ABSTRACT

DAVID, LISA ILLENE. The role of hormones in wound-stress response in *Arabidopsis thaliana.* (Under the direction of Dr. Eric Davies)

Wounding stress is a continuous threat to the survival of all organisms, and, in crop plants it leads to a dramatic reduction in crop yield. The signaling pathways that allow plants to respond to wounding stress are known to be complex. Many plant hormones such as jasmonic acid (JA), salicylic acid (SA), and ethylene have been identified as critical factors for the induction of wound-responsive genes in a number of plant species. However, the specific roles of each hormone *in vivo,* and how different hormones interact in the wound-stress response are largely unknown. Elucidation of the specific interactions of these plant hormones in response to wound stress is a major focus of wound research. The major goal of this research is to utilize Arabidopsis JA, SA, and ethylene biosynthetic and signaling mutants to analyze the role of each hormone separately and together in wound-responsive gene induction. Arabidopsis wild type, *ein2*, *npr1, jar1, npr1/ein2, and npr1/jar1* plants were wounded and sampled at different times, after which RNA was extracted, subjected to electophoresis, transferred to filters and probed for several putative wound-regulated transcripts, which included *PDF1.2, PR1*, *WAK1*, and, *LOX2*. The constitutive levels of transcript expression as well as patterns of accumulation of these transcripts in response to the wounding stimulus varied in the different mutants.

THE ROLE OF HORMONES IN WOUND-STRESS RESPONSE IN *ARABIDOPSIS THALIANA*.

by

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DEDICATION

To my beautiful daughter Ame. This, as with everything I do, is for you. I hope to make a better future for you and me. I hope that by doing this I can show you that if you stick to something long enough you will accomplish your goals. I hope that you will learn to become the strong, intelligent, tenacious woman that I want to be. As I write this you are only one month old. Yet, in your eyes I can see your future and it is just as clear and wondrous as your dark eyes. You are my sunshine. You are the shining light that guides me down the path less traveled. I love you with all my heart.

BIOGRAPHY

Lisa Illene David was born and raised in Minneapolis, Minnesota. She graduated from Mills College in Oakland, California in 1995 with a B.A. in Biology. She married her wonderful, hard-working, husband, Manabu Ishitani (a fellow plant scientist) on May 3, 2000 and gave birth to her daughter Ame Ishitani on October 1, 2003. After completing her master's degree she plans to continue working as a plant molecular biologist and join her husband at Centre International de Agriculture de Tropic (CIAT) in Cali, Columbia.

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Many people have helped me during my graduate school experience at NC State University. My advisor, Dr. Eric Davies, has supported me both intellectually and emotionally through the past two years and I wish to thank him immensely for that. My committee consisting of Dr. Eric Davies, Dr. Becky Boston, Dr. George Allen, and Dr. Jose Alonso, have offered me great suggestions for my project and have been incredibly giving with their time. Sue Vitello in the Botany office and Dr. Nina Allen have helped keep me on track with all of my graduate school requirements and endless red tape. Dr. Jenny Xiang and Dr. Chuanzhu Fan let me use their sequencing equipment in their lab for my project. Dr. Xinnian Dong at Duke University kindly provided me with the double mutant seeds that I used for this project. And, I never could have made it through without the help of my husband, Dr. Manabu Ishitani. In addition to providing loving encouragement to me, he also helped me with many technical problems in the lab, worked grinding tissue and running gels after I gave birth to our daughter, and supported our family financially through it all.

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13-HPLA: 13-hydroxyperoxylinolenic acid

ABA: abscisic acid

ACC: 1-aminocyclopropane-1-carboxylic acid

AdoMet: *S*-Adenosyl-methionine

ATA: aurintricarboxylic acid

DNA: deoxyribonucleic acid

EDTA: ethylenediamine tetraacetic acid

ein2: ethylene insensitive

ET: ethylene

JA: jasmonic acid

jar1: jasmonic acid resistant

LiCl: lithium chloride

MeJA: methyl jasmonate

MOPS: 3-(N-Morpholino) propanesulfonic acid

MS: Murashige and Skoog

NaCl: sodium chloride

NaH_2P0_4: sodium phosphate

npr1: nonexpresser of PR genes

OGAs: oligogalacturonides

PCR: polymerase chain reaction

PIN: proteinase inhibitor

PL: phospholipids

PR: pathogenesis-related

RNA: ribonucleic acid

SA: salicylic acid

SAR: systemic acquired resistance

SDS: sodium dodecyl sulfate

SSPE: buffer consisting of 3M NaCl, 0.2M NaH_2P0_4, 19.9 mM EDTA

UV: ultra violet

WR: wound-responsive

INTRODUCTION

Summary:

Wounding stress is a continuous threat to the survival of all organisms, and in crop plants it leads to a dramatic reduction in crop yield. In a world where people are poor and malnourished and world population for the year 2025 is projected to be 8 billion (Herrera-Estrella, 2000), scientists must find ways to increase food production. Because land and water are limiting resources in food production, the only solution is to increase yields on the available land. Bio-engineered crops are our best hope to meet current and future food supply demands. A better understanding of how plants respond to environmental stress factors, such as wounding, is necessary to engineer stress-resistant crops and thereby increase crop yield.

A complex number of different signals play regulatory roles in the wound stress response. Examples include reactive oxygen species, oligogalacturonides, electrical pulses, hydraulic waves, plant hormones, polyamines and systemin (an 18 amino acid peptide). Many plant hormones such as abscisic acid (ABA), jasmonic acid (JA), salicylic acid (SA), and ethylene have been identified as critical factors for the induction of wound-responsive genes in a number of plant species. However, the specific roles of each hormone and how different hormones interact in wound-stress responses are largely unknown.

Elucidation of the specific roles of these plant hormones and their interactions with one another in response to wound stress is a primary focus of this research. The major goals were to 1) use Arabidopsis hormone signaling mutants to analyze the role of each hormone in wound-responsive gene induction and 2) use double mutants that are

deficient in two hormone pathways to analyze the interactions of plant hormones in response to wound stress.

This research has several important implications including the elucidation of hormonal regulation of the wound stress response and the discovery of useful tools for genetic improvement of plants with enhanced resistance to wound stress. An understanding of how plants respond and defend themselves against wound stress is essential to the development of wound-stress resistant crops whose productivity is markedly reduced due to wounding stress.

Overview of wounding stress:

Because plants are sessile organisms, they are unable to avoid the constant onslaught of injuries that they incur from various factors in their environment. These include both mechanical damages from abiotic factors such as wind, sand, hail, and rain as well as biotic factors such as insect and herbivore feeding. The wounding stress produced by these various factors results in a substantial loss of yield in crops world wide. Herrera-Estrella (2000) estimated that pests destroy over half of all world crop production.

Wounding stress evokes defense responses in plants which rely on transcriptional activation of specific wound-responsive genes (Leon et al., 2001). The proteins encoded by wound-responsive genes have various roles to heal local damaged tissue and to prevent further systemic damage to the plant. Protein functions include repair, inhibition of insect predators, and activation of wound-defense signaling pathways (Leon et al., 2001). To date, the best characterized wound-induced proteins are the proteinase inhibitors (*Pin1*, *Pin2*) in the Solanaceae family. *Pin1* and *Pin2* are serine proteinase inhibitors, which disrupt digestion

in the insect's gut (Green and Ryan, 1972). These inhibitors are inactivators of proteolytic enzymes and they also trigger physiological feedback mechanisms that cause overproduction and secretion of digestive proteases and a decrease in appetite in the insects (Ryan, 1990). Homologs of *Pin1* and *Pin2* have not been found in Arabidopsis. Additionally, many genes whose expression is regulated by pathogen attack are also regulated by wounding stress (an example of this is the PR (pathogenesis-related) genes (Glazebrook, 1999).

Local vs. systemic response:

When a plant is wounded, different gene products are required in different areas of the plant body. At the site of the wound, proteins are needed that function in repair and inhibiting insect and pathogen predators as well as the generation of systemic signals to mobilize defenses in distant tissues. In distant, non-wounded tissues proteins involved in the activation of wound-defense signaling pathways are required to prevent further attack in these tissues or possibly prepare the tissue for wounding stress. Research in the area of wound-stress response in plants has shown that the wound signaling network is quite complex (Leon et al., 2001). Many structurally different molecules play regulatory roles in this response (Figure 1). These signals can be broadly grouped into those that act locally at the site of wounding and those that act systemically to confer resistance to non-wounded tissues. In a recent review, Leon et al. (2001) summarized local signals to include reactive oxygen species, and oligogalacturonides and mobile signals to include electrical pulses, hydraulic waves, plant hormones, and systemin.

Systemin is an 18 amino acid peptide generated from its precursor, prosystemin (McGurl and Ryan, 1992). Recently, a tomato mutant that is defective in systemin perception

and systemic wound signaling was identified (Lee and Howe, 2003). Although systemin has been well characterized as a primary long-distance transmittable signal in the Solanaceae (Ryan, 2000), the existence of a related peptide has so far not been found in *Arabidopsis*.

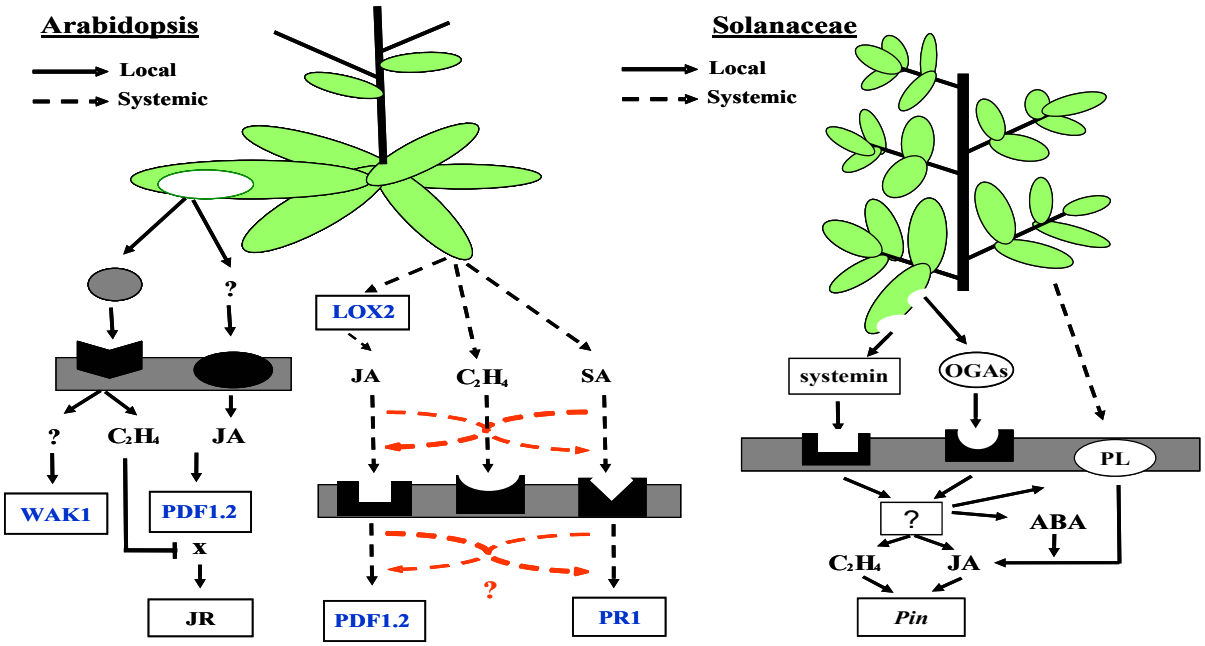

Figure 1. Proposed wound-induced signal transduction in *Arabidopsis* and Solanaceae. Response to wounding in plants is a complex process. The important components of the wound stress signaling pathway indicated are oligogalacturonides (OGAs), phospholipids (PL), systemin and its precursor prosystemin, membrane receptors and proton pumps, as well as hormones such as jasmonic acid (JA), abscisic acid (ABA), and ethylene (C_2H_4). Many genes responsive to these stresses are also indicated (Pin, JR3, PDF and general wound-responsive (WR) and jasmonic acid-responsive (JR) genes).

