MOLECULAR INSIGHTS INTO ARABIDOPSIS RESPONSE TO *MYZUS PERSICAE* SULZER (GREEN PEACH APHID)

by

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B.Sc., Osmania University, 1995
M.Sc., Hyderabad Central University, 1997

AN ABSTRACT OF A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Division of Biology
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2005

ABSTRACT

Phloem-feeding insects like aphids feed on a variety of crop plants and limit plant productivity. In addition they are vectors for important plant viruses. Efforts to enhance plant resistance to aphids have been hampered by lack of sufficient understanding of mechanisms of plant defense against aphids. I have utilized a plant-aphid system consisting of the model plant *Arabidopsis thaliana* and the generalist aphid, *Myzus persicae* Sulzer (green peach aphid [GPA]), to study plant response to aphids. These studies have demonstrated an important role of premature leaf senescence in controlling aphid growth in Arabidopsis. Molecular and physiological studies suggest that the Arabidopsis *PAD4 (PHYTOALEXIN DEFICIENT 4)* gene modulates the GPA feeding-induced senescence process. Furthermore, in comparison to the wild type plants, GPA growth was higher on *pad4* mutant plants, suggesting an important role for *PAD4* in plant defense against GPA. In contrast, constitutive expression of *PAD4* in transgenic Arabidopsis enhanced basal resistance against GPA. Unlike its involvement in plant defense against pathogens, the role of PAD4 in Arabidopsis resistance to GPA is independent of its involvement in phytoalexin biosynthesis and of its interaction with EDS1, a PAD4-interacting protein. Instead, the heightened resistance to GPA in these *PAD4* constitutively expressing plants was associated with the rapid activation of leaf senescence. The association of premature leaf senescence in basal defense against GPA is supported by our observation that in comparison to the wild type plant, GPA growth was restricted on the Arabidopsis hypersenescence mutants, *ssi2* and *cpr5*.

Gene expression studies suggested some overlap between plant responses to pathogens and aphids, for example, activation of genes associated with the salicylic acid (SA) signaling pathway. However, the characterization of aphid performance on Arabidopsis SA biosynthesis and signaling mutants have ruled out the involvement of SA signaling in controlling aphid growth.

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ACKNOWLEDGEMENTS

I would like to gratefully acknowledge my major advisor Dr. Jyoti Shah who has not only been a guru to me but also a great friend. I thank Dr. Shah for his patience, advice and for his assistance in my Ph.D. research program. I would also like to thank my committee members Dr. Muthukrishnan and Dr. Ruth Welti for their generous help in mentoring me in scientific writing. I am also deeply thankful to Dr. John Reese and Dr. Jan E Leach for their advice and helpful scientific discussions. I also wish to acknowledge my external chair Dr. Allan Fritz. I am thankful to Dr. Parker, for providing me with useful mutant lines.

I am also grateful to all the past and present members of the Shah Lab, for their generous help and support during my Ph.D. program, Dr. Ratnesh chaturvedi, Dr Aparna Patankar, Dr. Ragiba Makandar for useful scientific discussions and Dr. Ashis Nandi for showing me what it takes to be a successful post doctorate. I would also like to thank my fellow graduate and undergraduate students in the Shah Lab. Especially Kartik Krothapalli for his friendship, Caleb Knepper and Susan Park for their help during the mutant screening program. I would also like to thank, Jessica Mortan, Joe Louis, Vijay Singh and Vamsi Nalam for their help in proofreading my thesis. I would also like to thanks my old friends, Dr. Raghava Boddupalli, Dr. Santanu Dasgupta and Dr. Gerard F. Barry for motivating me towards Ph.D. studies. Lastly, I want to thank my dad, mom, sisters and my younger brother for always believing in me and extending their full support in every aspect of my life.

DEDICATION

I dedicate this thesis to my parents Mr. Pegadaraju Padmanabhan and Mrs. P. Rajeshwari my sisters, Aparna & Anitha, and my beloved younger brother Kamalakar.

LIST OF ABBREVIATIONS

ACC Oxidase: 1-aminocyclopropane-1-carboxylate- Oxidase

BGL2: β-1,3-glucanase

CPR5: *CONSTITUTIVE EXPRESSOR OF PR GENES 5*

DDRT-PCR: Differential-Display-Reverse Transcriptase –Polymerase Chain Reaction

DPR: *DEFENSE RELATED PROTEIN*

EDS5: *ENHANCED DISEASE SUSCEPTIBILITY 5*

GPA: Green peach aphid

HPL: hydrogen peroxide lyase

HR: hypersensitive response

IGL: INDOLE-3-GLYCEROL PHOSPHATE LYASE

IPT: *ISOPENTYL TRANSFERASE*

JA: Jasmonic acid

MeSA: Methyl Salicylate

Mi: *Meloidogyne incognita*

NO: Nitric oxide

NPR1: *NON EXPRESSOR OF PR GENES 1*

PAD3: *PHYTOALEXIN DEIFICENT 3*

PAD4: *PHYTOALEXIN DEFICIENT 4*

PI: protease inhibitors

PAL: phenylalanine ammonia lyase

PR1: PATHOGENESIS RELATED-1

SA: Salicylic acid

SAG101: SENESCENCE ASSOCIATED GENE 101

SID2: *SALICYLIC ACID INDUCTION DEFICIENT 2*

SLW1: *SLIVER WHITE FLY 1*

SLW3: *SLIVER WHITE FLY 3*

STC1: SESQUITERPENE CYCLASE GENE 1

SSI2: SUPPRESSOR OF SA INSENSITIVITY 2

VOC: Volatile organic compounds

Introduction and Objectives

INTRODUCTION

One of the practical means of attaining higher yields in crop plants is by minimizing the damage caused due to insect infestations. It is estimated that about 14% of losses of the total agricultural production is pest associated (Oerke et al., 1994). Worldwide crop losses to insects, despite the >$ 10 billion spent annually on chemical insecticides, are estimated to exceed $90 billion per annum (Shah et al., 1995; Demaagd et al., 1999; Sharma et al., 2000). Conventional methodologies such as breeding for insect resistance and usage of insecticides to control insect pests pose certain limitations. Hence, a major emphasis has been to utilize modern scientific approaches like biotechnology in crop protection programs. Integration of *Bt* (*Bacillus thuringiensis*) transgenic technology in routine crop improvement programs is proving to be successful, as evident from the FDA approval of several transgenic insect resistant crops. The global area of *Bt* transgenic crops has increased from 1.1 mha (million hectares) to 15.6 mha between 1996 to 2004 (James C, 2004). However, the currently available transgenic technology for insect control is exclusively targeted to chewing class of insects. None target phloem feeders like aphids which are major plant pests, and vectors for >100 economically important plant viruses (Kennedy et al., 1962; Blackman & Eastop, 1985; Matthews, 1991). Over 250 species of aphids feed on a wide variety of plants including cereals, fruits, vegetables and horticultural species (Blackman & Eastop, 1985). While some aphids have a narrow host range, others like the green peach aphid (*Myzus persicae* [GPA]) have a wide host range covering more than 50 families of plants. Hence, it is capable of spreading viruses amongst different plant species (Kennedy et al., 1962). Aphid infestation also increases damage caused by growth of fungi in the excreted

honeydew drops. Plant resistance to aphids will not only minimize losses but also control spread of important plant viruses. However, our lack of knowledge of plant defense response against aphids has hampered progress on this front.

Aphids belong to a broad category of piercing/sucking group of insects. The piercing/sucking group of insects pierce cells/tissues with stylets and consume copious amount of fluids. While some feed on mesophyll cells, others like thrips feed on epidermal and parenchymal cells (Walling, 2000). Aphids, by contrast selectively feed on the photoassimilate present in the sieve element. Aphids use their incredibly slender stylet to penetrate intercellular spaces between the epidermis and mesophyll cell to access the sieve element for feeding. Ocasssional puncturing of plant tissue by aphid stylets can result in injection of aphid salivary secretions into the plant cell and ingestion of the plant cell material (Tjallingii, 1990). Two types of saliva are injected by aphids into plants; gelling saliva which polymerizes to form a protective sheath around the stylets, and a watery saliva, which contain several enzymes like peroxidases, pectinases, cellulases, lipases and β-glucosidases, which are released into the phloem sieve element (Miles, 1999). A diagram depicting the intercellular mode of aphid feeding is shown in Fig. 1.

Mechanism(s) of Plant Response to Insect Herbivory

Our current understandings of plant defense against insects stem from studies that in- volve chewing insects. Plant defenses against insect pest can be broadly classified into two major categories: preformed and induced. The physical barrier provided by the cell wall and the cuticle, and the insecticidal allelochemicals (e.g. glucosinolates) are

examples of preformed factors. In addition, insect feeding activates several direct defenses for example, the synthesis of insecticidal molecules like phenolics, alkaloids, terpenoids & protease inhibitors (Karban and Baldwin, 1997). Glucosinolates posses dual roles; some of them serve as deterrents against generalist herbivores while others acts as attractants to insects that are specialized feeders on glucosinolates containing plants (Rask et al., 2000). Glucosinolates themselves are not insecticidal. The action of myrosinase on glucosinolates produces isothiocynates and nitriles which are biologically active as insecticides (Chew et al., 1988; Louda & Mole 1991; Rask et al., 2000). A recent study demonstrates that overexpression of a novel calmodulin–binding nuclear protein, IQD1 (IQ-Domain 1), stimulated glucosinolate accumulation in Arabidopsis and caused reduced growth of the generalist-phloem feeder green peach aphid (*Myzus persicae*) as well as against a generalist-chewing lepidopteran, cabbage looper (*Trichopulsia ni*).

In addition to direct defense, plants also activate indirect defenses that attract predators and parasitoids of herbivores to the infested plant. These indirect defenses include synthesis of various volatile organic compounds (VOC) that are emitted by the insect damaged plant (Pare et al., 1999). Some of these volatiles serve as systemic airborne signals, which activate systemic resistance to subsequent insect feeding (Hildebrand, 1993; Hardie, 1994; Walling et al., 2000; Vancanneyt, 2001). Since, this phenomenon reduces insect feeding on the plant; volatiles may be regarded as a component of the indirect defense system in the plants. An elicitor of volatile production, volicitin or N-(17-hydroxylinolenoyl)-L-glutamine, has been isolated from oral secretions

of beet armyworm (Alborn et al., 2000). When volicitin is applied to damaged maize leaves, it triggers the emission of parasitoid-attracting volatiles from the plant. The fatty acid component of this insect 'spit factor' (linolenic acid) is derived from the plant host, and glutamine is of insect origin. Volcitin induces the production of plant volatiles and insect infested plants results in a tritophic interaction which protects the plants by attracting insect parasitoids. Understanding the molecular basis of herbivore-induced VOC production in maize was advanced with the identification of the maize *STC1*(*SESQUITERPENE CYCLASE 1*) gene, which is involved in the synthesis of naphthalene-based sesquiterpenoid (Shen et al., 2000) and *IGL*, which encodes indole-3-glycerol phosphate lyase and is involved in the release of indole (Frey et al., 2000). Both *IGL* and *STC1* are induced by volicitin and are involved in the biosynthesis of components of maize's herbivore-induced VOC bouquet.

The usage of cDNA microarray techniques and Differential-Display-Reverse transcriptase-Polymerase Chain Reaction (DDRT-PCR) has provided additional glimpses on herbivore–induced gene expression in plants. Early microarray experiments to study plant-insect interactions, utilized a limited set of preselected 150 Arabidopsis genes to analyze plant response to *Pieris rapae* (cabbage white fly) larvae feeding. Comparison of Arabidopsis genes induced by wounding and herbivore feeding suggests that herbivore attack modifies wound responses (Reymond et al., 2000). For instance, expression of several mechanical wounding-activated genes were down regulated in *Pieris rapae* infested Arabidopsis, indicating that feeding by chewing insects is not equivalent with mechanical wounding event.

In contrast, to feeding by the chewing insects, many phloem–feeders cause minimal visible wounding damage to the plant. Consistent with this, wound-induced proteins PI (protease inhibitors) and leucine aminopeptidase are not activated by whitefly feeding (Walker and Perring, 1994). Likewise, phloem–feeding aphids do not increase *pin2* (proteinase inhibitor 2) mRNA levels in tomato plants (Fidantsef et al., 1999). However, GPA feeding on Arabidopsis induced the expression of pathogenesis–related genes (*PR1*, β-1,3-glucanase and chitinases) and the *PDF1.2* genes (Van der Westhuizen et al., 1998; Moran et al., 2002). Similarly, whitefly and potato aphid feeding on tomato activated the expression of the *PR* genes (Walling, 2000). In addition, limited studies demonstrate that plants also activate unique responses to phloem feeders (Van deVen et al., 2000). For instance, silver whitefly feeding on squash activated the expression of the *SLW1* and *SLW3* gene. Interestingly, both *SLW1* and *SLW3* (sliver white fly 1, 3) expression were not induced in response to the signaling molecules (NO, JA, ethylene, SA, abscisic acid), implicating that plants posses novel defense mechanism(s) that are induced in response to aphid feeding.

Resistance to Piercing/Sucking Herbivores

Although gene for gene for gene type resistance has been extensively reported and studied in plant-pathogen interactions, only a few cases of gene for gene type resistance mechanism have been reported for plant-aphid interaction. For example, the *Nr* gene in lettuce confers resistance to a single aphid species, *Nasonovia ribisnigri* (Helden et al., 1993). Likewise the *sd1* gene in apple mediates resistance to two biotypes of the aphid,

Dysaphis devecta (Roche et al., 1997) and *Mi1.2* of tomato that confers resistance to the potato aphid, *Macrosiphum euphorbiae* (Rossi et al., 1998). *Mi1.2* was the first aphid resistance gene to be cloned and it encodes a protein which exhibits homology to a nucleotide-binding site (NBS), leucine-rich repeat (LRR) resistant (R) gene. *Mi1.2* offers resistance to both root–knot nematode (*Meloidogyne incognita*) and to certain biotypes of potato aphid. *Mi1.2* causes an HR response against *M. incognita* feeding, but not against potato aphid. *Mi* protein resembles the *Prf* tomato gene that is required for *Pto*-mediated resistance against *Pseudomonas syringae* (Salmeron et al., 1996). *Pto* is also known to recognize two non-homologous avirulence gene products present in the same bacteria. It is likely that *Mi* also recognizes two distinct avirulence products, one from a nematode and the other from an aphid.

The central region of the *Mi* gene covers a 260 amino acid stretch which contains a NBS (nucleotide binding site) domain and the C-terminal region contains 14 highly imperfect copies of an LRR motif. Studies emerging from other R-gene characterizations, suggest that the LRR motif carries determinants for specificity of recognition (Ellis et al., 1999). Activation of R gene signals resistance, for example, transcripts of *PR-1* were detected earlier and accumulated to higher levels in the incompatible than in the compatible potato aphid /tomato interactions (Oscar et al., 2003). Phloem-feeding insects also trigger the production of a variety of lipid-derived C6 volatiles, which actively suppress aphid multiplication in addition to activating indirect defenses (Hildebrand et al., 1993, Hardie et al., 1994; Walling, 2000). Antisense suppression of a potato hydroperoxide lyase (HPL), which is involved in the production

of C6 volatiles, in potato resulted in reduced activity of HPL and allowed enhanced performance of green peach aphid (GPA) in comparison to the wild type plants. Aphid feeding triggered the release of methyl SA (MeSA), which is a strong aphid repellent (Hardie, 1994). In addition, SA and JA have been reported to modulate the emission of volatile compounds associated with defense against insects (VanPoecke and Dicke, 2004). For instance, the volatile emission generated during lima bean (*Phaseolus lunatus* L.)- two spotted spider mite (*Tetranychus urticae*) interactions is dependent on both SA- and JA- related signal transduction pathways and provide resistance to the plants (Ozawa et al., 2000). In addition, to regulating volatile emission, both SA and JA are also important activators/modulators of direct plant defenses. Activation of SA signaling is associated with elevated levels of expression of genes for the pathogenesis–related (PR) proteins in aphid infested plants (Moran et al., 2001; Bernasconi et al., 1998; Fidantsef et al., 1999).

Dynamics of Plant-Aphid Interaction

Both biochemical and molecular studies show that plant-aphid interactions are complex in nature. From a plant's perspective, aphids are metabolic sink organs which consume copious amount of photoassimilates. In order to survive, a plant has to effectively activate its defense machinery to minimize the flow of photoassimilates to the "false sinks". Likewise, success of an aphid is dependent on its ability suppress the effective host immune response and at the same time to manipulate host machinery to enhance the quality and quantity of their nutrition. Evolution has facilitated an optimal development of these strategies in both plants and aphids. For instance, the rose aphid,

Macrosiphum rosae, preferentially avoids feeding on old rose buds (Davidson, 1923) of a plant since it contains particularly large quantities of catechin, a phenolic monomer which is extremely toxic in nature. While in other instances, phenolic compounds have been detected in the gut and the honey dew secretions of rose aphids (Campbell & Eikenbary, 1990), which suggest that aphids might ingest some of these phenolics and detoxify them. Insect studies on artificial diets, suggest that aphids secrete enzymes like catechol oxidase into the artificial media to convert the toxic catechin into a less toxic form (Peng and Miles, 1988a, b). Additionally, the salivary secretions of the rose aphid have also been shown to contain a rapid-acting peroxidase, which, in the presence of traces of hydrogen peroxide, oxidizes various polyphenols, other phenolic derivatives and also aromatic amines (Campbell & Eikenbary, 1990). Some aphid species manipulate amino acid composition in the phloem (Sandstorm et al., 2000). Others such as gall forming *Pemphigus betae*, manipulate plant allocation patterns while competing for plant sinks for resources (Larson & Whitham, 1997).

The mechanisms responsible for these manipulations are largely unknown. Manipulation of phloem amino acid composition appears to influence the nutritional quality of plants for aphids, as supported in another correlated study in which the potato aphid and the green peach aphid performed better on pretuber-filling potato plants with high glutamine levels than on tuber-filling plants with low glutamine levels (Karley et al., 2002).

Role of Senescence in Plant-Aphid Interactions

Senescence is a complex, highly regulated, developmental phase in the life of a leaf. Unlike other developmental processes, which are composed of cell division, cell differentiation, and cell expansion, leaf senescence involves massive programmed cell death (Gan and Amasino, 1997). Leaf senescence is accompanied by decrease in photosynthetic rate, and an increase in other catabolic events such as chlorophyll, lipid, protein, and nucleic acid degradation. The released nutrients are remobilized subsequently to growing leaves, developing seeds, or storage tissues. Thus, leaf senescence is a nutrient-mining and–recycling process (Noodén, 1988; Buchanan-Wollaston, 1997; Quirino et al., 2000). The onset of leaf senescence can be regulated by an array of endogenous and external factors. Environmental cues such as drought, nutrient deficiency, and pathogen infection can readily trigger premature leaf senescence (Stoddart & Thomas, 1982).

Aphid feeding induces localized changes in the metabolism of their hosts which simulate senescence, and produce chlorotic lesions in plants. Similar to plant tissues undergoing natural senescence, the chlorotic tissue is higher in free amino acids and can benefit aphids (Dixon, 1975; Dorschner et al., 1987). *Rhopalosiphum padi* L., feeding on winter wheat, elevated γ-aminobutyric acid and aspartic acid levels but decreased the concentrations of glutamic acid, valine, leucine, isoleucine and tyrosine (Havlickova, 1987). A premature senescence independent of aphid feeding can also result form the accumulation of honeydew on leaf surface. Deposition of honey dew can reduce the photosynthesis and promote the growth of saprophytic fungi which subsequently promote

senescence (Bardner and Fletcher, 1974; Wratten, 1975; Rabbinge et al., 1981). However, in some cases senescence-associated processes may limit aphid performance. For example, premature senescence induced by a gall-aphid correlated with the reduced performance of another aphid, feeding on the same leaflet of *Pistacia palaestina* trees (Inbar et al., 1995). Moreover, GPA feeding causes loss of chlorophyll in Arabidopsis leaves (Fig. 4*A*, *B*), a typical symptom associated with senescence.

GPA-Feeding Induced Changes in Plant Gene Expression

Microarray studies to identify plant genes in response to aphid feeding have been successfully conducted in different plant-aphid models (Moran et al., 2002; Zhu-Salzman et al., 2004; Divol et al., 2005; DeVos et al., 2005). These studies suggest an overlap between plant response to pathogens and aphids. In addition, aphid feeding also altered expression of genes involved in diverse plant responses. For instance, array studies conducted in *M. persicae- Arabidopsis* revealed genes associated with oxidative stress (glutathione-S-transferase, superoxide dismutases), Ca^{2+}/calmodulin-related signaling genes, PR genes (BGL2, PR-1, hevein-like protein), ethylene biosynthesis genes (ACC oxidase 1) and aromatic biosynthesis genes (PAL2, chalcone synthase, tyrosine decarboxylase) to be up-regulated or down-regulated after 72-96 h of *M. persicae* attack.

