation of pre-existing components rather than involving the biosynthetic machinery of the cell. Among the reactions identified were: release of reactive oxygen species (ROS) termed the 'oxidative burst' [11–14], changes in exocellular pH and in membrane potentials [15,16], ion fluxes [17–19], changes in protein phosphorylation patterns [15,20,21], and the oxidative immobilization of plant cell wall proteins [22,23].

Most cells possess the ability to produce and detoxify ROS. In normal conditions ROS appear in cells as inevitable by-products formed as a result of successive one-electron reductions of molecular oxygen (O_2). Most cells have also acquired the relevant protective mechanisms to maintain the lowest possible levels of ROS inside the cell. In some cases, however, especially under stress conditions, these protective mechanisms are overridden by the rapid, transient, production of huge amounts of ROS, namely the oxidative burst. This reaction has been known for more than 30 years in mammals from studies on the 'respiratory burst' in phagocytes (reviewed in [24,25]). However, in plants the phenomenon was first demonstrated much later [26], and recently published data have indicated that the ROS produced in the oxidative burst could serve not only as protectants against invading pathogen, but could also be the signals activating further plant defence reactions, including the HR of infected cells [27].

This Review first describes the chemistry of the ROS, their role in the oxidative burst and in induction of other defence reactions. As the origin of ROS building the oxidative burst is now probably the major controversy in this research area, hypotheses trying to explain this phenomenon are reviewed and supporting results evaluated. Some of the data have been discussed pre-

Abbreviations used: DPI, diphenylene iodonium; ECM, exocellular matrix (in this Review the term 'exocellular matrix' has been adopted to denote the cell wall with the meaning proposed by Wyatt and Carpita [1]); HR, hypersensitive response; HRGP, hydroxyproline-rich glycoprotein; INA, 2,6-dichloroisonicotinic acid; OGA, oligo-1,4-α-D-galacturonide; PR, pathogenesis-related; PRP, proline-rich glycoproteins; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic acquired resistance; SOD, superoxide dismutase; $^{\bullet}O_2^-$, superoxide radical; $^{\bullet}OH$, hydroxyl radical.

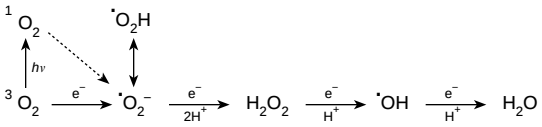
viously, and the reader is referred to excellent earlier reviews [11–14,28,29].

CHEMISTRY OF ROS

For the purpose of this Review, the term ROS is used to describe the products of the sequential reduction of molecular oxygen (Scheme 1a): superoxide radical ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$), species predominantly detected in plant–pathogen interactions. These ROS are routinely generated at low levels in non-stressed plant cells in chloroplasts and mitochondria, and also by cytoplasmic, membrane-bound or exocellular enzymes involved in redox reactions.

The generation of $^{\bullet}O_2^-$ requires a slight input of energy. In living cells $^{\bullet}O_2^-$ exists in equilibrium with its protonated form, the hydroperoxyl radical ($^{\bullet}O_2H$). The latter form is more hydrophobic than superoxide and can more easily penetrate the lipid bilayers of the membranes. However, at a physiological pH, $^{\bullet}O_2^-$

(a)



(b)

Superoxide disproportionation

$$2\ ^{\bullet}O_2H \rightleftharpoons H_2O_2 + O_2 \quad (1)$$

$$2\ ^{\bullet}O_2H + {}^{\bullet}O_2^- + H^+ \rightleftharpoons H_2O_2 + O_2$$

$$2\ ^{\bullet}O_2^- + 2\,H^+ \rightleftharpoons H_2O_2 + O_2$$

Catalase

$$H_2O_2 + H_2O_2 \longrightarrow 2H_2O + O_2 \quad (2)$$

Peroxidase

$$\text{Peroxidase } (Fe^{3+}) + H_2O_2 \longrightarrow \text{Compound I} \quad (3)$$

$$\text{Compound I} + RH_2 \longrightarrow \text{Compound II} + {}^{\bullet}RH$$

$$\text{Compound II} + RH_2 \longrightarrow \text{Peroxidase } (Fe^{3+}) + {}^{\bullet}RH$$

Generation of H_2O_2

$$^{\bullet}RH + O_2 \longrightarrow {}^{\bullet}O_2^- + RH \quad (4)$$

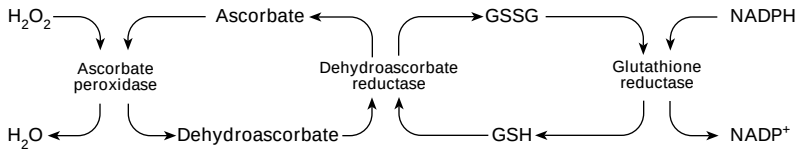
$$\text{Peroxidase} + {}^{\bullet}O_2^- \longrightarrow \text{Compound III}$$

$$\text{Compound III} + RH_2 \longrightarrow \text{Peroxidase } (Fe^{3+}) + {}^{\bullet}RH$$

$$2\ ^{\bullet}O_2^- + 2\,H \rightleftharpoons H_2O_2 + O_2$$

$$RH_2 + {}^{\bullet}O_2^- \longrightarrow {}^{\bullet}RH + H_2O$$

Halliwell–Asada pathway:



(5)

Haber–Weiss reaction:

$$H_2O_2 + {}^{\bullet}O_2^- \longrightarrow {}^{\bullet}OH + OH^- + O_2 \quad (6)$$

Fenton reaction:

$$H_2O_2 + Fe^{2+}(Cu^+) \longrightarrow Fe^{3+}(Cu^{2+}) + {}^{\bullet}OH + OH^- \quad (7)$$

$$^{\bullet}O_2 + Fe^{3+}(Cu^{2+}) \longrightarrow Fe^{2+}(Cu^+) + O_2 \quad (8)$$

Scheme 1 (a) Interrelationships between molecular oxygen and ROS generated in reactions likely to occur in living plant cells and (b) chemical equations depicting major reactions determining the fate and possible interconversions of reactive oxygen species in plants

is not very reactive against major macromolecular components of the cell. In aqueous solutions, at neutral or slightly acidic pH, this radical in either form disproportionates to H_2O_2 and O_2 (Scheme 1b, eqn. 1). This reaction either occurs spontaneously or is catalysed by superoxide dismutase (SOD) found in the cytosol, chloroplasts, mitochondria [28] and exocellularly [30]. The equation shows also that, in any system producing $^{\bullet}O_2^-$, substantial amounts of H_2O_2 are also formed.

H_2O_2 is a relatively stable ROS. Being not very reactive and electrically neutral, H_2O_2 is able to pass through cell membranes and reach cell locations remote from the site of its formation. In plant cells, newly formed H_2O_2 could be (a) disproportionated spontaneously or with the participation of catalase to form water and molecular oxygen (Scheme 1b, eqn. 2), (b) used as a substrate by various peroxidases (e.g. for the generation of phenoxyl radicals, which are building blocks for lignin synthesis; Scheme 1b, eqn. 3) or (c) detoxified by ascorbate peroxidase acting in concert with dehydroascorbate reductase and glutathione reductase in the Halliwell–Asada pathway (Scheme 1b, eqn. 5). Under appropriate conditions, however, with the supply of reductant, H_2O_2 could be generated by peroxidases in a series of reactions involving formation of Compound III (Fe^{II}–O–O; Scheme 1b, eqn. 4) [31,32].

The hydroxyl radical is the most reactive species among ROS discussed here. It could be formed upon direct reaction of H_2O_2 and $^{\bullet}O_2^-$ (Haber–Weiss reaction; Scheme 1b, eqn. 6). Under conditions normally found in plant cells, this reaction, however, proceeds rather slowly, and is ineffective in producing substantial amounts of $^{\bullet}OH$ [33]. On the other hand, significant levels of $^{\bullet}OH$ could be formed through the cycle of reactions involving oxidation of transition metals, such as Fe^{2+} or Cu^+ (Fenton reaction; Scheme 1b, eqn. 7), and subsequent regeneration of the oxidized ions to their reduced state via reaction with superoxide (Scheme 1b, eqn. 8). As transition metals act here as catalysts, their location and accessibility are probably the major factors determining the cellular site of $^{\bullet}OH$ formation [33–35]. The hydroxyl radical with its ability to initiate radical chain reactions is believed to be a major ROS responsible for the irreversible modifications of cellular macromolecules and damage of organelles. As its half-life is in the range of 10^{-9} s, both its detection and determining its role in plant–pathogen interactions have been unsuccessful. However, recent experiments on suspension-cultured rice (*Oryza sativa*) cells treated with *N*-acetylchito-oligosaccharide elicitor provided the evidence for the generation of $^{\bullet}OH$ in this model system [36].

OXIDATIVE BURST IN DEFENCE RESPONSES

In contrast with most animal systems, plants cells are able to produce ROS, mainly H_2O_2, constitutively in significant amounts, and this production is predominantly associated with the cell's exocellular compartment (exocellular matrix, ECM) and is regulated by developmental factors such as hormones, light and wounding. H_2O_2 is mainly detected in cells undergoing lignification, i.e. in tracheary elements and phloem fibres, and in some epidermal cells [37]. It is generally absent in cell walls of rapidly elongating cells, but its intensive production could be observed in wounded cells or cells subjected to mechanical stress [37,38].

