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Mini review

Induced resistance in plants and the role of pathogenesis-related proteins

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The nature of induced resistance

Resistance, according to Agrios (1988) is the ability of an organism to exclude or overcome, completely or in some degree, the effect of a pathogen or other damaging factor. Disease resistance in plants is manifested by limited symptoms, reflecting the inability of the pathogen to grow or multiply and spread, and often takes the form of a hypersensitive reaction (HR), in which the pathogen remains confined to necrotic lesions near the site of infection. Induced resistance is the phenomenon that a plant, once appropriately stimulated, exhibits an enhanced resistance upon 'challenge' inoculation with a pathogen. Although induced resistance has been attracting attention recently (Ryals et al., 1994; Hammerschmidt and Kuc, 1995), the first systematic enquiry into induced resistance was made by Ross (1961a,b). He observed that the inducible resistance response to tobacco mosaic virus (TMV) in *N* gene-containing, hypersensitively reacting tobacco was not confined to the immediate vicinity of the resulting local necrotic lesions, but extended to other plant parts. A ring of tissue around the developing lesions became fully refractory to subsequent infection (localized acquired resistance; Ross, 1961a), whereas challenge inoculation of distant tissues resulted in much smaller, and occasionally fewer, lesions (systemic acquired resistance (SAR); Ross, 1961b) than in non-induced plants. Even leaves that were mere initials at the time of the primary inoculation became induced, suggesting that as a result of the initial infection, a signal was generated, transported and propagated, that primed the plant to respond more effectively to subsequent infection (Bozarth and Ross, 1964). Treatments that influenced lesion size after primary infection had similar effects on lesions developing upon challenge inoculation (Ross, 1966), leading to the conclusion that the mechanisms responsible for resistance expression were the same under both conditions. Only upon challenge inoculation, defense mechanisms appeared to be expressed earlier and to a greater extent (De Laat and Van Loon, 1983; Dean and Kuc, 1987).

Subsequent work, notably by Kuc and co-workers, has shown that induction of disease resistance in plants by necrotizing pathogens is a general phenomenon, and that the induced resistance is non-specific with respect to both the inducing and the challenging pathogen (Hammerschmidt and Kuc, 1995). Thus, a primary infection of cucumber with the fungus *Colletotrichum lagenarium* or with tobacco necrosis virus (TNV) led to enhanced resistance against fungi, bacteria and viruses causing various foliar and root diseases (Kuc, 1982). In all cases symptom expression due to the challenging pathogen was substantially reduced, sometimes to the extent that infection was hardly apparent. These observations indicate that induced resistance constitutes a mechanism through which the level of general resistance to pathogens is increased. That this enhanced resistance depends on extant mechanisms is illustrated by examples showing their increased expression upon challenge inoculation. In tobacco the number of lesions developing after inoculation with TMV is a reflection of virus concentration and number of infectible sites on the leaves, which are influenced by the ambient conditions (Whenham and Fraser, 1981). No defense mechanism appears to operate against virus entry, which is considered passive. Consequently, induced resistance is not evident as a reduction in lesion numbers,

(unless lesions remain so small as to escape detection). In contrast, resistance does operate at the level of virus multiplication and/or spread, and enhanced resistance results in reduced lesion expansion (Ross, 1966; Van Loon, 1983a). Likewise, tobacco does not seem to mount a defense against tissue penetration by the blue mold fungus *Peronospora tabacina*, but formation of haustoria in the tissue can trigger cell wall lignification, impeding the establishment of an effective feeding relationship. In induced tissues, enhanced cell wall deposition severely restricted fungal development, indicative of an enhanced defensive response (Ye et al., 1992). On the contrary, infection of cucumber by *C. lagenarium* is already restricted at the level of tissue penetration, with formation of papillae beneath appressoria. Upon induction of resistance, a reduction of fungal development was observed both at the leaf surface and in the mesophyll, indicative of several induced defense reactions being activated simultaneously as a result (Kovats et al., 1991). Although it has occasionally been inferred that induced resistance involves masking of symptoms rather than pathogen restriction (Fraser, 1979; Doss and Hevisi, 1981), this is certainly not the case for the large majority of plant-pathogen combinations that have been studied in detail (Hammerschmidt and Kuc, 1995). For instance, in tobacco reduction in TMV lesion diameter was closely associated with a reduction in virus titer (Ross, 1966), and reduction of blue mold symptom severity with inhibition of fungal growth (Ye et al., 1992). It appears then that, indeed, induced resistance is operative through those mechanisms that function normally to restrict pathogen growth and spread, and that the effectiveness of those mechanisms is enhanced as a result of a primary necrotizing infection. A consequence of this conclusion would be that induced resistance should not be manifest after challenge inoculation with a pathogen that apparently circumvents triggering any resistance reaction in the host, i.e. in what is commonly considered to be a compatible reaction. This does not seem to be the case, however, because also virulent pathogens are often found to be restricted in their activity in induced tissues. This holds, e.g. for TMV in non-*N* gene-containing tobacco, where induction of resistance by TNV did reduce multiplication of TMV in inoculated leaves (Van Loon and Antoniw, 1982), be it without preventing the virus from escaping and inducing systemic mosaic symptoms in the young leaves. However, even in systemically reacting tobacco, resistance mechanisms are operative in those cells that give rise to the green parts of the mosaic tissue, where inhibition of viral replication is evident (Murakishi and Carlson, 1976). In compatible plant-fungus and plant-bacterium interactions resistance mechanisms may be activated too slowly to be effective or be suppressed by the invading pathogen, and in induced tissues the balance may be shifted in favour of the plant. Thus, even in seemingly non-resistant plants, a certain level of resistance may be extant or triggered, and this may be enhanced when resistance is induced by primary infection. The level of basic resistance may simply not be sufficient to halt infection and prevent extensive tissue colonization and symptom development. Often, defense mechanisms are found to be activated late in infection, when the plant can no longer benefit from these activities, because the pathogen had already colonized the tissue. An earlier and quicker response of the plant then can be effective in limiting tissue colonization. Upon induction, enhancement of resistance might have from substantial to only marginal effects, depending on the specific plant-pathogen relationship.

