Role of Reactive Oxygen Intermediates and Cognate Redox Signaling in Disease Resistance[1]

John J. Grant and Gary J. Loake*

Institute of Cell and Molecular Biology, University of Edinburgh, Daniel Rutherford Building, The King's Buildings, Edinburgh EH9 3JH, Scotland, United Kingdom

Plants have evolved a plethora of sophisticated defense mechanisms to resist their potential colonization by microbial pathogens and parasites. Preformed physical and chemical barriers constitute the first line of defense. Superimposed upon these is a battery of inducible responses, their engagement being dependent upon successful recognition of the invading pathogen (Hammond-Kosack and Jones, 1996). This exquisitely specific recognition event is thought to be mediated via the direct or indirect interaction between the product of a microbial avirulence (*avr*) gene and the corresponding plant disease resistance (*R*) gene product. This often results in the programmed execution of challenged host cells, producing a visible area of cell death, the hypersensitive response (HR), surrounding the site of attempted pathogen ingress (Dangl et al., 1996). Following HR formation is the establishment of immunity in systemic tissues to secondary infections termed systemic acquired resistance (SAR), which conveys protection against a broad spectrum of microbial pathogens.

One of the most rapid defense responses engaged following pathogen recognition is the so-called oxidative burst, which constitutes the production of reactive oxygen intermediates (ROIs), primarily superoxide (O_2^-) and H_2O_2, at the site of attempted invasion (Apostol et al., 1989). In addition, nitric oxide (NO), a key signal molecule in animal cells, has also been shown to accumulate during HR formation. The emerging evidence suggests the oxidative burst and cognate redox signaling may play a central role in the integration of a diverse array of plant defense responses. Here we will review the recent advances in the generation, regulation, and function of the oxidative burst and the possible mechanisms underlying the transduction of cognate redox signals.

CHEMISTRY OF ROIs

The oxidative burst was first reported by Doke (1983), who demonstrated that potato tuber tissue generated O_2^- following inoculation with an avirulent race of *Phytophthora infestans*. A virulent race of the same pathogen failed to engage O_2^- production. Subsequently, O_2^- generation has been identified in a diverse array of plant-pathogen interactions involving avirulent bacteria, fungi, and viruses. The production of ROIs is a double-edged sword because their concentration must be carefully regulated to avoid unwanted cellular cytotoxicity (Fig. 1; Halliwell and Gutteridge, 1990). The half-life of O_2^- is less than a second and is usually rapidly dismutated either nonenzymatically or via O_2^- dismutase (SOD) to H_2O_2, which is relatively stable. Protonation of O_2^- can produce the hydroperoxyl radical HO_2^-, which can convert fatty acids to toxic lipid peroxides, destroying biological membranes. Moreover, in the presence of divalent metal ions such as Fe^{2+}, H_2O_2 can undergo the Fenton reaction, producing the hydroxyl radical ($OH^{\bullet}$), the most reactive species known to chemistry. This ROI can initiate self-perpetuating lipid peroxidation and damage nucleic acids and proteins.

GENERATION OF ROIs

Following pathogen recognition, plants produce a biphasic oxidative burst (Fig. 2); the identity of the molecular machinery responsible, however, remains to be rigorously established. Recent evidence has implicated a number of possible mechanisms including a plasma membrane located NADPH-dependent oxidase (Groom et al., 1996; Keller et al., 1998), a cell wall peroxidase (Bolwell and Wojitaszek, 1997; McLusky et al., 1999), and apoplastic amine, diamine, and polyamine oxidase-type enzymes (Allan and Fluhr, 1997). Intracellular sources of ROI generation also exist and include the mitochondria, chloroplasts, and peroxisomes. Of these proposed mechanisms the NADPH-dependent oxidase system, similar to that present in mammalian neutrophils, has received the most attention. This complex is composed of an unusual b-type cytochrome with two subunits, $p22^{phox}$ and $gp91^{phox}$ (Segal and Abo, 1993). During neutrophil activation two cytosolic proteins, $p67^{phox}$ and $p47^{phox}$, the latter following phosphorylation, also associate to complete formation of the holoenzyme. Patients with defects in the $p22^{phox}$ subunit possess a

[1] This work was supported by the Biotechnology and Biological Science Research Council (BBSRC) and the Leverhulme Trust. J.J.G. is the recipient of a BBSRC postgraduate studentship.

* Corresponding author; e-mail gloake@srv0.bio.ed.ac.uk; fax 44–131–650–5392.

rare autosomal recessive condition termed chronic granulomatous disease manifested as a massively increased susceptibility to microbial infection. Genes homologous to $gp91^{phox}$ have been identified in rice and as a superfamily in Arabidopsis (Groom et al., 1996; Keller et al., 1998). Genes encoding the other components of this complex, however, have not been uncovered in plants. Thus it is becoming increasingly likely that the plant and animal NADPH oxidase complexes are regulated, at least in part, via different mechanisms. To confirm the composition of the plant holoenzyme, it will be important to functionally reconstitute the activity of this complex in vitro from its proposed composite proteins.