The oxidative burst is generally defined as a rapid production of high levels of ROS in response to external stimuli [7,12,16]. The first report on the rapid ROS production appeared in 1983, demonstrating the generation of $^{\bullet}O_2^-$ by potato (*Solanum tuberosum*) tuber discs only in response to inoculation with an incompatible race of *Phytophthora infestans* or with hyphal wall components, but not with a compatible race [26]. However, as mentioned previously, it was the use of suspension-cultured cells that has enabled intensive investigations of this phenomenon. As a result, although not initially appreciated, the oxidative burst is now considered as one of the earliest events following elicitation. This reaction was observed in plants challenged with pathogenic micro-organisms, including fungi, bacteria and viruses, as well as in cultured cells treated with elicitor preparations, pathogens or plant cell-wall fragments or in response to mechanical stress (Table 1). Most of the data seem to indicate that the major ROS building the oxidative burst is H_2O_2, with possible participation of $^{\bullet}O_2^-$. However, the inherent interrelationship between H_2O_2 and $^{\bullet}O_2^-$ generation (see Scheme 1b, eqn. 1), makes it sometimes difficult to identify clearly the ROS behind the oxidative burst. In the first experiments, Doke used the cytochrome *c* or NitroBlue Tetrazolium-reduction assays to demonstrate the generation and release of $^{\bullet}O_2^-$ as the major ROS [26,39,59]. Auh and Murphy identified the same phenomenon in rose (*Rosa* sp.) cells treated with *Phytophthora* elicitor monitoring the chemiluminescence of lucigenin [51]. However, when also analysing luminol-dependent chemiluminescence, they were able to observe the simultaneous accumulation of H_2O_2. As the accumulation of H_2O_2 was inhibited by the SOD inhibitor (*NN*-diethyldithiocarbamate), this suggested the origin of H_2O_2 was from the SOD-catalysed disproportionation of generated $^{\bullet}O_2^-$ [51]. In the tomato (*Lycopersicon esculentum*)–*Cladosporium fulvum* elicitor system, when suspension-cultured cells were used, both H_2O_2 and $^{\bullet}O_2^-$ were found to be produced [44], while in tomato hypocotyls, only $^{\bullet}O_2^-$ has been detected [45].However, in the latter case only the NitroBlue Tetrazolium staining assay was used, hence the relationship between these two ROS still remained unclear. In most systems studied, however, H_2O_2 was identified as a building force of the oxidative burst, either with the use of luminol-dependent chemiluminescence [16,18,40,43,46,49,50,54–56,58,62,63,65] or by other methods such as fluorescent dye quenching [41,42,60,64], scopoletin cleavage [57] or Phenol Red oxidation [61]. In all cases the oxidative burst was sensitive to the addition of catalase to the media, and insensitive to the presence of SOD or mannitol (Table 1). It should be noted, however, that failure to detect $^{\bullet}O_2^-$ accumulation might indicate that either $^{\bullet}O_2^-$ is not an intermediate or it is extremely rapidly dismutated, e.g. within plant cell-wall boundaries [57]. Moreover, the relative timing of the application of both the elicitor and catalase is of primary importance. Although catalase added before or together with the elicitor inhibited the generation of ROS, the same enzyme added 1 h after the elicitor application had no effect on the oxidative burst [41].

The occurrence of the transient increase in ROS production is usually very rapid, but could vary depending on the plant system studied, and on the challenging factor used (Figure 1). In suspension-cultured cells this response usually starts 1–2 min after the addition of fungal elicitors or plant-derived oligo-1,4-α-D-galacturonides (OGA) to the media, reaches its maximum several minutes later, and is completed within 30–60 min after elicitation [16,41,43,44,57,60,65]. It should be noted, however, that the observed oxidative burst is to some extent independent from the accumulation of free radicals, calculated from the enhanced $g = 2.00$ EPR signal, and detected usually over the period of several hours after elicitation, probably resulting from lipid peroxidation [43]. When plant segments were used to study the oxidative burst, this response was observed considerably later, within the first 8–12 h after elicitation [26,61], with the evident signs of ROS generation visible after 2–4 h [45]. Recent detection *in vivo* of the production of H_2O_2 by lettuce only 5 h after inoculation with incompatible race of *Pseudomonas syringae*

Table 1 Plant systems generating ROS when challenged with various pathogens/elicitors or in response to mechanical stress

Unless mentioned otherwise, suspension-cultured plant cells were used.

Plant	Pathogen/type of elicitor	AOS observed	Comments	Ref.
Plant–fungus interactions				
Potato tuber discs	*Phytophthora infestans* zoospores or hyphal wall components	$^{\bullet}O_2^-$	Reaction only to incompatible race or hyphal wall components	[26]
Potato protoplasts	*Phytophthora infestans* hyphal wall components	$^{\bullet}O_2^-$	ROS generation in the presence of external NADPH	[39]
Soybean	*Phytophthora megasperma* f.sp. *glycinea* cell-wall preparation	H_2O_2 and $^{\bullet}O_2^-$	Inhibited by catalase, SOD and peroxidase inhibitors	[40]
Soybean, cotton	*Verticillium dahliae* 277 crude elicitor	H_2O_2	Heterogeneity in oxidative processes; oxidative burst independent from phytoalexin formation	[41,42]
Dark red kidney bean	*Colletotrichum lindemuthianum* galactoglucomannan	H_2O_2, others?	Accumulation of free radicals over 12 h detected by EPR spectroscopy; cell death observed	[43]
French bean	*Colletotrichum lindemuthianum* crude cell-wall preparation; chitin or chitosan oligomers	H_2O_2	Transient alkalinization of apoplast, a prerequisite for oxidative burst; reaction independent from PAL activity or hypocotyl browning	[16,23]
Tomato	*Cladosporium fulvum* race-specific secreted elicitors	H_2O_2 and $^{\bullet}O_2^-$	Lipid peroxidation; increased peroxidase activity in incompatible interactions	[44]
Tomato cotyledons	*Cladosporium fulvum* race-specific elicitors	$^{\bullet}O_2^-$	Activation of *Cf-Avr*-mediated response results in severe oxygen stress	[45]
Spruce	*Amanita muscaria, Hebeloma crustuliniforme, Heterobasidion annosum* cell-wall components	H_2O_2	Elicitors from mycorrhizal fungi less active than from pathogens	[46]
	Rhizosphaera kalkhoffii crude cell-wall preparation	H_2O_2		[47]
Carrot protoplasts	*Pythium aphanidermatum* secreted compounds	None	Other responses (ion fluxes, 4-hydroxybenzoic acid synthesis) detected	[17]
Parsley	*Phytophthora megasperma* f.sp. *glycinea* crude elicitor or pure glycoprotein elicitor	H_2O_2		[18]
	Phytophthora sojae crude elicitor; chitosan	H_2O_2	Salicylic acid enhances the oxidative burst	[48]
Tobacco	*Phytophthora parasitica* var. *nicotianae* crude cell-wall elicitor	H_2O_2?	Only cryptogein active	[49]
	Phytophthora cryptogea, purified cryptogein	(Not identified)		
	Pure cryptogein and capsicein protein elicitors	H_2O_2 and $^{\bullet}O_2^-$?	ROS generation, lipid peroxidation and necrosis are directly related, but independent from phytoalexin synthesis	[50]
Rice	*N*-Acetylchito-oligosaccharides	$^{\bullet}O_2^-$, H_2O_2, $^{\bullet}OH$		[36]
Rose	*Phytophthora* sp. crude cell-wall preparation	H_2O_2 and $^{\bullet}O_2^-$	H_2O_2 accumulation is a result of SOD-catalysed dismutation of $^{\bullet}O_2^-$	[51]
Pueraria lobata	Commercial preparation of yeast extract	H_2O_2		[52]
Cucumber hypocotyls	*Phytophthora sojae* crude elicitor or partially purified oligoglucan mixture	H_2O_2	Competence for elicitation requires translational protein synthesis	[53]
Plant–bacterium interactions				
White clover	*Pseudomonas corrugata* cells	H_2O_2		[54]
Tobacco	*Erwinia amylovora* cells or purified harpin elicitor	Not identified		[55]
Tobacco	*Pseudomonas syringae* pv. *syringae* and *P. fluorescens*	Not identified	Bacterial *hrmA* mutants evoke normal phase II of ROS production, but no cell death observed	[56]
Soybean	*Pseudomonas syringae* pv. *glycinea*	H_2O_2	Psg(*avrA*) induces biphasic oxidative burst; cell death observed	[57]
Arabidopsis thaliana	Protein elicitor harpin	H_2O_2 and $^{\bullet}O_2^-$	Inhibited by inhibitors of SOD, NADPH oxidase or ROS scavengers; cell death observed	[58]
Plant–virus interactions				
Tobacco leaf discs	Tobacco mosaic virus	$^{\bullet}O_2^-$		[59]
Plant–plant cell-wall fragments				
Soybean and cotton	Oligogalacturonide	H_2O_2	Homologous and heterologous desensitisation observed	[42,60]
Soybean	Oligogalacturonide	H_2O_2	Time-course different from that evoked by fungal elicitor	[57]
Cucumber hypocotyls	Oligogalacturonide	H_2O_2	Part of ROS generation dependent upon *de novo* protein synthesis	[61]
Tobacco	Oligogalacturonide	H_2O_2	Inhibited by 6-dimethylaminopurine	[62]
Mechanical stress				
Eucheuma platycladum thalli		H_2O_2	Completely inhibited by catalase	[63]
Soybean		H_2O_2		[64]

