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Signal transduction in resistance to plant viruses

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Abstract

Salicylic acid is part of a signal transduction pathway that induces resistance to viruses, bacteria and fungi. In tobacco and Arabidopsis the defensive signal transduction pathway branches downstream of salicylic acid. One branch induces PR-1 proteins and resistance to bacteria and fungi, while the other triggers induction of resistance to RNA and DNA viruses. This virus-specific branch can be activated using antimycin A and cyanide, or inhibited with salicylhydroxamic acid, suggesting a role for alternative oxidase in resistance to viruses. The virus-specific defensive pathway activates multiple resistance mechanisms. In tobacco, salicylic acid induces resistance to systemic movement of cucumber mosaic virus but has no effect on its replication or cell-to-cell movement. However, in the case of tobacco mosaic virus in tobacco, salicylic acid appears to induce interference with the synthesis of viral RNA.

Abbreviations: AlMV – alfalfa mosaic virus; AOX – alternative oxidase protein or activity; *Aox* – gene sequence for AOX; CaMV – cauliflower mosaic virus; CMV – cucumber mosaic virus; GFP – green fluorescent protein; HR – hypersensitive response; INA – 2,6-dichloroisonicotinic acid; IVR – inhibitor of virus replication; LAR – local acquired resistance; PR – pathogenesis-related; PVX – potato virus X; ROS – reactive oxygen species; SA – salicylic acid; SAR – systemic acquired resistance; TMV – tobacco mosaic virus; TVCV – turnip vein clearing virus; VIGS – virus-induced gene silencing.

Introduction

Induced, or acquired resistance to plant pathogens can be triggered by chemicals (Kessmann et al., 1994; Sticher et al., 1997), or by an encounter with an avirulent pathogen (Baker et al., 1997; Staskawicz et al., 1995). In recent years, a great deal of progress has been made in understanding the signalling processes and the changes in gene expression that are associated with the induction of resistance. However, it is still not always clear which specific factors are directly responsible for the inhibition of a particular pathogen (Hammerschmidt, 1999a). This problem is highlighted when it comes to understanding induced resistance to viruses. Although a number of inducible factors like phytoalexins (Hammerschmidt, 1999b; Kuć, 1995) and pathogenesis-related (PR) proteins (van Loon and van Strien, 1999) are known to have inhibitory effects on at least some fungal and bacterial plant pathogens, none of these factors have been conclusively implicated in resistance to viruses.

It is perhaps ironic that relatively little information is available concerning the mechanisms of induced resistance to viruses. This is because several factors that have proved to be very important in our general understanding of induced resistance were discovered during studies focused on plant–virus interactions. Examples include the existence of PR proteins and the role of salicylic acid (SA) as a naturally occurring defensive signal chemical. In this article, we will review what

is currently known about induced resistance to plant viruses and suggest lines of future research that might repair some of the ambiguities and deficiencies in our knowledge of this phenomenon.

The hypersensitive response and acquired resistance

Much of the groundwork for studies of induced resistance to plant viruses was laid down in the early 1960s by A.F. Ross at Cornell. At that time it was already known that tobacco plants that possess the N resistance gene exhibited a hypersensitive response (HR) to tobacco mosaic virus (TMV). That is, instead of spreading systemically, the virus is limited to the vicinity of the inoculated cells, many of which undergo a controlled process of cell death. Ross found that NN genotype plants respond to a second encounter with TMV by producing smaller or fewer lesions (Ross, 1961a,b). This indicated that the first infection with TMV had enabled the plant to slow or restrict the replication and/or the spread of the virus in advance of the cell death event. He termed the induced resistance to TMV that occurred close to the initial infection sites 'local acquired resistance' (LAR) (Ross, 1961a), and to the induced resistance seen in distant, non-inoculated tissues of the plant he gave the name 'systemic acquired resistance' (SAR) (Ross, 1961b). The phenomena of LAR and SAR hinted at the existence of one or more mobile alarm signals capable of priming and enhancing plant defense.