Long vs. short time course:

Often when examining wound-responsive genes researchers have examined tissues (either local or systemic) collected at only one time point. This time point often represents a long-term response such as 24 or 48 hours after wounding. Recent research in tomato (*Lycopersicon esculentum*) has shown that transcript levels of some genes rapidly accumulate in systemic tissues. Davies et al. (1997) showed that when one tomato leaf was electrically stimulated or heat wounded many transcripts including those of proteinase inhibitor genes (*PIN*) were rapidly up-regulated in systemic leaves, peaking at 5 to 30 minutes after wounding. Their research showed a transient period of transcript accumulation (short time course response) preceding a period of transcript decrease, followed by a second period of transcript accumulation (long time course response). The identity of the systemic wound signal(s) for the short and long term responses are not yet known and it is possible that they are different. Candidates for systemic signaling include hormones transmitted via the phloem or the xylem, electrical signals in the phloem, and hydraulic pressure in the xylem among others (Stankovic and Davies, 1997).

The role of hormones:

Plant hormones have been shown to play a role in plant wound-stress response in a number of plant species. Elucidation of the specific roles of these plant hormones and their interactions with one another in response to wound stress is a major focus of research in the field of plant responses to wounding.

Jasmonic Acid:

Jasmonic acid is a plant hormone that affects plant growth, and is derived from the lipoxygenase-dependent oxidation of linolenic acid. It is thought to have analogies to prostaglandins which serve as stress signals in animals (Staswick, 1992). It has been suggested that JA arises from the release of cell membrane fatty acids through the action of lipase in response to wounding or autolytic events (Staswick, 1992). Jasmonic acid (JA) is synthesized from alpha-linolenic acid by a lipoxygenase (*LOX2*)-mediated oxygenation leading to 13-hydroxyperoxylinolenic acid (13-HPLA) which is then subsequently transformed to JA by the action of hydroperoxide-dehydrase activity and additional modification steps (Howe & Schilmiller, 2002). Jasmonic acid (JA) helps plants resist fungal infection and other stresses and induces plant production of protective secondary compounds such as alkaloids. Ample evidence suggests that JA is a key signal molecule in wound-response in multiple plant species (Reviewed in Wasternack and Parthier, 1997; Liechti and Farmer, 2002; reviewed in Stratmann, 2003; Li et al., 2003). Airborne methyl jasmonate and application of octadecanoid precursors of JA can induce the synthesis of *PIN* genes in plant species in the Solanaceae and Fabaceae families (Farmer and Ryan, 1990; Farmer and Ryan, 1992). Recently, wound-induced genes whose expression is regulated by a JA-independent signaling pathway have been identified in *Arabidopsis thaliana* (Titarenko et al., 1997).

Salicylic Acid:

SA, is a phenolic compound found in willow bark, and is thought to have been used as early as the 4th century B.C. by Hippocrates to aid in pain relief for women in child birth (Klessig and Malamy, 1994). Exogenous application of SA has been shown to affect a

variety of processes including stomatal closure, seed germination, fruit yield and glycolysis (Klessig and Malamy, 1994). SA also helps plants perceive pathogen attack (Klessig and Malamy, 1994).

SA is thought to be synthesized in plants from phenylalanine which is converted to trans-cinnamic acid by phenylalanine ammonia lyase (PAL). PAL is a key enzyme in the phenylpropanoid pathway that yields phytoalexins, lignins and hydroxybenzoic acids. Salicylic acid was found to prevent wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis (Pena-Cortes et al., 1993). In a review, Klessig and Malamy (1994), gave an overview of evidence supporting the role of SA in plant disease resistance. Because pathogens can enter through a wound site, researchers have also investigated the role of SA in wound-stress response and found it to play a critical role (Pena-Cortes et al., 1993).

Endogenous levels of SA positively correlate with expression of defense-related genes and development of systemic acquired resistance (SAR). SAR is the process by which non-infected tissue develops an enhanced level of resistance to the attacking pathogen (Klessig and Malamy, 1994). In addition, exogenous applications of SA induce defense responses and elimination of endogenous SA represses these responses (Gaffney et al., 1993). Genetic studies in *Arabidopsis thaliana* have shown that SA is required for the induction of local defense responses, for activation of numerous defense-related genes including a set of pathogenesis-related (PR) genes, and in the establishment of SAR (Kunkel and Brooks, 2002). Attempts by several laboratories to genetically dissect the SAR pathway downstream of the SA signal all resulted in the identification of numerous alleles of a single gene

designated *NPR1* (reviewed in Somssich, 2003). The *NPR1* gene was found to encode a novel protein containing ankyrin repeats (Cao et al., 1997).

Ethylene:

Ethylene biosynthesis is increased by stress conditions such as drought, flooding, chilling, or mechanical wounding (Taiz et al, 1998). During such stress responses, ethylene is produced by the usual biosynthetic pathway, and it has been shown to result, at least in part, from an increase in transcription of ACC (1-aminocyclopropane-1-carboxylic acid) synthase mRNA. ACC synthase catalyzes the conversion of *S*-adenosyl-methionine (AdoMet) to ACC, the immediate precursor of ethylene. Stress-induced ethylene is involved in the onset of stress responses such as abscission, senescence, wound healing, and increased disease resistance (Taiz and Zeiger, 1998). In addition to helping plants to perceive and respond to pathogen attack and mechanical stress, ethylene also promotes ripening of some fruits, promotes leaf and flower aging and leaf and fruit drop from plants, and affects cell elongation and seed germination (Taiz and Zeiger, 1998). Ethylene is also required in the transduction pathway leading from injury to *PIN* gene expression (O'Donnell et al., 1996).

Experimental approaches:

Many researchers have shown that hormones, including JA, SA, and ethylene play a role in the systemic response to wounding in plants (reviewed in Leon et al., 2001; Bell et al. 1995; Benedetti et al, 1998; Chen et al., 2002; Cheong et al., 2002; Farmer & Ryan, 1990; Klessig & Malamy, 1994; O'Donnell et al., 1996; Pena-Cortes et al., 1993; Titarenko et al., 1997). However, many of these results rely on the exogenous application of the hormone, or

some derivative of the hormone to the plant surface and it is difficult to relate this method of exposure to a hormone to what would naturally occur in the plant. The exogenous application process requires the absorption of the chemical through the leaf or root surface rather than the release or production of the hormone within the plant. It also means that the exact dosage of the hormone that would normally be supplied by the plant during stress is not administered and that the proper derivative of the hormone is perhaps not even applied. Yet, this is the process used by many, including those using functional genomic methods to identify genes responsive to these wound-induced hormones (Mahalingam et al., 2003, Reymond et al., 2000). For example some functional genomic studies claim the involvement of plant hormones by simply showing that genes in the hormone biosynthetic pathway were activated (Cheong et al., 2002). While such data provide additional evidence for the hypothesis that these hormones are involved in wounding stress response, this evidence is correlative. In contrast by eliminating the production of the hormone, or by using mutants defective in hormone signaling, conclusive evidence could be gathered to show whether these hormones are necessary for the transcription of wound-responsive genes. In this way, hormone mutants that are unable to produce or sense a particular hormone can be used to determine the possible involvement of a certain hormone in the stress response.

Researchers are now taking advantage of the large collection of Arabidopsis mutants available to the scientific community. Reymond et al. (2000) performed wounding stress on the *coi1* mutant, which is an Arabidopsis mutant that is coronatine- and jasmonate-insensitive, to show the importance of jasmonic acid in the wound stress response. Chen et al. (2002) used microarrays to search for transcription factors involved in environmental stresses in SA, JA, and ethylene mutants in Arabidopsis to look at altered expression of these transcription

factors in these mutants. Pena-Cortes et al. (1995) used tomato and potato ABA-biosynthetic mutants to show the involvement of ABA in activation of *Pin2* gene expression.

However, these studies have not addressed the important topic of potential cross-talk between these hormone pathways. In order to fully understand this stress response, we must understand how the important components in the signaling pathway interact with each other to create a defense response. This can be accomplished by the use of double mutants. To my knowledge, this thesis represents a novel approach to studying the cross-talk between these hormones during wound-stress response.

Potential crosstalk between hormone signals:

Because so many signaling molecules, including several different hormones, are involved in wound and pathogen stress responses, some researchers have become interested in the potential cross-talk between these signaling molecules. In a recent review, Gazzarrini and McCourt (2003) give an overview of how the concept of hormone interactions has developed. At first, genetic analyses seemed to suggest that hormones work through distinct pathways to elicit their responses and if interaction occurred it was distantly downstream of the primary response pathway. However, the recent development of hormone-response mutants indicates that different hormones could influence each other's synthesis and perhaps share signaling components. This led to a model in which hormones interact or cross-talk to form complex webs of overlapping signaling pathways (Figure 1). Kunkel and Brooks (2002) recently reviewed the evidence of cross-talk between hormone pathways specifically in pathogen defense. JA, SA and ethylene were specifically highlighted because they are the three major hormones known to be involved in plant defense. Cross-talk between these

hormones involves different pathways influencing each other through both positive (working synergistically to regulate the same genes) and negative (working antagonistically in gene regulation) interactions. Unfortunately, much of this evidence relies on results from microarray experiments in which the hormones were applied exogenously and transcript abundance was measured. Schenk et al. (2000) showed that exogenous application of JA or ethylene was able to induce the expression of a large number of the same defense-related genes. Their study also showed that SA and ethylene function together to coordinately induce the same genes, again, by exogenous treatment with these hormones. Other evidence of cross-talk comes from examining gene expression levels in single hormone mutants. For instance, levels of *PR1*, which requires SA for transcription, were lower in *ein2* mutant plants which are ethylene insensitive (Lawton et al., 1994). Also, Arabidopsis mutants that were impaired in SA accumulation were found to exhibit enhanced responses to inducers of JA-dependent gene expression (Gupta et al, 2000). However, to my knowledge, no one has utilized double mutant plants (impaired in two hormone signaling pathways) to examine the response of these plants to wounding stress. This research is designed to provide evidence of the interaction of these hormone pathways in response to wound stress *in vivo*.

