

Wound-Inducible Genes in Plants



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INTRODUCTION

All living organisms are involved in a constantly struggle with and against other organisms to exploit their environment. Every organism exploits its own environmental niche to gain nutrients for growth and development. However, when multiple organisms interact, then a direct competition is established between those organisms. The organism that is better able to compete usually has an evolutionary advantage and is assured of survival. Some organisms move when in direct competition, however, because of their sedentary lifestyle, plants generally can not. Instead, plants have developed very potent biochemical responses that serve to protect their integrity and to limit the invasive nature of the competing organisms.

Structurally, plants have a polyester coating composed of cutin and suberin (Kolatakuddy, 1980). This coating normally isolates the plant tissues from competing organisms and plants are therefore relatively immune from the presence of these competitors even on their surface. However, if a break or wound occurs in this surface coating, then competing organisms gain entrance into the plant's tissues where they can cause injurious damage to those tissues. Consequently, plants have developed a complex response to wounding that dramatically alters the cellular physiology of plant tissues and results in the production of defenses. These defenses are particularly potent against microorganisms and are even effective against small herbivores.

The response of plants to wounding has been studied since the early 1970s when Green and Ryan (1972) discovered that an inhibitor of chymotrypsin in tomato leaves accumulated in response to wounding. Further, because chymotrypsin-like proteins do not occur in plants, but are common in insect digestive

tracts, they concluded that this inhibitor was part of a wound-responsive plant defense system. Since that time at least 70 other proteins have been identified as also being wound-inducible.

Table I provides a list of different genes that have been demonstrated to be wound-inducible. This list is not meant to be all inclusive, but it does give a broad perspective of both the number and classes of plant genes that have been identified to date as being wound-inducible. Many of these genes are discussed in some detail below. In addition, this table provides additional information about the modes of regulation where known for each particular gene. In some cases, genes encoding a particular protein have been described from multiple species. There are sometimes differences in regulation of the genes between species for individual genes. In addition, many of the genes listed in Table I are members of multigene families. In these cases, the several members are often differentially regulated, with only one or a few members of the family being wound-inducible.

Because any attempt to adequately discuss the expression of 70 different proteins from at least 38 species across 20 families would result in a morass of contradictory information. We will, therefore, limit this article to two areas of discussion. First, because of this large number of proteins that are induced in response to a wound, we can identify the classes of proteins produced and begin to draw some conclusions about overall biochemical processes that are important in response to a wound. Secondly, there are a few wound-inducible proteins and their genes that have been studied in great detail, and the mechanisms of gene activation of several seemingly unrelated proteins (i.e., proteinase inhibitors of the solanaceae and vegetative storage proteins of the fabaceae) share many details of gene activation. Therefore, we will also examine the details of the mechanisms of gene activation for these well studied systems.

THE MULTIPLE PHASES OF A WOUND RESPONSE

Wounding results in the activation of many different genes within a plant. The types of genes and the timing of their activation allows the identification of different phases following a wound. Each of these phases of the wound-induction process biochemically solves a different problem that wounding causes the plant. These problems include: placing mechanical barriers to invading organisms, sealing the wound tissue, activating defensive compounds against invading organisms and recovering from the wound. The sum of these processes results in recovery from a wound and return to a normal physiology.

The hydrogen peroxide response

The initial phase of a wound-response is a rapid reaction to close the wound thereby protecting the plant from loss of cellular components and restricting entry of mi-

croorganisms into the plant tissues. This is composed of at least two general processes. Initially there is an almost immediate oxidative burst that results in a crosslinking of plant cell wall proteins (Bradley et al., 1992; Brisson et al., 1994). This oxidative burst can be detected within 15 seconds. This crosslinking of the cell wall proteins provides a structural barrier that inhibits the invasion of microorganisms. In addition, H_2O_2 from this oxidative burst is thought to activate some of the wound-inducible genes (Levine et al., 1994). Because hydrogen peroxide is itself toxic to plant cells (Lachman, 1986), there are numerous peroxidases that are produced in response to a wound to limit peroxide accumulation (Diehn et al., 1993; Mohan et al., 1993).

Up-regulation of phenylpropanoids

In addition to this peroxide response, there is a general up-regulation of genes encoding the phenyl propanoid pathway. The kinetics of this activation are also extremely rapid, with new mRNAs for these enzymes appearing within 15 minutes (Templeton and Lamb, 1988; Lawton et al., 1983). This up-regulation of the phenyl propanoid pathway genes may be regulated by the H_2O_2 burst because the direct addition of H_2O_2 to bean suspension cells induced the accumulation of mRNAs encoding phenylalanine ammonia lyase, chalcone synthase and chalcone isomerase (Mehdy, 1994). The function of the up-regulation of these genes is to provide the cell with lignin precursors which can reseal the wounded surface and to provide cells with precursors of phenolic plant defensive compounds. .

