Achieving Systemic Acquired Resistance In Sports Turf

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INTRODUCTION

Plants have evolved a number of inducible defense mechanisms against pathogen attack. Recognition of a pathogen often triggers a localized resistance reaction, known as the hypersensitive response (HR), which is characterized by rapid cell death at the site of infection. In the 1960s, research showed that tobacco plants challenged with tobacco mosaic virus (TMV) subsequently developed increased resistance to secondary infection in distal tissues. This spread of resistance throughout the plant's tissues was termed systemic acquired resistance (SAR). We now know that SAR can be activated in many plant species by pathogens that cause necrosis, either as part of the HR or as a symptom of disease. The resistance conferred is long-lasting, sometimes for the lifetime of the plant, and effective against a broad-spectrum of pathogens including viruses, bacteria, fungi, and oomycetes. More recently, within the last 5 years or so, research has shown that it is possible for turf grass plants to achieve systemic acquired resistance.

Molecularly, SAR is characterized by the increased expression of a large number of pathogenesis-related genes (*PR* genes), in both local and systemic tissues. PR proteins were first described in the 1970s by Van Loon, who observed accumulation of various novel proteins after infection of tobacco with TMV. Although many PR proteins have antimicrobial properties in vitro, the function of each in the defense response has not been clearly defined. It is generally thought that SAR results from the concerted effects of many PR proteins rather than a specific PR protein. Although their roles in establishing SAR are unclear, *PR* genes serve as useful molecular markers for the onset of SAR.

In 1979, White observed that PR protein accumulation and resistance to TMV could be induced by treatment of tobacco with salicylic acid (SA), aspirin (acetyl SA), or benzoic acid. Evidence that SA is a signal for the induction of SAR came from two studies published in 1990. Studies showed that the endogenous SA concentration rises in both local and systemic tissues after infection of tobacco with TMV and this rise correlates with *PR* gene inducticion. Researchers found that cucumber plants infected with either *Colletotrichum lagenarium* or tobacco necrosis virus (TNV) have considerably elevated levels of SA in the phloem sap. In a search for SA analogues that were less phytotoxic than SA, 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole S-methyl ester (BTH) were found to induce the same set of *PR* genes. A requirement for SA as an endogenous signal for SAR was proven by Gaffney et al. using a bacterial gene, *nahG*, encoding salicylate hydroxylase, which removes SA by conversion to

catechol. Transgenic tobacco and *Arabidopsis* expressing *nahG* accumulate very little SA after pathogen infection, fail to express *PR*genes, and are impaired in SAR (17, 35).

In the past 10 years, genetic analyses in the model plant *Arabidopsis* have identified additional components of SAR downstream of SA. Plants that are nonresponsive to SA were identified in a number of mutant screens and found to have mutations in the same gene, *NPR1/NIM1* (*NON-EXPRESSER OF PR GENES1/NONINDUCIBLE IMMUNITY1*). Considerable progress has been made in elucidating the role of NPR1 and associated proteins in the induction of SAR since the last Annual Review on SAR in 1997. We therefore focus on these recent molecular and genetic experiments that have contributed to our understanding of SAR.

NATURE OF THE SYSTEMIC SIGNAL

Early grafting experiments demonstrated that the infected leaf produces a systemic signal for SAR, and this signal is not species specific. The nature of the systemic signal has been a subject of controversy for many years.

Salicylic Acid

The detection of increased SA levels in systemic leaves and in the phloem led many researchers to believe that SA might be a systemic signal for SAR. The evidence for and against this hypothesis has been the subject of previous reviews. Labeling studies in TMV-infected tobacco showed that most of the SA (69%) accumulating systemically was made and exported from the inoculated leaf. Similarly, in cucumber infected with TNV, SA found in systemic leaves was both imported from the infected leaf and synthesized de novo. A more recent study suggests that signaling might occur through the conversion of SA to the volatile compound methyl salicylate, which could induce resistance not only in the uninfected parts of the same plant but also in neighboring plants.

A number of experiments argue against SA being the systemic signal. Detachment of *Pseudomonas syringae*-infected cucumber leaves before SA levels had increased in the petiole did not block the development of SAR. Furthermore, grafting experiments in tobacco between wild-type scions and *nahG*-expressing rootstocks showed that, although the rootstock was unable to accumulate SA, the SAR signal was still produced and translocated to the scion. The reciprocal grafting experiment showed that the systemic tissue must accumulate SA for the SAR signal to be perceived. p lants react to pathogen attack by activating an elaborate defense mechanism that acts both locally and systemically. In many cases, local resistance is manifested as a hypersensitive response, which is characterized by the development of lesions that restrict pathogen growth and/or spread 1 (Fig. 1). Associated with the hypersensitive response is the induction of a diverse group of defenserelated genes. The products of many of these genes play important roles in containing pathogen growth, either indirectly, by helping to reinforce the defense capabilities of host cell walls, or directly, by providing antimicrobial enzymes and secondary metabolites (Fig. 2). These products include cell wall polymers, such as lignin and suberin, as

well as phenylpropanoids and phytoalexins. Several faroilies of pathogenesis-related (PR) proteins are also induced during the hypersensitive response. Some of these proteins are hydrolytic enzymes [e.g. ~-l,3-glucanases (PR-2) and chitinases (PR-3)], but the functions of other PR proteins reviews have yet to be determined. Most of the PR proteins have been shown to possess antimicrobial activity in vitro or the ability to enhance disease resistance when overexpressed in transgenic plants 2'3. Additionally, the hypersensitive response is associated with a massive increase in the generation of reactive oxygen species (the oxidative burst), which precedes and then accompanies lesion-associated host cell death. Over a period of hours to days after the primary infection, systemic acquired resistance develops throughout the plant. The systemic acquired resistance is manifested as an enhanced and long-lasting resistance to secondary challenge by the same or even unrelated pathogens. The application of molecular, genetic and biochemical techniques has led to the identification of key components of the signaling pathways leading to defense responses. Here, we focus on recent advances, and discuss the central role of salicylic acid in resistance to pathogens. **Salicylic acid and disease resistanc** .The signaling pathways involved in the initiation and maintenance of the hypersensitive response and systemic acquired resistance are still poorly understood. Only recently has salicylic acid emerged as a key signaling component involved in the activation of certain plant defense responses. For several years, it was known that the application of salicylic acid or aspirin to tobacco induced PR gene expression, and enhanced resistance to pathogens such as tobacco mosaic virus (TMV). However, in the early 1990s, it became apparent that salicylic acid is an endogenous compound that operates in the signaling pathway for plant defense. After TMV infection, salicylic acid accumulates to high levels at the site of infection, with a subsequent, but much smaller rise, in the uninfected systemic tissues. In tobacco, this increase paralleled the transcriptional activation of PR genes in both the inoculated and un-inoculated leaves. Strikingly, exogenously supplied salicylic acid induced the same set of nine genes that are activated systemically upon TMV infection. An increase in salicylic acid levels in the phloem of cucumber plants infected with either tobacco necrosis virus or Colletotrichum lagenarium was also shown to precede the development of systemic acquired resistance 2,~. More recently, the participation of salicylic acid in plant defense responses has been demonstrated through analysis of transgenic tobacco and Arabidopsis expressing the nahG gene, which encodes the enzyme salicylate hydroxylase from Pseudomonas putida 4'~. These plants accumulate little, if any, salicylic acid, and as a consequence show reduced or no PR gene expression, fail to establish systemic acquired resistance, and are compromised in their ability to prevent pathogen growth and spread from the primary infection site. The importance of salicylic acid in the activation of resistance was further underscored by the demonstration that Arabidopsis plants become susceptible to avirulent fungal pathogens when phenylalanine-ammonia lyase (PAL) activity is specifically inhibited ~. Since PAL catalyzes the first step in salicylic acid biosynthesis, and resistance can be restored in PAL-inhibited plants by treatment with exogenous salicylic acid, increased susceptibility is presumably caused by a block in salicylic acid synthesis. Although salicylic acid and salicylic acid signal transduction pathways are involved in resistance to many pathogens, in some cases PR gene expression and resistance can be activated in a salicylic acid-independent manner. For Fig. 1. The hypersensitive response and systemic acquired resistance. Tobacco cultivars that carry a dominant resistance gene [e.g. N ('Nicotiana')] are able to restrict the spread of tobacco mosaic virus to a small zone of tissue around the point of entry, where a necrotic lesion later appears (right). This resistance phenotype, the hypersensitive response, is subsequently accompanied by the induction throughout the plant of systemic acquired resistance. Consequently, a secondary infection with the virus, occurring several days after the initial infection, results in much smaller lesions (left) as compared with those induced by the primary infection. The leaves are shown 4 d after infection. The activation of systemic induced resistance in Arabidopsis by root inoculation with the biocontrol bacterium P. fluorescens is not associated with increases in endogenous salicylic acid or PR gene expression 7. Additionally, the systemic resistance induced by P. fluorescens is manifested equally well in transgenic Arabidopsis