Terminology

Because of the enhanced protection afforded by induction of resistance through exposure to a pathogen, the term ‘induced resistance’ has been used synonymously with ‘acquired resistance’, ‘acquired immunity’ and ‘immunization’ (e.g. Kuc, 1983). The term immunization is misleading. Because plants, unlike animals, neither possess a circulatory system, nor immune surveillance, the mechanisms must be entirely different. Indeed, immunization of an animal leads to the production of antibodies that are highly specific for the antigen encountered, whereas induced resistance is non-specific. The result of animal immunization resembles more the phenomenon of ‘cross protection’, where a mild strain of a virus protects plants against severe isolates or strains of the same or closely related viruses (Urban et al., 1990), but the mechanisms again appear very different. Hence, the term immunization appears inadequate and should not be used to describe induced resistance in plants.

Induced disease resistance has been adopted as a general term and defined as ‘the process of active resistance dependent on the host plant’s physical or chemical barriers, activated by biotic or abiotic agents (inducing agents)’ (Kloepper et al., 1992). The term ‘induced resistance’ is not entirely unambiguous. It might seem to imply that resistance was absent, but became present as a result of the action of an inducing

agent. In fact, as discussed above, induced resistance is dependent on extant resistance mechanisms and, thus, resistance must be operative to begin with. Resistance to primary infection can result from the presence of preformed defensive barriers (Osbourn, 1996), but often depends on inducible resistance mechanisms, the infecting pathogen triggering defense responses through the release of elicitors which, in turn, lead to the expression of novel anti-pathogenic activities (Hammond-Kosack and Jones, 1996). The HR is a case in point, where resistance is expressed only as a result of the specific recognition between plant and pathogen (Crute and Pink, 1996). 'Induced resistance' appears to constitute another layer of interaction between pathogen and plant, requiring its induction along with the defenses triggered upon primary infection, but expressed only when challenge actually occurs. In that sense, induced resistance is the additional capacity for defensive activities resulting from the primary infection, and dependent on the concomitant triggering of resistance responses. Once a plant has been stimulated in this way, it can express this enhanced defensive capacity irrespective of whether the challenging pathogen gives rise to an incompatible or to a compatible interaction.

The term induced resistance emphasizes the fact that a triggering factor ('inducing agent') is needed to achieve this enhanced defensive capacity. The term 'acquired resistance', advocated by Ross (1961a,b), points to a change in the physiology of the plant resulting from an added property. Acquired resistance obviously is not the phenomenon that resistance was not present, but acquired only as a result of primary infection. In that respect, the term suffers from the same drawback as induced resistance. It does, however, circumvent the possible confusion arising from the occurrence of inducible defense responses to primary infection, and emphasizes that the enhanced defensive capacity resides in a plant property. Meanwhile, the term 'systemic acquired resistance' (SAR) has found widespread acceptance in describing the state of enhanced defensive responsiveness throughout a plant resulting from local infection with a pathogen inducing necrotic lesions, such as in a HR. The term is appropriate and emphasizes the historic link with the pioneering work of Ross (1961a,b; 1966). Whereas Kloepper et al. (1992) suggested the use of the term 'induced systemic resistance' as an all compassing designation, this has not been generally adopted. However, it can be appropriately used in a broader sense for phenotypically similar phenomena resulting from different types of agents or treatments. Another way to define induced resistance would be to allude to the induced state as the 'enhanced defensive capacity' (EDC) of the plant. This term avoids any confusion that may arise about the meaning of 'induced' or 'resistance', while stressing that it is a 'capacity' that is utilized only upon challenge inoculation.

