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## Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals

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**ABSTRACT** The activation of plant defensive genes in leaves of tomato plants in response to herbivore damage or mechanical wounding is mediated by a mobile 18-amino acid polypeptide signal called systemin. Systemin is derived from a larger, 200-amino acid precursor called prosystemin, similar to polypeptide hormones and soluble growth factors in animals. Systemin activates a lipid-based signaling cascade, also analogous to signaling systems found in animals. In plants, linolenic acid is released from membranes and is converted to the oxylipins phytodienoic acid and jasmonic acid through the octadecanoid pathway. Plant oxylipins are structural analogs of animal prostaglandins which are derived from arachidonic acid in response to various signals, including polypeptide factors. Constitutive overexpression of the prosystemin gene in transgenic tomato plants resulted in the overproduction of prosystemin and the abnormal release of systemin, conferring a constitutive overproduction of several systemic wound-response proteins (SWRPs). The data indicate that systemin is a master signal for defense against attacking herbivores. The same defensive proteins induced by wounding are synthesized in response to oligosaccharide elicitors that are generated in leaf cells in response to pathogen attacks. Inhibitors of the octadecanoid pathway, and a mutation that interrupts this pathway, block the induction of SWRPs by wounding, systemin, and oligosaccharide elicitors, indicating that the octadecanoid pathway is essential for the activation of defense genes by all of these signals. The tomato mutant line that is functionally deficient in the octadecanoid pathway is highly susceptible to attacks by *Manduca sexta* larvae. The similarities between the defense signaling pathway in tomato leaves and those of the defense signaling pathways of macrophages and mast cells of animals suggests that both the plant and animal pathways may have evolved from a common ancestral origin.

Plants, for hundreds of millions of years, have been evolving defensive strategies to protect themselves against herbivores and pathogens. As plant species evolved within their ecological niches, they developed their own unique arrays of defensive chemicals against the predators they confronted. Virtually thousands of chemicals are present among living plant species that are thought to be defense-related. The advantages of inducible defensive chemicals to plants is not totally clear, but it can be speculated that many plant species have evolved in very hostile environments in which their nutrients were limited, and the need to conserve energy in diverse ecological systems was essential. The inducible defenses have been of particular interest to biochemists and molecular and cell biologists because of the challenges to understand their complex signaling systems, and because of their potential for

genetically regulating defensive genes to improve plant productivity.

Defensive chemicals are synthesized in many plant species in response to damage inflicted by attacking herbivores (1, 2). In tomato plants, a signal (or signals) originates at the wound site that is transported throughout the plant where it activates the synthesis of defensive proteins that interfere with the digestive systems of the attacking herbivores (Fig. 1). These inducible defensive proteins have been identified as serine, cysteine and aspartyl proteinase inhibitors (2–4) and polyphenol oxidase (5). These proteins interact with the proteins and proteinases of herbivore guts and adversely affect proteolysis of the ingested food, reducing the availability of essential amino acids and retarding the growth and development of the herbivores (2, 5). The net effect of this process in a natural ecosystem is likely to result in a reduction of damage to the plant, either by killing the predator or by providing a longer period of exposure of surviving herbivores to their natural predators. Research on inducible plant defenses has been primarily concerned with defense against insect predators, but more recently, proteinase inhibitors in sedges and grasses have been found to be a major factor in regulating fluctuating populations of lemmings (6).

### Systemin: A Mobile Signal for Plant Defense Against Herbivores

The initial search for the systemic signal in tomato plants resulted in the finding that oligogalacturonides derived from the plant cell wall were inducers of the defensive proteinase inhibitor genes in excised tomato leaves (7). Subsequently, chitin and chitosan oligomers derived from fungal cell walls were also found to be active inducers (8). However, both classes of oligosaccharides were active only at relatively high concentrations (several hundred micrograms per plant) and did not move to distal leaves when placed on leaf wounds (9). The oligosaccharides are therefore considered to be among the signals produced at sites of pathogen attacks where they help produce localized defensive chemicals. A renewed search for the systemic signal in the soluble components of tomato leaves resulted in the isolation of an 18-amino acid polypeptide called systemin (Fig. 2) (10), that was nearly 1 million times more powerful than the oligosaccharides in inducing the synthesis of defensive genes in excised tomato leaves (11). Radiolabeled systemin, when applied to a fresh wound on a tomato leaf, is transported into the apoplast and xylem elements within 30 min, loaded into the phloem, and transported out into the upper, unwounded leaves of the plant within 60–90 min (11, 12). Full-length systemin was identified in phloem exudates from plants at various times following wounding (11). The mobility of systemin, together with its powerful inducing