Evidence has also been presented for the involvement of peroxidases in the generation of H_2O_2 during the oxidative burst. Peroxidase isoforms have been shown to produce H_2O_2 in vitro at an alkaline pH, which is characteristically found in the apoplast following pathogen recognition (Bolwell and Wojtaszek, 1997). Moreover, the directed secretion of peroxidases to sites of infection has also been demonstrated (McLusky et al., 1999). Other potential sources of ROIs include amine, diamine, and polyamine oxidases, which may generate apoplastic H_2O_2 in tobacco (Allan and Fluhr, 1997). In barley, a germin-like oxalate oxidase that generates H_2O_2 has been shown to accumulate during resistance to *Erysiphe graminis* f. sp. *hordei*, suggesting that alternative mechanisms of ROI generation could exist in monocots.

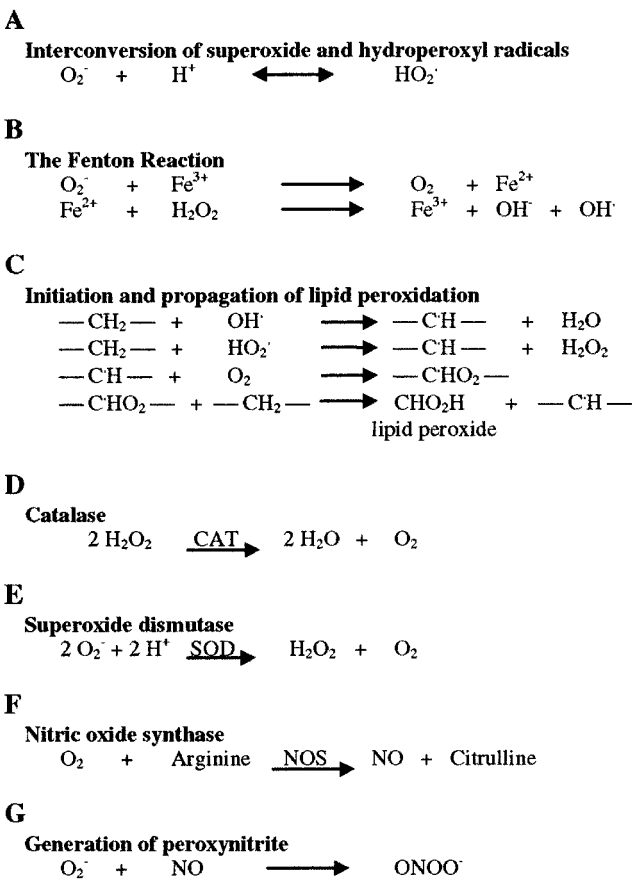

Figure 1. Key chemical reactions involving ROIs and NO in plants.

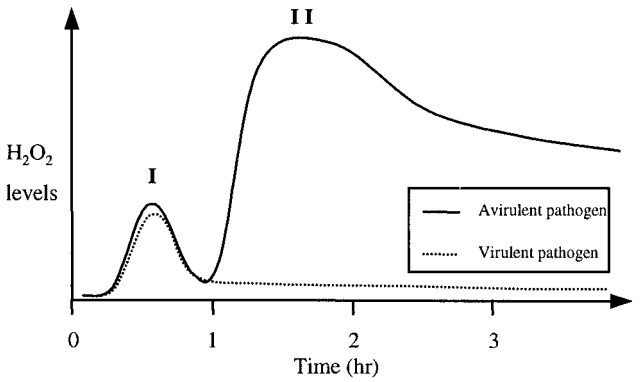

Figure 2. Biphasic accumulation of H_2O_2 during the plant oxidative burst in response to an avirulent or virulent microbial pathogen. Only phase II correlates with the establishment of disease resistance. Phase I is a non-specific response to a number of stress stimuli, including wounding.

Therefore, a number of potential mechanisms have now been proposed for the origin of the oxidative burst. It is probable that some of these mechanisms are not mutually exclusive and in this context, simultaneous ROI production from independent sources has been demonstrated (Allan and Fluhr, 1997). Alternatively, some plant species may have evolved different methods of ROI generation or distinct mechanisms could be deployed against different microbial pathogens. The analysis of gene knockout or antisense Arabidopsis lines is now urgently required to critically assess the potential role of these ROI generating systems in the oxidative burst against a range of microbial pathogens.

GENERATION OF NO

NO is a key signal in the immune, nervous and vascular system of animals and is produced by the action of NO synthase (NOS). In mammals there are three classes of NOS, which are complex P450 enzymes that require NADPH, FAD, FMN, and tetrahydrobiopterin for their activity. A number of recent observations have suggested NO may play an important role in the establishment of plant disease resistance. Thus NO has been shown to accumulate during incompatible, but not compatible, plant-pathogen interactions, and pharmacological inhibitors of NOS have been shown to ameliorate the establishment of plant disease resistance (Delledonne et al., 1998). Moreover, the transient accumulation of NO in tobacco plants via the injection of mammalian NOS elaborated resistance against a previously virulent strain of tobacco mosaic virus (Durner et al., 1998). To date, however, a plant gene encoding NOS has not

been identified. There are alternative mechanisms for NO generation in addition to NOS. Respiration, denitrification, and nitrogen fixation can all produce NO as a by-product derived from NO_2 accumulation, either nonenzymatically or via nitrate reductase activity. If a candidate NOS gene remains elusive, the biochemical purification of the enzyme responsible for NO production during the establishment of disease resistance will become an important future goal in this area.