A similar comprehensive array-analysis was used by Zhu-Salzman et al., 2004 to compare the transcriptional response in sorghum bicolor plants elicited either by greenbug (*Schizaphis graminae*), SA, and JA. Greenbug attack caused changes in the expression of defense genes (PRs, PIs and phenolics biosynthesis genes), antioxidant

genes (glutathione-*S*-transferase, lactoylglutathione lyase and catalase) abiotic stress-related genes (drought-, salt-, and low-temperature responsive genes, aldehyde oxidase). In addition, two greenbug-specific genes, leucine-rich repeat-containing protein and a defense-related protein (DPR) known to be induced by sugar depletion were identified in this current study.

We analyzed approximately 23,000 genes on the Arabidopsis whole genome chip (Affymetrix ATH1). Among the genes which were upregulated by aphid feeding, 200 genes showed an elevated expression (>2-fold) 48 hours post GPA-feeding. Further, we observed that about 95 genes were down regulated (<3-fold) in response to GPA feeding. Some of the GPA-activated genes are listed in Appendix, Table 1. GPA feeding caused the activation of genes involved in shikimate pathway (Fig. 2). For instance, At3g54640 which encodes tryptophan synthase α and At4g27070, which encodes the tryptophan synthase β chain, were induced 5-fold and 3-fold respectively in plants that had been subjected to aphid feeding(Appendix, table 1). Shikimate pathway is involved in the synthesis of several insecticidal secondary metabolites like flavanoids, indole-glucosinolates and alkaloids.

Similarly, the gene encoding indole-3-glycerol phosphate synthase was induced 4-fold in comparison to the uninfested samples; this gene is involved in the synthesis of indole/tryptophan (Appendix, Table.1). Furthermore, GPA feeding resulted in the 8-fold activation of a short chain alcohol dehdrogenase gene. Short chain alcohol dehdrogenases are involved in the synthesis of C6 volatiles, which are potent signal molecules in plant

defense and have anti-aphid properties (Hildebrand et al., 1993; Kasu et al., 1995; Walling, 2000). GPA feeding also activated a β-glucosidase (At3g57240). Similarly in squash silver leaf whitefly feeding activates the *SLW3* gene, which encodes a β-glucosidase (Van de Ven et al., 2000). β-glucosidases are also present in the aphid saliva and may suppress deposition of callose near feeding sites (Shiroada, 1993)

GPA feeding activated the expression of SA biosynthesis genes. For instance, *SID2 (SALICYLIC ACID DEFICIENCY 2)*, *EDS5 (ENHANCED DISEASE SUSCEPTIBILITY 5)* that are involved in SA synthesis and *NPR1* (*NON-EXPRESSOR OF PR-1*), which is involved in SA signaling were increased >3 fold in GPA infested plants (Fig. 2). Likewise, the expression of *PAD3 (PHYTOALEXIN DEFICIENCY 3)* and *PAD4 (PHYTOALEXIN DEFICIENCY 4)* gene involved in phytoalexin synthesis were induced 6-fold in response to GPA feeding (Fig. 2.). GPA feeding also activated gene associated with senescence mechanism such as *SAG13, 15, 18, 21, 25 27* and *29*. In addition, GPA-feeding triggered expression of genes potentially involved in signal transduction. For instance, one of the calcium binding proteins was induced as high as 37-fold more in GPA infested samples. The other putative calcium binding proteins include those encoded by the At5g39670 and At2g41410 genes.

GPA-feeding altered expression genes involved in source-sink relationships. For instance, the sucrose transporter, *SUC1* gene expression was elevated to 6-fold higher levels in the GPA-infested samples. Similarly, At*GPT2*, glucose-6-phosphate translocator was induced to about 6-fold. In contrast, the glucose transporter gene

At1g11260 was repressed to about 5-fold. Some of the other GPA–repressed genes are described in the (Appendix, Table 2). Two putative trehalose-6-phosphate (T6P) phosphatase genes (At2g18700 and At1g70290) were down regulated 9-and 6-fold respectively (Fig. 3). Expression of a myrosinase-binding protein (At1g52040), which might be involved in regulating myrosinase activity and thereby decrease production of glucosinolates, was also depressed in response to GPA feeding. A peroxidase and a hydrogen peroxide generating copperamine oxidase, was about down regulated 4-fold (Fig. 3).

Objective and Approaches

As mentioned above, a very little information is available on the molecular basis of resistance against phloem feeding insects. I utilized the Arabidopsis-green peach aphid interaction as a model system to characterize the molecular response in plant upon aphid feeding. Microarray technology was implemented for rapid characterization of Arabidopsis gene expression in response to the GPA-feeding (Appendix, Table 1, Table 2). Further, the availability of the Arabidopsis whole genome microarray chip (Affymetrix, ATH1) enabled the characterization of global genome changes in the plant in response to aphid feeding (Chapter 1).

A reverse-genetics approach was implemented to determine the functional significance of the microarray identified genes. A fast and efficient insect bioassay protocol, based on the no-choice test procedure was developed which enabled us to screen the genetic mutants. I screened several Arabidopsis mutants using this procedure and successfully identified seven new mutants on which GPA growth significantly differed in comparison to their respective controls. I have studied *pad4-1* mutant in greater detail. I determined that the Arabidopsis PAD4 modulated SA-signaling and camalexin biosynthesis did not have an important role in resistance against GPA (Chapter 2).

In addition, microarray data provided useful molecular markers (*SAG13, SAG21, SAG27*) and characterization of these *SAGs* (SENESCENCE ASSOCIATED GENES) gene expression in *pad4* mutant plants suggested that GPA-feeding induces a senescence-

like mechanism and *PAD4* is vital for modulating this event. These finding were further complemented with *PAD4* over expression studies (Chapter 3). Additional studies were also performed on hyperactivated senescence mutants such as *ssi2 and cpr5* to determine the role of senescence in plant-aphid interaction (Chapter 3).

Earlier, studies have shown that PAD4 functions along with, EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1) & SAG101 (SENESCENCE ASSOCIATED GENE 101) proteins to modulate basal resistance against obligate biotrophic and hemibiotropic pathogens (Feys et al., 2005). One of the objectives has been to determine if PAD4 has a similar or different mechanistic mode of action in aphid-mediated resistance (Chapter 4).

MATERIALS AND METHODS

Plant and Aphid Growth Conditions

Arabidopsis plants were grown in soil at 22°C in a growth chamber programmed for 14 h light (100 μE m^{-2} s^{-1}) and 10 h dark cycle. Approximately four-week-old Arabidopsis plants were used for all studies. A combination of commercially available radish (Early scarlet globe) and mustard (Florida broadleaf), at a 50:50 ratio, was used for the routine propagation of GPA at 22°C in a growth chamber programmed for 14 h light (100 μE m^{-2} s^{-1}) and 10 h dark cycle. Approximately 20-25 day old radish and mustard seedlings were used as the feeding material for aphids. Aphids were transferred to fresh batch of radish and mustard mix once every two weeks.

RNA extraction and RT-PCR analysis

Leaf material from uninfested and GPA-infested plants was harvested and quick frozen in liquid nitrogen. RNA was extracted as described by Das et al. (1990). Approximately 1gm of Arabidopsis leaf tissue was ground in the presence of liquid N_2 and was completely suspended in 5 ml of extraction buffer (4 M guanidium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M β-mercaptoethonol, 0.2 M sodium acetate). 2.5 ml of CHISM (24:1 mixtue of chloroform: isoamyl alcohol) was added to the samples and suspended in the extraction buffer. Samples were vortexed vigoursly for about 10-20 sec and finally centrifuged at 13,000 rpm for 15 min. RNA in supernanant was precipitated using isoproponal. RNA pellet was obtained after centrifugation at 15,000 rpm for 10 min. The isolated RNA was purified using the RNeasy Mini kit (Stratagene, CA), spectrophotometrically quantified at 260 nm and subsequently used in the RT-PCR reactions. RT-PCR analysis was performed with the Super

Script One-step RT-PCR kit (Invitrogen Life Technologies, city MD). The RT reaction was carried out at 50℃ for 30 min in a 20 μl reaction with 100 ng of the total RNA as template as recommended by the manufacturer. PCR conditions for the GPA-feeding induced and GPA-feeding repressed genes were as follows: 95℃ for 5 min followed by 28 cycles of 95℃ for 15 sec, 50 or 55℃ for 30 sec and 72℃ for 1 min with a final extension at 72℃ for 5 min. The primer sequences of the GPA-feeding induced & repressed genes are provided separately in the appendix section in Table 1 & Table 2. The amplified fragments were resolved on a 1.2% agarose gel, stained with ethidium bromide and visualized with a Gel Doc UVP BioDoc-It^{TM} system.

Chlorophyll extraction and estimation

Leaves were ground in a mortar with a pestle in the presence of liquid nitrogen. Chlorophyll was extracted once with an extraction buffer consisting of a 85:15 (v/v) mix of acetone: Tris-HCl (1 M; pH 8.0 in water). The absorbance of the extract was recorded at 663 nm and 647 nm against an extraction buffer control, and the chlorophyll content calculated as described by Lichtenthaler et al. (1987). The chlorophyll a and b content in the samples was measure using the following calculations. Chlorophyll a =12.25 * A_{663}- 2.79 * A_{647}; Chlorophyll b=21.50 * A_{647}- 5.10 *A_{663}; Total chlorophyll content = Chl a + Chl b.

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FIGURE LEGENDS

Fig. 1. The intercellular mode of aphid feeding. Diagram shows aphids use their slender stylets to penetrate between cells (intercellular). However, occasionally they may penetrate the cell wall and plasma membrane of the mesophyll and parenchyma cells. Intercellular stylet progression is often accompanied by active salivary secretions, the red color shows the saliva released during the aphid feeding. Two types of saliva are secreted; a watery saliva that contains pectinases and cellulases, which soften the cell wall and therefore may aid in the intercellular penetration of stylets. Secondly, the presence of gelling saliva which forms a sheath on the penetrating stylets has been proposed to prevent wounding responses by sealing off the ruptured cells and the damaged cell walls.

Fig. 2. Time course analysis on GPA-feeding induced in Arabidopsis genes. A total of about 200 genes were up-regulated (>2 fold) in the microarray experiment. RT-PCR analysis was carried out to validate the microarray data. RT-PCR analysis for 34 of those genes were performed on RNA extracted from GPA infested Arabidopsis samples at the following time points 12, 24, and 48 h post-infestations. RNA extracted from uninfested plants provided a negative control. GPA feeding induced the expression of genes belonging to SA-signaling, genes involved in signal transduction, secondary metabolite biosynthesis, DNA-binding proteins, and genes associated with sugar metabolism and senescence responses. *ACT8* gene served as a control for RNA quality in the RT-PCR reaction. Majority of these genes were analyzed in two repeated experiments and RNA used in each experiment was isolated independently.

Fig. 3. Time course analysis of GPA-feeding repressed Arabidopsis genes. Microarray experiment identified 95 genes, with >2fold down-regulation during the course of aphid feeding in Arabidopsis. RT-PCR analysis was performed on RNA extracted 12, 24, 48 h post-infestation (hpi). RNA extracted from uninfested plants provided a negative control. *ACT8* gene served as a control for RNA quality in the RT-PCR reaction. We performed the RT-PCR analysis for a majority of these genes in duplicate experiments and RNA used each time for analysis was isolated independently.

Fig. 4. GPA feeding causes activation of premature senescence in Plants. (*A*) Photograph of wild type (WT) Arabidopsis leaves infested with GPA, 7 day post-release of 15 mature insects. Uninfested WT plants of similar age served as negative control. (*B*) Comparison of total chlorophyll content in the WT plants infested with GPA, 2 days post-release of 15 mature insects and in uninfested WT plants of same age group. All values are the means of total chlorophyll from three plants $\pm$ SE. Different letters above the bars indicate values that are different from each other with a confidence of 95% with Student's *t*-test. These experiments were repeated twice and we obtained consistent results.

Fig. 1

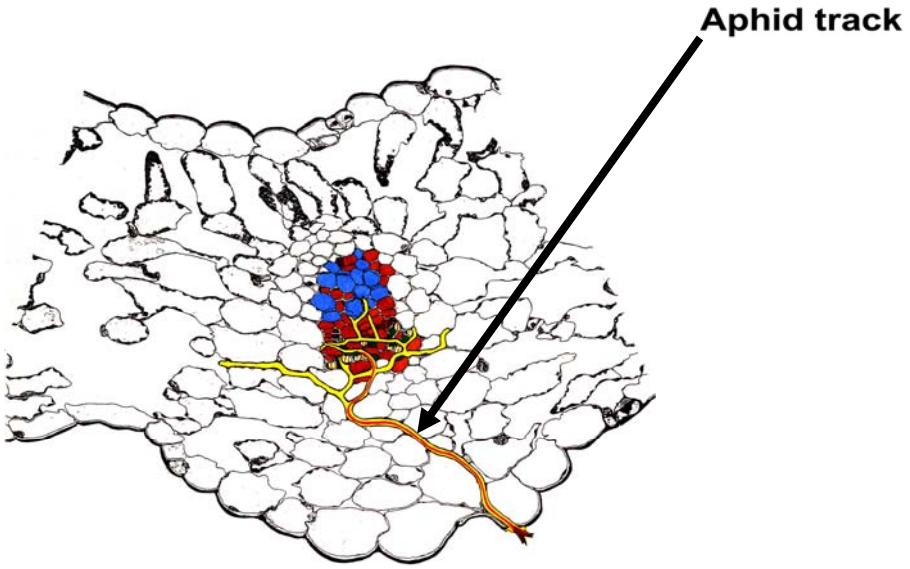
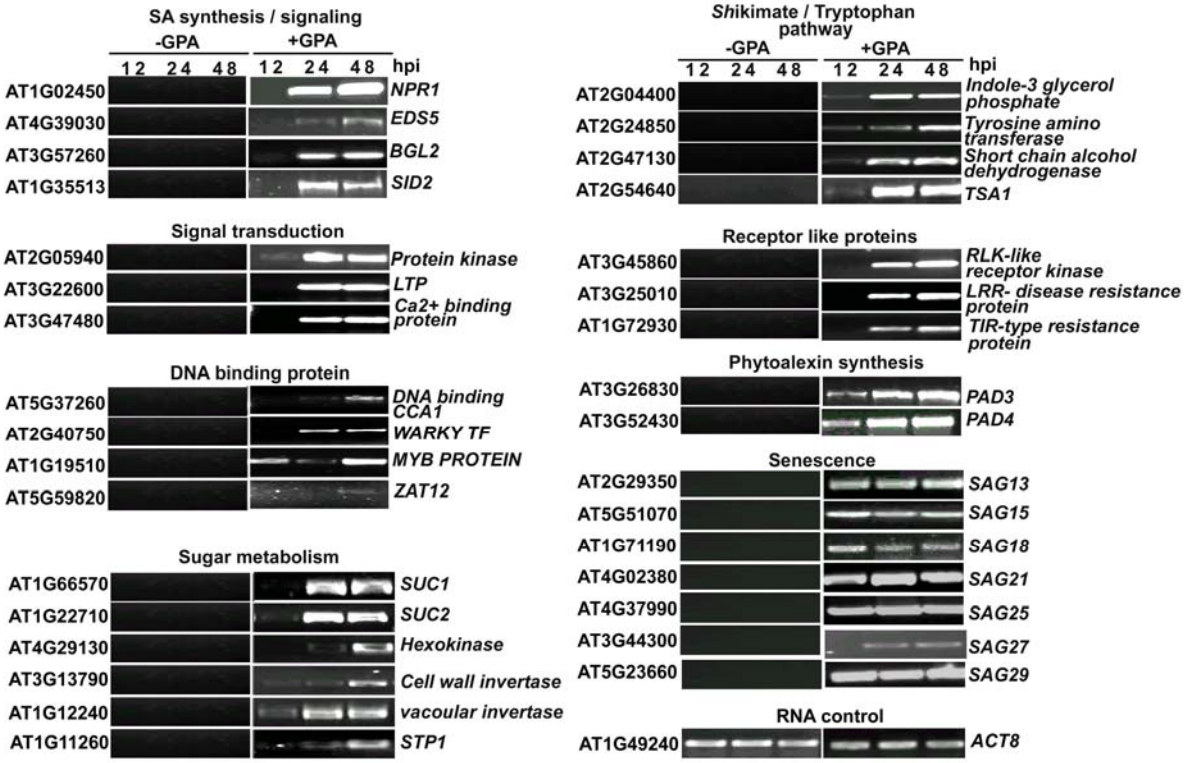


Image by Freddy Tjallingii

Fig. 2


SA synthesis / signaling
-GPA
+GPA
12 24 48 12 24 48 hpi
AT1G02450 NPR1
AT4G39030 EDS5
AT3G57260 BGL2
AT1G35513 SID2
Signal transduction
AT2G05940 Protein kinase
AT3G22600 LTP
AT3G47480 Ca2+ binding protein
DNA binding protein
AT5G37260 DNA binding CCA1
AT2G40750 WARKY TF
AT1G19510 MYB PROTEIN
AT5G59820 ZAT12
Sugar metabolism
AT1G66570 SUC1
AT1G22710 SUC2
AT4G29130 Hexokinase
AT3G13790 Cell wall invertase
AT1G12240 vacoular invertase
AT1G11260 STP1
Shikimate / Tryptophan pathway
-GPA
+GPA
12 24 48 12 24 48 hpi
AT2G04400 Indole-3 glycerol phosphate
AT2G24850 Tyrosine amino transferase
AT2G47130 Short chain alcohol dehydrogenase
AT2G54640 TSA1
Receptor like proteins
AT3G45860 RLK-like receptor kinase
AT3G25010 LRR- disease resistance protein
AT1G72930 TIR-type resistance protein
Phytoalexin synthesis
AT3G26830 PAD3
AT3G52430 PAD4
Senescence
AT2G29350 SAG13
AT5G51070 SAG15
AT1G71190 SAG18
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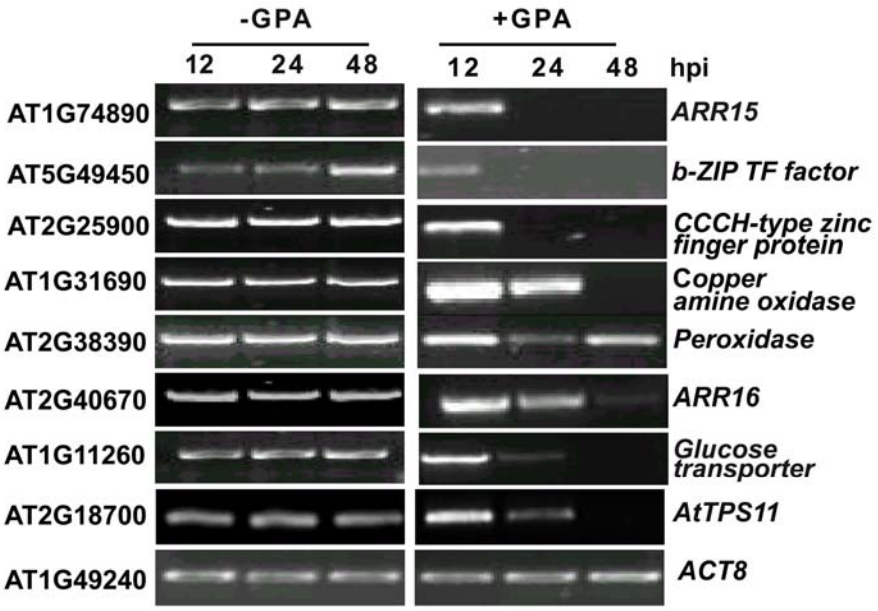
AT4G37990 SAG25
AT3G44300 SAG27
AT5G23660 SAG29
RNA control
AT1G49240 ACT8

Fig. 3


-GPA
+GPA
12
24
48
12
24
48
hpi
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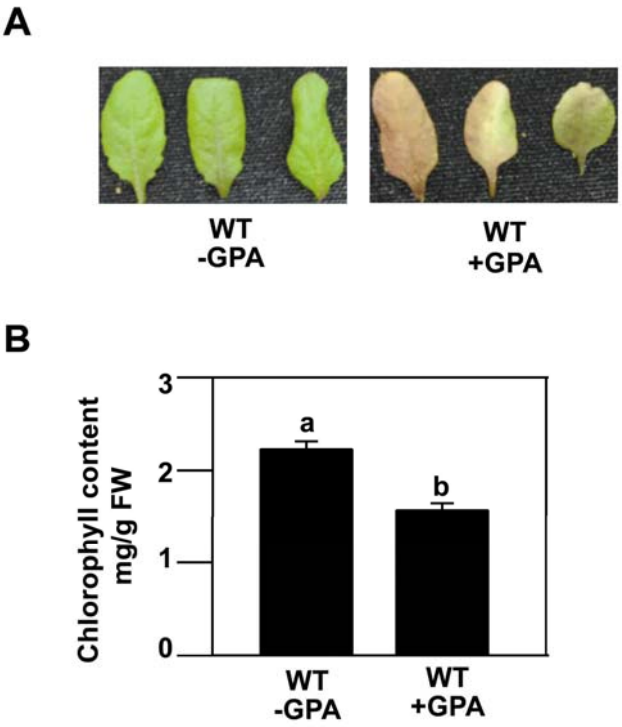
ARR15
AT5G49450
b-ZIP TF factor
AT2G25900
CCCH-type zinc finger protein
AT1G31690
Copper amine oxidase
AT2G38390
Peroxidase
AT2G40670
ARR16
AT1G11260
Glucose transporter
AT2G18700
AtTPS11
AT1G49240
ACT8

Fig.4


A
WT
-GPA
WT
+GPA
B
Chlorophyll content
mg/g FW
3
2
1
0
a
b
WT
-GPA
WT
+GPA

Chapter 2

A novel function for the *Arabidopsis thaliana PAD4* gene in basal resistance to green peach aphid

Results presented in this chapter are in press for publication in Plant Physiology.