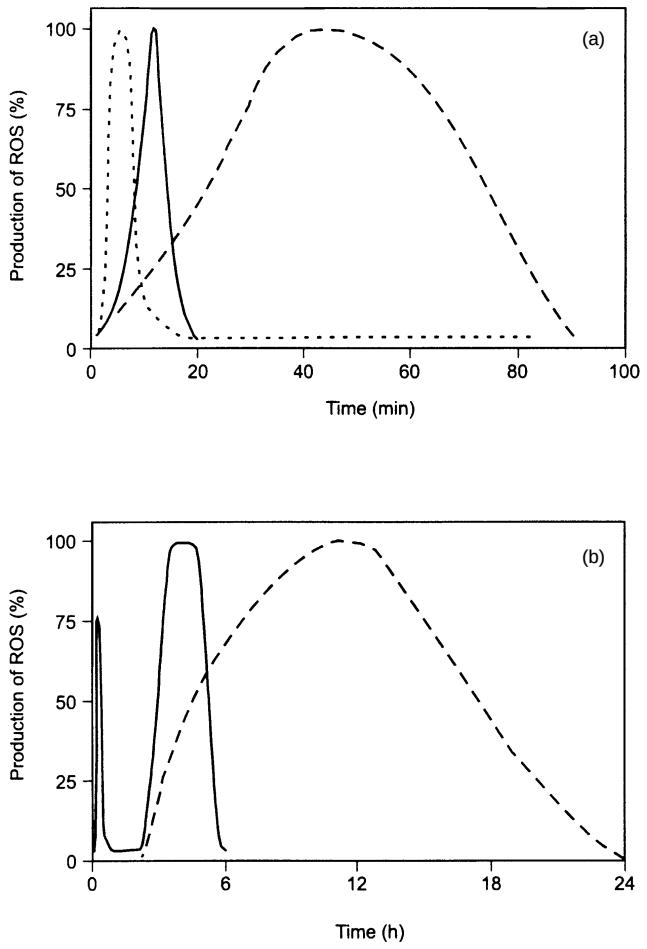


Figure 1 A schematic representation of the kinetics of the oxidative burst in plants or suspension-cultured plant cells following challenge with the various types of elicitor molecules

Note the differences in the time course of ROS generation evoked by pretreatment of plants ('preconditioning effect'; see the text). The idealized graphs of ROS generation presented are based on the experimental data presented in [16,53,55,57,61]. (**a**) Responses of suspension-cultured plant cells to fungal elicitors (——) and plant-derived oligogalacturonide fragments (·····) compared with the response of preconditioned plant to fungal elicitor (– – – –). (**b**) Biphasic reaction of plant cells to bacterial elicitation (——) and ROS generation by plants in response to treatment with OGA (– – – –).

pv. *phaseolicola* by cerium chloride staining, and transmission electron microscopy (Figure 1B by John Mansfield in [8]) seems to confirm this conclusion. However, other data indicated also that a more rapid response of cucumber (*Cucumis sativus*) hypocotyls to elicitor could be achieved by abrasion of the cuticle and conditioning of the segments with salicyclic acid (SA) or 2,6-dichloroisonicotinic acid (INA), which are inducers of systemic acquired resistance (SAR) [53,66]. In such preconditioned hypocotyls, the production of H_2O_2 reached its maximum about 30 min after elicitation and was completed within 90 min, thus greatly resembling the situation found in suspension-cultured cells (Figure 1). Moreover, the effect of hypocotyl preconditioning was completely inhibited by the addition of cycloheximide or puromycin, suggesting a requirement for translational protein synthesis [53]. The same requirement has been indicated for the H_2O_2 generation by OGA-treated cucumber hypocotyls [61]. Other similar observations had already suggested the existence of 'elicitation competency factors' required for induction of defence responses in soybean (*Glycine max*) [67]. However, as plant cells cultured in suspensions are subjected to constant mechanical stimulation as a result of stirring, such a 'preconditioning effect' is probably inherent in these model systems. Interestingly, in suspension-cultured cells, pretreatment with SA does not change the time course of the oxidative burst, but enhances the intensity of ROS generation [48]. In suspension-cultured plant cells treated with bacteria or bacterial elicitors, the ROS production was demonstrated as a biphasic process (Figure 1). Phase I is very similar in its timing to the reaction of plant cells to fungal elicitors, and is considered as a non-specific response. In incompatible interactions, however, Phase I is accompanied by a Phase II (relatively long-lived ROS generation occurring 1.5–6 h after elicitation) [13,55,57].

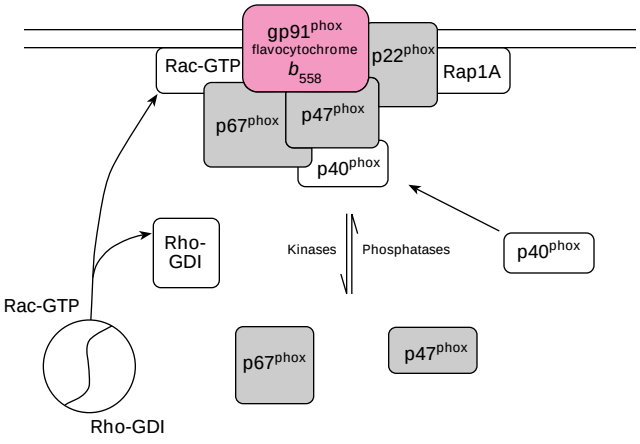
In plant systems, where the ability of various elicitor preparations to evoke the oxidative burst were compared, significant differences in time of occurrence and intensities of the ROS production were observed. In suspension-cultured soybean cells, plant-derived OGA evoked an oxidative burst very similar to that caused by treatment of cells with crude elicitor from *Verticillium dahliae* [41,60]. However, in the latter case it was demonstrated that only a carbohydrate, but not a protein, moiety of *V. dahliae* elicitor is responsible for the induction of H_2O_2 production [42]. A slightly different situation was noted in tobacco (*Nicotiana tabacum*) cells, where a crude elicitor from a tobacco pathogen, *Phytophthora parasitica* var. *nicotianae*, was ineffective in eliciting the oxidative burst, while cryptogein (a protein from the culture medium of the non-pathogen of tobacco *P. cryptogea*) was an effective inducer of the ROS generation [49]. OGA elicitor induced a more rapid and transient oxidative burst in suspension-cultured soybean cells when compared with that evoked by a glucan elicitor prepared from mycelial cell walls of *Phytophthora megasperma* f.sp. *glycinea* [57]. The opposite was true when both elicitors were used on the cucumber hypocotyl segments [61]. However, in that case the more rapid response (maximal at 4–6 h after elicitation) evoked by oligo-β-glucan elicitor reached only about 10–15 % of the maximum response (at 10–12 h post elicitation) induced by OGA. Four different elicitor preparations were tested on suspension-cultured bean cells [23]. Although the timing of the response was roughly the same, reactions of plant cells varied greatly in their intensities. A crude cell-wall elicitor preparation from *Colletotrichum lindemuthianum* was the strongest inducer of ROS. This elicitor when treated with agarose-bound wheat-germ agglutinin was 4-fold less active, indicating the importance of *N*-acetylglucosamine residues as inducers of the oxidative burst. However, when chitin or chitosan oligomers were used for elicitation, the intensity of the oxidative burst reached only 10 % or less of that evoked by the crude elicitor, suggesting the existence of additional moietes necessary for the induction of that response [23].

Results of early studies on the oxidative burst indicated the possibility that the reaction occurs at the cell surface. This conclusion was based on the analysis of the distribution of different components of the assay mixtures, e.g. reporter probes, such as scopoletin, which did not enter the cells [57] or carboxyfluorescein diacetate, which, when loaded into cells, did not serve as a substrate in a fluorescent-dye quenching assay [41]. This notion was stressed, and the ECM indicated as a site of the ROS production by circumstantial evidence coming from studies on protoplasted cells. Firstly, when protoplasts of *Chlamydomonas reinhardtii* cells are regenerating their cell walls, composed almost exclusively of hydroxyproline-rich (HRGP)-type proteins, the burst of H_2O_2 production is observed concomitantly with the insolubilization of wall components, but not in freshly protoplasted cells [68]. On the other hand, the $^{\bullet}O_2^-$ is generated by

elicited potato protoplasts [39] or even by plasma-membrane-rich fractions of elicited potato tuber tissues [69] or rose cells [70]. In all cases, however, in the absence of externally supplied reductant [NAD(P)H], no ROS production is detected (see also the discussion below). Accordingly, carrot protoplasts, when not supplied with the reductant, do not respond with the oxidative burst to treatment with a fungal elicitor from culture medium of *Pythium aphanidermatum*, although other elicitor-induced responses such as ion fluxes and 4-hydroxybenzoic acid synthesis are observed [17].

OXIDATIVE BURST AND OTHER PLANT RESPONSES

In mammals, neutrophils, eosinophils and macrophages phagocytose invading bacteria and kill them. This action is accompanied by a period of intense oxygen consumption, called 'the respiratory burst'. The plasma-membrane NADPH-dependent $^{\bullet}O_2^-$-generating oxidase was identified as a major contributor to the bactericidal capacity of phagocytes (reviewed in [24,25,71]). This enzyme is a multi-component complex composed of membrane-bound and cytosolic proteins (Scheme 2). At the heart of the NADPH oxidase lies the unusual heterodimeric NADPH-binding flavocytochrome b_{558}, consisting of glycosylated transmembrane protein gp91^{phox} and non-glycosylated p22^{phox} subunit. The flavocytochrome contains the entire electron-transport chain from NADPH to oxygen, which means other components of the system play the regulatory role. These cytosolic proteins include p47^{phox} and p67^{phox} phosphoproteins, probably associated with cellular cytoskeleton, p40^{phox}, a protein with as yet unidentified function, and two small GTP-binding proteins (p21^{rac}, complexed with its regulatory counterpart *rho*-GDI, and Rap1A [25,72,73]). Upon activation, p47^{phox} and p67^{phox} become phosphorylated, and translocate together with p40^{phox} and p21^{rac} to the membrane to form the active NADPH oxidase complex (Scheme 2). The superoxide thus generated, and its dismutation product, H_2O_2, are probably used to kill the phagocytosed bacteria, although the mechanism of killing is still the matter of debate [25,71]. p21^{rac} returns then to the cytosol, while *phox* proteins seem to remain on the membrane, although



Scheme 2 Proposed model of the NADPH oxidase complex derived from studies on the enzyme acting in mammalian phagocytes, based on the data presented in [25,72,73]

The plant proteins found to cross-react with the antibodies to respective mammalian elements of NADPH oxidase complex are indicated in grey. The gp91^{phox} glycoprotein whose homologous gene has been recently identified in plants [124] is marked in pink.

the events accompanying the termination of NADPH activity are not fully elucidated.