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Abbreviation: SWRP, systemic wound-response protein.  
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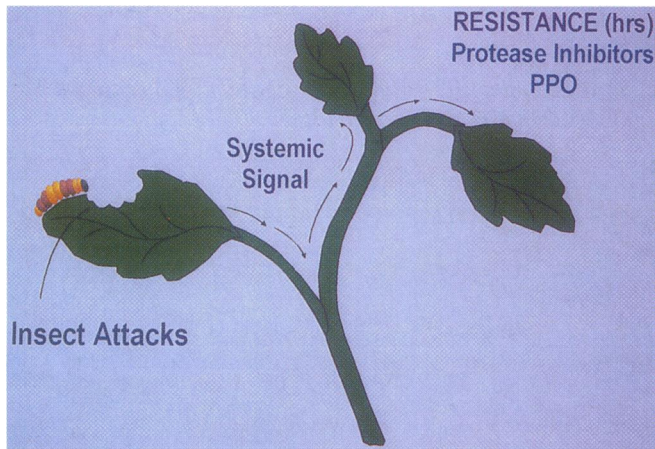
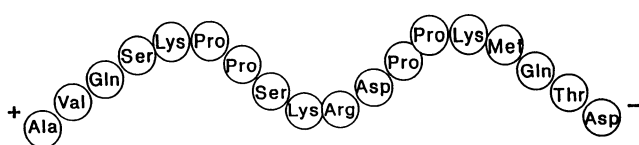


FIG. 1. Illustration demonstrating the systemic induction of defensive proteins in tomato leaves in response to insect attacks that is manifested within hours. PPO, polyphenol oxidase.

activity, made it a leading candidate for a systemic wound signal, and the first example of a polypeptide signal in plants.

Polypeptide hormones are a common feature of signaling systems in animals and yeast, and hundreds of polypeptide hormones and growth factors are known in higher animals (13, 14). Polypeptide hormones are nearly always derived from larger precursors and proteolytically processed in secretory vesicles by members of a family of site-specific subtilisin-related proteinases (13). Systemin was found to be derived from a larger precursor, a proprotein of 200 amino acids called prosystemin (Fig. 3) (15), but this prohormone does not exhibit the typical animal processing sites. The only potential processing site in prosystemin for the animal convertases is within the systemin polypeptide. This site does not exactly match the typical animal recognition sites, but systemin is cleaved at this site during its interaction with tomato plasma membranes (16). Thus, the processing sites within prosystemin that produce systemin remain to be identified. Additionally, prosystemin does not have an N-terminal signal sequence typical for proteins transported into the endoplasmic reticulum, as found with animal prohormones. While similar to animal and yeast polypeptide hormones in being processed from a prohormone precursor, the modes of synthesis and storage of prosystemin in plants remain to be established.

The functionality of prosystemin was demonstrated with tomato plants transformed with an antisense prosystemin cDNA driven by the constitutive cauliflower mosaic virus promoter (17). These transformed plants were found to be severely impaired in their systemic wound response. Transgenic tomato plants expressing the antisense gene not only accumulated low levels of proteinase inhibitors I and II in leaves of wounded plants, but lost their ability to mount inducible defenses against *Manduca sexta* larvae (17). Larvae feeding on the antisense plants attained weights over twice those of larvae feeding on wild-type plants. These experiments confirmed that prosystemin and systemin play a fundamental



## Systemin

FIG. 2. The amino acid sequence of systemin.