Associated authors are listed below

Venkatramana Pegadaraju, Caleb Knepper, John Reese and Jyoti Shah.

SUMMARY

Green peach aphid (GPA) is a phloem-feeding insect with a wide host range that includes *Arabidopsis thaliana*. Here we show that the Arabidopsis *PAD4* gene has an important role in Arabidopsis defense to GPA. GPA-feeding stimulated accumulation of the *PAD4* transcript. Moreover, in comparison to the wild type plant, growth of GPA was higher on the *pad4* mutant plant. *PAD4* is associated with camalexin synthesis and salicylic acid (SA) signaling. However, growth of GPA on the camalexin-biosynthesis mutant, *pad3*, and the SA-deficient *sid2* and *nahG* plants and the SA-signaling mutant, *npr1*, were comparable to that on the wild type plant, suggesting that camalexin and SA signaling are not important for restricting GPA growth in Arabidopsis. In comparison to the wild type plant, the increased susceptibility of the *pad4* mutant to GPA was paralleled by a delay in the activation of chlorophyll loss, cell death, and expression of the senescence-associated genes, *SAG13*, *SAG21* and *SAG27*. Our results suggest that in Arabidopsis *PAD4* modulates the activation of a senescence-like mechanism that contributes to the basal resistance to GPA.

INTRODUCTION

Aphids are a large class of piercing-sucking group of insects that feed on phloem sap (Pollard, 1973). Aphid feeding limits plant productivity (Dixon, 1998). In addition, aphids are vectors for several economically important plant viruses (Mathew, 1991). Our knowledge of plant defense against insects is based largely on studies involving chewing insects. However, due to their feeding behavior, unlike the chewing insects aphids do not cause extensive wounding to the plant host (Walling, 2000). Microarray studies suggest that host response to aphids does differ from that to chewing insects. For example, aphid feeding induces expression of genes known to confer resistance to pathogens (Fidantsef et al., 1999, Moran and Thompson, 2001, Moran et al., 2002; Zhu-Salzman et al., 2004). However, in general these responses are not stimulated by chewing insects (Reymond et al. 2000; Heidel and Baldwin, 2004; De Vos et al., 2005).

A few studies have identified plant genes and mechanisms associated with plant defense against aphids. For example, in tomato the *Mi 1.2* gene, which encodes a nucleotide binding site (NBS) leucine-rich repeat (LRR) protein mediates gene-for-gene resistance to the potato aphid *Macrosiphum euphorbiae*, in addition to nematodes (Rossi et al., 1998; Vos et al., 1998). Similarly, the lettuce *Nr* gene confers resistance to *Nasonovia ribisnigri* (Helden et al., 1993). Comparable to the function of *R* genes in plant response to pathogen infection (Bent, 1996; Hammond and Jones, 1996), interaction of aphid generated/derived signals with the *R* gene encoded activity may presumably activate signal transduction pathways that confer expression of appropriate defense responses. In wheat, resistance to Russian wheat aphid is accompanied by the activation

of cell death in the resistant wheat genotypes (Porter et al., 1997; Miles, 1999). Cell death has also been reported during aphid feeding on sorghum (Zhu-Salzman et al., 2004). A few studies have shown that oxylipin signaling contributes to plant defense against aphids. For example, the oxylipin, jasmonic acid (JA)- responsive genes were expressed at elevated levels in sorghum leaves infested with greenbug (Zhu-Salzman et al., 2004) and Arabidopsis infested with GPA (Moran et al., 2002). Moreover, methyl JA treatment caused a significant reduction in greenbug infestation on sorghum seedlings (Zhu-Salzman et al., 2004). The Arabidopsis *coi1* mutant, which is compromised in oxylipin signaling, supported increased growth of GPA than the wild type plant (Ellis et al., 2002), providing support to the involvement of oxylipins in plant defense against aphids. In addition, to JA signaling, aphid infestation also enhances expression of the SA-inducible pathogenesis-related (*PR*) genes (Moran et al., 2002; Zhu-Salzmann et al., 2004; Vander Westhuizen et al., 1998 a, b; V. Pegadaraju and J. Shah, unpublished). However, mutation in the Arabidopsis *NPR1* (*NON-EXPRESSER OF PR GENES 1*) gene, which is required for SA-signaling, and the *EDS5* (*ENHANCED DISEASE SUSCEPTIBILITY 5)* gene which is required for SA synthesis, do not compromise resistance to GPA (Moran et al., 2001) suggesting that SA and SA signaling may not have a major role in Arabidopsis defense against GPA.

The Arabidopsis *PAD4* (*PHYTOALEXIN DEFICIENCY 4*) gene regulates multiple defense mechanisms. Mutations in the *PAD4* gene compromise the synthesis of the phytoalexin, camalexin (Glazebrook et al., 1997). Phytoalexins are low molecular weight antimicrobial compounds which confer resistance against fungal pathogens.

Subsequently, PAD4 was also shown to modulate SA signaling and loss of PAD4 activity confered enhanced susceptibility to some bacterial and oomycete pathogens (Jirage et al., 1999). In addition, the *PAD4* gene is required for *R*-gene mediated resistance against some bacterial and oomycetes pathogens (Feys et al., 2001). Here we show that the Arabidopsis *PAD4* gene modulates basal resistance to GPA. We provide evidence that PAD4 modulates a senescence-like mechanism that is activated in plants in response to GPA feeding.

MATERIALS AND METHODS

Plant and Aphid Growth Conditions

Arabidopsis plants were grown in soil at 22°C in a growth chamber programmed for 14 h light (100 μE m^{-2} s^{-1}) and 10 h dark cycle. Approximately four-week-old Arabidopsis plants were used for all studies. A combination of commercially available radish (Early scarlet globe) and mustard (Florida broadleaf), at a 50:50 ratio, was used for the routine propagation of GPA at 22°C in a growth chamber programmed for 14 h light (100 μE m^{-2} s^{-1}) and 10 h dark cycle. Approximately 20-25 day old radish and mustard seedlings were used as the feeding material for aphids. Aphids were transferred to fresh batch of radish and mustard mix once every two weeks.

Arabidopsis Mutants

The *pad3-1* (Glazebrook and Ausubel, 1994), *pad4-1* (Glazebrook and Ausubel, 1994), *npr1-1* (Cao et al., 1994), and *sid2-1* (Wildermuth, 2001) mutants used in this study are in the ecotype Columbia background. The *nahG* mutants are in the ecotype Nössen background (Shah et al. 1999, 2001). The *pad4* T-DNA insertion line (SALK_089936) was identified from amongst the Salk collection (http://signal.salk.edu/).

No-choice test

A no-choice test was used to assay aphid growth on wild type and mutant plants. Approximately four-week-old Arabidopsis plants were used in the bioassay with a clonally propagated GPA. For the no-choice test each Arabidopsis plant received fifteen mature apterous (wingless forms) aphids at the center of the rosette and the plants were

incubated at 22° C in a growth chamber programmed for 14 h light (100 μE m^{-2} s^{-1}) and 10 h dark cycle. Growth of GPA on the Arabidopsis plants was measured 48 h post infestation. All values are the mean of 15 plants ± SE. Student's t-test was utilized to determine significance of difference between different treatments with a confidence of 95% and higher. Student t-tests were performed using Sigma plot V 5.0 (SPSS Inc, Chicago, IL). Each experiment was repeated thrice with similar results.

Histochemistry and microscopy

Leaf samples for trypan blue staining were processed and analyzed as described by Rate et al. (1999). Arabidopsis leaf samples infested with GPA for duration of 48h were harvested and vacuum infiltrated at ~50 mbar. Samples were subsequently boiled in the microwave for 1 min and cooled at room temperature for 1-2 min prior to the lactophenol treatment for 1 min at boiling temperature. Leaf samples were finally washed in 50% ethanol for 2-3 washes and mounted on glass slides for cell death visualization.

Chlorophyll extraction and estimation

Leaves were ground in a mortar with a pestle in the presence of liquid nitrogen. Chlorophyll was extracted once with a extraction buffer consisting of a 85:15 (v/v) mix of acetone: Tris-HCl (1M; pH 8.0 in water). The absorbance of the extract was recorded at 663 nm and 647 nm against an extraction buffer control, and the chlorophyll content was calculated as described by Lichtenthaler et al. (1987). The chlorophyll a and b content in the samples was measured using the following calculations. Chlorophyll a

=12.25 * A_{663}-2.79 * A_{647}; Chlorophyll b=21.50 * A_{647}- 5.10 *A_{663}; Total chlorophyll content = Chl a + Chl b.

DNA extraction and PCR analysis

DNA for the PCR analysis was extracted using a single leaf as previously described (Konieczny and Ausubel, 1993). A medium sized leaf of approximately 50 mg in weight was collected in a 1.5 ml microfuge tube and transferred into a container containing liquid nitrogen for about 10 min. Samples were ground in the presence of 200 µl of extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, 0.5% SDS). The extract was purified using the Tris-equilibrated phenol: chloroform mix (pH 7-8). DNA present in the supernatant was precipitated using equal volumes of isopropanol and finally suspended in sterile distilled water. A transgenic Arabidopsis line, which contains a T-DNA insertion within the *PAD4* (AT3G52430) gene was identified amongst the Salk collection (http://signal.salk.edu) obtained from the Ohio Stock Center. Multiplex PCR analysis was performed on the segregating plant material to identify plants homozygous for the T-DNA insertion. The PAD4-F (5'-GCTCTCCTCTGCTGGAAACC-3'), PAD4-R (5'-TTTTCTCGCCTCATCCAACCA-3') and T-DNA left border primer (5'- GCGTGGACCGCTTGCTGCAAC-3') were used at a concentration of 50ng/ul in the multiplex PCR. PCR was performed with the following conditions: 95°C for 5 min followed by 30 cycles of 95°C for 0.5 min, 65°C for 0.5 min and 72°C for 2 min, with final extension at 72 °C for 5 min. The PCR products were resolved on 1.2 % agarose gel, stained with ethidium bromide and visualized with a Gel Doc UVP BioDoc-It^{TM} system.

RNA extraction and RT-PCR analysis

Leaf material from uninfested and GPA-infested plants was harvested and quick frozen in liquid nitrogen. RNA was extracted as described by Das et al. (1990). Approximately 1 gm of Arabidopsis leaf tissue was ground in the presence of Liquid N_2 and was completely suspendend in 5 ml of extraction buffer (4M guanidium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1M β-mercaptoethonol, 0.2M sodium acetate). 2.5 ml of CHISM (chloroform: isoamyl alcohol) was added to the samples suspendend in the extraction buffer, samples were vortexed vigioursly for about 10-20 sec and finally centrifuged at 13,000 rpm for 15 min. RNA in the supernanant was precipitated using isopropanol. RNA pellet was obtained after centrifugation at 15,000 rpm for 10 min. The isolated RNA was purified using the RNeasy Mini kit (Stratagene, CA), spectrophotometrically quantified at 260 nm and subsequently used in the RT-PCR reactions. RT-PCR analysis was performed with the Super Script One-step RT-PCR kit (Invitrogen Life Technologies, MD). The RT reaction was carried out at 50℃ for 30 min in a 20 μl reaction with100 ng of the total RNA as template as recommended by the manufacturer. PCR conditions for the *ACT*8, *SAG13*, *SAG21* and *PAD4* were as follows: 95℃for 5 min followed by 25 cycles of 95℃ for 15 sec, 50 or 55℃ for 30 sec and 72℃ for 1 min with a final extension at 72℃ for 5 min. The ACT8-F (5'-ATGAAGATTAAGGTCGTGGCA-3') and ACT8-R (5'-TCCGAGTTTGAAGAGGCTAC-3'), SAG12-F(5'-TCTCGTCCACTCGACAATGAA-3') and SAG12-R(5' AGCTTTCATGGCAAGACCACA-3'), SAG13-F (5'-CAAGATGGAGTCTTGGAGGCA-3') and SAG13-R (5'-GGAAAAACCGTTAACAGTGGA-3'), SAG21-F (5'-CCAATGCTATCTTCCGACGTG 3') and SAG21-R (5'- GAACCGGTTTCGGGTCTGTAA 3'), SAG27-F(5'-TCCTGGCCCTGAAGTAGAAA-3') and SAG27-R(5'-

GTCCCGCAAGAACCTGTCC-3'), PAD4-F (5'-GCTCTCCTCTGCTCGGAAACC 3') and PAD4-R (5'-TTTTCTCGCCTCATCCAACCA 3') gene specific primers were used for PCR amplification of *ACT8* (238 bp), *SAG13* (761 bp), *SAG21* (181 bp), *SAG27*(523 bp) and *PAD4* (959 bp). The amplified fragments were resolved on a 1.2% agarose gel, stained with ethidium bromide and visualized with a Gel Doc UVP BioDoc-It^{TM} system.

RESULTS

PAD4 is Required for Basal Resistance to GPA.

Our initial microarray analysis suggested that *PAD4* gene was up-regulated in response to GPA feeding. This prompted us to test the requirement of *PAD4* gene in basal resistance against GPA. We observed that in comparison to an uninfested Arabidopsis plant, the *PAD4* transcript accumulated to a higher level in leaves exposed to GPA (Fig. 5*A*). Moreover, in a no-choice test, two days post release of GPA, aphid count was higher on the *pad4-1* mutant than on the wild type (WT) plant (Fig. 5*B*). Similarly, in comparison to the WT plant, GPA count was higher on a transgenic plant that contained a T-DNA insertion within the *PAD4* gene (Fig. 5*B*). To determine if the phenotype on *pad4* mutants was tolerant or susceptible, we analyzed the relative seed content in *pad4-1* and WT plants infested with GPA. Aphid-infested *pad4-1* plant produced 65% less seed than aphid-infested WT plant (Fig. 5*C*) suggesting that indeed *pad4-1* mutants were susceptible to GPA.

Camalexin and Salicylic Acid are not important for Basal Resistance to GPA

The enhanced susceptibility of the *pad4* mutants to GPA could be due to camalexin and/or SA deficiency. To ascertain if camalexin has a role in basal resistance to GPA, we compared aphid counts on the *pad3-1* mutant and WT plants, two days after release of GPA. The *PAD3* gene encodes a cytochrome P450 monooxygenase, which is required for camalexin biosynthesis (Zhou et al., 1999). Comparable number of aphids were present on the *pad3-1* mutant and WT plant (Fig. 6*A*), suggesting that camalexin is not important for basal resistance to GPA. Similarly, comparable numbers of aphids

were present on the WT, the SA-biosynthesis mutant *sid2* (Fig. 6*B*), the SA-insensitive *npr1* mutant (Fig. 6*B*) and the SA-deficient NahG transgenic plant (Fig. 6*C*), suggesting that SA accumulation and signaling is not critical for basal resistance to GPA. These results confirm and extend the observations of Moran and Thompson (2001), who noted no correlation between the loss of SA signaling and basal resistance to GPA in Arabidopsis.

GPA-feeding Activates a Senescence-like Process, which is Compromised in the *pad4* Mutant

We observed that despite the higher growth of GPA, *pad4-1* mutants infested with GPA stayed green for a longer duration of time in contrast to the WT plants. Therefore we wanted to evaluate if senescence phenomena was affected in the *pad4* mutant plants. In WT plants, GPA feeding results in chlorophyll loss (Fig. 7*A* and 7*B*), and ultimately death of the infested organs (V. Pegadaraju and J. Shah, unpublished). Microscopy of trypan blue stained leaves from WT plants revealed the presence of dead cells in GPA-infested leaves, two days after release of GPA (Fig. 7*C*). In addition, expression of the Arabidopsis senescence associated genes, *SAG13*, *SAG21* and *SAG27*, were induced in leaves of WT plants exposed to GPA (Fig. 7*D*), suggesting the activation of a senescence-like mechanism in GPA-infested leaves. However, in comparison to the WT plant, GPA-feeding induced chlorophyll loss, and expression of the *SAG13* and *SAG21* genes were delayed in the *pad4-1* mutant plant (Fig. 7*A*, 7*B* and 7*D*). Moreover, unlike the WT leaves, microscopic cell death was not evident in *pad4-1* leaves two days after

release of aphids (Fig. 7*C*). These results suggest that in Arabidopsis, PAD4 modulates a senescence-like cell death mechanism, which is activated in response to GPA-feeding.

DISCUSSION

In this chapter we have shown that the Arabidopsis *PAD4* gene is required for basal resistance to the phloem feeding insect, GPA. *PAD4* was previously shown to modulate camalexin synthesis and SA synthesis and signaling in plant defense against pathogens (Zhou et al., 1999, 1998). However, our analysis of GPA performance on the camalexin biosynthesis mutant, *pad3*, and the SA-deficient *sid2* and *nahG* plants and the SA-insensitive *npr1* mutant plant suggest that camalexin and SA do not have an important role in basal resistance to GPA. Similarly, an earlier study by Moran et al., (2001), found no correlation between the activation of SA signaling and basal resistance to GPA in Arabidopsis. Hence, we propose that the participation of *PAD4* in plant defense against GPA is independent of its involvement in camalexin synthesis and SA signaling. A similar association of *PAD4* in the expression of Arabidopsis genes, which is independent of its involvement in SA signaling, was observed in a microarray gene expression study (Glazebrook et al., 2003). Moreover, unlike the involvement of *PAD4* in SA signaling, which is dependent on the presence of a functional *EDS1* gene, we have observed that *EDS1* is not important for basal resistance to GPA (refer to chapter IV, Fig 15*A*, *B*).

Aphid infestation causes changes in resource allocation in the plant. For example, pea aphid infestation of alfalfa stem resulted in increased deposition of N in the aphid-infested tissue (Girousse et al., 2005). In contrast, the flow of nutrients to the resource demanding primary growth zones is reduced during aphid infestation of other parts of the plant (Mittler and Sylvester, 1961; Pollard, 1973). Furthermore, aphid-infestation converts the natural sink tissues into a source tissue (Girousse et al., 2005). Gene

expression studies have shown that aphid infestation may alter expression of plant genes that are potentially involved in the conversion of the feeding sites into metabolic sinks. For example, GPA feeding induces expression of the *STP4* gene, which encodes a monosaccharide H^+ symporter (Moran et al., 2001, 2002). In addition, expression of an extracellular acidic invertase is induced in the GPA-infested Arabidopsis leaves (V. Pegadaraju and J. Shah, unpublished). Previously, STP proteins along with invertases were shown to increase import and metabolism of carbohydrates into metabolically active wounded and pathogen-infested organs (Buttner et al., 2000). The alteration in source-sink relationships could increase the flow of nutrients to the aphid-infested organ, thus providing the aphid with a continued supply of resources. However, the plant host could counter the ability of aphid to increase the sink nature of an organ by activating a senescence-like mechanism in the aphid-infested organ. Indeed, our experiments show that GPA-feeding activates a senescence-like mechanism in Arabidopsis, which is associated with chlorophyll loss, microscopic cell death and the elevated expression of the senescence associated *SAG13* and *SAG21* genes. However, the GPA-infested *pad4-1* leaves stayed green for longer than the GPA-infested wt leaves. The GPA-feeding induced chlorophyll loss and microscopic cell death were delayed in the *pad4-1* mutant, suggesting that PAD4 promotes the activation of this senescence-like mechanism. The delay in the induction of expression of the senescence associated genes, *SAG13* and *SAG21*, in GPA-infested *pad4-1* plants, in comparison to GPA-infested wt plants, provides additional support for the involvement of *PAD4* in modulating a senescence-like mechanism during plant-aphid interaction. Previously, PAD4 was shown to modulate the spontaneous cell death phenotype associated with the Arabidopsis *lesion simulating*

disease 1 (Rustérucci et al., 2001), and *accelerated cell death 11* (Brodersen et al., 2002) mutants.

In conclusion, we demonstrate that although PAD4 is involved in SA signaling and phytoalexin metabolism, its involvement in basal resistance to aphids appears to be independent of its role in SA-signaling and phytoalexin synthesis. Instead *PAD4* modulates the activation of GPA induced senescence process in plants which may have role in basal resistance against phloem feeding insects.