There seems to be a great deal of similarity between plant and animal ROS-generating systems (see below). However, the intrinsic differences between both Kingdoms, stemming mainly from two factors [mobility of the cells and the presence of defined structural exocellular compartment (cell wall) in plants], are also reflected in the modes of defence responses. In mammals, spread of generated ROS is limited to phagocytotic vacuole: the invading bacteria are killed, while the phagocytes mostly remain alive. In plants, the successful limiting of the spread of pathogen is often manifested by HR, death of the cells at the site of pathogen infection, and this cell death is probably also the effect of the ROS generation. H_2O_2 has been shown to be directly toxic to micro-organisms [74], to drive the oxidative cross-linking of cell-wall (glyco)proteins [22,23], and so reduce their susceptibility to enzymic degradation [75], to induce SAR [76], and to orchestrate the hypersensitive cell-death response [27,57].

In long-term experiments, where the O_2 uptake was measured in elicited tomato cells 4 h after elicitation [44], no changes were observed in cells treated with elicitors from compatible pathogens. On the other hand, elicitors originating from the incompatible ones evoked the gradual decrease in the rate of oxygen uptake, starting by 1 h, and reaching the constant rate after 3 h. Similar kinetics was also observed in the *Fusarium oxysporum*-elicitor-treated carnation calli derived from a cultivar resistant to this fungus [77]. Interestingly, this decrease in the O_2 uptake was counteracted by catalase addition, but not by SOD or ascorbate [44]. In short-term experiments with elicitor-treated bean cells, a rapid and transient increase of O_2 uptake was noted, with the rate of uptake almost doubled to a maximum rate 8 min after elicitation, which preceded the peak of the oxidative burst at 12 min post elicitation. However, unlike the mammalian respiratory burst, which is sensitive to cyanide or azide [24], the observed increase of O_2 uptake was cyanide-sensitive, but totally inhibited by azide [16,65]. These observations seem to suggest that there are some fundamental differences between at least some of the components of the systems generating the ROS during the oxidative burst. Additional analyses of the levels of ATP and NADH/NAD^+ ratios indicated that the increased O_2 uptake is almost completely invested in the production of ROS, thus generating symptoms of the oxidative stress within the cells. This conclusion is also stressed by the demonstration of induction of alcohol dehydrogenase, an enzyme whose expression is stimulated by a low partial pressure of O_2 [65]. In this respect, the evidence that mannitol dehydrogenase is one of pathogenesis-related (PR) proteins is of particular interest, for, as mannitol could serve as an antioxidant, changes in its content could affect the extent of internal ROS production in response to pathogens [78] (reviewed in [79]).

Although the effects of elicitation on primary metabolism has been noted, the main switch observed is that to secondary metabolism, specific for plant cells (i.e. the production of phytoalexins, which are low-molecular-mass compounds with antimicrobial activity [80,81]). A connection between the ROS generation and the induction of the phytoalexin production was suggested [26,41], and direct application of H_2O_2 used to test this possibility. In soybean hypocotyls and radicles, H_2O_2 as well as fatty acid hydroperoxides elicited glyceollin accumulation [82,83]. An identical situation was found for suspension-cultured soybean cells, where the induction of phytoalexin production was inhibited by the addition of catalase, but not boiled enzyme [41]. These data seemed to demonstrate a direct causal link between those two phenomena. However, later results from the same laboratory

indicated that two different elements present in a crude elicitor preparation were responsible for parallel, but independent, induction of both responses [42] (see also above). Moreover, later studies on soybean hypocotyls/radicles identified lipid peroxidation, and not ROS generation, as a trigger sufficient for the induction of glyceollin synthesis [83]. Similarly, the experiments on defence reactions of white-clover or tobacco cells to pathogenic bacteria indicated that, although cells produced ROS in response to such challenge, this production was not an essential requirement for the induction of phytoalexin synthesis or HR. Also in this system, abiotic elicitation of plant cells with $HgCl_2$ evoked phytoalexin production, but not the oxidative burst [54]. Data from other laboratories confirm these latter observations. Elicited carrot protoplasts were able to accumulate 4-hydroxybenzoic acid, but failed to produce any detectable amounts of H_2O_2 [17]. In bean cells challenged with various elicitor molecules, the potential of a given elicitor to evoke the oxidative burst was completely unrelated to that for phenylalanine ammonia-lyase (first enzyme of the phenylpropanoid pathway) induction [23]. In tobacco cells treated with pure proteinaceous elicitors, phytoalexin synthesis was affected neither by inhibition of the ROS generation nor by suppression of the ROS accumulation in the medium [50]. Overall, results of recent experiments suggest that both responses are induced in parallel, probably by distinct recognition and signalling systems [84]. However, in leguminous plants, where flavonoids and isoflavonoids act as both phytoalexins [80,81] and antioxidants [85], the relative timing of ROS production and secretion of phenolic compounds towards the site of infection may be of crucial importance to the eventual magnitude of the oxidative burst.

SAR is one of the consequences of plant defence response. Since the infections are usually local events, the existence of systemic signals has been proposed. SA was initially identified as one of them (reviewed in [86]). However, SA was demonstrated later not to be a translocated signal of SAR [87], but its accumulation was indicated as an essential factor required for expression of multiple modes of plant disease resistance [88]. As the oxidative burst precedes the SA accumulation in infected tissues [89], the relationship between these two events has been studied in more detail. Infiltration of tobacco leaves with H_2O_2-activated benzoic acid 2-hydroxylase, an enzyme forming SA from benzoic acid, resulting in subsequent SA accumulation [90]. On the other hand, SA has been directly implicated in the ROS generation by findings demonstrating the cytoplasmic SA-binding protein to be in fact a catalase [76]. Both SA and its synthetic analogue, INA, were found to inhibit catalase and ascorbate peroxidase, two enzymes regulating the level of ROS in cells, but not guaiacol-utilizing peroxidases, participating e.g. in lignin synthesis [91,92]. These findings corroborate the observed effects of tissue pretreatment with SA or INA on the intensity of the oxidative burst [48,53,66] (see above), as both compounds would affect the ROS removal system, and not the ROS generation. However, the results obtained recently by Rüffer et al. questioned the inhibitory activity of SA on catalase, indicating the possibility that the binding of SA to catalase is in fact a specific binding to iron-containing enzymes found in plants, fungi and animals, but not to iron-free plant enzymes [93]. Yet another consequence of the eventual inhibitory activity of SA on catalase in plants would be the elevated level of H_2O_2 in the immediate vicinity of the infection site, and this level has been postulated to act as a second messenger of SA in the signal-transduction pathway leading to SAR and gene activation for PR proteins [86]. Although recent experiments indicated that the elevated levels of H_2O_2 as a consequence of catalase inhibition by SA are not required for the SAR induction [94], and the role of H_2O_2 in the induction of PR protein expression has also been questioned [95], the possibility that H_2O_2 could be one of the stimuli activating these responses has not been excluded [94].

Data obtained in two laboratories on elicitor-treated soybean and/or bean cells [22,23,75] provided a causal link between two known phenomena: the oxidative burst and the insolubilization (immobilization) of cell-wall proteins. Structural proteins become insolubilized upon arrival at the cell wall, and this process is believed to result from an isodityrosine ether linkage formed in a peroxidase-catalysed reaction in the wall [96]. However, although intramolecular isodityrosine cross-links in HRGPs have been demonstrated, the evidence for the intermolecular linkages is still lacking. Results obtained by Bradley et al. [22] showed the rapid disappearance of p35 and p100 (glyco)proteins from SDS extracts of crude cell-wall fractions from elicitor-treated soybean cells. The immunofluorescence labelling of these cells using anti-p35 antibodies indicated that these proteins are immobilized in the walls probably through covalent cross-links thus making them non-extractable with SDS. The loss of protein extractability followed the dynamics of the oxidative burst, starting 2 min after elicitation, and being completed within 20–30 min. That effect could be mimicked by the external application of H_2O_2 to the cells, and any insolubilization of cell-wall proteins was prevented by catalase or ascorbate [22]. The work of Wojtaszek et al. [23] shed more light on this phenomenon. In bean cells, five glycoproteins were demonstrated to be immobilized upon elicitation, with the kinetics of this process following that of the oxidative burst, and being completed within 15 min. Most of these proteins were demonstrated to be of HRGP/proline-rich glycoprotein (PRP) type and O-glycosylated. Probing of Western blots with concanavalin A or wheat-germ agglutinin revealed that N-glycosylated proteins were not immobilized. This immobilization of cell-wall proteins was demonstrated to be H_2O_2-driven and pH-dependent. However, the existence of another cross-linking mechanism that is lipid-peroxide-driven and pH-independent, and acts at the later stages post elicitation, was also indicated [23]. The process of induced oxidative cross-linking of plant cell-wall (glyco)proteins has some characteristics of previously identified, developmentally regulated and also H_2O_2-dependent insolubilization of sea-urchin coat proteins [97] and *Chlamydomonas* HRGP-type glycoproteins [68]. In plants, rapid immobilization of proteins may strengthen cell walls, thus slowing down the ingress of the invading pathogen [75]. Additionally, immobilized HRGP/PRP proteins could serve as an anchor for deposition of phenolic compounds and subsequent formation of papillae [23].