REGULATORY MECHANISMS MODULATING ROI PRODUCTION

Due to the highly cytotoxic and reactive nature of ROIs, their accumulation must be under tight control (Fig. 3). Studies employing pharmacological agents have shown the plant oxidative burst to be regulated, at least in part, by a phosphorylation/dephosphorylation poise (Levine et al., 1994). Phospholipases are thought to be intimately involved in the activation of the mammalian NADPH oxidase complex, which prompted studies to investigate if these enzymes have a similar function in plants. No role for phospholipase D has been identified, which is particularly important in mammals. However, inhibitors of phospholipase A have been shown to blunt the oxidative burst in tobacco suspension cells in response to specific recognition of the Cf-9 elicitor of *Cladosporium fulvum* (Piedras et al., 1998). Moreover, a role for phospholipase C-mediated production of inositol 1,4,5-triphosphate in engagement of the plant oxidative burst has also been proposed (Legendre et al., 1993). These observations are particularly pertinent because two Arabidopsis genes, designated *PAD4* and *EDS1*, which are required for resistance against virulent and avirulent pathogens, respectively, have been shown to encode products with homology to phospholipases (Falk et al., 1999; Jirage et al., 1999). However, a potential role for these proteins in the modulation of the oxidative burst remains to be investigated.

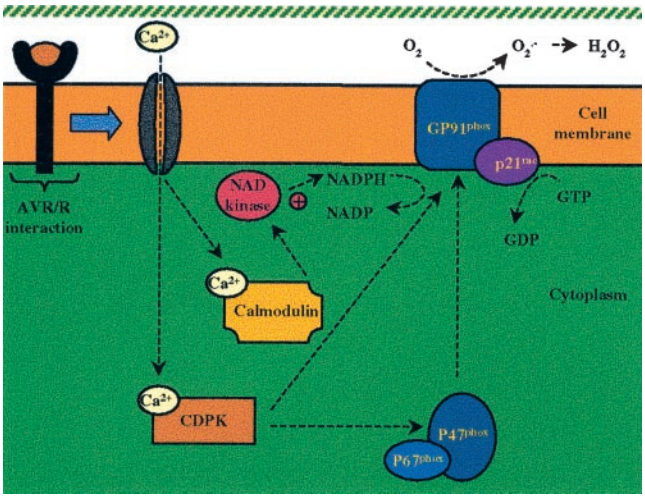

Figure 3. Schematic model for engagement of the NADPH oxidase-dependent oxidative burst in plants. Pathogen recognition results in an influx of Ca^{2+}, which may activate both the production of NADPH via NAD kinase and the translocation of $p67^{phox}$ and $p47^{phox}$ from the cytosol to the plasma membrane. Moreover, Ca^{2+} may also activate $gp91^{phox}$ directly, by binding to the two EF hand motifs present in this protein, or indirectly via phosphorylation, following the Ca^{2+}-mediated activation of a specific CDPK. The small GTP-binding protein, $p21^{rac}$, may also make an important contribution to the activation of the NADPH oxidase complex.

A striking feature of the plant homolog of $gp91^{phox}$, designated *rbohA*, is the presence of an extended amino terminus, which is followed by two Ca^{2+}-binding (EF) hand motifs, suggesting Ca^{2+} may play an important role in the regulation of NADPH oxidase activity (Keller et al., 1998). Structural predictions of rbohA suggest that the two EF hand motifs are located within the cytosol. The rapid accumulation of cytosolic Ca^{2+} in responses to elicitors and avirulent pathogens has been well documented (Zimmermann et al., 1997; Xu and Heath, 1998). Thus Ca^{2+} release following pathogen recognition may drive a conformation change in rbohA leading to transient O_2^- production. Recent evidence has also been presented suggesting that Ca^{2+} may modulate the NADPH oxidase complex via a more indirect route. NAD kinase catalyzes the final step in the production of NADPH and the activity of this enzyme is dependent on the Ca^{2+}-binding protein, calmodulin. Transgenic plants containing a constitutively active synthetic calmodulin have been generated and shown to possess increased basal and induced levels of NADPH in response to elicitor treatment, resulting in elevated levels of ROI production (Harding et al., 1997). Hence, Ca^{2+} may also regulate the NADPH oxidase complex indirectly by elevating the concentration of available NADPH via modulation of NAD kinase activity.

Biochemical studies of the human NADPH oxidase complex have identified two further cognate proteins: the small GTP-binding protein $p21^{rac}$ and the GDP-dissociation inhibitor factor rhoGDI. Thus GTP-binding and its subsequent hydrolysis to GDP may play an important role in modulating O_2^- production. Recently, a number of rice genes have been identified as homologs of human $p21^{rac}$ and dominant gain-of-function and dominant negative forms of one such gene, designated *OsRac1*, have been expressed in rice cell cultures and transgenic plants. It is intriguing that expression of constitutively active *OsRac1* resulted in the production of ROI, whereas expression of the dominant negative form ameliorated ROI generation (Kawasaki et al., 1999). Hence, OsRac1 may function to modulate the oxidative burst in rice by regulating the activity of an NADPH oxidase complex.

The active movement of the $p47^{phox}$ and $p67^{phox}$ components of the NADPH oxidase complex from the cytosol to the plasma membrane is thought to be

a key point of regulation in neutrophils. Antibodies raised against these proteins, which cross-react with proteins of a similar mass in plants, have been employed to show a similar translocation of these proteins. This process is thought to occur in response to race-specific elicitors of the tomato leaf mold pathogen *C. fulvum* (Xing et al., 1997). In animals, phosphorylation of these proteins has been proposed to initiate their translocation to the plasma membrane. In neutrophils, protein kinase C is thought to phosphorylate these proteins, whereas in tomato this process may be mediated via a Ca^{2+}-dependent protein kinase (CDPK; Xing et al., 1997).

