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PREVIEW

**CHARACTERIZATION OF WOUND-INDUCED ELECTRICAL SIGNALING
AND SYSTEMIC MOLECULAR RESPONSES IN ETIOLATED
PEA (*Pisum sativum*) EPICOTYLS**

by

Christopher Michael Smith

A DISSERTATION

**Presented to the Faculty of
The Graduate College at the University of Nebraska
In Partial Fulfillment of the Requirements
For the Degree of Doctor of Philosophy**

Major: Biological Sciences

Under the Supervision of Professor Eric Davies

Lincoln, Nebraska

December 1995

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Characterization of Wound-Induced Electrical Signaling and Systemic

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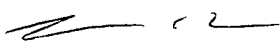
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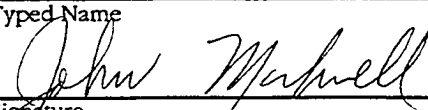
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UNIVERSITY OF NEBRASKA

CHARACTERIZATION OF WOUND-INDUCED ELECTRICAL SIGNALING
AND SYSTEMIC MOLECULAR RESPONSES IN ETIOLATED
PEA (*Pisum sativum*) EPICOTYLS

Christopher Michael Smith, Ph.D.

University of Nebraska, 1995

Advisor: Eric Davies

Plants are subjected constantly to an array of physical and chemical stresses from pathogens, herbivores, and an ever changing environment. In response, plants synthesize compounds, induce genes, activate proteins (enzymes), change physiological processes and anatomical features. In many cases the response is not restricted to the site of injury or for the duration of the insult, but occurs throughout the plant (systemically) and with long-lasting results. The observation that a wound in one part of the plant induces a response elsewhere suggests the presence of a systemically transmitted signal. Intercellular electrical potentials, hydraulic pressure gradients, and biocompounds have been hypothesized and to varying extent investigated as the signalling mechanism in plants. A recent report by Wildon et al. (1992, *Nature*, 360:315) presented convincing evidence that electrical potentials were one of the primary signaling mechanisms in tomato. Calmodulin (CaM) is a calcium-binding regulatory protein involved in a plethora of molecular processes. A variety of environmental stimuli and intrusive wounding induces CaM gene expression in a number of plant species. This study was undertaken to assess the relationship between wound-induced electrical activity, CaM gene expression, and changes in the polysome population in etiolated pea epicotyls. Electrical shock and excision wounding of etiolated epicotyls did not elicit intercellular electrical signalling, although changes in polysomes and CaM gene expression were observed in the later. Scorching and crushing of etiolated epicotyls induced an intercellularly propagated electrical signal, which was followed by marked changes in polysomes and CaM gene expression. Acetylsalicylate, a known inhibitor of wound-induced gene expression in other plants, inhibited scorch-induced electrical signaling, but had little effect on changes in polysomes and CaM gene expression. These results suggest that electrical activity may play a part in the systemic signaling response, but is not the primary signal.

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List of Abbreviations

ABA	Absciscic Acid
ABRE	ABA-Responsive Element
ACC	1-Aminocyclopropane-1-Carboxylic Acid
ASA	Acetylsalicylic Acid
CaM	Calmodulin
CAM	Crassulean Acid Metabolism
CDS	DNA Coding Sequence
CHI	Chalcone Isomerase
CHS	Chalcone Synthase
4CL	4-Coumarate:CoA Ligase
DAHPh	3-Deoxy-Arabinohexulosonic Acid-7-Phosphate
DEPC	Diethyl Pyrocarbonate
DOC	Deoxycholic Acid (disodium salt)
EGBF	Extensin Gene Binding Factor
EDTA	Ethylenediamino Tetraacetic Acid (disodium salt)
HRGP	Hydroxyproline-Rich Glycoprotein
LZP	Leucine Zipper Protein
MOPS	3-(N-Morpholino) Propanesulfonic Acid
PAL	Phenylalanine Ammonia Lyase
PIN	Proteinase Inhibitor
PR	Pathogenesis-Related
PTE	Polyoxyethylene 10-Tridecyl Ether
SDS	Sodium Dodecyl Sulfate