Association of pathogenesis-related proteins with induced resistance

The notion that the enhanced resistance apparent upon challenge inoculation depends on the same defense mechanisms as expressed after primary infection led to the identification of common metabolic alterations induced systemically in response to local infection. Whereas induction of phytoalexins and cell wall rigidification are local reactions, accumulation of pathogenesis-related proteins (PRs) extends into non-inoculated plant parts that, upon challenge, exhibit acquired resistance (Van Loon and Van Kammen, 1970; Ryals et al., 1996). The proteins themselves are not transported from the primary inoculated leaves, as demonstrated elegantly by Gianinazzi and Ahl (1983) through the analysis of reciprocal grafts of *Nicotiana* species expressing electrophoretically different proteins. While a link between PRs and acquired resistance in virus-infected tobacco was immediately hypothesized (Van Loon and Van Kammen, 1970; Kassanis et al., 1974; Van Loon, 1975), Fraser (1982) pointed out that PRs became apparent in non-inoculated leaves distinctly later than acquired resistance appeared manifest. However, in tissues already primed to express PRs, challenge inoculation might lead to their earlier and faster accumulation. Moreover, a hybrid between *N. glutinosa* and *N. debneyi* constitutively expressed PRs and was highly resistant against TNV (Ahl and Gianinazzi, 1982).

Induction of PRs has since been found to be invariably linked to necrotizing infections giving rise to SAR, and has been taken as a marker of the induced state (Ward et al., 1991; Uknes et al., 1992; Kessmann et al., 1994). This notion has been reinforced by the characterization in *Arabidopsis* of mutants that either are compromised in both the production of PRs and the induction of SAR (*npr1*; Cao et al., 1994, *nim1*; Delaney et al., 1995), or are constitutive expressors of PR genes as well as SAR (*cpr1*; Bowling et al., 1994). PRs have been defined as plant proteins that

are induced in pathological or related situations (Van Loon et al., 1994). Although they are implicated in plant defense, they have not been identified because of their anti-pathogenic action, but solely because of their accumulation in infected plants. Eleven families of PRs have now been officially recognized (Van Loon et al., 1994), but additional pathogen-induced proteins with potential anti-pathogenic action keep being described (e.g. Broekaert et al., 1995). PRs have been identified in at least nine plant families, with those in tobacco and tomato characterized best. It is now known that they comprise four families of chitinases (PR-3, -4, -8 and -11), one of β-1,3-glucanases (PR-2), one of proteinase inhibitors (PR-6), and one specific peroxidase (PR-9), as well as the PR-1 family with unknown biochemical properties, the thaumatin-like PR-5 family, and the birch allergen *Betv1*-related PR-10 family. Not all families are represented in any plant species, but each family may comprise several members.

Together the PRs form a set of pathogen-induced proteins that may be considered as stress proteins. In the past decades it has become evident that plants, when exposed to various environmental stresses, respond by synthesizing sets of specific proteins. Well-known are the heat-shock proteins, that appear to be common to all living organisms, and are transiently induced when ambient temperature exceeds some critical limit (Vierling, 1991). Different sets of proteins are induced by e.g. drought stress or freezing temperatures. For instance, during cold acclimation hardy cultivars of alfalfa synthesize a number of proteins that supposedly function in reducing the deleterious effects of low temperature on plant membranes. The plant hormone abscisic acid (ABA) induces a partly similar set of proteins and increases resistance to freezing stress, indicating that acclimation is hormone-controlled (Mohapatra et al., 1988; Heino et al., 1990). Similar proteins are induced by ABA during the acquisition of desiccation tolerance in developing seeds and upon drought stress of leaves (Skriver and Mundy, 1990). PRs may be considered as stress proteins produced in response to, particularly necrotizing, infections by viruses, viroids, fungi and bacteria, and thought to function in the acquired resistance against further infection (Van Loon, 1989). However, in contrast to most other types of stress proteins, they accumulate in plant tissues to levels that are easily detectable on gels by general protein stains. Why these inducible PRs may individually reach up to 1% of the total soluble protein in leaves, is unclear.

Some of the PRs possess potential antipathogenic activities (Linthorst, 1991; Van Loon et al., 1994). Chitinases, together with glucanases, could be directed against fungal cell walls and, perhaps, insects. Insects are likely to be affected by proteinase inhibitors. Bacteria may be inhibited by the PR-8 family of chitinases, which also possesses lysozyme activity. The PR-9 peroxidase is of the lignin-forming type and could be involved in the strenghtening of cell walls. The PR-10 family has sequence similarity to ribonucleases and is the only family consisting of cytoplasmic proteins, but there is no evidence that PR-10 proteins are active against e.g. viruses. The PR-1 and PR-5 proteins are often strongly induced and seem to affect membranes, but their precise actions have not been elucidated.