expressing nahG. Similarly, the presence of the nahG gene does not compromise either Cf-2 or Cf-9 gene-mediated resistance to Cladosporium fulvum in tomato s. Is salicylic acid the mobile signal? It has been known for some time that the signal for establishing systemic acquired resistance is transported from the pathogen-inoculated leaf to uninoculated leaves via the phloem. It was suggested that salicylic acid might be the systemic signal for systemic acquired resistance following reports of salicylic acid accumulation occurring in parallel to or even preceding PR gene activation, and the development of systemic acquired resistance in uninfected leaves of TMVinoculated tobacco, combined with the detection of salicylic acid in the phloem of pathogen-infected tobacco or cucumber 2'3's. However, while these experiments clearly demonstrated a correlation between salicylic acid and systemic acquired resistance, they do not prove that salicylic acid is the long-distance mobile signal. Currently, strong evidence that salicylic acid may be this long-distance signal has come from an elegant experiment in which the translocation of labeled salicylic acid was monitored in TMV-infected tobacco 9. This analysis made use of the fact that the final step in salicylic acid biosynthesis in tobacco is the O2-dependent hydroxylation of benzoic acid, catalyzed by benzoic acid 2-hydroxylase. It was therefore possible to label the salicylic acid synthesized in TMV-inoculated lower leaves by enclosing July 1997, Vol. 2, No. 7 267 reviews Systemic;, f'" 1 /.."acquired i ~t j/ resistance~ i …….. Hypersensitive i ~__-~ Me~yl ~ / j , ……. i , } response i ~ ~ ~ sal!~ylate Salicylic i ~lnfection-–"-0~ ",. ,~ / ~t ac,d / Methl , '. / l i " / "~ Salicylic …… [~Y. "~.. -- Sa eylic acid \L acid sa,icyl~m ~/ …….. ~]glucoside I \. t',. / Salioyli~ acid \. 'k.~, ~ , B_glucos'l-de ~ ~-~ _~...,,J I i Fig. 2. Defense responses to pathogen infection. The infection of resistant plants by pathogens generally results in the hypersensitive response - the formation of necrotic lesions and restricted pathogen growth and spread. A variety of defense responses is induced locally around the sites of infection. An oxidative burst precedes the formation of necrotic lesions. Additional defense responses in surrounding cells include the induction of genes for pathogenesisrelated (PR) proteins, peroxidases and enzymes involved in cell wall strengthening and the biosynthesis of phytoalexins. Some of these genes are also activated systemically, and are believed to play a role in the development of systemic acquired resistance. The synthesis and accumulation of salicylic acid appear to be necessary for the activation of several of these defense responses, both locally and systemically. A substantial amount of salicylic acid is converted to salicylic acid ~-glucoside, a probable storage form 2''~. It is still unclear whether salicylic acid is a long-distance mobile signal in systemic acquired resistance. Most recently, methyl salicylate, which is synthesized from and metabolized to salicylic acid, was shown to act as an airborne signal that activates defense mechanisms in distal leaves and possibly even neighboring plants 11. However, at room temperature, methyl salicylate is a liquid and could be translocated through the vascular system of the plant, just as salicylic acid. them in an lSQ-rich environment. Subsequent analysis of the upper uninoculated leaves indicated that almost 70% of the salicylic acid was 1SO-labeled and had therefore been synthesized in and transported from the TMV-inoculated leaf. The biosynthesis and transport of salicylic acid have been studied by administering 14C benzoic acid to cucumber cotyledons infected with C. lagenarium ~°. In these experiments, 14C-labeled salicylic acid was detected in upper uninoculated leaves before the development of systemic acquired resistance. Recently, it was shown that methyl salicylate, produced from salicylic acid upon TMV infection of tobacco, may function as an airborne signal '1. Alternatively, it may be translocated through the vascular system. After the conversion of methyl salicylate back to salicylic acid, it activates defense responses in uninfected tissues and possibly even neighboring plants (Fig. 2). Despite the strong correlations from these studies on salicylate biosynthesis and transport, they do not rule out the possibility that salicylates are simply translocated in parallel with an unknown signal molecule. This possibility is supported by the observation that the signal for the development of systemic acquired resistance moved out of P. syringae-infected cucumber leaves before any increase in salicylic acid level could readily be detected in the phloem sap 12. Grafting experiments between NahG and wild-type tobacco have also suggested that salicylic acid is not the long-distance signal s. When a

NahG rootstock (which is unable to accumulate salicylic acid) was inoculated with TMV, the uninoculated leaves of the wild-type scion still showed systemic acquired resistance. However, these results need to be interpreted with caution. Although it has been shown that catechol, produced from salicylic acid via salicylate hydroxylase, cannot substitute for salicylic acid 13, it is unclear whether the residual salicylic acid in NahG plants is able to act as a long-distance messenger. Studies of transgenic tobacco expressing the cholera toxin gene (a known modulator of signaling pathways dependent on heterotrimeric G proteins) also suggest that salicylic acid is not the translocated systemic acquired resistance signal '4. These plants constitutively accumulate high levels of salicylic acid, express PR genes, show enhanced resistance and develop spontaneous lesions. However, systemic acquired resistance was not observed when a wild-type scion was grafted onto a transgenic rootstock, even though the rootstock accumulated high levels of salicylic acid. Thus, further work, such as the isolation of salicylic acid biosynthesis genes and the identification of mutations targeting the pathways for salicylic acid metabolism and transport, are required to clarify whether salicylic acid functions as a long-distance signal. Mechanisms of action Whether or not salicylic acid emerges as the mobile systemic acquired resistance signal, it does appear to be required for establishing and maintaining systemic acquired resistance. This conclusion is based on results from the NahG grafting experiments already described ~. Systemic acquired resistance did not develop in an NahG scion after infection of the wild-type rootstock with TMV. However, the mechanism by which salicylic acid induces systemic acquired resistance is still unclear. Previous studies have demonstrated that salicylic acid binds and inhibits tobacco catalase activity both in vitro and in vivo 1~'16. Thus, one possible function of salicylic acid is to inhibit the hydrogen peroxide (H202)-degrading activity of catalase, thereby leading to an increase in the endogenous level of H2Q, which is generated by photorespiration, photosynthesis, oxidative phosphorylation and the hypersensitive responseassociated oxidative burst. The H2Q , and other reactive oxygen species derived from it, could then serve as second messengers to activate the expression of plant defenserelated genes, such as PR-1. This hypothesis is currently the subject of intense debate. Reactive oxygen species and plant defense In plants, H202, superoxide radicals (02") and hydroxyl radicals (OH') are thought to play key roles in defense responses. Following infection, plants resistant to the invading pathogen develop a sustained increase in reactive oxygen species. In a manner analogous to their participation in macrophage or neutrophil action, these re active .oxygen species might be involved in directly killing invading pathogens. In addition, increases in H202 have been shown to induce the crosslinking of cell wall proteins ~7 and to enhance the peroxidase-catalyzed synthesis of lignin, thereby creating a physical barrier against pathogens ~. Reactive oxygen species can also serve as second messengers for the activation of defense gene expression. For example, elevated reactive oxygen species levels induce the genes for glutathione-S-transferase, glutathione peroxidase and polyubiquitin, as well as peroxidases, catalases and other enzymes involved in scavenging reactive oxygen species, chilling tolerance and pathogen resistance. Currently, the mechanism(s) by which redox signaling activates these genes is a matter of debate. The ability of reactire oxygen species, and thus the cellular redox state, to activate plant defenses may parallel the mechanism by which oxidative stress induces the genes associated with animal immune and inflammatory responses. Activities of at least two transcription factors, NF-KB and AP-1, have been shown to be regulated by the cellular redox state; whether these proteins are activated directly by H~O2, or indirectly by thiol metabolites, such as glutathione, is unclear. To date, the only genes shown to be regulated directly by reactive oxygen species are those in the bacterial oxyR and sox regulons ~s. Another line of early plant defense that may be triggered by reactive oxygen species is cell death. Treatment of soybean suspension cells with high concentrations of H20 2 (6-10 raM) was shown to cause cell death, which could be enhanced by the addition of salicylic acid or the catalase inhibitor 3-aminotriazole ~9. In contrast, another study has suggested that O z" , but not H202, is crucial for the induction of cell death. To determine the mechanism by which spontaneous lesions develop on the leaves of the Arabidopsis lsdl ('lesion

