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REVIEWS

Systemic Acquired Resistance in Crop Protection: From Nature to a Chemical Approach

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Plant natural resistance to potential parasites is regulated by two fundamental mechanisms: the "nonhost" and the "gene-for-gene" resistance, respectively. The latter is relevant when a cultivar resistant (R) gene product recognizes an avirulence gene product in the attacking pathogen and triggers an array of biochemical reactions that halt the pathogen around the site of attempted invasion. To cope with virulent pathogens, plants may benefit by some temporary immunity after a challenge triggering such an array of defense reactions, following a localized necrotizing infection as a possible consequence of a hypersensitive response (HR). This process, mediated by accumulation of endogenous salicylic acid (SA), is called systemic acquired resistance (SAR) and provides resistance, to a certain extent even against unrelated pathogens, such as viruses, bacteria, and fungi, for a relatively long-lasting period. SAR may be more potently activated in plants pretreated with chemical inducers, most of which appear to act as functional analogues of SA. This review summarizes the complex aspects of SAR as a way to prevent crop diseases by activating the plants' own natural defenses. The following outline is taken: (1) introduction through the historical insight of the phenomenon; (2) oxidative burst, which produces high levels of oxygen reactive species in a way similar to the inflammation state in animals and precedes the HR to the pathogen attack; (3) SAR as a coordinate action of several gene products leading to the expression of defenses well beyond the time and space limits of the HR; (4) jasmonic acid (JA) and ethylene as other endogenous factors mediating a different pahway of induced resistance; (5) pathogenesis related proteins (PR proteins) de novo synthesized as specific markers of SAR; (6) exogenous inducers of SAR, which include both synthetic chemicals and natural products; (7) the pathway of signal transduction between sensitization by inducers and PR expression, as inferred by mutageneses, a process that is still, to a large extent, not completely elucidated; (8) prospects and costs; (9) final remarks on the state-of-the-art of the topic reflecting the chemical view of the author, based on the more authoritative ones expressed by the authors of the reviewed papers.

Keywords: Systemic acquired resistance; H_2O_2; HR; peroxidase; catalase; chemical inducers; salicylic acid; jasmonic acid; ethylene; BTH; sensitization

1. INTRODUCTION

In nature most plants resist the attack of potential parasites by developing a variety of biochemical responses that often lead to a hypersensitive reaction around the sites of attempted penetration. That is the case of an incompatible interaction as the result of a genetic predisposition of the plant to promptly respond to the invader by putting in place effective barriers to its entry. Actually, the activation of the earliest responses to the attack may be triggered by some interaction between the products of plant resistance (R) genes and pathogen avirulence genes or, more often, by a general inherent capability of the plant to act as a nonhost. The differences between these two genetically distinct types of resistance are matters typically reserved to expert phytopathologists (*1*), but, for the scope of this review, only the gene-for-gene response is relevant.

Evolution has allowed some microorganisms to establish a compatible interaction with host plants by acting as biotrophs or necrotrophs, so eluding or preventing the host's natural defenses and making it susceptible to infection. As part of the fight against these pathogens, pioneering investigations were started to determine whether plant defenses could be affected or enhanced as a nonbiocidal alternative to pesticides in crop protection.

The concept that a sort of immune system could be at work in plants was first proposed by Ross to explain systemic resistance to virus infection observed in plants previously

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inoculated with tobacco mosaic virus (TMV) (*2*). Works by Kuc, among others, drew attention to the reactions induced by the much more complex and highly structured fungal pathogens in compatible and incompatible relationships with challenged plants. Local accumulation of phytoalexins appeared to be the first, important, coordinate expression of antimicrobial response to the attack by various fungi, although they were also produced under abiotic stress (*3*, *4*). Later, it became apparent that phytoalexins represented only one of the many events responsible for successfully halting the pathogen invasion (*5*). Enhancement of several enzyme activities, including β-1,3-glucanase, chitinase, peroxidase, lipoxygenase, and catalase, which may contribute, directly or indirectly, to pathogenesis resistance, has been observed during the first stages of the infection process (*6*).

The immunity that a plant may acquire subsequently to a local necrotic lesion by an avirulent pathogen and develop in distal, untreated, parts against unrelated virulent pathogens has been clearly demonstrated by several authors. This proves that the original observation by Ross is a general phenomenon occurring with several species of host-plants and pathogens. This type of immunity is called systemic acquired resistance (SAR) and differs from animal immunity in both its apparent lack of specificity (absence of antibody induction) and its lesser efficacy and duration (*7*). Proteins synthesized de novo by plants during infection with various pathogens are called pathogenesis-related proteins (PR proteins) and have been extracted mainly from dicotyledonous plants. Some of them show properties related to the above-mentioned enzymes, whereas others have a direct antifungal activity (*8*). Genes encoding these proteins were shown to be induced, through their RNA messengers, at the onset of the SAR (*9*).

Mechanisms by which SAR is induced and established are largely unknown. In induced resistance processes, more than one biochemical pathway appears to be activated, based on the requirement of different signal transduction pathways depending on salicylic acid (SA) or jasmonic acid and ethylene (*10*). Early studies showed that SAR was mediated by SA, following the observation that exogenous treatment with salicylates induced PR protein synthesis and enhanced resistance to a number of different pathogens, including TMV (*11*). Later, and more importantly, synthetic chemicals were discovered that may be considered as functional analogues of SA but act more potently than SA and prime mono- and dicotyledonous plants to resist a wide spectrum of pathogens (*12*).

This review gives a brief account of some recent and significant contributions to the research on SAR but does not claim to be comprehensive.

2. REACTIVE OXYGEN SPECIES AND THE OXIDATIVE BURST

In animals the inflammation state is a general reaction of tissues to infection. This state is characterized, inter alia, by an abnormal increase of oxidation reactions, the so-called "respiratory burst", that, initially, is not very dissimilar from what occurs in plants during the stages preceding the hypersensitive reaction.

Hydrogen peroxide, oxygen superoxide ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), and singlet oxygen ($O{=}O$) are the four reactive species of oxygen that, when overproduced under pathological conditions, must be promptly destroyed to prevent damage to the tissues. Ascorbic acid reacts with all of these species and is converted to the mildly reactive monodehydroascorbyl radical by most of them.

Hydrogen peroxide is the most diffusible and chemically stable of all partially reduced forms of oxygen. Its production in isolated, perfused, rat liver was estimated to be of the order of 10^{-9} mol g^{-1} s^{-1}, and in tissues where removal of H_2O_2 is not so effective, such as the lens of the human eye, its steady-state concentration was found to be in the range of $(1-2.5) \times 10^{-5}$ M (*13*). Under pathological conditions, rapid generation of the superoxide, $O_2^{\bullet -}$, and accumulation of H_2O_2 at levels of 10^{-3} M occur in activated neutrophils as a response of the human immune system to tissue injury during the inflammation state. The oxidative burst taking place under these conditions appears to be initiated by an NADPH-dependent oxidase complex associated with the plasma membrane and responsible for the overall reduction of O_2 into $O_2^{\bullet -}$:

$$NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^{\bullet -}$$

$O_2^{\bullet -}$ is then converted to H_2O_2 by a spontaneous sequence of reactions with the intermediate formation of its protonated form ($^{\bullet}OOH$) or, much more efficiently, by superoxide dismutase (SOD):

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} O_2 + H_2O_2$$

$O_2^{\bullet -}$ can also bring about the reduction of Fe^{3+} to Fe^{2+}, which is potentially damaging.

Reminiscent of the above-mentioned oxidative burst is the hypersensitive response (HR) taking place in plants inoculated with avirulent pathogens. Whereas with virulent pathogens the response is generally weak and transient, with avirulent ones the response is followed by a second, heavier, and longer lasting phase of oxidative burst that leads to rapid cell death around the sites of attempted invasion (*14*). In their notable work with tobacco and soybean cell suspensions, Baker and Orlandi demonstrated that the second phase of oxidative burst against bacterial pathogens was triggered by an interaction between an avirulence gene in the pathogen and a corresponding resistance gene in the plant cultivar (*15*). During this phase, which occurred 2−3 to 5−6 h after inoculation, the levels of H_2O_2 were found to rise up to several micromoles per liter exclusively in incompatible interactions, whereas hypersensitive cell death occurred much later, starting after the seventh hour. The ultimate cause of the hypersensitive cell death remains an open question. In reviewing these and other works on the oxidative burst, the above-mentioned authors stated that the production of active oxygen species detected during the first few hours after the bacterial treatment may be one of a series of events leading to HR, but not itself the direct cause of hypersensitive cell death (*15*).

The process leading to plant-cell death may follow quite different morphological and biochemical modes of expression, ranging between two extreme models, which are described as necrosis and programmed cell death (PCD), respectively.

Necrosis is generally considered to result from severe injury of tissues and may be triggered, for example, by the formation of toxic lipid hydroperoxides. Various authors have shown that necrotic HR closely followed the occurrence of lipid peroxidation, this being a possible cause of membrane damage and cell death (*14*). In contrast, PCD recalls the programmed cell suicide known as apoptosis in animals. It is characterized by the formation of the so-called apoptotic bodies and a typical DNA fragmentation. In reviewing the most recent investigations on this type of HR, Beers and McDowell outlined a number of possible pathways that are assumed to regulate the PCD genetically with the interplay of reactive oxygen

species ($O_2^{\bullet -}$, H_2O_2), nitric oxide (NO), SA, jasmonic acid, ethylene, and strategic proteins (*16*). NO has been recently recognized as an important signal in plant hypersensitive response and to play a role in cooperation with other reactive oxygen species.