Inactivation of photosynthetic translation

The second phase of the wound response particularly in monocots, is a turn-off of photosynthetic protein translation by arresting the translation of nuclear encoded photosynthetic genes (Criqui et al., 1992; Reinbothe et al., 1993c). Because maintenance of the photosynthetic apparatus represent a major expenditure of cellular energy, repressing the synthesis of new proteins would save energy for the plant following the wound. Among these down-regulated proteins are those for the small subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (SSU, rbcS gene product) and several light harvesting chlorophyll protein complex apoproteins (LHCPs, cab gene products). However, the changes in protein synthesis do not correspond to equivalent changes in the rbcS and cab transcript levels. Rather, these mRNAs are shifted to smaller polysomes in methyl jasmonate-exposed leaf tissues (Reinbothe et al., 1993a). Control mRNAs encoding leucyl-tRNA synthetase (LRS1, lrs1 gene product) neither changed its abundance nor its association with polysomes in methyl jasmonate-treated leaves and was translated into the corresponding polypeptide.

Several mechanisms are responsible for this altered regulation of the photosynthetic machinery. First, methyl jasmonate induces a shift in the 5' untranslated region of the *rbcl* transcript (Reinbothe et al., 1993b). The primary transcript is initiated at -316 from the translation start codon. Under normal conditions, the 5' end of the mature *rbcl* transcript is processed to yield a mRNA with a 59 bp 5' untranslated region. Following jasmonate treatment, the mRNA is alternatively processed to give a 94 bp untranslated region. This alternatively spliced transcript contains within the 5' untranslated region, a 35-base motif that has high complementarity to the 3' terminus of the 16S rRNA. This portion of the 16S rRNA is involved in intramolecular base pairings within the ribosome and can associate with 30S but not with 70S complexes. Normal transcripts lacking this 35-base motif are active in terms of translation initiation. However, those transcripts having this sequence interfere with translation initiation by competing for ribosome binding at the Shine-Delgarno sequence of the *rbcl* transcript leading to down regulation of the Large subunit which in turn leads to regulation of the Small subunit.

A second method that plants use to alter protein synthesis in stressed plant tissues involves the expression of ribosome-inactivating proteins. These have also been most highly studied in barley. One of these proteins, previously identified as a 60 kDa jasmonate-induced protein (JIP60), has been shown to cleave polysomes into ribosomal subunits (Chaudhry et al., 1994; Reinbothe et al., 1994).

Finally, chaperonins that interact with ribulose bis phosphate carboxylase/oxygenase are also strongly repressed following wounding (Zabaleta et al., 1994), thereby further indicating the role of wounding on inhibition of photosynthesis.

Induction of Ethylene Biosynthesis

In addition to being developmentally regulated, ethylene is synthesized following wounding. SAM synthase catalyzes the formation of S-adenosylmethionine from methionine and ATP. Ethylene is then formed from S-adenosylmethionine in two steps (Kende, 1989). The first step is catalyzed by the enzyme ACC Synthase and the second step by ACC Oxidase.

These genes usually are developmentally expressed in fruit; however, each of the steps in this pathway is also wound-inducible. This wound induction is apparently self-propagating because these enzymes are also regulated by ethylene itself (O'Donnell et al., 1996). Because these genes rely on the synthesis of ethylene to regulate their wound-inducibility, they are often referred to as ethylene-related genes. Varieties of fruit that produce the highest levels of ethylene also induce higher levels of these ethylene-related genes. Some of these ethylene-related genes have unknown functions (Parsons and Mattoo, 1991).

Induction of plant defenses

A major phase of the wound-response is a generalized activation of plant defenses. Because the majority of microbial infections occur in plants following a wound, plants have developed a variety of biochemical defenses to combat invading pathogens and even small herbivores. The accumulation of phytoalexins after wounding has been a particularly rich area for study. Many different plant species have been shown to activate the synthesis of phytoalexins after a wound or after methyl jasmonate treatment [Methyl jasmonate has a proposed role in the regulation of defense genes. see below]. Phytoalexins are plant synthesized small molecular weight defensive compounds that have biological activity against microorganisms or herbivores. These include phenolic, terpenoid, and alkaloid compounds that are a major component of plant secondary metabolism.

In the recent literature, some of these induced phytoalexins have been shown to include furanocoumarin biosynthesis in *Apium graveolins* leaves (Miksch and Boland, 1996); taxol biosynthesis in *Taxus cuspidata* suspension cultures (Mirjalini and Linden, 1996); momilactone in suspension cultured rice cells (Nojiri et al., 1996) and alkaloid synthesis in *Catharanthus roseus* (Aerts et al., 1996). In these cases, wounding or treatment with jasmonates activates the genes encoding the biosynthetic pathways for these different biochemicals; however, for many of these the individual biochemical steps leading to phytoalexin biosynthesis are not known or have not been examined.

Some secondary metabolites are even effective against large phytophagous insects. Ramputh and Brown (1996) report on the accumulation of the inhibitory neurotransmitter, GABA, following mechanical damage of soybean leaves. These authors also demonstrated that increasing levels of GABA decreased the survival of larvae and increased the length of time that larvae required to pupate.

Leaf damage by herbivores in *Nicotiana sylvestris* produces a damage signal that dramatically increases *de novo* nicotine synthesis in the roots. The increased synthesis leads to increases in nicotine pools, which is then transported up the plant. This results in increased nicotine pools throughout the plant making plants more resistant to further herbivore attack (Baldwin et al., 1994).