The mutants:

Hormone mutants used in this study were selected based on previous research on the role of hormones in the wound-stress response (Leon et al, 2001). All single mutants are genetically recessive, were produced by ethylmethane sulfonate (EMS) mutagenesis, and are freely available from the TAIR Arabidopsis Seed Stock Center. All double mutants were

created in the lab of Dr. Xinnian Dong at Duke University. The mutants used were *npr1, jar1, ein2*, and *npr1/ein2* and *npr1/jar1* double mutants.

npr1

The *NPR1* gene was originally identified in a screen for *Arabidopsis thaliana* mutants that were unable to mount a general defense against pathogen attack (Cao et al., 1994). In response to an attack by pathogens, plants have the ability to defend uninfected parts of the plant by a process called systemic acquired resistance (SAR). SAR is characterized by the expression of pathogenesis-related (PR) genes. SAR can be induced by treatment with either salicylic acid (SA) or 2,6-dichloroisonicotinic acid. The *npr1* (nonexpresser of PR genes) mutant was identified by its inability to induce the expression of PR genes via a SAR response to an avirulent pathogen attack (Cao et al., 1994). The *NPR1* gene was later cloned and found to encode a novel protein containing ankyrin repeats. A mutation in the ankyrin sequence leads to disruption in *NPR1* function. Therefore, the ankyrin repeats are thought to be important for *NPR1* function. Mutants with defects in *NPR1* fail to respond to various SAR-inducing treatments, display little expression of pathogenesis-related (PR) genes and have increased susceptibility to infections (Cao et al., 1997).

jar1

Jasmonic acid and its methyl ester, methyl jasmonate (MeJA), affect plant growth including primary root growth in wild type *Arabidopsis thaliana*. The *jar1* (jasmonic acid resistant) mutant was isolated from a screen for mutagenized plants that exhibited a decreased sensitivity to MeJA in terms of root growth. In addition, the *jar1* mutant had

decreased accumulation of JA-responsive proteins in both roots and leaf tissue (Staswick et al., 1992).

ein2

The *EIN2* gene was discovered in an *Arabidopsis thaliana* mutant screen for ethylene-insensitive phenotypes (Guzman and Ecker, 1990). In the dark, the presence of ethylene causes what is known as the "triple response" in seedlings. In Arabidopsis, the triple response consists of radial swelling of the hypocotyls, exaggeration of the apical hook, and inhibition of hypocotyl and root elongation (Chen & Bleecker, 1995). In the presence of ethylene, *ein2* mutants do not exhibit the triple response. Alonso et al. (1999) recently cloned the *EIN2* gene and found that, at the amino acid sequence level, it appears to be structurally unique. However, they did identify a hydrophobic region that allows *EIN2* to function as an integral membrane protein. They also found sequence similarity between *EIN2* and the Nramp family of proteins, several of which function as transporters of divalent cations. They also suggested that *EIN2* may be functionally equivalent to the yeast glucose sensors Snf3 and Rgt2. They presume that, like these yeast glucose sensors, *EIN2* is able to perceive an unknown stress signal and then interact with downstream components in the pathway.

npr1/ein2 and npr1/jar1 double mutants:

The double mutants were created in the lab of Dr. Xinnian Dong at Duke University. The *npr1*/*ein2* double mutant was created by crossing homozygous *npr1* and *ein2* plants. The F2 seed was plated on MS plates containing 50 uM 1-amino-cyclopropane-1-carboxylic acid (ACC) and placed in a growth chamber. After 5 days in the dark, the seedlings were scored

for the presence or absence of the ethylene (ET)-induced triple response (Guzman and Ecker, 1990). The *ein2* mutant, being ET insensitive, does not display the triple response. F2 plants that lacked the triple response were collected and transferred to soil. F3 seed of putative double mutants were collected, rescreened on MS-ACC plates and then the cleaved amplified polymorphic sequence marker for *npr1* (Cao et al., 1997) was used to confirm homozygosity at the *npr1* locus. For the *npr1*/*jar1* mutant F2 progeny were grown on MS plates containing 50 μM jasmonic acid (JA) and assayed for the lack of JA-induced responses, which include inhibition of root growth and excessive accumulation of anthocyanin (Staswick et al., 1992). F2 plants that lacked the JA-induced root and anthocyanin phenotypes were transferred to soil and confirmed for the *npr1* mutation as stated above for the *npr1*/*ein2* mutant.

Transcripts up-regulated by wound stress:

PDF1.2:

PDF1.2 is induced by aphid feeding and encodes an antimicrobial protein, plant defensin, a peptide involved in JA- and ethylene-dependent responses (Moran et al., 2001). Infection by the fungus *Alternaria brassicicola* leads to sustained increases in *PDF1.2* mRNAs both locally and systemically. This mRNA increase is thought to take place in a jasmonic acid (JA)- and ethylene-dependent manner (Penninckx et al., 1998; Thomma et al., 1998; Pieterse and van Loon, 1999). In the Arabidopsis mutant *coi1-1*, which is affected in a signal-transduction component acting downstream of JA, *Alternaria brassicicola* inoculation could not induce *PDF1.2* transcript accumulation (Thomma et al., 1998).

PR1:

PR (pathogen responsive) genes encode some of the plant proteins that break down cellular constituents of pathogens or aid in signaling. After infection by *Pseudomonas syringae* bacteria, *Peronospora parasitica* fungi, and other pathogens, expression of *PR* genes increases via a SA-dependent response pathway (Glazebrook, 1999). *PR* genes are induced by aphid feeding and associated with SA-dependent responses to pathogens (Moran et al., 2001). *PR-1* gene expression is correlated to SAR to further pathogen infection (Ryals et al., 1992). *PR-1* can be used as a marker of SA-dependent induction in Arabidopsis (Uknes et al, 1992; Rogers and Ausubel, 1997).

WAK1:

WAKs are a family of wall-associated serine-threonine kinases that are thought to be involved in signaling between the cell wall and the cytoplasm. They are linked directly to the cell wall, span the plasma membrane and have a cytoplasmic kinase domain (Cheeseman & Kohorn, 1999). WAKs are very strongly associated with the cell wall, as enzymatic digestion of the carbohydrate or boiling in detergent are required to release them from the cell wall (Kohorn, 2002). There are five WAKs in *Arabidopsis*. *WAK1* is induced during pathogen and wound response and is required for the plant to respond to pathogen infection (He et al., 1999). *WAK1* is one of the most ubiquitous and abundant WAKs.

LOX2:

Plant lipoxygenases (LOX) are thought to be involved in the biosynthesis of lipid-derived signaling molecules. Lipoxygenases are involved in the synthesis of jasmonic acid (JA) and are up-regulated by leaf wounding. One specific lipoxygenase, *LOX2* in Arabidopsis is induced by aphid feeding and encodes a JA-inducible lipoxygenase (Moran et al., 2001). Characterization of *LOX2* showed that the protein is targeted to the chloroplasts (Bell et al, 1995). *LOX2* was also found to be required for the wound-induced synthesis of JA in some plant tissues (Bell el al., 1995).

MATERIALS AND METHODS

Growth of Arabidopsis:

Arabidopsis thaliana plants were grown at the Phytotron facility at North Carolina State University. Temperature, humidity and light were held constant in the growth chambers and were as follows: temperature 22º C, humidity 65%, light cycle 16 hours of light at 200 μE followed by 8 hours of dark. Seeds were sown into 3 inch pots containing Metro-mix 360 soil. Seeds were vernalized on wet soil at 4º C for 3 days then moved to the growth chambers. After germination, extra seedlings were removed so that only one plant remained per pot. Trays of pots were covered with transparent domes to increase humidity until true leaves had formed on the seedlings. Plants were sub-irrigated every 2 days. After the formation of true leaves, plants were treated with 20-20-20 Peters fertilizer (Scotts #91010) once a week by sub-irrigation. Plants were collected for the wound-stress assay when the primary bolts were approximately 2 inches long and before the rosette leaves began to senesce. The *ein2* mutant was late-flowering. Other than that, there were no developmental differences between the mutants and wild type plants.

Wound-stress assay:

Wounding stress was performed on wild type, single hormone mutant and double hormone mutant plants (Figure 2) using a soldering iron (Michigan Industrial Tools, 117V, 30W, no. 7266). The soldering iron had a 0.2cm tip and was held on the wounded leaf for 5 seconds. A soldering iron was used because it creates a specific wound which can be precisely replicated on each sample plant, and, unlike flame wounding, does not produce

light which could activate light-responsive genes. One of the primary four rosette leaves was wounded and other leaves were collected (Figure 2). Systemic response was measured by analyzing the wound-induced gene transcript levels in the non-wounded leaves. After wound stress, non-wounded leaves were collected at the following time points: 5 min., 15 min., 30 min., 60 min., and 360 min. after wounding and immediately frozen in liquid nitrogen. These time points were selected to represent both the fast and slow kinetic response to wound stress (Davies, 1987; Davies et al., 1997; Vian et al., 1999). The wounding experiment was repeated three to six times with three replicates each containing five to six plants per treatment.

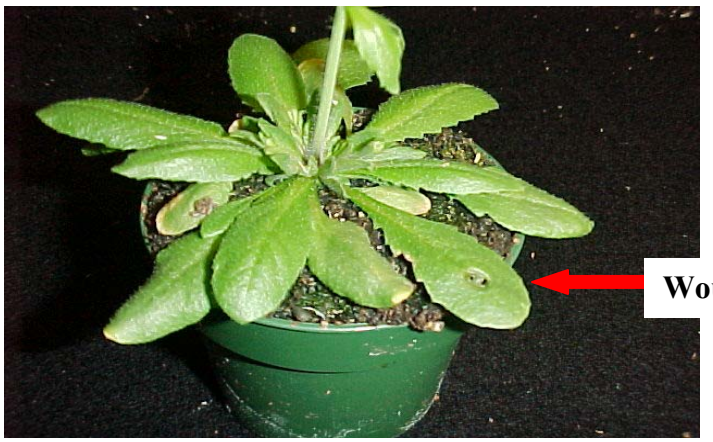

Figure 2. Wounded Arabidopsis plant

Wounding was performed using a soldering iron. This created a precise hole in one of the rosette leaves. The other rosette leaves were then collected by cutting with scissors at different time points after wounding and immediately frozen in liquid nitrogen. For control plants (0 min) the rosette leaves were collected by cutting with scissors without wounding with the soldering iron.

RNA extraction and gel electrophoresis:

Total RNA of wounded and control (unwounded) plants was extracted from non-wounded leaves of *Arabidopsis thaliana* (Columbia ecotype) using a method previously described in Liu and Zhu (1997) and Carson and Botha (2000). Approximately 1 gram of tissue from non-wounded leaves was ground in liquid nitrogen using a mortar and pestle immediately after collection. Ground tissue was then added to a tube on ice with 4.5 ml of extraction buffer containing 50 mM Tris-HCl, pH 8.0 (at room temperature), 300 mM NaCl, 5 mM EDTA, 2% SDS, 2 mM aurintricarboxylic acid, and 10 mM β-mercaptoethanol. After the addition of 0.7 mL of cold 3 M KCl, the mixture was incubated on ice for 15 min and then centrifuged at 9000g for 20 min. RNA was precipitated from the supernatant by the addition of 2 mL of 8 M LiCl and incubation at 4C overnight. The precipitate was pelleted by centrifugation at 9000g for 20 min and then resuspended in 2 mL of water. The suspension was extracted with phenol and chloroform and RNA was precipitated from the aqueous phase with ethanol. The pellet was washed with 80% ethanol, air-dried, and resuspended in 50 μL of water. Total RNA from a total of three to six replicates from each time point was used for each mutant and wild type plant. RNA was separated on formaldehyde-agarose gels and blotted onto positively charged nylon membranes overnight using 5X SSPE solution. Gels for Northern analyses contained 1.4% agarose and 1X MOPS buffer. RNA was then UV cross-linked (using the autolink function) to the membranes before hybridization with the radio-labeled probes.

PCR amplification for making probes:

Probes consisted of cDNA of putative wound and stress responsive genes including *LOX2*, *PDF1.2*, *PR1*, and *WAK1*, and were selected based on previous research in the wound-stress field. The cDNA sequences for all of the probes have been published and are available on the PubMed website. *Arabidopsis thaliana* genomic DNA was used as a template for the PCR reaction. Probes were made by PCR amplification using primers specific for the 3' non-coding region of the published cDNA sequence. Primers contained approximately 50% GC content. All primer sequences were verified for specificity using BLAST searches. Primers were as follows:

LOX2 forward primer 5' GCCTGAAGACTTTGGACCAG 3',

LOX2 reverse primer 5' GGTGTTTGGGAAGGCAAGTA 3',

PDF1.2 forward primer 5' GGAAACAGTAATGCATGCAAGAA 3',

PDF1.2 reverse primer 5'TATTATTGTAACAACAACGGGA 3',

PR1 forward primer 5' GAGACTCGGATGTGCCAAAGTGA 3',

PR1 reverse primer 5' TCTCGTAATCTCAGCTCTTATTTGT 3',

WAK1 forward primer 5' AATGAAAGTTTCAGTATCAC 3',

WAK1 reverse primer 5' GCAGTTAACTTGTTGTTGGC.

Purification of PCR products:

The double-stranded PCR products were cleaned using 20% polyethylene glycol 8000/2.5 mol/L NaCl (Morgan and Soltis, 1993; Soltis and Soltis, 1997).