Being itself a free radical, NO may, in part, scavenge the deleterious hydroxyl radical by the reaction

$$^{\bullet}N{=}O + {}^{\bullet}OH \rightarrow HO{-}N{=}O$$

or react with the anionic superoxide to produce the reactive peroxynitrite:

$$^{\bullet}N{=}O + O_2^{\bullet -} \rightarrow O{=}N{-}O{-}O^{-}$$

In animals, peroxynitrite appears to be the effective inducer of cytotoxicity and apoptosis through DNA strand breakage (*17*). In plants, however, peroxynitrite apparently is not the essential intermediate of NO-mediated cell death, as demonstrated in soybean cells (*18*). Rather, the hypersensitive disease response appears to depend on a correct balance between the concentrations of NO and H_2O_2. In accurate experiments with soybean cell suspensions challenged with avirulent *Pseudomonas*, hypersensitive cell death was triggered only when the ratio [NO]/[H_2O_2] was between 0.25 and 2. An increase of either of the two species altering the ratio beyond these limits resulted in a suppression of cell death, which, however, was restored by the addition of a corresponding amount of the other species. This finding suggests that a close cooperation between NO and H_2O_2 is needed to determine the hypersensitive cell collapse, possibly as the result of a reaction between the two species (*18*).

In this regard, it must, however, be noted that, despite its typical occurrence in gene-for-gene resistance, hypersensitive cell death may not be an absolute requirement for the induction of SAR. As will be also reported in section 7, certain mutants of *Arabidopsis* have been isolated that effectively respond to a gene-for-gene interaction with avirulent bacteria by induction of *PR-1* and glucanase without HR expression. According to Bent and co-workers, it is conceivable that a constitutive elevation of salycilate, observed in these mutants, may substitute for the HR cell death in potentiating the defense response (*19*).

All of the above-mentioned facts suggest that, despite the absence of a specialized immunity system, such as in mammals, plants conserve a common, primitive method of defense, which appears to be initiated also by NADPH oxidase: when elicited by an incompatible pathogen, the host-plant generates reactive oxygen species, the most abundant of which is H_2O_2. In several cases accumulation of H_2O_2 is inhibited by diphenylene iodonium, a scavenger of $O_2^{\bullet -}$, suggesting that it is produced via this intermediate, which is originated by NADPH oxidase (*14*). In addition, or as an alternative, to this mechanism, some authors propose a direct generation of H_2O_2 on the apoplastic side, induced by pathogen elicitors through the mediation of plant-cell wall peroxidases (*20*).

Hydrogen peroxide may have several effects, some of which are highly toxic to the cell. The most serious of these is the Fenton reaction with Fe^{2+}, which may produce the extremely reactive hydroxyl radical (*13*, *21*). Various iron-containing enzymes, thiol-rich proteins, and unsaturated lipids may be severely damaged by H_2O_2. To avoid this damage, plant metabolism produces strategic antioxidants, such as ascorbic acid and glutathione, and related enzymes to replace the oxidizing potential of H_2O_2 by progressively milder oxidants (*22*). The oxidative burst plays a substantially beneficial role by virtue of a balanced development of counteracting/defense means to contrast the pathogen attack. Peroxidases are particularly important in this task, and an appraisal of their mechanism is relevant to our understanding of some hypotheses on the signal transduction of SAR that will be considered later.

The catalytic cycle of a typical peroxidase is outlined in **Figure 1**, where a ferric protoporphyrin is drawn in stylized form to indicate the active site, with omission of the fifth axial ligand. The cycle starts with the reaction of the ferric state with H_2O_2 to give, via a transient hydroperoxide, the first significant intermediate in the form of a green complex, called compound **I**. This important step involves the heterolytic cleavage of the O−O bond, which is made easier by the catalytic role of suitably positioned side chains of amino acids serving as proton donors. The heme of compound **I** is therefore in a redox state oxidized by two equivalents above the ferric state and bears a single oxygen atom bound to iron. All peroxidases, in the full sense of the term, allow a reducing substrate, generically indicated as SH_2, to transfer electrons and protons in two subsequent and distinct one-electron steps. The first of these steps affords a free radical $^{\bullet}SH$ together with the second significant intermediate, called compound **II**, which retains only one oxidizing equivalent. In the subsequent, final step, this hydroxo-ferryl species is reduced in a rate-limiting reaction, by the uptake of an electron and a proton from a second molecule of substrate generating another radical $^{\bullet}SH$. The nature of the products strongly depends on that of the substrate, which may pertain to a wide variety of reducing chemicals.

When the substrate is ascorbate, the specific enzyme is ascorbate peroxidase (APX) and the two, intermediate, one-electron-oxidized species $^{\bullet}SH$ are resonance-stabilized, monodehydroascorbyl free radicals, which by disproportionation afford dehydroascorbic acid as the final oxidized product.

According to a simpler point of view, catalase may be considered as a special case of peroxidase in that it uses one molecule of H_2O_2 as oxidant and a second molecule of H_2O_2 as the reducing substrate. Catalase, in fact, utilizes a similar high-valent oxo intermediate **I** as in peroxidases but, in this case, compound **I** acts in a fast, two-electron oxidation of the second molecule of H_2O_2, which thus loses its O_2 in a single step (*23*). A tentative visualization of the two-electron reduction of compound **I** by H_2O_2 is given at the bottom of **Figure 1**. Therefore, the mechanism of catalase differs from that of a peroxidase essentially in the reduction of compound **I**, the formation of which is common to both types of enzymes.

However, catalase may also effect slow peroxidatic reactions in which a transient catalase−compound **II** is spectroscopically observed. This occurs, for example, with phenolic substrates and indicates that an oxidizing process may take place under these conditions, with formation of free radical intermediates (*24*).

Hydrogen peroxide may also be generated, in particular conditions, by cell wall peroxidases, responsible for the apoplastic burst in well-characterized models (*20*). These peroxidase isoforms have been shown to have their optimum at pH ≥ 7, and this was in accordance with the transient alkalinization in the apoplast observed in response to pathogen recognition. The subject has been thoroughly investigated by Bolwell and co-workers, who isolated and cloned a cell wall peroxidase extracted from French bean. This isoform was capable of generating H_2O_2 in vitro when the pH was raised to 7.2 and cystein was added as a reductant model, so mimicking the in vivo oxidative burst even if the actual reductant substrate remained elusive (*25*). A mechanism through which a peroxidase may generate H_2O_2 at neutral or basic pH has been postulated

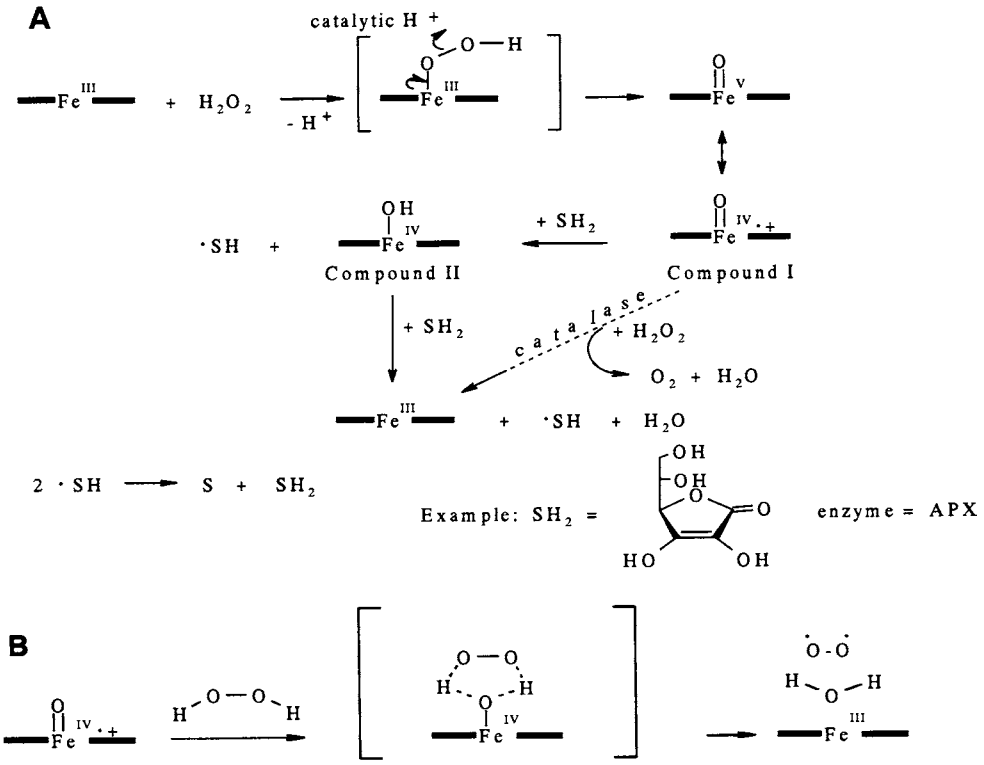

Figure 1. (A) Catalytic cycle of a peroxidase. The peroxidatic mechanism is exemplified for APX (SH_2 = ascorbic acid; SH• = monodehydroascorbyl free radical; S = dehydroascorbate). When H_2O_2 acts also as reducing substrate, the enzyme is a catalase (dashed arrow). (B) At the bottom a tentative visualization of H_2O_2 decomposition by compound I of catalase is given (the real mechanism is still open to discussion).

to involve a ferrous dioxygen intermediate (compound **III**), which requires a suitable hydrogen donor RH_2 as substrate, in the wall, to renew the ferric state of the enzyme:

$$[Fe^{III}-O-O^{\bullet}]^{2+}\ (\text{compound } \mathbf{III}) + RH_2 \rightarrow Fe^{III} + R^{\bullet -} + H_2O_2$$

Compound **III** may be generated when a free radical with good electron donor properties (e.g., NAD•) reduces O_2 to $O_2^{\bullet -}$ and the latter reduces the ferric state of peroxidase:

$$NAD^{\bullet} + O_2 \rightarrow NAD^{+} + O_2^{\bullet -}$$

$$O_2^{\bullet -} + Fe^{III} \rightarrow [Fe^{III}-O-O^{\bullet}]^{2+}$$

In addition to antimicrobial effects against bacterial and fungal pathogens, H_2O_2 has been credited with an important role in the oxidative cross-linking of structural proteins in cell walls of challenged plants, thus restricting pathogen development (*14*). Extracellular peroxidases are probably involved to mediate this process. Another effect triggered by the oxidative burst is the localized elicitation of phytoalexins, the antifungal properties of which are well-known.