Wound/Stress-Induced Signaling and Molecular Responses in Plants: A Review

I. Introduction and Research Objectives

Unlike their terrestrial counterparts, plants lack the physical capacity to remove themselves from hostile or life-threatening environmental conditions. Therefore there has been strong evolutionary pressure toward the survival of plant lines that are best adapted to a given climatic condition (Ray et al., 1983; Valentine, 1978). Examples include, the succulent cacti of desert terrains, the broadleaf plants of the sub-tropical rain forest, and the conifers of the northern taiga. Plants in a given climatic region possess anatomical, physiological, and molecular attributes that provide the plant with the tools needed to survive the physical conditions of their environment. A classic example is the evolution of desert succulents, cacti, which are characterized anatomically by their fleshy, parenchymatous (water retaining) tissue and thick epidermal cuticle (water loss reduction, thermal tolerance), physiologically by their ability to rapidly uptake and store copious amounts of water, and molecularly by a novel mechanism of carbon (dioxide) fixation, crassulacean acid metabolism (CAM). Although these features provide the plant with distinct advantages for survival in a given climate, they alone are not sufficient to accommodate local variations in their environment (e.g., a hail storm, predation, pathogenesis, pollution, etc.). Thus evolutionary pressure has also selected for plants that possess molecular mechanisms to deal with constant barrage of local environmental stresses.

Modern plants of all climatic regions share some common mechanisms, long- and short-term, to adjust to local disturbances in their environment. Mechanisms that have terminal effects or provide long-term protection include localized necrosis or senescence to isolate a pathogen infected or injured region (or appendage) from the uninjured parts of the plant.

and production of compounds that provide increased resistance or tolerance to subsequent insult (e.g., systemic acquired resistance to pathogens (Ryals et al., 1994) and heavy metals (Jackson et al., 1985)). Other mechanisms provide transient protection. In response to insect herbivory, some plants produce compounds that make the plant tissue unpalatable or reduce its digestibility by the herbivore (Green and Ryan, 1972; Ryan, 1990). Plants also are able to produce substances that aid in the repair, neutralize, and/or minimize the detrimental or potential damaging effects of environmental stimuli. For example, the production of UV absorbing flavanoids in response to potential DNA damaging UV irradiation (Koostra, 1994), and thermally induced heat shock proteins that appear to provide some degree of thermotolerance (Key et al., 1981; Lin et al., 1984).

Although, local stimuli induce local responses and systemic stimuli induce systemic responses, an unexpected observation was that some local stimuli produce systemic responses. These responses include: the emission and changes thereof of volatile aromatic compounds (Loreto and Sharkey, 1993; Turlings and Tumlinson, 1992), the production of disease/stress resistance compounds (phytoalexins), the activation of enzymes (Hammerschmidt et al., 1982; Metraux, 1986), shifts in the levels and distribution of polyribosomes (Davies and Schuster, 1981), and changes in the rate and kinds of proteins synthesized (Davies et al., 1986) and transcription (Margis-Pinheiro et al., 1993; Wingender et al., 1989). One of the earliest recognized and most studied systemic response has been the systemic activation of proteinase inhibitors in potato and tomato plants in response to localized wounding in a single leaf (Bowles, 1990; Pena-Cortes et al., 1988; Ryan and An, 1988; Ryan, 1990, 1992). Subsequently, systemic responses in plants have been studied for a number of environmental stimuli and the molecular responses they induce (Davies, 1987, 1993; Linthorst, 1991; Parsons et al., 1989).

The observation that a wound in one part of a plant induces a response elsewhere suggests that there is a signaling mechanism that transmits the wound stimulus throughout