The inducible PRs are mostly acidic proteins that are secreted into the intercellular space of the leaf. Both through cDNA sequence homologies and on the basis of similar enzymatic activities, additional basic counterparts have been identified. These basic PRs occur at relatively low levels in the vacuole and, besides being induced upon infection, are expressed in a tissue-specific and developmentally-controlled manner in leaves, roots and floral parts (Eyal and Fluhr, 1991; Linthorst, 1991). Specific activities vary greatly, i.e. from 5 nKat/mg with laminarin as a substrate for the inducible, acidic glucanase PR-2a to 23 and 1300 nKat/mg for -2b and -2c, respectively, and 1100 nKat/mg for the developmentally-controlled basic glucanase PR-2e (Kauffmann et al., 1987). It has been suggested that in induced plants the accumulated intercellular proteins form the first line of defense to a challenging pathogen and, if this fails and the tissue is disrupted, the release of the vacuolar PRs functions as a second line, engulfing the pathogen with lytic enzymes (Mauch and Staehelin, 1989). The constitutive expression of several of the basic proteins in older leaves, roots and developing flowers could be similarly considered as a protective mechanism against pathogen invasion, possibly contributing to the often observed increase in resistance with plant age. However, the organ-specific expression of specific PR genes suggests that the proteins also play roles in normal developmental processes.

Effects of pathogenesis-related proteins on expression of resistance

Constitutive expression of individual PRs in transgenic plants can lead to reduced pathogen growth and symp-

tom expression, consistent with a role of PRs in the expression of acquired resistance (Ryals et al., 1994). However, such effects are by no means general and pathogens may have evolved mechanisms to reduce the effects of PRs. Thus, many chitin-containing fungi are not inhibited by chitinases, presumably because the chitin in their cell walls is shielded by a protective layer. Such a layer may be less developed at growing hyphal tips, which can be lysed (Schlumbaum et al., 1986). Significant suppression of disease symptoms caused by the soil-borne fungus *Rhizoctonia solani* was demonstrated in tobacco or canola expressing a vacuolar (class I) chitinase from bean (Broglie et al., 1991), the basic tobacco chitinase PR-3c (Lawton et al., 1993; Vierheilig et al., 1993), tobacco or cucumber PR-8 (Lawton et al., 1993), or the (class II) barley chitinase (Jach et al., 1995), but enhanced chitinase levels caused no significant protection against *Cercospora nicotianae* (Neuhaus et al., 1991; Nielsen et al., 1993; Zhu et al., 1994) or *Fusarium oxysporum* (Van den Elzen et al., 1993; Jongedijk et al., 1995). The reduction of *R. solani* in vacuole-targeted class I chitinase-transformed plants is fairly unexpected, because tobacco roots constitutively express high levels of their own class I chitinase but, nevertheless, are fully susceptible.

Antifungal activity of chitinases can be synergistically enhanced by β-1,3-glucanases, both in vitro and in vivo. Thus, co-expression of chitinase and glucanase genes in tobacco enhanced resistance against *C. nicotianae* (Zhu et al., 1994; Jach et al., 1995). In tomato, simultaneous expression of the basic tobacco chitinase PR-3d and glucanase PR-2e afforded substantial protection against *F. oxysporum* f.sp. *lycopersici*, whereas transgenic plants expressing either one of these genes were not protected (Jongedijk et al., 1995). Targeting the proteins to the apoplast was not more effective, indicating that tonoplast leakage must occur sufficiently early to halt pathogen progress. Combinations of the acidic tobacco chitinase PR-3a and glucanase PR-2b were not effective when accumulating either in the apoplast or in the vacuole, nor were combinations of an acidic glucanase with a basic chitinase, or vice versa. Thus, the inducible acidic PRs associated with SAR were ineffective, whereas the constitutively expressed basic PRs were.

The situation appears largely similar for the PR-1 and -5 proteins, which have been found to possess antifungal activity against oomycetes, which lack chitin in their cell walls. Constitutive expression of PR-1a in tobacco reduced symptoms caused by *P. tabacina* (Alexander et al., 1993; Lawton et al., 1993) and *Phytophthora parasitica* f.sp. *nicotianae* (Alexander et al., 1993), but not those provoked by the non-oomycete *C. nicotianae*, or the bacterial pathogen *Pseudomonas syringae* pv. *tabaci* (Alexander et al., 1993). The various tomato and tobacco PR-1 proteins displayed inhibitory activity on the growth of *P. infestans* in tomato leaf disc assays, with tomato PR-1c and tobacco PR-1g being the most effective family members (Niderman et al., 1995). Basic PR-5 ('osmotin') likewise has antifungal activity against *P. infestans* (Vigers et al., 1992; Zhu et al., 1996), but in transgenic tobacco no delay in symptoms caused by *P. parasitica* f.sp. *nicotianae* was apparent (Liu et al., 1994). So far, no results have been published on suppression of pathogens in transgenic plants expressing PR-4, but basic tobacco PR-4c exhibits antifungal activity in vitro against certain fungi and under these conditions has been found to act synergistically with basic tobacco PRs -2 and -3 (Ponstein et al., 1994). An additional 'SAR gene' in tobacco, SAR 8.2. when expressed constitutively in transgenic tobacco, was also found to reduce disease caused by *P. parasitica*, but the protein has not been characterized (Lawton et al., 1993).