Additionally, the oxidative burst taking place at the site of an immunizing inoculation has the effect of inducing systemic "micro-HRs". This implies the generation of a mobile signal able to cover relatively long distances, although its nature has not yet been determined (*14*).

The array of reactions elicited in plants as defense from pathogen invasion has been intensively investigated in past decades and recently aroused great interest with the discovery of sensitizers that can successfully stimulate the natural defenses even against virulent pathogens. The effects of these sensitizers include, in part, some of those elicited in the oxidative burst, but they extend well beyond the limits of time and space usually observed in the gene-to-gene HR.

The following paragraphs summarize the main outlines of this progress.

3. SYSTEMIC ACQUIRED RESISTANCE

In the past decade several authors have published evidence suggesting that salicylic acid (SA), a metabolite downstream of the biosynthetic pathway initiated by phenylalanine ammonia-lyase (PAL), plays the role of endogenous signal when plants are primed to resist pathogens. This evidence rests on different converging facts, the comprehension of which requires that the type of resistance involved, called systemic acquired resistance (SAR), be first defined. According to Hammerschmidt, this type of resistance develops locally or in distal parts of the plant in response to a pathogen that causes a necrotic lesion, this being the result of either an infection or a hypersensitive response (*26*). As a result, the plant acquires a sort of aspecific immunization against challenge infections, even though this protection is not transmitted through the seeds. From the point of view of the practical use of this acquired resistance, the most important fact which has recently emerged is that it can also be induced, or enhanced, by exogenous application of SA or synthetic compounds that may have similar or more powerful effects (*26*). Although originated, or extended, from a type of natural resistance against certain strains of a pathogen recognized by the plant through a gene-for-gene process, SAR is generally expressed against a broad spectrum of pathogens that may include viruses, bacteria, and fungi. Once established, SAR may last for a certain period of time (from weeks to months), during which any attempted invasion by a virulent pathogen is hampered as though the pathogen were an avirulent one.

Usually treatment with SA or a synthetic inducer does not necessarily produce the apparent biochemical changes typical

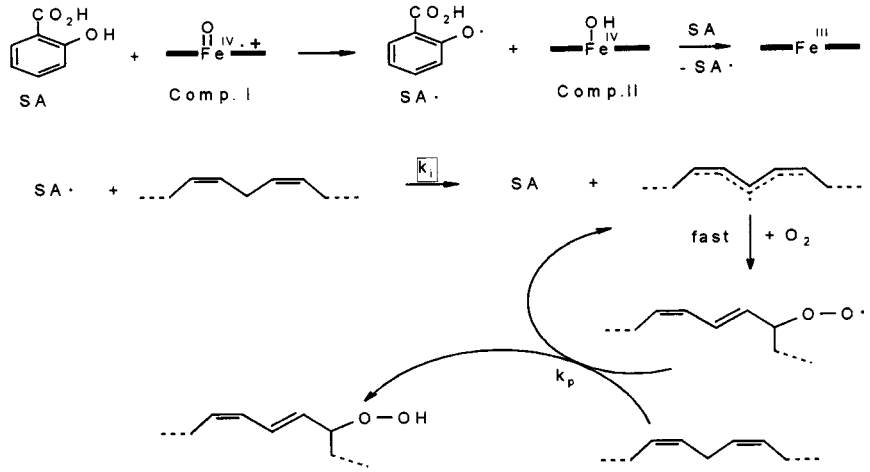

Figure 2. A single SA free radical, produced by catalase (or a peroxidase), may initiate the autoxidation of a large number of polyunsaturated side chains of membrane lipids (adapted from ref *35* with permission). Symbols: k_i = specific rate of initiation step; k_p = specific rate of propagation step; k_p[R–O–O•] assumed to be $\gg k_i$[SH•].

of SAR as long as the plant is not challenged with a pathogen. As soon as this occurs, the plant is primed to react more efficiently than without the treatment. As a typical example, it has been reported that cell cultures of parsley, after pretreatment with either SA or the synthetic inducer 2,6-dichloroisonicotinic acid (INA), gave rise to strongly enhanced production of coumarins and incorporation of phenolics into their cell walls only when challenged with elicitors from *Phytophthora megasperma* (*27*, *28*). This means that the phenylpropanoid biosynthetic pathway, which is responsible for the production of phytoalexins, lignins, and SA itself, is alerted to respond more rapidly to the pathogen challenge by a pretreatment with the chemical inducers. Lignification is a well-known natural mechanism by which the plant cell tries to resist pathogen invasion by setting up a barrier to its entry. At low concentrations, SA enhances the expression of defense genes, but, as kindly remarked by a referee, at higher concentrations, it can directly induce their expression. Molecular genetics offer important means to understand some crucial aspects of such effects. Transgenic plants of tobacco expressing the salicylate hydroxylase gene *NahG* are unable to accumulate SA because this enzyme catalyzes the decarboxylative hydroxylation of SA to catechol. These plants failed to express SAR (*29*). Other experiments showed that, when PAL is genetically suppressed or chemically inhibited, SAR is abolished (*10*). All of these facts support the crucial role of SA as a signal transducer between pathogen elicitation and plant resistance, even if the response of distal organs of the plant probably requires other, rapid translocating signals, which are still unknown.

The most distinctive expression of SAR is the rapid induction of several genes that remain latent as long as the plant is not challenged. Accumulation of SA in plant tissues, either as the effect of a necrotic lesion or, more potently, by pathogen challenge after exogenous application of the same inducer (or sensitizer), is followed by the expression of a set of genes encoding the so-called pathogenesis-related (PR) proteins. These include glucanases, chitinases, and peroxidases. Some of these proteins may have their own role in contrasting the development of fungal or bacterial pathogens via hydrolytic action on their cell walls. However, they are only part of the metabolic battery induced by SAR as shown by the finding that transgenic plants expressing PR genes do not necessarily become more resistant to all pathogens (*30*). Interrelated with the role of SA is the high endogenous level of H_2O_2 during the expression of SAR. Salicylic acid is a known chelator of iron and, thus, it may inhibit several heme-based enzymes including catalase. An increase in the concentration of H_2O_2 may be produced by this inhibition, which has also been reported to result from a binding of SA to a protein with catalase activity. This protein (SABP) was found to bind SA with an affinity (K_D = 14 μM) thought to be consistent with the physiological concentrations of SA during SAR (*31*). [Afterward, in TMV-infected tobacco leaves, SA levels were reported to increase 10−100-fold and range from 7 to 56 μM (*32*).] It was then proposed that SA-dependent elevated levels of H_2O_2, or reactive species derived from it, might act as systemic mediators leading to SAR (*31*).

This hypothesis was subsequently questioned by several authors on the basis of the following pieces of contrary evidence:

(a) SA binds aspecifically to iron-containing enzymes, but the endogenous concentrations of SA necessary for catalase inhibition (1 mM) are hardly ever reached within the plants (*33*).

(b) Artificially produced H_2O_2 was unable to induce PR-1 gene expression in NahG transgenic plants (*34*).

(c) As a consequence, H_2O_2 appears to act upstream of SA in the signal transduction cascade, rather than (or in addition to) acting downstream of SA.

Pursuing the aim of discovering significant effects of SA as a mediator of SAR, Klessig and co-workers obtained spectroscopic evidence that SA blocks the catalytic cycle of a peroxidase at the level, and with accumulation, of compound **II** (see **Figure 1**). Together with other results, this implies the formation of a free radical SA• in the interaction with both catalase and APX (*32*). Another notable observation was that, even at relatively low concentrations, SA and biologically active analogues are able to induce lipid peroxidation in tobacco cell cultures. This fact was considered to be strictly connected with the induction of PR-1 gene expression, because such activation was also induced by exogenous application of 13(*S*)-hydroperoxylinoleic and 13(*S*)-hydroperoxylinolenic acids. Conversely, the induction of PR-1 genes by SA was inhibited by diethyldithiocarbamic acid, a reagent that, among various other possible effects, is able to reduce hydroperoxides. In a study in which lipid peroxidation was circumstantially analyzed as a possible way to activate PR genes, Anderson, Chen, and Klessig proposed that an autoxidation of the polyunsaturated chains of fatty acids could be initiated by some free radical SA• (see **Figure 2**). They speculated that this may occur even in uninoculated leaves of infected plants of tobacco, where the

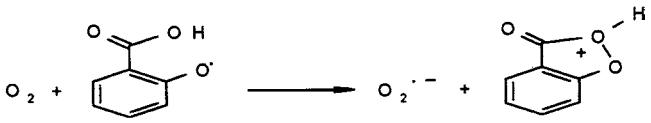

Figure 3. Role proposed for SA• as electron donor to O_2 (*37*).

SA levels are low (0.5−9 μM) and the increase of H_2O_2 level is weak (*35*).

These studies tend to reconcile the original view of SA-inhibited catalase with the arguments summarized in points a−c (above) and introduce a new hypothesis on the role of SA in the systemic part of SAR induction.

Independent studies by other laboratories support the view that the interaction of SA with peroxidases must produce transient, phenolic, free radicals with an important role in SAR establishment (*36*).

Recent experiments with cell suspension cultures of tobacco showed that SA induces a rapid generation of $O_2^{\bullet -}$ mediated by a peroxidase. Enhancement of reactive oxygen species, except for •OH, was in fact observed by the addition of horseradish peroxidase, the function of which was thought to be that of catalyzing the transient formation (from SA + a trace of H_2O_2) of a free radical SA•, which would then act as an electron donor to O_2 (see **Figure 3**). The resulting $O_2^{\bullet -}$ would then be converted to H_2O_2 and trigger an increase of cytosolic Ca^{2+} concentration (*37*).

Ca^{2+} is another important strategic signal in SAR and is involved in the activation of the oxidative burst culminating with local cell death.

In addition to the interactions with catalase and peroxidases discussed above, SA has also been shown to make a further specific binding of higher affinity with a soluble protein (SAPB2) in tobacco, which will be commented later on.