TOPO TA cloning:

The PCR products were purified and cloned to competent *E. coli* cells using TOPO TA cloning techniques (Invitrogen Life Technologies, Carlsbad, California, USA 92008). The transformed *E. coli* were plated and cultured according to TOPO TA cloning kit specifications. The growing colonies were screened for positive transformants using PCR amplification by probe specific primers (described above). Two to four positive transformants were inoculated to multiply the cells. Plasmid DNAs were extracted and purified using Promega Minipreps DNA purification system (Promega, Madison, Wisconsin 53711-5399, USA). The purified plasmid DNA products were directly sequenced.

Sequencing:

Plasmid products were used as the templates for sequencing using the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, USA). Cycle-sequencing reactions (10 μL) were prepared by combining 2 μL terminator ready reaction mix, 2 μL sequencing buffer (200 mmol/L Tris-pH8.0, 5mmol/L $MgCl_2$), 0.6 μL primer (5 μmol/L), 2 μL or 4 μL of 200 ng/μL cleaned PCR products or plasmid DNA, 0.5 μL DMSO, and 0.9μL DI water. Cycle-sequencing was conducted on a PTC-100 Programmable Thermal Controller (MJ Research Inc., Watertown, MA, USA) as follows: 25 cycles of 96ºC for 30 sec, 50ºC for 15 sec, and 60ºC for 4 min. Products of cycle-sequencing were cleaned using ethanol/sodium acetate precipitation (ABI applied Biosystems, Foster City, California 94404, USA) with an additional 95% ethanol wash. The cleaned sequencing products were analyzed on an ABI-377 automated sequencer

(Applied Biosystems, Foster City, California 94404, USA). The sequence chromatogram output files for all samples were checked to ensure that the sequence was from the probe of interest. Band size was confirmed on a 2% agarose gel stained with ethidium bromide (Figure 3). The putative wound-induced genes that were used as probes are indicated in Table 1.

Table 1. Probes used to determine transcript levels.

cDNA probes for the wound-responsive genes *LOX2, PDF1.2, PR1*, and *WAK1* were created by PCR amplification using 3' specific primers from each gene sequence. All fragments used were 3' non-coding region of each gene, had approximately 50% GC content and were verified for specificity using a BLAST search. a – Bell et al. (1995), b – Epple et al. (1997), c – Carr and Klessig (1989), d – He et al. (1996).

Gene	Protein	Response/ Induction	Gene ID/ Accession #	PCR product size	mRNA length	Chromosome	References
LOX2	Lipoxygenase	Pathogen, JA, Aphid	L23968	202 bp	2.8 kb	III	a
PDF1.2	Defensin	Pathogen, JA, Aphid	At5g44420	242 bp	0.5 kb	V	b
PR1	Pathogenesis related protein	Pathogen, SA	At2g14610	255 bp	0.7 kb	II	c
WAK1	Wall associated kinase	Pathogen, Wound	AJ009696	235 bp	3.9 kb	I	d

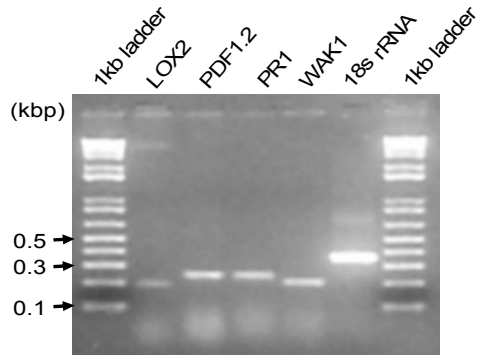

Figure 3. Agarose gel of PCR products used as probes.

5 µl of the PCR products of the wound-responsive genes and 18S rRNA fragment used as a loading control for quantification were run on a 2% agarose gel and stained with ethidium bromide to confirm the size of the PCR products. All probes used were between 200 and 250 nucleotides.

Radio-isotope labeling of probes for RNA gel blots:

The PCR products were ^{32}P-labled and used for hybridization to membranes containing total RNA from sample plants as follows. Purified PCR products from the wound-responsive genes were labeled with ^{32}P labeled dCTP using DECAprimeII Random Priming DNA Labeling Kit (Ambion, #1455, Austin, Texas 78744-1832, USA). Ambion's DECAprime II DNA labeling kit is optimized to produce high specific activity probes of ≥ 2-3 x 10^9 cpm/µg. Reaction products were purified using Micro Bio-Spin Chromatography Columns (BioRAD, #732-6200, Hercules, California 94547, USA), then denatured by boiling for 3 minutes and placing on ice for 3 minutes before hybridization to the membrane.

These radio-labeled probes were then hybridized to the RNA-containing membranes using ULTRAhyb Ultrasensitive Hybridization Buffer (Ambion, #8670, Austin, Texas 78744-1832, USA). Hybridizations were overnight according to Ambion kit instructions at 42º C for the wound-responsive probes and 5 hours for the rRNA control probe. Membranes were washed according to Ambion kit instructions in 2X SSPE/0.1% (w/v) SDS solutions at 42º C and then exposed to x-ray film. The x-ray film was imaged using a Kodak Digital Science 1D Image Analysis Software (Eastman Kodak Company, Scientific Imaging Systems, Rochester, NY 14650). The pixel intensity of each band on the x-ray film was quantified using this software program and these data were used as the intensity values of each probe.

RESULTS

Basal level of gene expression in different mutants:

The levels of all of the transcripts in all of the control (unwounded) plants in all of the experiments were collected and the results recorded in Table II. The level of transcript encoding the plant defense protein, *PDF1.2* was lowest in the wild type, intermediate but not significantly different in both the jasmonic acid (*jar1*) and salicylic acid (*npr1*) mutants, and about 65% higher than wild type in the ethylene-insensitive mutant, *ein2*. In contrast, the level of the transcript encoding pathogenesis-related protein, *PR1*, was highest but rather variable in the *jar1* mutant, intermediate in wild type and *npr1*, but lowest in *ein2*. It was rather surprising to us that *PR1* was higher in the *npr1* mutant, which was discovered through its low level of expression of *PR* genes (Cao et al., 1994) than in *ein2*. The level of the transcript encoding the wall-associated kinase, *WAK1*, was virtually the same in wild type, *jar1* and *npr1*, and statistically lower in *ein2* than in wild type. The level of the lipoxygenase transcript, *LOX2*, was significantly higher in *ein2*, intermediate in wild type and *npr1* and lowest in *jar1*. Apparently the *ein2* mutation is having the most effect, since in two cases (*PR1*, *WAK1*) it had the lowest level of expression, while in two others (*PDF1.2, LOX 1*) it showed the highest level. Although the specific activity of the probes was not measured, they are similar in size, they were all made in the same way, and always used fresh, and there is no reason to suspect that their specific activity was very different from each other. If that is the case, then the total level of expression will be related to the size of the values in Table II. Thus it appears as though *PDF1.2* is the most abundant transcript, *LOX2* and *PR1* intermediate, and *WAK1* the least abundant.

The level of most transcripts was far lower in the double mutants than in the single mutants or in wild type (Table II). For instance, *PDF1.2* was totally absent in both *npr1/ein2* and in *npr1/jar1*. This is surprising because it was higher in all of the single mutants that in wild type. For some reason it appears as though knocking out (perception of) two hormones has a negatively synergistic effect of expression of this gene. The *PR1* transcript was unexpectedly higher in the *npr1/ein2* double mutant than in either of the single (*npr1, ein2*) mutants. Again, for some reason it seems as though inability to perceive two hormones has a synergistic (this time positive) effect on the basal level of gene expression. The *PR1* transcript was totally absent in the *npr1/ein2* mutant, and this was unexpected from the results with the single mutants, both of which were almost as high as, or higher than, the level in wild type. However, these results with *PR1* are more in keeping with current thought insofar as *npr1* was discovered because of its low level of *PR* gene expression (Cao et al., 1994). The level of *WAK1* transcript was always lower in any single mutant than in wild type, and even lower in both of the double mutants. Again, any two hormones (or mutants lacking the ability to perceive any two hormones), seemed to interact synergistically to prevent expression of the gene. The results with *LOX2* were almost as striking as with *PDF1.2*, insofar as the level of expression was very low in the *npr1/ein2* double mutant, and zero in the *npr1/jar1* double mutant. As with *PDF1.2* the result with *ein2* was unexpected since the level of this transcript was highest in *ein2* but very low in *npr1/ein2*.

Table II: Constitutive expression of putative wound-induced transcripts in wild type, single and double mutants.
The numbers indicate band intensity (± standard deviation) of each specific probe divided by band intensity of the 18S rRNA control probe for four transcripts in five mutants plus wild type.

	Intensity of each specific probe with standard deviation			
***Arabidopsis* plants**	***PDF1.2***	***PR1***	***WAK1***	***LOX2***
wild type	1.15±0.25	0.47±0.17	0.37±0.08	0.42±0.10
ein2	1.89±0.20	0.12±0.04	0.19±0.01	1.27±0.14
jar1	1.58±0.48	0.97±0.54	0.26±0.24	0.16±0.06
npr1	1.73±0.42	0.38±0.15	0.35±0.24	0.63±0.11
npr1/ein2	0±0	0.65±0.04	0.13±0.09	0.09±0.04
npr1/jar1	0±0	0±0	0.09±0.13	0±0

Changes in expression of the Plant Defense gene, *PDF1.2*, in response to wounding:

For these experiments, one rosette leaf of WT or of various signaling mutant Arabidopsis plants was heat-wounded and other leaves harvested immediately (zero time control) or at various times after wounding. RNA was extracted, subjected to electrophoresis, transferred to filters, and the membrane probed with the *PDF1.2* probe and then with a ribosomal RNA probe. Data from 3 to 6 samples were included in calculations for each time point for each mutant (plus WT) and the results (means plus/minus standard deviations) are shown in Figure 4A-F.

In wild type, the basal level of *PDF1.2* expression was the lowest (Fig 4A, Table II), remained unchanged for the first 5 minutes after wounding, before declining to very low levels by 15 minutes and then rising to almost double the initial value by 6 hours (Fig 4A). With the single mutants, the pattern of expression was quite similar in *ein2*, except that the initial level was highest, the decline between 5 and 15 minutes was more dramatic, and the later accumulation was much reduced (Fig 4B). The overall pattern was also quite similar in *jar1,* except there was less initial decline, but a much larger late accumulation to levels about 3-fold above the initial (Fig 4C). In contrast, the pattern was quite different in *npr1*, where

the level was essentially constant over the entire time period except for a transient peak at 15 minutes (Fig 4D). With the double mutants, the pattern was very different. The *PDF1.2* transcript was totally absent and failed to accumulate in both the *npr1/ein2* (Fig 4E) and *npr1/jar1* (Fig 4F) plants.