Unfortunately, plant gene homologs encoding $p47^{phox}$ and $p67^{phox}$ have yet to be uncovered. In this context an alternative mechanism for the activation of the plant NADPH oxidase independent of these proteins has been proposed. The *R* gene-dependent activation of a CDPK that functions upstream of the oxidative burst has recently been reported (Romeis et al., 2000). Although the molecular target of this CDPK remains enigmatic, it may phosphorylate the plant NADPH oxidase, as this protein possesses CDPK phosphorylation signature sites. Hence, this kinase may directly contribute to the activation of NADPH oxidase activity (Fig. 3).

Mechanisms regulating the production of ROIs via the modulation of cell wall-associated peroxidase activity are also now beginning to emerge. Peroxidase isoforms have been isolated that actively produce H_2O_2 in vitro at an alkaline pH (Bolwell and Wojtaszek, 1997). This is an important criterion, because following pathogen recognition, there is a rapid alkalization of the apoplast. Hence, an increase in apoplastic pH, mediated via plasma membrane ion channels, could engage the activity of the relevant peroxidase isoforms driving the production of H_2O_2. The delivery of substrate(s) to the apoplast may provide another powerful mechanism for the regulation of peroxidase-dependent ROI production, although the existence of such a regulatory mechanism remains to be established. A recent study has highlighted the possible colocalization of peroxidase activity and H_2O_2 accumulation at *Botrytis allii* infection sites in onion epidermal cells (McLusky et al., 1999). The directed secretion of peroxidases to sites of attempted pathogen infection could thus provide an elegant mechanism for regulating peroxidase-dependent ROI generation. Finally, like the NADPH oxidase complex, the activation of apoplastic peroxidase activity may also be coupled to Ca^{2+} fluxes, as Ca^{2+} is required for optimal enzyme activity.

ROI-MEDIATED OXIDATIVE CROSS-LINKING

The plant cell wall is a dynamic molecular machine comprised of a complex structure of polysaccharides, phenolic compounds, and structural proteins. One of the most rapid defensive strategies identified to date is the oxidative cross-linking of cell wall structural proteins, which is initiated within 2 to 5 min in elicitor-treated soybean and bean cells (Bradley et al., 1992). The accumulation of extracellular H_2O_2 following the oxidative burst has been proposed to drive the formation of protein cross-links, thereby strengthening the resistance of this key physical barrier against attempted pathogen penetration. The molecular nature of the cross-links responsible for the rapid insolubilization of these proteins remains unclear. Isodityrosine, an oxidatively coupled dimer of Tyr, could be generated quickly and could elaborate cross-linking. Interpolypeptide isodityrosine is therefore a strong candidate for this covalent cross-link. Unfortunately, analysis of soluble oligopeptides released from the cell wall by protease digestion has identified only intrapolypeptide loops of isodityrosine. More recently, however, evidence has been presented that di-isodityrosine, a novel tetrameric derivative of Tyr, may constitute this intermolecular cross-link (Brady and Fry, 1997).

In addition to driving the cross-linking of cell wall structural proteins, ROIs generated from the oxidative burst may also cross-link cell wall-bound phenolics. Polysaccharides with ester-linked feruloyl side chains are abundant in the plant cell wall. In the Gramineae, feruloyl groups are predominantly attached to Ara side chains of arabinoxylans. The production of diferuloyl groups may act as covalent cross-links between polysaccharide chains within the cell wall, thereby strengthening cell wall structure. Recently, evidence has been presented that H_2O_2 accumulation may elaborate the extraprotoplastic oxidative coupling of polysaccharide-bound feruloyl residues (Fry et al., 2000). Hence, the oxidative burst may also drive the cross-linking of polysaccharide chains within the cell wall, thus strengthening this physical barrier against attempted pathogen ingress.

FUNCTION OF ROIs IN HR FORMATION

ROIs generated via the oxidative burst have been proposed to play a central role in the development of host cell death during the HR. The deployment of antioxidant enzymes or scavengers has been shown to blunt the development of cell death during a number of incompatible plant-pathogen interactions. Moreover, the inhibition of endogenous antioxidant mechanisms using specific and non-specific pharmacological agents, thus increasing the concentration of ROIs, resulted in elevated levels of host cell death (Levine et al., 1994). These observations have more recently been extended to include experiments employing antisense technology to suppress the expression of key antioxidant genes. Thus transgenic plants with reduced amounts of catalase activity, which catalyzes the decomposition of H_2O_2, accumulate increased levels of ROIs and develop HR-like lesions when exposed to high light intensities (Chamnong-

pol et al., 1998). This phenomenon probably reflects a bona fide defense response because gene expression analysis has demonstrated the presence of transcripts from key defense marker genes. In this context, the pivotal defense metabolite salicylic acid (SA) has been postulated to both inhibit the activity of some antioxidant enzymes and potentiate the production of NADPH oxidase-dependent O_2^- via a positive feedback loop (for review, see Van Camp et al., 1998).