Changes in membrane permeability and the resulting ion fluxes, mainly Ca^{2+} and H^+ influx, and K^+ and Cl^- efflux, are among the most rapid responses of plant cells to elicitation [5,6]. In parsley (*Petroselinum crispum*) cells these fluxes are initiated 2–5 min after elicitation, and a transient extracellular alkalinization is observed [19]. Similar situation was noted in elicited tomato [15] and French-bean [16] cells. In elicitor-treated protoplasted carrot cells, in the absence of an oxidative burst, Ca^{2+} influx and K^+ efflux were still observable [17]. The involvement of Ca^{2+} ion-channel activation in the induction of the oxidative burst and other defence responses has also been indicated [19]. Recently the importance of the transiency of Ca^{2+} fluxes was demonstrated in isolated plasma-membrane-rich fraction of potato tuber tissues [69]. In this system, activation of the NADPH-dependent $^{\bullet}O_2^-$-generating reaction which occurred after treatment with elicitor was strictly dependent upon the presence of Ca^{2+} ions, but the elicitor-enhanced $^{\bullet}O_2^-$-generating activity was Ca^{2+}-independent [69]. Changes in pH of various plant cell compartments resulting from ion fluxes seem to play an

important role in the regulation of plant defence responses, with the ROS production among them. Although little or no changes in vacuolar or cytoplasmic pH have been found in soybean cells in response to elicitation [98], changes in external (apoplastic + extracellular) pH were found to be closely related to the intensity of the oxidative burst in elicitor-treated bean cells. Moreover, in bean cells both the oxidative burst and the immobilization of cell-wall proteins are evoked simply by transferring the cells into the medium buffered at higher pH [16,23], while changing the external pH by use of monensin, nigericin or valinomycin eliminates the transient pH change and abolishes the generation of H_2O_2 [16]. Interestingly, the binding of specific fungal oligopeptide elicitor to parsley membranes was found to be pH-dependent, with little binding at acidic and neutral pH values, and a huge increase in binding at pH above 7.0 [18]. In mammalian phagocytotic cells, pH changes have also been proposed to modulate the oxidative burst [99].

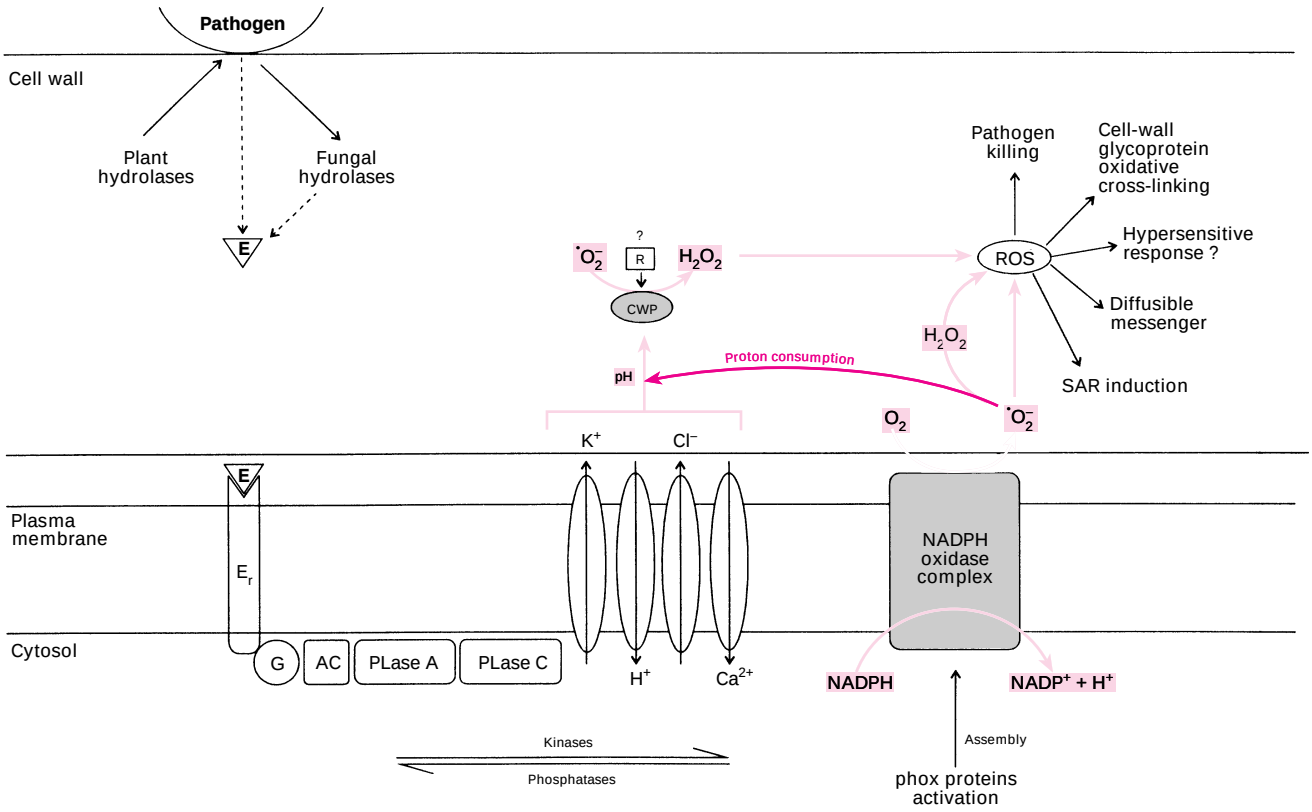
Recently, a further role for H_2O_2 was proposed as the key component in the orchestration of the hypersensitive cell death [27,57]. In suspension-cultured soybean cells treated with isogenic lines of *Pseudomonas syringae* pv. *glycinea*, cell death only occurred upon inoculation with an avirulent strain, but not with a virulent one. This could be inhibited by diphenylene iodonium (DPI) addition (a suicide substrate inhibitor of mammalian NADPH oxidase), stimulated by 3-amino-1,2,4-triazole addition (an inhibitor of catalase), and it could be mimicked by the extracellular addition of H_2O_2. Moreover, the factor triggering cell death was found to be diffusible, and destroyed by catalase. This diffusible signal, presumably H_2O_2, induced also the expression of genes encoding cellular protectants like glutathione S-transferase or glutathione peroxidase. The gene activation was achieved at 2 mM H_2O_2, while cell death appeared to represent a threshold response with abrupt transition between 4 and 6 mM H_2O_2 [57]. However, estimated release of H_2O_2 by 1 ml of elicited soybean cell culture is approx. 0.12 μmol [60], well below the observed threshold value. Moreover, the results of Levine et al. [57] demonstrated characteristic biphasic production of ROS, and the difference between virulent and avirulent strain of bacteria used for inoculation was noted only in the lack of Phase II for a virulent strain, in accordance with data from other systems (reviewed in [13]; for discussion see above). This means that the ROS were generated during Phase I in both systems, yet only the avirulent strain caused the measurable death of soybean cells. Although these results might be interpreted as an indication of the accumulative oxidative stress leading to plant cell death in incompatible interactions, results of recent experiments with *P. syringae* pv. *syringae* mutants contradict such conclusion. These *hrmA* mutants, despite eliciting the normal biphasic ROS production in tobacco cells, caused neither HR on tobacco leaves nor the hypersensitive cell death of suspension-cultured cells [56]. Taking together these data do not support the role for H_2O_2 as a molecule orchestrating the hypersensitive cell death. However, it seems possible and justified to consider this ROS as an diffusible messenger involved in the activation of gene expression for cellular protectants in cells adjacent to the site of infection, possibly via the induction of nuclear transcription factors analogous to the NF-κB or AP-1 factors found in animal cells [100,101]. The identification of plant mutants compromised for the control of the disease lesion formation [102–105] (reviewed in [9]) opens new possibilities in determining the role of ROS in the development of HR. Interestingly, the role for $^{\bullet}O_2^-$, and not H_2O_2, in initiation of cell death and lesion formation was demonstrated recently in one of these mutants (*lsd1*) in *Arabidopsis* [106]. As strong similarities have been drawn between the HR in plants and programmed cell death (apoptosis) of animal cells (reviewed in [7,9]), it is worth mentioning that some recent data from animal systems indicated that ROS may not be required for the programmed cell death (reviewed in [107]).

SOURCES OF ROS IN THE OXIDATIVE BURST

The most challenging issue in studies on the oxidative burst is the question of the origin of ROS building this phenomenon. Several possibilities have been presented, but only the two that are currently receiving the most attention will be discussed here, namely the action of NADPH oxidase system analogous to that of animal phagocytes and, secondly, the pH-dependent generation of H_2O_2 by cell-wall peroxidase. A third system, germin/oxalate oxidase, although not related to the oxidative burst, but producing H_2O_2 in response to pathogens, will be also briefly presented. Lipoxygenase was also proposed as a possible source of ROS in the oxidative burst, but recent data indicated that, in most systems studied, ROS production preceded lipoxygenase activity [13,108].