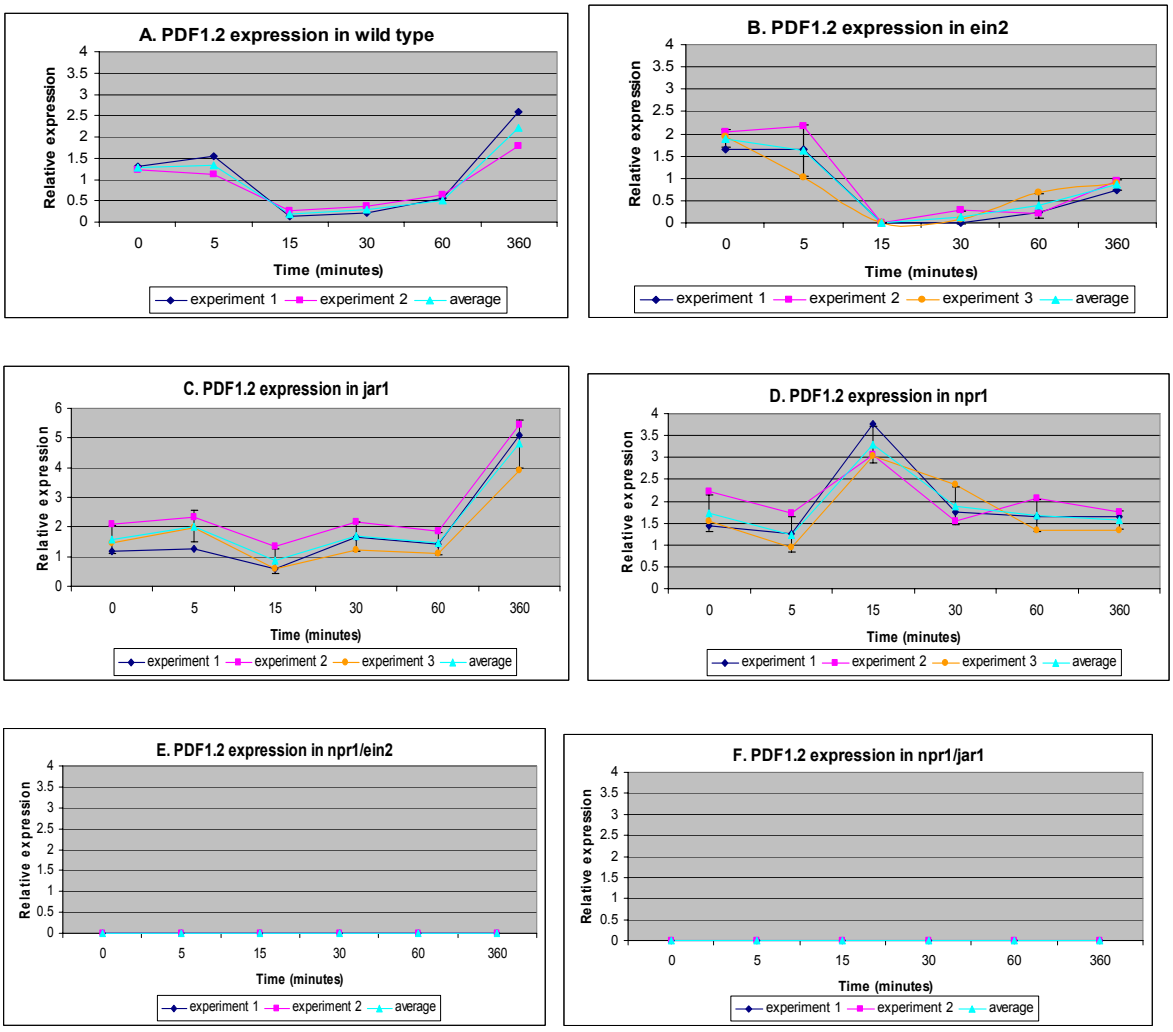

Figure 4: Changes in transcript abundance of *PDF1.2* (Plant defensin) after wounding in wild type (WT) and several signaling mutants of Arabidopsis thaliana

Wild type *Arabidopsis thaliana* plants were grown in the NCSU Phytotron, heat-wounded for different periods of time, harvested, frozen in liquid nitrogen, and RNA extracted, subjected to electrophoresis and transferred to filters. The membranes were probed with *PDF1.2*, then

washed and re-probed with 18S RNA as a control. Samples from 3 separate experiments were analyzed simultaneously and the data averaged for each time point for each mutant. However, in experiments with wild type and both double mutants one experiment gave inconsistent results and so these data were not included in the figures. Note however, these "outlier" values are included in the appendix (Table III). A corresponds to wild type, B to *ein2,* C to *jar1,* D to *npr1,* E to *npr1/ein2* and F to *npr1/jar1* plants. It should also be noted that the scale is less sensitive for *jar1* and more sensitive for both double mutants in order to more easily visualize the patterns.

Changes in expression of the pathogen-responsive gene, *PR1,* in response to wounding:

In most cases, the wound-induced changes in expression of *PR1* (Figure 5) were quite different from those of *PDF1.2* (Figure 4). In wild type, the level was low initially, increased more than 5-fold between 5 and 15 minutes, and then remained constant (Fig 5A). Similarly, the initial level in *ein2* was very low, but increased more than 10-fold within 5 minutes and then stayed almost constant over the duration of the experiment (Fig 5B). Interestingly, in *jar1*, the expression pattern of *PR1* (Fig 5C) was essentially identical to that of *PDF1.2* (Fig 4C) insofar as it was constant over the first hour, before increasing several fold by 6 hours. The level of expression of *PR1* was very low initially in *npr1* (Fig 5D) and disappeared almost entirely after wounding, exhibited a sharp drop over the first 15 minutes followed by a minor recovery at 6 hours. In the double mutant *npr1/jar1*, the level of *PR1* transcript was low initially as expected, and showed only a minor increase after wounding (Fig 5F), whereas it was substantially higher in the *npr1/ein2* mutant and showed a slight transient

increase in response to wounding (Fig 5E). Apparently the *ein2* mutation outweighs the *npr1* mutation and allows expression of the gene for this pathogenesis-related protein.

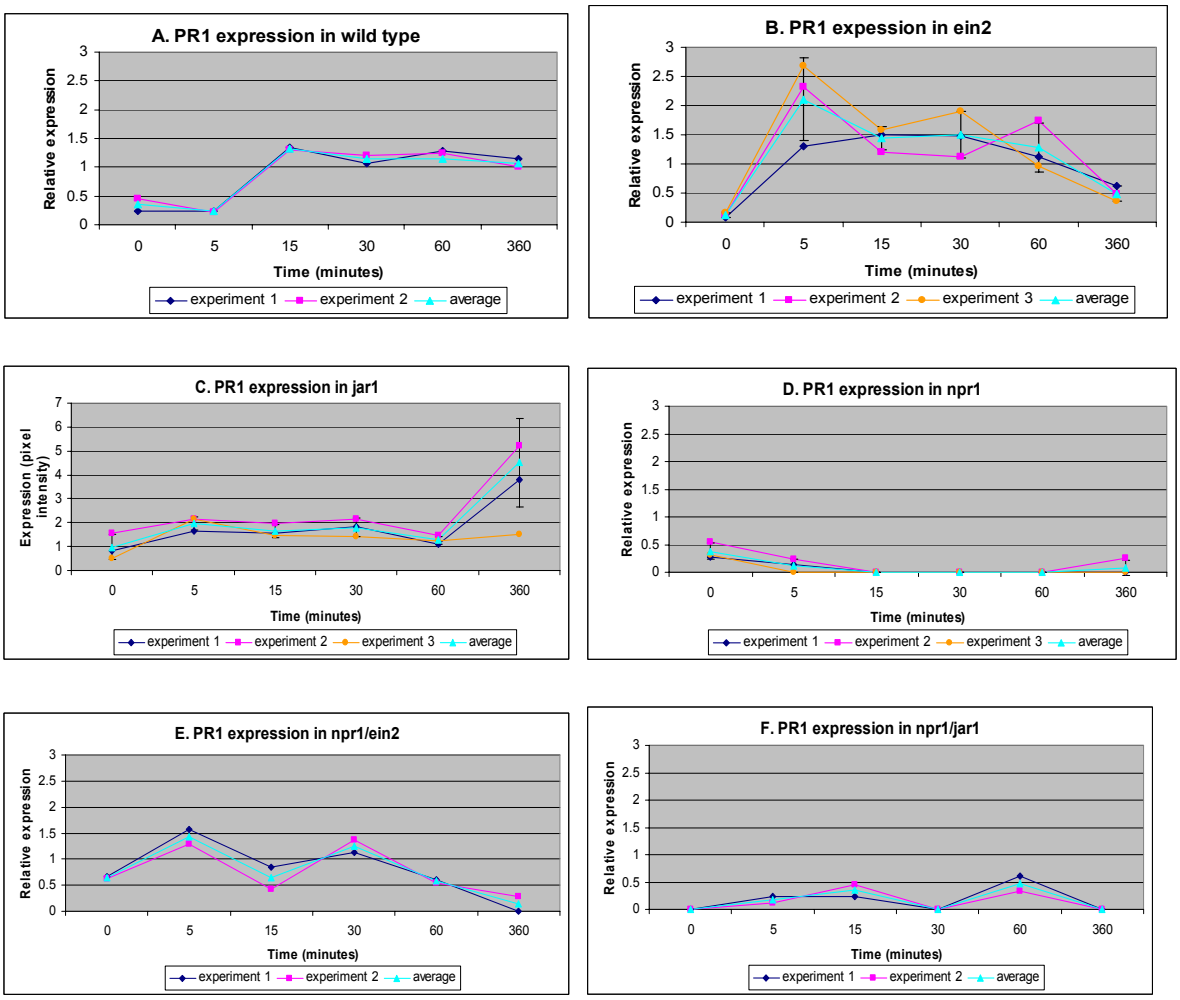

Figure 5: Changes in transcript abundance of *PR1* (Pathogenesis-related 1) after wounding in wild type (WT) and several signaling mutants of Arabidopsis thaliana.

Wild type Arabidopsis thaliana plants were grown in the NCSU Phytotron, heat-wounded for different periods of time, harvested, frozen in liquid nitrogen, and RNA extracted, subjected to electrophoresis and transferred to filters. The membranes were probed with *PR1*, then washed and re-probed with 18S RNA as a control. Samples from 3 separate experiments

were analyzed simultaneously and the data averaged for each time point for each mutant. However, in experiments with wild type and both double mutants one experiment gave inconsistent results and so these data were not included in the figures. Note however, these "outlier" values are included in the appendix (Table III). A corresponds to wild type, B to *ein2,* C to *jar1,* D to *npr1,* E to *npr1/ein2* and F to *npr1/jar1* plants. It should also be noted that the scale is less sensitive for *jar1* in order to more easily visualize the patterns.

Changes in expression of the wall-associated kinase gene, *WAK1*, in response to wounding:

Again, the wound evoked changes in expression of *WAK1* (Fig 6) were somewhat different from those of *PDF1.2* (Fig 4) and *PR1* (Fig 5). In wild type, *WAK1* transcript accumulated slowly over the first hour and then dropped sharply by 6 hours in both wild type (Fig 6A) and in *ein2* (Fig 6B), a pattern distinct from any others seen so far in this study. However, the response of WAK1 in *jar1* (Fig 6C), was again almost identical to *PDF1.2* (Fig 4C) and *PR1* (Fig 5C), being almost constant for the first hour before rising sharply by 6 hours. The pattern of expression in the *npr1* mutant (Fig 6D) was very similar to wild type (Fig 6A) showing only minor changes in response to wounding. In the double mutant, *npr1/ein2*, the basal level of expression was low and there was a slight transient increase at 15, and then a gradual decline (Fig 6E). In contrast, in the *npr1/jar1* double mutant, the pattern was similar up to 30 minutes, but then the level increased massively by 6 hours (Fig 6F).