Despite the notable advances achieved in this field, the real mechanism by which SAR can be induced to successfully prevent plant infection by pathogens remains so far unknown. A tentative connection between the different metabolic branches involved in the hypersensitive response (HR) has been outlined in a recent review by Lamb and Dixon, and the related networks are reproduced in the scheme of **Figure 4** (*14*).

The oxidative burst can be impaired by the addition of indole carbazole alkaloids, such as K-252a and staurosporine, which are known to inhibit the phosphorylation of serine and threonine hydroxyl groups by related protein kinases. Therefore, a phosphorylation step must be involved as a link between elicitor perception and NADPH oxidase (*38*, *39*).

Other steps of phosphorylation/dephosphorylation are involved in the regulatory system controlling the expression of PR proteins, as mentioned later on.

According to the scheme of **Figure 4**, besides inducing PR protein genes, SA is assumed to act before the divergence of the two metabolic branches leading to the phenylpropanoid pathway and the activation of the oxidative burst, respectively. In such a way, SA amplifies its own synthesis, which, in addition, is stimulated by H_2O_2.

However, an alternative route to SA may also be available upstream of cinnamic acid. Recent evidence showed that, in the chloroplast of *Arabidopsis*, SA is synthesized from isochorismate through the following pathway: chorismate → isochorismate → SA + pyruvate. This route, typical of bacteria, seems to be required even in other plants to produce SA for defense against pathogens (*40*).

When induced by exogenous application of synthetic activators, SAR may benefit from more powerful effects with respect to the necrotizing infection, as reported in section 7 for individual inducers. Additionally, this treatment offers the opportunity to investigate the activation process in two distinct stages: the plant conditioning, taking place between the application and the inoculation, and the subsequent response to the pathogen attack. The first stage takes several days to prime the plant to dispose of all defenses available for an adequate reponse. From what is known so far, mainly by the use of plant cell suspension cultures, the chemical priming appears to play a double role: (1) It directly induces a first series of reactions, including activation of *PR* genes and synthesis of anionic peroxidase. (2) A second effect would consist of the progressive rise of hypothetical cellular components, able to act in cooperation with signals subsequently induced by the pathogen for the enhanced expression of other defense genes. These would include, for example, the PAL genes in parsley and *Arabidopsis* and those responsible for the callose deposits (*41*). Ionic fluxes across the membrane (H^+/K^+, Ca^{2+}) and oxidative burst are also enhanced, but hypersensitive cell death is not an usual event.

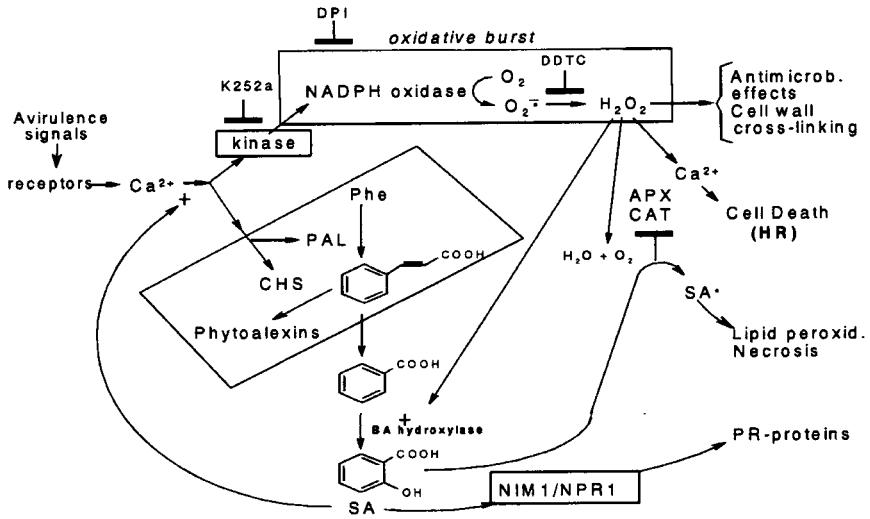

Figure 4. Signal networks in the HR (adapted with permission from ref 14). Abbreviations and symbols: APX, ascorbate peroxidase; BA, benzoic acid; CAT, catalase; CHS, chalcone synthase; DDTC, diethyldithiocarbamate; DPI, diphenylene iodonium; NIM1 = NPR1, protein mediating signal transduction for expression of *PR* genes; PAL, phenylalanine ammonia-lyase; Phe, phenylalanine; ⊥, impairment.

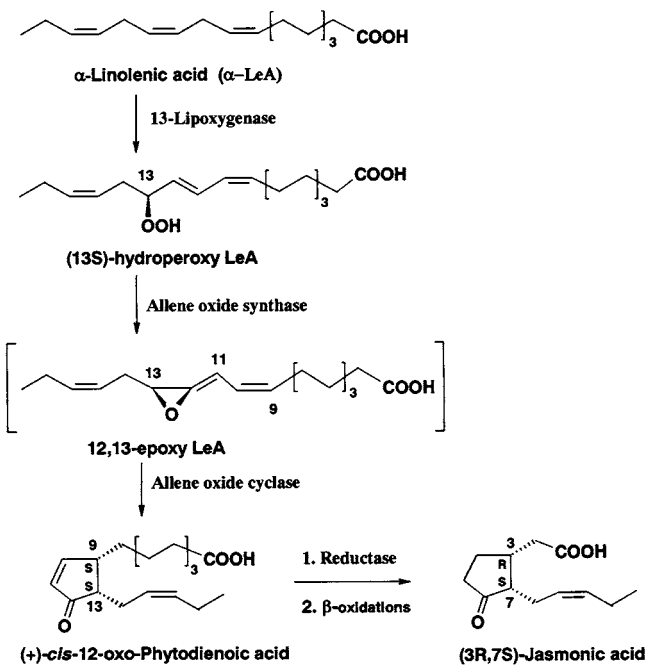

Figure 5. Biosynthetic pathway of jasmonic acid (reproduced in part from ref *48*).

4. JASMONATE AND ETHYLENE IN PLANT DEFENSES

Other signaling pathways involved in induced resistance have been shown to depend on jasmonic acid (JA) and ethylene. JA and its methyl ester have been mainly implicated as mediators of plant responses triggered by wounding and insect feeding, but their involvement in resistance against pathogens has also been proved. For example, parsley suspension cell cultures were primed by methyl jasmonate to respond with enhanced phenylpropanoid production to fungal elicitation (*42*). This effect was similar to those observed after pretreatments with SA or INA, but most of the studies on plant give evidence that the two pathways of SAR mediated by SA and JA must be clearly distinct, with general effects that may be antagonistic or additive (*43*).

One of the most notable natural defense phenomena is the so-called induced systemic resistance (ISR) elicited by root-colonizing bacteria through a pathway requiring cooperation of JA and ethylene. In recent studies, where this type of resistance was induced in *Arabidopsis thaliana*, the level of the two hormones was not increased with respect to noninduced plants, but was greatly enhanced upon pathogen attack (*44*). At the same time, the SA-dependent SAR appeared to be simultaneously activated, so providing an additive effect of the two pathways (*45*).

However, more frequently the two pathways seem to not act independently but rather affect each other, with potentially negative consequences when plants must cope with different enemies (*46*). Early evidence with tomato plants showed that SA, or its acetyl derivative aspirin, can impair the wound-induced gene expression generally induced by JA, and this impairment was ascribed to the inhibition of JA biosynthesis by SA (*47*).

Biosynthesis of JA follows a route (see **Figure 5**) starting with a regio- and stereospecific dioxygenation of α-linolenic acid (α-LeA) at C-13 (*48*). This is followed, among several other fates, by the transient formation of an unstable allene oxide (12, 13-epoxyLeA) under the action of an atypical P-450 enzyme, called allene oxide synthase (AOS). This intermediate may spontaneously degrade to ketols together with a minor cyclization to a racemic 12-oxo-(10,15*Z*)-phytodienoic acid. However, in the presence of a second enzyme, called allene oxide cyclase (AOC), the ring closure takes place exclusively and affords the enantiomeric 12-oxo-(10,15*Z*)-phytodienoic acid [*cis*-(+)-12-oxo-PDA] with a stereochemistry characterized by an *S*,*S*(*cis*)-configuration of the two stereogenic centers of the cyclopentenone ring (*48*). Interestingly, this compound was found to be, per se, a signaling factor in plant mechanotransduction as well as in defense gene expression (*49*). Reduction of the ring double bond and three rounds of β-oxidation finally afford jasmonic acid with retention of the configuration at the two stereogenic centers in the ring, named (+)-7-*iso*-jasmonic acid or (3*R*,7*S*)-JA.

More than one step of this biosynthetic pathway has been reported to be affected by SA, but the effects appear to be more complex than what a direct inhibition of enzymatic activities involved would suggest (see ref *46* and references cited therein). In wounded leaves of flax plants the gene expression of AOS and the JA accumulation were found to be blocked by SA (*50*). In contrast, in leaves of *A. thaliana* SA not only induced AOS transcript and protein accumulation but also triggered a steady increase in the levels of 12-oxo-PDA, with no apparent effect on the levels of JA (*51*). Moreover, in barley leaf segments the SA treatment induced a shift in the fate of (13*S*)-hydroperoxyLeA toward a reduction branch leading to the accumulation of (13*S*)-hydroxy LeA (*52*). At present the conflicting results in the interactions between the SA and JA patways and their consequences on induced defenses against pathogens and plant-feeding insects are not clearly understood.

With regard to ethylene, in addition to regulating many physiological processes in plant growth and development, it also appears to play a role following wounding, infection, or treatment with elicitors, in which its production is often associated with that of JA. Apparently, the transduction pathways of the two phytohormones can interact with each other in the coordinate expression of some defense-related *PR* genes. These, as well as the positive and negative interactions with the SA-dependent pathway, have been recently outlined in a review on the ethylene production and signaling systems (*53*). Key enzymes and precursors in the biosynthesis of ethylene may be involved in its controversial effects on SAR.

