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Signal transduction in the wound response of tomato plants

Dianna Bowles

The Plant Laboratory, Department of Biology, University of York, PO Box 373, York YO1 5YW, UK

The wound response of tomato plants has been extensively studied, and provides a useful model to understand signal transduction events leading from injury to marker gene expression. The principal markers that have been used in these studies are genes encoding proteinase inhibitor (pin) proteins. Activation of *pin* genes occurs in the wounded leaf and in distant unwounded leaves of the plant. This paper reviews current understanding of signalling pathways in the wounded leaf, and in the systemically responding unwounded leaves. First, the nature of known elicitors and their potential roles *in planta* are discussed, in particular, oligogalacturonides, jasmonates and the peptide signal, systemin. Inhibitors of wound-induced proteinase inhibitor (pin) expression are also reviewed, with particular reference to phenolics, sulphydryl reagents and fusicoccin. In each section, results obtained from the bioassay are considered within the wider context of data from mutants and from transgenic plants with altered levels of putative signalling components. Following this introduction, current models for *pin* gene regulation are described and discussed, together with a summary for the involvement of phosphorylation–dephosphorylation in wound signalling. Finally, a new model for wound-induced *pin* gene expression is presented, arising from recent data from the author's laboratory.

Keywords: signalling; wound; tomato; gene regulation; jasmonates; proteinase inhibitors

1. INTRODUCTION

Leaf injury to tomato plants has been used as a model to study plant wound responses for many years. Green & Ryan (1972) were the first to demonstrate the systemic nature of the response, and it is this characteristic in particular that has continued to fascinate researchers for several decades. Thus, application of a wound, such as caused by crushing the leaf lamina with blunt forceps, leads to changes throughout the wounded lamina and throughout the rest of the plant. Generally, the systemic response is defined as events in distant unwounded leaves, but equally, changes are now known to occur also in the stem, petioles and root system. One of the central issues in this response is the probability of many signals emanating from the wound challenge. Changes in the dying cells at the injury site will take place sequentially, leading to the likely release of molecular species capable of acting as signals over an extended time-course. In this context, it is of interest that the main 'marker' for the wound response, expression of genes encoding proteinase inhibitor (pin) proteins, is relatively slow: with steady-state levels of transcripts detectable only after several hours. The systemic response in this model is also clearly different in timing from that of systemic-acquired resistance (SAR) in which rapid events at the site of pathogen challenge lead to the appearance of systemic changes several days later. In the wound response, timing of the systemic response, using *pin* gene expression as the marker, occurs in parallel to the local response and some data have suggested it can be even faster.

Ryan's group devised the bioassay when they discovered that removal of the root system from a young tomato plant by an excision at the base of the stem, did not lead to induction of *pin* gene expression in the leaves. The controls involved incubation in water, and these were compared with the consequences of applying a range of compounds to the excised plants through the transpiration stream. While it is useful to identify elicitors and inhibitors of the response, data from the bioassay can be difficult to interpret because the applied compounds can in theory act anywhere in the plant from the site of application through to local action if/when they are mobile in the transpiration stream and are carried to the leaves. Increasingly, mutants and transgenics in which levels of putative causal signals are up- or down-regulated, are being used to confirm or further define the involvement of agents identified in the bioassay.

An aim of this paper it to review critically what is now known, or not known, of the local and systemic transduction pathways leading to the wound-induced regulation of *pin* gene expression. Recent data from my own laboratory will then be discussed within that context.

2. ELICITORS OF *PIN* GENE EXPRESSION

A total of three distinct classes of elicitors have been identified through their ability to induce *pin* gene expression when applied in bioassays to tomato leaves: glycans, lipids and peptides. There is good experimental evidence to show that each of these exist in the plant and could therefore represent endogenous intermediates in wound-related signal transduction pathways. In addition, the

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aminopeptidase inhibitor, bestatin, has also been shown to induce *pin* gene expression in the bioassay.

(a) *Glycans*

Early work demonstrated that an active factor in a leaf hydrolysate capable of inducing *pin* gene expression was a glycan, most probably derived from the cell wall polysaccharide, pectin (Bishop *et al.* 1981, 1984). At that time there was growing awareness that plant cell wall fragments could have bioactivity in regulating plant defence (e.g. Hahn *et al.* 1981; Jin & West 1984; reviewed by Darvill & Albersheim (1984)). The cell wall was described as representing a reservoir of latent signals in the form of diverse polysaccharides that acted as structural elements when intact, but on chemical or enzymic hydrolysis released fragments capable of triggering a range of events related to developmental and defence responses (for a review, see Bowles 1990; Darvill *et al.* 1992; Van Cutsem & Messiaen 1994; Aldington & Fry 1996). There is now good evidence that in defence responses, these fragments may originate from plant cell walls and from walls of plant pathogens. Plant enzymes with an appropriate specificity to release pathogen-derived fragments have been identified, as well as pathogen endohydrolases of pectins (e.g. Di Pietro & Roncero 1996; reviewed by Kombrink & Somssich (1995)). Significantly, plant-encoded inhibitor proteins of fungal endohydrolases have also been identified that *in planta* could regulate the size and half-life of any pectin fragments generated (for a review, see Hahn *et al.* 1989; Cervone *et al.* 1996).

In the context of *pin* gene expression, both the oligogalacturonide (OGA) family of plant cell wall fragments and the chitosan family of fragments representing putative pathogen-derived elicitors are active (Walker-Simmons *et al.* 1984; Walker-Simmons & Ryan 1984). Most work has focused on the OGAs. These have been analysed in a range of assays using oligomers of defined degree of polymerization (DP), as well as mixtures of oligomers of different sizes. For example, different size-ranges of OGAs have been shown to induce rapid depolarization of membrane potential, that in turn has been suggested as an early event in wound transduction (Thain *et al.* 1990, 1995; Mathieu *et al.* 1991). Closely related epimers of galacturonides, the guluronides and mannuronides, have also been used to define structural requirements (Kohn 1985) and to indicate whether Ca^{2+}-chelation is likely to be necessary for galacturonide activity in the plant.

In the bioassay, all size-ranges of OGAs from the 2-mer through to the 18–20-mers induce *pin* gene expression. As yet, however, no constitutively expressed leaf endohydrolase capable of producing such fragments from the wall pectins has been purified, although enzyme activity has been shown to modify OGAs applied to the transpiration stream in the bioassay (MacDougall *et al.* 1992). Nevertheless, the current view exemplified in models remains that OGAs act as elicitors of *pin* gene expression only if the tomato plants are wounded by insect pests or challenged by pathogens that could secrete the necessary enzymes to release the plant cell wall fragments (see § 5). However, in this respect, several lines of evidence suggest that the effects of pathogens or insect damage can be clearly distinguished from those of abiotic injury (see, for example, Pautot *et al.* 1991; Korth & Dixon 1997).

Early studies (Farmer *et al.* 1989, 1991) showed that large OGAs (with a DP of more than 12) induced phosphorylation of a membrane protein in the leaves. As large oligomers of guluronic acid were also active, whereas those of mannuronic acid were inactive, they suggested that Ca^{2+}-chelation might be involved *in planta* because only the OGAs and guluronic acid oligomer would be structurally capable of forming 'egg-box' complexes with the metal ion. Whether the phosphorylation event is involved in OGA-mediated *pin* gene expression is questionable, given that both the guluronic and mannuronic epimers can act as elicitors in that capacity. Also, OGAs of a DP of less than ten were incapable of inducing phosphorylation, yet are known to be active in the induction of *pin* gene expression. Recently, Farmer's laboratory has purified the OGA-binding protein that undergoes phosphorylation, and shown it to be a hydrophilic protein that associates strongly as a peripheral component of the plasma membrane (Reymond *et al.* 1995, 1996).