To elaborate a working model of the ROS generation in the oxidative burst, several recent papers [5,8,9,12,14,18,109,110] have drawn strong analogies with the NADPH oxidase system of mammalian cells [24,25]. According to this model (Scheme 3), an elicitor molecule is recognized by an appropriate receptor located on plasma membrane. Several putative receptors have been identified and at least partially characterized so far. Further interactions based on the available data implicate the involvement of GTP-binding proteins [60,111–113], ion channels (especially Ca^{2+}) [18,46,55,69,114–116], protein kinases and protein phosphatases [20,46,48,55,57,58,62,113,115–119], phospholipases A and C [120,121] and possibly cyclic AMP [122] along the signalling pathway leading to the activation of NADPH oxidase, generation of $^{\bullet}O_2^-$, and its dismutation to H_2O_2. In parsley cells the oxidative burst was strictly dependent upon the presence of Ca^{2+} in the culture medium, was inhibited by the ion-channel inhibitors, and initiated by compounds stimulating ion fluxes [19]. Protein kinase inhibitors (K-252a and staurosporine) were found to inhibit the generation of ROS, while protein phosphatase inhibitors (calyculin A, okadaic acid, cantharidin) acted as stimulators of the oxidative burst in soybean cells [57,117]. The NADPH oxidase complex has not been purified so far from plant cells, but its presence has been assumed from various pharmacological, immunological and reconstitution experiments in model plant systems [39,51,58,59,69,70]. Likewise, although phosphorylation–dephosphorylation has been suggested as a mechanism activating this enzyme, no direct evidence for that event have been provided. However, DPI and α-naphthol, inhibitors of the phagocytous enzyme, are also inhibitors of the oxidative burst in soybean cells [57,123]. Recent demonstrations of the cross-reactivity of antibodies against components of the mammalian enzyme with the proteins of the same molecular mass from several plant cell lines (anti-$p22^{phox}$ antibodies, [27]; anti-$p47^{phox}$ and anti-$p67^{phox}$ antibodies, [58,123]) indicated the presence of the NADPH oxidase proteins in plants. The strongest argument for the existence of the complex comes from the identification of a rice gene, *rboh A*, similar to the gene coding for the mammalian $gp91^{phox}$ protein [124]. Additionally, the presence of at least two other elicitor-induced enzymes capable of the ROS generation has been indicated, namely NADH oxidase [70,113] and NADH-dependent cytochrome *c* reductase [113].

An alternative model of the generation of H_2O_2 by a pH-dependent cell-wall peroxidase has been proposed recently [16] (see Scheme 3). Opposite to the previous one, which stresses the importance of the signalling events leading to the activation of



Scheme 3 Schematic representation of major hypotheses describing the possible origin of ROS building the oxidative burst

Upon arrival of the pathogen, both plant and fungal hydrolases release cell-wall fragments (elicitors). These molecules when bound to respective receptors located at the plasma membrane initiate the cascade(s) of signalling events leading to the activation of either NADPH oxidase and/or cell-wall-bound peroxidases (marked in grey) generating ROS (pink) used further in various aspects of the plant defence response. The key element of the 'unifying' hypothesis proposed in this Review is shown in red. All the major elements of the signalling pathway are presented although the differences in the active elements of the pathway with respect to plant species and/or elicitor applied have been found. Abbreviations used: AC, adenylate cyclase; CWP, cell-wall-bound peroxidase; E, elicitor; E_r, receptor; G, GTP-binding protein(s); PLase A and PLase C, phopholipases A and C; R, reductant.

NADPH oxidase, this one, although not excluding a signalling pathway, emphasizes the importance of pre-existing components, especially those present in the plant ECM. According to this model, an elicitor arriving at the cell surface is recognized by the appropriate receptor molecule, and this event leads to the activation of ion channels. The movement of the ions (Ca^{2+}, K^+, H^+, Cl^-) results in a transient alkalinization of ECM, which leads to an activation of pH-dependent cell-wall peroxidase. A change in the enzyme's active site occurs, the peroxidase compound III (Fe^{II}–O–O), and subsequently $^{\bullet}O_2^-$ is formed, which can be initially reduced by oxidation of cysteine residues adjacent to the haem site. With the supply of reductant, the generation of H_2O_2 can be sustained for a longer period of time [16]. The generation of H_2O_2 by peroxidases (Scheme 1b, eqn. 4) has been previously demonstrated and characterized during lignification [31,32,125,126]. Control by pH of cell-wall peroxidase activity involved in lignification was also previously demonstrated, both at the level of the coniferyl alcohol oxidation and H_2O_2 production, with increased activities at more alkaline pH [127]. The model presented was based on the data obtained from French bean cells, and a cationic cell-wall-bound peroxidase from this source has been identified [128] and its pH-dependence in H_2O_2 generation demonstrated [16]. Moreover, transferring the cells to a buffered higher-pH medium is sufficient to induce the H_2O_2 generation. Although the reductant has not been identified yet, preliminary data indicate it is not NADPH, NADH, ascorbate, glutathione or cysteine. Moreover, time-course experiments showed the reductant to be present at high levels in apoplastic fluids of French-bean cells just before the start of the oxidative burst, suggesting its elicitor-induced secretion into ECM, its synthesis in the ECM or its release from wall-bound stores [129]. The validity of this model has been recently confirmed in *in vitro* experiments on oxidative cross-linking of cell-wall glycoproteins using cell-wall peroxidase [128], three HRGP/PRP-type wall proteins, and apoplastic fluid isolated from elicited French-bean cells, and carrying the cross-linking reaction at pH 7.5 (P. Wojtaszek, J. Trethowan and G. P. Bolwell, unpublished work).

Each model presents a slightly different point of view on the source of ROS in the oxidative burst and, depending on the plant species studied and type of elicitor used, the results obtained seem to conform to one of these models. This is not surprising, taking into account the multiplicity of recognition and signalling events leading to the activation of either one or both of the ROS generating systems. However, there are results which suggest that it should be possible to propose a unified model utilizing the main features of both hypotheses presented (see Scheme 3). Upon recognition of elicitor by an appropriate receptor molecule, the signalling pathway is induced, leading to the activation of NADPH oxidase. Along this pathway, the ion channels are also activated and, due to their action and subsequent ion movement, the ECM becomes transiently alkalic. According to the model of

NADPH oxidase action proposed for the mammalian enzyme [24,25], the $^{\bullet}O_2^-$ generation requires transfer of electrons, unaccompanied by protons, across the plasma membrane. The $^{\bullet}O_2^-$ thus produced becomes protonated, consuming protons in phagocytous vacuole, and inducing vacuolar proteinases active at higher pH. In plants this process would eventually further participate in the alkalinization of the ECM, driving pH up to 7.0–8.0. Hence the activity of *both* components, ion channels and NADPH oxidase, would result in a transient pH increase in ECM. The resultant alkalinization activates pH-dependent cell-wall peroxidase and the generation of H_2O_2 in the presence of an unknown reductant by the mechanism presented above. Additionally, although it is a highly speculative proposition, the other model of mammalian NADPH oxidase activity [71,130] might probably explain the transience of the observed ECM alkalinization and eventual termination of the oxidative burst. According to this view, transfer of electrons and subsequent $^{\bullet}O_2^-$ generation are compensated for by the outward H^+ translocation via an H^+ channel, the opening of which lags behind the activation of oxidase [130]. The activity of this channel may thus provide firstly H^+ for $^{\bullet}O_2^-$ protonation and, when proceeding further, one means to acidify ECM and terminate the oxidative burst as either NADPH oxidase or cell-wall peroxidase are active at higher pH. In this respect it should be noted that the existence of elicitor-induced plasma-membrane H^+-ATPase has been already indicated in plants [119,131]. Moreover, tobacco plants expressing a bacterial proton pump show symptoms of HR in the absence of a pathogen [132]. The presented model fits well with the data describing a possible heterogeneity of the elicitor-induced oxidative pathways, noted, for example, in soybean cells [41]. It might also explain the results where H_2O_2 is the major ROS observed upon elicitation, while NADPH oxidase, generating primarily $^{\bullet}O_2^-$, is identified as a source of ROS. It is noteworthy that, in cells cultured in aqueous suspensions, water is easily available, both as a medium for ROS diffusion and as a source of protons, even in cell walls, thus making dismutation of the generated $^{\bullet}O_2^-$ sufficiently easy. On the other hand, plant cell walls are relatively hydrophobic [133–135], and only a minor proportion of the protons in the walls are mobile [136], making an environment where the protonation of $^{\bullet}O_2^-$ might be quite difficult. Under these conditions the operation of the peroxidative pathway might be prevalent. There are still, however, a few more points of differentiation making comparisons between various experimental systems rather complex. Of the most importance is probably the tissue origin of the callus, and subsequently cell-culture lines, where at least three factors might affect the observable output of elicitation, when the oxidative burst is considered. Firstly, as the distribution of peroxidases in cell walls is tissue-specific [37,137], the abundance of the enzyme(s) in different cell lines might vary. Secondly, the availability of the suitable reductants in cell walls might be different. Thirdly, although this applies mainly to the situation found *in planta*, a distinct apoplastic pH might be observed in various plant tissues or even in cell walls of different cells within a given tissue [138].

The significance of germin/oxalate oxidase system seems to be limited to interactions with pathogens involving cereals, although some preliminary evidence suggests this protein to be present also in dicotyledoneous plants (P. Wojtaszek, M. Piślewska, G. P. Bolwell, M. Stobiecki and C. Gerrish, unpublished work). The oxalate oxidase, which releases H_2O_2 and CO_2 from oxalic acid, is also known as germin (extracellular glycoprotein marker of early plant development) [139,140]. Although the oxalate oxidase system is not fully characterized, especially with respect to the relative location of the protein and its substrate, several observations have been presented demonstrating probably the first example of the activation of a H_2O_2-generating enzyme in response to pathogenic infection. In barley plants, oxalate oxidase is expressed constitutively at low basal levels, but some expression is developmentally regulated. Its activity, however, greatly increases in response to inoculation with the pathogenic fungus *Erysiphe graminis* f.sp. *hordei*, but not in response to wounding, with a timing of a response paralleling that of chitinase [141] or even preceding accumulation of PR-1 protein [142]. Additionally, there are indications that this pathogen-response activity differs from that observed constitutively in barley roots [142]. In wheat plants both the level of germin mRNA and the oxalate oxidase activity were found to be induced by infection with *Erysiphe graminis* f.sp. *tritici*. Germin gene expression was observed in both resistant and susceptible wheat cultivars, and it has been suggested that oxalate oxidase activity is a marker of general defence response rather than cultivar resistance [143].