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1  MGTPSYDIKN KGDDMQEEP KVLHHEKGGD EKEKIEKET PSQDINNKDT
51  ISSYVLRDDT QEIPKMEHEE GGYVKEKIVE KETISQYIIK IEGDDDAQEK
101  LKVEYEEEEE EKEKIVEKET PSQDINNKGD DAQEKPKVEH EEGDDKETPS
151  QDIKMEGEG ALEITKVVCE KIIVREDLAV QSKPPSKRDP PKMQTDNNKL
                                SYSTEMIN

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FIG. 3. The amino acid sequence of prosystemin.

role in the systemic signaling pathway for activating defense genes in response to herbivore attacks.

## Systemin Activates Defense Genes Via the Octadecanoid Pathway

As systemin was being isolated and characterized as a powerful elicitor of proteinase inhibitor synthesis in tomato plants, we found that exposure of tomato plants to methyl jasmonate and jasmonic acid also powerfully activated the synthesis of proteinase inhibitors I and II (18, 19). Several years before, a postdoctoral fellow in our laboratory, M. K. Walker-Simmons, had observed that linolenic acid, when applied to the surface of tomato leaves, was a potent activator of proteinase inhibitor synthesis. At that time we had no clue as to the mechanism of its biological activity. In the 1980s, Vick and Zimmerman (20) elucidated the biosynthetic pathway for jasmonic acid, which originates from linolenic acid via a series of steps that included lipoxidation, cyclization, and  $\beta$ -oxidation. Intermediates of the pathway between linolenic acid and jasmonic acid were subsequently found to activate the defensive genes (19), which indicated that the octadecanoid pathway may be involved in

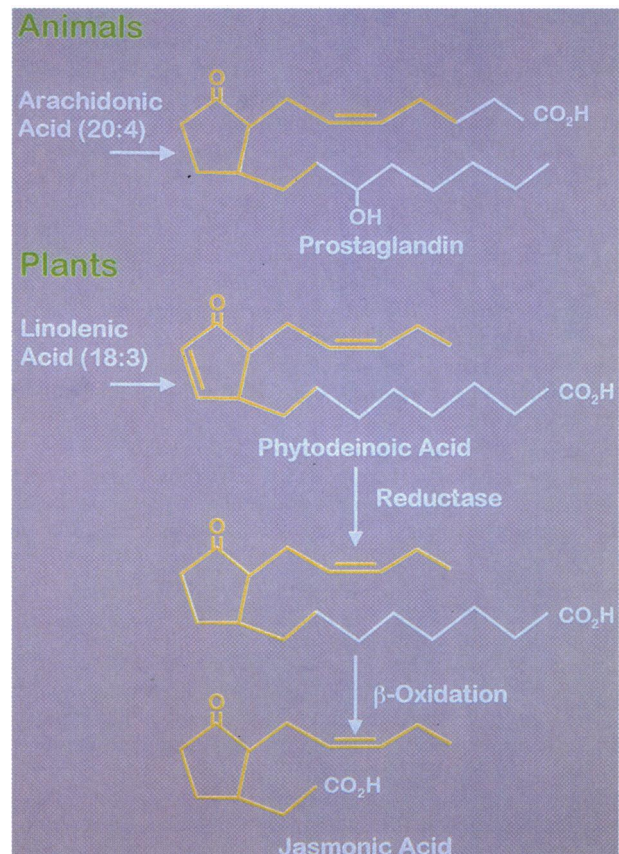


FIG. 4. Comparison of the structures of phytodienoic acid and jasmonic acid, derived from linolenic acid, with the structure of a prostaglandin derived from arachidonic acid. The similar structural features are indicated in yellow.