Using the bioassay, individual OGAs across the size-range DP 2–15 have been analysed for their effects on ethylene, as it is now known that ethylene synthesis and action are required for *pin* gene expression in the wounded leaf (O'Donnell *et al.* 1996; § 5). Only the 4–6-mers were active, both in inducing ethylene, and in the up-regulation of expression of the gene encoding the terminal enzyme in ethylene biosynthesis, 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (Simpson *et al.* 1998). These data suggest it may be possible that, dependent on size, OGAs induce *pin* gene expression through an ethylene-dependent and ethylene-independent pathway. Alternatively, the activity of the large oligomers in inducing *pin* gene expression may arise from their hydrolysis after application to the plant. In this context, the study by MacDougall *et al.* (1992) clearly demonstrated that OGAs were modified after their uptake via the transpiration stream. Using 21-day-old plants, the 6-mer was converted rapidly to the 4–5-mers, whereas in 40-day-old plants, 3-mers only were recovered. Therefore, in addition to evidence of apoplastic esterification (by a suggested methyl transferase mechanism), the study also showed that exo- and endopolygalacturonase activity may be constitutively present in the unchallenged plant.

There are no data available as yet on any putative receptor system for OGA activity in relation to their induction of *pin* gene expression, although clearly, given the hydrophilic nature of the elicitors and their probable release from the cell wall in the apoplast, some mechanism of transduction across the surface membrane must occur. The size-range of active oligomers in the bioassay is very broad, but with the exception of the study of MacDougall *et al.* (1992), the extent of degradation *in planta* is unknown, and in turn the size of the OGAs actually eliciting the effects in the leaves is uncertain. As only the large OGAs induce phosphorylation, the data from Farmer's laboratory strongly suggest that degradation, if it were to occur generally, will be incomplete.

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(b) *Lipids*

Methyl jasmonate applied as a spray or as an airborne volatile in a closed container was found to induce *pin* gene expression in tomato plants (Farmer & Ryan 1990, 1992). This first observation led to discussion that volatile jasmonates acted as interplant communicators in the wound response. However, co-incubation of wounded and unwounded plants in a closed container did not lead to transfer of a wound-inducible signal from one population to another (Farmer & Ryan 1990). Application of jasmonic acid (JA) either directly to the leaf surface or through the transpiration stream is also active in eliciting *pin* gene expression (Farmer *et al.* 1992). From a first observation that a prostaglandin-like metabolite of linolenic acid could be produced by flax seed extracts (Zimmerman & Feng 1978), octadecanoids are now known to occur and to have several functions in plants, being implicated in a diverse range of developmental events, responses to abiotic stresses and responses to pests and pathogens (see, for example, Gundlach *et al.* 1992; for a review, see Farmer 1994; Creelman & Mullet 1995; Baldwin *et al.* 1996).

Stages in the biosynthetic pathway leading from linolenic acid (18:3) to JA are summarized in figure 1 (Vick & Zimmerman 1984), and have been extensively studied and reviewed (see, for example, Vick 1993; Ward & Beale 1993; Taapken *et al.* 1994; Mueller 1997). As yet, the lipase(s) responsible for the release of the fatty acid from the membrane and the location of that membrane within the cell are ill-defined. There is some evidence that a phospholipase A_2 is activated by elicitors that have also been shown to induce *pin* gene expression (Chandra *et al.* 1996). From studies on *Arabidopsis*, a lipoxygenase (LOX) involved in wound-related changes in JA and wound-induced gene expression was found to be located in the chloroplast (Bell *et al.* 1995), and in spinach, the site of metabolism of fatty-acid hydroperoxides was shown to be the chloroplast envelope membrane (Blée & Joyard 1996). Purification and cloning of the allene oxide synthase and allene oxide cyclase from several plant species have shown that both gene products possess plastid-targeting signals (Song & Brash 1991; Song *et al.* 1993; Laudert *et al.* 1996; Ziegler *et al.* 1997). Thus, at least three of the early biosynthetic steps are likely to occur in the chloroplast and certainly, the main location of 18:3 in plants is known to be the galactolipids of chloroplasts. Purification, cloning and sequencing of the 12-oxo-phytodienoic acid (12-oxo-PDA) reductase from *Arabidopsis* has not revealed any obvious targeting information, suggesting the enzyme is cytoplasmic (Schaller & Weiler 1997*a*,*b*). As β-oxidation is thought to reside exclusively in peroxisomes (see, for example, Gerhardt 1983), the formation of JA by three rounds of β-oxidation is likely to occur in that organelle. Taken together, these data imply that the functioning of at least three compartments is involved in the production of octadecanoid signals. Involvement of the chloroplasts in stress signalling in such a fundamental way ensures the response of the plant will be coordinated with photosynthetic capacity and carbon partitioning. The close interrelatedness of these metabolic events and stress signalling is also suggested by the known effects of octadecanoids on chloroplast functions, as well as the effects of chloroplast metabolites on stress-related gene expression.

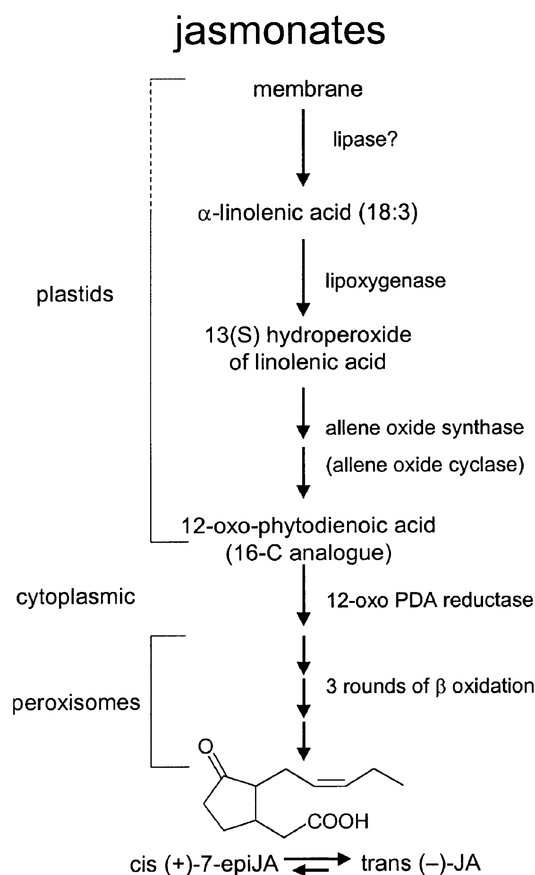

Figure 1. The biosynthetic pathway leading to synthesis of jasmonic acid.

In addition to JA, the 18:3, 13(S) hydroperoxylinolenic acid and 12-oxo-PDA were also shown to be active elicitors of *pin* gene expression in tomato plants (Farmer & Ryan 1992). This suggests that the enzymes responsible for JA biosynthesis are constitutively present in the leaves. Whereas a number of fatty acids other than the 18:3 were inactive (12:0, 16:0, 18:0, 20:4), surprisingly at the time, this early study showed that linoleic acid (18:2) was as active as wounding in the induction of *pin* gene expression.

The first evidence that endogenous octadecanoids might be involved in wound signalling came from experiments with diethyldithiocarbamic acid (DIECA) (Farmer *et al.* 1994). Pre-treatment with DIECA before wounding, OGAs, or systemin, blocked *pin* gene expression, whereas exogenous JA continued to be effective. As the effect of LA was also inhibited while the 13(S) hydroperoxide and 12-oxo-PDA were as active as JA, the compound appeared to block directly upstream of 13(S) hydroperoxide function. Farmer *et al.* (1994) went on to show that DIECA interacted with the 13(S) hydroperoxide of the 18:3 fatty acid, converting it into an hydroxyl derivative and therefore shunting it out of the biosynthetic pathway to JA. In mammalian systems, DIECA is used routinely to inhibit the Cu–Zn superoxide dismutase, and it is also likely that the compound will inhibit any other enzyme-protein dependent on Cu coordination for its bioactivity.