'Novel' proteins and chemical signals

Following on from the work of Ross (Ross, 1961a,b) several groups attempted to find evidence of changes in host gene expression specifically associated with the HR and the induction of SAR in TMV-inoculated tobacco. For example, Loebenstein and co-workers identified an extracellular protein produced after the TMV-induced HR in resistant plants which they called inhibitor of virus replication (IVR) (Loebenstein and Gera, 1981; Spiegel et al., 1989). A cDNA clone encoding IVR was recently isolated by this group (Akad et al., 1999). With this clone now available, it should soon be possible to determine definitively whether IVR has a role in LAR or SAR against viruses.

PR proteins, as they are now called (van Loon and van Strien, 1999), were discovered at around the same time in the laboratories of S. Gianinazzi (see Gianinazzi et al., 1970) and L.C. van Loon (see van Loon and van Kammen, 1970). Both groups found that 'novel', host-encoded proteins accumulated in the leaves of NN genotype tobacco plants inoculated with TMV and in leaves expressing SAR (Gianinazzi et al., 1970; van Loon and van Kammen, 1970). The PR proteins were not observed in healthy plants, or in TMV-susceptible tobacco plants infected with the virus (Gianinazzi et al., 1970; van Loon and van Kammen, 1970). For several years, it was thought possible that PR proteins might play a role in resistance to viruses. However, this view, based on correlative evidence, was controversial (Fraser and Clay, 1983) and it finally became untenable when transgenic plants with modified levels of *PR* gene expression were produced (Cutt et al., 1989; Linthorst et al., 1989). Although such transgenic plants have, in some cases, improved resistance to fungal pathogens (for example, see Alexander et al., 1993), they do not have enhanced resistance to viruses (Cutt et al., 1989; Linthorst et al., 1989).

One of the most influential studies of the relationship between SAR and PR protein induction was that of White (1979). He demonstrated that aspirin (acetylsalicylic acid), a synthetic derivative of SA, could induce a SAR-like state (reduced HR lesion size in response to TMV), and the production of PR proteins, in tobacco. Based on this and subsequent studies, it was suggested that aspirin was mimicking a natural benzoic acid-like defensive signal (van Loon, 1983). This hypothesis was vindicated by the discovery that SA is a key component of the defensive signal transduction pathway that leads to the induction of SAR and the activation of a number of *PR* genes (Malamy et al., 1990; Métraux et al., 1990; Delaney et al., 1994).

A role for cell death in resistance to viruses?

Cell death during the HR is a very obvious, visible manifestation of resistance to pathogens. Unfortunately, the death of host cells during the HR is not sufficient in itself to limit the spread of a virus in a resistant host plant. Figure 1 shows a sequence of images taken during the development of a necrotic lesion on a leaf of an NN-genotype tobacco plant that was inoculated with a strain of TMV modified to express the jellyfish green fluorescent protein (GFP). Although most of the cells that were infected with TMV–GFP die off during the HR, a few fluorescent (virus-infected) cells at the periphery of the lesion remained alive (Figure 1).