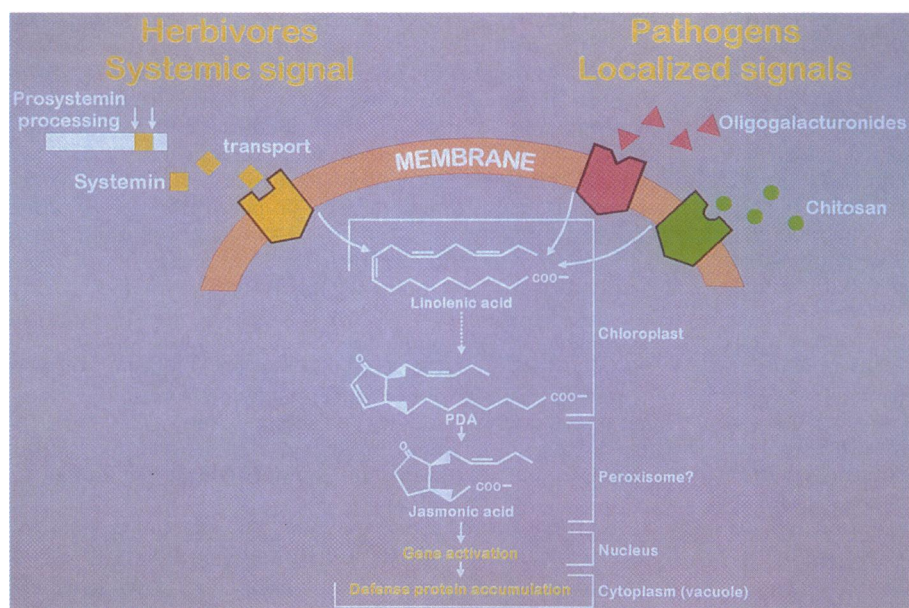


FIG. 5. Proposed model for the activation of defensive genes in tomato plants in response to herbivore and pathogen attacks. Systemin is released in response to wounding by herbivores. Oligouronide fragments from the plant cell wall are produced by extracellular pectin-degrading enzymes from pathogens, whereas chitin and chitosans are fragments of fungal cell walls, produced by the action of plant  $\beta$ -glucanases during pathogen attacks.

the wound-inducible and systemin-inducible signaling pathway for defensive genes. Because of the similarity of the structure of jasmonic acid and its precursor phytodienoic acid to the structures of some prostaglandins (Fig. 4), and from knowledge of animal systems that prostaglandins are derived from arachidonic acid released from membranes by phospholipase  $A_2$  (21), we proposed a model in which wounding and systemin activated a lipase in receptor cell membranes resulting in the release of linolenic acid, the production of jasmonic acid, and the activation of proteinase inhibitor genes (Fig. 5) (19). In this model, oligosaccharides are localized signals, whereas systemin is the systemic signal that activates the defense signaling pathway.

Several lines of evidence have supported this lipid-based pathway in plants, including the identification of diethyldithiocarbamic acid (22) and salicylic acid (23–25) as inhibitors of the octadecanoid pathway, and the identification of a tomato signaling mutant (26) that is deficient in a pathway component. This mutant, now called defenseless 1 (*def1*) (27), only weakly responds to wounding, systemin, and oligosaccharide elicitors, but responds normally to phytodienoic acid and jasmonic acid. As predicted by the model, the mutant produces only low levels of jasmonic acid in response to wounding or systemin. When compared with wild-type tomato plants, the *def1* mutant has lost its ability to defend itself against *M. sexta* larvae (27). The larvae induced only small increases in proteinase inhibitors in the leaves of the *def1* plants (less than 10% of the levels induced in wild-type plants), and after several days of feeding, the average insect weights were two- to three-fold higher than those of larvae feeding on wild-type plants.

#### Systemic Wound Response Proteins

Tomato plants were transformed with a constitutively expressed prosystemin (28) with the expectation that systemin release would be amplified when the plants were wounded. Unexpectedly, the transformed plants exhibited a phenotype in which the defensive genes were constitutively expressed, indicating that the high constitutive expression of prosystemin resulted in an abnormal release of systemin in the absence of wounding. The plants produced the two serine proteinase inhibitor I and II proteins constitutively, and with time, the levels of inhibitors increased to extraordinary levels in older leaves, reaching over 1 mg of the inhibitors per ml of leaf juice, a level 4–5 times those induced in leaves of young plants by wounding. In comparing the electrophoretic protein profiles of