CONCLUSIONS

The role of ROS not only as toxic by-products, but also as an important component of the plant defence response to pathogenic infection, is now well established. However, the particular mechanisms governing the observable output of this reaction, such as the nature of the elicitor and receptor molecules participating in the recognition events, the elements and the arrangement of the signalling pathway and the source of ROS in the oxidative burst, are only partially elucidated, and much more work is needed in order to present a more detailed picture of the oxidative burst and related events, especially hypersensitive cell death. Apart from classical biochemical, biophysical and molecular data, two recently developed approaches seem to be the most promising, namely studies of mutants compromised for the control of HR [102–105] (reviewed in [9]), and research on transgenic plants with a changed level of the generated ROS, e.g. by introduction of a gene encoding H_2O_2-generating glucose oxidase [144], or transgenic plants showing symptoms of HR in the absence of a pathogen [132].

I am indebted to many colleagues for providing reprints, and particularly to Dr. J. Dangl and Dr. K. Hammond-Kosack for providing preprints of their work. I am especially grateful to the anonymous reviewers of Biochemical Journal for their critical and helpful comments. I thank Dr. G. P. Bolwell for many stimulating discussions. I thank Dr. Zbigniew Michalski for the preparation of Figures and Schemes. This work was made possible by the State Committee for Scientific Research grant 401/P04/95/08.

REFERENCES

1 Wyatt, S. E. and Carpita, N. C. (1993) Trends Cell Biol. **3**, 413–417
2 Dixon, R. A., Harrison, M. J. and Lamb, C. J. (1994) Annu. Rev. Phytopathol. **32**, 479–501
3 Benhamou, N. (1996) Trends Plant Sci. **1**, 233–240
4 van de Rhee, M. D., Linthorst, H. J. M. and Bol, J. F. (1994) in Stress-Induced Gene Expression in Plants (Basra, A. S., ed.), pp. 249–284, Harwood Academic Publishers, Lausanne, New York, Philadelphia and Reading
5 Kombrink, E. and Somssich, I. E. (1995) Adv. Bot. Res. **21**, 1–34
6 Atkinson, M. M. (1993) Adv. Plant Pathol. **10**, 35–64
7 Jones, A. M. and Dangl, J. L. (1996) Trends Plant Sci. **1**, 114–119
8 Hammond-Kosack, K. E. and Jones, J. D. G. (1996) Plant Cell **8**, 1773–1791
9 Dangl, J. L., Dietrich, R. A. and Richberg, M. H. (1996) Plant Cell **8**, 1793–1807
10 Ebel, J. and Cosio, E. G. (1994) Int. Rev. Cytol. **148**, 1–36
11 Sutherland, M. W. (1991) Physiol. Mol. Plant Pathol. **39**, 79–93
12 Mehdy, M. C. (1994) Plant Physiol. **105**, 467–472
13 Baker, C. J. and Orlandi, E. W. (1995) Annu. Rev. Phytopathol. **33**, 299–321
14 Low, P. S. and Merida, J. R. (1996) Physiol. Plant. **96**, 533–542
15 Felix, G., Regenass, M. and Boller, T. (1993) Plant J. **4**, 307–316
16 Bolwell, G. P., Butt, V. S., Davies, D. R. and Zimmerlin, A. (1995) Free Radical Res. **23**, 517–532