In addition to the accumulation of the small molecular weight phytoalexins, plants also activate the synthesis of proteins following a wound. Many of these wound-inducible proteins are directly active against the growth of herbivores and microorganisms. Among these are the serine proteinase inhibitors (Ryan, 1981), -amylase inhibitors (Ishimoto and Chrispeels, 1996), chitinases (Brogliè et al., 1991), -glucanases (Mauch et al., 1988), osmotin (Grosset et al., 1990) lectins (Casalagué and Pont Lezica, 1985) and others. Each of these enzymes or inhibitors performs a specific function in combating the invading herbivore or pathogen.

The serine proteinase inhibitors and the α -amylase inhibitors are particularly effective against insects. These proteins block the digestive processes that liberate free amino acids or glucose in an insect's digestive tract. By blocking these processes, the plant limits the nutrition that an insect can glean from the tissue it eats. While these processes may be rather ineffective against single insects that may move from plant to plant, they very effectively reduce the fecundity of developing larvae that grow and develop on a single plant. It should also be pointed out that while plants have many serine proteinase inhibitors, the presence of serine proteinases in plants is rare (Ryan, 1981). Thus, plants apparently lack the specific target enzymes of these inhibitors. These enzymes are however very rich in the digestive tract of insects, and this has led to the conclusion that these inhibitors are targeted against insects.

Chitinases and α -1,3-glucanases are other defensive enzymes that have no natural target in plants. Chitin does not exist in plants and α -1,3-glucans are not major components of plant cells. Chitin and α -1,3-glucans are; however, extensively found in the cell walls of fungi. Thus, these defensive compounds are apparently directed against invading yeast and fungal microorganisms. The expression of these enzymes limits the growth and development of these microorganisms, especially during spore germination.

Additional defensive proteins that accumulate in plants following a wound target other specific features of microorganisms or herbivores to limit their growth and development. Note that antibacterial responses and antiviral responses apparently require specific interactions with surface or intracellular receptors in plants that activate the hypersensitive responses (Ritter and Dangl, 1996; Reuber and Ausubel, 1996). These responses are mediated by different signal transduction pathways than the classical wound-induction pathways and in general do not cross communicate. Recently, however, studies on the overexpression of small GTP binding proteins have demonstrated that altered regulation of these G-proteins can lead to cross-signaling between these two pathways (Sano et al., 1994; Sano and Ohashi, 1995).

Induction of storage proteins

In plant families, vegetative storage proteins accumulate in leaves prior to anthesis, decline during pod filling and then accumulate again after seed maturation (Staswick, 1989). In woody species such as poplar trees, a similar set of proteins termed bark storage proteins accumulate in the autumn months in the protein storage vacuoles of the inner bark parenchyma and xylem ray cells (Coleman et al., 1994). These proteins are remobilized during the spring bud burst when active growth dictates a need for nitrogen. This pattern of expression is consistent with the role of these storage proteins as a temporary sink for nitrogen in the growing tissues.

In addition to this developmental mode of gene regulation, these proteins are also induced by wounding and by jasmonates (Staswick et al., 1991; Mason et al.,

1992). While the teleological reason for induction of these storage proteins following a wound is unclear, perhaps, these storage proteins serve to temporarily store nitrogen and carbon following a wound. This storage would help protect from the loss of these metabolites during the wound response. These wound-induced reserves could later serve as a source for new growth after the wound-recovery phase.

Return to normal physiology

The final phase of the wound-response is a recovery phase that returns the plant cell to a normal physiology. This phase is much longer in duration than the earlier phases of the wound-response, generally lasting from days to a week or so after the wound.

Several unique processes occur during this phase. One of these processes includes the uptake of carbohydrates into the wounded tissues. It is known that both extracellular invertases (Sturm and Chrispeels, 1990) and sugar transporters (Truernit et al., 1996) are induced following wounding. The extracellular invertases cleave extracellular sucrose into its component sugars. The sugar transporters then re-internalize the monosaccharides that may have been spilt by the wound. This process thereby limits the free carbohydrate content of the extracellular milieu for any invading microorganisms.

Thus, wounding of plant tissues produces a large scale alteration of plant metabolism that is initiated almost immediately following a wound. Numerous formerly quiescent genes are activated following a wound that mediate this altered metabolism. The changes include sealing the wound at the surface of the cell, limiting photosynthetic translation, induction of hormone biosynthesis, producing secondary metabolites and defense proteins, producing storage proteins, and finally recovery after the wound to return to a normal physiology.

MECHANISM OF WOUND INDUCTION

Because of the wide number of genes that are activated and the very different time frames during which these genes become activated, it is certain that numerous mechanisms are responsible for wound-inducible gene expression in plants. While some of these mechanisms may involve peroxide-induction of gene expression (Levine et al., 1994), or ethylene (O'Donnel et al., 1996) perhaps the best characterized of the wound-inducible genes are the proteinase inhibitor genes of solanaceous plants and the vegetative storage protein genes that are similarly regulated. The remainder of this article will discuss the mechanism of wound-induction of the proteinase inhibitor and vegetative storage protein genes.

SYSTEMIC SIGNAL

One of the most striking characteristics about the wound-inducibility of the proteinase inhibitor genes in solanaceous plants is the fact that local wounding triggers expression of these genes at a distal site. Currently there are two mechanisms that have been proposed to trigger the wound-induced systemic accumulation of these proteinase inhibitor genes. These mechanisms are mediated by either electrical or chemical signals.