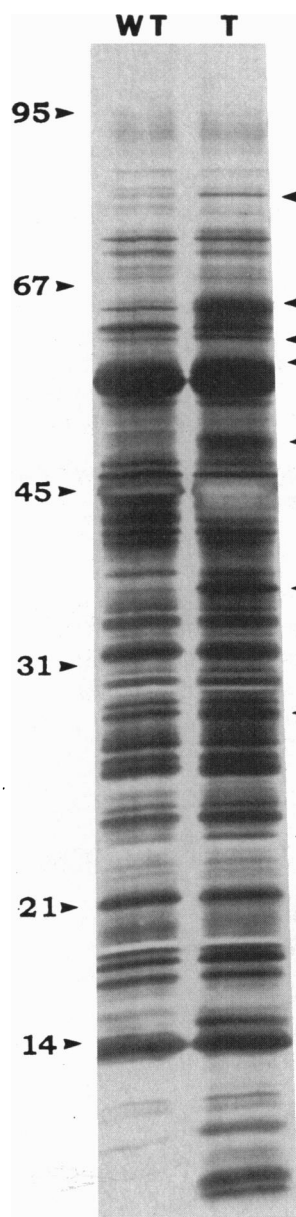


FIG. 6. Electrophoretic profiles of the soluble proteins from leaves of wild-type tomato plants (Left), and transgenic tomato plants transformed with a gene containing a prosystemin cDNA driven by a constitutive cauliflower mosaic virus (CaMV 35S) promoter. The transgenic plants overexpressed prosystemin, resulting in the constitutive synthesis of wound-inducible defensive genes. Several of the overproduced proteins in the transgenic plants are marked with arrowheads.

these plants with wild-type plants (Fig. 6), it was apparent that the prosystemin-overexpressing plants were accumulating not only proteinase inhibitor I and II proteins, but numerous other proteins as well. Several wound-inducible proteins and cDNAs had been reported previously; a carboxypeptidase enzyme (29), a polyphenol oxidase (5), prosystemin (15), an aspartic proteinase (30), an aspartic proteinase inhibitor (3), a novel cysteine proteinase (D.R.B. and C.A.R., unpublished data), a cysteine proteinase inhibitor (3, 4), a leucine aminopeptidase (3, 31), threonine deaminase (3), nucleotide diphosphate kinase (32), and lipoxygenase (T. Heitz and C.A.R., unpublished data).

We began a systematic analysis of the leaf proteins whose accumulation was significantly enhanced in transgenic plants, as compared with the levels of proteins in leaves of wild-type plants (Fig. 7). As a general strategy, individual overexpressed proteins were partially purified and recovered from gels and their N-terminal amino acid sequences were determined. From these sequences, oligonucleotide primers were synthesized for PCR. The amplified DNA fragments were sequenced and used as probes to obtain cDNAs from a library prepared using mRNA from the transgenic plants.

The first of the overexpressed proteins that was isolated and characterized was a polyphenol oxidase (5). Enzymatic analyses also showed that the activity of this enzyme was enhanced by wounding and by systemin (5). Other overexpressed proteins and cDNAs have now been identified and include the previously identified carboxypeptidase, aspartic proteinase inhibitor, cysteine proteinase inhibitor, leucine aminopeptidase, lipoxygenase, and threonine deaminase. We have additionally identified calmodulin, a ubiquitin-like protein, a nucleotide diphosphate kinase, and an acyl CoA-binding protein as well as 85-, 46-, and 38-kDa proteins that are presently being analyzed (cf. Fig. 6). Systemin, therefore, appears to be a "master switch" that activates a host of genes that are involved in reprogramming the protein synthesis machinery in response to wounding and systemin. The gene products (SWRPs), are grouped into four categories in Fig. 7: (i) those that are involved directly in defense against herbivores, including serine, cysteine, and aspartyl proteinase inhibitors and poly-

### Systemic Wound Response Proteins (SWRPs) In Tomato Plants

#### Defensive Proteins

Serine Proteinase Inhibitor I (Inh I)  
Serine Proteinase Inhibitor II (Inh II)  
Cysteine Proteinase Inhibitor (CYS)  
Aspartic Proteinase Inhibitor (CDI)  
Polyphenol Oxidase (PPO)

#### Signal Pathway-Associated

Prosystemin (ProSYS)  
Lipoxygenase (LOX)  
Calmodulin (CAM)  
Nucleotide Diphosphate Kinase (NDPK)  
Acyl CoA-Binding Protein (ACBP)