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In summary, application of octadecanoids to tomato plants induces *pin* gene expression and inhibition of biosynthetic steps beyond the LOX-catalysed step, and inhibits wound-induced expression. Subsequently, a number of studies showed that endogenous jasmonate levels increase after wounding, as well as after elicitor induction (see, for example, Peña-Cortes *et al.* 1993; Doares *et al.* 1995*a*), and most recently, free LA and linoleic acids were both also found to increase in wounded leaves (Conconi *et al.* 1996). The increase in 18:3 and 18:2 fatty acids in the wounded leaf, together with the earlier data showing that 18:3 and 18:2 are effective elicitors of *pin* gene expression suggest that both JA and dihydro JA may be involved in wound signalling in tomato. The latter compound is known to exist naturally (Miersch *et al.* 1989) and has been shown in early studies to have bioactivity (Ravid *et al.* 1975). In this context, the gene encoding allene oxide synthase from *Arabidopsis* has been recently cloned and expressed in *E. coli* (Laudert *et al.* 1996). At least *in vitro*, the recombinant enzyme could convert 18:3- as well as 18:2-derived 13-hydroperoxides to allene oxides. These data again suggest that two routes to bioactive jasmonates may be involved *in planta*, with potentially very different cell specificities, intracellular locations, and mechanisms of regulation.

The situation is potentially even more complicated, with 12-oxo-PDA also known to have bioactivity in its own right in other systems (Weiler *et al.* 1993; reviewed by Blechert *et al.* 1995), and a new hexadecanoid signal (dinor-oxo-PDA) recently being discovered in both *Arabidopsis* and potato, and shown to increase in level upon wounding (Weber *et al.* 1997). In addition, conjugates of 1-oxo-indane-4-carboxylic acid, structural analogues of the amino-acid conjugates of JA that are active agents in stimulating volatile emissions from plant leaves (Hopke *et al.* 1994; Krumm *et al.* 1995; Piel *et al.* 1997), have also now been shown to induce *pin* gene expression in the bioassay (Dorans *et al.* 1998). Recently, Mullet and coworkers have demonstrated that jasmonates are essential for insect defence in *Arabidopsis* (McConn *et al.* 1997).

These data imply that potentially, a very wide range of bioactive signals originate from the unsaturated octadecanoic acids, 18:3 and 18:2, in the wounded plant. The precise role of the individual molecular species *in planta* during the wound response remains unknown. Many of the genes encoding enzymes involved in jasmonate biosynthesis and metabolism, are themselves induced by octadecanoid signals, again suggesting very considerable complexity in regulation and functionality of this pathway (see, for example, Grimes *et al.* 1992; Melan *et al.* 1993; Feussner *et al.* 1995; Heitz *et al.* 1997).

(c) *Peptides*

An 18-mer peptide, named systemin (see figure 2), was purified from tomato leaves by Ryan and colleagues and shown to be an active elicitor of *pin* gene expression in the bioassay at femtomole quantities (Pearce *et al.* 1991). This was the first demonstration of a bioactive peptide in plants. The systemin sequence is found towards the carboxy (C)-terminus of a protein, encoded by a gene which has been called prosystemin on the tacit assumption that it represents an inactive precursor which is cleaved to release the active peptide. The sequence of the

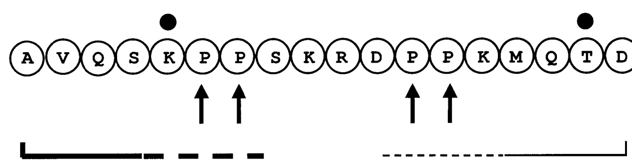

Figure 2. Sequence of the 18-mer peptide systemin. The C-terminal part of the peptide has been shown to be critical for induction of pin gene expression, whereas the N-terminal part is critical for binding to the kex2-like protease.

prosystemin cDNA contains no suggestive processing or targeting information, implying that in the cell, newly synthesized protein will remain in the cytoplasm following translation (McGurl *et al.* 1992).

Prosystemin transcripts can be detected at low levels in the aerial regions of an unwounded tomato plant, but the single-copy gene is not expressed in the root system. On leaf wounding, steady-state levels increase both at the injury site and systemically in unwounded tissues. Using expression of a reporter gene driven by the prosystemin promoter, β-glucuronidase (GUS) activity was found to be located only in the phloem companion cells and associated parenchyma (Jacinto *et al.* 1997). In the same study, antibody (Ab) staining of prosystemin protein was confined to those cell types. Thus, expression of the prosystemin gene is highly localized to specific cells in the vascular system, and while up-regulated on wounding, the tight cell-specificity is maintained.

Transgenic tomato plants either constitutively expressing the prosystemin gene at high levels or expressing an antisense gene of prosystemin have been constructed (McGurl *et al.* 1992, 1994). The former plants constitutively express *pin* genes, whereas the latter were shown to be be defective in the systemic expression of *pin* genes (expression in the wounded leaf was not investigated or reported).

Application of radiactively labelled systemin to a wound site in the leaf has clearly shown that the 18-mer peptide is mobile in the plant and can be transported from its site of application on the wound to other leaves and tissues (Narváez-Vásquez *et al.* 1995).

Elegant studies using L-alanine (Ala) substitutions along the 18-mer sequence have defined residues and stretches of sequence important or critical for inducing *pin* gene expression in the bioassay (Pearce *et al.* 1993). Thus, substitutions in the amino (N)-terminal half of the peptide have little or no effect on this activity. In contrast, removal of the last amino acid, or substitution with an Ala at position 17 (Ala17) abolishes the activity. These data, together with analyses of smaller sections of the peptide, have led to an awareness that the C-terminal half of systemin is critically important for *pin* gene expression. In fact, the C-terminal tetrapeptide is able itself to induce *pin* gene expression, albeit at much higher concentrations than the 18-mer. Significantly, while Ala17 is inactive, the peptide can be used as an antagonist in the bioassay. Pretreatment of plants with Ala17 prior to systemin blocks the action of systemin and no *pin* gene expression is induced (Pearce *et al.* 1993).

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Application of systemin to suspension culture cells of *L. peruvianum* was found to induce ethylene biosynthesis (Felix & Boller 1995). Exogenous systemin applied to plants in the bioassay also induces ethylene synthesis (O'Donnell *et al.* 1996).

While prosystemin has been cloned and expressed in *E. coli* to produce protein for antibody production (Jacinto *et al.* 1997), no data have been published as yet on the bioactivity of the entire recombinant protein. As described in § 5, there are also no data to show that systemin is released from prosystemin *in planta*, nor of the existence and characteristics of processing enzyme(s) or receptor(s). A tomato homologue of the mammalian and yeast prohormone convertase (kex2P) class of enzyme has been identified, but binding and processing assays have as yet only been published on the enzyme's interactions with the 18-mer systemin (Schaller & Ryan 1994). In this context, the N-terminal half of the peptide is important for kex2 binding, with Ala substitution at position 5 abolishing the interaction, whereas Ala17 remains as active in binding as native systemin. Binding of the systemin to the kex2P protease did lead to release of two peptides, probably at position Arg^{10}-Asp^{11}, which has the characteristic of a kex2 cleavage consensus site (Schaller & Ryan 1994). In this context also, a wound-induced leucine aminopeptidase has been discussed as a potential processing enzyme of peptide signals in tomato (Gu *et al.* 1996*a*,*b*). Structural analysis of the systemin 18-mer by NMR revealed no obvious 3D features that could explain its properties (Russell *et al.* 1992).

(d) *Bestatin*

Application of bestatin, an inhibitor of aminopeptidases when applied to excised tomato plants, was found to induce *pin* gene expression (Schaller *et al.* 1995). The compound did not lead to elevated JA, nor was its activity inhibited by the systemin antagonist Ala17. Thus, either its effect arose from triggering a systemin- and JA-independent pathway to *pin* gene expression, or its site of action (and presumably the aminopeptidase(s) which are inhibited) lies downstream of jasmonates.

3. INHIBITORS OF *PIN* GENE EXPRESSION

A wide range of chemicals has been assayed for their effects in the bioassay. The use of DIECA in relation to the involvement of jasmonates in wound signalling has been described in § 2. Inhibitors of protein kinases and protein phosphatases will be described in a later section, within the context of the involvement of phosphorylation–dephosphorylation in the regulation of the response. This section will focus on four classes of compound that have been shown to act as inhibitors: phenolics, inhibitors of mammalian enzymes that mediate inflammatory responses, sulphydryl reagents and the fungal toxin, fusicoccin, known to deregulate the protein ATPase. In addition, there is good evidence suggesting that auxin may be involved *in planta* in repressing *pin* gene expression in leaves (Kernon & Thornburg 1989; Thornburg & Li 1991).