ACKNOWLEDGEMENTS

We thank Sharon McClung for providing us with the *Myzus persicae* colony, Drs. Ratnesh Chaturvedi and Ragiba Makandar, for reading this chapter, and the Arabidopsis Biological Resource Center (ABRC) at Ohio State University for Arabidopsis seeds. This work was supported by a grant from the Cooperative State Research, Education, and Extension Service, U.S. Department of Agriculture, under agreement 2004-35301-14506, a grant from the National Science Foundation (MCB0416839) and by a seed grant from the Kansas State University Plant Biotechnology Center. Fellowships from the Kansas Biomedical Research Infrastructure Network and the Terry Johnson Cancer Center provided support for CK.

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FIGURE LEGENDS

Fig. 5. *PAD4* expression is required for resistance to GPA. (*A*) RT-PCR analysis of *PAD4* and *ACT8* expression in GPA-infested Arabidopsis leaves. RT-PCR was performed on RNA extracted 3, 6, 12, 24 and 48 h post infestation (hpi). RNA extracted from uninfested plants provided a negative control. (*B*) Comparison of GPA numbers on WT and *pad4-1* mutant (left panel), and WT and a transgenic line (*pad4*Δ) that contains a T-DNA insertion within *PAD4* (right panel), 2 days after release of 15 insects per plant. All values are the mean of 15 plants ± SE. (*C*) Seed yield from uninfested and GPA-infested WT and *pad4-1* mutant plants. All values are the mean of seed yield from five plants ± SE. Different letters above the bars indicate values that are different from each other with a confidence of 95% or greater with Student's *t*-test. The results on the seed yield were concluded based on data from two independent experiments.

Fig. 6. Camalexin and SA are not required for basal resistance to GPA. (*A*) Comparison of GPA numbers on WT and the camalexin deficient *pad3* mutant, (*B*) WT and the SA-deficient *sid2* and the SA-insensitive *npr1-1* mutant plants, (*C*) WT and the transgenic *nahG* plants which accumulates lower levels of SA, 2 days after release of 15 insects per plant. All values are the mean of 15 plants ± SE. Different letters above the bars indicate values that are different from each other with a confidence of 95% or greater with Student's *t*-test. Results were concluded with data obtained from three independent experiments.

Fig. 7. PAD4 modulates a senescence-like process that is activated in response to GPA-feeding. (*A*) Photograph of WT and *pad4-1* leaves, 7 days after release of GPA. The aphids were removed before the leaves were photographed. (*B*) Relative chlorophyll content in GPA infested leaves of WT and *pad4-1* plants, two days after release of 15 insects per plant. The chlorophyll values in the GPA infested WT and *pad4-1* plants are relative to that in the corresponding uninfested WT and *pad4*-1 plants, which were assigned a value of 100. (*C*) Trypan blue staining of leaves from uninfested WT and *pad4-1* plants and from GPA-infested WT and *pad4-1* plants, 2 days after release of insects. The arrows point to the intensely stained dead cells. (*D*) RT-PCR analysis of *SAG13* (761bp), *SAG21* (181bp), *SAG27* (523bp) and *ACT8* (238bp) expression in leaves from uninfested WT and *pad4-1* plants and leaves from GPA-infested WT and *pad4-1* plants, 3, 6, 12, 24, 48 and 72 h post infestation (hpi) by GPA.

Fig. 5

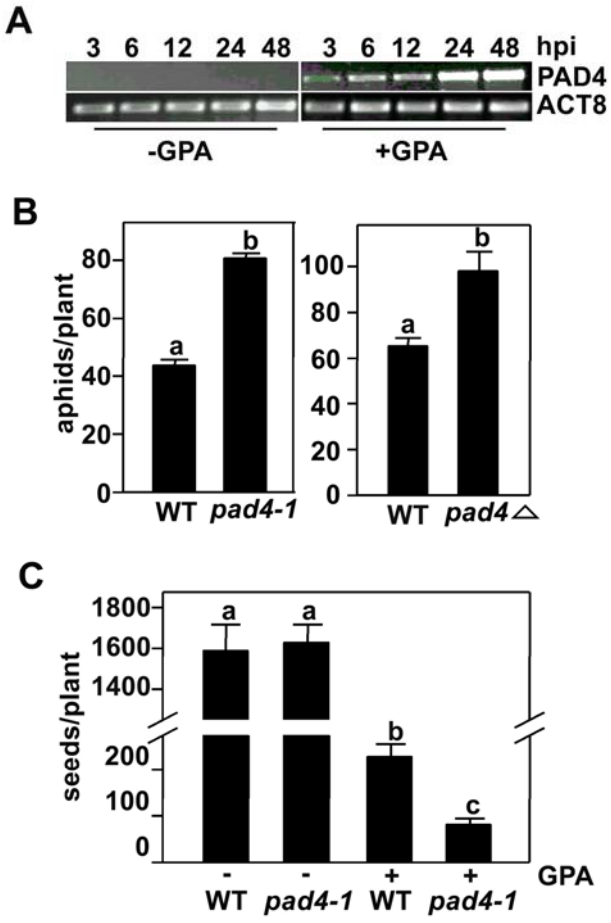

A
3 6 12 24 48 3 6 12 24 48 hpi
PAD4
ACT8
-GPA
+GPA
B
aphids/plant
0 20 40 60 80
a b
WT pad4-1
0 20 40 60 80 100
a b
WT pad4△
C
seeds/plant
0 100 200 1400 1600 1800
a a b c
- - + + GPA
WT pad4-1 WT pad4-1

Fig. 6

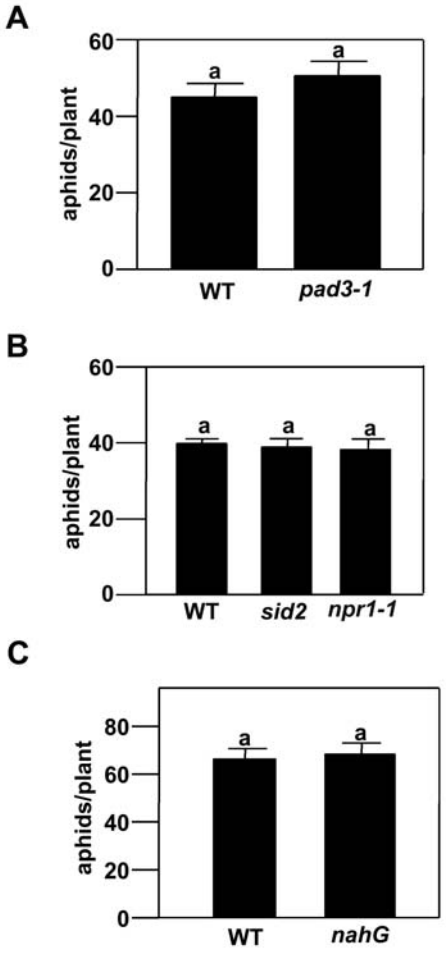
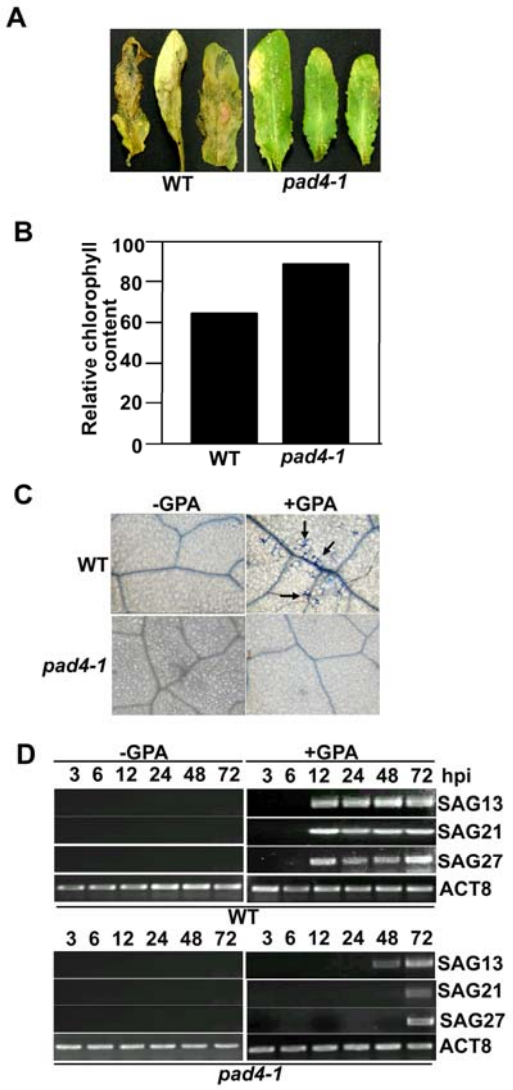

A
60
40
20
0
aphids/plant
a
a
WT
pad3-1
B
60
40
20
0
aphids/plant
a
a
a
WT
sid2
npr1-1
C
80
60
40
20
0
aphids/plant
a
a
WT
nahG

Fig. 7


A
WT
pad4-1
B
Relative chlorophyll content
100
80
60
40
20
0
WT
pad4-1
C
-GPA
+GPA
WT
pad4-1
D
-GPA
+GPA
3 6 12 24 48 72 3 6 12 24 48 72 hpi
SAG13
SAG21
SAG27
ACT8
WT
3 6 12 24 48 72 3 6 12 24 48 72
SAG13
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ACT8
pad4-1

Chapter III

A *PAD4* modulated senescence mechanism is associated with basal resistance against green peach aphid

SUMMARY

GPA feeding activates premature leaf senescence, which is modulated by the Arabidopsis *PAD4* gene. Here we show that in comparisons to the WT plants, GPA growth was restricted on the Arabidopsis hypersenescent mutants, *ssi2* and *cpr5*. The *ssi2*-conferred resistance against GPA and constitutive high level expression of the *SAG* genes were compromised in the *ssi2 pad4* double mutant, suggesting that the wild type *SSI2* and *PAD4* gene have opposing effects on basal resistance against GPA. Furthermore, GPA-feeding-induced senescence is hyperactivated in transgenic Arabidopsis plants that constitutively overexpress the *PAD4* transcript. This hypersensitivity of the *PAD4* overexpressing lines to GPA-feeding is paralleled by a heightened resistance to GPA in these *PAD4* overexpressing plants than the WT plants. We suggest that *PAD4* modulates the activation of premature leaf senescence that contributes towards controlling GPA growth.

INTRODUCTION

Senescence is a developmental process that results in the ordered disassembly and degradation of cellular components for the recovery and translocation of the nutrients within the plant body (Gan & Amasino, 1997; Noodén et al., 1997). During the final stages of leaf senescence chromatin condensation and DNA laddering which are hallmarks of apoptotic cell death can be observed (Delorme et al., 2000; Simeonova et al., 2000). Leaf senescence is characterized by the turnover of cellular chlorophyll, protein, and lipid degradation (Hörtensteiner & Feller, 2002; Thompson et al., 1998). Furthermore, leaf senescence is an active process that involves transcription activation and downregulation of senescence–associated genes (*SAG*s) (Davies and Grierson, 1989; Bate et al., 1991; Thomas, 1992; Becker and Apel, 1993; Hensel et al., 1993; Buchanan-Wollaston, 1994; Lohman et al., 1994; Smart et al., 1995).

SAG gene expression is known to be regulated temporally. Smart et al. (1994) proposed six classes of *SAG* genes, which differ in the pattern of gene expression and function. The class I genes constitute the housekeeping genes that are expressed at a constant level throughout the life cycle of the leaf, whereas, the transcription of class II and class III genes are required only for the onset of senescence, but not during the later stages of senescence. In contrast, the class IV genes, which encode regulatory proteins are turned on only after the onset of senescence and are expressed only for a short period of time. Class V and VI genes encode proteins involved in the nutrient mobilization process and are activated at the onset of senescence and continue till the death of the leaf. For instance, the *SAG12*, a class V member encoding a cysteine protease, is expressed

only in the yellowing tissue during the late stages of leaf senescence (Lohman et al., 1994). An autoregulated induction of *IPT* (*ISOPENTYL TRANSFERASE*) gene using the *SAG12* promoter increased the leaf number and seed yield by delaying the senescence mechanism in plants (Gan and Amasino, 1995).

Infection of plants by incompatible pathogens induces programmed cell-death process, referred to as the hypersensitive response (HR), which is believed to contribute to pathogen defense (Greenberg, 1997; Heath, 2000). At the molecular and physiological level both leaf senescence and HR have some similarities. For example, both processes involve the induction of pathogenesis-related genes and the accumulation of salicylic acid (SA) and reactive oxygen species (Quirino et al., 2000, Morris et al., 2000; Prochazkova et al., 2001). Moreover, expression of some SAG genes is also induced by SA application. Therefore, it seems that there is a significant overlap between signaling pathways involved in the plant defense against the biotic stress and leaf senescence. Several mutants of Arabidopsis have been identified, in which cell death is inappropriately activated. For example *ssi1*, *ssi2*, *ssi4*, *cpr1*, *cpr5*, *acd11* mutants constitutively exhibit cell death and SA mediated defense response against pathogenes and are resistant to virulent strains of *Pseudomonas syringae* and *Peronospora parasitica* (Bowling et al., 1997; Shah et al., 1999, 2000; Shirano et al., 2002; Brodersen et al., 2002).

The *hys1* mutation, which is an allele of *cpr5*, was independently identified in a screen for hypersenescent mutants, also (Yoshida et al., 2002). The *hys1* mutant exhibits

premature expression of dark-induced and age-dependent leaf senescence which is accompanied by the expression of several SAG genes (Yoshida et al., 2002). *HYS1*/*CPR5* gene encodes a novel membrane protein that has a nuclear-localisation signal, suggesting that it functions in signal transduction, although the biochemical function of this gene remains to be elucidated (Yoshida et al., 2002). Cell death events during HR do show some differences from leaf senescence. For instance, the Arabidopsis *accelerated cell death 11*(*acd11*) mutant, which carries a mutation in a sphingosine transferase protein, exhibits a spontaneous cell death phenotype accompanied by the expression of *SAG13* but not *SAG12*, which is a late marker for senescence (Brodersen et al., 2002). This lack of *SAG12* expression in *acd11* has been taken as evidence to support the idea that cell death events can be uncoupled from the age-dependent leaf senescence.

Aphid feeding alters C and N allocation and source-sink relationship throughout the plant, resulting in the induction of a strong sink in the infested organ (Pollard, 1973; Girousse et al., 2005). Aphid infestation induces premature senescence of plant organs (Inbar et al., 1995). The increased proteolytic activity associated with the manifestation of senescence could increase the availability of amino acids for the aphid. However, in some cases senescence-associated processes may limit aphid performance. For example, premature senescence induced by a gall-aphid correlated with the reduced performance of another aphid, feeding on the same leaflet of *Pistacia palaestina* trees (Inbar et al. 1995).

Here we demonstrate that GPA growth is limited on the Arabidopsis *ssi2* and *cpr5* mutants, which constitutively manifest senescence associated characteristics like cell death and *SAG* expression. *ssi2*-conferred resistance to the GPA was compromised in the *ssi2 pad4* double mutant. Further, GPA growth was also limited in the *PAD4* over expressing plants which also hyperactivate leaf senescence response to GPA feeding.

MATERIALS AND METHODS

Plant and Aphid Growth Conditions

Arabidopsis plants were grown in soil at 22 ℃ in a growth chamber programmed for 14 h light (100 μE m^{-2} s^{-1}) and 10 h dark cycle. Approximately four-week-old Arabidopsis plants were used for all studies. A combination of commercially available radish (Early scarlet globe) and mustard (Florida broadleaf), at a 50:50 ratio, was used for the routine propagation of GPA at 22 ℃ in a growth chamber programmed for 14 h light (100 μE m^{-2} s^{-1}) and 10 h dark cycle. Approximately 20-25 day old radish and mustard seedlings were used as the feeding material for aphids. Aphids were transferred to fresh batch of radish and mustard mix once every two weeks.

No-choice test

A no-choice test was used to assay aphid growth on wild type and mutant plants. Approximately four-week-old Arabidopsis plants were used in the bioassay with a clonally propagated GPA. For the no-choice test each Arabidopsis plant received fifteen mature apterous (wingless forms) aphids at the center of the rosette and the plants were incubated at 22° C in a growth chamber programmed for 14 h light (100 μE m^{-2} s^{-1}) and 10 h dark cycle. Growth of GPA on the Arabidopsis plants was measured 48 h post infestation. All values are the means of 15 plants $\pm$ SE. Student's t-test was utilized to determine significance of difference between different treatments with a confidence of 95% and higher. Student t-tests were performed using Sigma plot V 5.0 (SPSS Inc, Chicago, IL). Each experiment was repeated thrice before we finally concluded our results.

Histochemistry and microscopy

Leaf samples for trypan blue staining were processed and analyzed as described by Rate et al. (1999). Arabidopsis leaf samples infested with GPA for duration of 48 h were harvested and vacuum infiltrated at ~50 mbar. Samples were subsequently boiled in the microwave for 1 min and cooled at room temperature for 1-2 min prior to the lactophenol treatment for 1 min at boiling temperature. Leaf samples were finally washed in 50% ethanol for 2-3 washes and mounted on glass slides for cell death visualization.

RNA extraction and RT-PCR analysis

Leaf material from uninfested and GPA-infested plants was harvested and quick frozen in liquid nitrogen. RNA was extracted as described by Das et al. (1990). Approximately 1 gm of Arabidopsis leaf tissue was ground in the presence of Liquid N_2 and was completely suspendend in 5 ml of extraction buffer (4 M guanidium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1M β-mercaptoethonol, 0.2M sodium acetate). 2.5 ml of CHISM (chloroform: isoamyl alcohol) was added to the samples suspendend in the extraction buffer, samples were vortexed vigioursly for about 10-20 sec and finally centrifuged at 13,000 rpm for 15 min. RNA in the supernanant was precipitated using isopropanol. RNA pellet was obtained after centrifugation at 15,000 rpm for 10 min. The isolated RNA was purified using the RNeasy Mini kit (Stratagene, CA), spectrophotometrically quantified at 260 nm and subsequently used in the RT-PCR reactions. RT-PCR analysis was performed with the Super Script One-step RT-PCR kit (Invitrogen Life Technologies, MD). The RT reaction was carried

out at 50℃ for 30 min in a 20 μl reaction with100 ng of the total RNA as template as recommended by the manufacturer. PCR conditions for the *ACT*8, *SAG13*, *SAG21* and *PAD4* were as follows: 95℃ for 5 min followed by 25 cycles of 95℃ for 15 sec, 50 or 55℃ for 30 sec and 72℃ for 1 min with a final extension at 72℃ for 5 min. The ACT8-F (5'-ATGAAGATTAAGGTCGTGGCA-3') and ACT8-R (5'-TCCGAGTTTGAAGAGGCTAC-3'), SAG12-F(5'-TCTCGTCCACTCGACAATGAA-3') and SAG12-R(5' AGCTTTCATGGCAAGACCACA-3'), SAG13-F (5'-CAAGATGGAGTCTTGGAGGCA-3') and SAG13-R (5'-GGAAAAACCGTTAACAGTGGA-3'), SAG21-F (5'-CCAATGCTATCTTCCGACGTG 3') and SAG21-R (5'- GAACCGGTTTCGGGTCTGTAA 3'), SAG27-F(5'-TCCTGGCCCTGAAGTAGAAA-3') and SAG27-R(5'-GTCCCGCAAGAACCTGTCC-3'), PAD4-F (5'-GCTCTCCTCTGCTCGGAAACC 3') and PAD4-R (5'-TTTTCTCGCCTCATCCAACCA 3') gene specific primers were used for PCR amplification of *ACT8* (238 bp), *SAG13* (761 bp), *SAG21* (181 bp), *SAG27*(523 bp) and *PAD4* (959 bp). The amplified fragments were resolved on a 1.2% agarose gel, stained with ethidium bromide and visualized with a Gel Doc UVP BioDoc-It^{TM} system.

RESULTS

Constitutive cell death mutants of Arabidopsis display heightened resistance to GPA.

We had previously observed that GPA feeding results in premature leaf senescence. To test the hypothesis that premature leaf senescence may be a means by which plants counter the ability of aphids to alter plant metabolism and hence growth of aphids, we monitored GPA performance on the Arabidopsis *ssi2* and *cpr5* mutants, which spontaneously develop lesions containing dead cells (Bowling et al., 1997; Shah et al., 2001) and constitutively express the *SAG13, 21, 27* gene at elevated level (Fig. 8*C*). As shown in the Fig. 8, in comparison to WT plants, GPA counts were lower on the *ssi2* and *cpr5* mutant plants (Fig. 8*A*, *B*). The *ssi2* and *cpr5* mutants also accumulate high levels of SA and are dwarfs (Bowling et al., 1997; Shah et al., 2001). However, GPA growth on the *ssi2* and *ssi2 nahG* mutants, which accumulate higher basal levels of *SAG13* transcripts were comparably lower (Fig. 8*B*, *C*, *D*), suggesting that the accumulation of the high levels of SA is not important for the *ssi2*-conferred resistance to GPA. In addition, GPA counts on the *snc1* mutant, which is a dwarf and accumulates elevated levels of SA, like the *ssi2* and *cpr5* mutants, but does not constitutively express the *SAG* genes, was comparable to that on the WT plants (Fig. 8*A*, *B*), thus supporting our hypothesis that GPA performance is not restricted by the dwarf stature or elevated SA content of the *cpr5* and *ssi2* mutants.

pad4 compromises *ssi2*-conferred *SAG* gene expression and resistance against GPA.