Ethylene biosynthesis is known to follow a route starting from methionine (Met) via *S*-adenosylmethionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) (see **Figure 6A**).

ACC synthase mediates the rate-limiting step of the process. It requires pyridoxal phosphate as an essential cofactor to facilitate the deprotonation of C-1 and thereby induce the intramolecular, nucleophilic displacement of methylthioadenosine (MTA), which then enters into a separate cycle to restore methionine. The second key enzyme of the process converts ACC to ethylene and has been the object of intensive research to be recognized as an oxidase that uses ascorbate as cosubstrate (*54*). Molecular genetics had an important role in the discovery of this enzyme, now called ACC oxidase, but its mechanism has not been clearly elucidated yet. According to an early suggestion by Yang, the ethylene-forming enzyme implies the N-hydroxylation of ACC to drive the fragmentation reactions (see **Figure 6B**) (*54*).

ACC oxidase is described as a nonheme ferrous protein, working best at pH 7.2, which is activated by CO_2, inhibited

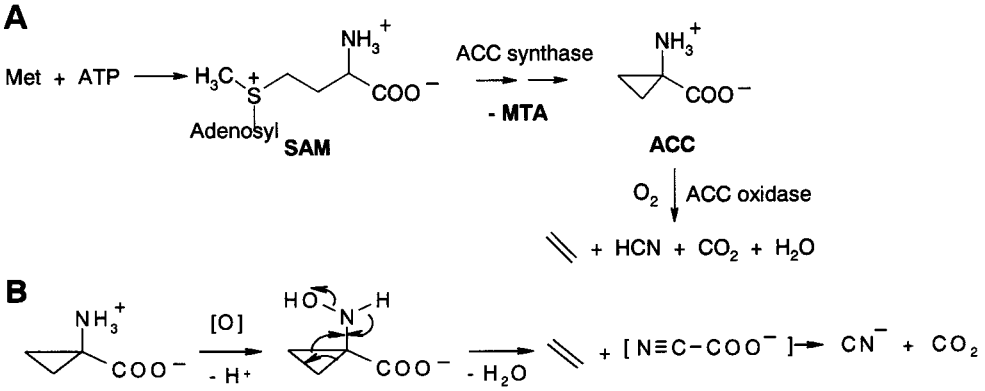

Figure 6. (A) Biosynthetic pathway of ethylene (symbols: ATP, adenosine triphosphate; Met, methionine; SAM, *S*-adenosylmethionine; MTA, methylthioadenosine; ACC, 1-aminocyclopropane-1-carboxylic acid). (B) Postulated mechanism for ACC oxidase and ethylene-forming reaction (*54*).

by iron chelators, free radical scavengers, and several metal ions (Co^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+}), and totally abolished by 0.8 mM H_2O_2 (*55*).

In sunflower cell suspensions, ethylene and the precursor ACC appeared to play opposite roles in the regulation of chitinase and β-1,3-glucanase triggered by the fungal elicitor Pmg, prepared from *Phytophthora megasperma* f. sp. *glycinea* (*56*).

5. PATHOGENESIS-RELATED PROTEINS

According to current recommendations, PR proteins are defined as proteins produced by the host plant under stress conditions. This definition does not credit them with a necessary role in the resistance to pathogens. Nevertheless, their induction in local as well as in distal parts of the injured or infected plant tissues and their association with the development of SAR suggest a probable role in this type of resistance. On the basis of amino acid sequence and properties, PR proteins have been classified into 14 families, denoted PR-1 to PR-14, in varying amounts detected in tobacco, tomato, cucumber, parsley, radish, *Arabidopsis*, and barley. Families from PR-2 to PR-14 have been characterized as being responsible for a specific function or enzymatic activity, including, for example, a β-1,3-glucanase (PR-2), chitinases (PR-3, PR-4, PR-8, PR-11), and a peroxidase (PR-9) (*9*).

Circumstantial evidence suggests for PR-10 a ribonuclease-like function. PR-10 proteins are homologous to tree-pollen allergens and have been found to cause allergic reactions (*57*). PR-1 proteins form the most important group and are abundantly induced in tomato and tobacco plants as homologous members with acid or basic properties. Surprisingly, their specific functions have not yet been clearly established. However, two basic PR-1 proteins isolated from the above-mentioned plants were shown to display high fungicidal activity against *Phytophthora infestans* both in vitro, through inhibition of zoospore germination, and in vivo (*58*).

General characteristics of PR proteins are their accumulation in extracellular or vacuolar spaces, stability to low pH values, and resistance to proteases.

Evidence that genes encoding PRs are expressed under a specific pathological condition or by a suitable elicitor is generally based on blot analysis of their related mRNAs and/or direct recognition of the induced proteins by monoclonal antibody methodology.

Accumulation of PR-1 proteins is currently regarded as the most reliable biochemical marker for SAR.

6. EXOGENOUS INDUCERS OF SAR

To be considered a true activator, or elicitor, of plant defense reactions in crop protection, a compound must fulfill the preliminary condition that no antimicrobial activity is to be displayed either by the compound itself or by its possible metabolites. Although the first of these criteria can be easily verified by in vitro tests, the second one is sometimes cumbersome. As a typical example, fosetyl-Al, $Al(PO_3)_3$, was long considered to act as an elicitor of defense reactions in plants until it was proved to release the fungitoxic H_3PO_3 as a metabolite in low-phosphate media (*59*). Nevertheless, it is fair to acknowledge that part of the protection exerted by certain systemic fungicides and other currently used pesticides has been proved to involve plant defenses triggered by elicitors released during their action on pathogens. According to an early example, defense mechanisms were suggested to contribute to the inhibition of *Phytophthora megasperma* in soybeans by metalaxyl as inferred from the production of glyceollin associated with the control (*60*). More recently, the antifungal activity of metalaxyl, fosetyl-Al, and $Cu(OH)_2$ was shown to depend, in part, on the SAR defense system when tested in *Arabidopsis* plants for the control of *Peronospora parasitica*. In fact, their activity was reduced in SAR-compromised NahG and nim1 plant. Together with other pieces of evidence, this suggested a contribution of the SA-dependent SAR for the exploitation of all their antifungal potential in wild-type plants (*61*).

Curiously, the activation of SAR was also found to occur when protoporphyrinogen IX oxidase (PPO), the enzyme target of a class of herbicides (*62*), was impaired. Plants with antisense PPO showed, in fact, resistance against *P. parasitica* according to activation of SAR (*63*).

Plant resistance activators can more properly be considered some old compounds with weak or no direct fungitoxic activity, which have been proved to potentiate defense responses. These include probenazole and 2,2-dichloro-3,3-dimethylcyclopropane carboxylic acid (DDCC), both of which protect rice against *Magnaporthe grisea*, and a synthetic amino acid endowed with a broader spectrum of action, β-aminobutyric acid (BABA) (see **Figure 7**). Literature up to 1987 on the mode of action of these and other non-fungitoxic compounds, including melanine biosynthesis inhibitors, has been reviewed by Sisler and Ragsdale (*64*).

Probenazole (Oryzemate). Probenazole appears to enhance some of the resistance mechanisms associated with the oxidative burst after infection by the rice-blast agent. Moreover, it induces the accumulation of unsaturated fatty acids acting as anticonidial factors (*9*). Rice plants conditioned with this compound have

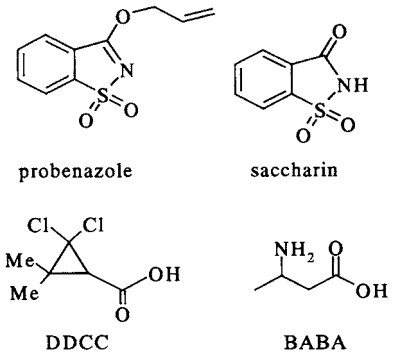

Figure 7. Early non-fungitoxic compounds that appear to control fungal diseases via enhanced resistance of the host plant.

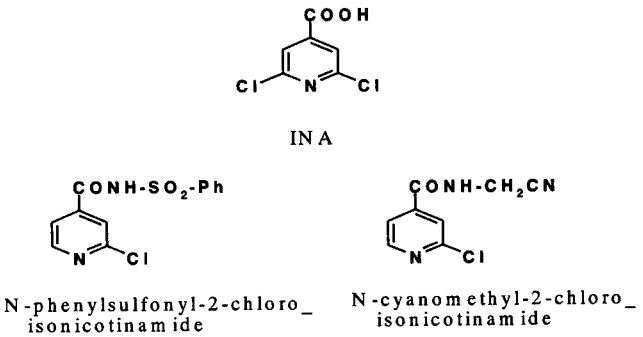

Figure 8. Structures of INA and analogues with similar activity as inducers of SAR.

been reported to react with rapid lignification to inoculation by *Pyricularia oryzae*. At the same time, peroxidase activities were strongly enhanced with respect to the control in response to an elicitor isolated from the fungal mycelium. Conversely, when lignin biosynthesis was impaired by inhibitors of PAL or of cinnamyl alcohol dehydrogenase, treated plants became more susceptible to the infection (*65*).

In cultured parsley cells, substances that are known as typical inducers of SAR have been shown to strongly enhance the production of furanocoumarin-phytoalexins in response to the addition of a low dose of an elicitor preparation from cell walls of *Phytophthora sojae*. Spectrophotometric measurement of coumarins absorbance has then been used in a screening system for potential SAR-inducing compounds (*66*). Unexpectedly, probenazole was not active in this test, but saccharin, which is a major metabolite of probenazole in rice plants, was found to be very effective. Saccharin was then proposed to be the active principle responsible for SAR induction nominally ascribed to probenazole in rice plants.

DDCC. DDCC has been found to induce suppression of hyphal development in rice plants as a result of enhanced accumulation of momilactones around the invasion site (*67*).

Peroxidase activities in response to elicitor application have also been observed in rice plants pretreated with this compound, similar to what was observed with probenazole (*65*). Another argument suggesting that DDCC acts through host plant defenses stems from the hypersensitive reaction of pretreated plants to picolinic acid, reputed to be a pathotoxin produced by *P. oryzae* (*68*).