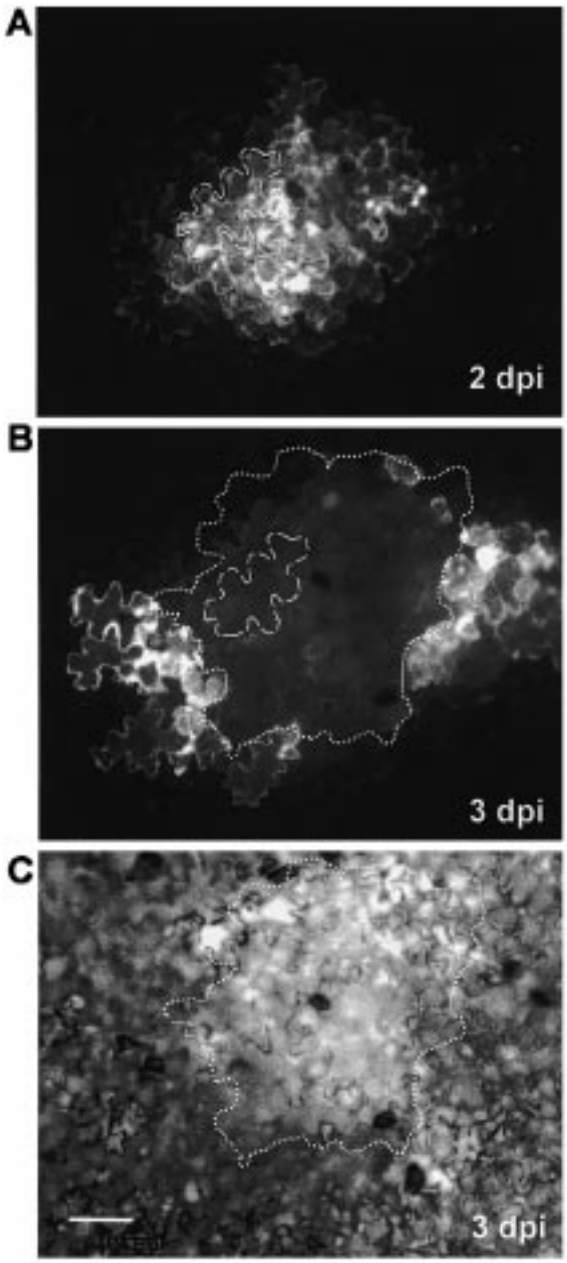

Figure 1. Development of a hypersensitive response lesion in response to inoculation with TMV–GFP. The surface of NN-genotype tobacco leaf tissue was inoculated with TMV–GFP. (A). Two days post-inoculation a typical TMV–GFP infection site was located by epifluorescence microscopy and imaged digitally. The outline of an epidermal cell that contains GFP is indicated with a broken line. At this point in time, no cell death had occurred. (B). Twenty-four hours later, the same infection site was located. By this time, a section of leaf tissue, including the outlined cell had died. The extent of cell death is indicated by the dotted line. Although there was no GFP signal apparent in cells that had died, TMV–GFP was present in living cells surrounding the lesion. The TMV–GFP did not spread out into the surrounding tissue but remained apparent in living cells at the periphery of the lesion for at least a further two days after lesion formation. (C). For comparison, a bright field image of the area seen in panel (B) is also shown. The dead lesion tissue is transparent to transmitted light and therefore appears bright in this image. Scale bar equal to 100 μm.

This, and similar results obtained with TMV expressing β-glucuronidase (Arce-Johnson et al., 1995), show graphically that not all virus-infected cells are destroyed during the HR, yet the virus is nonetheless delimited to the immediate vicinity of the lesion.

Additional lines of evidence show that cell death is not directly responsible for virus resistance. For example, growth of NN-genotype tobacco plants in a low-oxygen atmosphere abolishes the cell death associated with the HR but does not prevent the restriction of TMV (Mittler et al., 1996). In addition, application of aspirin to nn-genotype plants reduces the yield of TMV in these plants in the absence of any cell death (White et al., 1983).

Until quite recently, it was thought possible that cell death might be of central importance in resistance to nonviral pathogens (Dangl et al., 1996). However, this now seems less likely following the isolation and characterisation of an Arabidopsis mutant, *dnd*1, that cannot exhibit HR but retains resistance to an avirulent strain of the bacterial pathogen, *Pseudomonas syringae* (Yu et al., 1998). It seems that the role of programmed cell death in defense, against pathogens in general, not just viruses, needs to be re-evaluated.

The effects of SA on virus replication and movement

If neither cell death nor any of the known PR proteins limit viral spread either during the HR, or in plants that are expressing induced resistance, what is responsible? A pioneering study carried out in the mid-1980s by J.F. Bol and colleagues (Hooft van Huijsduijnen et al., 1986) showed that alfalfa mosaic virus (AlMV) replication was inhibited in SA-treated protoplasts. However, this work was not followed up at the time and for several years it was not clear if SA inhibited the replication of all viruses, or only some.

Our group has investigated the effects of SA on a number of plant viruses, concentrating for the most part

on the infection of tobacco with the well-understood viruses, TMV and CMV (Murphy et al., 1999). More recently, we began to investigate the effects of SA on the infection of *Arabidopsis thaliana* by turnip vein clearing tobamovirus (TVCV) and cauliflower mosaic virus (CaMV) (Carson, 1999; Wong, Carson, Carr, unpublished).

