Reversal of an immunity associated plant cell death program by the growth regulator auxin

Suresh Gopalan*

DOE-Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, USA, and Department of Plant Pathology, University of Kentucky, Lexington, KY 40546, USA

*Present Address: Department of Molecular Biology, Massachusetts General Hospital & Department of Genetics, Harvard University, Boston, MA 02114, USA.
email: gopalan@molbio.mgh.harvard.edu

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ABSTRACT

One form of plant immunity against pathogens involves a rapid host programmed cell death at the site of infection accompanied by resistance, termed the hypersensitive response (HR). Here it is shown that the HR programmed cell death program initiated by the bacterial type III secretion system dependent proteinaceous elicitor harpin from *Erwinia amylovora* can be reversed till very late in the process by the plant growth regulator auxin. Early inhibition or late reversal of this cell death program does not affect marker genes tightly correlated with local and systemic resistance. Cross-regulation between cell death programs and growth regulators is prevalent in different kingdoms. Thus, the concept that cell death program can be reversed till late provides a framework for further investigation of such phenomena, in addition to having utility in choosing better targets and strategies for treating mammalian and agricultural diseases.

INTRODUCTION

A well studied form of immunity against pathogens in the plant kingdom involves a rapid programmed cell death at the site of infection by the pathogen, associated with restriction of multiplication and spread of the pathogen, termed the hypersensitive response (HR) (Dangl & Jones, 2001). Often this HR cell death is accompanied by induction of broad spectrum resistance in uninfected parts of the plants, which is referred to as systemic acquired resistance (SAR). The cell death program and its constituent components in plants is less well understood than the corresponding phenomena (e.g., of aptoptosis) in mammals, though existence of mechanistic parallels with the animal kingdom has been demonstrated. For example, caspase inhibitors can inhibit certain forms of the plant cell death program, a mammalian equivalent of a caspase has not been identified (Hatsugai et al, 2004; Lam, 2004). In addition, this process is mechanistically similar to the requirement of immune activation for effective apoptosis based treatment of certain tumors (Apetoh et al, 2007).

The response of the plant host to a pathogen is intricately dependent on the physiological and developmental status of the plant, that are in turn controlled by signaling by different growth hormones and environmental conditions. Many gram-negative bacterial pathogens of plant, animal and human hosts e.g., *Pseudomonas*, *Erwinia*, *Xanthomonas*, *Ralstonia, Yersinia*, *Shigella* and Salmonella species encode a secretion system, termed the type III secretion system (TTSS) that enable them to secrete effector proteins, many of which are directly translocated into host cells. These effector molecules have been shown have to a variety of functions, often involving modulation of host signaling and cytoskeletal structural components (Galan & Collmer, 1999). In the case of plants, the recognition of several bacterial effectors have evolved through a class of often intracellular LRR containing receptors and/or kinases, termed resistance (R) genes that recognize specific effectors (Dangl & Jones, 2001). The specific

genetic or biochemical recognition of the bacterial component by the plant host triggers the rapid HR cell death program.

Harpins, unlike many other type III effectors were identified as proteins that are secreted by the bacteria into the media via the TTSS and by their ability to effect host cell death when purified protein is injected into the host apoplastic space (intercellular space) of leaves (He et al, 1993; Wei et al, 1992). Despite this main difference between harpins and other type III effectors such as the *Pseudomonas syringae* AvrRpt2 and AvrRpm1 proteins, many lines of evidence support the conclusion that harpin elicited cell death is programmed and shares many aspects of physiological, transcriptional and resistance associated events that are hallmarks of many resistance associated cell death phenomena in plants (Baker et al, 1993; Gopalan et al, 1996; Strobel et al, 1996). While the receptor or specific genetic components that recognize harpins are yet to be reported, a ca. 25 kDa protein is recognized in the membrane enriched fraction of *Nicotiana tabacum* by an anti-idiotypic antiserum to harpin from *Erwinia amylovora* ($harpin_{Ea}$) (S. Gopalan and SY. He, unpublished results).

RESULTS AND DISCUSSION

Based on the well established concept that organismal homeostasis and key processes are controlled by the delicate balance between antagonistic death and survival signals, the hypotheses that the HR cell death program induced by a purified bacterial elicitor – harpin, can be reversed by a key plant growth regulator, auxin was tested. To examine the effect of auxin on HR cell death, 50 μM auxin (2,4-D) and purified $harpin_{Ea}$ were coinfiltrated into the apoplast of *Nicotiana tabacum* cv. Samsun NN leaves. Whereas treatment of harpin in buffer elicited cell death - Figure 1A-1, auxin completely inhibited harpin elicited cell death Figure 1A-2. Inhibition could also be observed by co-infiltrating indole acetic acid (IAA) at 50 μM and harpin (data not shown).