Taken together, these observations do not indicate a significant role of the major, pathogen-inducible PRs in the enhanced resistance expressed upon challenge inoculation of plants with SAR. Additional SAR genes, comprising both minor, developmentally-controlled PRs and those encoding mRNAs for which the protein has not been identified (SAR 8.2; Ward et al., 1991, glycine-rich protein; Van Kan et al., 1988; Linthorst, 1991) could have more effect. However, in as far as the activities of PRs have been determined, these are directed only against fungi; PRs or similar proteins effective against bacteria or viruses have, so far, not been identified. A role of proteinase inhibitors against insect attack is well established (Ryan, 1990), but SAR is not commonly associated with enhanced protection against insects. Screening specifically for antifungal, antibacterial and antiviral activities in plants has yielded PR-like proteins with antifungal properties related to PR-families -4 (Hejgaard et al., 1992) and -5 (Vigers et al., 1991), as well as ribosome-inactivating proteins, thionins, lectins and defensins (Linthorst, 1991). These proteins commonly occur in storage organs, such as seeds and tubers, but may also be induced in leaves following pathogen attack (Broekaert et al., 1995).

Judging from the synergistic actions of some of these proteins when expressed together, it may be expected that when multiple SAR genes are coordinately expressed, such as in a HR, complementary

actions of the resulting proteins could yield a strong anti-pathogenic potential. Moreover, when resistance responses are activated upon challenge inoculation, PRs are induced again, and more quickly and strongly than in non-induced plants. However, the apparent lack of PRs or other induced defensive proteins with activity against bacteria and viruses is difficult to reconcile with the non-specificity of acquired resistance. It is not inconceivable that, besides the conspicuous PRs, several other compounds are induced that have anti-pathogenic activities, but are yet to be discovered. The significance of the inducible PRs then becomes difficult to assess at present. It is intriguing that the thaumatin-like PR-5 family is expressed not only in response to pathogens, but also during osmotic stress. Thus, osmotin (tobacco PR-5c) and its homolog NP24 (PR-5a) in tomato were independently identified as being induced by salt stress (Singh et al., 1987) and infection (Stintzi et al., 1991). Interestingly, lipid transfer proteins can also be induced by infection, have antifungal activity, and are expressed during drought stress (Kader, 1997). Such observations support a role for PRs as stress proteins with functions that exceed their involvement in plant-pathogen interactions.

Systemic signalling

Observations that exogenously applied salicylic acid (SA) induces both acquired resistance and PRs in e.g. tobacco, tomato and *Arabidopsis*, that SA accumulates endogenously in tobacco and cucumber expressing a HR and developing SAR, and that transgenic tobacco and *Arabidopsis* plants expressing the salicylate hydroxylase gene *NahG* from *Pseudomonas putida* do not accumulate SA and are incapable of expressing PRs or SAR in response to pathogen infection, have provided proof that SA acts as a signal in the induction of acquired resistance (Gaffney et al., 1993). Moreover, *NahG*-containing plants are more susceptible to a variety of fungal, bacterial and viral pathogens (Delaney et al., 1994). Thus, SA is required for the expression of resistance, as well as for the enhanced defensive capacity of tissues with acquired resistance (Ryals et al., 1994, 1996). Salicylate is synthesized from cinnamic acid and dependent on the functioning of the phenylpropanoid pathway (Yalpani et al., 1993). Although accumulation of SA is required for the development of SAR, and it may be transported from infected leaves (Shulaev et al., 1995; Mölders et al., 1996), it does not appear to be the primary long-distance signal for systemic induction (Rasmussen et al., 1991; Vernooij et al., 1994).

Induction of acquired resistance and PRs is often accomplished by spraying plants with SA solution and assaying of the sprayed leaves. Such an experimental set-up is inadequate for testing systemic effects, because SA is applied at the site of challenge. In experiments in which SA was injected into three leaves of a tobacco plant, and challenge inoculation was carried out on non-treated upper leaves, in 6 out of 12 experiments no induction of SAR was apparent, and in none were PRs recognizable in the upper leaves. In those experiments in which acquired resistance was apparent in upper leaves, veins were likely to be hit during the injection procedure. When SA was watered on the soil, acquired resistance was apparent in upper leaves, indicating that SA was absorbed by the roots and transported throughout the plant (Van Loon and Antoniw, 1982). In contrast, upon careful injection, SA does not seem to move beyond the injected leaf and under those conditions, induction of acquired resistance and PRs were found to be confined to the treated leaf. Thus, the effect of SA is local, even though it is required for acquired resistance to be expressed.