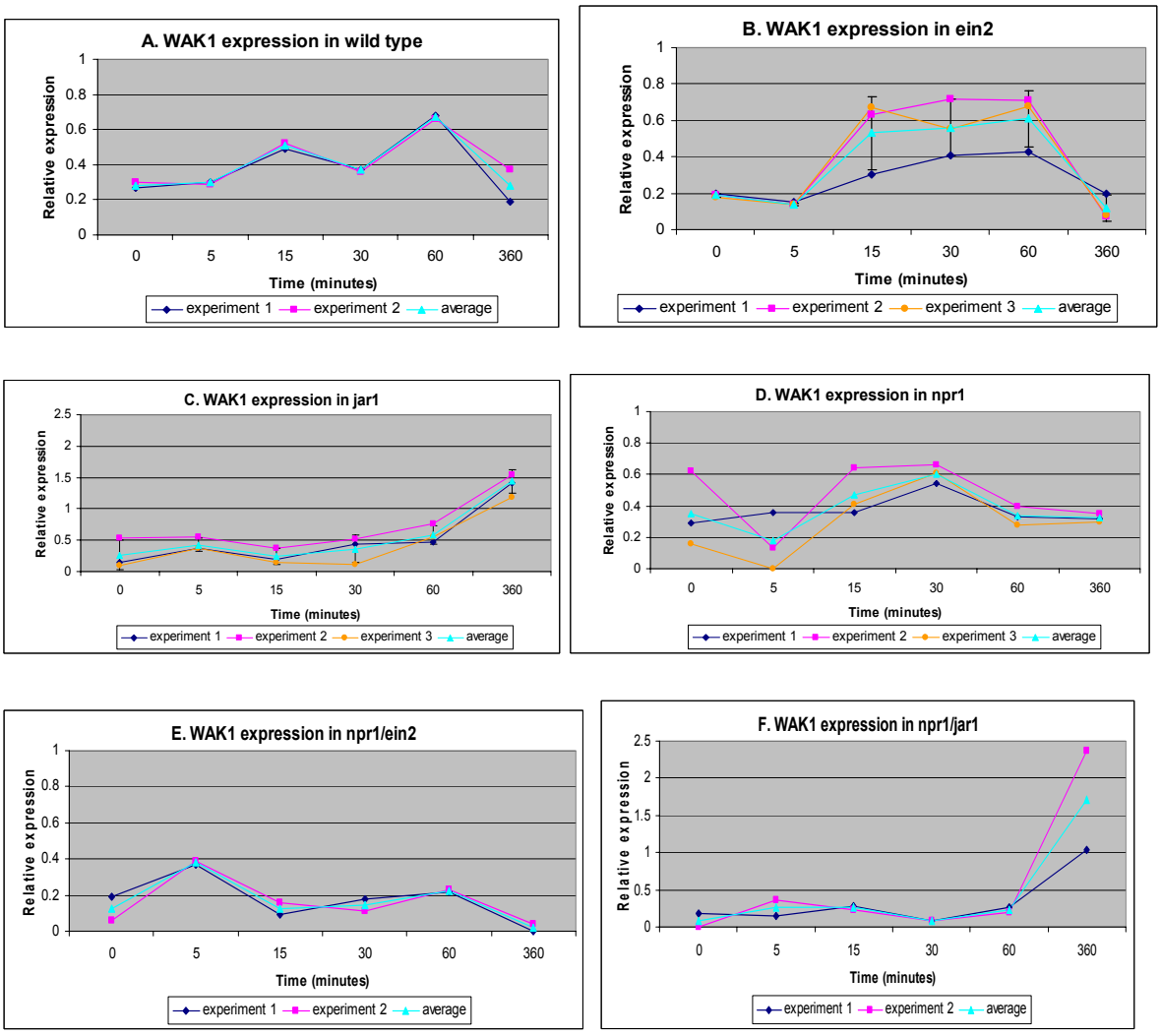

Figure 6: Changes in transcript abundance of *WAK1* (Cell wall-associated kinase 1) after wounding in wild type (WT) and several signaling mutants of *Arabidopsis thaliana*.

Wild type Arabidopsis thaliana plants were grown in the NCSU Phytotron, heat-wounded for different periods of time, harvested, frozen in liquid nitrogen, and RNA extracted, subjected to electrophoresis and transferred to filters. The membranes were probed with *WAK1*, then washed and re-probed with 18S RNA as a control. Samples from 3 separate experiments

were analyzed simultaneously and the data averaged for each time point for each mutant. However, in experiments with wild type and both double mutants one experiment gave inconsistent results and so these data were not included in the figures. Note however, these "outlier" values are included in the appendix (Table III). A corresponds to wild type, B to *ein2,* C to *jar1,* D to *npr1,* E to *npr1/ein2* and F to *npr1/jar1* plants. It should also be noted that the scale is less sensitive for *jar1* and *npr1/jar1* in order to more easily visualize the patterns.

Changes in expression of the lipoxygenase gene, *LOX2*, in response to wounding:

In wild type, *LOX2* was rather low initially, declined until 30 minutes after wounding and then increased to double the zero time value by 6 hours (Fig 7A). This pattern was every similar to *PDF1.2* in wild type (Fig 4A). In the single mutant, ein2, the basal level was much higher, but there was a (slightly erratic) decline until 60 min before showing a slight recovery (Fig 7B). As with wild type, the pattern in *ein2* was very similar to the corresponding *PDF1.2* pattern (Fig 4B). In *jar1*, there was a large accumulation in the first 5 minutes, a decline to basal levels and then a delayed increase until 6 hours (Fig 7C). This up-down-up pattern is very reminiscent of that seen in systemic wounding of tomato (Davies et al., 1997). In *npr1*, there was a slight decrease at 5 minutes, then a large, transient peak at 15 minutes, followed by a decline to below basal levels (Fig 7D). Again, the pattern was very similar to the *PDF1.2* pattern in the same mutant (Fig 4D). In both double mutants the level was lower initially, with very little change after wounding in *npr1/ein2* (Fig 7E) and a delayed increase until 1-6 hours in *npr1/jar1*. Although the absolute level was lower with *LOX2* (Fig 7) than

with *WAK1* (Fig 6), the patterns of response of the double mutants were amazingly similar (cf: Fig 7E, 7F with Fig 6E, 6F).

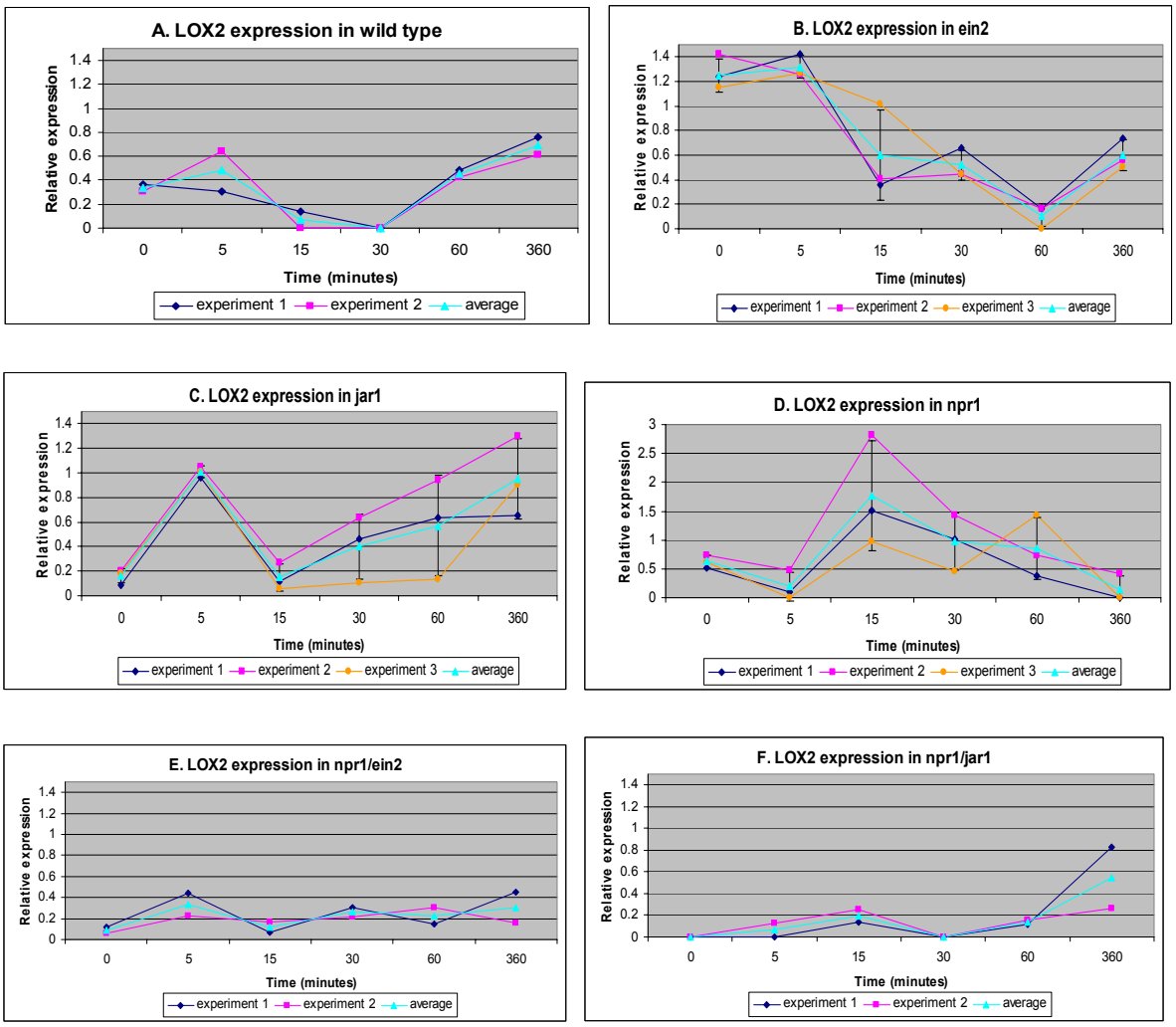

Figure 7: Changes in transcript abundance of *LOX2* (Lipoxygenase 2) after wounding in wild type (WT) and several signaling mutants of *Arabidopsis thaliana*.

Wild type Arabidopsis thaliana plants were grown in the NCSU Phytotron, heat-wounded for different periods of time, harvested, frozen in liquid nitrogen, and RNA extracted, subjected to electrophoresis and transferred to filters. The membranes were probed with *LOX2*, then washed and re-probed with 18S RNA as a control. Samples from 3 separate experiments

were analyzed simultaneously and the data averaged for each time point for each mutant. However, in experiments with wild type and both double mutants one experiment gave inconsistent results and so these data were not included in the figures. Note however, these "outlier" values are included in the appendix (Table III). A corresponds to wild type, B to *ein2,* C to *jar1,* D to *npr1,* E to *npr1/ein2* and F to *npr1/jar1* plants. It should also be noted that the scale is less sensitive for *npr1* in order to more easily visualize the patterns.

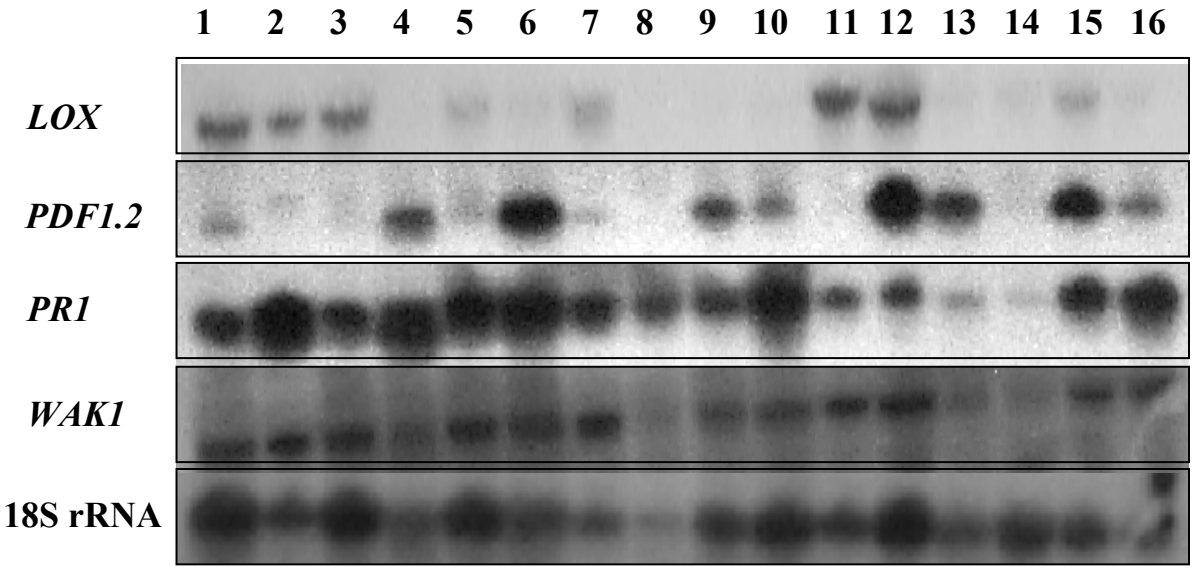

Figure 8: Typical northern blot for all of the probes used

The Northern blots shown in Figure 8 provide examples of all of the probes used for the specific transcripts and for the control 18S rRNA probe used as assays in this thesis. In all cases shown here, the probe furnished a distinct band in the appropriate location on the gel, and band intensity never exceeded the linear range. On occasion the bands were smeared towards lower molecular mass (indicating RNA degradation) and thus these samples were excluded from analysis. It should be noted that the background was lower for membranes

probed first (*LOX1, PDF1.2*, and *PR1*) then became higher for those probed later (*WAK1*, 18S rRNA).