In contrast to the hypersenescence and GPA resistant phenotype of the ssi2 mutant, mutations in the *PAD4* gene delay the activation of GPA-induced leaf senescence and permit higher growth of GPA (Refer Chapter 2, Fig. 5*B,* 7). To understand the genetic relationship between *ssi2* and *pad4*, we crossed the *pad4-1* mutant (in ecotype Columbia) with the *ssi2* mutant (in ecotype Nössen) to generate the *ssi2 pad4* double mutant plants. Since, the *ssi2* and *pad4-1* mutants differ in their genetic background, we performed our studies on progeny derived from multiple F2-plants (#2, 9 & 36) derived from this cross. As shown in Fig. 9*B*, presence of the *pad4-1* allele, compromised the *ssi2*-conferred constitutive expression of the *SAG* genes in the *ssi2 pad4* double mutant plants. In parallel, a no-choice test comparison of GPA growth between the *ssi2* and *ssi2 pad4* plants (#2, 9 & 36) indicated that GPA growth was higher on the *ssi2 pad4* double mutant plants than on the *ssi2* single mutant (Fig. 9*C*). However, amongst the three *ssi2 pad4* lines, variation in the extent of GPA growth was observed. The *ssi2 pad4* line #9 supported growth of GPA that was higher than observed on the WT and *ssi2* mutant plants, and comparable to that seen on the *pad4-1* single mutant plant. In contrast the *ssi2 pad4* line #s 2 and 36 had GPA growth that was intermediate between those on the *ssi2* single mutant and the WT plant. In all three *ssi2 pad4* lines, the *ssi2* conferred spontaneous cell death phenotype was prevalent, albeit at lower levels (Fig. 9*A*). These results suggest that *pad4-1* is episatic to *ssi2* for SAG expression. Furthermore, although *pad4-1* did compromise the *ssi2*-conferred resistance against GPA in one double mutant line, other loci from the ecotype Columbia and Nössen do impact the overall strength of GPA resistance in these hybrid plants. These results also suggest that cell death per se

may not be a factor contributing to the enhanced GPA resistance observed in the *ssi2* mutant.

GPA feeding triggered an early senescence response in the constitutively expressing *PAD4* lines.

To determine if *PAD4* indeed modulates the activation of premature leaf senescence in response to GPA feeding, we tested if constitutive expression of *PAD4* would hyperactivate the GPA-feeding induced premature leaf senescence. Transgenic Arabidopsis plants that constitutively express the wild type *PAD4* gene under the control of ubiquitously expressed constitutive cauliflower mosaic virus 35s gene promoter and nopaline synthase terminator were used for this study. These transgenic plants are in the *pad4-5* mutant background; hence, the *PAD4* transcript that accumulates in these plants is derived from the chimeric transgene. As shown in the Fig. 10*B*, *PAD4* constitutively accumulates in the transgenic plants, as opposed to the negligible expression of the endogenous *PAD4* gene in the WT plant. Morphologically, the constituitve *PAD4* expressing plants are indistinguishable from the WT plants (Fig. 10*A*). However, as shown in Fig. 11*C*, expression of SAG genes in response to GPA feeding was induced faster in the constituitve *PAD4* expressing plants, than in the WT plant. Both *SAG13* and *SAG21* expression was observed as early as 3 hpi in these transgenic lines, as opposed to 12 hpi in the WT plant. In addition, cell death in the transgenic line was also observed as early as 3 hpi, as opposed to 48 hpi in WT plants (Fig. 11*B*). These results support our hypothesis that *PAD4* modulates the activation of leaf senescence in response to GPA-feeding.

Constitutive expression of Arabidopsis PAD4 confers enhanced resistance against GPA.

We next tested if hyperactivation of GPA-feeding induced premature leaf senescence in the PAD4 overexpressing plants was paralleled by heightened resistance to GPA. A no-choice test was performed to compare the GPA growth on the transgenic PAD4 overexpressing lines and the WT plants. Indeed, as shown in Fig. 11, GPA growth was significantly lower on the transgenic constituitve *PAD4* expressing plants than on the WT plants (Fig. 11*A*), confirming the importance of PAD4 in modulating the activation of defense mechanisms that contribute to controlling GPA growth.

DISCUSSION

Aphid feeding alters source-sink relationships (Girousse et al., 2005), by converting the aphid infested organ into sink organ. This might be attained through suppressing the transcription of host defense genes in the local feeding tissue coupled with increased transcription of plant metabolic genes, required for enhancing the nutritional quality of the sink tissues (Voelckel et al., 2004). In *Myzus nicotianae-Nicotiana attenuata* interactions, aphid feeding caused down-regulation of the gene encoding a 13-lipoxygenase, a defense related protien and upregulation of a gene that encodes a protein involved in amino acid synthesis (Fd-GOGAT) in sink tissues as opposed to the source tissues. Aphid feeding also induces premature senescence in the plant organs (Inbar et al., 1995) possibly as a means to increase the availability of amino acids for aphids. Senescence is a catabolic process associated with increased turnover of proteins, lipids, and carbohydrates. While senescence could be beneficiary in some cases of plant-aphid interaction, in other cases it could also limit the performance of aphid. For instance, induction of premature senescence by gall-aphid feeding reduced the growth and development of another aphid feeding on the same leaflet of *Pistacia palaestina* trees (Inbar et al., 1995). The premature leaf senescence that is observed in GPA-infested plants could counter these aphid-induced changes. We therefore hypothesized that premature leaf senescence may be a defense mechanism utilized by Arabidopsis to counter GPA. In support of this hypothesis we found that GPA growth in comparison to the WT plants was lower on the Arabidopsis *ssi2* and *cpr5* mutants, which constitutively express the *SAG13, SAG21* and *SAG27* genes, and develop lesions containing dead cells.

In the *ssi2 pad4* mutants, *pad4-1* allele suppressed the *ssi2* conferred senescence aspect but, not the *ssi2* cell death properties. Double mutants of *ssi2 pad4* displayed loss of *ssi2*-confered GPA resistance; however these mutants varied in their levels of GPA counts. Since, the double mutants of *ssi2 pad4* were generated from a cross between two different backgrounds; there is a high likelihood of different genetic loci influencing the overall strength of the mutant phenotype. Thereby, the currently available data does not provide sufficient evidence to determine if PAD4 can be positioned either upstream or down stream of SSI2. Through our model proposed in Fig. 12, we suggest that both PAD4 and SSI2 participate in regulating the senescence mechanism in plants, in which PAD4 and SSI2 have opposite roles. Feeding by GPA triggers *PAD4* gene expression which positively modulates the senescence phenomenon. In contrast, a SSI2 derived product is involved in negative regulation this event. Alternatively, since *SSI2* encodes a fatty acid desaturase, a lipid that aberrantly accumulates in the *ssi2* mutant could constitutively activate plant defense against GPA. .

We also demonstrate that the constitutive expression of *PAD4* gene in Arabidopsis enhanced the basal resistance in plants by causing an early activation of senescence mechanism in plants. Interestingly, constitutive activation of *PAD4* in the uninfested transgenic lines was not associated with constitutive activation of SAG genes or cell death phenotype. This would suggest that PAD4 is dependent on an unknown factor to modulate senescence process. The candidate elicitor of this senescence response in plants may be a factor(s)originating either from the GPA or a plant-derived compound. Salivary secretion released by aphid posses cell wall degrading enzymes such as

pectinases and cellulases, which could generate potent elicitors of plant response (McAllan and Adams, 1961 Cambell and Dreyer, 1985).

In conclusion, manipulation of leaf senescence through breeding or genetic engineering might help to improve crop yields by keeping leaves photosynthetically active for longer. However, our results suggest that it might have a negative implact on crop plants. Since, delaying in senescence can predispose plants towards insect infestations especially towards phloem feeders like aphids.

ACKOWLEDGEMENTS

We thank Sharon McClung for providing us with the *Myzus persicae* colony, Dr. Jane Parker for providing us with constitutive *PAD4* expressing transgenic Arabidopsis lines, and the Arabidopsis Biological Resource Center (ABRC) at Ohio State University for Arabidopsis seeds.

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FIGURE LEGENDS

Fig. 8. Constitutive activation of a senescence-like mechanism is associated with enhanced resistance against GPA in the *ssi2* and *cpr5* mutant plants. (*A*) Comparison of GPA growth on the WT ecotype Columbia and *cpr5* mutant plant (left panel), and the WT ecotype Columbia and *snc1* mutant plant, 2 days after release of 15 insects per plant. The *cpr5* and *snc1* mutants are in the ecotype Columbia background. (*B*) Comparison of GPA growth on the WT ecotype Nössen, and the *ssi2*, *ssi2 nahG* and *nahG* mutant plants, 2 days after release of 15 insects per plant. The mutants are in the ecotype Nössen background. In *A* and *B*, all values are the mean of 15 plants $\pm$ SE. Different letters above the bars indicate values that are different from each other with a confidence of 95% or greater with Student's *t*-test. (*C*) RT-PCR analysis of *SAG13*, *SAG21*, *SAG27* and *ACT8* expression in leaves from four week old WT ecotype Columbia and Nössen plants, and the *ssi2* and *cpr5* mutant plants. The *ssi2* and *cpr5* mutants are in the ecotype Columbia and Nössen, respectively. (*D*) RT-PCR analysis of *SAG13* and *ACT8* expression in leaves from four week old GPA-uninfested WT ecotype Nössen, *ssi2*, *ssi2nahG*, *nahG* plants.

Fig. 9. Genetic interaction of *SSI2* and *PAD4*. (*A*) Trypan blue staining of leaves from uninfested *pad4-1*, *ssi2*, *ssi2pad4-1* line-2,-9, -36 and their respective WT plants. Homozygous lines for the *ssi2* and *pad4-1* were confirmed by performing a CAPS PCR analysis. The *pad4-1* and *ssi2* mutants are in the ecotype Columbia and Nössen, respectively. (*B*) RT-PCR analysis of *SAG13* (761bp), *SAG21* (181bp), *SAG27* (523bp) and *ACT8* (238bp) expression in leaves from four week old uninfested WT ecotype

(Columbia and Nössen plants), and the *ssi2*, *pad4-1*, *ssi2pad4* line -2,-9,-36 mutant plants. (*C*) Comparison of GPA growth on the WT (Columbia and Nössen), and *ssi2*, *pad4-1, ssi2 pad4-1* line #2, -9, -36 mutant plants, 2 days after release of 15 insects per plant. All values are mean of 15 plants ± SE. Different letters above the bars indicate values that are different from each other with a confidence of 95% with student's t-test. Data was concluded based on two independent experimental results.

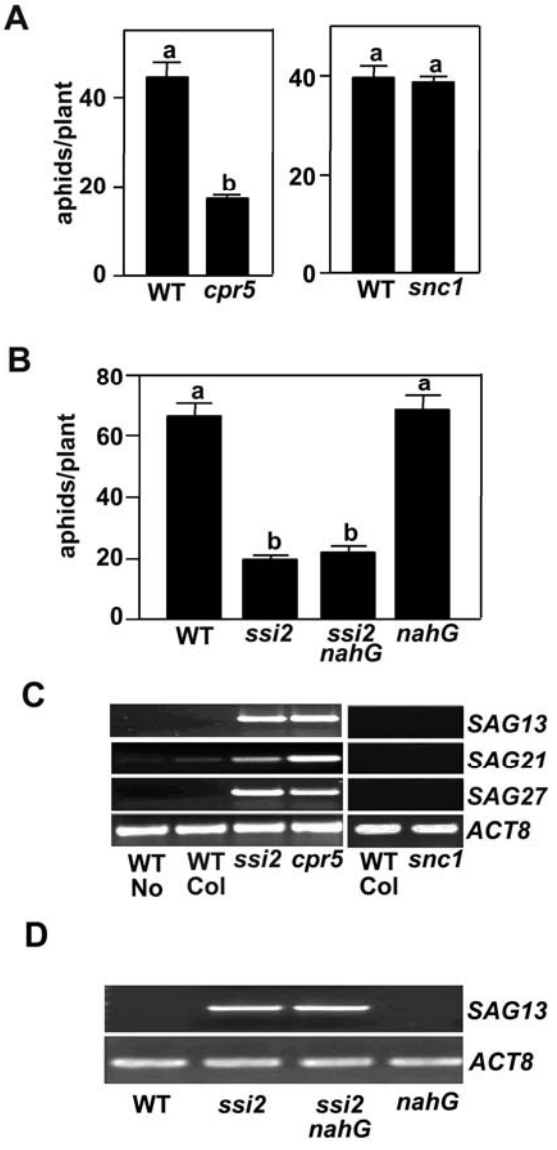
Fig. 10. Constitutive expression of PAD4 in Arabidopsis plants. (*A*) Photograph shows Arabidopsis WT plants (Ws-0, left panel) and *PAD4* over expressing plants. Both the WT and the constitutive *PAD4* expressing plants were grown at 22ºC, for three week prior to photograph. (*B*) RT-PCR analysis of *PAD4* and *ACT8* expression in WT and *PAD4* constitutively expressing lines infested with GPA. RT-PCR was performed on RNA extracted 3, 6, 12, 24, 48 h post infestation (hpi). RNA extracted from uninfested plants provided as negative control. *ACT8* gene served as a control for RNA quality in the RT-PCR reaction. These experiments were repeated twice and RNA used in each experiment was isolated independently.

Fig. 11. Enhanced basal resistance in the constitutive PAD4 expressing line is associated with early activation of senescence event. (*A*) Comparison of GPA numbers on WT and *PAD4* overexpressing lines, 48h after release of 15 insects per plant. All values are mean of 15 plants ± SE. Different letters above the bars indicate values that are different from each other with a confidence of 95% with student's t-test. (*B*) Trypan blue staining of leaves from uninfested WT and *PAD4* overexpressing lines and from GPA–

infested WT and *PAD4* over expressing plants, 3, 6, 12, 24, 48 h post GPA feeding. (*C*) RT-PCR analysis of *SAG13* (761 bp), *SAG21* (181 bp) and *ACT8* (238 bp) expression in leaves from uninfested WT and the constitutive *PAD4* expressing plants (left panel), and leaves from GPA-infested WT and the constitutive *PAD4* expressing plants, 3, 6, 12, 24, 48 h post infestation (hpi) by GPA. *ACT8* gene served as a control for RNA quality in the RT-PCR reaction. RT-PCR experiments were repeated twice and RNA used in each experiment was isolated independently.

Fig. 12. Model for GPA-induced senescence mechanism in Arabidopsis. GPA-feeding induces expression of the *PAD4* gene, which subsequently modulates senescence like mechanism in plants to provide resistance against GPA. Based on the studies of *ssi2* and *cpr5* mutants, *SSI2* and *CPR5* are shown to function by negatively regulating this event in WT Arabidopsis plants.

Fig. 8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b
a
WT
ssi2
ssi2 nahG
nahG
C
SAG13
SAG21
SAG27
ACT8
WT No
WT Col
ssi2
cpr5
WT Col
snc1
D
SAG13
ACT8
WT
ssi2
ssi2 nahG
nahG

Fig. 9

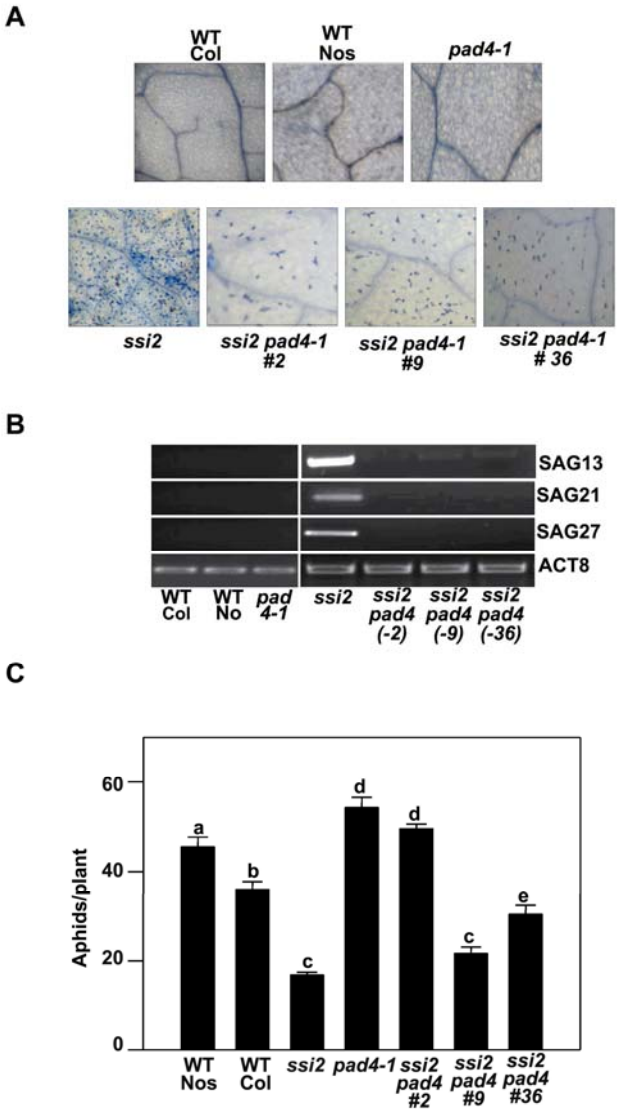

A
WT Col
WT Nos
pad4-1
ssi2
ssi2 pad4-1 #2
ssi2 pad4-1 #9
ssi2 pad4-1 # 36
B
SAG13
SAG21
SAG27
ACT8
WT Col
WT No
pad 4-1
ssi2
ssi2 pad4 (-2)
ssi2 pad4 (-9)
ssi2 pad4 (-36)
C
Aphids/plant
60
40
20
0
a
b
c
d
d
c
e
WT Nos
WT Col
ssi2
pad4-1
ssi2 pad4 #2
ssi2 pad4 #9
ssi2 pad4 #36