It has been described that SA coordinately induces the full spectrum of SAR genes (Ward et al., 1991), encompassing all well-characterized PRs. This appears correct, but the relative amounts of individual PR-mRNAs or proteins differ greatly from those seen during a HR. For instance, in *N* gene-containing tobacco SA induces PR-1a, -1b, -1c, -2a and -2b to the same extent as TMV, but -2c, -3a, -3b, -4 and -5 remain at low levels and only reach high levels in the presence of additional signalling compounds, such as ethylene (Van Loon, 1977) or jasmonic acid (Xu et al., 1994). Differential induction of PR genes by SA has also been demonstrated at the mRNA level (Brederode et al., 1991), indicating that SA cannot be the only signal in the induction of PRs. Moreover, necrotic lesion formation in tobacco is associated with the induction of all ten PRs mentioned above, but in non-inoculated leaves expressing SAR only nine accumulate, with PR-2c missing (Van Loon and Gerritsen, 1989). The latter observation clearly suggests that systemic induction of PR-2c is regulated independently.

A reason for the repeated statements that SA induces the full set of SAR genes may be the toxicity of the high concentrations commonly used for spraying. In tobacco, 1 mm proved to be at the border of toxicity, as determined by the tendency of the

stress enzyme peroxidase to increase (Van Loon and Antoniw, 1982). However, SA is often sprayed at a concentration of 5 mM when studying SAR induction, and growth retardation, leaf yellowing and even marginal necrosis are not uncommon, particularly on sensitive plants, such as *Arabidopsis*. A slow, progressive necrosis, by itself, is a powerful inducing condition of both acquired resistance and PRs (cf. Hammerschmidt and Kuc, 1995). This is particularly evident in the so-called *acd* (accelerated cell death; Greenberg et al., 1994) and *lsd* (lesions simulating disease; Dietrich et al., 1994) mutants of *Arabidopsis*, that form spontaneous lesions resembling a HR, express SAR genes and exhibit enhanced resistance. In tobacco, necrosis-inducing peptide elicitors (elicitins) from *Phytophthora* species likewise induce both PRs and SAR (Ricci et al., 1989). PRs have been described to be induced by numerous conditions and chemical compounds, including senescence, callus culture, UV light, wounding, plasmolysis, polyacrylic acid, auxin, cytokinin, heavy metal salts, mannitol, amino acids, thiamine, arachidonic acid, ozone, hydrogen peroxide, etc. (Van Loon, 1983b; Kessmann et al., 1994; Ryals et al., 1996). Whilst some of these treatments may directly affect components of the signal-transduction pathway, others are likely to act by stressing the plant or through their toxic action, even without visible necrosis becoming apparent. During a HR, such as in tobacco, necrotic lesions, once formed, tend to expand for a few days before finally being limited (Ross, 1966). Thus, at least during the first few days, necrotic cells are surrounded by a ring of tissue in which cells are strongly reacting before being overtaken and succumbing. During this slow necrotization process, there must be ample opportunity for the release of eliciting and signalling compounds to neighbouring cells and distant tissues.

Induction of systemic resistance by non-pathogenic micro-organisms

Although SAR is usually associated with localized necrosis, compatible interactions can also lead to SAR (Kuc, 1982; Ryals et al., 1994) and necrosis is not required for SAR induction. Davis and Ross (1968) demonstrated that acquired resistance was evident in tobacco displaying mosaic symptoms due to infection with potato virus Y, and such infection was subsequently shown to be accompanied by the presence of at least two PRs (Van Loon, 1975). Localized symptomless (starch) lesions due to TMV on cucumber were reported by Roberts (1982) to be associated with SAR induction. A mutant of *Arabidopsis* developing disease symptoms following inoculation with a normally avirulent bacterium developed SAR, but exhibited a weaker response when challenged, indicating that a HR contributes to, but is not essential for, the induction of SAR (Cameron et al., 1994). Similarly, some of the chemicals indicated above, e.g. amino acids, as well as specific benzoic acid derivatives, can induce resistance without any necrosis developing. Particularly evident is systemic resistance induced by non-pathogenic, rhizosphere-colonizing bacteria of the genus *Pseudomonas*. These bacteria are often referred to as plant growth-promoting rhizobacteria (PGPR), because they are able to suppress deleterious micro-organisms in the soil and, thereby, improve plant stand. Such induced resistance has been demonstrated conclusively in test systems in which the inducing bacterium and the challenging pathogen remained spatially separated for the duration of the experiments, and any direct interference of the bacteria with the activity of the pathogens was ruled out.