simulating disease') mutant in the absence of pathogen infection, these plants were treated with O2"-or H20 2 generating or scavenging systems 2°. Strikingly, elevated levels of O2"-, but not H20 2, were able to induce lesion formation. Despite repeated suggestions that reactive oxygen species are involved in the signaling pathways leading to apoptosis and/or programmed cell death in animals ~s, there is still no conclusive evidence that they are required for actually killing the cells. Indeed, it has recently been suggested that reactive oxygen species may be associated with, but not directly responsible for, apoptosis in animal cells 2~. 1-1202 and salicylic acid: which is the source and which is the signal? It has been hypothesized that salicylic acid binds to catalase, inhibits its activity and thereby increases the intracellular concentration of H2Q, which might then serve as a second messenger for the induction of a defense response 1~. In contrast, recent reports have suggested that PR gene induction during the hypersensitive response and systemic acquired resistance may not be activated by salicylic acidmediated increases in H=O 2. At the site of infection, salicylic acid levels can reach 150 llM, a concentration sufficient to cause substantial inhibition of catalase and ascorbate peroxidase, the other major H2Q-scavenging enzyme 4'1~'~6'22'~3. However, no decrease in catalase activity could be detected in pathogen-inoculated leaves ~3'24. In contrast, the concentration of salicylic acid in uninfected systemic tissue is probably too low to increase H2Q levels through the inhibition of catalase or ascorbate peroxidase, unless salicylic acid is concentrated in a subcellular compart/nent. Recent studies using transgenic tobacco plants have also suggested that the salicylic acid-mediated inhibition of catalase and increased level of H20 2 are not involved in the activation of defense responses. When catalase expression was suppressed in leaves of transgenic plants through sense cosuppression or antisense suppression, most plants failed to show constitutive PR gene expression 25'26. Additionally, H20 2 and H2Q-inducing chemicals were unable to induce PR expression in NahG plants, although they could activate PR-1 genes in wild-type tobacco 13'27. Based on these results, salicylic acid appears to act downstream of H20 2, rather than the reverse. Furthermore, it was recently demonstrated that high levels of H20~, as well as ozone or ultraviolet treatment, stimulate salicylic acid biosynthesis 24'27'2s. Thus, H20 2 might play a role in the activation of PR genes by increasing salicylic acid levels. It is believed that all organisms utilize signaling cascades to transduce oxidative stress, and that they frequently respond to stress by strengthening their anti-oxidative systems. Whether salicylic acid plays a role in either process in plants is unknown. It has been proposed that alterations in the cellular redox state, as a result of changes in the glutathione or plastoquinone pools or the levels of metabolites such as nicotinamide or salicylic acid, serve as redox messengers in plants 15,1s'2~. On the other hand, phenol-based anti-inflammatory drugs (including salicylic acid and aspirin) are thought to act, at least in part, as anti-oxidative compounds and direct scavengers of reactive oxygen species 3°. Thus, it is possible that salicylic acid functions, in part, by acting as an anti-oxidant. In this capacity, it could help contain the oxidative damage associated with lesion formation and/or spread during the hypersensitive response. This would closely resemble the anti-oxidative role of salicylic acid in inflamed mammalian tissues. It is noteworthy that, in NahG plants, lesions that develop upon TMV infection are larger than in wild-type plants ~. However, an anti-oxidative role of salicylic acid in the living plant remains to be proven. If the predominant mechanism by which salicylic acid induces defense responses is not through increased H20 2 levels caused by the inhibition of catalase and ascorbate peroxidase, how is the salicylic acid signal perceived and transmitted? One possibility is through the generation of salicylic acid radicals, a likely by-product of the interaction of salicylic acid with catalase and peroxidases 31 (see Box 1). Free radicals derived from phenolic compounds can induce lipid peroxidation, and the products of this reaction, such as lipid peroxides, are potent signaling molecules in animals and possibly also in plants. For example, it is known that salicylic acid induces lipid peroxidation in tobacco suspension cells, and that lipid peroxides activate PR-1 genes in these cells 32. Salicylic acid may also interact with other effector proteins, besides those involved in redox regulation. Recently, a soluble, high-affinity, salicylic acid-binding protein (SABP2) was identified, which reversibly binds biologically active, but not inactive, analogs of salicylic