DISCUSSION

Role of hormones in endogenous transcript levels:

In this study we measured the abundance of four wound/pathogen-related transcripts (plant defensin, non-expresser of pathogenesis related genes, wall-associated kinase, lipoxygenase) in wild type *Araboidopsis* plants and in mutants impaired in their capacity to perceive three individual hormones (ethylene, jasmonic acid, salicylic acid) and two hormone combinations (ethylene/salicylic acid and jasmonic acid/salicylic acid). Transcript abundance was measured in unwounded plants to determine whether the hormones were necessary for their endogenous expression and also after wounding to see if any of these hormones might be involved in the wound signaling. It should be noted that transcript abundance is the overall net difference between its transcription and its degradation, and so when the level increases transcription must exceed degradation, while when the level declines, degradation must exceed synthesis.

The basal (endogenous) expression of none of these transcripts was totally dependent on the ability to perceive any individual hormone (Table II). For instance with *PDF1.2*, the basal level in wild type was lower than in all three mutants, while with *PR1* and *LOX2* wild type was intermediate and only with *WAK1* was wild type highest. The ethylene mutant, *ein2*, showed the highest basal level of *PDF1.2* and *LOX2*, and the lowest level of *PR1* and *WAK1*. Ethylene perception appears not to be necessary for (and may be slightly inhibitory to) the constitutive levels of *PDF1.2* and *LOX2*, whereas it is a definite aid to constitutive levels of *PR1* and *WAK1*. Similarly, jasmonic acid perception seems unnecessary for expression of *PDF1.2*, *WAK1* and *LOX2*, but it appears inhibitory to *PR1* transcript abundance. The

salicylic acid perception mutant, *npr1*, had the least effect on endogenous transcript levels, never being significantly different from wild type. (Table II).

Transcript abundance in the double mutants was far from being a straightforward combination of the ability of the individual mutants from which they were derived. In one case (*PR1* in *npr1/ein2*) transcript level was higher than in either parent even though both of these were lower than wild type. This implies that the inability to perceive both ethylene and salicylic acid enhances the capacity to accumulate transcript, i.e., that the combination of hormone insensitivities removes a block on transcript abundance. Again, this was surprising in that an *npr1* (double) mutant would accumulate more *PR1* than wild type, especially considering that the other parent (*ein2*) had the lowest endogenous level of all. However, in all other cases (all four transcripts in *npr1/jar1*, three out of four transcripts in *npr1/ein2*, transcript level was lower than in either of the individual mutant parents and in many cases it was totally absent (Table II). Indeed, the *npr1/jar1* (salicylic acid/jasmonic acid) mutant totally eliminated expression of *PDF1.2, PR1*, and *LOX2* and clearly reduced the accumulation of *WAK1*. This implies that elimination of sensitivity to either of these two hormone combinations has a synergistic (and deleterious) affect on gene expression. There must, therefore, be major cross-talk between the hormone signaling pathways, where (in seven out of eight cases) transcript abundance was severely repressed, while in the other, abundance was strongly enhanced. Although hormone cross-talk is obviously occurring, we are unable to speculate meaningfully on whether the effect is through transcript synthesis or degradation. In this work we measured levels of four transcripts in wild type single and double mutants assaying their constitutive level, their short term response to wounding, and

their long term response to wounding. Accordingly, we find it impossible to generate a model which could encompass the complexity of the work performed here.

Role of hormones in patterns of wound-induced gene expression:

Wild type. In wild type, *PDF 1.2* showed little or no change in the first 5 minutes, a rather sharp drop to almost zero by 15 minutes followed by a steady increase until 6 hours where is was double the initial level. This we call is a "level, down, up" pattern. The decrease is similar to that seen by Gutierrez et al. (2002) in responses to mechanical wounding in Arabidopsis, and thus might result primarily from enhanced degradation. *PR1* showed a similar 5 min lag, but then increased about 5-fold between 5 and 15 minutes and then remained constant. This we call a "level, up, level" pattern. This 5-fold increase in a 10 minute period implies a large increase in the rate of synthesis, decrease in rate of degradation, or both. In contrast *WAK1*showed a slow, unsteady 2-fold increase from zero time to 60 minutes followed by a decline to basal levels. This we call an "up, down" response and implies a relatively short-lived response to wounding. *LOX2* showed a "level, down, up" response basically similar to *PDF1.2*, but rather less dramatic.

***ein2*.** In *ein2*, *PDF1.2* had a similar overall "level, down, up" response as in wild type, but the level was higher to start with, the drop was precipitous (many-fold between 5 and 15 minutes) and the recovery was less. Again, this might indicate a degradation (RNase) response as seen by Gutierrez et al. (2002). The pattern of *PR1* response in *ein2* was different from any other pattern. Here, the level increased many-fold within the first 5

minutes, and then was maintained for 1 hour before declining. This was an "up, level, down" response, and the initial, massive accumulation of transcript was typical of man heat-wound gene responses in tomato and implies (but does not prove) a role for enhanced RNA synthesis. Regardless of the mechanism behind the increase in transcript, these data do reinforce the notion that some very rapid signal is being transmitted from the wounded leaf to the recipient leaf. It is unlikely that this is a chemical transported in the phloem (Wildon et al., 1992), but is much more likely to be a hydraulic surge transmitted through the xylem and affecting gene expression in adjacent living cells (Davies, 1993; Stankovic et al., 1998). Lawton et al. (1994) found that levels of *PR1* transcript were lower in the *ein2* mutant compared to wild type. The results here indicate that *PR1* expression was lower at the long time point (360 minutes) but not at the shorter time points (0-60 minutes) for *ein2* compared to wild type. *WAK1* in *ein2* exhibited the "level, up, down" response seen with *WAK1* in wild type, although the variation between 15 and 60 minutes almost masked this pattern. *LOX2* showed a "level, down, up" pattern of expression similar to that of wild type, but with a 3-fold higher initial level, a bigger initial drop, and a smaller late recovery.

jar1. In wild type, the expression of *PDF1.2* was different from any other seen so far, and showed essentially no change over the first 60 minutes followed by a 3-fold increase between 1 and 6 hours. This "level, up" pattern is more typical of the "genuine" slowly-accumulated wound genes (Davies, 1997). *PR1* had an almost identical pattern to *PDF1.2*, with no change for 1 hour and then a 2-3-fold increase at 6 hours. Interestingly, *WAK1* also had a very similar "level, up" response with the majority of the increase occurring between 1 and 6 hours. One way to interpret these results is to suggest that inability to sense jasmonic

acid prevents any kind of response to the rapid (hydraulic) signal, but permits a response to a later (chemical?) signal (Stankovic et al., 1998). However, this interpretation is rendered unlikely by the results with *LOX2*, which shows the typical "up, down, up" pattern seen in many of the tomato genes that respond to the rapid wound signal (Davies, 1997). Indeed, the 10-fold increase in transcript accumulation within the first 5 minutes is as large as any seen in the tomato system, and it is these transiently-increase transcripts that do not get recruited into polysomes, but are degraded before they can be translated (Stankovic & Davies, 1997). We have no proven explanation for this transient accumulation, but suspect that it might be part of a general defense mechanism where mRNAs unprotected by ribosomes (including viral mRNAs, newly-arrived through a wound site) would be preferentially degraded.

***npr1*.** The level of *PDF1.2* in *npr1* was more-or-less unchanged except for a transient 2-fold increase at 15 minutes, i.e., a "level, up, down, level" response. Expression *PDF1.2* is believed to be a JA-dependent (Penninckx et al., 1998) and Gupta et al. (2000) found that mutants that were impaired in SA accumulation exhibited enhanced responses to inducers of JA-dependent gene expression. These results indicate that the *npr1* mutant had increase expression of *PDF1.2* compared to wild type at 0-60 minutes but not at 360 minutes after wounding. *PR1*, which, by definition, would be expected to be low in the *npr1* mutant, showed a steady decline in response to wounding until it disappeared by 15 minutes. In contrast, *WAK1* showed a "down, up, down" pattern, that was much less obvious than *PDF1.2*. However, *LOX2* showed a much more exaggerated "down, up, down" pattern than even *PDF1.2*, with a 10-fold increase between 5 and 15 minutes followed by a 10-fold decline thereafter. It would appear that the ability to perceive salicylic acid is needed for the

wound-induced increase in the pathogenesis-related protein, *PR1*, but not for the rapid (15 min), transient increase in the other three transcripts.

npr1/ein2. As seen in Table II, *PDF1.2* was absent from this mutant at zero time, although there was a small, delayed increase peaking at 60 minutes, showing a "level, up, down" response. The absence of *PDF1.2* at zero time was unanticipated, since both of the parents (*ein2, npr1*) had abundant levels initially, but the transient increase was not totally unexpected since one of the parents (*ein2*) was unchanged over the first 5 minutes, while the other parent (*npr1*) showed a transient 2-fold increase at 15 minutes. In marked contrast, all of the other transcripts all had a similar pattern of "up, down, level/down" with a 3-4-fold peak at 5 minutes followed by a slow decline in *PR1* and *WAK1* or maintenance in *LOX2* over the next 6 hours. The expression of *PR1* in the double mutant was quite similar to that in the *ein2* parent, but different from the *npr1* parent, since it was essentially absent there, indicating that ethylene perception is a more potent factor than salicylic acid perception. The expression of *WAK1* in *npr1/ein2* was somewhat similar to that in the parents, except that in both parents the level peaked at 15-30 minutes, whereas it peaked at 5 minutes in the double mutant. *LOX2* expression was also somewhat similar to both parents. In *npr1/ein2* it peaked at 5 minutes before declining; in *ein2* it was level for 5 minutes before declining; and in *npr1* it peaked at 15 minutes before declining. Apparently neither hormone plays a dominant role in control of *LOX2* expression. Schenk et al. (2000) found that SA and ethylene function together to coordinately induce the same genes by exogenous treatments with these hormones. In this work, the results for *PDF1.2* expression support the work by Schenk et al. (2000), in that expression of *PDF1.2* was altered in both the *npr1* and *ein2* single mutants but almost

eliminated in the *npr1/ein2* double mutant. This indicates that cross-talk between these two hormones in necessary for expression of this gene. However, results from the other three genes (*PR1, WAK1* and *LOX2*) do not indicate that cross-talk between SA and ethylene is necessary for transcript expression.

npr1/jar1. *PDF1.2* was present initially in trace amounts in *npr1/jar1* and disappeared immediately on wounding ("down-level"). This was totally different from both parents, where it was abundant initially and then in *jar1* it stayed level until peaking at 6 hours, while in *npr1* it peaked at 15 minutes. Again it seems that mutants lacking perception for two hormones are far less able to accumulate transcript than either single mutant. *PR1* was low initially but showed an "up, down, up" pattern of expression with the early peak at 15 minutes and the maximal level at 6 hours. *PR1* expression in the double mutant was quite similar to that in *jar1*, except that there was no huge increase at 6 hours. It was totally different from the *npr1* parent where it was almost totally absent. It seems as though inability to perceive jasmonic acid overcomes the inhibition wrought by the inability to perceive salicylic acid. Although the basal of both *WAK1* and *LOX2* was lower in *npr1/jar1* than in either parent, the pattern of expression was almost identical to that of *jar1*, where there was relatively little change over the first hour, but quite a large accumulation at 6 hours. For both of these transcripts it appears as though inability to perceive jasmonic acid is plays a major role, primarily by delaying the major response until after a slow (chemical) signal has reached the recipient leaf.