In tobacco, SA appears to affect both the replication and movement of viruses. Experiments with TMV (Chivasa et al., 1997) and potato virus X (PVX) (Naylor et al., 1998) showed that in leaf discs that had been pre-treated with SA the overall level of viral RNA accumulation was reduced. Furthermore, in SA-treated tissue the ratios of plus- to minus-sense, and of viral genomic to subgenomic mRNA, were different from those seen in untreated tissues after inoculation with virus (Chivasa et al., 1997; Naylor et al., 1998). On the face of it, these results suggest that the major effect of SA is to induce inhibition of viral RNA synthesis (replication and gene expression). However, this may not be the whole story. In recent work (A.M. Murphy, unpublished), epifluorescence and confocal scanning laser microscopy were used to image the infection of tobacco leaf tissue with TMV–GFP. Preliminary results suggest that the distribution of the GFP-tagged virus between epidermal and mesophyll cells differs considerably between infection sites on untreated versus SA-treated plants. Further work is in progress to determine whether this difference in the distribution of the virus is due to an effect on virus cell-to-cell movement, or to an indirect effect of inhibition of virus replication in the initially inoculated epidermal cells.

Naylor et al. (1998) found that in tobacco, SA did not significantly inhibit the accumulation of CMV in directly inoculated tissue. However, plants treated with SA, and plants that were expressing SAR due to a prior inoculation with TMV, showed a significant delay in the onset of systemic disease symptoms (Naylor et al., 1998). A time-course study of CMV coat protein accumulation in inoculated and uninoculated tissues of untreated and SA-treated plants showed that the delay in the onset of disease in SA-treated plants was most likely due to an inhibition of the systemic movement of the virus through the host's vascular system (Naylor et al., 1998). Thus, depending upon the virus, SA can induce at least two forms of resistance to viruses: either an inhibition of replication and/or cell-to-cell movement at the site of inoculation, or an inhibition of viral long-distance movement.

How does SA induce resistance to viruses?

It is becoming increasingly clear that defensive signal transduction pathways overlap and branch (Genoud and Métraux, 1999; Moller and Chua, 1999). Indeed, our group has found evidence that in the signal transduction pathway responsible for the activation of SAR, there is, downstream of SA, a distinct branch that leads to the induction of resistance to viruses (Murphy et al., 1999). The evidence for this arose fortuitously during a series of experiments designed to assess the possibility that there might be a connection between SAR and a plant mitochondrial enzyme, the alternative oxidase (AOX).

The mitochondria of plants can use two respiratory electron transport chains. In the conventional pathway, electron flow through a series of carriers to cytochrome oxidase is coupled to ATP synthesis. However, AOX can 'siphon off' electron flow from one of the electron carriers, ubiquinone, reducing molecular oxygen to water (Laties, 1982; Siedow and Moore, 1993; Vanlerberghe and McIntosh, 1997). This 'alternative' pathway is not coupled to ATP generation and the reaction generates heat. It has been known for several years that AOX activity is responsible for heat generation in specialised floral tissues of thermogenic plants belonging to the Araceae (Meeuse and Raskin, 1988; Raskin et al., 1987). More recently, AOX was shown to play a role in controlling the levels of reactive oxygen species (ROS) in plant tissues (Maxwell et al., 1999). This is a function of much wider physiological importance and may also have some bearing on plant defence, given the likely importance of ROS as resistance signals (Draper, 1997; Dempsey et al., 1999; McDowell and Dangl, 2000).

However, even before this information was published, we were intrigued by certain parallels between the regulation of AOX activity and gene expression, and the induction of SAR. For example, *Aox* gene expression can be stimulated by SA (Rhoads and McIntosh, 1993). In addition, AOX activity in thermogenic plants is triggered naturally by SA (Raskin et al., 1987) and can be triggered artificially using the synthetic inducer of resistance to pathogens, 2,6-dichloroisonicotinic acid (INA) (Chivasa et al., 1999).