#### Proteolysis-Associated

Leucine Aminopeptidase (LAP)  
Carboxypeptidase (SerCP)  
Aspartic Proteinase (AspP)  
Cysteine Proteinase (CysP)  
Ubiquitin-like Protein (UBQ-like)

#### Other

Threonine Deaminase (TD)

FIG. 7. Systemic wound-response proteins (SWRPs) systemically induced in tomato leaves by herbivore damage or other severe mechanical wounding.

phenol oxidase; (ii) proteins and enzymes that appear to be associated with the signaling pathway; (iii) several proteolytic enzymes whose specific roles are still unknown but that may be involved in enhancing protein turnover or in processing prosystemin; and (iv) proteins whose identities and possible roles in the wound response remain to be established.

PCR-generated DNA fragments, together with cDNA sequences isolated from the library prepared from leaves of the transgenic plants, were employed to assay wound-inducible and systemin-inducible mRNA levels in leaves of wild-type plants. The mRNAs coding for the SWRPs shown in Fig. 6 are induced by wounding and systemin (Fig. 8). In contrast, messenger RNAs coding for the pathogenesis-related (PR) proteins PR3 and PR3a that are induced during systemic acquired resistance did not accumulate in response to wounding or to systemin (ref. 33, and data not shown).

### Analogy Between Plant and Animal Defense Signaling

Several features of the plant signal transduction system for defense against herbivores are strikingly similar to the host defense signaling systems of animals against pathogens and parasites. Interactions of macrophages with pathogens trigger the release of the polypeptide cytokine tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) that circulates in the blood stream and to nearby cells (Fig. 9) (34). TNF- $\alpha$  can interact with cells in the brain stem to trigger the intracellular activation of phospholipase A<sub>2</sub>,

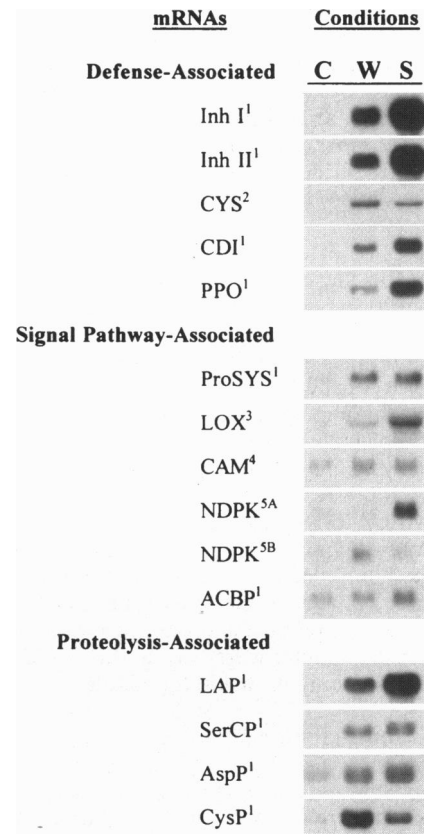


FIG. 8. Northern blots of mRNAs coding for systemic wound responsive proteins in leaves of tomato plants. Lanes: C, unwounded control plants; W, plants wounded on the lower leaf and the upper leaf was assayed for mRNA at the times indicated below; and S, small tomato plants excised and supplied with systemin for 1 h and then water for the following times (indicated in superscript in the mRNA column): 1, 8 h; 2, 14 h; 3, 2 h; 4, 1 h following wounding or 8-h constant supply of systemin; 5A, 8 h; and 5B, 4 h. Messenger RNAs from excised control plants at the times indicated did not exhibit levels over those found in intact unwounded control plants (data not shown).