17 Bach, M., Schnitzler, J.-P. and Seitz, H. U. (1993) Plant Physiol. **103**, 407–412
18 Nürnberger, T., Nennstiel, D., Jabs, T., Sacks, W. R., Hahlbrock, K. and Scheel, D. (1994) Cell **78**, 449–460
19 Hahlbrock, K., Scheel, D., Logemann, E., Nürnberger, T., Parniske, M., Reinold, S., Sacks, W. R. and Schmelzer, E. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 4150–4157
20 Dietrich, A., Mayer, J. E. and Hahlbrock, K. (1990) J. Biol. Chem. **265**, 6360–6368
21 Felix, G., Grosskopf, D. G., Regenass, M. and Boller, T. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 8831–8834
22 Bradley, D. J., Kjellbom, P. and Lamb, C. J. (1992) Cell **70**, 21–30
23 Wojtaszek, P., Trethowan, J. and Bolwell, G. P. (1995) Plant Mol. Biol. **28**, 1075–1087
24 Segal, A. W. and Abo, A. (1993) Trends Biochem. Sci. **18**, 43–47
25 Wientjes, F. B. and Segal, A. W. (1995) Semin. Cell Biol. **6**, 357–365
26 Doke, N. (1983) Physiol. Plant Pathol. **23**, 345–357
27 Tenhaken, R., Levine, A., Brisson, L. F., Dixon, R. A. and Lamb, C. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 4158–4163
28 Scandalios, J. G. (1993) Plant Physiol. **101**, 7–12
29 Foyer, C. H., Descourviéres, P. and Kunert, K. J. (1994) Plant Cell Environ. **17**, 507–523
30 Streller, S. and Wingsle, G. (1994) Planta **192**, 195–201
31 Gross, G. G., Janse, C. and Elstner, E. F. (1977) Planta **136**, 271–276
32 Halliwell, B. (1978) Planta **140**, 81–88
33 Bartosz, G. (1995) Druga Twarz Tlenu, Wydawnictwo Naukowe PWN, Warszawa
34 Gille, G. and Sigler, K. (1995) Folia Microbiol. **40**, 131–152
35 Kaim, W. and Rall, J. (1996) Angew. Chemie Int. Ed. Engl. **35**, 43–60
36 Kuchitsu, K., Kosaka, H., Shiga, T. and Shibuya, N. (1995) Protoplasma **188**, 138–142
37 Olson, P. D. and Varner, J. E. (1993) Plant J. **4**, 887–892
38 Schopfer, P. (1994) Plant Physiol. **104**, 1269–1275
39 Doke, N. (1983) Physiol. Plant Pathol. **23**, 359–367
40 Lindner, W. A., Hoffman, C. and Grisebach, H. (1988) Phytochemistry **27**, 2501–2503
41 Apostol, I., Heinstein, P. F. and Low, P. S. (1989) Plant Physiol. **90**, 109–116
42 Davis, D., Merida, J., Legendre, L., Low, P. S. and Heinstein, P. F. (1993) Phytochemistry **32**, 607–611
43 Anderson, A. J., Rogers, K., Tepper, C. S., Blee, K. and Cardon, J. (1991) Physiol. Mol. Plant Pathol. **38**, 1–13
44 Vera-Estrella, R., Blumwald, E. and Higgins, V. J. (1992) Plant Physiol. **99**, 1208–1215
45 May, M. J., Hammond-Kosack, K. E. and Jones, J. D. G. (1996) Plant Physiol. **110**, 1367–1379
46 Schwacke, R. and Hager, A. (1992) Planta **187**, 136–141
47 Messner, B. and Boll, M. (1994) Plant Cell Tissue Organ Cult. **39**, 69–78
48 Kauss, H. and Jeblick, W. (1995) Plant Physiol. **108**, 1171–1178
49 Bottin, A., Véronési, C., Pontier, D., Esquerré-Tugayé, M.-T., Blein, J.-P., Rustérucci, C. and Ricci, P. (1994) Plant Physiol. Biochem. **32**, 373–378
50 Rustérucci, C., Stallaert, V., Milat, M.-L., Pugin, A., Ricci, P. and Blein, J.-P. (1996) Plant Physiol. **111**, 885–891
51 Auh, C.-K. and Murphy, T. M. (1995) Plant Physiol. **107**, 1241–1247
52 Park, H.-H., Hakamatsuka, T., Sankawa, U. and Ebizuka, Y. (1995) Z. Naturforsch. **50c**, 824–832
53 Fauth, M., Merten, A., Hahn, M. G., Jeblick, W. and Kauss, H. (1996) Plant Physiol. **110**, 347–354
54 Devlin, W. S. and Gustine, D. L. (1992) Plant Physiol. **100**, 1189–1195
55 Baker, C. J., Orlandi, E. W. and Mock, N. M. (1993) Plant Physiol. **102**, 1341–1344
56 Glazener, J. A., Orlandi, E. W. and Baker, C. J. (1996) Plant Physiol. **110**, 759–763
57 Levine, A., Tenhaken, R., Dixon, R. and Lamb, C. (1994) Cell **79**, 583–593
58 Desikan, R., Hancock, J. T., Coffey, M. J. and Neill, S. J. (1996) FEBS Lett. **382**, 213–217
59 Doke, N. and Ohashi, Y. (1988) Physiol. Mol. Plant Pathol. **32**, 163–175
60 Legendre, L., Reuter, S., Heinstein, P. F. and Low, P. S. (1993) Plant Physiol. **102**, 233–240
61 Svalheim, O. and Robertsen, B. (1993) Physiol. Plant. **88**, 675–681
62 Mathieu, Y., Sanchez, F. J., Droillard, M.-J., Lapous, D., Laurière, C. and Guern, J. (1996) Plant Physiol. Biochem. **34**, 399–408
63 Collén, J. and Pedersén, M. (1994) Physiol. Plant. **92**, 417–422
64 Yahraus, T., Chandra, S., Legendre, L. and Low, P. S. (1995) Plant Physiol. **109**, 1259–1266
65 Robertson, D., Davies, D. R., Gerrish, C., Jupe, S. C. and Bolwell, G. P. (1995) Plant Mol. Biol. **27**, 59–67
66 Kauss, H. and Jeblick, W. (1996) Plant Physiol. **111**, 755–763
67 Graham, M. Y. and Graham, T. L. (1994) Plant Physiol. **105**, 571–578
68 Waffenschmidt, S., Woessner, J. P., Beer, K. and Goodenough, U. W. (1993) Plant Cell **5**, 809–820
69 Doke, N. and Miura, Y. (1995) Physiol. Mol. Plant Pathol. **46**, 17–28
70 Murphy, T. M. and Auh, C.-K. (1996) Plant Physiol. **110**, 621–629
71 Jones, O. T. G. (1994) BioEssays **16**, 919–923
72 Leto, T. L., Adams, A. G. and De Mendez, I. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 10650–10654
73 De Leo, F. R., Ulman, K. V., Davis, A. R., Jutila, K. L. and Quinn, M. T. (1996) J. Biol. Chem. **271**, 17013–17020
74 Peng, M. and Kuc, J. (1992) Phytopathology **82**, 696–699
75 Brisson, L. F., Tenhaken, R. and Lamb, C. (1994) Plant Cell **6**, 1703–1712
76 Chen, Z., Silva, H. and Klessig, D. F. (1993) Science **262**, 1883–1886
77 Trillas, M. I. and Azcón-Bieto, J. (1995) Plant Physiol. Biochem. **33**, 47–53
78 Williamson, J. D., Stoop, J. M. H., Massel, M. O., Conkling, M. A. and Pharr, D. M. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 7148–7152
79 Stoop, J. M. H., Williamson, J. D. and Pharr, D. M. (1996) Trends Plant Sci. **1**, 139–144
80 Dixon, R. A. and Paiva, N. L. (1995) Plant Cell **7**, 1085–1097
81 Smith, C. J. (1996) New Phytol. **132**, 1–45
82 Montillet, J.-L. and Degousée, N. (1991) Plant Physiol. Biochem. **29**, 689–694
83 Degousée, N., Triantaphylidès and Montillet, J.-L. (1994) Plant Physiol. **104**, 945–952
84 Jakobek, J. L. and Lindgren, P. B. (1993) Plant Cell **5**, 49–56
85 Rice-Evans, C. A., Miller, N. J., Bolwell, G. P., Bramley, P. M. and Pridham, J. B. (1995) Free Radical Res. **22**, 375–383
86 Klessig, D. F. and Malamy, J. (1994) Plant Mol. Biol. **26**, 1439–1458
87 Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., Uknes, S., Kessmann, H. and Ryals, J. (1994) Plant Cell **6**, 959–965
88 Delaney, T. P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., Ward, E. and Ryals, J. (1994) Science **266**, 1247–1250
89 Hammond-Kosack, K. E., Silverman, P., Raskin, I. and Jones, J. D. G. (1996) Plant Physiol. **110**, 1381–1394
90 León, J., Lawton, M. A. and Raskin, I. (1995) Plant Physiol. **108**, 1673–1678
91 Conrath, U., Chen, Z., Ricigliano, J. R. and Klessig, D. F. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 7143–7147
92 Durner, J. and Klessig, D. F. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 11312–11316
93 Rüffer, M., Steipe, B. and Zenk, M. H. (1995) FEBS Lett. **377**, 175–180
94 Neuenschwander, U., Vernooij, B., Friedrich, L., Uknes, S., Kessmann, H. and Ryals, J. (1995) Plant J. **8**, 227–233
95 Bi, Y.-M., Kenton, P., Mur, L., Darby, R. and Draper, J. (1995) Plant J. **8**, 235–245
96 Iiyama, K., Lam, T. B.-T. and Stone, B. A. (1994) Plant Physiol. **104**, 315–320
97 Shapiro, B. M. (1991) Science **252**, 533–536
98 Horn, M. A., Meadows, R. P., Apostol, I., Jones, C. R., Gorenstein, D. G., Heinstein, P. F. and Low, P. S. (1992) Plant Physiol. **98**, 680–686
99 Segal, A. W., Geisow, M., Garcia, R., Harper, A. and Miller, R. (1981) Nature (London) **290**, 406–409
100 Meyer, M., Schreck, R. and Baeuerle, P. A. (1993) EMBO J. **12**, 2005–2015
101 Anderson, M. T., Staal, F. J. T., Gitler, C., Herzenberg, L. A. and Herzenberg, L. A. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 11527–11531
102 Greenberg, J. T. and Ausubel, F. M. (1993) Plant J. **4**, 327–341
103 Greenberg, J. T., Guo, A., Klessig, D. F. and Ausubel, F. M. (1994) Cell **77**, 551–563
104 Dietrich, R. A., Delaney, T. P., Uknes, S. J., Ward, E. R., Ryals, J. A. and Dangl, J. L. (1994) Cell **77**, 565–577
105 Johal, G., Hulbert, S. H. and Briggs, S. P. (1995) BioEssays **17**, 685–692
106 Jabs, T., Dietrich, R. A. and Dangl, J. L. (1996) Science **273**, 1853–1856
107 Jacobson, M. D. (1996) Trends Biochem. Sci. **21**, 83–86
108 Rosahl, S. (1996) Z. Naturforsch. **51c**, 123–138
109 Jones, J. D. G. (1994) Curr. Biol. **4**, 749–751
110 Lamb, C. J. (1994) Cell **76**, 419–422
111 Bolwell, G. P., Coulson, V., Rodgers, M. W., Murphy, D. L. and Jones, D. (1991) Phytochemistry **30**, 397–405
112 Legendre, L., Heinstein, P. F. and Low, P. S. (1992) J. Biol. Chem. **267**, 20140–20147
113 Vera-Estrella, R., Higgins, V. J. and Blumwald, E. (1994) Plant Physiol. **106**, 97–102
114 Tavernier, E., Wendehenne, D., Blein, J.-P. and Pugin, A. (1995) Plant Physiol. **109**, 1025–1031
115 Miura, Y., Yoshioka, H. and Doke, N. (1995) Plant Sci. **105**, 45–52
116 Salzer, P., Hebe, G., Reith, A., Zitterell-Haid, B., Stransky, H., Gaschler, K. and Hager, A. (1996) Planta **198**, 118–126
117 Chandra, S. and Low, P. S. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 4120–4123
118 Viard, M.-P., Martin, F., Pugin, A., Ricci, P. and Blein, J.-P. (1994) Plant Physiol. **104**, 1245–1249

119 Vera-Estrella, R., Barkla, B. J., Higgins, V. J. and Blumwald, E. (1994) Plant Physiol. **104**, 209–215
120 Legendre, L., Yueh, Y. G., Crain, R., Haddock, N., Heinstein, P.F and Low, P. S. (1993) J. Biol. Chem. **268**, 24559–24563
121 Chandra, S., Heinstein, P. F. and Low, P. S. (1996) Plant Physiol. **110**, 979–986
122 Bolwell, G. P. (1995) Trends Biochem Sci. **20**, 492–495
123 Dwyer, S. C., Legendre, L., Low, P. S. and Leto, T. L. (1996) Biochim. Biophys. Acta **1289**, 231–237
124 Groom, Q. J., Torres, M. A., Fordham-Skelton, A. P., Hammond-Kosack, K. E., Robinson, N. J. and Jones, J. D. G. (1996) Plant J. **10**, 515–522
125 Elstner, E. F. and Heupel, A. (1976) Planta **130**, 175–180
126 Mäder, M. and Amberg-Fisher, V. (1982) Plant Physiol. **70**, 1128–1131
127 Pedreño, M. A., Ros Barceló, A., Sabater, F. and Muñoz, R. (1989) Plant Cell Physiol. **30**, 237–241
128 Zimmerlin, A., Wojtaszek, P. and Bolwell, G. P. (1994) Biochem. J. **299**, 747–753
129 Bolwell, G. P. (1996) Biochem. Soc. Trans. **24**, 438–442
130 Henderson, L. M., Chappell, J. B. and Jones, O. T. G. (1987) Biochem. J. **246**, 325–329
131 Xing, T., Higgins, V. J. and Blumwald, E. (1996) Plant Cell **8**, 555–564
132 Mittler, R., Shulaev, V. and Lam, E. (1995) Plant Cell **7**, 29–42
133 Grignon, C. and Sentenac, H. (1991) Annu. Rev. Plant Physiol. Plant Mol. Biol. **42**, 103–128
134 Bolwell, G. P. (1993) Int. Rev. Cytol. **146**, 261–324
135 Canny, M. J. (1995) Annu. Rev. Plant Physiol. Plant Mol. Biol. **46**, 215–236
136 MacKay, A. L., Bloom, M., Tepfer, M. and Taylor, I. E. P. (1982) Biopolymers **21**, 1521–1534
137 Smith, C. G., Rodgers, M. W., Zimmerlin, A., Ferdinando, D. and Bolwell, G. P. (1994) Planta **192**, 155–164
138 Hoffmann, B. and Kosegarten, H. (1995) Physiol. Plant. **95**, 327–335
139 Lane, B. G. (1994) FASEB J. **8**, 294–301
140 Lane, B. G., Dunwell, J. M., Ray, J. A., Schmitt, M. R. and Cuming, A. C. (1993) J. Biol. Chem. **268**, 12239–12242
141 Dumas, B., Freyssinet, G. and Pallett, K. E. (1995) Plant Physiol. **107**, 1091–1096
142 Zhang, Z., Collinge, D. B. and Thordal-Christensen, H. (1995) Plant J. **8**, 139–145
143 Hurkman, W. J. and Tanaka, C. K. (1996) Plant Physiol. **111**, 735–739
144 Wu, G., Shortt, B. J., Lawrence, E. B., Levine, E. B., Fitzsimmons, K. C. and Shah, D. M. (1995) Plant Cell **7**, 1357–1368