Summary and Future Considerations:

Although this work is a promising step toward understanding hormone cross-talk during stress perception, much remains to be done. Only four wound/pathogen responsive genes were examined in this work and, as is often the case, some of the results contradicted previous findings. A more in depth approach can be taken by using microarray to study the wound response in these double mutants and others. Although the transcription of many genes is regulated by wounding stress, as seen in this work, different wound-responsive genes can respond very differently in terms of transcript levels and kinetics of expression. A genomic approach would help to unravel these complexities.

Also, it is not know how these changes in transcript abundance, particularly the rapid changes, occur. The mechanisms of transcription and degradation would be interesting to investigate and could perhaps be performed by isolation of polysomes. This again would best be done on a genomic scale using microarray techniques.

Additionally, this work provides evidence that hormones are an important component of wound-stress response and that cross-talk between hormones in necessary for systemic accumulation of wound-responsive gene transcripts. However, it does not show that hormones are the mobile signals that travel from the local wound site to the systemic tissue. It is possible that other signals, such as electrical pulses, are the systemic signal but hormones must be present in the systemic tissue to receive the signal. It would be interesting to examine the cross-talk between hormones and other stress signals such as electrical pulses, using these and other single and double hormone mutants.

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APPENDICIES

Table III. Full data set of transcript abundance used for figures and table in the thesis. These values in this table include all of those used in the figures and tables in the results section and also values from some experiments which gave very different results. These different results are noted with an asterisk (*) and provided for the reader to have access to the full data set. Averages that include these outlier results are also marked with an asterisk (*).

Wild Type	PDF1.2	PR1	WAK1	LOX2
0 min	1.3	0.24	0.27	0.37
	1.24	0.46	0.3	0.31
	0.27*	0.16*	0.43*	0.81*
average 1	1.27	0.36	0.28	0.34
average 2	0.94*	0.27*	0.33*	0.50*
	1.28	0.46	0.53	0.57
	0.78	0.92	0.55	0.36
average 3	1.15	0.52	0.41	0.4
average 4	0.97*	0.65*	0.44*	0.54*
5 min	1.56	0.24	0.3	0.31
	1.11	0.22	0.29	0.64
	0.17*	1.21*	0.59*	1.01*
average 1	1.33	0.23	0.3	0.48
average 2	0.94*	0.55*	0.39*	0.59*
15 min	0.13	1.34	0.49	0.14
	0.26	1.3	0.52	0
	0*	1.08*	0.37*	0.59*
average 1	0.2	1.32	0.51	0.07
average 2	0.13*	1.24*	0.46*	0.24*
30 min	0.22	1.07	0.37	0
	0.37	1.21	0.36	0
	0.22*	0.99*	0.24*	0*
average 1	0.3	1.14	0.37	0
average 2	0.27*	1.09*	0.32*	0*
60 min	0.55	1.28	0.68	0.48
	0.64	1.25	0.66	0.42
	0.93*	1.44*	0.59*	0.07*
average 1	0.51	1.14	0.67	0.45
average 2	0.62	1.22*	0.64*	0.32*
	0.35	0.9	0.48	0.35

npr1	PDF1.2	PR1	WAK1	LOX2
0 min	1.45	0.27	0.29	0.52
	2.22	0.55	0.62	0.74
	1.53	0.31	0.16	0.62
	1.09*	0*	0.02*	0.12*
average 1	1.75	0.38	0.36	0.63
average 2	1.58*	0.28*	0.27*	0.5*
5 min	1.26	0.13	0.36	0.09
	1.73	0.23	0.13	0.47
	0.94	0	0	0
	5.02*	0*	1.72*	4.56*
average 1	1.24	0.12	0.18	0.19
average 2	2.19*	0.09*	0.55*	1.28*
15 min	3.77	0	0.36	1.51
	3.06	0	0.64	2.82
	3.03	0	0.41	0.98
	4.52*	0.43*	0.66*	3.73*
average 1	3.29	0	0.47	1.77
average 2	3.59*	0.11*	0.52*	2.26*
30 min	1.76	0	0.54	1.02
	1.55	0	0.66	0.46
	2.37	0	0.61	1.44
	2.52*	0.26*	0.59*	1.97*
average 1	1.89	0	0.6	0.97
average 2	2.05*	0.07*	0.6*	1.22*
60 min	1.64	0	0.33	0.38
	2.06	0	0.4	0.73
	1.34	0	0.28	1.44
	2.24*	0.17*	.35*	1.86*
average 1	1.35	0	0.34	0.85
average 2	1.57*	0.04*	0.34*	1.1*

360 min	2.58	1.14	0.19	0.76	360 min	1.65	0	0.32	0
	1.79	1.01	0.37	0.61		1.74	0.25	0.35	0.41
	1.02*	1.13*	0*	1.13*		1.33	0	0.3	0
average 1	2.21	1.07	0.28	0.69		2.06*	0.3*		0.40*
average 2	1.92*	1.1*	0.19*	0.83*	average 1	1.57	0.08	0.32	0.14
	2.27	1.05	0.27	0.85	average 2	1.69*	0.14*		0.2*
ein2	PDF1.2	PR1	WAK1	LOX2	*npr1/ein2*	PDF1.2	PR1	WAK1	LOX2
0 min	1.66	0.09	0.2	1.24	0 min	0	0.67	0.19	0.12
	2.05	0.12	0.19	1.42		0	0.62	0.06	0.06
	1.94	0.16	0.18	1.15		0*	0.75*	0.09*	0.06*
	0.62*	0.26*	0.15*	0.94*	average 1	0	0.64	0.13	0.09
average 1	1.84	0.12	0.19	1.25	average 2	0	0.68	0.11	0.08
average 2	1.56*	0.16*	0.18*	1.18*					
5 min	1.66	1.31	0.15	1.42	5 min	0	1.58	0.37	0.44
	2.17	2.33	0.14	1.26		0	1.29	0.39	0.23
	1.03	2.69	0.14	1.27		0*	4.09*	0.39*	0.64*
	0.59*	1.12*	0.21*	1.14*	average 1	0	1.44	0.38	0.34
average 1	1.62	2.11	0.14	1.32	average 2	0*	2.32*	0.38*	0.44*
average 2	1.37*	1.86*	0.16*	1.27*					
15 min	0	1.51	0.3	0.36	15 min	0	0.85	0.09	0.07
	0	1.21	0.63	0.41		0	0.42	0.16	0.17
	0	1.59	0.67	1.02		0*	0.63*	0.2*	0.18*
	0.14	1.65*	0.11*	1.35*	average 1	0	0.64	0.13	0.12
average 1	0	1.44	0.53	0.6	average 2	0*	0.63*	0.15*	0.14*
average 2	0.05*	1.49*	0.43*	0.79*					
30 min	0	1.48	0.41	0.66	30 min	0	1.13	0.18	0.3
	0.28	1.12	0.72	0.45		0	1.36	0.11	0.22
	0.07	1.91	0.55	0.45		0*	0.7*	0.09*	0.14*
	0*	1.01*	0.38*	0*	average 1	0	0.16	0.15	0.26
average 1	0.12	1.5	0.56	0.52	average 2	0*	1.06*	0.13*	0.22*
average 2	0.09*	1.38*	0.52*	0.39*					
60 min	0.23	1.11	0.43	0.16	60 min	0	0.61	0.22	0.15
	0.21	1.74	0.71	0.16		0	0.55	0.23	0.3
	0.68	0.96	0.68	0		0.04*	1.07*	0.16*	0.38*
	0.32*	1.09*	0.38*	0*	average 1	0	0.58	0.23	0.23
average 1	0.38	1.28	0.61	0.11	average 2	0.01*	0.74*	0.2*	0.28*
average 2	0.36*	1.22*	0.55*	0.08*					
360 min	0.73	0.63	0.2	0.74	360 min	0	0.01	0	0.45
	0.93	0.49	0.07	0.56		0	0.28	0.04	0.16
	0.89	0.35	0.08	0.5		0.01*	0*	0*	0*
	0*	0.1*	0.47*	0.22*	average 1	0	0.15	0.02	0.31
average 1	0.85	0.49	0.12	0.6	average 2	0*	0.1*	0.01*	0.2*
average 2	.64*	0.39*	0.21*	0.51*					

jar1	PDF1.2	PR1	WAK1	LOX2	*npr1/jar1*	PDF1.2	PR1	WAK1	LOX2
0 min	1.19	0.82	0.15	0.09	0 min	0	0	0.18	0
	2.09	1.56	0.53	0.2		0	0	0	0
	1.49	0.52	0.1	0.18		0.04*	0.23*	0.1*	0*
	0.37*	1.63*	0.01*	1.63*	average 1	0	0	0.09	0
average 1	1.62	0.97	0.26	0.16	average 2	0.01*	0.08*	0.09*	0*
average 2	1.31*	1.38*	0.2*	0.55*					
5 min	1.27	1.63	0.38	0.96	5 min	0	0.23	0.15	0
	2.31	2.14	0.56	1.05		0	0.12	0.37	0.13
	1.97	2.13	0.38	1.01		0*	0*	0.18*	0.11*
	0.41*	1.38*	0.12*	0.18*	average 1	0	0.18	0.26	0.07
average 1	2.02	1.97	0.43	1.01	average 2	0*	0.12*	0.23*	0.07*
average 2	1.87*	1.82*	0.36*	0.8*					
15 min	0.61	1.55	0.19	0.12	15 min	0	0.23	0.28	0.14
	1.33	1.95	0.38	0.27		0	0.46	0.23	0.25
	0.61	1.45	0.14	0.06		0*	0*	0.15*	0.13*
	0.31*	0.62*	0.05*	0*	average 1	0	0.35	0.26	0.2
average 1	0.85	1.85	0.24	0.15	average 2	0*	0.23*	0.22*	0.07*
average 2	0.72*	1.36*	0.19*	0.11*					
30 min	1.66	1.83	0.44	0.46	30 min	0	0	0.09	0
	2.18	2.17	0.52	0.63		0	0	0.09	0
	1.21	1.41	0.11	0.11		0*	0*	0.12*	0.2*
	0.43*	1.77*	0*	0*	average 1	0	0	0.09	0
average 1	1.68	1.8	0.36	0.4	average 2	0*	0*	0.1*	0.12*
average 2	1.37*	1.8*	0.27*	0.3*					
60 min	1.43	1.12	0.43	0.63	60 min	0	0.6	0.27	0.12
	1.86	1.45	0.76	0.94		0	0.33	0.19	0.16
	1.11	1.23	0.56	0.13		0*	0*	1.21*	0.77*
	0.63*	0.59*	0.08*	0*	average 1	0	0.47	0.23	0.14
average 1	1.45	1.27	0.58	0.57	average 2	0*	0.31*	0.56*	0.36*
average 2	1.26*	1.10*	0.46*	0.43*					
360 min	5.11	3.82	1.42	0.65	360 min	0	0	1.04	0.83
	5.45	5.21	1.55	1.3		0	0	2.37	0.26
	1.98*	1.52*	1.17*	0*		0*	1.57*	1.04*	0.23*
average 1	5.28	4.51	1.49	0.97	average 1	0	0	1.71	0.55
average 2	4.18*	3.51*	1.43*	0.66*	average 2	0*	0.52*	1.48*	0.34*