(a) *Phenolics*

The first class of compounds identified by their inhibition of wound- or elicitor-induced *pin* gene expression, were phenolics, including salicylic acid (SA), acetyl salicylic acid (ASA) and related benzoic acids (Doherty *et al.* 1988). In this early study, the structural specificity shown for inhibitor action in tomato was found to be near-identical to that shown for induction of pathogenesis-related (PR) gene expression in the closely related plant species, tobacco. Given that endogenous SA and other phenolics are known to be involved in the regulation of pathogen-related responses, it is possible that the inhibitor data gained from the bioassay do reflect physiological events in the wound response *in planta* (Malamy & Klessig 1992). For the inhibitory effect, the compounds had to be applied prior to wounding, with application at 30 s post-wounding already ineffective. This suggested that the event(s) that was inhibited occurred at a very early stage of the response.

Subsequently, a number of studies have analysed the effects of SA and ASA in the bioassay in more detail, both in relation to other elicitors of *pin* gene expression and to other parameters in the response (Peña-Cortes *et al.* 1993, 1995; Doares *et al.* 1995*b*). While aspirin was also found to block the action of systemin and OGAs in eliciting *pin* gene expression, its effect on *pin* induction by octadecanoid signals is more controversial. Using dark-incubated plants, work from Peña-Cortes & Willmitzer's laboratory (1993) showed that the ASA block could be overcome by JA and its direct precursor 12-oxo-PDA, whereas 18:3 and the 13-hydroperoxide of 18:3 were inactive. It was suggested that the target for ASA inhibition was the allene oxide synthase. Subsequently, an additional target was found downstream of JA synthesis (Doares *et al.* 1995*b*), and most recently, a further effect of ASA was shown to be the complete inhibition of the ethylene synthesis induced by wounding or by the elicitors: OGA, systemin and JA (O'Donnell *et al.* 1996). In this last study, recovery of *pin* gene expression post-ASA treatment was achieved by joint application of ethylene and JA, not by application of either agent separately.

While ASA and the related phenolics act at several sites in the plant cell, it is probable that at least some of their effects arise from their actions as weak acids. In this context, reduction in wound-induced reactive oxygen species (ROS) across the injured leaf lamina can be abolished by benzoic acids, as well as several other weak acids such as pivulic acid (Marttila & Bowles 1998). It is highly likely that oxidative stress will be an essential step in wound signalling (Inzé & Van Montague 1995; Allan & Fluhr 1997), although as yet, surprisingly, this aspect has not been greatly studied in the tomato wound response.

(b) *Additional inhibitors of LOX- and cyclooxygenase- (COX) mediated reactions*

In addition to ASA, Peña-Cortes *et al.* (1995) investigated other inhibitors of mammalian LOX and COX reactions, and demonstrated that propyl gallate, salicyl hydroxamic acid (sham) and zk139 were equally effective as ASA in blocking *pin* gene expression in the bioassay. Interestingly, in their study, the effect of each of the compounds could be overcome by either 12-oxo-PDA or JA, suggesting a similar inhibitory mechanism.

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(c) *Sulphydryl reagents*

Ryan's group have demonstrated that the sulphydryl reagent, p-chloromercuribenzene sulphonic acid (pCMBS) acted as an inhibitor in the bioassay to prevent systemin, or wound-induced, *pin* gene expression (Narváez-Vásquez *et al.* 1994). The inhibition was reversed if systemin was applied with reducing agents such as dithiothreitol or glutathione. Whereas induction by the peptide elicitor was inhibited, induction by chitosan, OGAs, 18:3 or methyl JA was unaffected. These data were taken to support different receptor mechanisms for OGAs and systemin at the level of the plasma membrane, with the lack of pCMBS on octadecanoid induction reflecting an intracellular site of action for jasmonates. Given the nature of pCMBS and the known effects of sulphydryl reagents on very many different cellular events involving proteins whose confirmation–function depends on disulphide bridges, interpretation of these data is as difficult as those obtained with ASA. Nevertheless, the reagent was useful in distinguishing the effects of wounding and different elicitors in the bioassay.

(d) *Fusicoccin*

The fungal toxin fusicoccin (FC) is also a potent inhibitor of wound induction of *pin* gene expression in the bioassay (Doherty & Bowles 1990). Fusicoccin effects are mediated through FC-binding protein(s), now identified as members of the 14-3-3 protein family (Kourhout & de Boer 1994; Marra *et al.* 1994; Oecking *et al.* 1994). Many years ago, Marrè and co-workers first established that application of FC deregulated the plant plasma membrane H^+-ATPase (for a review, see Marrè 1979). This enzyme plays a central role in the cell biology and physiology of plants because its activity governs the electrochemical gradient across the plasma membrane, which in turn controls many aspects of ion transport and the regulation of cytoplasmic pH. Treatment with FC causes membrane hyperpolarization leading to a wide range of downstream effects including those on cell expansion, nutrient uptake and stomatal regulation. The effect of FC on the kinetics of the H^+-ATPase resembles that of treatments which displace the C-terminal autoinhibitory domain of the pump (Rasi-Caldognov *et al.* 1993). In this context, there is now convincing evidence that 14-3-3 proteins can form a complex with the C-terminus in the presence of FC, and that this leads to deregulation of the enzyme (Jahn *et al.* 1997; Oecking *et al.* 1997). 14-3-3 proteins have been extensively characterized in mammalian systems, as it has been shown that members of the family play key roles in the coordination of protein–protein interactions in signalling pathways involving protein kinases and phosphatases.

In relation to wound signalling in the tomato model, inhibition of wound-induced *pin* gene expression by FC suggests both that changed membrane potential is important in the transduction events, and that the *14-3-3* gene family will be useful targets to manipulate those events (Roberts & Bowles 1998).

4. THE USE OF MUTANTS AND TRANSGENICS TO UNDERSTAND THE WOUND RESPONSE OF TOMATO AND POTATO

Increasingly, mutants and transgenics with modified signalling pathways are being used to understand wound-induced events. Hopefully, this approach will clarify the meaning of the biochemical and physiological evidence that has accumulated to-date. For example, use of the transgenic tomato plants expressing a prosystemin antisense gene provided clear evidence that expression of prosystemin is required for wound-induced *pin* gene expression in the systemic leaves. Similarly, as described in § 6, transgenics expressing an ACC oxidase antisense gene, were useful in defining a role for that gene product in wound-induced *pin* gene expression. Use of the classical ABA-deficient mutants of potato and tomato have also proved instrumental in bringing ABA into the discussion of wound signalling. This section will focus on the two classes of mutants that have been studied in most detail.

(a) *ABA-deficient mutants*

Use of the classical ABA-deficient mutants of tomato and potato by the laboratory of Peña-Cortes & Willmitzer have led to an awareness that ABA is involved at some level in wound signalling and the regulation of *pin* gene expression (Peña-Cortes *et al.* 1989, 1991, 1996; Hildmann *et al.* 1992; Herde *et al.* 1995, 1996; reviewed by Peña-Cortes *et al.* (1995)). They showed that wounding the mutants did not lead to increased *pin* gene expression, whereas application of ABA or JA to the plants rescued the pin response. Local application of ABA or JA to the surface of one leaf was sufficient to induce local and systemic *pin* gene expression. These data have been interpreted to indicate that both compounds are mobile and that JA is downstream of ABA in the wound signal cascade. Surprisingly, application of exogenous systemin to the ABA-deficient mutants of tomato and potato was ineffective in the induction of *pin* gene expression (Peña-Cortes *et al.* 1996). Wounding had been shown previously to be inactive, yet application of 18:3 to the mutants was sufficient to induce elevated JA and *pin* gene expression. Thus, the enzymes leading from 18:3 to JA were functional in the mutants, and providing the plants with 18:3 was sufficient to rescue the pin response. These data suggest that ABA is required for the release of 18:3, and in turn, show that exogenous systemin is inactive if the ABA-regulated step is absent. The membrane in question is now recognized to be, most probably, the chloroplast.