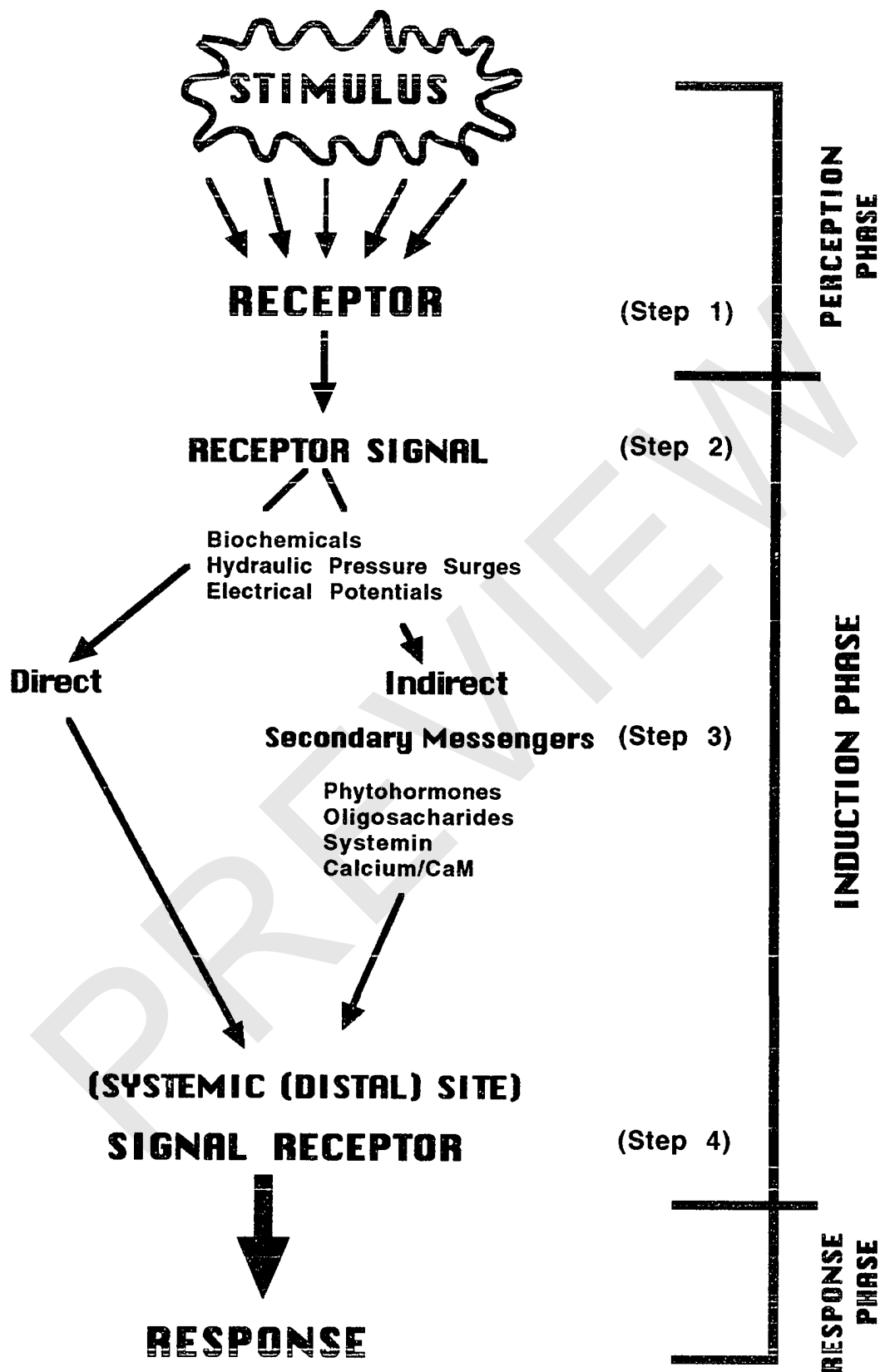
the plant (see Figure 1). Intercellular electrical gradients, hydraulic pressure gradients, chemical, and proteinaceous factors have been hypothesized and to varying extent investigated as the signaling mechanism in plants (Bowles, 1993; Davies, 1987; Ryan, 1992; Ryan and Farmer, 1991; Wildon et al., 1992). Potential chemical, proteinaceous, and hydraulic signals have received most of the plant-related signal transduction research focus over the years. The significance of electrical signals has moved to the forefront these past few years, especially after the findings of Wildon and associates (Wildon et al., 1992). They demonstrated a correlation between wound-induced electrical signals and protein inhibitor (pin) gene expression in young tomato plants. Despite this and many related findings, there still exists much controversy over the existence of various signals (e.g., Ricca's factor, Traumatol), the influence of any one signaling mode, or the possible interaction of various modes on signal transduction in plants. Since not all plants, or for that matter any one plant respond to various stimuli in only one way, it is conceivable that signal transduction in plants, may be stimulus-dependent and/or involve the concerted action of various signaling mechanisms.

The focus of this thesis is to assess in a qualitative sense the extent and correlation between stimuli (wound)-dependent (scorching, excision, crushing, electroshock) intercellular electrical signaling and systemic calmodulin and chalcone synthase gene expression in pea. Since the electrical activity (Pickard, 1971, 1973; Stahlberg and Cosgrove, 1992) and various molecular and physiological characteristics (Davies and Schuster, 1981; Davies et al., 1986; Davies and Larkins, 1973; Schuster and Davies, 1983a, 1983b) have been well characterized in etiolated pea epicotyls, they were utilized in these studies. Additionally, wound-induced calmodulin gene expression has been thoroughly investigated in *Arabidopsis* (Braam, 1992a; Braam and Davis, 1990a) and to a lesser extent in pea (Liu, 1990; Wood, 1990). Wound-induced chalcone synthase gene

expression has also been studied in a variety of plants, including pea (Wood, 1990). Calmodulin and chalcone synthase message (mRNA) were used in this study as the "response" (gene expression) marker.