Electrical signals

Wildon et al., (1992) have showed that wounding of the cotyledons of a young tomato plant results in a slow moving action potential that propagates away from the site of the wound toward the upper leaves. In all cases, this action potential correlates with the induction of proteinase inhibitor genes. Plants are unique, in that they have symplastic connections that continue throughout the organism. These connections are made by plasmodesmata, and are well suited for electrical signals.

This work has been confirmed (Herde et al., 1995; Stankovic and Davies, 1995) and expanded (Rhodes et al., 1996). Herde et al., (1995) showed that the electrical induction of the proteinase inhibitor genes correlated with alterations of the stomatal aperture. Stankovic and Davies (1995) showed that both electrically stimulated action potentials and flame-induced hydraulic signals could induce high levels proteinase inhibitor mRNA. Rhodes et al., showed that the electrical signals traveled from the wounded cotyledon to distant unwounded leaves along sieve-tubes and companion cells.

While it is clear that such an electrical action potential stimulates the activation of the proteinase inhibitor genes *in planta*, the mechanisms that translate this action potential into a chemical form that activates gene transcription have not been fully elucidated. Recently, Herde et al., (1995) have shown that electrical current and localized heating induce the accumulation of ABA and jasmonate in wild type plants to levels that approach that of wounding. They also demonstrate that ABA deficient plants are able to synthesize jasmonate in response to heat, but not in response to wounding. While the mechanism of electrical signal transduction is unknown, there have been several ion channels identified in plants (Maathuis and Sanders, 1995; Lurin et al., 1996) that could possibly participate in this process. Additionally, one of the inhibitors of wound-inducible gene expression, acetylsalicylic acid, is known to disrupt H^+/K^+ transporters at the plasma membrane (Glass and Dunlop, 1974). Also an induced oxidative stress has been shown to be the result of electrical pulses in maize plants (Sabri et al., 1996). It is also not clear whether the electrical stimulation of proteinase inhibitor gene induction is capable of inducing the wide a variety of genes that wounding induces.

Systemin

One of the most intriguing recent findings in the area of plant biochemistry is the finding that polypeptide signals may function in activation of plant defense genes such as proteinase inhibitors in animal cells (Berger et al., 1996). These studies were initiated by the original finding that a polypeptide from tomato leaves at very low concentrations was capable of initiating the signal transduction cascade leading to the expression of proteinase inhibitor genes in the absence of a wound (Pearce et al., 1991). A synthetic polypeptide identical to the one purified from plants was also active in proteinase inhibitor gene induction. Further this synthetic polypeptide was readily mobile in the phloem, as opposed to oligosaccharide signals (Baydoun and Fry, 1985).

The cDNA and gene encoding the signaling molecule, systemin, have been isolated and characterized (McGurl et al., 1992; McGurl and Ryan, 1992). The signaling molecule, systemin, is synthesized from a 200 amino acid pro-protein termed prosystemin that is encoded in 11 exons. The mRNA is found throughout the tomato plants with the exception of the roots. Its expression was also wound-inducible in leaves indicating that its expression provides a self-amplification of the wound signal.

Systemin must be proteolytically processed to release the active systemin peptide. Recently, Gu et al., (1996) reported on the wound induction of a leucine aminopeptidase that accumulates in tomato leaves. These authors speculate that this amino peptidase activity may be important for plant-defense response possibly by processing of prosystemin to systemin.

A correlation of the activity of the systemin polypeptide with its structure has been examined (Pearce et al., 1993). Alanine scanning mutations revealed two regions required for activity: the first at Pro¹³ and the other at Thr¹⁷ near the carboxyl terminus of the peptide. Modifications at or near the carboxyl terminus were especially effective in reducing the activity of the polypeptide although these modified systemins could compete with the native systemin interactions with its receptor.

Alteration of systemin expression has been examined in transgenic tomato plants. Plants transformed with an antisense copy of prosystemin cDNA showed a dramatic suppression of proteinase inhibitor expression in the leaves of the transgenic plants (McGurl et al., 1992). An over expression of prosystemin cDNA in tomato plants resulted in a constitutive expression of proteinase inhibitor proteins in leaves (McGurl et al., 1994). These plants were still wound-inducible, expressing high levels of proteinase inhibitors both locally and systemically following wounding. Systemin also is capable of inducing other plant defensive proteins including polyphenol oxidase (Constabel et al., 1995), indicating that systemin has a role in signaling plant defensive genes other than proteinase inhibitors. In this same study, these authors also grafted non-transformed, wild type scions onto the transgenic root stock and demonstrated elevated levels of proteinase inhibitors in the non-transformed scions. These studies demonstrated

that a signal could be transmitted from root stock transformed with the prosystemin cDNA through a graft junction to non-transformed leaves in the absence of wounding.

To further investigate this systemic mobility of the systemin polypeptide, Narvaez-Vasquez et al., (1994) have used p-chloromecuribenzene sulfonic acid (PCMBs), an inhibitor of active apoplastic phloem loading. PCMBs was shown to be a powerful inhibitor of wound-induced and systemin-induced activation of proteinase inhibitor synthesis tomato leaves. When placed on fresh wounds, PCMBs severely inhibited systemic induction of proteinase inhibitors, in both the presence and absence of exogenous systemin. This process could be reversed by addition of various sulfhydryl compounds.