The authors interpreted the data as showing that 'the site of systemin action is located upstream of the site of ABA action and the site of JA action is downstream of ABA and systemin . . .' This interpretation depends on the three signalling components residing in a linear transduction pathway. However, recently, the laboratory of Sanchez-Serrano (Dammann *et al.* 1997) has further analysed ABA and JA signalling in potato plants. Whereas pretreatment with the protein phosphatase inhibitor, okadaic acid, blocked JA induction of *pin* genes, the compound had no effect on their induction by ABA which, rather, was blocked by the protein kinase inhibitor, staurosporine. These data suggest that there are two independent pathways to *pin* gene expression in potato leaves: one involving JA, affected by a protein phosphatase inhibitor, and one involving ABA, affected by a protein kinase inhibitor.

Importantly, should these experiments give identical results in tomato, they will call into question current

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interpretations of bioassay data involving exogenous JA. Rescue experiments with JA have always been interpreted as indicating that JA is downstream of the site blocked by the inhibitor or defective in the mutant-transgenic. These interpretations have been questioned previously (Bowles 1993), as the data could have been interpreted equally to mean that JA was inducing *pin* gene expresion via an independent pathway(s). Certainly, the JA rescue in the ABA-related mutant of tomato may now be owing to the involvement of separate pathways rather than the relative position of ABA and JA in a linear signalling cascade.

The study of Dammann *et al.* (1997) also showed that exogenous ABA activated a number of separate signalling pathways in parallel, leading to the induction of several different sets of genes. To quote, 'exogenous ABA application activates all of the pathways, leading to a non-selective expression of ABA-responsive genes involved in different responses to stresses such as water deficit, salt or wounding'. Thus, whereas highly effective in a bioassay, application of exogenous ABA overrides the selectivity of response normally in place *in planta*. In relation to studies in tomato plants, it is equally possible that application of elicitors such as exogenous OGAs, JA, or systemin in a bioassay, will cause many more events to occur than those induced by the highly selective cell-specific and transient changes during a wound response.

(b) *The JL5 mutant of tomato*

There were two non-allelic recessive mutants first described by Lightner *et al.* (1993) and as the pin response in each could be rescued by exogenous JA, both lines were suggested to have defects in the pathway between injury and JA. Further studies by Howe *et al.* (1996) on the JL5 mutant included an analysis of the response of the plants to elicitors and jasmonate precursors. In contrast to their effect in wild-type, the three elicitors (systemin, OGAs, and chitosan) were largely ineffective in the JL5 phenotype. Application of 18:3 or its hydroperoxide was similarly ineffective, but 12-oxo-PDA was as active as JA. These data were interpreted as a defect in the mutant at the level of the allene oxide synthase–cyclase steps in the jasmonate pathway. Injury led to lower levels in the wounded JL5 leaves compared with the wild-type (measured at 90 min), and in particular, the mutant line was incapable of synthesizing JA in response to exogenous systemin. Therefore, with respect to wounding, JL5 does not make the appropriate level of jasmonates.

(c) *Transgenics with increased levels of jasmonates*

The cloning of the allene oxide synthase provided the foundation for analysing the effects of its overexpression in tomato plants (Harms *et al.* 1995). Whereas up-regulation of the gene led to a massive rise in endogenous JA in the plant, no constitutive expression of *pin* genes was observed. Injury was still required to trigger the pin response. Since, as described in O'Donnell *et al.* (1996), both JA and ethylene are required, it is possible that simply elevating endogenous JA does not induce ethylene, and in the absence of either one of the putative signals, the response is not triggered.

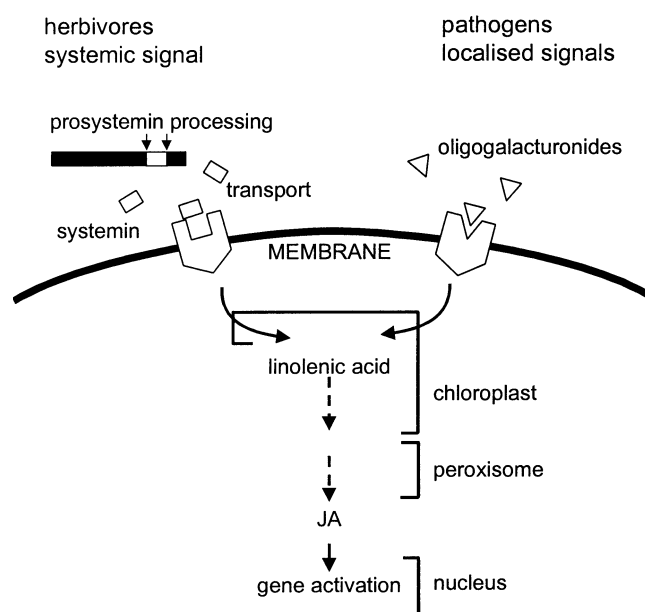

Figure 3. A model, taken from Bergey *et al.* (1996), providing their current framework for the relative positions of OGAs, systemin and jasmonates in pin gene regulation. It shows the following points: (i) systemin is released from prosystemin and is transported as the systemic signal; (ii) systemin and OGAs have different receptors; (iii) system and OGA transduction pathways are via JA; (iv) JA synthesis occurs at the local and systemic sites; and (v) JA is directly upstream of gene activation.

5. CURRENT MODELS FOR *PIN* GENE REGULATION

(a) *A framework to understand the relative involvement of OGAs, systemin and jasmonates*

The first model incorporating OGAs, systemin and jasmonates was proposed by Ryan and colleagues and was published in Farmer & Ryan (1992). The most recent update (Bergey *et al.* 1996) is shown in figure 3. Systemin and OGAs are each shown interacting with their specific receptors at the external face of the surface membrane. As a consequence of these interactions, 18:3 is released from the chloroplast and is converted along the biosynthetic pathway to JA within the cytoplasm, which interacts with a receptor leading to *pin* gene activation. The model shows systemin and OGAs in the apoplast, each has a specific receptor, and on receptor-binding of either the peptide or the glycan, JA levels are elevated, and JA is the causal signal immediately upstream of *pin* gene transcription. As shown in the model by Bergey *et al.* (1996), there is a distinction made between herbivores and the systemic signal involving the systemin receptor, and pathogens and localized signals involving an OGA receptor and/or a chitosan receptor. The biosynthetic pathway from 18:3 to JA remains common to both local and systemic events, and JA is the terminal signal of the cascade.

Central to this model are three proposals. First, octadecanoid signals are required for *pin* gene expression, whether in the wounded leaf or in distant unwounded leaves. Second, cell wall fragments and systemin, are both located in the apoplast, interact with a membrane to give

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rise to elevated intracellular JA, and do so via independent receptor-recognition systems. Third, systemin, encoded by prosystemin is proteolytically released from the larger protein at damaged cells and the 18-mer represents the mobile systemic signal that is transported around the plant.

The evidence to support octadecanoid involvement in *pin* gene regulation is based on (i) the bioassay, in which OGAs and systemin induce elevated JA; (ii) measurement of JA levels in wounded leaves, in which levels are shown to rise prior to the increase in steady-state levels of *pin* transcripts; (iii) the use of inhibitors applied in the bioassay such as ASA and DIECA that prevent the rise in JA together with inhibition of *pin* gene expression; and (iv) the use of the mutant JL5 in which JA synthesis is compromised. These independent lines of evidence are convincingly persuasive that jasmonates are involved in events leading to *pin* gene expression *in planta*. However, to-date there have been no published data on changes in JA levels in distant leaves, nor have 18:3 levels been measured in unwounded tomato leaves, even though Conconi *et al*. (1996) measured a time-course of these changes in wounded leaves and discussed them in the context of both the local and systemic response. As will be described in § 8, recent evidence shows that there is no rise in JA in unwounded leaves.

In relation to the second proposal, there are no data, such as using appropriate antisera, on the existence of cell wall fragments or the systemin peptide at the wound site or elsewhere. Use of the Ala-17 homologue as an antagonist can be used to identify events that involve the systemin–Ala17 receptor system. As Ala17 pre-treatment blocks wound-induced *pin* gene expression, there is the clear implication that wound-released signals leading to *pin* gene expression are transduced through the systemin receptor that is antagonized by Ala17. This receptor has not been identified. However, as will be described in § 8, recent data show that treatment of plants with Ala17 before treatment with OGAs also blocks the glycan induction of *pin* gene expression. This result indicates that the OGA effect is mediated through a recognition–transduction system that at some level is antagonized by Ala17, implying either that a/the systemin 'receptor' is downstream of OGAs, or an identical receptor can be activated by both peptides and glycans.