(D,L)-3-Aminobutyric Acid. Racemic β-aminobutyric acid is a nonprotein amino acid, long known to protect pea plants against the oomycete *Aphanomyces euteiches* by soil drench application and more recently thoroughly studied by several research groups (*69*−*71*). It is endowed with a broad spectrum of activity against diseases caused by downy mildews as well as by necrotrophic fungi, bacterial, and viral pathogens and nematodes in several crops. The absence of direct toxicity on pathogens and the lack of metabolites in protected plants supported the belief that the compound must act as an inducer of SAR by virtue of its own structure. In tobacco, resistance against *Peronospora tabacina* was uniquely activated by the *R*-enantiomer of BABA (*70*).

Many experiments have been carried out with the aim to understand the mechanism of action of BABA, but the interpretation of the results was made difficult by the diverse effects observed depending on the plant and pathogen species, as well as on the application mode. When applied to noninoculated tomato plants as a foliar spray, it was found to rapidly enhance PR-1 accumulation (*72*). When applied to tobacco leaves at 10 mM, the compound was found to locally develop HR-like lesions as a consequence of the oxidative burst and related events (cell death, lipid peroxidation, and callose formation), followed by a local and systemic increase in SA content and expression of PR-1. The enhancement of resistance to TMV appeared to closely depend on the SA-mediated pathway as it was abolished in transgenic NahG plants (*73*). On the other hand, necrotic lesions were not observed when the inducer was injected into the tobacco stem, and no accumulation of acid soluble PR proteins was detected under these conditions, although the plants were well protected against infection by *P. tabacina* (*70*). The mechanism of systemic induced resistance in tobacco by this type of application was then inferred to differ from that induced by SA via PR protein accumulation. A similar behavior was observed after a soil drench application of BABA to *Arabidopsis* plants, which were induced to react with a fast HR and papilla formation when challenged with *P. parasitica* (*74*).

A surprising aspect for the mode of action of this inducer of systemic resistance is the statement that it may also have a curative effect (*70*). However, when applied to *Arabidopsis* after *P. parasitica* had colonized the leaves, no curative effect was observed (*74*).

INA. More similar to the mode of action of salicylic acid is the protection afforded by 2,6-dichloroisonicotinic acid (INA) and some of its derivatives (see **Figure 8**) to various dicotyledonous and monocotyledonous plants against a wide spectrum of pathogens (*10*). INA induces the expression of SAR genes, sometimes before the challenging inoculation and, in other cases, after pathogen attack only. It acts independently of the presence of SA, and this is the most notable distinction between the two compounds. INA, indeed, is still effective in NahG plants unable to accumulate SA. This indicates that, in the sequence of events leading to SAR, it acts at, or downstream of, the site sensitive to SA.

The use of INA as an inducer of SAR in mutant screens led to the discovery of a regulatory gene essential for SAR signal transduction (*75*, *76*) (see section 7).

BTH. Further screening by Novartis singled out the methyl ester of benzo(1,2,3)-thiadiazole-7-carbothiolic acid (BTH, acibenzolar-*S*-methyl) as a particularly efficient inducer of SAR at doses low enough to avoid phytotoxic effects (*77*). This compound was developed as an immunizing agent to sensitize various crops against pathogen infections. Its mode of action shares with INA the property of activating SAR downstream of SA (*76*). A structure−activity relationship, involving a high number of analogues, showed that the activity is substantially restricted to the benzothiadiazole skeleton bearing a carboxyl group, either free or esterified, on position 7 (see **Figure 9**). The phenyl ring can be replaced by a pyridine one as long as the N is located in suitable positions: it may replace 4-CH or

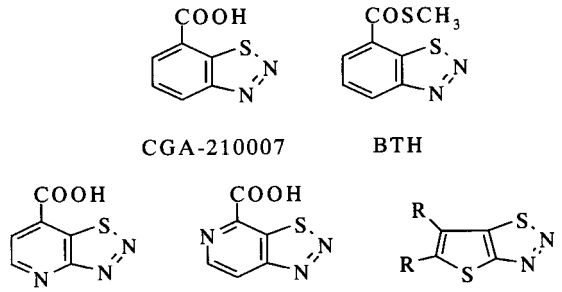

Figure 9. Structures of benzo(1,2,3)thiadiazole-7-carboxylic acid derivatives and analogues.

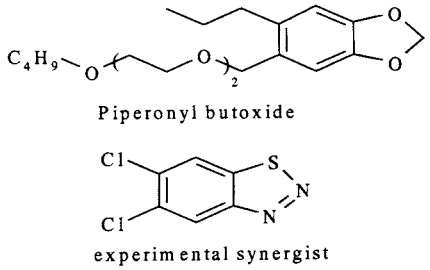

Figure 10. Structure of representative insecticide synergists that inactivate microsomal oxidases by reacting with the heme of cytochrome P-450.

6-CH but, when it replaces the 5-CH position, the activity is lost (*78*). Recently some thieno[2,3-*d*][1,2,3]thiadiazole-6-carboxylate derivatives have also been synthesized as bioisosteric models of BTH (*79*).

Three decades ago a number of benzo(1,2,3)thiadiazoles were extensively studied as synergists of insecticides. This type of action rests on their ability to inhibit insect mixed-function oxidases, preventing the oxidative inactivation of insect control agents. The best-known prototype of this synergistic activity is piperonyl butoxide, the structure of which contains the methylenedioxyphenyl ring (see **Figure 10**).

Similarly to the 1,3-dioxol ring, the 1,2,3-thiadiazole moiety was thought to interact with the active site of the cytochrome P-450 of the microsomal oxidases, thus inhibiting their enzymatic turnover (*80*−*82*).

However, the structural requirements for the microsomal oxidase inhibition by the benzo(1,2,3)thiadiazoles differ remarkably from those necessary for the SAR induction activity by the BTH analogues. A large number of substituents are tolerated on the benzene ring of the former class, whereas the activity of the latter is strictly dependent on the presence of a 7-carboxylic group (*78*).

Nevertheless BTH, together with INA and SA, has the potential for interacting with heme, or nonheme, iron-containing enzymes. In fact, BTH, SA, and INA were all found to inhibit catalase as well as ascorbate peroxidase (APX). At first, these inhibitions appeared to suggest an increase of the levels of H_2O_2 and other reactive oxygen species that could serve as second messengers of SAR (*32*, *83*). However, as discussed earlier, other studies by the same and other authors brought forth evidence that H_2O_2 acts upstream of SA, and the view of the role played by SA was adjusted in favor of that of a slow, alternative, reducing substrate for these enzymes (*84*−*86*).

By analogy, it may tentatively be remarked that the structure of BTH bears elements which might predispose it to serve as a similar reducing substrate for enzymes of this type.

In a recent study, the fate of BTH was investigated in tomato leaves together with the systemic protection it affords against *Pseudomonas syringae*. BTH was found to rapidly translocate to apical leaves and totally disappear 3 days after application. The free carboxylic acid (CGA 210007) was detected as a metabolite in the treated leaves and found to degrade at a similar rate. Infection degree and bacterial viability were reduced at best 7−8 days after inoculation of plants pretreated, 5 days earlier, with either BTH or its acid analogue. Because protection was observed long after both compounds had been degraded, the activation of plant defense was tentatively ascribed to an unfavorable environment created in the intercellular space by the treatments (*87*).

In tobacco plants BTH was reported to induce systemic resistance against a broad spectrum of pathogens, including *Cercospora nicotianae*, *Peronospora tabacina*, *Phytophthora parasitica*, the bacterial agent *Pseudomonas syringae*, and the tobacco mosaic virus (TMV). On the contrary, it failed to induce resistance against the necrotrophic fungi *Alternaria alternata* and *Botrytis cinerea*. This spectrum of pathogen control coincided with that observed in SAR induced by local pre-inoculation with TMV. A number of genes that code for PR proteins were coordinately expressed in tobacco leaves 7 days after treatment with BTH and reached the maximum level of *PR-1* mRNA accumulation at a BTH concentration of ∼36 μM. As previously shown with SA, this expression of the *PR-1* gene was blocked by cycloheximide, suggesting that a de novo protein synthesis was required for activation (*77*).

In *Arabidopsis thaliana*, expression of *PR-1*, *PR-2*, and *PR-5* genes was detected, with maximum accumulation of related mRNAs 1 day after treatment with 0.3−1 mM BTH. Resistance was then activated against *Peronospora parasitica* and *P. syringae*. Accumulation of *PR-1* mRNA and resistance to *P. parasitica* were also induced in transgenic plants expressing the *nahG* gene, which indicated that, as for INA, BTH activation does not depend on accumulation of SA (*88*).

Wheat was protected against powdery mildew by treatment with 30 g of BTH/ha. Five new genes, indicated as WCI-1−5 (for wheat chemically induced), were strongly activated a few days after inoculation with *Erysiphe graminis*. Two of them, WCI-1 and WCI-4, appeared to encode a lipoxygenase and a cysteine proteinase, respectively. Induction of the WCI genes was much greater in wheat plants treated with 0.3 mM BTH or INA than with 3 mM of SA, and the time course of their transcript levels appeared to be correlated with the protection efficacy in the order BTH > INA ≫ SA. Although activated even in plants grown and treated under sterile conditions, the WCI genes were considered as pertaining to the PR superfamily (*15*). However, PR-1 genes, which are generally considered the best markers for SAR, did not respond in wheat to any of the three above-mentioned compounds (*89*).

In some plants a dose-dependent growth reduction following treatment with BTH has been observed, with effects similar to those caused by sterol biosynthesis inhibitors (*90*).

In a very recent work, attention was given to the early biochemical effects produced by BTH (0.3 mM) in leaves of French bean primed to resist *Uromyces appendiculatus*. An oxidative burst was found to take place, with high levels of H_2O_2 and peroxidase activity. Interestingly, no evidence of cell death emerged after any of the applications with BTH effective concentrations. Neither was cell death observed after challenging inoculation with the pathogen, indicating that resistance to bean rust had been induced through a manner not mimicking an incompatible interaction (*91*).