Pseudomonas fluorescens strain WCS417 was applied to the roots of carnation, and plants were challenged one week later by stem inoculation with *F. oxysporum* f.sp. *dianthi*. As a result, both the number of diseased plants and disease severity were significantly reduced compared to plants not treated with the bacteria (Van Peer et al., 1991). Similar observations have been made in cucumber (Wei et al., 1991), tobacco (Maurhofer et al., 1994), radish (Leeman et al., 1995a), *Arabidopsis* (Pieterse et al., 1996) and tomato (Duijff et al., 1997). Although in some of these and other studies spatial separation of the inducing and challenging micro-organisms was not proven, many reports can be reasonably explained by assuming a plant-mediated enhanced defensive capacity as a result of root bacterization. Seed bacterization has likewise been shown to be effective and, under those conditions, bacteria are able to colonize not only the emerging roots, but also to some extent the developing shoot (Raaijmakers et al., 1995a). Due to competition and nutrient limitation, bacterial numbers on cotyledons or leaves remain generally low. In contrast, root exudates stimulate multiplication of bacteria in the rhizosphere (Lynch, 1976). Once induction of resistance has occurred, the numbers of bacteria may dwindle without their protective effect being lost. Direct application of bacteria to roots during transplanting, or transplanting seedlings into bacterized soil, are also used for induction. Under those

conditions, inducing bacteria have not been recovered from the above-ground parts, and reduced symptom expression upon challenge with foliar pathogens can be attributed only to plant-mediated induced systemic resistance (ISR). To be able to conclude that ISR is the mechanism by which PGPR suppress root diseases, it must be shown that on the root system no contact between the inducing bacteria and challenging pathogen occurs. This has been demonstrated in split-root experiments (Liu et al., 1995a), as well as in a separate inoculation system (Leeman et al., 1995a), in which roots are placed horizontally on rockwool cubes. Adjoining rockwool cubes are compartmented through enclosure in plastic bags, only a small excision in the bags allowing protrusion of the radicle into the next compartment. Routinely, the lower part of the root in one compartment is treated with a bacterial suspension in peat or talcum emulsion. After a few days, the upper part of the root in another compartment is inoculated with the challenging pathogen. In this way, induction of systemic resistance has been demonstrated in radish (Leeman et al., 1995a) and *Arabidopsis* (Van Wees et al., 1997) against *F. oxysporum* f.sp. *raphani*. In no case were inducing bacteria found to be present in the compartment harbouring the challenging pathogen. That protection is plant-mediated rather than the result of inhibition of the pathogen by a transported bacterial metabolite, was borne out by the induction of systemic resistance through application of heat-killed bacteria (Van Peer and Schippers, 1992) or purified bacterial lipopolysaccharide (LPS) (Leeman et al., 1995b). Apparently, the outer membrane LPS is recognized by the plant root, and triggers one or more signals leading to an enhanced resistance resembling SAR. From a few days to a week are commonly needed for ISR to develop (Leeman et al., 1995a). This demonstrates that, like in SAR, the plant needs time to respond and reach the induced state.

PGPR show little specificity in their colonization of roots of different plant species. However, induction of resistance appears highly specific with individual bacterial isolates being active on some species, but not others (Table 1). Moreover, within a plant species, genetic variation appears to exist with regard to inducibility by rhizobacteria. Thus, *P. fluorescens* strain WCS417 induced substantial resistance against *F. oxysporum* f.sp. *dianthi* in the relatively resistant carnation cultivar Pallas, and less so in the relatively susceptible cultivar Lena (Van Peer et al., 1991). This observation is consistent with the notion that the induced state constitutes an enhancement of the extant defensive capacity. However, no difference in inducibility was apparent in radish cultivars ranging from susceptible to resistant against *F. oxysporum* f.sp. *raphani* (Leeman et al., 1995a). In *Arabidopsis*, inducibility was found to be ecotype-dependent, with Columbia (Col) and Landsberg *erecta* (La-*er*) being inducible by strain WCS417, but RLD not. Treatment of RLD with SA did induce SAR, indicating that the pathway leading to SAR in RLD is unimpaired (Van Wees et al., 1997). Root colonization of RLD by WCS417 bacteria was of the same order as on Col en La-*er*, suggesting that RLD may lack (a) receptor(s) for the recognition of bacterial determinant(s) required for resistance induction.

The spectrum of pathogens to which PGPR-mediated ISR is active, has not been studied as extensively as in the case of SAR. Yet, individual bacterial isolates have been shown to protect e.g. radish against the fungi *F. oxysporum* and *Alternaria brassicicola* and the bacterium *P. syringae* pv. *tomato* (Hoffland et al., 1996), and cucumber against the fungi *Colletotrichum orbiculare* (Wei et al., 1991) and *F. oxysporum* (Liu et al., 1995a), as well as the bacterium *P. syringae* pv. *lachrymans* (Liu et al., 1995b). A paper by Maurhofer et al. (1994) concerns induction of systemic resistance in tobacco against TNV, resembling the classic system for studying SAR. It can be concluded, therefore, that also rhizobacterially-induced systemic resistance is non-specific in affording enhanced protection against different types of pathogens.