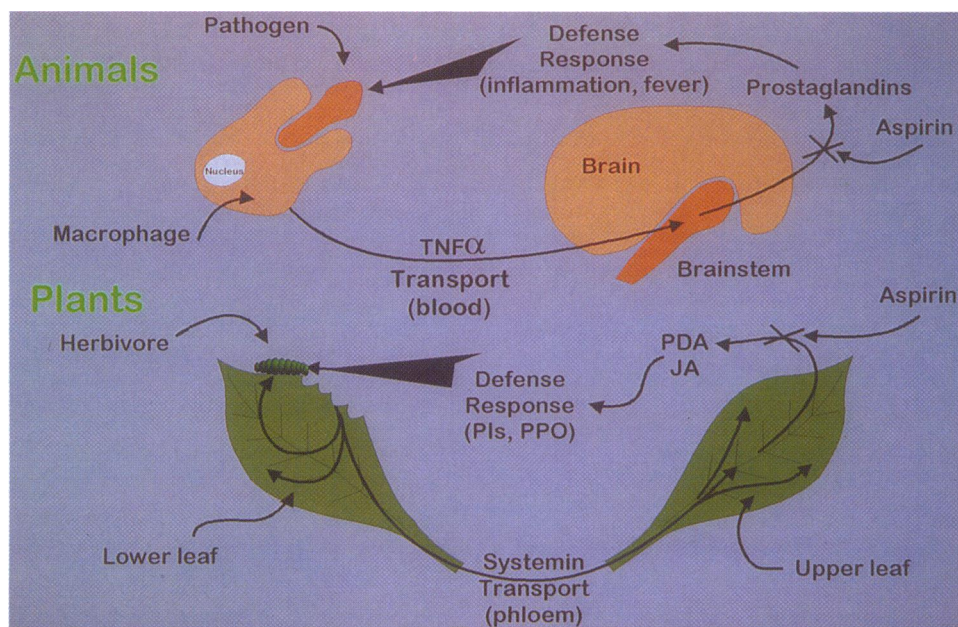


FIG. 9. Illustration comparing the similarities in the defense signaling of macrophages from animals in response to a pathogen attack with the response of leaves of tomato plants to an insect attack. PDA, phytodienoic acid; JA, jasmonic acid.

which releases arachidonic acid from the plasma membranes. Arachidonic acid is converted to prostaglandins which trigger fever to fight off the infection. TNF- $\alpha$  is also a signal released from mast cells in response to bacterial infections (35, 36) and activates the inflammatory response. In plants (Fig. 9), which do not synthesize arachidonic acid (20:4), linolenic acid (18:3) is released in response to the polypeptide wound signal, systemin, that is proposed to be transported through the phloem to distant leaf cells where it activates an intracellular lipase (19). Linolenic acid is subsequently converted to phytodienoic acid and jasmonic acid, both of which are powerful activators of an array of defense-related genes leading to the synthesis of SWRPs. In one animal model, phospholipase A<sub>2</sub> is activated after phosphorylation by a mitogen-activated protein kinase (MAPK or ERK kinase) in the presence of calcium (37). Two recent reports have demonstrated the activation of MAPK activity in tobacco and tomato leaves in response to wounding (38, 39), and increases in intracellular calcium have been documented in defense gene activation in response to oligouronides and chitin oligomers (40, 41). Also, systemin has recently been shown to effect rapid increases in ion fluxes in tomato leaf cells (42, 43). The overall polypeptide- and lipid-based strategies of the plant and animal systems, together with the analogous structures of prostaglandins and phytodienoic acid and jasmonic acid, strongly suggest that the two systems have developed through divergent evolution over the past several hundred million years from an ancestral organism that had fundamental components from which each system was derived. But the divergence must have occurred at a very early stage of evolution, since different plant family members appear to utilize the octadecanoid pathway to activate defense genes (44). It is possible that other defensive systems in plants, such as systemic acquired resistance, in which several signaling systems appear to be coordinated to mobilize defenses against pathogens, may also involve yet to be discovered polypeptide- and lipid-based signaling systems. In animals, an array of cytokines mediate several aspects of defense against both pathogens and parasites. An alternative explanation is that the similarities between animal and plant defensive systems have resulted from convergent evolution and that the analogies are extraordinarily coincidental. As more information concerning the intracellular biochemical events of the

plant signaling pathway become available, such as the identification of the lipase and the mode of its activation, the events leading to the evolution of the plant and animal defense signaling pathways may become more apparent.

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