Localized interactions

Once the long distance systemic signal reaches its local site of action, that signal (whether electrical or chemical) must be transduced to the nucleus of the cell where gene transcription occurs. Electrical signals are known to open ion channels in cells that could lead to a transducing chemical signal; but, the involvement of such ion channels have not been demonstrated with any of the chemical signals known to induce wound inducible genes. Typically, chemical signals interact with a cell surface receptor that then transmits chemical energy across the membrane to the cytoplasm.

Because of the variety and chemical diversity of the signals that are known to activate wound-inducible genes [polyanionic, plant cell wall fragments, (Bishop et al., 1981, 1984); polycationic, fungal cell wall fragments (Walker-Simmons and Ryan, 1984); and the polypeptide, systemin (Pearce et al., 1991)] there should be numerous cell surface receptors. However to date, no cell surface receptor has been identified. There are; however, intriguing findings that imply the existence of such receptors. For example, elicitation of *Eschscholtzia* cell cultures (Blechert et al., 1995) or tomato cells (Felix et al., 1993) leads to a rapid alkalinization of the growth medium, possibly implying the involvement of membrane transport or ion movement. This alkalinization of the medium occurred prior to jasmonate formation and inhibition of this alkalinization process by the protein kinase inhibitor staurosporine also inhibited jasmonate formation (Blechert et al., 1995).

In addition, the interaction of oligosaccharide elicitors with cells leads to several alterations in the plasma membrane. It is known that wounded plant cells have increased membrane fragility (Walker-Simmons et al., 1984) perhaps due to phospholipase action. Further, elicitor treatment of cells lead to the phosphorylation of various plant plasma membrane proteins in both potato and tomato (Farmer et al., 1989; Felix et al., 1993) In tomato both a 34 kDa and 29 kDa proteins were phosphorylated, but in potato only a 34 kDa phosphoprotein was detected. In contrast to this, the elicitation with systemin resulted in the hyperphosphorylation

of a 27 kDa protein. These studies indicate that protein kinases may play an important role in the mechanism of signal transduction leading to defense gene expression. Indeed, Bögre et al., (1997) have recently reported that the MMK4 MAP kinase is activated within one minute of wounding. This kinase shows maximal activity by 5 minutes after wounding and then activity disappears by 40 minutes after wounding. The specific role of this or other kinases in wound-induction is unknown, however, protein kinase inhibitors such as staurosporine can block the synthesis of jasmonates which are intermediates in the signal transduction pathway (Blechert et al., 1995).

Recent evidence provided by Damman et al., (1997) demonstrate that an okadiac acid sensitive protein phosphatase is involved in jasmonate induced signal transduction in leaves; however, jasmonate induced gene activation in roots does not require this protein phosphatase to activate gene transcription in roots. Thus, multiple pathways of signal transduction occur in different tissues.

Oxylipins

As mentioned earlier, jasmonic acid and its methyl ester, methyl jasmonate, are active in inducing the accumulation numerous wound-inducible genes in plants. Northern analysis of methyl jasmonate-induced inhibitors I and II mRNAs in tomato leaves, and of alfalfa trypsin inhibitor mRNA in alfalfa leaves, indicated that nascent inhibitor mRNAs were transcriptionally regulated in a manner similar to wounding (Farmer and Ryan, 1990). Further, this induction was systemic (Farmer et al., 1992).

After jasmonates were identified as potential mediators of the wound-response, numerous investigators examined the levels of jasmonates in wounded plants. Creelman et al., (1992) used isotopically labeled standards to demonstrate that wounded soybean stems rapidly accumulated jasmonic acid and methyl jasmonate. Albrecht et al., (1993), used an ELISA to show that levels of jasmonic acid rose immediately and transiently in leaves as a consequence of wounding. The rapid, but transient, synthesis of *cis*-jasmonic acid was demonstrated after insect attack and by microbial elicitor in plant suspension cultures (Blechert et al., 1995). Leaf damage in *Nicotiana sylvestris* rapidly causes the level of shoot jasmonic acid pools to rise rapidly (<0.5 hr). Root jasmonic acid pools also rise in response to leaf damage, but more slowly (<2 hrs). The levels of jasmonic acid remain elevated for 24 hrs in shoots and 10 hrs in roots (Baldwin et al., 1994).

The pathways of jasmonic acid biosynthesis

The synthesis of jasmonic acid requires that starting products be liberated from membrane phospholipids. Ryu and Wang, (1996) have demonstrated that phospholipase D is rapidly activated by wounding in the leaves of castor bean result-

ing in an accumulation of phosphatidic acid and free choline throughout the leaf. New synthesis of phospholipase D mRNA was not observed following a wound, but rather, the wound-activation of the phospholipase resulted from intracellular translocation of the protein from the cytosol to membranes. Conconi et al., (1996) have found that the levels of linolenic acid (18:3) and linoleic acid (18:2) increased within 1 hour of a wound. Presumably this is due to phospholipase A₁ or A₂ activity; although induction of these activities following a wound has not been demonstrated. After 1 hour, they found a 15-fold excess of 18:3 over that required to account for the levels of newly synthesized jasmonic acid.

The intracellular location of jasmonate biosynthesis is thought to be the chloroplast envelope membranes (Blée and Joyard, 1996). It is currently unclear whether the free fatty acids are liberated from chloroplast phospholipids or from other membranes and are transported to the chloroplast via lipid transfer proteins.