Initial experiments made use of an inhibitor of AOX activity, salicylhydroxamic acid (SHAM). It was found that SHAM was able to antagonise SA-induced resistance to TMV in tobacco (Chivasa et al., 1997). However, SHAM did not prevent the induction of *PR-1* gene

expression by SA or INA (Chivasa and Carr, 1998; Chivasa et al., 1997). This was surprising because *PR-1* gene expression is a widely accepted molecular marker for the induction of SAR and chemically-induced resistance. Given this unexpected finding with *PR* gene expression, it was decided to see what effect SHAM had on resistance to non-viral pathogens.

Two non-viral pathogens were examined: a fungus, *Botrytis cinerea*, and a bacterium, *Erwinia carotovora* (Chivasa et al., 1997). The progress of invasion by *B. cinerea* is significantly slowed in tobacco seedlings sprayed with SA (Chivasa et al., 1997; Murphy et al., 2000). Addition of SHAM to the spraying mix did not inhibit the induction of resistance to the fungus (Chivasa et al., 1997). Similarly, SHAM was not able to inhibit SA-induced resistance to the soft rot disease caused by *E. carotovora* (Chivasa et al., 1997). Thus, it appeared that in tobacco, SA-induced resistance to TMV, as well as to CMV and PVX (Naylor et al., 1998), could be antagonised by SHAM but that SA-induced *PR-1* gene induction and resistance to non-viral pathogens could not.

Further experiments using tobacco showed that non-lethal concentrations of two inducers of AOX activity and gene expression, cyanide and antimycin A, were able to induce resistance to TMV, without triggering *PR-1* gene induction (Chivasa and Carr, 1998). Subsequent experiments with *Arabidopsis thaliana* have shown that SA and cyanide can induce resistance to a DNA virus CaMV and to TVCV, an RNA virus closely related to TMV (Carson, Wong, Carr, unpublished). Induction of resistance with cyanide does not induce PR-1 expression in Arabidopsis and SHAM can inhibit the induction of resistance by SA and cyanide, although less reliably than in tobacco (Carson, Wong, Carr, unpublished).

These pharmacological experiments imply that, downstream of SA, the defensive signal transduction pathways of tobacco and Arabidopsis split into distinct branches. One branch is responsible for induction of *PR-1* gene expression and resistance to bacterial pathogens, while the other leads to induction of resistance to viruses. Recently, this idea has received backing from experiments carried out using Arabidopsis mutants (Kachroo et al., 2000). In plants carrying the *HRT* resistance gene for resistance to turnip crinkle virus (TCV), the induction of resistance to that virus was dependent on SA, but was independent of the *NPR1* gene (also called *NIM1*: (Cao et al., 1997; Ryals et al., 1997)). The significance of this result is that NPR1 is

Figure 2. Branching of the defensive signal transduction pathway downstream of salicylic acid (SA) in tobacco and Arabidopsis. Increased levels of SA due to chemical treatment or resulting from pathogen attack activate defensive signal transduction. Downstream of SA, the defensive pathway appears to divide. One branch leads to the induction of extracellular PR proteins and is dependent on NPR1 (Kachroo et al., 2000). The other branch leads to the induction of resistance to viruses. It has been suggested that the virus-specific branch may be dependent on the activity of the AOX. However, this has not yet been definitively demonstrated (Murphy et al., 1999) and hence a question mark appears in the diagram.

a protein that plays a key role, downstream of SA, in the induction of *PR* gene transcription (Després et al., 2000; Zhou et al., 2000). Therefore, this work is consistent with the hypothesis that resistance to viruses is dependent on a separate, virus-specific, branch of the defensive signal transduction pathway (Figure 2).

Is AOX involved in resistance to viruses?

Based on pharmacological (Chivasa and Carr, 1998; Chivasa et al., 1997; Murphy et al., 1999; Naylor et al., 1998) and genetic (Kachroo et al., 2000) evidence, it appears safe to conclude that induced resistance to viruses is triggered by a distinct branch of the defensive signal transduction pathway. But how safe is it to conclude that AOX is involved in resistance to viruses?