acid in vitro 3~. Additionally, it has a 15-fold higher affinity for the plant protecting agent benzothiadiazole (BTH), which is consistent with the greater efficacy of BTH in inducing plant defense responses ~3'34 (Box 2). However, whether SABP2 is a true receptor or another member of the class of salicylic acid-binding metalloproteins is currently unclear. Moreover, it is likely that future analyses will identify other July 1997, Vol. 2, No. 7 269 reviews Box 1. Proteins that interact with salicylic acid The discovery that salicylic acid (SA) inhibits tobacco catalase(s ~ stimulated discussion concerning both the biological significance of this interaction and the mode of inhibition. The complexity of the redox chemistry of the enzyme allows for many possibilities. H20 + 02 H202 (a) Ferric enzyme . "\"-- J = Compoundl / H202 H20 (b) Ferric enzyme ~-- ~ = Compound I ~\ H202 H20 // SA" \ ~ SA (+H20)~"~ / \, / Compound I1 The issue as to how salicylic acid could inhibit catalase Was recently clarified by the demonstration that salicylic acid acts as a one electron-donating substrate that siphons catalase from the extremely rapid catalytic cycle (a) into a much slower peroxidative cycle (b), which is a secondary acti,dty of catalase ~1 (compound II is another enzyme intermediate of different oxidation state from ferric enzyme and compound I). A consequence of the interaction of salicylic acid with catalase and peroxidases is the formation of a salicylic acid radical (SA'). The ability of salicylic acid to serve as an electron donor for heme proteins is not restricted to catalase. Many protei n~ with peroxidase function have been shown to interact with salicylic acid (although this does not necessarily imply inhibition), Some of the potential targets of salicylic acid in mammalian cells include: ….. • Prostagtandin H synthetase. • Lactoperoxidase*. • Myeloperoxidase% • Catalase* • Aconitase~ • Methemoglobin*, • Metmyoglobin*. • Potential targets in plants include: • Catalase*. • Aconitase. • Leghemoglobin*. (a) The catalytic cycle of catalase, in which hydrogen peroxide • Aminocyclopropane carboxylic acid (ACC) oxidase. (H~Q) is converted to H20 and 02 Ferric enzyme and compound I are enzyme intermediates of different oxidation states. Among An asterisk denotes proteins for which salicylic acid has been the suggestions as to how salicylic acid could inhibit catalase shown or suggested to act as an electron donor; all of these are have been chelation of the home iron and a novel atlosteric home proteins. In other cases, salicylic acid might chelate the binding site at the surface of catalase s1"5~, iron of an FeS protein or simply block a substrate binding site. salicylic acid effector proteins that might play roles in disease resistance and/or other salicylic acid-mediated responses (e.g. thermogenesis). Salicylic acid and gene expression. In plants, salicylic acid has been shown to induce the expression of many defense-related genes, as well as to potentiate the production of H202, the induction of cell death and the activation of several genes induced by fungal elicitors and wounding 19'35'~6. The genes induced by salicylic acid can be grouped into two broad classes. The first class consists of genes whose expression is insensitive to protein synthesis inhibitors, such as the glutathione-S-transferase genes, the 35S promoter of cauliflower mosaic virus and the nopaline and octopine synthase genes of Agrobacterium. Promoters of this class of genes contain copies of as-l-like cis elements, which mediate salicylic acid-induced expression. Several transcription factors belonging to the TGA family of bZIP proteins have been identified and shown to bind these elements 37. It was also recently demonstrated that salicylic acid or cycloheximide treatment of tobacco leaves increases an as-1 binding activity, and that phosphatase treatment of nuclear extracts decreases it 38. From these results, it was proposed that the as-1 binding activity is sequestered by an inhibitory protein that is released after salicylic acid treatment, probably via a phosphorylation event(s). This in turn leads to activation of promoters containing the as-l-like element. A MAP kinase that can be activated by salicylic acid and TMV has been identified and purified from tobacco extracts 39, but it is not known whether this kinase is involved in the salicylic acid-mediated activation of this DNA-binding protein. The second class of salicylic acid-inducible genes includes the acidic (class II) PR genes, whose induction by salicylic acid is sensitive to inhibitors of protein synthesis. Promoters of the tobacco PR-la and PR-2 genes have been studied by several groups. However, no common cis elements involved in the salicylic acid-inducible expression of these genes have yet been defined. A 10 bp TCA element that is common to the promoters of several tobacco PR genes, as well as

several stressinduced genes, was shown to bind a 40 kDa nuclear protein in a salicylic acid-dependent manner 4°. However, this TCA element was neither sufficient nor required for salicylic acidmediated induction of the tobacco PR-2d promoter in vivo 41. In contrast, in vivo analysis of the PR-2d promoter has identified a 25 bp element that is involved in salicylic acidinducible expression. This element contains the sequence TTCGACC, which is related to the W-boxes present in the promoters of several elicitor- and wound-induced genes 42. Induction of some of these genes by pathogens, elicitors or 270 July 1997, Vol. 2, No. 7 reviews Significant progress has been made in the development of transgenic plants with enhanced resistance to microbial attack. For example, overexpression of genes encoding antifungal enzymes such as chitinase and ~-l,3-glucanase has shown considerable promise. Their introduction and successful use in field crops is anticipated in the near future. Significant advances in the understanding of signal trm~sduction pathways that mediate disease resistance could lead to the next generation oftransgenic plants in which manipulation of key signaling components results in the activation of a broad array of host defenses. Alternatively, the signaling pathway might be altered so that it is primed more rapidly and effectively to activate these defense arsenals upon infection. Another approach to enhance resistance is through treatmerit with compounds that activate part or all of the host defense arsenals. In addition to salicylic acid and aspirin, two such 'plant defense activators' have been identified and characterized: 2,6-diehloroisonicotinic acid and benzothiadiazole. Both appear to be functional analogs of salicylic acid, and the latter is being used commercially as a plant protecting agent 34. Further research on salicylic acid and its cellular targets should facilitate the development of compounds that mimic endogenous messengers and thereby induce disease resistance. 0 0 H ~ C OH C OH 0 ....OH /O C OH 3 Salicylic acid Asoirin O O H C--OH C S -- OH 3 2.6-Dichloroisonicotinic Benzothiadiazole acid wounding is potentiated by pretreatment with salicylic acid ~9'35'3~. It may be that related factors are involved in the expression of the elicitor-induced defense genes as well as the salicylic acid-induced PR-2d gene. The tobacco PR-la gene promoter contains several binding sites for Myb proteins, redox-regnlated transcription factors found in plants and animals. Some of these sites can be bound by recombinant tobacco Mybl protein in vitro 43. Binding sites for Myb proteins are also present in the promoters of PAL genes, whose expression is potentiated by salicylic acid 35'37. Expression of the tobacco Mybl gene is rapidly induced by salicylic acid, with kinetics similar to those of genes containing the as-1 element. Thus, it is possible that Mybl binding activity is involved in transducing the salicylic acid signal to the PAL and PR promoters. However, Mybl by itself may not be sufficient for salicylic acid inducibility of the PR-la gene, because in vivo analysis of this promoter has suggested that more than one region is involved in the salicylic acid-mediated activation. GT-l-like proteins have also been shown to bind various fragments of the tobacco PR-la promoter in vitro 44. Their binding activity is reduced in extracts from salicylic acid-treated or TMVinfected leaf tissue. Although it is tempting to speculate that the Mybl and GT-l-like proteins might be involved in the salicylic acid-dependent expression of the PR-la gene, there is still no evidence in vivo. Genetic approaches for understanding the role of salicylic acid in defense responses In addition to environmental stress, such as exposure to ultraviolet light and ozone, the inappropriate expression or repression of endogenous or foreign genes in plants can lead to the constitutive expression of defense genes, the activation of systemic acquired resistance and, in several cases, the spontaneous development of lesions like those of the hypersensitive response (Table 1). In most of these cases, the constitutive systemic acquired resistance and spontaneous lesion phenotypes are associated with elevated levels of endogenous salicylic acid. However, none of these transgenes are anticipated to participate directly in salicylic acid biosynthesis. Rather, their expression may induce metabolic stress, which in turn elevates salicylic acid levels, resulting in constitutive systemic acquired resistance. Alternatively, it has been suggested that in some cases expression of these transgenes mimics part of the defense signaling pathway, which then activates salicylic acid biosynthesis s'45. Several Arabidopsis mutants have been identified that constitutively exhibit systemic acquired resistance and contain