The third proposal is unsubstantiated for events *in planta*. There is convincing evidence that radioactively labelled systemin peptides applied to the wound site on a leaf can be recovered in exudate from the petiole, and this indicates that the exogenous 18-mer peptide is mobile in the plant. However, there are no data on the course of events in the wounded plant to show that prosystemin is cleaved, that systemin is 'released' from damaged cells, that transport is apoplastic, and that the endogenous systemin peptide is the systemic signal moving around the plant.

A key experiment in this context would involve the transgenic tomato plants expressing a prosystemin antisense gene. As described earlier, these transgenics have been constructed, and their analyses showed that wound-induced systemic *pin* gene expression was inhibited. No data was shown on *pin* gene expression in the wounded leaves, and the phenotype was discussed solely in the context of systemin as the mobile systemic signal. If transgenic plants were grafted onto a wild-type rootstock, a central prediction of Ryan's model is that wounding the transgenic leaf would be unable to release mobile systemin owing to the absence of its precursor, prosystemin. As a consequence, systemic *pin* gene expression in the wild-type plants would not occur. However, these crucial experiments have not been published. In contrast, grafting experiments with transgenics constitutively expressing a prosystemin gene have been done, but this phenotype is highly abnormal given that the transgenic plants also constitutively express *pin* genes. As a consequence, interpretation of the graft results in which wild-type also start to constitutively express *pin* genes is more complicated because there are no data on the extent of changes in the transgenics, such that JA might also be elevated and mobile across the graft union.

(b) *Inclusion of ABA in the framework*

As described previously, ABA has been implicated in wound signalling, through the use of ABA-deficient mutants and their rescue by application of ABA or JA. Figure 4 shows a model proposed by Peña-Cortes *et al.* (1995), incorporating ABA into the framework discussed in § 5a, and also incorporating electrical signals as discussed in the next section.

(c) *The route and nature of the systemic signal*

In 1992, Wildon *et al.* (1992) provided data to show that a rapidly transmissible signal leading to *pin* gene expression in distal leaves could exit from a mechanically injured or heat-challenged cotyledon or leaf, through a cold block on the petiole. Experiments with C^{11} indicated the signal was transmitted under conditions in which phloem translocation was blocked. Given that *pin* gene expression was induced under these conditions, the data implied that the systemic signal was not exiting from the wounded leaf in the phloem. Evidence was also presented to show that on wounding, an electrical signal could be detected with the characteristic of an action potential and this was propagated through the cold block. Thus, as there was a close correlation between passage of the electrical signal and induction of *pin* gene expression in the unwounded leaves, a role for electrical signalling in the systemic response was suggested. This study is cited very often as showing the systemic signal is an electrical signal. However, as noted in the paper, the data were only correlative. In contrast, definite evidence was provided to show the causal systemic signal was not transmitted with photosynthate through the phloem.

The lack of evidence for phloem translocation provided by Wildon *et al.* (1992) contrasted with many earlier suggestions that the systemic response was induced by a mobile chemical transported in the phloem. Initially, it had been suggested that cell wall fragments were mobile in the phloem, but this was countered by Baydoun & Fry (1985), showing that application of pectic fragments to a wound site did not exit the leaf under conditions in which sucrose was transported. Subsequently, systemin was suggested to be the mobile chemical signal. As described in § 2c, exogenous systemin applied to a wound site is

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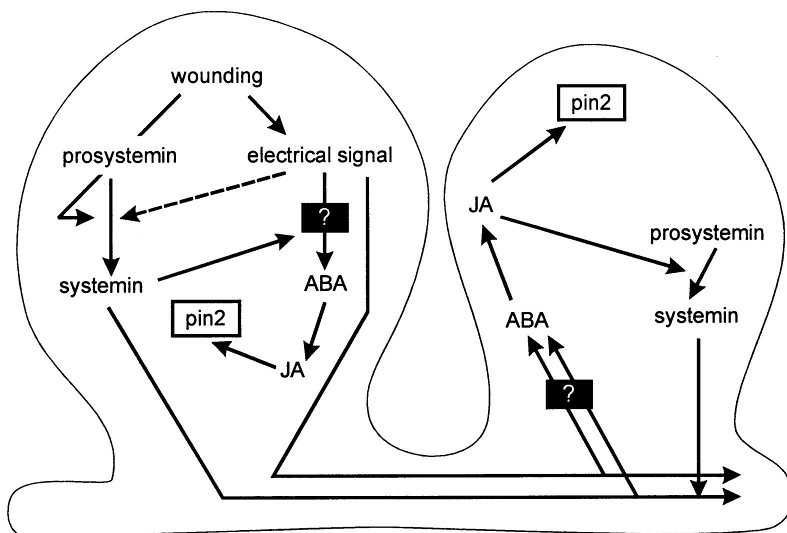

Figure 4. A model, taken from Peña-Cortes *et al*. (1995), describing their framework for including ABA and electrical signals in *pin* gene regulation.

mobile, does exit the leaf, and is transported around the plant (Narváez-Vásquez *et al.* 1995). While pCMBS has been suggested to exert its inhibitory effect on systemic (and local) *pin* gene expression through affecting active loading of metabolites into the phloem (Narváez-Vásquez *et al.* 1994), there remains no direct evidence that endogenous systemin is mobile in the wounded plant. Most recently, Stratmann & Ryan (1997) have shown that wounding induces an elevated kinase activity in the local and systemic leaves, and as the causal signal(s) leaving the injury site is not affected by stem-girdling, the data imply it is transmitted in the xylem.

Further studies on long-range electrical signalling in tomato have been done in several laboratories (Peña-Cortes *et al.* 1995; Herde *et al.* 1995, 1996; Rhodes *et al.* 1996). The study of Herde *et al.* (1995), addressed a range of issues, comparing mechanical stimulation with heat stimulation and, importantly, showing that treatment of leaves with an electrical current led to the local and systemic expression of *pin* genes. In particular, they found evidence for two classes of electrical signal. A fast signal with the characteristics of an action potential that occurred on mechanical wounding, and which also was propagated following electrical stimulation. In addition, a second electrical signal, with a much slower time constant was observed, and was suggested to have a hydraulic component. This implies that both action potentials, as well as variation potentials arising from the electrical activity of xylem-transmissible chemicals, may be triggered *in planta* upon leaf injury. The study of Herde *et al.* (1995) also showed that the pattern of electrical events induced by mechanical injury was very different from those induced by heat stimulation, although both challenges led to *pin* gene expression. This study was followed by Herde *et al.* (1996), which again confirmed that the transduction pathway leading from mechanical injury to *pin* gene expression differed from that induced by a heat stimulus. Mechanical damage and electrical stimulation of *pin* gene expression were similar, heat stimulation was different. Therefore, while Rhodes *et al.* (1996) have provided convincing evidence that an action potential triggered by a heat stimulus is propagated through the sieve tube–companion cell complex, the relation of this action potential to the causal signal induced by a mechanical stimulus is unknown.

Therefore, at this time, there is substantial evidence that leaf wounding induces the propagation of electrical signals, as described in the references given and in a range of earlier work, such as that of Pickard's laboratory, cited in those references. There is also evidence that electrical stimulation can induce local and systemic *pin* gene expression. Thus, it is possible that the electrical signal(s) observed on mechanically wounding the plant is, or are, also involved in the systemic transduction events.

Malone has also worked extensively on the route and nature of the systemic signal in the tomato wound response, and has been instrumental in drawing attention to the possible involvement of hydraulic signals (see, for example, Malone *et al.* 1994*a*,*b*; Malone & Alarcon 1995). Malone *et al.* (1994*a*) initially suggested that hydraulic signals were the systemic signal in *pin* gene expression; however, because cutting a tomato plant stem and removal of the root system does not induce *pin* gene expression, release of hydraulic tension *per se* cannot be the causal signal for systemic signalling. The bioassay is based on this procedure and the plants are not excised under water.