ROIs may also mediate the induction of a cytoplasmic Ca^{2+} influx during the establishment of disease resistance. Elegant studies of the interactions of cowpea with the rust fungus *Uromyces vignae* identified a prolonged elevation of cytosolic Ca^{2+} during resistant, but not susceptible responses, suggesting a key role for cytoplasmic Ca^{2+} in HR development (Xu and Heath, 1998). Recent studies have begun to highlight a role for H_2O_2 in the initiation of this Ca^{2+} influx. The deployment of fluorescence ratio imaging techniques has shown that H_2O_2 accumulation results in a dose-dependent increase of cytoplasmic Ca^{2+} (Levine et al., 1996). This dramatic increase in cytoplasmic Ca^{2+} was subsequently shown to be an important factor in the development of ROI-mediated cell death. Moreover, specific inhibitors of Ca^{2+} channels, which block Ca^{2+} entry, blunted the development of cell death in soybean cells, mediated by either *Pseudomonas syringae* pv *glycinea* or H_2O_2. The location of this Ca^{2+} pool is presumably extracellular because the addition of Ca^{2+} ionophores, which open Ca^{2+} channels, were sufficient to invoke cell death in the presence, but not absence, of extracellular Ca^{2+}. Thus an ROI-stimulated Ca^{2+} influx is a key determinant in HR development and may reflect a requirement for Ca^{2+} in the activation of cell death effectors.

Genetic evidence for the involvement of ROIs in the HR has been provided by studies employing the recessive *lsd1* lesion mimic mutant of Arabidopsis. The accumulation of O_2^- preceded the onset of cell death and the local accumulation of O_2^-, but not H_2O_2 in *lsd1* was sufficient to initiate the development of runaway cell death in this mutant (Jabs et al., 1996). Thus runaway cell death in *lsd1* plants probably reflects abnormal accumulation of O_2^- and an inability to respond to signals derived from it. *lsd1* has been shown to encode a zinc finger transcription factor and may function by monitoring a O_2^--dependent signal and negatively regulating a cell death pathway. Recently, some of the targets of *lsd1* gene function have begun to emerge; *lsd1* has been shown to be required for the induction of CuZnSOD in response to SA accumulation (Kliebenstein, et al., 1999). Thus the runaway cell death phenotype in *lsd1* probably reflects the accumulation of O_2^- to a critical threshold concentration, due to a reduction in CuZnSOD activity, which engages the mechanism(s) underlying runaway cell death in this mutant. However, it is currently unclear how this mechanism operates because O_2^- production is presumed to be extracellular and the target CuZnSOD intracellular. Furthermore, O_2^- will not typically cross biological membranes due to its inherent charge.

The first suspicions that ROIs may not be sufficient for the complete host cell death response came from experiments employing a battery of *P. syringae* pv *syringae hrp* gene mutants. This gene cluster encodes a type III secretory system, conserved among gram-negative plant pathogenic bacteria, which may function as a conduit for the delivery of microbial AVR proteins directly into plant cells. Mutation of the *hrmA* gene within this cluster, which encodes a regulatory function, decreased the development of cell death in tobacco suspension cells, but had no impact on the magnitude of the oxidative burst (Glazener et al., 1996). In contrast, mutations in the remaining genes within this cluster abolished both responses. Data supporting these observations has recently emerged from studies of the oxidative burst engaged by tomato suspension cultures in response to the AVR9 elicitor of *C. fulvum*. A substantial increase in the level of ROIs was measured in this system, but no cell death was detected (Piedras et al., 1998). Thus ROIs generated via the oxidative burst may not be sufficient for the complete host cell death response during the development of the HR, at least in cell suspension cultures, suggesting other mechanisms may contribute to this cellular execution process.

Recent evidence has suggested that NO may make a pivotal contribution to HR formation. In this context NO has been shown to potentiate ROI-mediated induction of cell death in soybean cells (Delledonne et al., 1998). Thus ROIs may function in combination with NO to drive the development of host cell death during the formation of the HR, possibly after reacting together to form highly toxic peroxynitrite ($ONOO^-$). However, the identity of the agent(s) that directly execute plant cells still remains to be rigorously established.

ROI SIGNAL FUNCTION IN SAR

A potential role for local ROI accumulation in systemic signaling leading to the establishment of SAR was highlighted by an elegant series of experiments in transgenic tobacco plants, which contained an antisense catalase gene (Chamnongpol et al., 1998). Exposure of these plants to high light levels for 2 d resulted in visible necrosis and induced pathogenesis-related (PR) proteins in adjacent, light-shielded, local, and systemic leaf tissues. In contrast, exposure to high light levels for 4 h induced PR proteins in adjacent, light-shielded local, but not systemic tissue, in the absence of necrosis. Thus the ROI-mediated activation of SAR genes could be uncoupled from cell death in local tissues. However, local ROI-mediated cell death was necessary for the accumulation of PR proteins in systemic tissues.

Studies employing Arabidopsis have placed similar observations in a more biological context (Alvarez et al., 1998). Engagement of a local oxidative burst in response to an avirulent isolate of *P. syringae* pv *tomato*-induced "microbursts" in systemic leaf tissue. These microbursts drove the formation of "micro-HRs," which preceded the establishment of SAR. Co-infiltration of the NADPH oxidase inhibitor, diphenylene iodinium, with avirulent *P. syringae* pv *tomato* ameliorated engagement of a local oxidative burst and blocked the formation of systemic microbursts and the development of SAR. In the corresponding gain-of-function experiment local infiltration of an H_2O_2-generating system induced systemic microbursts and subsequently SAR. Hence, an ROI-mediated systemic signaling network may also mediate the establishment of plant immunity. ROI accumulation may, therefore, integrate a plethora of local and systemic defense responses (Fig. 4).