The conversion of free 18:3 fatty acids into jasmonic acid occurs in five steps through an oxidative pathway. The intermediates are termed oxylipins. Initially, lipoxygenase catalyzes the incorporation of molecular O₂ into certain polyunsaturated fatty acids having a cis, cis 1,4-pentadiene system to form a fatty acid hydroperoxide. Typically in plants, there are numerous lipoxygenases and only some isoforms of these enzymes are themselves wound-inducible (Royo et al., 1996). Thus, like many of the wound-inducible target genes, those genes which participate in the activation process are also wound-inducible. In addition, many of these lipoxygenases are induced by a variety of biochemical components such as fungal elicitor, plant and fungal cell wall oligosaccharides, and methyl jasmonate (Bohland et al., 1997). In *Arabidopsis*, the lipoxygenase involved in jasmonate biosynthesis is LOX2 (Bell et al., 1995). Cosuppression of LOX2 in transgenic plants leads to reduced levels of jasmonate biosynthesis as well as reduced levels of wound-inducible gene expression. The *Arabidopsis* lipoxygenase LOX2 that is involved in jasmonate biosynthesis is chloroplastic (Bell et al., 1995).

Following the formation of 13-hydroperoxylinolenic acid, the enzyme allene oxide synthase forms an epoxide intermediate termed allene oxide. The flax allene oxide synthase contains a 58 amino acid chloroplast transit peptide (Harms et al., 1995). These same authors constitutively overexpressed the flax allene oxide synthase cDNA in transgenic potato plants. This expression led to an increase in the endogenous level of jasmonic acid within the plants. However, despite the fact that the transgenic plants had levels of jasmonates similar to those found in nontransgenic wounded plants, the wound-inducible *pin2* genes were not constitutively expressed in the leaves of these plants (Harms et al., 1995). The reason for this lack of expression is not clear, but perhaps compartmentalization of the signaling factors is involved.

Following the formation of allene oxide, a cyclooxygenase acts to form 12-oxo-phosphodienoic acid (12-oxo-PDA). Originally the substrate for this enzymatic step was thought to be the 13-hydroperoxylinolenic acid (Vick et al., 1980); but, Harms et al., (1995) indicate that allene oxide may be the substrate for the cyclization. The ring double bond of the 12-oxo-PDA is then reduced by a NADP⁺ utilizing enzyme to form 12-oxo-PMA. This is the rate limiting step of jasmonate biosynthesis (Vick and Zimmerman, 1986). Utilization of NADP⁺ is consistent with the localization of these enzymes in the chloroplast. Finally jasmonic acid is synthesized from the 12-oxo-PMA by three rounds of β -oxidation. It is not clear whether a novel fatty acid oxidase functions in the synthesis of jasmonic acid or even whether Coenzyme A derivatives or acyl carrier proteins are involved.

It has also been proposed that a second oxylipin cascade exists in plants starting from linoleic acid via 15,16-dihydro-12-oxo-phytodienoic acid to 9,10-dihydrojasmonate (Blechert et al., 1995). Recently, the cDNA encoding allene oxide synthase has also been isolated from *Arabidopsis thaliana* (Laudert et al., 1996). After expression of this enzyme in *E. coli*, the protein was enzymatically active with substrates derived from either linolenic acid or linoleic acid, verifying that there are indeed duplicate pathways to the synthesis of jasmonic acid and dihydrojasmonic acid.

In addition, to the synthesis of jasmonates, a wide variety of other oxylipin products from *n*-hexenal to ketols to traumatic acid are also derived from these same intermediates (Avudiusenko et al., 1995; Blée and Joyard, 1996). Whether these intermediates also have gene regulatory activity will require further examination. It is known; however, that *n*-hexenal accumulates in the volatile head-gas of wounded plants and there has been speculation that this may be involved in rejection of plants by insects (Röse et al., 1996).

Inhibitors of oxylipin metabolism

Numerous inhibitors of the expression of wound-inducible genes have been reported. By far the majority of these inhibitors support occur in the oxylipin pathway. Inhibitors of lipoxygenases that inhibit wound inducible gene expression include phenidone (Farmer et al., 1994), SHAM and ZK139817 (Peña-Cortés et al., 1993). Propyl gallate and piroxicam (Peña-Cortés et al., 1993) and salicylic acid (Doherty et al., 1988; Peña-Cortez et al., 1993; Doares et al., 1995) are inhibitors of hydroperoxide dehydrase (cyclooxygenase). Numerous studies involving salicylic acid have demonstrated that this compound blocks activation of proteinase inhibitor genes by electrical signals (Doherty et al., 1988), oligouronide induction, systemin induction, and linolenate induction (Doares et al., 1995) as well as transcription of the genes encoding proteinase inhibitor II, cathepsin D inhibitor, and threonine deaminase (Peña-Cortés et al., 1993).

Metabolism of jasmonates

The synthesis of jasmonates is a relatively transient response. Usually, jasmonate levels decline rapidly following the burst of synthesis (Albrecht et al., 1993; Bleichert et al., 1995; Conconi et al., 1996); yet many plant responses remain activated for many hours. In an attempt to explain this phenomenon, Krumm et al., (1995) have investigated the role of amino acid conjugation of jasmonates. These authors have prepared many jasmonate-amino acid conjugates. They have shown that many of these amino acid conjugates are inactive, however conjugates of leucine and isoleucine retain their activity. These authors speculate that these active conjugates may function in the long-term maintenance of jasmonate-mediated signaling in plants.