To examine how farther down the process this inhibition can be achieved, auxin or buffer was infiltrated 6 h post-infiltration of harpin, in the same area where harpin was initially infiltrated. Under the conditions of the experiments, harpin elicited visible tissue flaccidity about 8 h post-infiltration (pi). Interestingly, the area infiltrated with auxin 6 h after initial infiltration did not show any cell death symptoms, whereas the buffer infiltrated area showed visible tissue flaccidity 3 h later and eventually showed HR symptom (Figure 1B). These data indicate that the cellular commitment to die and the cell death program can be reversed by appropriate signals till late in the process.

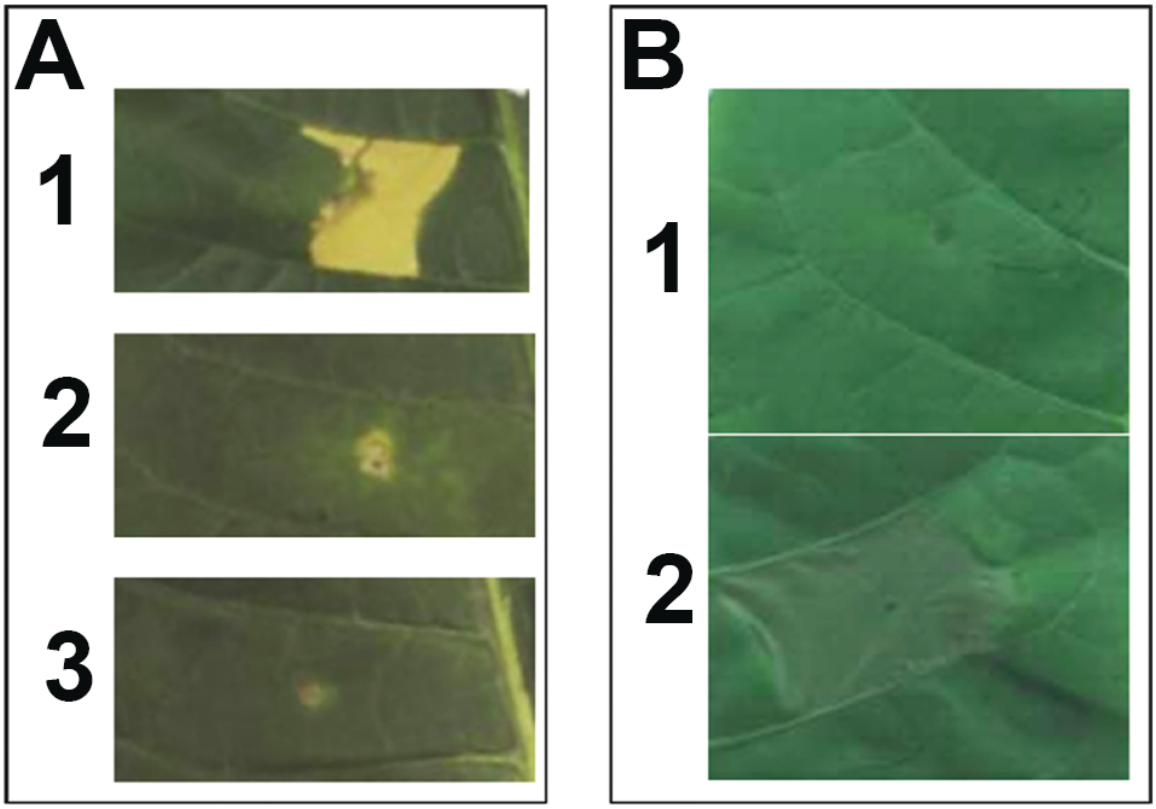

Figure 1 Early inhibition and late reversal of harpin initiated HR cell death program by growth regultor auxin. **(A)** Inhibition of HR cell death by auxin. Harpin (panel 1), Harpin in 50 μM 2,4-D (panel 2), or 50 μM 2,4-D (panel 3) was infiltrated into the apoplastic space of leaves of *Nicotiana tabacum* CV. Samsun NN, and symptom development was recorded 36 h later. **(B)** HR cell death program can be reversed till late in the process. Auxin (50 μM 2,4-D)- (panel 1) or buffer (panel 2) was infiltrated in the same area where harpin was infiltrated 6 hours earlier. Symptoms were recorded 24 h after the first infiltration. Harpin was used at 1-2 μM in 10 mM $MgCl_2$.