Fig. 10

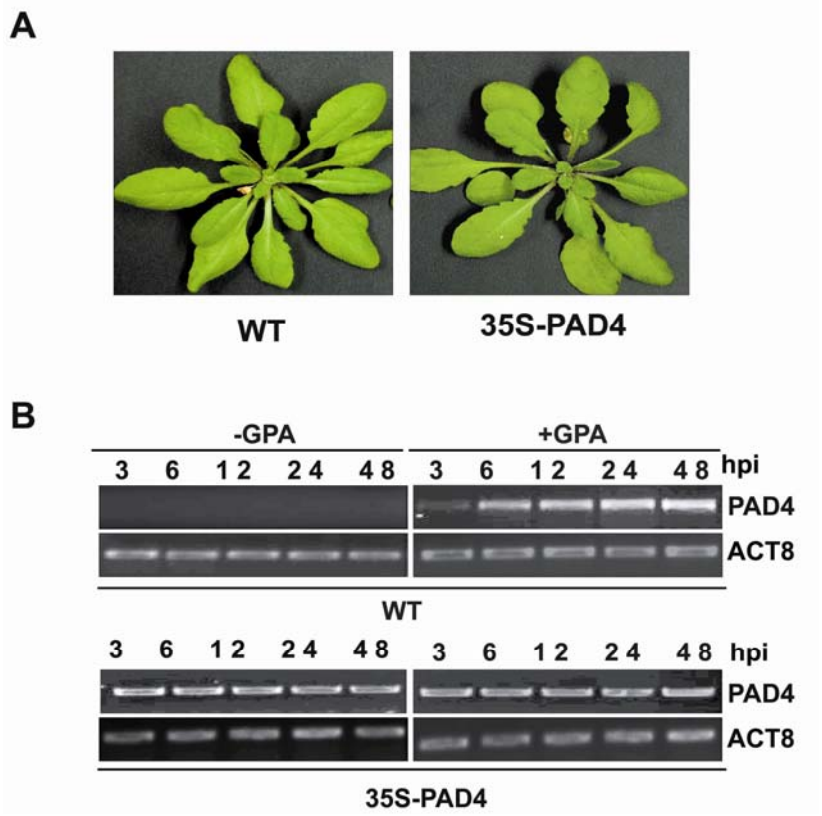

A
WT
35S-PAD4
B
-GPA
+GPA
3 6 12 24 48 3 6 12 24 48 hpi
PAD4
ACT8
WT
3 6 12 24 48 3 6 12 24 48 hpi
PAD4
ACT8
35S-PAD4

Fig. 11

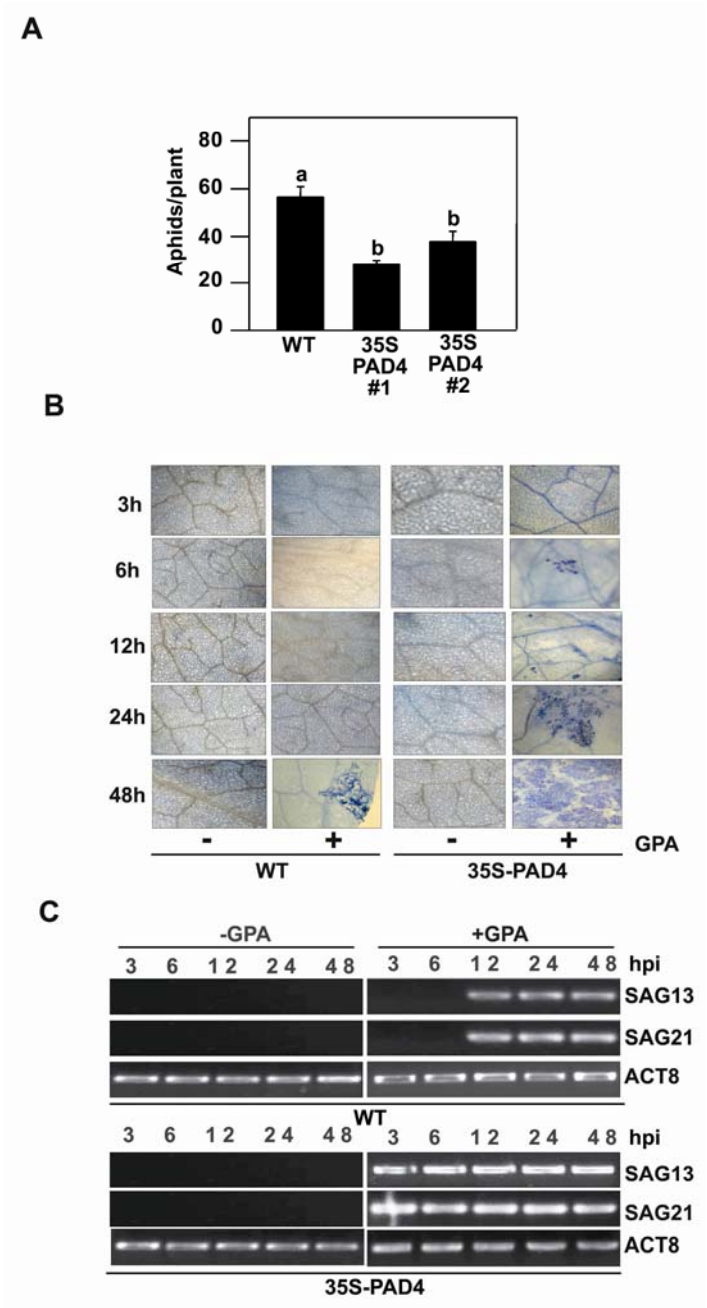
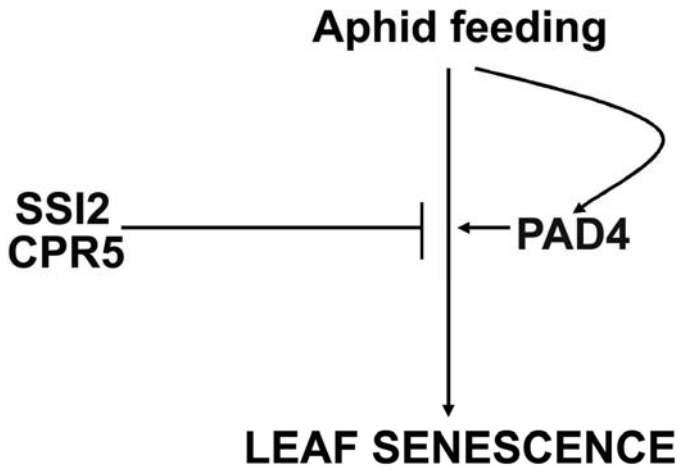

A
Aphids/plant
80
60
40
20
0
a
b
b
WT
35S PAD4 #1
35S PAD4 #2
B
3h
6h
12h
24h
48h
-
+
-
+
GPA
WT
35S-PAD4
C
-GPA
+GPA
3 6 12 24 48 3 6 12 24 48 hpi
SAG13
SAG21
ACT8
WT
3 6 12 24 48 3 6 12 24 48 hpi
SAG13
SAG21
ACT8
35S-PAD4

Fig.12



Chapter IV

PAD4's Involvement in Plant-Aphid Interaction is Independent of EDS1 and SAG101

SUMMARY

Previously, the *ENHANCED DISEASE SUSCEPTIBILITY 1(EDS1)* and *SENESCENCE – ASSOCIATED GENE 101* (*SAG101*), which encodes a EDS1-binding protein) were shown to required for accumulation of the PAD4 protein in uninfested Arabidopsis. Furthermore, elimination of *EDS1* and *SAG101* resulted in the reduction of PAD4 protein levels in uninfested plants. Here we show that *EDS1* and *SAG101* null mutants did not display enhanced susceptibility to GPA like the *pad4* mutant. Furthermore, eliminating both PAD4 and SAG101 activity in the *pad4 sag101* mutant did not result in a additive effect in a no-choice test; GPA growth was similar in both *pad4-1* and in *pad4-1 sag101-1* mutants, confirming that *sag101* is not required for the basal resistance against GPA. Furthermore, unlike in the *pad4* mutant, GPA feeding triggered cell death response and caused the activation of SAG13 and *SAG21* expression in a Col-0 *eds1* RNAi line, in the *sag101-1* mutant and in the WT plants. These results strongly support the hypothesis that, both EDS1 and SAG101 functions are not required either in PAD4 mediated resistance mechanism against GPA or in GPA-induced activation of premature leaf senescence.

INTRODUCTION

PAD4 is involved with gene-for-gene resistance against bacterial and oomycete pathogens that is mediated by a variety of *Resistance* (*R*) genes that contain the intracellular Toll-interleukin receptor, nucleotide binding/leucine-rich repeat(TIR-NBS-LRR) domains (Feys et al., 2001). The EDS1 (*ENHANCED DISEASE SUSCEPTIBILITY 1*) gene is also required for gene-for-gene resistance mediated by *R* gene pathways that require PAD4. In addition, EDS1 is required for resistance conferred by other R genes, which are not dependent on PAD4 function (Feys et al., 2001), suggesting that EDS1 signaling in plant defense may be mediated via a PAD4 independent mechanism, in addition to PAD4-dependent mechanism. Furthermore, EDS1 and PAD4 can be co-immunoprecipitated from extract of Arabidopsis leaves (Feys et al., 2001). Genetic analysis positioned EDS1 and PAD4 downstream of several TIR-NBS-LRR class of R proteins (Zhang et al., 2003; Zhou et al., 2004). Furthermore, EDS1 and PAD4 are required for SA accumulation and for defense potentiation involving the processing of ROI-derived signals around the site of infection foci (Feys et al., 2001; Rustérucci et al., 2001). Moreover, SA itself regulates the expression of *EDS1* and *PAD4* as a part of a positive feed back loop that is most likely important in defense amplification. (Zhou et al., 1998; Falk et al., 1999; Jirage et al., 1999; Shirano et al., 2002; Xiao et al., 2003). In addition, the role of EDS1and PAD4 was also found necessary in regulating abiotic stress response. For instance, the *lsd1* mutants of Arabidopsis which fail to acclimate to excess excitation energy (EEE) and exhibit symptoms such as reduced stomatal conductance were dependent on EDS1 and PAD4 functions (Mateo et al., 2004).

EDS1 and PAD4 have pockets of homology to eukaryotic lipases (Falk et al., 1999; Jirage et al., 1999). The other Arabidopsis member which shares a significant homology to the EP-domain (novel conserved domain in the C-terminus of EDS1 and PAD4) of EDS1 and PAD4 is the *SENESCENCE-ASSOCIATED GENE101* (*SAG101*). *SAG101* was originally identified in a screen for mutants with altered senescence properties; antisenes suppression of *SAG101* gene in Arabidopsis led to the delay in age-dependent leaf senescence (He and Gan, 2002). More recently, SAG101 has been identified as a new *in-vivo* interactor of EDS1 (Feys et al., 2005). SAG101 participates in the EDS1- directed defense signaling, however in a PAD4- independent manner. EDS1, forms distinct complexes with PAD4 and SAG101 which can be purified away from each other. Furthermore, while the EDS1-SAG101 complex localized inside the nuclear compartment, the EDS1-PAD4 and EDS1-EDS1 complexes could be detected both in nucleus as well as cytosol.

Both PAD4 and SAG101 regulate the levels of EDS1 protein accumulation *in vivo.* The *hypersusceptibility* of the *pad4 sag101* double mutant plant to *P. parasitica* could be due to severe depletion of EDS1 protein level in the double mutant plant as opposed to the individual mutants (Feys et al., 2005). This regulation is achieved by EDS1 at the posttranscriptional stage; the level of *SAG101* and *PAD4* mRNA remains unaffected in the *eds1* null mutant (Feys et al., 2005). It is likely that EDS1 acts as an adaptor or scaffold for PAD4 and SAG101 to ensure appropriate signal relay (Park et al., 2003; Feys et al., 2005).

Earlier, we showed that *PAD4* has an important role in basal resistance against green peach aphid (Chapter 2, Fig.1); Loss-of-function mutations in *PAD4* enhanced susceptibility to GPA. In contrast, constitutive expression of *PAD4* in Arabidopsis enhanced the basal resistance in plants against GPA. This reduced growth of GPA in the overexpressing lines was accompanied by an early triggering of GPA-induced senescence event in the plants. These results strongly favor the hypothesis that *PAD4* function in basal resistance against GPA is predominantly through modulating leaf senescence in plants. However, it is not clear if as in plant-pathogen interaction the physical interaction between PAD4, EDS1and SAG101 is important for Arabidopsis resistance against GPA Here, we provide genetic evidence that PAD4 modulates premature leaf senescence and GPA resistance occurs independently of EDS1 and SAG101 function.

MATERIALS AND METHODS

Plant and Aphid Growth Conditions

Arabidopsis plants were grown in soil at 22° C in a growth chamber programmed for 14 h light (100 μE m^{-2} s^{-1}) and 10 h dark cycle. Approximately four-week-old Arabidopsis plants were used for all studies. A combination of commercially available radish (Early scarlet globe) and mustard (Florida broadleaf), at a 50:50 ratio, was used for the routine propagation of GPA at 22° C in a growth chamber programmed for 14 h light (100 μE m^{-2} s^{-1}) and 10 h dark cycle. Approximately 20-25 day old radish and mustard seedlings were used as the feeding material for aphids. Aphids were transferred to fresh batch of radish and mustard mix once every two weeks.

No-choice test

A no-choice test was used to assay aphid growth on wild type and mutant plants. Approximately four-week-old Arabidopsis plants were used in the bioassay with a clonally propagated GPA. For the no-choice test each Arabidopsis plant received fifteen mature apterous (wingless forms) aphids at the center of the rosette and the plants were incubated at 22° C in a growth chamber programmed for 14 h light (100 μE m^{-2} s^{-1}) and 10 h dark cycle. Growth of GPA on the Arabidopsis plants was measured 48 h post infestation. All values are the mean of 15 plants $\pm$ SE. Different letters above the bars indicate values that are different from each other with a confidence of 95% or greater with Student's *t*-test. Student t-tests were performed using Sigma plot V 5.0 (SPSS Inc, Chicago, IL). Each experiment was repeated thrice before we finally concluded our results.

Histochemistry and microscopy

Leaf samples for trypan blue staining were processed and analyzed as described by Rate et al. (1999). Arabidopsis leaf samples infested with GPA for duration of 48 h was harvested and vacuum infiltrated at ~50 mbar. Samples were subsequently boiled in the microwave for 1 min and cooled at room temperature for 1-2 min prior to the lactophenol treatment for 1 min at boiling temperature. Leaf samples were finally washed in 50% ethanol for 2-3 washes and mounted on glass slides for cell death visualization.

RNA extraction and RT-PCR analysis

Leaf material from uninfested and GPA-infested plants was harvested and quick frozen in liquid nitrogen. RNA was extracted as described by Das et al. (1990). Approximately 1 gm of Arabidopsis leaf tissue was ground in the presence of Liquid N_2 and was completely suspendend in 5 ml of extraction buffer (4M guanidium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1M β-mercaptoethonol, 0.2M sodium acetate). 2.5 ml of CHISM (24:1 mix of chloroform: isoamyl alcohol) was added to the samples suspendend in the extraction buffer, samples were vortexed vigioursly for about 10-20 sec and finally centrifuged at 13,000 rpm for 15 min. RNA in the supernanant was precipitated using isopropanol. RNA pellet was obtained after centrifugation at 15,000 rpm for 10 min. The isolated RNA was purified using the RNeasy Mini kit (Stratagene, CA), spectrophotometrically quantified at 260 nm and subsequently used in the RT-PCR reactions. RT-PCR analysis was performed with the Super

Script One-step RT-PCR kit (Invitrogen Life Technologies, MD). The RT reaction was carried out at 50℃ for 30 min in a 20 μl reaction with100 ng of the total RNA as template as recommended by the manufacturer. PCR conditions for the *ACT*8, *SAG13*, *SAG21* and *PAD4* were as follows: 95℃ for 5 min followed by 25 cycles of 95℃ for 15 sec, 50 or 55℃ for 30 sec and 72℃ for 1 min with a final extension at 72℃ for 5 min. The ACT8-F (5'-ATGAAGATTAAGGTCGTGGCA-3') and ACT8-R (5'-TCCGAGTTTGAAGAGGCTAC-3'), SAG12-F(5'-TCTCGTCCACTCGACAATGAA-3') and SAG12-R(5' AGCTTTCATGGCAAGACCACA-3'), SAG13-F (5'-CAAGATGGAGTCTTGGAGGCA-3') and SAG13-R (5'-GGAAAAACCGTTAACAGTGGA-3'), SAG21-F (5'-CCAATGCTATCTTCCGACGTG 3') and SAG21-R (5'- GAACCGGTTTCGGGTCTGTAA 3'), SAG27-F(5'-TCCTGGCCCTGAAGTAGAAA-3')and SAG27-R(5'-GTCCCGCAAGAACCTGTCC-3'), PAD4-F (5'-GCTCTCCTCTGCTCGGAAACC 3') and PAD4-R (5'-TTTTCTCGCCTCATCCAACCA 3') gene specific primers were used for PCR amplification of *ACT8* (238 bp), *SAG13* (761 bp), *SAG21* (181 bp), *SAG27*(523 bp) and *PAD4* (959 bp). The amplified fragments were resolved on a 1.2% agarose gel, stained with ethidium bromide and visualized with a Gel Doc UVP BioDoc-It^{TM} system.

RESULTS

EDS1 function is not required for the PAD4-conferred resistance against GPA.

We observed that in comparison to the WT uninfested plants, both the *EDS1* and *PAD4* gene transcripts accumulated to higher levels in the GPA–infested plants (Fig 14). Induction of *EDS1* and *PAD4* occurred as early as 3 hours post release of GPA. To further determine if PAD4 is dependent on functional EDS1 for modulating basal resistance against GPA, a no-choice test was performed on *EDS1*, (Col-0 *eds1* RNAi, Ws-0 *eds1-1*) and *PAD4* (Col-0 *pad4-1*, Ws-0 *pad4-5*) mutants. Levels of *EDS1* mRNA were almost undetectable in the col-0 *eds1* RNAi line in comparison to the WT plants (Feys et al., 2005) In comparison to the WT plants, as reported earlier, *pad4-1* mutants supported greater growth of GPA. However, GPA growth was comparable between the WT and Col-*eds1* RNAi line (Fig. 15*A*). The Col-0 line in which the endogenous *EDS1* was stably silenced using the double-stranded RNA interference (dsRNAi) is not a complete null mutant of the *EDS1* gene (Feys et al., 2005). To further characterize the GPA growth phenotype on the complete null mutants of *EDS1* we performed no-choice analysis for GPA-resistance between WT and the *eds1-1* mutant. Similar to our observation with the *eds1* RNAi line GPA growth between the *eds1-1* mutant and WT were comparable. Furthermore, we observed that the GPA growth patterns on the *pad4-5/eds1-1* double mutants were similar to *pad4-5* mutants (Fig. 15*B*). The *pad4-5* allele is a null allele of PAD4 in the Ws ecotype background.

PAD4 functions independently of SAG101 in modulating basal resistance against GPA

Unlike, *EDS1* and *PAD4* gene expression, *SAG101*expression could not be detected in the GPA infested samples (Fig.14). Furthermore, loss of *SAG101* gene function did not cause higher growth of GPA (Fig. 16). GPA growth on the *sag101-1* mutants was comparable to that on the WT plant. Overlap exists between PAD4 and SAG101 requirement in plant-pathogen interaction (Feys et al., 2005). To determine if similarly PAD4 and SAG101 may have overlapping roles in plant-aphid interactions, we performed the no-choice test in Arabidopsis to compare GPA growth on the *pad4-1, sag101-1* single mutants and *pad4-1 sag101-1* double mutant. As shown in Fig.16 there were no significant differences in GPA growth between *pad4-1* and *pad4-1 sag101-1* mutants. These results strongly favor the hypothesis that PAD4 functions independently of EDS1 and SAG101 to mediate resistance in plants against GPA.

GPA-induced senescence occurs in PAD4 dependent, EDS1 and SAG101 independent manner

To assess weather, EDS1 and SAG101 participate in the GPA –induced senescence mechanism in Arabidopsis; we analyzed the senescence responses in the GPA-infested *EDS1, PAD4* and *SAG101* mutants. Microscopy of trypan blue stained leaves from WT, Col-0 *eds1*RNAi line and *sag101-1* mutant plants revealed the presence of dead cells in GPA-infested leaves, two days after release of GPA (Fig. 17*A*). In contrast, as shown previously the GPA feeding did not trigger the activation of cell death response in the *pad4-1* mutant plants. Furthermore, visible signs of senescence, such as yellowing of leaf could be observed on WT (La-er, Col -0), *eds1-2,* and *sag101* mutant plants 7days after release of GPA (Fig. 17*B*). However, *pad4-2* mutants stayed green at

the end of 7dpi with GPA, suggesting that *pad4-2* mutants loose less chlorophyll compared to the WT, *eds1-2* and *sag101-1* mutants. Also refer to Fig. 18 which shows the GPA growth on the *eds1-2* and *pad4-2* mutants, when compared to the WT plants GPA growth was higher in the *pad4-2* mutants, however the GPA growth was comparable in the *eds1-2* mutants. Further, to determine if GPA–feeding induced *SAG* gene expression on these mutants, RT–PCR analysis was performed with the SAG primers (Fig. 17*C*). As shown, GPA feeding, caused the induction of *SAG13* and *SAG21* gene expression in the WT, Col-0 *eds1* RNAi line and sag101-*1* mutants, however, a similar expression could not be detected in the *pad4-1* mutants.

DISCUSSION

In this study we have shown that Arabidopsis PAD4 modulates Arabidopsis defense against GPA, independent of its genetic interaction with the EDS1 and SAG101 genes. Additionally, the GPA-induced senescence events in Arabidopsis were not dependent on functions of EDS1 and SAG101. Previously, Feys et al., 2005, had shown that *in planta*, the basal levels of EDS1 protein are regulated by PAD4 and SAG101. The basal levels of EDS1 protein decreased in an incremental manner in the *sag101*, *pad4*, and *pad4 sag101* mutants, the lowest levels being found in the *pad4 sag101* double mutant. Our results suggest that the higher growth of GPA on the *pad4* or *pad4 sag101* mutants is unlikely to be due to the impact of *pad4* and *sag101* mutations on the basal levels of the EDS1 protein; GPA growth was comparable between the *pad4* single mutant and the *pad4 eds1* double mutant plant. Similarly, the EDS1 protein is required for the basal level of PAD4 and SAG101 protein accumulation in WT Arabidopsis. Despite this growth of GPA on null mutants of *PAD4* and *EDS1* differed significantly. We believe that upon GPA-infestation the increase in *PAD4* expression results in increased accumulation of PAD4 protein. This GPA-induced accumulation of PAD4 may not absolutely require EDS1. We also suggest that the increase in *EDS1* gene transcripts in GPA-infested plants may be associated with the activation of SA-mediated antimicrobial defense in WT Arabidopsis. Aphid feeding is known to trigger SA responses in plants (Fidantsef et al., 1999, Moran and Thompson, 2001, Moran et al., 2002; Zhu-Salzmann et al, 2004). Furthermore, SA is known to induce expression of the *EDS1* gene, which subsequently participates in the SA accumulation and signaling (Feys et al., 2001).