ROI-MEDIATED REDOX SIGNALING

Redox signaling mediated via ROIs is thought to be effected by the oxidation of nucleophilic centers or coordinate interactions with transition metals strategically located at either the allosteric or active sites of target proteins, resulting in a modulation of their activity (Stamler, 1994). More exotic redox switches may also include production of the less oxidized forms of Cys, such as sulfenic and sulfinic acids, whose formation is also reversible. Thus ROIs signal functions are manifested as a consequence of their ability to act as mobile carriers of an unpaired electron. In animals there is compelling evidence for both direct and indirect mechanisms for the regulation of gene expression in response to changes in cellular redox status. In response to severe hyperoxic states key Cys residues of redox modulated transcription factors may become oxidized, effecting changes in the expression profile of their target genes. HoxB5, a member of the mammalian homeodomain gene family and Sp-1, a zinc finger protein, are examples of transcription factors that are positively and negatively regulated by oxidation respectively (Galang and Hauser, 1993; Wu et al., 1996).

In contrast, signal transmission in response to lower levels of ROIs may require the action of specific protein kinases. In animal cells ROIs have been shown to engage specific mitogen activated protein kinase (MAPK) cascades (Klotz et al., 1999). The emerging evidence suggests that MAPKs will play an important role in the establishment of plant disease resistance. For example, in parsley, a cytosolic MAPK has been shown to translocate to the nucleus in response to a fungal elicitor, where it may phosphorylate target transcription factors (Ligterink et al., 1997). However, this MAPK is thought to act independently or upstream of the oxidative burst. More recently, ROIs have been shown to mediate the expression of redox responsive defense genes via the engagement of an alternative MAPK module (J. J. Grant and G. J. Loake, unpublished observations). Hence, significant parallels may exist in the transduction of stress induced redox cues between plants and animals.

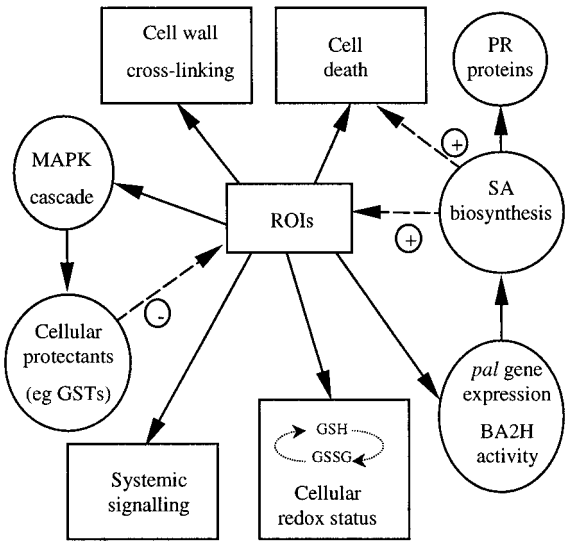

Figure 4. Functional integration of defense responses by ROIs during the establishment of plant disease resistance. BA2H, Benzoic acid 2-hydroxylase; *pal*, Phe ammonia lyase; GSH/GSSG, reduced and oxidized forms of glutathione, respectively.

REDOX SIGNALING VIA NO

Superimposed upon ROI signal function during HR formation is redox signaling mediated via NO and reactive nitrogen intermediates (RNIs). The preferred targets of NO are proteins containing iron (II) porphyrins, such as heme. The prototypic target in animals is guanylate cyclase, which elicits many cellular responses to NO via the secondary messenger cyclic GMP (cGMP), including vasorelaxation and inhibition of platelet aggregation (Stamler, 1994). During the establishment of plant disease resistance, NO production is also thought to activate this key enzyme, leading to increased levels of cGMP, which may function as a downstream messenger activating the expression of key plant defense genes including *PR1*, in an SA-dependent manner (Durner et al., 1998).

NO has also been shown to regulate the generation of animal prostaglandins, lipid-based signals produced during the inflammatory response, by modulating the activity of cyclooxygenase (Salvemini et al., 1993). A plant homolog of cyclooxygenase designated PIOX has recently been isolated from tobacco (Sanz et al., 1998). Therefore, NO may regulate lipid signal function during the HR via a similar mechanism. It is interesting that the expression of PIOX is activated by pathogens and ROIs. However, the impact of ROIs or

RNIs, if any, on the activity of the corresponding gene product remains to be determined.

ROI AND NO REGULATORY INTERPLAY

The interactions between ROIs and NO during the establishment of disease resistance is likely to be complex. Conceptually, various positive and negative regulatory functions based on this interplay could be built into the underlying signaling circuitry. For example, NO antagonizes the action of O_2^-, driving its activity away from metal clusters toward sulfhydryl groups, which are the preferred targets of $OONO^-$, formed from NO and O_2^-. It is intriguing that the O_2^--producing NADPH oxidase may be a target for inactivation by NO during HR formation. In neutrophils, the activity of this oxidase is blunted by NO accumulation, decreasing tissue inflammation (Clancy et al., 1992). Thus NO inhibition of O_2^- production during the establishment of disease resistance may function to limit the extent of cell death mediated via the production of $OONO^-$. Conversely, NO is known to function as a potent activator of $p21^{ras}$ G-proteins, possibly by inducing an *S*-nitrosylation-induced conformational change, promoting the rate-limiting release of GDP (Lander et al., 1993). Hence, NO could activate *Os-Rac1*, the *ras*-related plant homolog of $p21^{rac1}$, the G-protein associated with the NADPH oxidase complex, driving the formation of O_2^-. This apparent regulatory conflict could be reconciled if the modulation of these redox switches was temporally resolved during HR formation via their differing sensitivities to NO. This regulatory mechanism could be amplified further, because both catalase and γ-glutamylcysteinyl synthetase, which catalyzes a key step in glutathione biosynthesis, a pivotal antioxidant, are both known to be targets for NO inactivation in animals (Halliwell and Gutteridge, 1990). Disabling these antioxidant systems in plants during HR formation would presumably further increase ROI levels. Finally, NOS itself is a P450 enzyme and would consequently be a potential target for NO inactivation, possibly during the later stages of a resistant response. Inactivation of NOS could therefore function as a further brake on lesion development. Unraveling the interplay between ROIs and NO in the context of redox signal transduction should provide significant insights into the establishment of plant disease resistance.