constitutively high levels of salicylic acid (Fig. 3; Table 2). Interestingly, the lsd, cepl ('constitutive expression of PR genes') and acd2 ('accelerated cell death') mutants also spontaneously develop hypersensitive response-like lesions, while the cprl ('constitutive expresser of PR genes') and cim3 ('constitutive immunity') mutants do not 2'32'4~. Because the constitutive systemic acquired resistance phenotype of these mutants is suppressed by the presence of the nahG gene, elevated levels of salicylic acid appear to have a causal role in the development of constitutive systemic acquired resistance (Fig. 3; Table 2). In contrast to these constitutive systemic acquired resistance mutants are the systemic acquired resistancecompromised mutants (Table 2), of which only the allelic nprl/sail/niml (npr, 'nonexpresser of PR genes'; sai, 'salicylic acid insensitive'; nim, 'noninducible immunity') class appears to affect the salicylic acid signal transduction pathway 4~-4s. Mutations in this gene prevent both the development of systemic acquired resistance and the induction of PR genes by salicylic acid. These mutations are recessive, suggesting that the wild-type protein acts as a positive regulator of the salicylic acid signal transduction pathway. Recently, NPR1 was shown to encode a unique 60 kDa soluble protein containing ankyrin repeats, which facilitate protein-protein interactions. Because one of the nprl mutations is in these repeats, Nprl probably functions in signal transduction by interacting with other proteins. The nprl/sail/niml mutants are also insensitive to the plant activators 2,6-dichloroisonicotinic acid (INA)and benzothiadiazole 4s (Box 2), which supports the biochemical evidence of stress response salicylic acid metabolism \ / acd2 ~ cprl --~ Salicylic ~- --~ nprl/sail/ lsd cim3 acid K] "~ nim 1 /# cep l nah G Metabolic stress and ,. ,. . ,. SIS gene expression pathogen infection and disease resistance Fig. 3. Arabidopsis mutants with altered disease resistance. Vm4ous signals can lead to the accumulation of salicylic acid and a corresponding increase in systemic acquired resistance-associated gene expression and disease resistance. The mutants acd2 ('accelerated cell death'), lsd ('lesion simulating disease'), cepl ('constitutive expression of PR genes'), cprl ('constitutive expresser of PR genes') and cim3 ('constitutive immunity') are placed upstream of salicylic acid, because they constitutively accumulate high levels of salicylic acid and their systemic acquired resistance phenotypes are suppressed by the presence of the nahG gene, which encodes salicylate hydroxylase from Pseudomonas putida 2'3~'4~. Evidence for feedback regulation of salicylic acid accumulation comes from studies of the sail-1 ('salicylic acid insensitive') mutant, which accumulates salicylic acid upon pathogen infection to much higher levels than the wild-type plants 4s, and the allelic nprl-1 ('nonexpresser of PR genes') mutant, which is more sensitive to exogenous salicylic acid 4~. Additionally, the action of salicylic acid is under feedback control. This is evident from studies of the lsdl and lsd6 mutants. The spontaneous lesion phenotypes of lsdl and lsd6 are suppressed by the presence of the nahG gene. However, the application of salicylic acid or 2,6-dichloroisonicotinic acid (INA) restores the spontaneous lesion phenotype of these lsdl and lsd6 nahG plants. The SIS ('salicylic acid-independent, systemically induced') genes are predominantly induced by a pathway independent of salicylic acid; they are activated equally well in pathogen-infected wild-type and NahG plants. However, treatment with salicylic acid can also induce transient expression of these genes. Expression of systemic acquired resistance genes and disease resistance that all three compounds induce plant defense responses via the same signal transduction pathway. Although salicylic acid appears to be required for systemic acquired resistance, analyses of mutants and transgenic plants have suggested that salicylic acid is not an absolute requirement for the hypersensitive response. The lsd2 and lsd4 mutants are capable of developing the hypersensitive response in the presence of the nahG gene. Moreover, TMV infection leads to the hypersensitive response in NahG tobacco plants 4'~. These lesions, however, are larger and more diffuse than those shown by wildtype plants. Furthermore, the lesions eventually spread to the stems, suggesting that the NahG plants have lost their ability to limit the hypersensitive response. Hence, although salicylic acid may not play a causal rote in lesion development, it might participate in restricting the spread of lesions. Concluding remarks: The ability to respond rapidly and effectively to environmental signals and pathogens is essential to the survival of all organisms. This review has Transgene Encoded protein and/or function Salicylic acid Pathogenesis-

related Comments Refs levels gene expression Halobacterium Proton pump. Cauliflower mosaic Inclusion-body Not Constitutively high Develop spontaneous 45 virus gene VI matrix protein, determined lesions. Cholera toxin Inhibits GTPase Elevated Constitutively high Develop spontaneous 45 subunit A1 gene activity of G proteins lesions. Rice rgpl gene Ras-like G protein. Elevated upon High upon wounding Develop systemic 45 wounding acquired resistance upon wounding. Tobacco DS22 cDNA Mitogemactivated Elevated upon High upon wounding Wounding systemically 52 protein (MAP) kinase, wounding induces salycylic acid Mutant ubiquitin Interferes with ubiquitinubR48 gene dependent proteolysis. Elevated Constitutively high Tobacco catalase Hydrogen peroxide Elevated Constitutively high gene degradation (note that conditional) plants are catalase under high deficient), light) Yeast invez~ase gene Hexose transport. Elevated Constitutively high Pseudomonas putida Converts salicylic acid to Low Not inducible by salicylate hydroxylase catechol~ salicylic acid; (nahG) gene INA-indnsible accumulation and PR gene expression. Develop spontaneous 45 lesions; develop fewer lesions upon tobacco mosaic virus infection. Develop spontaneous lesions (conditional): smaller and fewer lesions upon tobacco mosaic virus infection. …….. 45 lesions. Enhanced susceptibility to pathogens. Mutant" Dominant/ Salicylic acid Pathogenesis-related Comments recessive levels gene expression Refs nprl. sail, Recessive Normal Salicylic acid/ ntm l INA-noninducible eds Recessive Normal Normal ~drl Recessive Not Normal determined acdl Recessive Not Normal determined acd2 Recessive Elevated Constitutively high lsdl, lsd3, Recessive Elevated Constitutively high lsd5 cepl Recessive Elevated Constitutively high lsd2, lsd4, Dominant Elevated Constitutively high lsd6. lsd7 cprl Recessive Elevated Constitutively high cim3 Dominant Elevated Constitutively high Enhanced susceptibility to aviz~lent pathogens; normal hypersensitive response. Enhanced susceptibility to pathogens. 45 Enhanced susceptibility to pathogens; normal hypersensitive response to most avirutent pathogens tested. Develop spontaneous lesions and has enhanced susceptibility to pathogens. Develop spontaneous lesions and has enhanced resistance to pathogens. Develop spontaneous lesions and have enhanced resistance to pathogens, Develop spontaneous lesions and have enhanced resistance to pathogens; constitutive PR expression suppressed by nahG. Develop spontaneous lesions and have enhanced resistance to pathogens; all phenotypic properties suppressed by nahG in lsd6 and lsd7; lesion formation not suppressed by nahG in lsd2 and lsd4. Enhanced resistance to pathogens; all phenotypic properties suppressed by nahG. Enhanced resistance to pathogens; all phenotypic 2 properties suppressed by nahG. ~npn, "nonexpresser of PR genes'; sai, 'salicylic acid insensitive'; nim, 'noninducible immunity'; eds, 'enhanced disease susceptibility'; ndr, "nonrace specific disease resistance': acd.'accelerated cell death'; lsd~ 'lesion simulating disease'; cprl, 'constitutive expresser of PR genes'; cep. "constitutive expression of PR genes': cim3. "constitutive immunity' focused mainly on salicylic acid and its importance in plant disease resistance, but this compound and its signaling pathways are only one aspect of the many responses activated by pathogen attack. In recent years, several key components involved in the perception and transduction of resistance signals have been identified. Strikingly, at least some of these components appear to be conserved among eukaryotes. For example, the product of the tobacco disease resistance gene N ('Nicotiana') has similarity with the interleukiml receptor (IL-1R) and the Toll protein, which activate defense responses in mammals and Drosophila, respectively 5°. Moreover, the parallels between the cytokine-mediated activation of NF-KB and the Tollmediated induction of dorsal/dif suggest the presence of an ancient regulatory cascade that may be conserved in plants and might function during N-gene-mediated resistance. Further comparative analyses of animal and plant defense responses should improve understanding of plant disease resistance. In addition, they may provide insights that can be used both to engineer plants that are better able to defend themselves against a wide spectrum of pathogens and to develop compounds capable of inducing systemic resistance.