PAD4 and *SAG101* are known to have overlapping roles in Arabidopsis defense against pathogens (Feys et al., 2005) and *PAD4* can compensate for *SAG101* in plant-pathogen interaction. For instance, loss of *SAG101* gene function can be compensated by *PAD4* in both TIR-NB-LRR-type R gene- triggered and in basal resistance. However, *SAG101* is not efficient in compensating for the absence of *PAD4* (Feys et al., 2005). Unlike, the PAD4 and EDS1 gene, SAG101 transcripts could not be detected in GPA-infested WT plants. This suggested that SAG101 may not be important for Arabidopsis defense against GPA. However, SAG101 does not influence the accumulation of the PAD4 proteins in healthy unstressed plants (Feys et al., 2005). It is conceivable that the absence of SAG101 protein could result in lowered PAD4 protein levels and thus affect GPA growth. However, GPA growth in *sag101-1* mutants was comparable to that on the WT plants. Moreover, GPA growth on the *pad4-1* and *pad4-1 sag101-1* mutants was comparable. Together, these results conclusively demonstrate that a *SAG101*- mediated mechanism(s) in not vital for defense against GPA.

Our earlier studies have shown that constitutive expression of *PAD4* gene caused an early triggering of GPA–induced senescence in plants, which was associated with GPA resistance (Chapter III, Fig. 11*B*, 11*C*). Unlike, the situation observed in the *pad4* mutants, GPA-feeding induced loss of chlorophyll and microscopic cell death in the *eds1* and *sag101* mutants. Furthermore, GPA feeding induced the expression of *SAG*s (*SAG13* and *SAG21*) genes, in both col-0 *eds1* RNAi line and the *sag101* null mutant. Thus, the GPA-induced senescence is also not dependent on EDS1 and SAG101, as senescence was activated even in their absence. In conclusion, PAD4 seems to posses a novel

function even at the molecular level of operation for GPA resistance in Arabidopsis. Further studies on differences in gene expression profiles between uninfested and GPA-infested WT, *pad4* mutant and *PAD4* overexpressing lines will aid in understanding how PAD4 modulates Arabidopsis defense against GPA and also identify new players in Arabidopsis resistance against GPA.

ACKNOWLEDGEMENTS

We thank Sharon McClung for providing us with the *Myzus persicae* colony, Dr. Jane Parker for genereously providing us with the various *EDS1* and *PAD4* seed material, and the Arabidopsis Biological Resource Center (ABRC) at Ohio State University for Arabidopsis seeds.

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FIGURE LEGENDS

Fig. 13. A model depicting the mechanistic mode of PAD4 action in modulating defense in plants against pathogens.

Arabidopsis PAD4, EDS1 and a newly identified *in vivo* EDS1-interactor, SAG101, constitute a regulatory node that links R-protein mediated pathogen recognition to activation of basal defenses. EDS1 and PAD4 function in concert and required for SA accumulation and for defense potentiation involving the processing of ROI-derived signals around the site of infection. EDS1 protein exists in different molecular forms ranging from monomeric, homomeric and heteromeric complexes. A major pool of EDS1 is available in the form of EDS1-SAG101 hetero-dimer, which localizes to the nucleular compartment, whereas the EDS1-PAD4 complex is relatively a small pool and is detected both inside the nucleus and cytosol. SAG101 do not physically interact with PAD4, however, can still influence the PAD4 protein accumulation in the cell. Further, SAG101 has a partially overlapping function with PAD4 both in basal and TIR-NB-LRR mediated resistance.

Fig. 14. Accumulation of *PAD4, EDS1* and *SAG101* transcripts in GPA-infested Arabidopsis plants. (*A*) RT-PCR analysis of *PAD4*, *EDS1*, *SAG101* and *ACT8* expression in GPA-infested Arabidopsis leaves. RT-PCR was performed on RNA extracted 3, 6, 12, 24 and 48 h post infestation (hpi). RNA extracted from uninfested plants provided a negative control.

Fig. 15. EDS1 function in not required for basal resistance against GPA. (*A*) Comparison of GPA numbers on WT (Col-0), *pad4-1* and Col-0 *eds1* RNAi mutant line 2 days after release of 15 insects per plant. (*B*) GPA growth recording on WT (Ws-0), *eds1-1* and *pad4-5* mutants, 2 days after release of 15 insects per plant. All values are the mean of 15 plants $\pm$ SE.

Fig. 16. PAD4 is independent of SAG101 function in modulating basal resistance against GPA. Comparison of GPA numbers on WT, *pad4-1, sag101-1, sag101-1/pad4-1* mutant lines, 2 days after release of 15 insects per plant. All values are the mean of 15 plants $\pm$ SE.

Fig. 17. GPA-feeding triggered senescence response in Col-o*eds1* RNAi, *sag101* Arabidopsis plants. (*A)* Trypan blue staining of leaves from uninfested WT, *pad4-1*, Col-0 *eds1* RNAi line, *sag101-1* plants and from GPA-infested WT, *pad4-1,* Col-0 *eds1* RNAi line, *sag101-1* plants, 2 days after release of insects. (*B)* Photograph of WT, *eds1-2*, *pad4-2* and *sag101-1* plants showing the chlorophyll loss in GPA infested samples, 7days post release of GPA. *eds1-2* and *pad4-2* are in the ecotype Lar-er and *sag101-1* is in Columbia ecotype. (*C)* RT-PCR analysis of *SAG13*, *SAG21*, *SAG27* and *ACT8* expression in leaves from uninfested WT, *pad4-1,* Col-0 *eds1* RNAi line, *sag101-1* plants and leaves from GPA-infested WT, *pad4-1*, Col-0 *eds1* RNAi line, *sag101-1* plants, 48h post infestation (hpi) by GPA.

Fig. 13

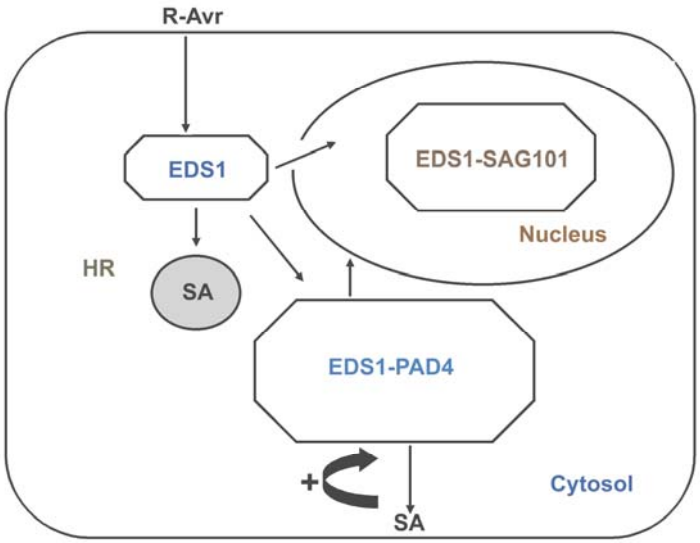


Fig. 14

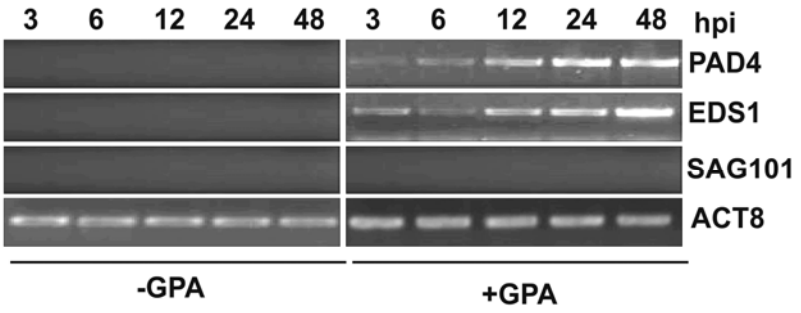


Fig.15

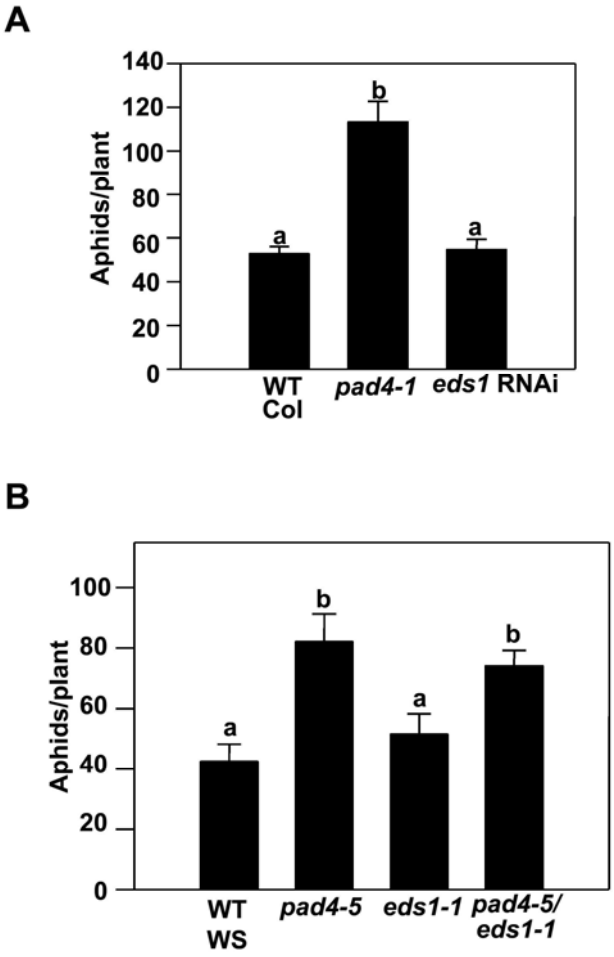

A
Aphids/plant
140
120
100
80
60
40
20
0
a
b
a
WT Col
pad4-1
eds1 RNAi
B
Aphids/plant
100
80
60
40
20
0
a
b
a
b
WT WS
pad4-5
eds1-1
pad4-5/ eds1-1

Fig. 16

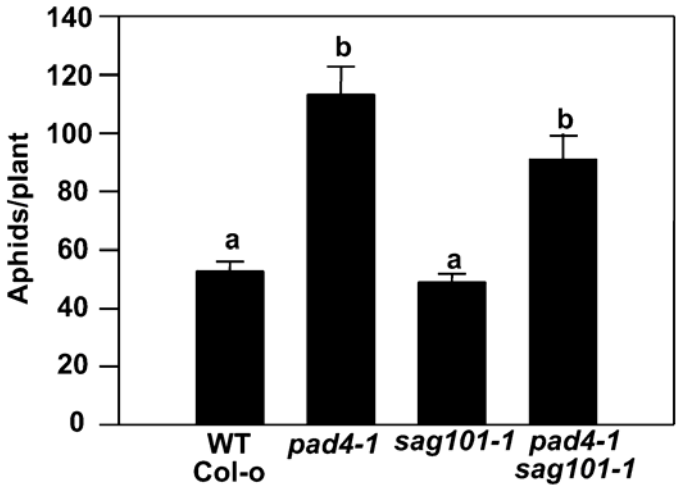
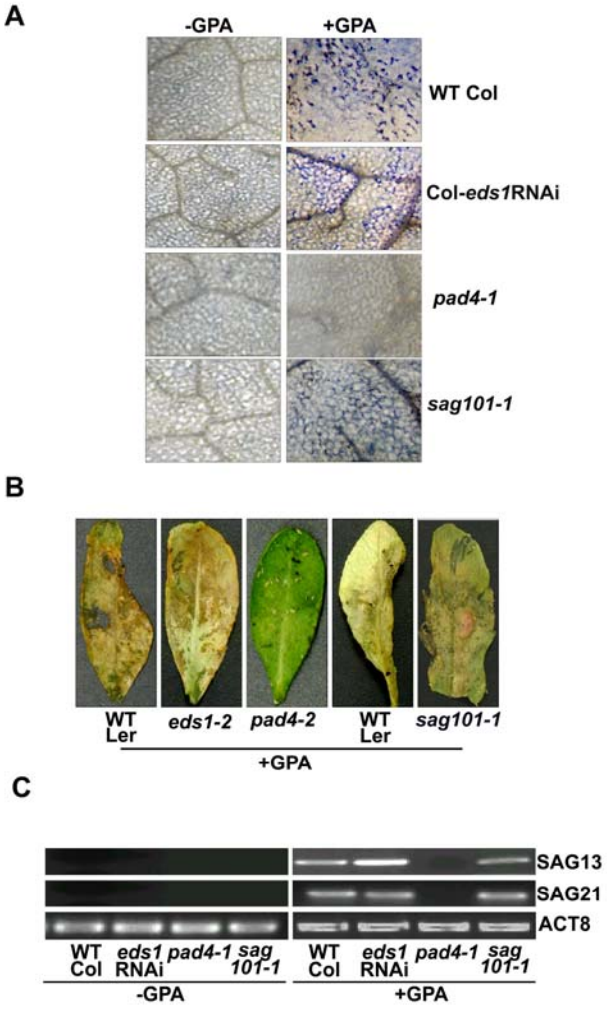

140
120
100
80
60
40
20
0
Aphids/plant
a
b
a
b
WT
Col-o
pad4-1
sag101-1
pad4-1
sag101-1

Fig. 17


A
-GPA
+GPA
WT Col
Col-eds1RNAi
pad4-1
sag101-1
B
WT Ler
eds1-2
pad4-2
WT Ler
sag101-1
+GPA
C
SAG13
SAG21
ACT8
WT Col
eds1 RNAi
pad4-1
sag 101-1
WT Col
eds1 RNAi
pad4-1
sag 101-1
-GPA
+GPA

FUTURE PERSPECTIPES

Our research findings emphasize the role of premature senescence in plant defense to aphids. We have identified PAD4 as an essential modulator of GPA-induced senescence mechanism in plants. Interestingly, the mechanistic mode of PAD4 action seems to be largely different in plant-aphid interaction in contrast to its earlier define role in plant-pathogen interactions. Identification of other players in the GPA-induced senescence pathway will increase our understanding of how senescence is activated in plants by phloem-feeding insects. The characterization of the elicitor(s) from aphid salivary secretions that trigger plant senescence could be important. This signal or signals might be enzymes, peptides or low molecular weight compounds. Such a signal could be used as a pest control spray to activate defense reaction in plants.

Suppressor or enhancer genetic screens can be initiated in Arabidopsis for identification of other functionally related genes in *PAD4* assisted GPA-induced senescence pathway. Such a strategy has been successfully implemented, previously in our lab to define components of defense signaling pathway (Nandi et al., 2003). Arabidopsis *pad4-5* mutant plants can be EMS (ethylene methyl sulfonate) mutagenized and genetic revertants which have lost the delayed senescence phenotype can be selected and further characterized. Subsequently, a map-based cloning procedure can be implemented for isolating the gene/s which participates in *PAD4* modulated senescence pathway.

Recent studies have shown that PAD4 protein localizes to the nuclear compartment of cell (Feys et al., 2005) PAD4 could potentially influence the transcription of other genes, involved in plant-aphid interactions. Microarray technology can be implemented for identification of target gene regulated by PAD4. This technology offers an advantage to study global changes in expression of thousand genes covering the entire genome in a rapid manner (Brown, et al., 1999, Richmond et al., 2000). Previously, we successfully utilized microarray approach to identify Arabidopsis genes differentially expressed in response to GPA feeding and identified PAD4 gene as an important modulator of plant defense against GPA. A similar experimental design to compare the gene expression profile between GPA infested WT plants and *pad4* mutant plants can be conducted to identify the target genes specific to PAD4 protein.

Additionally, the currently available fundamental knowledge can be translated for applied purposes. We are interested in identifying *PAD4* crop homolog in various economically important crop plants such as wheat, sorghum, tomato and rice. Amenable transformation protocols are available for these crop plants, there by resistance against aphids could be engineered by over expressing *PAD4* gene with the aid of strong tissue specific promoters.

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Richmond T, Somerville S (2000) Chasing the dream: plant EST microarray. Current Opinons in Plant Biology **3**: 108-116

APPENDIX

Table 1. GPA feeding–activated genes

Array Element	Locus Identifier	*Gene description*	Fold Induction
263693_at	AT1G31200	Expressed protein	2.44
261692_at	AT1G08450	calreticulin 3 (CRT3)	2.54
246518_at	AT5G15770	GCN5-related N-acetyltransferase (GNAT) family protein	2.06
259179_at	AT3G01660	Expressed protein	2.67
251422_at	AT3G60540	Sec61beta family protein	6.2
252649_at	AT3G44680	Histone deacetylase	2.10
258791_at	AT3G04720	hevein-like protein	3.39
246196_at	AT4G37090	Expressed protein	2.08
251673_at	AT3G57240	Beta-1, 3-glucanase (BG3)	9.49
264172_at	AT1G02120	GRAM domain-containing protein	1.97
251370_at	AT3G60450	Expressed protein	2.09
256442_at	AT3G10930	Expressed protein	3.34
258046_at	AT3G21220	mitogen-activated protein kinase kinase (MAPKK	2.43
254624_at	AT4G18580	Expressed protein	2.97
250435_at	AT5G10380	Zinc finger (C3HC4-type RING finger) family protein	4.84
262953_at	AT1G75670	Expressed protein similar to DNA-dependent RNA polymerase	2.19
245993_at	AT5G20700	Senescence-associated protein	2.23
252327_at	AT3G48740	nodulin MtN3 family protein	2.28
256453_at	AT1G75270	dehydroascorbate reductase, putative similar to GI:6939839 from [Oryza sativa]	2.00
249918_at	AT5G19240	Expressed protein	2.77
248440_at	AT5G51260	acid phosphatase	2.07
257154_at	AT3G27210	Expressed protein	2.33
265025_at	AT1G24575	Expressed protein	4.54
246998_at	AT5G67370	Expressed protein similar to unknown protein (gb|AAC18972.1)	3.488
248551_at	AT5G50200	Expressed protein	2.20
248335_at	AT5G52450	MATE efflux protein	2.93
264968_at	AT1G67360	Rubber elongation factor (REF) family protein contains Pfam profile: PF05755 rubber elongation factor protein (REF)	3.22
246071_at	AT5G20150	SPX (SYG1/Pho81/XPR1) domain-containing protein	3.72
257960_at	AT3G25570	adenosylmethionine decarboxylase family protein	2.60
260943_at	AT1G45145	thioredoxin H-type 5	3.99
265722_at	AT2G40100	Chlorophyll A-B binding protein	2.32
248092_at	AT5G55170	Small ubiquitin-like modifier 3 (SUMO)	2.93
262177_at	AT1G74710	isochorismate synthase 1 (ICS1) / isochorismate mutase	2.99
256299_at	AT1G69530	expansin	2.30
261221_at	AT1G19960	Expressed protein	2.56
262396_at	AT1G49470	Expressed protein	2.10
246860_at	AT5G25840	Expressed protein	2.00

257100_at	AT3G24954	[AT3G24954, leucine-rich repeat family protein	4.99
246744_at	AT5G27760	Hypoxia-responsive family protein	2.98
252603_at	AT3G45050	Expressed protein	2.29
256366_at	AT1G66880	Serine/threonine protein kinase family protein	4.22
253361_at	AT4G33100	Hypoxia-responsive family protein	1.94
259925_at	AT1G75040	Expressed protein	9.98
249777_at	AT5G24210	Serine/threonine protein kinase family	5.32
255341_at	AT4G04500	Expressed protein contains	2.67
259479_at	AT1G19020	Pathogenesis-related protein 5	2.70
263842_at	AT2G36835	Lipase class 3 family protein contains	2.30
256062_at	AT1G07090	Protein kinase family	2.71
266371_at	AT2G41410	Expressed protein	4.21
250992_at	AT5G02260	Expressed protein	2.31
261409_at	AT1G07640	Expressed protein	2.65
257818_at	AT3G25120	calmodulin, putative	2.09
257844_at	AT3G28480	expansin, PMID:11641069	3.09
252827_at	AT4G39950	Dof-type zinc finger domain-containing protein	2.70
266017_at	AT2G18690	Mitochondrial import inner membrane translocase	4.68
256766_at	AT3G22231	oxidoreductase, 2OG-Fe(II)	1.97
263583_at	AT2G17130	cytochrome P450	2.10
260551_at	AT2G43510	Expressed protein	2.31
253228_at	AT4G34630	Expressed protein	2.07
262314_at	AT1G70810	Isocitrate dehydrogenase subunit 2 / NAD+	3.16
262119_s_at	AT1G02920	trypsin inhibitor	3.72
257228_at	AT3G27890	Expressed protein	2.11
261456_at	AT1G21050	C2 domain-containing protein	2.34
266615_s_at	AT2G29720	[AT1G02920, glutathione S-transferase	2.12
248970_at	AT5G45380	Sodium: solute symporter family protein	3.16
250314_at	AT5G12190	NADPH-dependent FMN	2.57
249417_at	AT5G39670	Expressed protein	5.67
259765_at	AT1G64370	monooxygenase family protein	2.60
260852_at	AT1G21900	Sodium:solute symporter family protein	2.15
260904_at	AT1G02450	RNA recognition motif (RRM)-containing protein	7.79
266181_at	AT2G02390	Calcium-binding EF hand family protein	3.80
262374_s_at	AT1G72910	Disease resistance protein (TIR-NBS class),	2.96
248330_at	AT5G52810	Expressed protein	4.59
246099_at	AT5G20230	Emp24/gp25L/p24 family protein	2.55
254673_at	AT4G18430	NPR1/NIM1-interacting protein	2.41
259626_at	AT1G42990	Glutathione S-transferase	2.19
252411_at	AT3G47430	Disease resistance protein (TIR-NBS class	2.22
245052_at	AT2G26440	pectinesterase	4.22
247487_at	AT5G62150	ornithine cyclodeaminase/mu-crystallin family protein	4.22
262382_at	AT1G72920	plastocyanin-like domain-containing protein	4.13
266037_at	AT2G05940	Ras-related GTP-binding protein	3.25
251447_at	AT3G59810	bZIP transcription factor family protein	1.966
266291_at	AT2G29300	peroxisomal biogenesis factor 11 family protein	1.98
252619_at	AT3G45210	pectinesterase family protein	2.46
260881_at	AT1G21550	peptidoglycan-binding LysM domain-containing protein contains Pfam profile PF01476: LysM domain	2.98
259033_at	AT3G09410	Disease resistance protein (TIR-NBS class),	2.98