As mentioned above the HR cell death is associated with local and systemic resistance in plants. Thus, the induction of marker genes strongly correlated with both these processes were tested. Induction of both the genes tested, *HIN1*- a gene induced locally at the site of HR cell death (Gopalan et al, 1996), and *PR1* - an established marker gene whose induction is tightly correlated with SAR, were not affected by inhibition of HR cell death by auxin – Figure 2. While the precise contribution of HR cell death to resistance is not known, there are examples of cell death elicitation (spontaneous and induced) that can lead to resistance (Dietrich et al, 1994; Greenberg & Ausubel, 1993). In contrast, there are also examples of constitutive resistance without cell death (Bowling et al, 1994; Yu et al, 1998). A striking example of separate of HR cell death and resistance programs is the R gene (Rx) mediated recognition of potato virus X (PVX) (Bendahmane et al, 1999). Collectively, these data indicate that resistance phenomena while tightly associated with certain cell death processes, is

triggered through a divergent signaling process that is strongly modulated by signals from cell death programs.

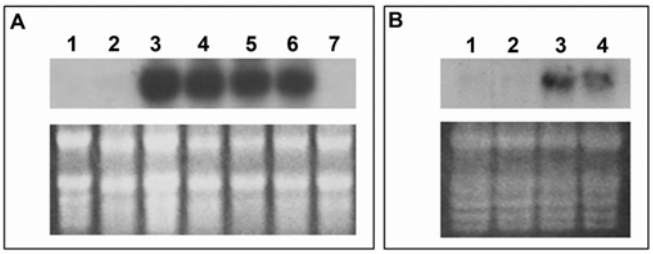

Figure 2 Induction of genes indicative of local and systemic defense are not affected by early inhibition or late reversal of harpin initiated cell death by auxin.

(**A**) Expression of *HIN1* mRNA. Northern blot using RNA from leaves infiltrated with lane 1: Buffer 4.5 h; lane 2: 50 μM 2,4-D 4.5 h; lane 3: Harpin 4.5 h; lane 4: Harpin + 50 μM 2,4-D 4.5 h; lane 5: Harpin + 50 μM 2,4-D 24 h; lane 6: harpin - necrotic (9 h); lane 7: 50 μM 2,4-D 24 h. (**B**) Expression of *PR1* mRNA. Lane 1: Buffer 24 h; lane 2: 2,4-D 24 h; lane 3: harpin 24 h; lane 4: Harpin + 2,4-D 24 h. 10 μg total RNA per lane from infiltrated area (Panel A) or 1 cm region surrounding the infiltrated region (Panel **B**) were used for the analysis. For Panel **B**, leaf tissue was collected from region surrounding the infiltrated region because *PR1* was not induced in the infiltrated area at these time points. rRNA bands of the RNA gel used for the Northern blot, visualized by staining with ethidium bromide, is shown in the lower frame of each panel.

While the mode of action of the reversal of this cell death program need to be worked out, some clues suggested by signaling and physiological effects of auxin are, (i) involvement of ubiquitin mediated proteolysis, as signaling through many short-lived transcription factors is a hallmark of auxin response and the fact that TIR1, a ubiquitin ligase, is an auxin receptor mediating these responses (Dharmasiri et al, 2005; Kepinski & Leyser, 2005), (ii) auxin causes extracellular acidification and cell wall loosening as opposed to alkalinization and reinforcement and fortification through crosslinking of cell wall during HR. An example relevant to this aspect is a pathogen effector AvrPtoB from *Pseudomonas syringae* pv. *tomato* that harbors an N-terminal cell death domain and a C-terminal E3 ubiquitin ligase activity that inhibits the cell death (Abramovitch et al, 2006). These data imply that pathogens have also evolved to utilize the fine balance between death and survival signals in host to enhance their own survival.