Both chitin and chitosan have demonstrated antiviral, antibacterial, and antifungal properties, and have been explored for many agricultural uses. They have been utilized to control disease or reduce their spread, to chelate nutrient and minerals, preventing pathogens from accessing them, or to enhance plant innate defenses.

When used to enhance plant defenses, chitin and chitosan induce host defense responses in both monocotyledons and dicotyledons. These responses include lignification, ion flux variations, cytoplasmic acidification, membrane depolarization and protein phosphorylation, chitinase and glucanase activation phytoalexin biosynthesis generation of reactive oxygen species, biosynthesis of jasmonic acid and the expression of unique early responsive and defense-related genes. In addition, chitosan was reported to induce callose formation, proteinase inhibitors, and phytoalexin biosynthesis in many dicot species. The response to chitin, chitosan, and derived oligosaccharides varies with their acetylation degree. This review summarizes some of the uses of these natural products in agriculture and gives an overview of the mechanisms involved.

2. Antimicrobial Properties of Chitosan

Chitosan exhibits a variety of antimicrobial activities, which depend on the type of chitosan (native or modified), its degree of polymerization, the host, the chemical and/or nutrient composition of the substrates, and environmental conditions. In some studies, oligomeric chitosans (pentamers and heptamers) have been reported to exhibit a better antifungal activity than larger units. In others, the antimicrobial activity increased with the increase in chitosan molecular weight, and seems to be faster on fungi and algae than on bacteria.

2.1. Against viruses

Chitosan was shown to inhibit the systemic propagation of viruses and viroids throughout the plant and to enhance the host's hypersensitive response to infection. The level of suppression of viral infections varied according to chitosan molecular weight. Similar observations were reported with the potato virus X, tobacco mosaic and necrosis viruses, alfalfa mosaic virus, peanut stunt virus, and cucumber mosaic virus.

2.2. Against bacteria

Chitosan inhibits the growth of a wide range of bacteria. The minimal growth-inhibiting concentrations vary among species from 10–1,000 ppm. Quaternary ammonium salts of chitosan, such as *N*,*N*,*N*-trimethylchitosan, *N*-propyl-*N*,*N*-dimethylchitosan and *N*-furfuryl-*N*,*N*-dimethylchitosan were shown to be effective in inhibiting the growth and development of *Escherichia coli*, especially in acidic media. Similarly, several derivatives of chitin and chitosan were shown to inhibit *E. coli, Staphylococcus aureus*, some *Bacillus* species, and several bacteria infecting fish.

2.3. Against fungi and oomycetes

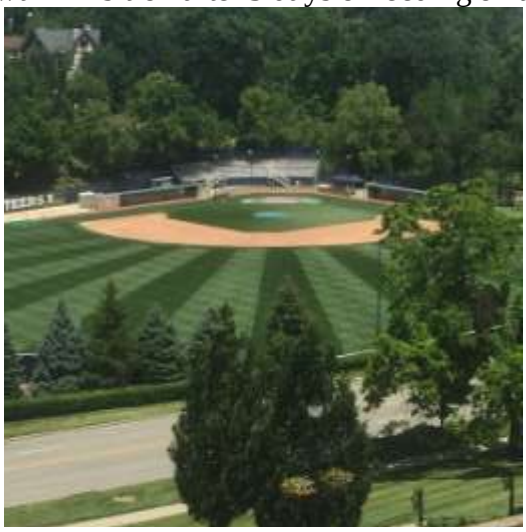
Fungicidal activity of chitosan has been documented against various species of fungi and oomycetes. The minimal growth-inhibiting concentrations varied between 10 and 5,000 ppm. The maximum antifungal activity of chitosan is often observed around its *pKa* (pH 6.0).

Studies have been reported on the fungicidal activity of 24 new derivatives of chitosan (*i.e.*, *N*-alkyl, *N*-benzylchitosans) and showed, using a radial hyphal growth bioassay of *B. cinerea* and *P. grisea*, that all derivatives have a higher fungicidal action than the native chitosan. *N*-dodecylchitosan, *N*-(*p*-isopropylbenzyl)chitosan and *N*-(2,6-dichlorobenzyl)chitosan

were the most active against *B. cinerea*, with EC_{50} values of 0.57, 0.57 and 0.52 $g.L^{-1}$, respectively. Against *P. grisea*, *N*-(*m*-nitrobenzyl)chitosan was the most active, with 77% inhibition at 5 $g.L^{-1}$. *O*-(decanoyl)chitosan at mol ratio of 1:2 (chitosan to decanoic acid) was the most active compound against *B. cinerea* (EC_{50} = 1.02 $g.L^{-1}$) and *O*-(hexanoyl)chitosan displayed the highest activity against *P. grisea* (EC_{50} = 1.11 $g.L^{-1}$). Some of the derivatives also repressed spore formation at rather high concentrations (1.0, 2.0 and 5.0 $g.L^{-1}$). Recently, research demonstrated that chitosan is able to permeabilize the plasma membrane of *Neurospora crassa* and kills the cells in an energy-dependent manner.

In general, chitosan, applied at a rate of 1 mg/mL, is able to reduce the *in vitro* growth of a number of fungi and oomycetes except Zygomycetes, which have chitosan as a component of their cell walls. Another category of fungi that seems to be resilient to the antifungal effect of chitosan, the nemato-/entomo-pathogenic fungi that possess extracellular chitosanolytic activity.

more and more derivatives of chitosan (*i.e.*, *N*-alkyl-, *N*-benzylchitosans) are made available through chemical synthesis, their insecticidal activities are being reported using an oral larvae feeding bioassay [37,38]. Twenty four new derivatives were shown to have significant insecticidal activity when administered at a rate of 5 $g \cdot kg^{-1}$ in an artificial diet. The most active derivative, *N*-(2-chloro-6-fluorobenzyl)chitosan, caused 100% mortality of larvae and its LC_{50} was estimated at 0.32 $g.kg^{-1}$. All synthesized derivatives highly inhibited larvae growth as compared to chitosan by 7% and the most active derivative was the *O*-(decanoyl)chitosan, with 64% growth inhibition after 5 days of feeding on the treated artificial diet.



3. Applications of Chitosan in Plant Disease Control

Chitosan used to control plant pathogens has been extensively explored with more or less success depending on the pathosystem, the used derivatives, concentration, degree of deacylation, viscosity, and the applied formulation (*i.e.*, soil amendment, foliar application; chitosan alone or in association with other treatments). For example, researchers tested the effectiveness of five chemically-modified chitosan derivatives in restricting the growth of *Saprolegnia parasitica*. Results indicated that methylpyrrolidinonechitosan, *N*-phosphonomethylchitosan, and *N*-carboxymethylchitosan, as opposed to *N*-dicarboxymethylchitosan, did not allow the fungus to grow normally.

Substratum amendment with chitosan was reported to enhance plant growth and suppress some of the notorious soil-borne diseases. For example, in soilless tomato, root rot caused

by *Fusarium oxysporum* f. sp. *radicis-lycopersici* was suppressed using chitosan amendments. Similarly, in order to control post-harvest diseases, addition of chitosan stimulated microbial degradation of pathogens in a way resembling the application of a hyper-parasite. This area of application is important because it suggests alternatives to the use of pesticides on fresh produce in storage. Recent investigations on coating tomatoes with chitosan have shown that it delayed ripening by modifying the internal atmosphere, which reduced decays due to pathogens. Various methods of application of chitosan and chitin are practiced to control or prevent the development of plant diseases or trigger plant innate defenses against pathogens.

3.1. Applied as seed coating agents

Researchers examined the use of chitosan to prime maize seeds. Although chitosan had no significant effect on germination under low temperatures, it enhanced germination index, reduced the mean germination time, and increased shoot height, root length, and shoot and root dry weights in two tested maize lines. In both tested lines, chitosan induced a decline in malonyldialdehyde content, altered the relative permeability of the plasma membrane and increased the concentrations of soluble sugars and proline, and of peroxidase and catalase activities.