Elicitors applied in an agar block to a cut surface were inactive in inducing *pin* gene expression in the absence of basipetal mass-flow (Malone *et al.* 1994*b*). This led to the suggestion that systemic *pin* gene expression *in planta* involved the uptake of active elicitors at the wound site into the xylem and transport through the xylem to the rest of the plant. As wound-induced mass-flow is transient, solutes will only be drawn into the xylem if they are present at or immediately after injury. Malone & Alarcon (1995) went on to show that changing shoot water status and therefore xylem tension, has an important effect on systemic signalling. When xylem tension was negligible, systemic wound-induced hydraulic events were absent, and no *pin* gene expression, measured as pin activity, was detected in the unwounded leaves. Data on the local wound-site were not shown. Steam-(solder-)girdling was also used to show that causal signals leading to systemic *pin* gene expression transversed the heat-killed section of the petiole.

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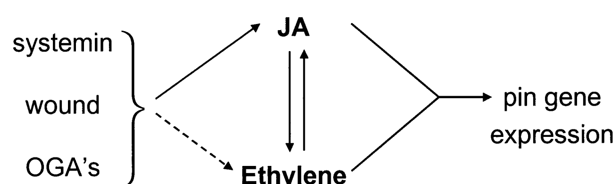

Figure 5. A model take from O'Donnell *et al*. (1996), describing their framework for including ethylene in events leading to *pin* gene expression.

Taking these data together with those arising from the earlier studies, our current understanding of systemic signals leading to *pin* gene expression, is that (i) transport of a mobile signal in the phloem is not the causal systemic signal; (ii) action potentials and variation potentials are triggered by mechanical injury and *pin* gene expression can be induced by electrical stimulation, but the relatedness of these events to those *in planta* during a systemic wound response is unknown; and (iii) xylem-transmissible signal(s) certainly occur on wounding, can induce *pin* gene expression and on current evidence are most closely correlated with the systemic signal *in planta*.

(d) *The role of ethylene*

Stress ethylene induced by localized damage of vegetative tissue was discovered many years ago (reviewed in Kende (1993); Fluhr & Mattoo 1996; Morgan & Drew 1997). The synthesis of ethylene under these circumstances is independent of ethylene perception–action, whereas its down-regulation is an ethylene response. The expression of both ACC synthase (ACS) and ACO is up-regulated by wounding, is restricted to the wounded leaf, and is transient, with, for example, steady-state levels of ACO transcripts decreasing within 2–4 h of injury.

The three classes of elicitor leading to *pin* gene expression in the bioassay were found to be active also in ethylene production (O'Donnell *et al.* 1996). While ASA inhibited the synthesis of ethylene, most significantly, *pin* gene expression by wounding or elicitor application could be abolished by pretreatment of the plants with silver thiosulphate, or with norbornadiene (NBD). The inhibitory effect of NBD could be overcome by exogenous ethylene, confirming the likelihood that ethylene action was required for wound-induced *pin* gene expression. These data, and those from wound challenge of transgenic tomato plants constitutively expressing an ACO antisense gene, implicated the involvement of ethylene in *pin* gene regulation for the first time. The hormone had been analysed in early studies for its effect on the response, when gassing plants had been shown to have no effect on *pin* gene expression. However, application of exogenous ethylene to unwounded, or to excised plants incubated in H_2O, does not alone induce a rise in endogenous jasmonates.

Interestingly, ethylene is now known to play a regulatory role in the accumulation of endogenous jasmonates in the wounded leaf, or in elicitor-treated plants (O'Donnell *et al.* 1996, 1998*a*,*b*). In this context, the rapid and massive rise in JA that can be measured within 30 min of wounding consists of an ethylene-dependent component (amounting to some 80% of the total) and a component that is unaffected by ethylene synthesis–action inhibitors. The model suggested by these observations is shown in figure 5. These observations relate only to the leaf that is wounded, because, as described in §6, unwounded leaves show no ethylene transient nor any increase in JA.

(e) *The role of protein phosphorylation and dephosphorylation*

In recent years there has been a growing realization that signalling mechanisms in plants share many common features with those in other eukaryotic cells. This is particularly so in relation to the mitogen-activated protein (MAP) kinase cascade, comprising a functional module of three linked protein kinases, activated in animals, in response to a range of stimuli including mitogens and diverse stresses (reviewed in Hunter & Karin (1992); Ahn 1993; Jonak *et al.* 1994). The module terminates in the serine–threonine MAP kinase (MAPK), which is activated by phosphorylation on tyrosine and threonine residues, mediated by a single activator protein kinase, the MAPK kinase (MAPKK). Activation of that kinase occurs by phosphorylation on serine residues by other protein kinases, the MAPKK kinase (MAPKKK) class. A feature of the terminal MAPK which has been used diagnostically in a number of recent plant studies is that it can phosphorylate the artificial substrate myelin basic protein (MBP) *in vitro*, but not casein, nor histone (Ray & Sturgill 1988; Gotoh *et al.* 1990). Generally, activation of MAPK activity is rapid and is post-translational, depending on stimulation of the phosphorylation cascade by an external stimulus and involving pre-made and constitutively expressed upstream kinases. Often, activation of the *MAPK* gene is one target of its increased enzyme activity.

There have been several reports of a wound-induced MAPK (Suzuki & Shinshi 1995; Usami *et al.* 1995; Seo *et al.* 1995; Bögre *et al.* 1997; Stratmann & Ryan 1997). Of these reports, three use a tobacco model, one an alfalfa model and the last cited, a tomato model. In each model, there is rapid up-regulation of MAPK transcripts, as well as a rapid increase in kinase activity, as determined using the method of in-gel phosphorylation of MBP. Until now in these studies, the in-gel assay has only involved one-dimensional (1D) gel electrophoresis, and given the close simularity in molecular size of the entire family of MAPKs, analyses using 2D electrophoresis will be required to confirm whether different stimuli do indeed induce the same enzyme activity. In the tomato and tobacco models, the response of the kinase(s) to an injury stimulus was both local in the wounded leaf and systemic in distant unwounded leaves (Seo *et al.* 1995; Stratmann & Ryan 1997). Increased steady-state levels of mRNA were detected within minutes, locally and systemically, indicating a very rapid systemic signalling mechanism must be involved.

In the tomato study, application of exogenous systemin or OGAs also induced MBP kinase activity with an identical mobility in the 1D in-gel assay, and it was reported that Ala17 pre-treatment blocked the systemin induction. No data were presented on the effect of Ala17

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on wound induction of the kinase activity, nor the effect of Ala17 on OGA induction (§ 8).

Both the study of Bögre *et al.* (1997) and that of Stratmann & Ryan (1997), showed that exogenous JA and 12-oxo-PDA did not induce MBP kinase activity. This suggests that either elevation in JA and JA-mediated events are downstream of the kinase(s), or they trigger the response via an independent pathway (see § 4). Data from leaf injury in alfalfa (Bögre *et al.* 1997) also showed that ABA was unable to induce the MAPK that they had identified on wounding the tissue. However, ABA has been shown to induce a MAPK acitivity in other model systems (Knetsch *et al.* 1996), and many of the responses thought to be mediated by ABA, such as cold stress and drought, have recently been shown to induce up-regulation of a *MAPK* gene, as well as activation of enzyme activity (Jonak *et al.* 1996; Mizoguchi *et al.* 1996). If ABA is shown to induce these responses in the potato–tomato model, it would offer one explanation for the staurosporine inhibition of ABA induction of *pin* gene expression (Dammann *et al.* 1997), and in turn, albeit highly speculatively, why systemin was inactive in an ABA-deficient background (Peña-Cortes *et al.* 1996).

Also of relevance to the involvement of a MAPK cascade in wound signalling is the homology of *CTR1*, a negative regulator of the ethylene response pathway in *Arabidopsis*, to the Raf family of MAPKKKs (Kieber *et al.* 1993). *CTR1* lies downstream of *ETR1*, the ethylene response gene (Chang *et al.* 1993). Given that *pin* gene expression in the wounded leaf is an ethylene response (O'Donnell *et al.* 1996), the ethylene transduction pathway and its regulation will impact on the wound signalling leading to *pin* gene transcription.