Riboflavin. The soluble vitamin riboflavin was found to display properties similar to those of the synthetic SAR inducers, and its foliar applications control several diseases of tobacco (*92*). In addition, mixtures of riboflavin with methionine, metal

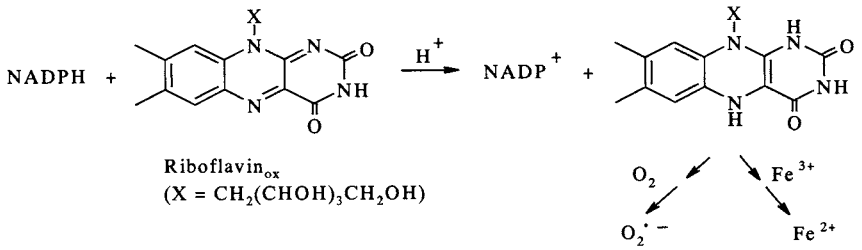

Figure 11. Redox-type reactions of riboflavin.

ions, and surfactant reduce powdery mildew infection in strawberry plants (*93*). Systemic resistance of *Arabidopsis* plants to *P. parasitica* and of tobacco to TMV and *Alternaria alternata* was most strongly activated when the plants were inoculated 4−5 days after treatment with 0.5 mM riboflavin (*94*).

Riboflavin is a known photosensitizer and may produce singlet oxygen or, in the presence of reducing substrates, oxygen superoxide. Part of the resistance of rice to rice blast by exogenous application of riboflavin appeared to be photoactivated, suggesting a mediation of oxygen reactive species via a hypersensitive reaction (*95*). Moreover, because NADPH-oxidase is a flavoprotein containing FAD, it is conceivable that riboflavin might potentiate the oxidative burst through redox-type reactions, as outlined in **Figure 11**. Interestingly, riboflavin was reported not to cause microscopic cell death in plants at rates that induce resistance in tobacco and *Arabidopsis*, so it was stated to activate resistance mechanisms in an HR-independent manner (*94*).

Chitosan. Oligomers of chitosan result from chemical or enzymatic deacetylation of chitin, the linear polymer of (1→4)-β-linked *N*-acetyl-D-glucosamine. The processes may afford products differing in both the average molecular weight and the extent of acetylation but sharing the property to assume polycationic nature under physiological conditions.

When in contact with *Fusarium oxysporum*, antimicrobial properties of chitosan have been described as typical structural and molecular alterations induced to fungal cell walls and regarded as disturbance effects in the regulation of chitin synthase (*96*). Partial deacetylation of chitin to chitosan has been observed in cell walls of *U. maydis* under the antifungal action of an ergosterol biosynthesis inhibitor (*97*).

On the mere basis of these in vitro effects, chitosan would not fulfill the most important criterion for being considered a plant resistance inducer. It is included in this review due to the growing body of evidence that it can effectively act as an elicitor capable of sensitizing susceptible plants to react to pathogen attack by accumulation of callose and phenolics. Chitosan has been studied, together with other products of fungal and non-fungal origin, as an elicitor of the transient production of H_2O_2 by abraded cucumber hypocotyls, a process highly enhanced by preconditioning the hypocotyls with SAR inducers (*98*).

Partially acetylated chitosans, when injected into wheat leaves, have been shown to elicit both PAL and peroxidase activity together with an increase of the lignin content in the cell walls and the appearance of necrotic symptoms (*99*). A number of studies have shown the induction of local and systemic acquired resistance against viral infections in chitosan-treated plants, with efficacy depending more on plant species than on type of virus. Histochemical and cytochemical observations on bean leaves revealed a homogeneous network of HR-like microscopic lesions, following chitosan treatments, which induced resistance to challenge by tomato bushy stunt tombus virus. This protecting effect was evident in both treated and, although only partially, even upper untreated leaves (*100*). A potent induction of JA has been observed from 15 to 60 min after treatment of rice leaf segments with a 0.1% chitosan solution, implying a role for JA downstream of a "pathogen-derived" signal (*101*).

The antiviral activity of chitosan in animals, plants, and microorganisms has been recently reviewed (*102*).

7. SIGNALING PATHWAYS BETWEEN INDUCER SENSITIZATION AND PR PROTEINS

The activation of the genes that encode the pathogenesis-related (PR) proteins is probably the most intimate and peculiar aspect of SAR. At present the possible pathways from inducer sensitization up to expression of these genes are actively pursued in plants where the genetics of the PR proteins are more conveniently investigated. Several authors made use of *Arabidopsis* mutants containing recessive mutations in a regulatory gene, known as *NIM1* or *NPR1*, to probe its role in the signal transduction of SAR. These mutants, called *nim1* (= *n*on inducible *im*munity) and *npr1* (= *n*on expresser of PR genes), showed normal, pathogen-induced accumulation of SA, but greatly reduced induction of genes PR-1, after treatment with all chemical inducers (*75*, *76*). As a matter of fact, *nim1*/*npr1* plants could be considered to be "immune compromised" with respect to SAR because they did not respond to these treatments by activating resistance to pathogens as would occur in wild-type plants (*103*). The *Arabidopsis NPR1* gene was mapped by Cao et al. and found to code for a 65-kDa protein (NPR1) containing "ankyrin repeats", which were disrupted in *npr1* mutants (*104*). The amino acid sequence of the protein encoded by the *Arabidopsis* gene *NIM1* was independently investigated by Ryals et al. and found to be identical to that encoded by *NPR1*, suggesting that the two genes were allelic (*105*). Both working groups emphasized the fact that the protein NIM1/NPR1 showed a partial, but significant, structural homology to the mammalian proteins I_kB_α, which act as inhibitors of the transcription factor NF-$_k$B. When phosphorylated by a kinase complex, these proteins are known to release the transcription factor enabling its translocation into the nucleus of animal cells, where it activates the defense gene expression involved in various pathogeneses (*104*).

Suggestive, as it is, of a common origin or design, the partial homology between NPR1 and I_kB_α raises the interesting question of how they apparently respond to the same chemical signal but trigger opposite effects: in mammals salycilate and aspirin act as anti-inflammatory drugs and SA has been reported to prevent the activation of defense reactions triggered by the I_kB/NF-$_k$B transcription (*106*). To the contrary, in plants SA activates the transduction signal depending on NPR1 and elevates oxygen reactive species, which are typical of HR, the counterpart of animal tissue inflammation.

The real function of NPR1 has not yet been fully understood. According to Cao et al., "it might affect the expression of PR genes indirectly by regulating the nuclear localization of a transcription factor through a protein−protein interaction medi-

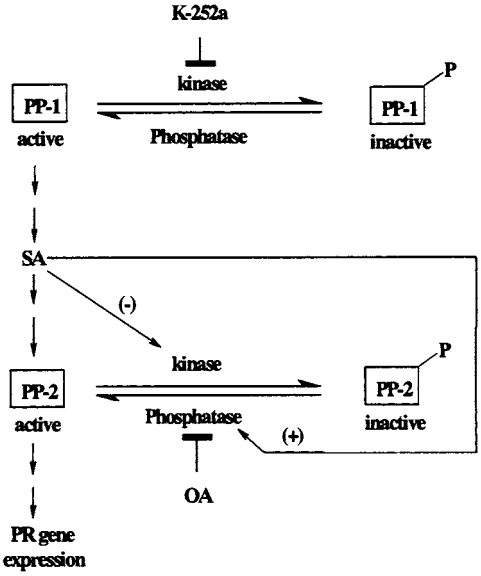

Figure 12. Tentative scheme showing involvement of two phosphoproteins (PP-1, PP-2) in the signal transduction pathway leading to PR gene expression (reproduced with permission from ref *113*; copyright 1997 Blackwell Science Ltd.).

ated by the ankyrin repeat motifs" (*104*). Subsequent work showed that, unlike the I_kB protein, which serves in animals as a repressor of gene expression, NPR1 accumulates in the nucleus of plant cells in response to SAR activators, and this localization is essential in inducing *PR* genes (*107*).

Other authors have circumstantially shown that SA and its transcriptional regulator gene *NPR1* can modulate opposing signals that promote or repress cell growth as well as cell death. For example, genetic and phenotypic studies with an aberrant double mutant of *Arabidopsis* revealed a role for *NPR1* in suppressing hypersensitive cell death (*108*).

In recent years, experimental evidence has been building up indicating that protein phosphorylation or dephosphorylation must take place when plants are induced to manifest an oxidative burst or a hypersenstive response (*109*, *110*). Moreover, functional protein kinases were found to be coded by a disease-related gene in tomato (*111*, *112*). In addition, various protein kinases appear to be involved in the signal transduction process leading to SAR and PR-1 gene expression in tobacco.

In a study based on suitable inhibitors of phosphorylation/dephosphorylation, Conrath, Silva, and Klessig addressed the way in which these types of enzymes might be implicated in SAR (*113*). Induction of PR-1 genes was shown to be enhanced by the protein kinase inhibitor K-252a, an indole carbazole alkaloid, and blocked by phosphatase inhibitors such as okadaic acid (OA). Some counteracting effects of these inhibitors, such as stimulation of SA production by K-252a and suppression of K-252a-induced PR-1 gene expression by OA, led the authors to postulate a signal transduction pathway based on two phosphoproteins. According to the scheme they proposed (see **Figure 12**), one of these phosphoproteins (PP-1) acts upstream of SA, whereas the other (PP-2) is SA-inducible and leads to PR gene expression. Both phosphoproteins are subject to equilibria between an active and an inactive form. The latter is produced by a phosphorylation of the former, which is catalyzed by a protein kinase. The reaction is reversed by phosphatase-mediated dephosphorylation. Such a scheme would explain the effect of K-252a in the stimulation of SA and in the PR gene induction. K-252a is a known, potent inhibitor of plant and animal phosphorylation of serine and threonine residues by protein kinases. Its action would move the equilibrium between the two forms of PP-1 toward the dephosphorylated active one. The active form of the second phosphoprotein, PP-2, would be suppressed by phosphatase inhibitors, such as OA, explaining the block of PR-1 genes induced by SA (*113*).