In other studies, seed priming with chitosan improved the vigor of maize seedlings. It was also reported to increase wheat seed resistance to certain diseases and improve their quality and/or their ability to germinate. Similarly, peanut seeds soaked in chitosan were reported to exhibit an increased rate of germination and energy, lipase activity, and gibberellic acid and indole acetic acid levels. Scientists showed that rice seed coating with chitosan may accelerate their germination and improve their tolerance to stress conditions. In carrot, seed coating helps restrain further development of Sclerotinia rot. Chitosan has also been extensively utilized as a seed treatment to control *F. oxysporum*in many host species.

Foliar application of chitosan has been reported in many systems and for several purposes. For instance, foliar application of a chitosan pentamer affected the net photosynthetic rate of soybean and maize one day after application. This correlated with increases in stomatal conductance and transpiration rate. Chitosan foliar application did not have any effect on the intercellular CO_2 concentration. The authors reported that the observed effect on the net photosynthetic rate is, in general, common in maize and soybean after foliar application of high molecular weight chitosan. Foliar applications of these oligomers did not, on the other hand, affect maize or soybean height, root length, leaf area, or total dry mass.

Researchers suggested that chitosan might be an effective anti-transpiring to preserve water resources use in agriculture. In their investigation, they examined the potential of foliar applications of chitosan on pepper plants transpiration in the growth room and in the field. In both experiments, the authors monitored plant water use directly and indirectly. The plant biomass and yield were determined to calculate biomass-to-water ratios and the differences in canopy resistance between control and chitosan-treated plants were analyzed. Using scanning electron microscopy and histochemical analyses, stomata were shown to close in response to treatment with chitosan, resulting in a decrease in transpiration. Reduced water use of pepper plants upon treatment with chitosan was estimated at 26–43%, while there was no change in biomass production or yield.

Scientists unveiled some of the aspects through which chitosan was able to reduce transpiration in bean plants after being used as a foliar spray. The authors showed that this activity was likely occurring thanks to the increase in abscisic acid (ABA) content in the treated leaves. Using scanning electron microscopy and other histocytochemistry techniques, the authors

showed that upon treatment and increase in ABA content, a partial stomatal closure occurred and led, among others, to a decrease in conductance for water vapor and in the overall transpiration rate. Interestingly, the authors revealed a new chitosan anti-transpirant mechanism in bean plants that was not described by their commercial supplier Vapor Gard®, and in which a formation of a thin anti-transpirant film at the surface of the leaves was much more efficient than stomatal closure. This difference in mechanisms also suggested an important consideration for the environmental conditions under which chitosan is applied as shown by the authors but may also depends on the intrinsic properties of the tested plant species.

Chitosan has also been extensively utilized as a foliar treatment to control the growth, spread and development of many diseases involving viruses, bacteria, fungi and pests. It has also been used to increase yield and tuber quality of micropropagated greenhouse-grown potatoes. Similarly, researchers showed that the use of chitosan applied as a foliar spray on barley reduced locally and systemically the infection by powdery mildew pathogen *Blumeria graminis* f. sp. *hordei*.

3.3. Applied as soil amendment

Chitosan utilized as a soil amendment was shown to control *Fusarium* wilts in many plant species. Applied at an optimal concentration, this biomaterial is able to induce a delay in disease development, leading to a reduced plant wilting. Similar results were reported in forest nurseries suffering from *F. acuminatum* and *Cylindrocladium floridanum* infections. These infections were dramatically reduced upon the use of chitosan as soil amendment. *Aspergillus flavus* was also completely inhibited in field-grown corn and peanut after soil treatment with chitosan. Part of the effect observed by chitosan on the reduction of soilborne pathogens comes from the fact that it enhances plant defense responses. The other part is linked to the fact that this biopolymer is composed of polysaccharides that stimulate the activity of beneficial microorganisms in the soil such as *Bacillus,* fluorescent *Pseudomonas*, actinomycetes, mycorrhiza and rhizobacteria. This alters the microbial equilibrium in the rhizosphere disadvantaging plant pathogens. Beneficial organisms, on the other hand, are able to outcompete them through mechanisms such as parasitism, antibiosis, and induced resistance.

Several researchers reported on the effect of chitin amendment on actinomycetes in soil and on the infection of potato from susceptible cultivar 'Bentje' by *Streptomyces scabies*, the causal agent of tuber scab. The percentage scab on tubers from the control and the soil amended with antagonist was about 22 % while only 4% of the tubers from the soil amended with chitin and chitin with antagonist had scab at harvest. After planting these tubers, for a second time, the scab was 21% on tubers from untreated soil and 9.5 % from soil amended with chitin. Investigation of the effect of chitin amendment on the actinomycete population in the soil, a few months after chitin amendment, revealed that chitin had a greater increase in total actinomycete population (24–30 times as compared to the untreated control). The study also showed that some actinomycetes (*i.e.*, *Micromonospora*) had disappeared, while others including *S. scabies* were isolated less frequently.

4. Mechanisms of Action of Chitosan in Reducing Plant Diseases

Although the exact mechanisms of action of chitosan in reducing plant disease are currently not fully understood, there is growing evidence showing its action through direct toxicity or chelation of nutrients and minerals from pathogens. Because of its biopolymer properties, this compound can also form physical barriers around the penetration sites of pathogens, preventing them from spreading to healthy tissues. This and bioactive derivatives can activate H^+-ATPases,

depolarizing biological membranes and inducing other series of events. Chitosan is known to induce reactions locally and systemically that involve signaling cascades, and the activation and accumulation of defenses-related antimicrobial compounds and proteins.

4.1. Direct activity against pathogens

Direct activity of chitosan against viruses and viroids has been shown to vary according to molecular weight. However, none of the studies that investigated this effect has clearly proven the ability of chitosan in completely inactivating viruses or viroids. Most literature reported on the inactivation of replication, which lead to the stoppage of multiplication and spread. This could be linked to the fact that upon penetration into plant tissues, chitosan nanoparticles tightly bind nucleic acids and cause a variety of damages and selective inhibitions. For instance, the selectively exerted inhibition could inactivate the synthesis of essential mRNA encoded by various genes required for important metabolic and infectious processes of the virus or viroid. These properties have been largely explored in gene therapy and gene silencing.

Against, bacteria, fungi, oomycetes and other pests, it seems that chitosan is likely to operate indirectly *via* other means such as the enhancement of host resistance. However, a number of studies have shown that chitosan, at defined concentrations, presents antimicrobial propel. For instance, chitosan was reported to exert an inhibitory action on the hyphal growth of numerous pathogenic fungi, including root and necrotrophic pathogens, such as *Fusarium oxysporum*, *Botrytis cinerea*, *Monilina laxa*, *Alternaria alternata* and *Pythium aphanidermatum* besides inhibiting spore germination in some of them.

Chitosan is often used in plant disease control as a powerful elicitor rather than a direct antimicrobial or toxic agent. Its direct toxicity remains dependent on properties such as the concentration applied, the molecular weight, degree of acetylation, solvent, pH and viscosity. The degree of acetylation defines the sites with which nucleophilic groups could react and viscosity provides an environment that could extend the duration and intensity of reactions.

4.2. Physical barrier around pathogen penetration sites

Chitosan, when applied to plant tissues, often agglutinate around the penetration sites and has two major effects. The first one is the isolation of the penetration site through the formation of a physical barrier preventing the pathogen from spreading and invading other healthy tissues. This phenomenon resembles the abscission zones often observed on leaves preventing several necrotrophic pathogens from spreading further. It is widely observed on potato tubers for example. Around the isolated zones, often an elicitation of a hypersensitive response occur with the accumulation of H_2O_2 that helps in cells wall fortification and serve as an alert signal for other healthy parts of the plant. The second effect is due to the chitosan' ability to bind various materials and initiate fast the wound healing process.