Of the four possible stereoisomers of jasmonic acid growth inhibitory activity was associated with both of the 1R stereoisomers, however there was no observed difference between the inhibition of straight growth of oat coleoptiles indicating that there may be multiple receptors mediating jasmonate activities (Koda et al., 1992). Further, stereochemically-locked *cis*- and *trans*-7-methyl derivatives of methyl jasmonate have low biological activity suggesting that the introduction of the locking methyl group at position 7 considerably lowers affinity for the jasmonate receptor, presumably owing to a steric effect (Koda et al., 1995).

Mutant in jasmonic acid synthesis and action

An ethylmethanesulfonate mutant (*jar1*) of *Arabidopsis thaliana* has been isolated that showed decreased sensitivity to methyl jasmonate inhibition of root elongation (Staswick et al., 1992). The jasmonate-inducibility of leaf proteins was 4-fold less in the *jar1* mutants than in the wild type *Arabidopsis* plants.

Signaling mutants have also been prepared in tomato (Lightner et al., 1993). These mutants, JL1 and JL5, were blocked in the induction of proteinase inhibitor genes. These mutants were deficient in the systemic-induction of both Proteinase Inhibitor I and II; however, these mutants showed some localized induction of proteinase inhibitors. These results were interpreted as suggesting that multiple signaling pathways (one systemic and another local existed in response to wounding). Further, these mutants were fully responsive to the addition of methyl jasmonate, indicating that the lesion in these mutants was located somewhere upstream of the final step in jasmonate biosynthesis. Recently, Howe et al., (1996) demonstrated that the JL5 mutant are affected in octadecanoid metabolism between the synthesis of hydroperoxylinolenic acid and 12-oxo-phytodienoic acid.

Other mutants have been selected using coronatine. Coronatine is a chlorosis-inducing phytotoxin produced by several pathovars of *Pseudomonas syringae*. In tomato, coronatine induces the accumulation of proteinase inhibitors (Palmer and Bender, 1995), but they are not protective against the *Pseudomonas* patho-

gen. Treatment of *Arabidopsis* plants with coronatine leads to inhibited root growth, anthocyanin accumulation and the induction of two proteins of 31 and 29 kDa (Feys et al., 1994). Similar responses are induced in response to jasmonates. *Arabidopsis* mutants have been isolated that are resistant to this phytotoxin (Feys et al., 1994) and these mutants are also insensitive to methyl jasmonate inhibition of root growth. These *coi1* mutants were all male sterile, producing abnormal pollen and had reduced levels of the 31 kDa protein. These authors conclude that the *coi1* protein controls jasmonate perception or response and also participates in flower development.

Jasmonates affect transcription

After jasmonates are synthesized, it is unclear how the biological activity of these compounds are transmitted to the promoters of the various genes that they activate. However, there is a recent report of a jasmonate binding protein that mediates the wound inducible regulation of transcription of the potato proteinase inhibitor 2 gene (Gurevich et al., 1996). In this work, a fragment of the *pin2* gene was isolated by PCR and used as an affinity sorbent. Nuclear proteins were bound and the sorbent was eluted with physiological concentrations of jasmonate. Four proteins were isolated by this procedure. The characterization of these proteins will require further studies.

Another factor that affects proteinase inhibitor expression downstream of jasmonates was discovered by Schaller et al., (1995). These authors found that an inhibitor of some aminopeptidases, bestatin, was able to induce proteinase inhibitor genes without affecting systemin, octadecanoids, or jasmonate. Furthermore, defense genes were induced by bestatin in the JL5 mutant tomato line that has a defect in the octadecanoid pathway. Thus, bestatin appears to function close to the level of transcription of wound-inducible genes. These authors speculate that a regulatory protease may be involved.

INVOLVEMENT OF ADDITIONAL HORMONE FACTORS

ABA

There is significant evidence that the initial stages of wound induction require the initial biosynthesis of abscisic acid prior to transcription of wound-inducible genes (Peña-Cortés et al., 1989, 1991; Hildmann et al., 1992). These studies demonstrate that exogenous application of abscisic acid induces a systemic pattern of Proteinase Inhibitor II mRNA accumulation that is identical to mechanical wounding. Numerous other wound-inducible genes are known to also be induced by ABA (see Table 1). These same authors also demonstrated that ABA-deficient plants do not respond to wounding unless ABA is supplied exogenously. There is also an increase in ABA in the leaves of tomato, potato and tobacco plants follow-

ing a wound (Sanchez-Serrano et al., 1991). In contrast to this, no increase in ABA was observed in leaves incubated with jasmonic acid, suggesting that jasmonates act after abscisic acid (Hildmann et al., 1992). Recently, Peña-Cortés et al., (1995) have shown that either electrical signals or systemin leads to an increase in ABA which in turn leads to an increase in jasmonic acid which then regulates gene transcription. According to this hypothesis, all jasmonate regulated genes should also be ABA regulated. Lee et al., (1996); however, have identified four genes by differential display which are regulated by jasmonate but are not regulated by ABA indicating that the signaling pathways for ABA and jasmonates function independently and not sequentially.