This study left open several questions. In particular, it was not clear whether SA was acting through signals generated by its inhibition of iron-based enzymes or through another SA-binding protein. In subsequent papers from the same laboratory, two novel proteins were reported to be involved in SAR. One of these was discovered by Du and Klessig as a soluble, high-affinity SA-binding protein of 25 kDa, present in small amounts in tobacco leaves (*114*). It was found to reversibly bind SA with an apparent dissociation constant of 90 nM and an affinity 150-fold greater than that between SA and catalase. Experiments carried out with labeled SA bound to this new protein (called SABP2) showed that the competition by most analogues of SA for the binding site correlated with their ability to induce defense gene expression and enhanced resistance. Accordingly, BTH was the strongest competitor, with an $IC_{50} = 0.1\ \mu M$, whereas unlabeled SA exhibited an IC_{50} of 1.5 μM. INA was a poor competitor ($IC_{50} = 70\ \mu M$) (*114*) (see **Table 1**). In further work, a 48 kDa protein kinase was found to be rapidly and transiently activated by SA treatment. It was identified as a member of the mitogen-activated protein (MAP) kinase family, and subsequent studies showed it was also involved in multiple signaling pathways that included eliciting stimuli from pathogens and injuries (*115*).

A negative regulation of SA-inducible defenses was uncovered in a MAPKK kinase by Frye et al. starting from a recessive mutation of *Arabidopsis*, which made the defense responses more easily induced by a virulent pathogen (*116*, *117*). This mutation, called *edr1* (for enhanced disease resistance), was found to deprive the plants of a constitutive expression of the *PR-1* gene, although it was more rapidly induced 3 days after inoculation with *Erysiphe cichoracearum*, which was blocked just before formation of conidiophores. Genetic investigation led to *EDR1* gene identification, showing it encodes a putative

Table 1. Comparison of Some Biochemical Assays of SAR Inducers with Their Induction of PR-1 Genes in Tobacco

	inhibition of APX[a]	inhibition of catalase[b]	competition for binding to SABP2[c]	inhibition of BTH-binding protein kinase[d]	maximum fold induction of *PR-1* genes	
compound	IC_{50}[e] (μM)	IC_{50} (μM)	IC_{50} (μM)	IC_{50} (μM)	Ind C_{50}[d,f] (μM)	Ind C_{50}[b,f] (μM)
SA	78	>100	1.5	150	3.4	65
INA	95		70	20	10	
BTH	145[b]	48	0.1	>1000	0.2	2.5
CGA 210007				90	0.2	

[a] Reference *32*. [b] Reference *83*. [c] Reference *114*. [d] Reference *118*. [e] IC_{50} = concentration required to inhibit by 50% the activity of APX[a](*32*) or catalase[b] (*83*), the binding of labeled SA to SABP2[c] (*114*), or the kinase activity of a BTH-binding protein[d] (*118*). [f] Ind C_{50} = concentration required for half-maximum induction of *PR-1* in tobacco.

mitogen-activated kinase kinase kinase (MAPKKK), assumed to play the role of a negative regulator of SA-inducible defense responses. Mutant *edr1* caused a loss of *EDR1* function by cutting off the kinase domain from the EDR1 protein, and this apparently allowed the plant to turn on the SA-associated defenses when elicited by pathogen-associated signals (*116*, *117*).

On the basis of a possible analogy between SAR and the mechanisms of defense-gene expression in mammals, protein kinases extracted from tobacco were investigated as possible mediators between SAR inducers and NIM1. In a study based on affinity chromatography, these kinases were selected by elution on a column of Sepharose bound via epoxide to the 5-hydroxy derivative of 1,2,3-benzothiadiazole-7-carboxylic acid. A BTH-binding protein kinase (called BBPK) able to phosphorylate the NIM1 protein as possible substrate was isolated. Although its functional role in vivo was not ascertained, the selectivity of its enzyme activity for certain types of proteins, including I_kB_α and NIM1, was thought to suggest a possible role of BBPK in the regulation of NIM1 (*118*). The main aim of this work was to search for a putative target site of SAR inducers. Therefore, various SAR inducers and their analogues were assayed as inhibitors of the kinase activity of BBPK in vitro, and their corresponding IC_{50} values were compared with those for the PR-1 induction in tobacco. However, no correlation was found between the two effects. Surprisingly, INA was found to be the most effective inhibitor of the cell-free activity of BBPK, with an IC_{50} ($= 20\ \mu M$) in the same range as that for in vivo PR-1 induction. In contrast, BTH, a much stronger inducer of PR-1 than INA, had a poor inhibiting effect on the kinase activity of BBPK, which, however, was moderately inhibited by the free carboxylic acid analogue CGA 210007 ($IC_{50} = 90\ \mu M$). SA was an even weaker inhibitor of the BBPK activity ($IC_{50} = 150\ \mu M$). In conclusion, BBPK was suggested as the most probable target site of INA but apparently not the primary target of BTH and SA.

8. PROSPECTS AND COSTS

After many wrong or uncertain starts, the idea of protecting crops by chemical activation of their own defenses appears to have a chance of being put into practice. The progress achieved with compounds emulating the role of endogenous inducers indicates that these treatments may lead to acceptable results in the context of integrated pest management. They meet the prospects of integrating, or limiting, the use of traditional pesticides in favor of nonbiocidal, presumably nontoxic, low-rate products. As such, these should enjoy an easier route to registration and might be better accepted by farmers and public opinion. More importantly, they should be less prone to generate resistance by pathogens than the systemic fungicides.

Increasing knowledge of the genetics involved in chemically induced resistance might also offer a bridge toward new strategies for crop protection, based on crops that in the future could be genetically engineered to constitutively express the traits of resistance against pathogens. However, some drawbacks that may be associated to induced resistance cannot be ignored, especially in a context of transgenic approaches. As already mentioned in section 4, when different types of attackers, such as insects and pathogens, simultaneously occur in the field, potentially tradeoffs may arise from negative interactions between the two defense pathways mediated by JA and SA, respectively. These conflicts may potentially increase vulnerability to insects when plants are induced to express SAR to pathogens (*119*).

A more general question entailed from induced resistance is whether it incurs fitness costs. This term is intended to cover all costs that may affect the fitness of resistant plants with respect to less resistant ones when compared under enemy-free conditions. These costs are difficult to evaluate and require a great deal of special indoor and field experiments. So far they have been extensively studied and often found to be relevant in applications of JA and its methyl ester against phytophagous insects, but recent evidence suggests that also SA-dependent SAR might incur fitness costs (*120*). Several mutants of *Arabidopsis* expressing constitutively elevated levels of SA exhibited stunted phenotypes. A particularly severe dwarfism was observed in *Arabidopsis* plants manipulated by an engineered bacterial salicylate synthase working via isochorismate, which showed high levels of SA and enhanced resistance to *Peronospora parasitica*. This dwarfism was tentatively ascribed to a depletion of the chorismate/isochorismate pools in the chloroplasts rather than to SA itself (*121*). In fact, in a similar experiment, other authors tested transgenic tobacco plants in which the enzymes for the synthesis of isochorismate and its lyase had been separately targeted to chloroplasts. These plants accumulated up to 1000 times more SA than control plants without deleterious effects on plant growth. However, when only the second of these enzymes, isochorismate pyruvate lyase, was expressed, the plants showed severely retarded grow (*122*).

Reduced growth and seed set were also observed in potted wheat plants treated with BTH and grown under limited nitrogen availability. The result was consistent with a resource allocation cost associated with resistance elicitation (*123*). In other words, fitness costs of chemical induction of SAR seem to become evident only when the elicitor is applied under conditions in which processes involved in plant growth are forced to compete with the synthesis of defense-related compounds for limited resources.

A further note worthy of great caution comes from the finding that many PR proteins display allergenic properties. Plant-derived allergens have, in fact, been identified with sequence similarity to PR protein families 2−5, 8, 10, and 14. Most of these groups have been reported to contain food allergens (*124*). If constitutively expressed by genetically modified plants, instead of being induced only after contact with a pathogen, they might involve a further unacceptable cost.

In view of the above-mentioned drawbacks and uncertainties, more basic research is needed to assess if the principle of an engineered constitutive disease resistance can really be applied with benefits overcoming the inherent costs.

9. FINAL REMARKS

Research in the field of new activators of SAR is just entering its infancy and, to proceed on solid bases, it needs a better understanding of how the plant−pathogen relationships may be affected in the activated tissues. A rational approach to synthetic inducers of SAR requires knowledge of the target on which their efficacy depends, but the symptoms so far detected rather suggest that elicitation of SAR might depend on multiple targets. This complicates the approach. The capability of acting as potential activators of plant-defense responses to both biotic and abiotic challenges is not infrequent even among systemic fungicides, as already observed with metalaxyl in soybeans and, more recently, with tetraconazole in corn plants (*60*, *125*). The peculiarity of an ideal inducer of SAR is that it should activate only the defense genes required to arrest the pathogen development when this is challenging the plant, without inducing undesirable side effects. However, the identification and expres-

sion of all "right genes" is a formidable task, as the intense research on *Arabidopsis* genetics is showing. Recent papers on this topic have gathered growing evidence that PR expression and disease resistance may also be activated by *NPR1*-independent pathways (*126*). This indicates the presence of different signaling networks and stresses the complexity of the interactions between diverse host defense pathways.

Molecular genetics and mutants are powerful tools to understand resistance mechanisms, but great caution is needed to avoid unnatural scenarios (*12*). If not properly integrated with biochemical and phytopathological insights, they may rather raise questions than give answers. A better knowledge of the molecular mechanisms responsible for the "priming" is clearly needed and has been recently invoked (*127*). Progress in this area is expected to offer a substantial, rational contribution to the chemical approach in crop protection.

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