4.3. Chelation of nutrients and minerals

Chitosans are well used in the fresh and salt water purification process as chelators for minerals and metals. These abilities are also explored when chitosan is applied to plants to prevent diseases because it can chelate nutrients and minerals (*i.e.*, Fe, Cu), preventing pathogens from accessing them. These polysaccharide molecules were also reported to bind mycotoxins, which may reduce damage to the host tissues due to toxins. In the beverage industry, for example, chitosan and derivatives are often used for their antimicrobial properties linked to their chelating abilities of nutrient and minerals, thus reducing fungal spoilage.

4.4. Effect on H^+-ATPase and depolarization of biological membranes

Various researchers reported on the early events that occur during the elicitation of plant defenses using chitosan. They showed that this molecule was able to trigger, in a dose-dependent

manner, a quick and transient depolarization of *Mimosa pudica* motor cell membranes. These modifications were also accompanied by a transient rise in pH. Using plasma membrane vesicles, the authors determined the site of action of this polysaccharide to be the plasma membrane H^+-ATPase due to the inhibitory effect observed on the proton pumping and the catalytic activity of the enzyme. Chitosan was also shown to alter many other H^+-mediated processes. For example, the uptake of certain carbohydrate and amino-acids was altered because of their dependence on co-transporters involving an exchange with H^+. Similarly, the light-induced H^+/K^+-mediated turgor reaction was shown to be inhibited in *M. pudica* motor cells in response to the treatment with chitosan.

Ultra-structural studies have shown that treatment with chitosan induces a series of morphological and structural modification, leading to disorganized hyphae associated with inhibition of fungal growth. This was linked to the polycationic properties of chitosan, allowing for changes in terms of membrane permeability and cytoplasmic aggregation. As a consequence, the activities of a number of enzymes involved in the synthesis and assembly of cell wall polymers are disturbed.

Chitosan and derivatives are known to act as potent inducers, enhancing a battery of plant responses both locally around the infection sites and systemically to alert healthy parts of the plant. These include early signaling events as well as the accumulation of defense-related metabolites and proteins such as phytoalexins and PR-proteins. Modulation of plant responses using chitosan has been reported in many pathosystems involving various plant species and a diverse range of pathogens, including virus and viroids, bacteria, fungi, nematodes and other pests. This biopolymer was shown to be an effective inducer of phytoalexins synthesis and accumulation in various host cells, and triggers callose formation, lignification responses, and the production of proteinase inhibitors.

Researchers studied the effect of chitosan in date palm in response to *Fusarium oxysporum* f. sp. *albedinis*, the causal agent of a major wilt in this crop. Beside a direct toxicity of the molecule on the fungus, the authors showed an enhancement of essential components of the host resistance. When injected into the roots at various concentrations, chitosan elicited date palm peroxidase and polyphenoloxidase activities, and increased the level of phenolic compounds. Among the accumulated phenolics, there was an increase in content of specific non-constitutive hydroxycinnamic acid derivatives, known to be of great importance in the resistance of this plant to this vascular fusariosis. Similarly, treatment of wheat seeds with chitosan revealed an increase in hydroxycinnamic (*i.e.*, *p*-coumaric, caffeic and ferulic) and benzoic (*i.e.*, benzoic, protocatechuic and gallic) acid derivatives, leading to an increase in lignin synthesis and accumulation. PAL activity was also reported to increase in response to elicitation with chitosan in many host species.

Researchers used a microarray consisting of 2,375 EST clones representing putative defense-related and regulatory genes to characterize changes in the gene expression patterns of *A. thaliana* in response to treatment with chitin. The authors reported that 71 ESTs, representing 61 genes, were altered three-fold or more in their transcript levels in chitin-treated seedlings as compared to the control. Interestingly, the levels of transcription of numerous genes were revealed to be altered as early as 10 min after exposure to chitin, hence translating the earliest changes that may occur in chitin-treated plants. These genes included commonly elicited defense-related genes (*i.e.*, phenylalanine amonia-lyase, chitinase, peroxidase) as well as other genes with function not yet identified. Among the transcriptional regulators, the authors reported on the increase in transcript accumulation of elements at the promoters region rich in W-boxes

along with other unknown regulatory elements. In parallel, researchers showed a decrease in transcript abundance of a number of genes encoding cell wall strengthening and wall deposit proteins. These genes were all downstream the chalcone synthase promoter, suggesting their potential suppression during plant x pathogen interactions. The authors also examined the genes based on their controlling pathways. They found that among the up-regulated genes in response to treatment with chitin, there were 43% that were also up-regulated with salicylic acid, 39% with methyl jasmonate and another 36% with ethylene. Among the down-regulated genes in response to chitin, 7% shared the down-regulation with salicylic acid, 9% with methyl jasmonate and 14% with ethylene.

Similarly, researchers examined the expression of defense-related genes in rice treated with *N*-acetylchitooctaose, using microarray analysis consisting of 8,987 randomly selected expressed sequence tags. In their experiments, the authors reported on the significant up-regulation of 166 genes and down-regulation of 93 genes. Out of the 259 responsive ESTs to *N*-acetylchytooctaose identified, the authors highlighted 18 putative genes related to signal transduction, including five calcium-dependent protein kinases (CDPKs).

4.6. Chitosan–A general pathogen-associated molecular pattern

Plants possess mechanisms by which they recognize their intruders. They are thought to have trans-membrane pattern recognition receptors (PRRs) able to interact with pathogen/microbe-associated molecular patterns PAMPs/MAMPs. PAMPs/MAMPs can be any effectors secreted by the pathogens or released from the cell wall of the host upon attack on the infection site. Cell wall polysaccharides such as glucans and chitosan have been reported to act as PAMPs/MAMPs in many pathosystems. Chitosan presents the advantage of being recognized by plant PRRs and triggers a panel of defense responses. Researchers reported that chitosan behaves like a PAMPs/MAMPs or a general elicitor, inducing non-host resistance and priming systemic immunity. The defense responses enhanced by chitosan application include the increase in H^+ and Ca^{2+} influx into the cytosol, the activation of MAP-kinases, callose apposition, oxidative burst, hypersensitive responses, the synthesis of abscisic acid, jasmonates, phytoalexins, and PR-proteins.

It was long believed that the elicitor activity of chitosan is mediated through the interaction of this polycationic molecule with negatively-charged phospholipids, rather than a specific interaction with a receptor-like molecule. However, researchers, examining the expression patterns of two GRAS family genes responsive to chitosan, have suggested that these two genes were regulated, at least partially, by high-affinity chitin-binding proteins localized in the plasma membrane of rice. Recently, several chitosan-binding proteins have been isolated and described as putative receptors for chitosan. These proteins are thought to bind also to chitin and have been called chitin elicitor-binding protein (CEBiP). However, the biological activity of chitosan, as a general elicitor, remains tied to its physicochemical properties such as the molecular weight, deacetylation degree and viscosity. These properties can make the difference between cytotoxicity due to higher concentrations and the priming of resistance using appropriate molecular weight, deacetylation degree, viscosity and concentration.

4.7. Effect on nuclear distortion and cell death

Chitosan induces programmed-cell death (PCD) and hypersensitive-associated responses in plants. It induced chromatin condensation and marginalization followed by a destruction of the nuclei and subsequent inter-nucleosomal DNA fragmentation. It did not affect stomatal guard cells but affected epidermal cells. Anaerobic conditions prevented the chitosan-induced destruction of epidermal cells' nuclei. The antioxidants nitroblue tetrazolium or mannitol