Because resistance-inducing PGPR are naturally occurring rhizosphere soil inhabitants, the question arises whether plants grown under field conditions are likely to be induced already. No studies specifically addressing this question appear to have been conducted. However, in field experiments clear differences have been seen between bacterized and non-bacterized plots, or between plants grown from bacterized and non-bacterized seeds. While such differences might also be attributed to antagonism, a minimal concentration of 10^5 colony-forming units per g root appears to be required for induction of systemic resistance in e.g. radish (Raaijmakers et al., 1995b). Although total bacterial populations in the rhizosphere can exceed these levels by far, bacterial diversity is immense and any inducing strain that may be naturally present, is unlikely to exceed this threshold.

Table 1. Systemic resistance induced by selected strains of *Pseudomonas* bacteria in different plant species

Bacterial strain	Carnation/ *F. oxysporum*	Radish/ *F. oxysporum*	*Arabidopsis*/ *F. oxysporum* *P. syringae*
P. putida WCS358	–	–	+
P. fluorescens WCS374	nd	+	–
P. fluorescens WCS417	+	+	+

PGPR-mediated induced systemic resistance is not commonly associated with pathogenesis-related proteins

The phenotypic resemblance between SAR and bacterially-induced systemic resistance has prompted research into the possible involvement of PRs in the latter. Induction of resistance by *P. fluorescens* strain CHA0 in tobacco against TNV was reported to be associated with the occurrence of all major acidic PRs (Maurhofer et al., 1994). However, the induced plants were slightly stunted. Strain CHA0 is a producer of the antibiotics diacetylphloroglucinol and pyrrolnitrin, as well as of HCN, substances with toxicity to plants. Stress imposed on the bacterized plants may, therefore, have contributed to the induction of PRs. *Pseudomonas aeruginosa* strain 7NSK2 is a producer of SA and its induction of resistance against *Botrytis cinerea* in bean was reported to be reduced when it had lost the ability to produce SA (De Meijer and Höfte, 1997). *P. fluorescens* strains WCS374 and WCS417 are likewise able to produce SA under iron-limiting conditions. Mutants that have lost the O-antigenic side-chain of the LPS no longer induced resistance in radish under iron-sufficient conditions, but did so in the presence of an iron-chelating compound, indicating an additional bacterial determinant to be active under low-iron conditions (Leeman et al., 1996). Application of SA in low concentrations to radish roots induced resistance against *F. oxysporum* f.sp. *raphani*, but only at higher concentrations PRs serologically related to tobacco PRs -1 and -5 became apparent (Hoffland et al., 1995). In contrast, when bacterial application induced resistance no PR induction at either the protein or the mRNA level was detectable. These observations demonstrate that induction of resistance by PGPR can occur without induction of PRs. These findings were corroborated and extended in assays using *Arabidopsis* challenged with the same fungal root pathogen or with the bacterial leaf pathogen *P. syringae* pv. *tomato*. Substantial induction of systemic resistance was obtained in ecotypes Col and La-*er* without PRs being induced (Pieterse et al., 1996). Moreover, the bacterial strain with the largest capacity to produce SA, WCS374, was ineffective in inducing resistance in *Arabidopsis* (Table 1). Evidence that SA does not play a role in the induction of this type of resistance was obtained using *NahG*-transformed plants. Resistance induced by strains WCS417 or WCS358 was as effective as on non-transformed plants. Thus, SA is not involved in the expression of systemic resistance induced by these PGPR-strains. Because systemic resistance induced by these rhizobacteria appears to differ mechanistically from SAR, it should not be denoted as such. Instead, it has been designated ISR (Pieterse et al., 1996), in accordance with the earlier proposal of Kloepper et al. (1992). The term ISR will also be useful in distinguishing PGPR-mediated induced systemic resistance from classic SAR.

Although the extent of induced resistance attained by SAR and ISR can be similar, ISR usually affords lesser protection than SAR. This is in line with the conclusion of Cameron et al. (1994), discussed above, that a HR contributes to the level of resistance achieved. It is not clear yet whether ISR is as broad-spectrum as SAR is. Preliminary findings suggest that resistance induced by PGPR can be further boosted by application of SA, suggesting that bacteria do not activate the full spectrum of responses induced by pathogens. Indeed, PRs are induced only by a few PGPR strains among those capable of inducing resistance, and no evidence is available that these bacteria stimulate the plant to produce antimicrobial compounds, such as phytoalexins. In fact, these bacteria appear completely harmless, do not cause any symptoms and, yet, induce substantial resistance against different pathogens. In contrast, strong induction of SAR requires a necrotizing pathogen and is associated with the full range of defense reactions characteristic of a HR. The latter situation obviously confounds the search for the

basic molecular-genetic mechanisms that underly the induced state. It can now be said definitively that accumulation of PRs is not a prerequisite for the induction of resistance. Nevertheless, because of the antipathogenic actions of at least some among the PRs, those are likely to contribute to the protective state against challenging pathogens. Together with other SA-induced antipathogenic activities, they could be responsible for the higher level of induced resistance associated with SAR and tissue necrosis.

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