In addition to MAP kinase cascades, there are many other events in metabolic regulation that are controlled by phosphorylation and dephosphorylation. Many of these are of direct relevance to understanding the wound response. A principal site of regulation is the activity of the plasma membrane H^+-ATPase. Control of the membrane potential and its depolarization or hyper-polarization in response to external stimuli is known to be one of the earliest events in a wide range of signal transduction pathways in plant cells, particularly in this context, leading from pathogens and pathogen-derived elicitors (see, for example, Xing *et al.* 1996). Membrane depolarization is also one of the first responses to cellular injury. In tomato, both OGAs and systemin have been shown to induce rapid depolarization (Thain *et al.* 1990, 1995; Moyen & Johannes 1996), and this has been implicated in spread of the wound response through a mechanism analogous to epithelial conduction (Wildon *et al.* 1992).

Regulation of the H^+-ATPase is through phosphorylation–dephosphorylation (reviewed by Assmann & Haubrick (1996)). Treatment with kinase or phosphatase inhibitors will most probably affect this regulation, and as described earlier, FC, a fungal toxin that acts through a 14-3-3-mediated pathway to deregulate the H^+-ATPase, has been shown to be a potent inhibitor of wound-induced *pin* gene expression in tomato (Doherty & Bowles 1990). FC can also inhibit OGA or systemin induction of *pin* gene expression, but does not affect induction by JA (O'Donnell 1994). This pattern of inhibition can be interpreted to mean that transduction of the two hydrophilic signals depends on an appropriate membrane potential, and its depolarization, whereas JA action is downstream. Alternatively, exogenous JA may induce *pin* gene expression through an independent pathway to wounding, OGAs and systemin.

Phosphorylation–dephosphorylation has also been shown to play a central role in the regulation of ethylene synthesis through the turnover and regulation of ACC synthase (see, for example, Spanu *et al.* 1994; Fluhr & Mattoo 1996), as well as in the regulation of many different ion channel activities, including those involved in Ca^{2+} uptake and K^+ efflux (for examples, see Conrath *et al.* 1991; Kauss & Jeblick 1991), and anion efflux through the slow anion channel (Schmidt *et al.* 1995; for a review, see de Boer & Wegner 1997). Thus, there are many different sites of potential regulation by kinases and phosphatases in addition to those mediated directly by the MAPK cascade.

6. A NEW MODEL FOR *PIN* GENE REGULATION

A principal distinction between the proposed model and the general framework described in the models of Ryan and colleagues and Peña-Cortes and colleagues, is that there are two quite different signal transduction pathways leading to up-regulation of *pin* genes, only one of which involves elevated jasmonate levels.

We have found that in the wounded leaf, *pin* gene expression can be regarded as an ethylene response because treatments such as blocking ACO expression, the stress ethylene transient, or ethylene action, are inhibitory (O'Donnell *et al.* 1996). At least one function of ethylene action is the enhanced synthesis of JA, and on wounding there is a positive correlation between these high JA levels and *pin* gene expression. We have found that only *ca.* 20% of the total JA accumulates in wounded transgenics expressing an ACO antisense gene, following silver and NBD pre-treatment of wild-type, or in the JL5 mutant background. Near-identical low levels are also caused by pre-treatment of the plants with cycloheximide (CHX), suggesting that *de novo* protein synthesis is required to attain the high JA levels correlated with *pin* gene expression (O'Donnell *et al.* 1998*a*).

In contrast to these data, when we measured ethylene synthesis or JA accumulation in systemically responding leaves, we found no change in ethylene, nor in levels of JA. Thus, the events in the unwounded leaves clearly differ from those at the injury site, and this is reflected both in the lack of ethylene and the lack of JA (O'Donnell *et al.* 1998*a*). These data imply that the transduction pathway of the systemic signal does not involve increased levels of JA, and may not require ethylene action. In this context, the systemic expression of *pin* genes is much less affected in an ACO antisense genetic background than in the wild-type.

We used Ala-17 to investigate the nature of the responses transduced through the systemin receptor antagonized by the inactive Ala17 homologue (O'Donnell *et al.* 1998*b*). As shown previously, pre-treatment with Ala17 blocked wound- and systemin-induced *pin* gene expression. Jasmonates have always been placed downstream of systemin in the earlier models. This site has

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been proposed for several reasons, for example: exogenous systemin induces the accumulation of JA; exogenous JA is active in prosystemin antisense transgenics; and exogenous JA is unaffected by an Ala17 pre-treatment.

However, we have discovered that pre-treatment with Ala17 prior to wounding, while inhibiting *pin* gene expression, has no effect on levels of wound-induced JA (O'Donnell *et al.* 1998*b*). Therefore, we can uncouple the effect of Ala17 on *pin* gene expression from the rise in endogenous jasmonates. This indicates that the ethylene-dependent rise in JA does not depend on a transduction path involving the systemin (Ala17) receptor, but *pin* gene up-regulation does. In turn, this implies that the ethylene-dependent JA is irrelevant in the wound transduction pathway to *pin* genes, and/or that the receptor antagonized by Ala17 is actually downstream of the jasmonate response.

Surprisingly, we also found that Ala-17 blocked OGA-induction of *pin* gene expression, suggesting that the transduction pathway from exogenous OGAs to *pin* gene transcription must also transverse through the systemin receptor antagonized by Ala-17. In an identical manner to wounding, Ala-17 pre-treatment prior to OGAs again had no effect on JA accumulation (O'Donnell *et al.* 1998*b*). Our interpretation of these data is that the ethylene-dependent JA induced by injury or by OGAs reflects the events in the wounded leaf. These will occur, but cannot lead to *pin* gene expression because the only path to that is blocked by the systemin antagonist. Systemin is involved locally, but the systemin–Ala17 receptor system is downstream of jasmonates. In the plant, such a JA-independent pathway leading from systemin to *pin* gene expression would be compatible with the observations in systemically responding leaves. From studies on prosystemin-antisense transgenics, systemin is known to be necessary for systemic *pin* gene expression, yet from our data there is neither a change in ethylene nor in JA at the systemic site. This implies that systemin can induce *pin* gene expression in the absence of changed levels of ethylene and JA.

How is this interpretation reconciled with the data arising from application of exogenous systemin or exogenous JA? First, there is a clear difference in the way in which wounding induces JA and exogenous systemin induces JA. This is now demonstrated by the Ala17 data but, in earlier studies on JL5 (Howe *et al.* 1996), and our own work on CHX effects (O'Donnell *et al.* 1998*a*), differences were also detected. Whereas wounding led to some JA in JL5, systemin produced none. We have confirmed that data and shown it to be the wound-induced ethylene-dependent JA that is affected in JL5. We have also found that CHX completely inhibits systemin induction of JA, but only affects the ethylene-dependent wound JA.

Therefore, it is possible that data obtained from the high levels of systemin applied in the bioassay are difficult to relate directly to data arising from events in the wounded plant, particuarly given the very tight cell-specificity of prosystemin expression and the extremely low levels of naturally occurring systemin, even in the wounded plant. Similar difficulties may again be encountered when exogenous JA is applied to the entire plant in the high levels of the bioassay. *In planta*, levels only rise in the wounded lamina and very transiently. Perhaps in some way, application of high levels of the compound bypass the need for systemin, and as described earlier, may even initiate an independent signalling pathway (Dammann *et al.* 1997).

If one focuses solely on wound-induced events in the plant, an ethylene-dependent increase in JA in the wounded lamina can be correlated with *pin* gene expression in that leaf. From the Ala17 antagonist data, the JA increase in that leaf would seem to be independent of systemin. In unwounded leaves, the lack of change in ethylene and JA suggests that the pathway to *pin* gene expression is different to that in the wounded leaf. If it involves (pro)systemin, which data from Ryan's laboratory suggest it does, then this induction is via an ethylene–JA-independent pathway. While it is clear that the systemin peptide is mobile in the plant, as yet there is no evidence to distinguish between systemin as the actual transported systemic signal, and systemin as the local effector of a systemic signal.

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