Several studies have now shown that *Aox* gene expression is increased either in tobacco and Arabidopsis plants undergoing the HR (Chivasa et al., 1997; Lacomme and Roby, 1999; Lennon et al., 1997; Simons et al., 1999). Similarly, *Aox* gene expression and/or activity is increased in plants, organs or tissues treated with SA or the synthetic resistance-inducing chemical, INA (Chivasa et al., 1999; Chivasa and Carr, 1998; Xie and Chen, 1999). Based on these studies, plus those that show an effect of AOX activators or inhibitors on induced resistance to viruses (Chivasa and Carr, 1998; Chivasa et al., 1997; Murphy et al., 1999; Naylor et al., 1998), it might be reasonable to suggest a role for AOX in induced resistance. However, this hypothetical connection between AOX and resistance to viruses is based solely on pharmacological or correlative evidence, and must therefore be treated with

extreme caution. For example, AOX may not be the authentic target of SHAM since this chemical can also affect the activity of lipoxygenases (Preisig and Kuć, 1987), as well as certain peroxidases (Møller et al., 1988). Furthermore, although it has been shown that triggering of the HR by TMV in NN genotype tobacco resulted in an increase in AOX protein, this did not cause a detectable increase in AOX activity (Lennon et al., 1997).

Currently, our group is taking two approaches to determine definitively whether AOX does, or does not, play a role in the induction of resistance to viruses. In the first approach, nn- and NN-genotype lines of tobacco, as well as Arabidopsis, have been transformed with sense and antisense *Aox* cDNA sequences under the control of a constitutive promoter (D.P. Singh and C.E. Wong, unpublished). T2 generation lines of these plants are now becoming available for experimentation. Our aim will be to determine whether constitutively higher or lower levels of *Aox* gene expression will affect AOX activity and whether that will, in turn, affect the plants' responses to viral infection in the presence or absence of resistance-inducing chemicals or the hypersensitive response.

The second approach involves modifying *Aox* gene expression in plant tissue using viral vectors. TMV vectors expressing sense and antisense *Aox* gene sequences have already been constructed and are being used to investigate their pathogenesis in *Nicotiana benthamiana*. However, a very powerful method for selectively knocking out the expression of a gene is virus-induced gene silencing (VIGS: reviewed by Baulcombe, 1999). This has proved to be a more reliable method of knocking out gene expression than transformation of plants with antisense gene constructs (Baulcombe, 1999). Therefore, the construction of VIGS vectors carrying *Aox* gene sequences has begun.

Future work

Once a role for AOX in the induction of resistance to viruses has been either confirmed, or refuted, there are two key aspects of virus-specific signalling that will need to be addressed. First, signalling components that are downstream of the cyanide-inducible/SHAM-sensitive step in virus-specific signal transduction need to be identified. Our laboratory has initiated work on the development of a screen for Arabidopsis mutants that are defective in virus-specific signalling (C.E. Wong, C.A. Clarke, J.P. Carr, unpublished).

Second, plant genes that are up- or down-regulated by the virus-specific signal pathway need to be identified. Currently, the cDNA-AFLP (amplified fragment length polymorphism) technique of Bachem et al. (1998) is being used to attack the problem (J. Hayward, J.P. Carr, unpublished). This method has been used very successfully to profile changes in gene expression during a fungus-induced HR (Durrant et al., 2000). We are using it to compare gene expression profiles in healthy tobacco leaf tissue with that in tissue that has been treated with antimycin A, a chemical that stimulates virus-specific defensive signal transduction. However, in the near future it may be possible to incorporate the even more powerful new technology of DNA microarrays into this project.

There are two reasons why these studies are important. Firstly, genes that are up- and down-regulated by the virus-specific defense pathway may encode, respectively, factors that inhibit or support virus replication or movement. Identification and characterisation of these factors will allow us to understand how induced resistance to viruses works at a mechanistic level. Secondly, identification of genes that act as specific markers for defence against viruses will contribute to the development of cybernetic models that may one day explain and predict the behaviour of the dynamic signal transduction network that exists in plants (Genoud and Métraux, 1999).

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