CONCLUSIONS

The emerging evidence suggests ROIs and NO integrate a plethora of diverse defense responses during the establishment of local *R* gene-mediated resistance and the development of systemic immunity. However, a number of fundamentally important questions remain unanswered. The mechanism of ROI generation still remains to be rigorously established. In this context the analysis of knockouts and antisense plants defective in the activity of isoforms of RbohA and specific cell wall-associated peroxidases should shed light on this question. Moreover, although there is compelling evidence for NO function in disease resistance, to date, no genes encoding either NOS, guanylate cyclase, or the cognate phosphodiesterase responsible for cGMP degradation have been identified. Our appreciation of the complex interplay between ROIs and NO still remains at a rudimentary level and advances in this area are likely to represent a formidable task. However, the ground has been broken by data suggesting that the synergistic interaction of these molecules mediates host cell execution. Although the targets of NO are known or can be speculated, the signal functions of ROIs are only just beginning to emerge. As our appreciation of the underlying biology increases, we anticipate that significant insights will emerge for the development of novel strategies for disease control based on the manipulation of the oxidative burst and the cognate redox signaling network.

ACKNOWLEDGMENTS

We thank our colleagues for communicating results prior to publication.

Received February 15, 2000; accepted April 17, 2000.

LITERATURE CITED

Allan AC, Fluhr R (1997) Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. Plant Cell **9:** 1559–1572

Alvarez ME, Pennell RI, Meijer PJ, Ishikawa A, Dixon RA, Lamb C (1998) Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. Cell **92:** 773–784

Apostol I, Heinstein PF, Low PS (1989) Rapid stimulation of an oxidative burst during elicitation of cultured plant-cells: role in defense and signal transduction. Plant Physiol **90:** 109–116

Bolwell GP, Wojitaszek P (1997) Mechanisms for the generation of reactive oxygen species in plant defense: a broad perspective. Physiol Mol Plant Pathol **51:** 347–366

Bradley DJ, Kjellbom P, Lamb CJ (1992) Elicitor-induced and wound-induced oxidative cross-linking of a proline-rich plant-cell wall protein: a novel, rapid defense response. Cell **70:** 21–30

Brady JD, Fry SC (1997) Formation of di-isodityrosine and loss of isodityrosine in the cell walls of tomato cell-suspension cultures treated with fungal elicitors or H_2O_2. Plant Physiol **115:** 87–92

Chamnongpol S, Willekens H, Moeder W, Langebartels C, Sandermann H, Van Montagu A, Inze D, Van Camp

W (1998) Defense activation and enhanced pathogen tolerance induced by H_2O_2 in transgenic tobacco. Proc Natl Acad Sci USA **95:** 5818–5823

Clancy RM, Leszczynskapiziak J, Abramson SB (1992) Nitric-oxide, an endothelial-cell relaxation factor, inhibits neutrophil superoxide anion production via a direct action on the NADPH oxidase. J Clin Investig **90:** 1116–1121

Dangl JL, Dietrich RA, Richberg MH (1996) Death don't have no mercy: cell death programs in plant-microbe interactions. Plant Cell **8:** 1793–1807

Delledonne M, Xia YJ, Dixon RA, Lamb C (1998) Nitric oxide functions as a signal in plant disease resistance. Nature **394:** 585–588

Doke N (1983) Involvement of superoxide anion generation in the hypersensitive response of potato-tuber tissues to infection with an incompatible race of *Phytophthora-infestans* and to the hyphal wall components. Physiol Plant Pathol **23:** 345–357

Durner J, Wendehenne D, Klessig DF (1998) Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. Proc Natl Acad Sci USA **95:** 10328–10333

Falk A, Feys BJ, Frost LN, Jones JDG, Daniels MJ, Parker JE (1999) EDS1, an essential component of R gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. Proc Natl Acad Sci USA **96:** 3292–3297

Fry SC, Willis SC, Patterson AEJ (2000) Intraprotoplasmic and wall-localized formation of arabinoxylan-bound diferulates and larger ferulate coupling products in maize cell-suspension cultures. Planta (in press)

Galang CK, Hauser CA (1993) Cooperative DNA-binding of the human Hoxb5 (Hox-2.1) protein is under redox regulation *in-vitro*. Mol Cell Biol **13:** 4609–4617

Glazener JA, Orlandi EW, Baker CJ (1996) The active oxygen response of cell suspensions to incompatible bacteria is not sufficient to cause hypersensitive cell death. Plant Physiol **110:** 759–763

Groom QJ, Torres MA, Fordham-Skelton AP, Hammond-Kosack KE, Robinson NJ, Jones JDG (1996) Rboha a rice homologue of the mammalian $gp91^{Phox}$ respiratory burst oxidase gene. Plant J **10:** 515–522