In contrast to inhibition and reversal of the harpin elicited cell death program by exogenous auxin, transgenic plants constitutively modulated in their endogenous levels of auxin (either upregulated by 7 fold or downregulated by 10 fold, (Romano et al, 1993; Romano et al, 1991) were neither affected in their ability to elicit harpin or the bacteria *Pseudomonas syringae* pv. *syringae* 61 (Pss61) induced cell death or the timing induction of death symptoms. Similarly, exogenous auxin could not prevent cell death mediated by bacterial pathogens

tested (*Erwinia amylovora* and Pss61). Earlier, it had been shown that auxin produced by some bacteria, can inhibit cell death elicited by an incompatible bacterial pathogen (Robinette & Matthysse, 1990). These data suggest the following testable hypotheses, (i) signals reprogrammed by constitutive modulation of endogenous auxin levels is not sufficient or equivalent to exogenous application of auxin in inhibition and reversal of cell death program, (ii) alternatively, the signaling modules are buffered (thus not able to express signals contributing to inhibition of the cell death program) during constant modulation of auxin levels, and (iii) the difference in the inhibition of bacterially elicited HR cell death by auxin and by purified elicitor possibly reflects the extended and continuous delivery of cell death elicitors or auxin by bacteria, as opposed to single delivery of the elicitor and auxin in this study.

In summary, these data demonstrate that a form of programmed cell death program in plants can be reversed till very late in the process and can be separated from the induction of associated disease resistance processes as evidenced by induction of strongly correlated transcriptional markers. The inhibition by auxin is akin to inhibition of apoptosis induced by several agents by growth factors, such as the antagonistic processes between TNF induced cell death program and prosurvival signals induced by growth factors EGF and insulin (Gaudet et al, 2005). These and other extensive studies of the molecular mechanisms of programmed cell death have identified many antagonistic signals, control points and gateways (Danial & Korsmeyer, 2004), but the point until which the cell death program can be reversed has not been specifically addressed. For example, the activation of the executioner caspases during apoptosis, unless inhibited rapidly by an inhibitor such as XIAP or degraded, is considered a point of no return in the cell death program (Riedl & Salvesen, 2007). The data presented here highlight the concept that a cell death program can be reversed till very late in the process and provides a framework to decipher the mechanism. Given the mechanistic conservation of signaling themes in different kingdoms, understanding the mechanism of such reversal of cell death program till late in the process would have significant implications in understanding and treating mammalian and agricultural diseases and modulation of other cellular programs that involve certain forms of programmed cell death, and dissociating beneficial programs concomitantly activated with the cell death program in certain disease conditions. One example of such utility would be to choose drug targets downstream of immune activation in adjacent cells through signals from the dying cells (for e.g., through TLR4) that have been shown to be important for effective apoptotic therapy of certain tumors (Apetoh et al, 2007).

METHODS

Bacterial growth, elicitor preparation and plant inoculation

Bacteria were grown in LM (Bacto tryptone 10 g l^{-1}, yeast extract 6 g l^{-1}, K_2HPO_4 1.5 g $^{-1}$, NaCl 0.6 g l^{-1}, $MgSO_4.7H_2O$ 0.4 g l^{-1}) with appropriate antibiotics. $Harpin_{Ea}$ was purified from DH5α (pCPP2139) as decribed earlier (Gopalan et al, 1996). Plants leaves were infiltrated with elicitors, bacterial suspensions and buffer, auxin into the apoplastic space using needleless syringe (Wei et al, 1992). Harpin and bacteria were diluted in 10 mM $MgCl_2$. Harpin was used at 1-2 μM. Tobacco plants (*Nicotiana tabacum* cv. Samsun NN, and the transgenic plants) were grown in artificial soil mix in a greenhouse.

RNA isolation and Northern blot analysis

Leaf tissue infiltrated as described were collected at time points indicated and frozen in liquid nitrogen and maintained at -70^{o}C until use. For analysis of *pr-1* induction, leaf tissue from about 1 cm region sorrounding the infiltrated region was collected. Total RNA was isolated using RNAgents total RNA isolation system (Promega). RNA was quantified using Gene Quant DNA/RNA calculator (Pharmacia). Ten micrograms of total RNA were fractionated in agarose formaldehyde gels (Ausubel et al, 1994) and transferred to Immobilin-N membrane (Millipore). The blots were cross-linked using Stratalinker (Stratagene). Hybridization and washes were carried out as described earlier (Gopalan et al, 1996). DNA probes used for *hin1* and *pr-1* were prepared as described in (Gopalan et al, 1996; Strobel et al, 1996) respectively.

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