PREVIEW

Figure 1. Schematic representation of the basic plant processes (steps) involved in external stimuli reception (“perception phase”), signal transduction (“induction phase”), and molecular response (“response phase”).



II. Wound-Induced Responses in Plants

a. Translation and Polysomes

Plant responses to environmental stimuli and pathogenesis are multifaceted, involving complex interrelated cellular and molecular processes. Rapid and dramatic changes in the protein composition and rates of protein synthesis are universal indicators of stress or wounding in most organisms. These observations suggest that there are comparable changes in the translational machinery. At the molecular level, plants respond to stress by switching their translational machinery from messages for housekeeping proteins to mRNAs to stress-related proteins (Ho and Sachs, 1989). Generally, stress-related proteins are translated from newly synthesized messages (Key et al., 1981). In symphony with the translational switch, stress-induced and wounding also cause dramatic changes in the polysome/monosome population. Anaerobiosis and water-stress cause a rapid transition from polysomes to monosomes in soybean and maize seedlings (Lin and Key, 1967; Hsiao, 1970), and mechanical wounding of root tissue results in a dramatic desegregation of polysomes (Theillet et al. 1982). In contrast, mechanical wounding of soybean hypocotyls and aged pea seedlings results in increased polysome formation (Travis et al., 1973; Davies and Schuster, 1981; Schuster and Davies, 1983a). The dramatic changes in polyribosome populations are an easily assessable indicator of wounding and environmental stress in etiolated pea epicotyls (Davies and Larkins, 1973; Davies, 1976; Davies and Schuster, 1981, 1983b; Davies et al., 1986), maize leaves (Hsiao, 1970; Wu et al., 1988), soybean root (Lin and Key, 1967), tobacco (Roberts and Wood, 1981), and barley (Dyer and Scott, 1972).

Stress-induced elevation of translational activity apparently is also accompanied by the transcriptional induction of cyclophilin genes (Gasser et al., 1990; Marivet et al., 1992). Cyclophilins (Cyp) are ubiquitous proteins that facilitate protein folding (analogous to chaperons) and thus are generally associated with protein synthesis machinery. Although,

the Cyp genes are constitutively expressed at basal levels in most tissues, consistent with its molecular function, elevated levels of Cyp transcripts have been reported in young, developing tissue (Marivet et al., 1992; Marty et al., 1993), and in damaged and stress-induced tissue (Marivet et al., 1992, 1993; Marty et al., 1993; Sykes et al., 1993), where they likely accelerate folding and maturation of newly synthesized polypeptides.

b. Local and Systemically-Induced Transcription

Plants respond to environmental stimuli, pathogenesis, herbivory, and mechanical damage by activating a number of defense and repair systems involving physiological, biochemical, and genetic processes. These processes provide mechanisms by which plants can minimize genetic and cellular damage (e.g., production of UV absorbing flavanoids and stress-related proteins [Bailey-Serres et al. 1988; Howarth, 1991; Linthorst, 1991; Kootstra, 1994]), ward off or make plants less attractive to herbivores (e.g., production of glycosides, volatile compounds, insecticidal proteins [Ryan, 1973, 1990; Turlings and Tumlinson, 1992]) and pathogens (e.g. production and/or activation of phytoalexins, thionins, and cell wall degrading enzymes [Bohlmann et al., 1988; Boller et al., 1983; Darvill and Albersheim, 1984; Dixon, 1986]), and strengthen or repair damaged tissue (e.g. accumulation of callose, lignin, and hydroxyproline-rich-glycoproline compounds [Kahl, 1973; Hammerschmidt et al., 1984]).

These responses typically require increases in the production or *de novo* synthesis of macromolecular compounds, requiring biosynthetic enzymes that may or may not be part of the normal housekeeping repertoire. For most responses, this translates into a change in transcriptional activity, either as a selective increase in the rate of transcription of nascent mRNAs (e.g., for phenylpropanoid biosynthesis enzymes; phenylalanine ammonia-lyase, chalcone synthase [Lawton and Lamb, 1987]), or a complete redirection of transcriptional activity toward the *de novo* synthesis of new messages (e.g., mRNAs

for heat shock proteins [Key et al. 1981]). The changes in transcriptional activity occur both locally and systemically in response to stimuli. The differential, temporal, and spatial expression of wound-induced genes argues for a complex, yet wound- and gene-specific mechanism of induction. The discussion hereafter will generally focus on plant genes that are systemically induced by wounding/stress, and the mechanism(s) by which the plant senses an insult and transduces a signal(s) throughout the plant. Stress-induced plant gene expression, not necessarily systemic, have been reviewed elsewhere (Bowles, 1990; Matters and Scandalios, 1986; Sachs and Ho, 1986).