Halliwell B, Gutteridge, JMC (1990) Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol **186:** 1–85

Hammond-Kosack KE, Jones JDG (1996) Resistance gene-dependent plant defense responses. Plant Cell **8:** 1773–1791

Harding SA, Oh S-H, Roberts DM (1997) Transgenic tobacco expressing a foreign calmodulin gene shows an enhanced production of active oxygen species. EMBO J **16:** 1137–1144

Jabs T, Dietrich RA, Dangl JL (1996) Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. Science **273:** 1853–1856

Jirage D, Tootle TL, Reuber TL, Frost LN, Feys BJ, Parker JE, Ausubel FM, Glazebrook J (1999) *Arabidopsis thaliana PAD4* encodes a lipase-like gene that is important for salicylic acid signaling. Proc Natl Acad Sci USA **96:** 13583–13588

Kawasaki T, Henmi K, Ono E, Hatakeyama S, Iwano M, Satoh H, Shimamoto K (1999) The small GTP-binding protein Rac is a regulator of cell death in plants. Proc Natl Acad Sci USA **96:** 10922–10926

Keller T, Damude HG, Werner D, Doerner P, Dixon RA, Lamb C (1998) A plant homologue of the neutrophil NADPH oxidase $gp91^{Phox}$ subunit gene encodes a plasma membrane protein with Ca^{2+} binding motifs. Plant Cell **10:** 255–266

Kliebenstein DJ, Dietrich RA, Martin AC, Last RL, Dangl JL (1999) LSD1 regulates salicylic acid induction of copper zinc superoxide dismutase in *Arabidopsis thaliana*. Mol Plant-Microbe Interact **12:** 1022–1026

Klotz LO, Pellieux C, Briviba K, Pierlot C, Aubry JM, Sies H (1999) Mitogen-activated protein kinase (p38-, JNK-, ERK-) activation pattern induced by extracellular and intracellular singlet oxygen and UVA. Eur J Biochem **260:** 917–922

Lander HM, Sehajpal PK, Novogrodsky A (1993) Nitric-oxide signaling: a possible role for G-proteins. J Immunol **151:** 7182–7187

Legendre L, Yueh YG, Crain R, Haddock N, Heinstein PF, Low PS (1993) Phospholipase-C activation during elicitation of the oxidative burst in cultured plant-cells. J Biol Chem **268:** 24559–24563

Levine A, Pennell RI, Alvarez ME, Palmer R, Lamb C (1996) Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. Curr Biol **6:** 427–437

Levine A, Tenhaken R, Dixon R, Lamb C (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell **79:** 583–593

Ligterink W, Kroj T, zur Nieden U, Hirt H, Scheel D (1997) Receptor-mediated activation of a MAP kinase in pathogen defense of plants. Science **276:** 2054–2057

McLusky SR, Bennett MH, Beale MH, Lewis MJ, Gaskin P, Mansfield JW (1999) Cell wall alterations and localized accumulation of feruloyl-3′-methoxytyramine in onion epidermis at sites of attempted penetration by *Botrytis allii* are associated with actin polarization, peroxidase activity and suppression of flavonoid biosynthesis. Plant J **17:** 523–534

Piedras P, Hammond-Kosack KE, Harrison K, Jones JDG (1998) Rapid, *Cf-9*- and *Avr9*-dependent production of active oxygen species in tobacco suspension cultures. Mol Plant-Microbe Interact **11:** 1155–1166

Romeis T, Piedras P, Jones JDG (2000) Resistance gene-dependent activation of a calcium-dependent protein kinase (CDPK) in the plant defense response. Plant Cell **12:** 803–815

Salvemini D, Misko TP, Masferrer JL, Seibert K, Currie MG, Needleman P (1993) Nitric-oxide activates cyclooxygenase enzymes. Proc Natl Acad Sci USA **90:** 7240–7244

Sanz A, Moreno JI, Castresana C (1998) PIOX, a new pathogen-induced oxygenase with homology to animal cyclooxygenase. Plant Cell **10:** 1523–1537

Segal AW, Abo A (1993) The biochemical basis of the NADPH oxidase of phagocytes. Trends Biochem Sci **18:** 43–47

Stamler JS (1994) Redox signaling: nitrosylation and related target interactions of nitric-oxide. Cell **78:** 931–936

Van Camp W, Van Montagu M, Inze D (1998) H_2O_2 and NO: redox signals in disease resistance. Trends Plant Sci **3:** 330–334

Wu XS, Bishopric NH, Discher DJ, Murphy BJ, Webster KA (1996) Physical and functional sensitivity of zinc finger transcription factors to redox change. Mol Cell Biol **16:** 1035–1046

Xing T, Higgins VJ, Blumwald E (1997) Race-specific elicitors of *Cladosporium fulvum* promote translocation of cytosolic components of NADPH oxidase to the plasma membrane of tomato cells. Plant Cell **9:** 249–259

Xu HX, Heath MC (1998) Role of calcium in signal transduction during the hypersensitive response caused by Basidiospore-derived infection of the cowpea rust fungus. Plant Cell **10:** 585–597

Zimmermann S, Nurnberger T, Frachisse JM, Wirtz W, Guern J, Hedrich R, Scheel D (1997) Receptor-mediated activation of a plant Ca^{2+} permeable ion channel involved in pathogen defense. Proc Natl Acad Sci USA **94:** 2751–2755