Specific roles for ABA have been proposed. ABA might lead to the activation of a lipoxygenase that generates hydroperoxides from free fatty acids within the cell (Peña-Cortés et al., 1995). Further evidence to support this hypothesis is provided by Abián et al., (1991), who demonstrated alterations in oxylipin metabolism in maize embryos in response to ABA. It is also known that water-stress also causes accumulation of ABA and activates a set of water-stress genes; however it does not induce wound-inducible genes (Hildmann et al., 1992). Thus different signal transduction mechanisms must regulate the ABA induction of these different sets of genes.

Mutants affecting ABA induction

Because ABA has been identified as a factor involved in the activation of proteinase inhibitor gene activation following wounding, there have been several investigations examining wounding in ABA deficient plants (Peña-Cortés et al., 1989, 1991). Several different ABA deficient plant lines have been used to evaluate the involvement of ABA in wound-inducible gene expression. The tomato mutants used for these studies are *flaca* and *sitiens* and the potato mutant is *droopy*. In all of these plants, proteinase inhibitor genes are not expressed unless abscisic acid is added. However, care should be taken in interpretations of the data derived from these hormone deficient plants, because they are often pleiotrophic mutations. For example the tomato mutant, *flaca*, is known to have elevated levels of IAA in addition to reduced levels of ABA (Tal and Imber, 1970). Further, there are numerous examples in the literature that exogenous application of ABA to plant tissues can cause alterations in endogenous levels of IAA within those tissues (Chang and Jacobs, 1973; Anker, 1975; Wodzicki and Wodzicki, 1981; Terek, 1982; Pilet and Rebeaud, 1983; Dunlap and Robacker, 1990).

Ethylene

As mentioned above, ethylene is synthesized following a wound and many wound-inducible genes are also responsive to ethylene. Recently, O'Donnell et al., (1996) have demonstrated that ethylene is absolutely required for wound-induction of

the proteinase inhibitor genes of tomato. These authors use norbornadiene, which is an inhibitor of ethylene synthesis (Sisler et al., 1990), and silver thiosulphate, which disrupts binding of ethylene to its receptor (Veen, 1987), to demonstrate that both jasmonic acid as well as ethylene are required for proteinase inhibitor gene expression. They propose that both ethylene and jasmonates are co-stimulatory for the other hormone, that is, after wounding the synthesis of ethylene induces higher jasmonate levels, and endogenous jasmonates induce higher ethylene levels. In this way, a sufficient amount of these hormones accumulate to regulate the wound-process (O'Donnell et al., 1996).

Additional studies in support of this hypothesis come from the study of a tomato ethylene mutant, termed *Never-ripe* (NR), which have a partial loss of ethylene sensitivity (Yen et al., 1995). In these plants, the wound-induced accumulation of proteinase inhibitor transcripts is significantly delayed. Also, transgenic tomato plants expressing an antisense ACC oxidase do not accumulate proteinase inhibitor transcripts in response to wounding. Thus, these studies also suggest that ethylene is required for wound-inducible gene expression of the proteinase inhibitor genes.

Auxin

Auxin has also been demonstrated to prevent expression of wound-inducible proteinase inhibitors (Kernan and Thornburg, 1989). This inhibition of expression occurs both in tissue cultured cells as well as in whole plants. It was specific for biologically active auxins and occurred at near physiological IAA concentrations. Auxin inhibition of gene expression has been demonstrated for a number of wound-inducible genes (see Table 1). Auxin also inhibits other chemical inducers. Auxin has also been shown to strongly inhibited methyl jasmonate-induced wound-inducible gene expression in soybean suspension-cultured cells (DeWald et al., 1994) and the expression of β -glucanase in response to fungal elicitor in tobacco and soybean cells (Jouanneau et al., 1991).

Thornburg and Li (1991) have also demonstrated that IAA in bulk leaf tissues declines by two to three fold following a wound and that the kinetics of IAA decline inversely correlate with the induction of wound-inducible gene expression.

Other cellular machinery required for induction of wound-inducible genes has not been fully elucidated, however, recent work indicates that this is a rich field for study. It is known that small GTP-binding proteins can mediate cross-signaling between the wound- and pathogen-induced signal transduction pathways (Sano et al., 1994; Sano and Ohashi, 1995). More recently, these authors demonstrated that these transgenic plants overexpressing this small GTP bind-

ing protein can synthesize jasmonates more rapidly than control plants. They also provide evidence based upon competition with 2-chloro-4-cyclohexylamino-6-ethylamino-s-triazine (a potent cytokinin antagonist) that cytokinins may be essential for accumulation of wound-inducible proteinase inhibitor transcripts (Sano et al., 1996). Indeed it has been previously suggested that wounding enhances endogenous cytokinin activity in cucumber (Crane and Ross, 1986).

From all of these studies, we can see the involvement of multiple long range signals, both chemical and electrical, multiple short range signals of plant and fungal origin, several signal transduction cascades involving GTP binding proteins, kinases, and phosphatases along with variations in multiple plant hormones, ethylene, cytokinins, auxin, and abscisic acid in addition to the biosynthesis of jasmonates. All of these factors clearly do play a role in the transcriptional activation of wound-inducible genes. It cannot be argued that these factors are coordinated in a vastly complex, well regulated network of responses leading to gene activation. In spite of all that is currently known about the expression of these genes, there is a long way to go before we fully understand wound-inducible gene expression in plants.

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