Mechanical wounding (crushing, excision, rubbing) and pathogenesis has been shown to induce the systemic expression of a number of genes including *Atvsp*, *Arabidopsis thaliana* vegetative storage protein acid phosphatase (Berger et al., 1995); CHS, chalcone synthase (Lawton and Lamb, 1987; Wingender et al., 1989); DAHP, 3-deoxy-arabinoheptulosonic acid-7-phosphate synthase, the first enzyme in the shikimate pathway - which produces aromatic amino acids and their derivatives (Dyer et al., 1989); PIN, proteinase inhibitors (Bowles, 1990; Pena-Cortes, 1995; Ryan 1990); *WIP1*, *WIN*, and *WUN-1* wound-inducible proteins (Bradshaw et al., 1989; Longemann et al., 1988; Parsons et al., 1989; Rohrmeir and Lehle, 1993); CHI, chalcone isomerase (Lamb et al., 1989; Wood, A.J., 1990); PR proteins, pathogenesis-related proteins (Brederode et al., 1991); 4CL, 4-coumarate:CoA ligase (Lee et al., 1995); *hev1*, hevein, a chitin-binding protein (Broekaert et al., 1990); extensin (Holdsworth and Laties, 1989; Zhou et al., 1992); PAL, phenylalanine ammonia-lyase (Lawton and Lamb, 1987; Sturm, 1992); HRGP, hydroxyproline-rich glycoproteins (Corbin et al., 1987; Lawton and Lamb, 1987; Lundevid et al. 1992); *tap1*, an anionic peroxidase (Mohan et al., 1993); *rbcs*, ribulose 1,5-bisphosphate carboxylase small subunit (Kombrink and Hahlbrock, 1990); chitinases (Mettraux, 1986); ACC (1-aminocyclopropane-1-carboxylic acid) synthase, an enzyme in the ethylene biosynthesis pathway (Li et al., 1992); and calmodulin (Braam and Davies, 1990; Liu, 1990).

Cold-stress induces the *de novo* synthesis of a variety of transcripts that encode proteins that are thought to be associated with cold acclimation or hardening in alfalfa (Monroy, et al., 1993; Wolfrain et al., 1993) and *Arabidopsis* (Welin et al., 1994). The primary physiological inducer of the chilling response appears to be calcium (Ca^{2+}). Chill-induced increases in cytosolic free Ca^{2+} (caused by cold-induced reduction in the basal rate at which Ca^{2+} exudes from the cell and/or increases in its influx by cold activated voltage-dependent cation channels [Minorsky, 1985]) may create osmotic and/or electrical potential gradients which serve as systemic signal(s).

Water deficit, which is a function of the water status (hydraulic pressure, turgor), is in many ways a (long-lasting) hydraulic signal, and causes the induction of a number of different genes in as many organisms (Skriver and Mundy, 1990), e.g., the *Lea* gene family of cotton (Galau et al., 1993), β -tubulin, actin, HPRG, vegetative storage proteins and a plasma membrane proton ATPase in soybean (Surowy and Boyer, 1991; Creelman and Mullet, 1991), and betaine alcohol dehydrogenase in barley (Ishitani et al., 1995). At the other extreme, water-saturation (e.g. root drowning) and the anaerobiosis that usually follows causes the systemic induction of alcohol dehydrogenases and several other genes (Matton et al., 1990; Bailey-Serres et al., 1988). Ultraviolet light induces the transcription of bean chitinases (Margis-Pinheiro et al., 1993), alfalfa chalcone reductase (Sallaud et al., 1995), and many of the enzymes involved in phenylpropanoid biosynthesis (e.g., phenylalanine ammonia-lyase and chalcone synthase) (Chappell and Hahlbrock, 1982).

A novel transcript (C-*Lea*-5), related to the osmotic-stress-related *Lea* genes of cotton, is systemically induced in the leaves of Citrus (*Citrus sinensis*) trees subjected to saline conditions (Naot et al., 1995). Salinity also causes differential mRNA transcription in barley (Ramagopal, 1987) and the transition from C_3 to CAM photosynthesis, inducing the expression of genes for phosphoenolpyruvate carboxylase, pyruvate, orthophosphate dikinase and other CAM-related enzymes (Michalowski et al., 1989). Chemical agents,