245385_at	AT4G14020	Protein kinase	5.91
261440_at	AT1G28510	Small nuclear ribonucleoprotein F	2.00
257083_s_at	AT3G20590	tropinone reductase, putative / tropine dehydrogenase	2.79
264014_at	AT2G21210	auxin-responsive protein	2.55
260002_at	AT1G67940	Expressed protein	3.00
259841_at	AT1G52200	Calcium-binding protein	2.23
266267_at	AT2G29460	pectinacetylesterase family protein	5.9
258179_at	AT3G21690	Rapid alkalinization factor (RALF) family protein	2.15
249204_at	AT5G42570	Expressed protein	2.1
260635_at	AT1G62420	Non-race specific disease resistance protein,	2.08
253486_at	AT4G31600	auxin-responsive protein	2.15
251790_at	AT3G55470	ABC transporter family protein	3.11
266184_s_at	AT2G38940	Expressed protein	3.83
256677_at	AT3G52190	Glutathione S-transferase, putative	2.10
261038_at	AT1G17490	MATE efflux family protein	2.22
258881_at	AT3G06310	Expressed protein	2.35
253917_at	AT4G27380	Expressed protein contains Pfam profile PF04720: Protein of unknown function (DUF506)	2.68
256583_at	AT3G28850	UDP-glucuronic acid/UDP-N-acetylgalactosamine transporter	2.32
251621_at	AT3G57700	C2 domain-containing protein	2.538
264616_at	AT2G17740	Phosphate transporter	2.14
254266_at	AT4G23130	transducin family protein	2.67
266596_at	AT2G46150	Expressed protein	2.37
260804_at	AT1G78410	NADH-ubiquinone oxidoreductase	4.93
253303_at	AT4G33780	Expressed protein	2.17
248820_at	AT5G47060	glutaredoxin family protein	2.14
253898_s_at	AT4G27070	Protein kinase	2.94
249606_at	AT5G37260	DC1 domain-containing protein	3.89
247602_at	AT5G60900	Receptor-like protein kinase 6	3.10
254211_at	AT4G23570	Expressed protein and genefinder	1.97
255479_at	AT4G02380	VQ motif-containing protein	3.15
258002_at	AT3G28930	Expressed protein	2.45
252076_at	AT3G51660	Senescence-associated protein	2.74
266257_at	AT2G27820	tryptophan synthase, beta subunit 2 (TSB2) identical to SP|25269	1.98
248381_at	AT5G51830	myb family transcription factor	2.98
260076_at	AT1G73630	lectin protein	2.51
265837_at	AT2G14560	phosphatase-related low	4.41
247696_at	AT5G59780	Late embryogenesis abundant 3 family protein	4.32
263807_at	AT2G04400	AvrRpt2-induced AIG2 protein (AIG2)	3.55
251733_at	AT3G56240	Macrophage migration inhibitory factor family protein	2.48
262832_s_at	AT1G14870	prephenate dehydratase family protein	11.00
257382_at	AT2G40750	pfkB-type carbohydrate kinase family protein	10.64
266355_at	AT2G01400	Calcium-binding protein)	2.19
254784_at	AT4G12720	Expressed protein	2.14
262160_at	AT1G52590	myb family transcription factor (MYB59)	2.31
261110_at	AT1G75440	Indole-3-glycerol phosphate synthase (IGPS)	2.22
249021_at	AT5G44820	Copper homeostasis factor / copper chaperone	2.86
258614_at	AT3G02770	Expressed protein	2.34
253950_at	AT4G26910	WRKY family transcription factor	2.10

249346_at	AT5G40780	Expressed protein	2.98
257206_at	AT3G16530	MutT/nudix family protein	3.36
256178_s_at	AT1G51780	Expressed protein	2.50
261485_at	AT1G14360	ubiquitin-conjugating enzyme 16	1.99
245265_at	AT4G14400	Expressed protein	3.21
262408_at	AT1G34750	dimethylmenaquinone methyltransferase family protein	2.29
260418_s_at	AT1G69750	2-oxoacid dehydrogenase family protein	2.62
253992_at	AT4G26060	Lysine and histidine specific transporter	2.87
258201_at	AT3G13910	Legume lectin family protein	2.75
259632_at	AT1G56430	IAA-amino acid hydrolase 5 / auxin nonconsensus AT acceptor splice site at exon3	3.20
248908_at	AT5G45800	UDP-galactose/UDP-glucose transporter,	2.04
260409_at	AT1G69935	ankyrin repeat family protein	2.27
258275_at	AT3G15760	Protein phosphatase 2C	2.49
253806_at	AT4G28270	Cox19 family protein	2.60
259058_at	AT3G03470	Expressed protein	2.26
255011_at	AT4G10040	Expressed protein	2.81
263129_at	AT1G78620	nicotianamine synthase	2.07
266746_s_at	AT4G02520	leucine-rich repeat transmembrane protein kinase, putative	2.19
266097_at	AT2G37970	Expressed protein	2.70
245353_at	AT4G16000	Expressed protein	3.91
246055_at	AT5G08380	Zinc finger (C3HC4-type RING finger)	1.99
247641_at	AT5G60540	cytochrome P450	1.90
257745_at	AT3G29240	cytochrome c	2.12
248327_at	AT5G52750	Integral membrane family protein	4.87
259272_at	AT3G01290	Glutathione S-transferase,	2.40
254833_s_at	AT4G12280	Copper amine oxidase	2.89
249580_at	AT5G37740	SOUL heme-binding family protein weak similarity to SOUL protein	2.032
266553_at	AT2G46170	Expressed protein	1.94
265742_at	AT2G01290	Alpha-galactosidase, putative / melibiase,	3.19
261443_at	AT1G28480	SNO glutamine amidotransferase family protein	2.65
251400_at	AT3G60420	Expressed protein	5.17
257375_at	AT2G38640	Heavy-metal-associated domain-containing protein	2.23
252549_at	AT3G45860	Band 7 family protein	6.72
259105_at	AT3G05500	Copper amine oxidase family	2.36
265067_at	AT1G03850	C2 domain-containing protein similar to zinc finger	2.44
263875_at	AT2G21970	reticulon family protein (RTNLB5	3.00
254805_at	AT4G12480	Expressed protein	2.36
259228_at	AT3G07720	glutaredoxin family protein	2.28
246293_at	AT3G56710	Expressed protein	3.01
252417_at	AT3G47480	Expressed protein	36.9
250881_at	AT5G04080	Receptor-like protein kinase	2.53
264590_at	AT2G17710	Rubber elongation factor (REF)	2.59
261339_at	AT1G35710	glutaredoxin family	2.97
260051_at	AT1G78210	Stress enhanced protein 2 (SEP2)	2.13
245178_at	AT5G12390	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	2.30
245711_at	AT5G04340	kelch repeat-containing	2.96

262656_at	AT1G14200	sigA-binding protein	2.25
257686_at	AT3G12800	Calcium-binding EF hand family protein	2.20
250796_at	AT5G05300	Expressed protein	2.39
248322_at	AT5G52760	Expressed protein	10.5
266155_at	AT1G64950	leucine-rich repeat transmembrane protein kinase	2.10
253534_at	AT4G31500	hydrolase, alpha/beta fold family protein	2.18
251638_at	AT3G57490	Expressed protein	2.83
264195_at	AT1G22690	Zinc finger (C2H2 type) family protein	2.40
259766_at	AT1G64360	Zinc finger (C3HC4-type RING finger)	3.38
257061_at	AT3G18250	Short-chain dehydrogenase/reductase (SDR)	8.01
262607_at	AT1G13990	Expressed protein	2.19
265460_at	AT2G46600	Heavy-metal-associated domain-containing protein	2.53
265670_s_at	AT2G32210	cytochrome P450]	1.98
266292_at	AT2G29350	cytochrome P450	3.28
251847_at	AT3G54640	40S ribosomal protein S2 (RPS2D)	4.71
246749_at	AT5G27830	gibberellin-responsive protein	2.66
252117_at	AT3G51430	Expressed protein	2.56
248686_at	AT5G48540	Expressed protein	5.37
266882_at	AT2G44670	Expressed protein	2.29

Table 2. GPA feeding–repressed genes

Array Element	Locus Identifier	*Gene description*	Fold repression
264146_at	At1g02205	hypothetical protein	8.11
248606_at	At5g49450	putative protein	4.74
247013_at	At5g67480	putative protein	4.35
252563_at	At3g45970	putative protein cim1 induced allergen	6.45
252415_at	At3g47340	Glutamine-dependent asparagine synthetase	4.58
245928_s_at	At5g24780	vegetative storage protein Vsp1	4.18
256020_at	At1g58290	glutamyl-tRNA reductase	4.08
255064_at	At4g08950	putative phi-1-like phosphate-induced protein	5.79
254815_at	At4g12420	putative pollen-specific protein	5.48
255962_at	At1g22335	glycine-rich RNA-binding protein	4.40
247878_at	At5g57760	unknown protein	5.07
247925_at	At5g57560	TCH4 protein	4.10
248094_at	At5g55220	trigger factor-like protein	4.51
247954_at	At5g56870	beta-galactosidase	4.56
252367_at	At3g48360	putative protein MEL-26	4.47
264238_at	At1g54740	hypothetical protein	4.46
264339_at	At1g70290	trehalose-6-phosphate synthase	5.50
246487_at	At5g16030	putative protein .	4.35
249923_at	At5g19120	conglutin gamma - like protein	4.52
256848_at	At3g27960	hypothetical protein	13.49
253136_at	At4g35470	putative protein	4.45
53340_s_at	At4g33260	WD-repeat protein -like protein	14.98
249065_at	At5g44260	putative protein	6.08
253285_at	At4g34250	fatty acid elongase	11.28
262914_at	At1g59750	auxin response factor 1	5.27
248584_at	At5g49960	putative protein	4.45
262814_at	At1g11660	putative heat-shock protein	5.427
262871_at	At1g65010	hypothetical protein	19.96
262984_at	At1g54460	hypothetical protein	7.66
263953_at	At2g36050	hypothetical protein	19.43
262600_at	At1g15340	unknown protein	6.94
263118_at	At1g03090	putative 3-methylcrotonyl-CoA carboxylase	5.23
261625_at	At1g01930	hypothetical protein	5.7
249467_at	At5g39610	NAM / CUC2 - like protein	4.64
261567_at	At1g33055	Expressed protein	12.30
250823_at	At5g05180	putative protein	11.05
256727_at	At3g52240	hypothetical protein	8.90
256613_at	At3g29290	hypothetical protein	10.11
248509_at	At5g50335	Expressed protein	12.19
256300_at	At1g69490	unknown protein	14.82
256254_at	At3g11290	hypothetical protein	10.74
253558_at	At4g31120	kinase binding protein	5.48
257181_at	At3g13190	hypothetical protein	5.88

265511_at	At2g05540	putative glycine-rich protein	5.83
265539_at	At2g15830	unknown protein	6.27
264802_at	At1g08560	putative syntaxin-related protein	4.43
251977_at	At3g53250	putative protein auxin-induced	32.55
251791_at	At3g55500	expansin-like protein	5.03
254954_at	At4g10910	hypothetical protein	7.69
255807_at	At4g10270	probable wound-induced protein	8.96
258736_at	At3g05900	unknown protein	17.86
258601_at	At3g02760	putative histidyl tRNA synthetase	8.17
266899_at	At2g34620	hypothetical protein	6.12
246704_at	At5g28090	putative protein	4.89
265387_at	At2g20670	unknown protein	16.67
264352_at	At1g03270	unknown protein	10.74
264353_at	At1g03260	hypothetical protein	6.37
266313_at	At2g26980	putative protein kinase	4.30
267562_at	At2g39670	unknown protein	4.53
245313_at	At4g15420	UFD1 like protein	6.66
265058_s_at	At1g52040	myrosinase-binding protein homolog	7.08
251415_at	At3g60380	putative protein	5.42
257817_at	At3g25150	putative RNA-binding protein	19.02
259609_at	At1g52410	myosin-like protein	12.55
252954_at	At4g38660	putative thaumatin-like protein	4.02
252512_at	At3g46290	receptor protein kinase -like	4.32
258125_s_at	At3g23520	hypothetical protein	14.69
252997_at	At4g38400	putative pollen allergen	8.86
248163_at	At5g54510	auxin-responsive-like protein	5.13
260717_at	At1g48120	serine/threonine phosphatase PP7	8.04
264266_at	At1g09160	putative protein phosphatase 2C	4.53
263337_at	At2g04990	hypothetical protein	4.68
260380_at	At1g73870	hypothetical protein	11.05
260944_at	At1g45130	beta-galactosidase	18.93
261480_at	At1g14280	phytochrome kinase substrate 1	5.74
264157_at	At1g65310	xyloglucan endotransglycosylase	6.20
265908_at	At4g00270	contains similarity to S. cerevisiae ADR1 gene	19.88
246278_at	At4g37190	tubulin-like protein	5.29
247324_at	At5g64190	putative protein	6.31
264633_at	At1g65660	step II splicing factor	8.88
250591_at	At5g07720	alpha galactosyltransferase protein	6.24
266419_at	At2g38760	putative annexin	7.68
264788_at	At2g17880	putative DnaJ protein	20.79
264913_at	At1g60770	hypothetical protein	5.44
245422_at	At4g17470	putative protein	33.06
247176_at	At5g65110	acyl-CoA oxidase	4.43
266656_at	At2g25900	putative CCCH-type zinc finger protein	4.02
			4.82
259640_at	At1g52400	beta-glucosidase	
266072_at	At2g18700	putative trehalose-6-phosphate synthase	8.62

262456_at	At1g11260	glucose transporter	4.36
256772_at	At3g13750	galactosidase	4.69
266295_at	At2g29550	tubulin beta-7 chain	4.84

Table 3. RT-PCR primers of GPA feeding–activated genes

Gene name	**Forward primer sequence 5'-3'**	**Reverse primer sequence 5'-3'**
NPR1	GAAAGAGACGATCAGGGTTG	GCCTTGTCTTCGTTTCGCTCT
EDS5	CCAAACACTGGTCGCAGAATC	GCGAAAGAAGCCGTCGTAGAT
SID2	TACCGACATTGATCCCATTGC	TGCACTCTTGCAAGCTTCCTC
PAD3	TATAAGCTTCCTCCGGGTCCA	TCTTTGGCTTCCTCCTGCTTC
PAD4	AGTTCATGCAACACCGCAAAT	TCCATTGCGTCACTCTCATCA
At3G640	TCTTCTTCCTCCAATCCCCAA	GCGACTTTGCATCACCCAATA
AT4G27070	TCCTCCGTTTCAGCTTCTTCC	GTCGCCTCTCCCACTGAAGTT
AT2G04400	GGCTTCACCTTCTCTGTATCGG	GGTCGTTCTGCTTCACAATGG
AT3G45860	CCTTCATCTTTCATTTGCCGA	CATCGACTGAACAAAGAGCGG
AT1G72930	GCATGACACTCGCCACAACTT	TGTCCACCAGCTTCGAGTCAT
AT3G25010	CCCCTTCAAGCTTCGTCACTT	CCAAGTAACACTCCAACCCCA
AT5G37260	GGCTATGCAGGAACGTTGTGA	GGCATGAACCCTCTCATGTTG
AT5G59820	ATCGGAGATCAAGTCGACGGT	AAGCCACTCTCTTCCCACTGC
AT2G40750	CGACCAACTTGTCGAAGGCTA	CATCGTTGTCGATGAAACCAAA
AT2G05940	TCCAGAAACCCTAATGGCATC	AACGGCGAATTCACTCCATTT
AT3G47480	GCCCAATCTCTCTCCTCACCA	CCTTAACCATCTTCCTACACTCCA
AT2G47130	TCAGATTGGATGGCAAAATCG	GCTATAACCCCCGTCAACAGC
AT3G22600	CTGCTCAGTCAAGCTGCACAA	TAGGAAACCGCGGAGAAGAAG
SAG21	CCAATGCTATCTTCCGACGTC	GAACCGGTTTCGGGTCTGTAA
SAG27	TCCTGGCCCTGAAGTAGAAA	GTCCCGCAAGAACCTGTCC
SAG13	GGAAAACCGTTAACAGTGGCA	CAAGATGGAGTCTTGGAGGGA
SAG15	ACGATCCACCGCTTCTCCACAACT	GCCGGCGCTACCATCATCAAC
SAG25	AGGCGGTTTAGGTCATGTAGGAGTG	GGCGGTGTTGACATAATCGGCAGAG
SAG27	TTGGCCCTGAAGTAGAAA	GTCCCGCAAGAACCTGTCC
SAG29	CCCTATGTGGTGGCGCTCTTCAG	CCGACGGCGTTTTGCAGTATTTG
SAG18	ATCGTCACTCCCACAATCCCT	ATGCTCGAACCGTTCTTCCTC
AT3G13790	GTCGTCAATCTTGAAGCCTCG	GATTTGGGCAGAGTTCATGCT
AT1G22710	TCCAATGGAGAAAGCTGCAAA	ACACCGTCAACGCCAATACAC
AT1G66570	GCCATCTCCGCTGAGAAAGAT	GCCACAATACTGCTGACTGCC
AT4G29130	GTTGGAGCGACTGTTGTTTGC	GCCTGATCCATCATTGGAGTG

Table 4. RT-PCR primers of GPA feeding-repressed genes

Gene ID	Forward primer sequence 5'-3'	Reverse primer sequence5'-3'
AT2G25900	ATCCAACGGCCACAATCTCTT	TCTCCGCTCTCAGCTCTTTCC
AT1G69420	GGCAGCTTCCATACCATCCTC	TCCACCAGATGCATCATAGCC
AT1G14280	TGCCAGATCCAGAAGTTCCAA	CGATTGCGTGTGAGAAACAGA
AT1G74890	CTCCGGAGTTGCATGTTCTTG	CGTTTTGAACATGAAGAGTCCTTG
AT1G19050	TGCCGTTGATGATAGTTTCGTG	TCGTCATCAAGGGAGGAAACA
AT2G40670	CGTCTTTAATGGATGTGGTGGC	GCAAGCTTCAAAGGCTTCTGC
AT2G38390	GCAGTGCTATGGGAGCCCTAA	TCGTCGTTCTCCACAACCCTA
AT1G31690	GATGGGCAAATTGGGAGTTTC	GAAAAAGTTGGTTGGCCGAAG
AT2G18700	TCGTTCGAATTGGAGAGCGTA	GCATCGATGTTCCACGGATTA

Fig. 18

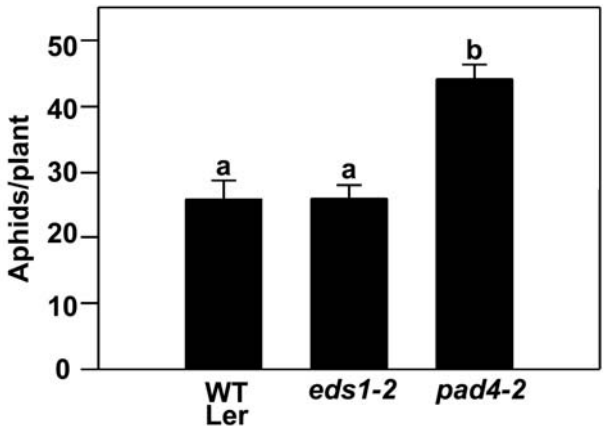

FIGURE LEGENDS

Fig. 18. Comparison of GPA numbers on WT, *eds1-2* and *pad4-2* mutant. 2 days after release of 10 insects per plant. All values are the mean of 10 plants ± SE.