Molecular analysis of early-responsive genes of chickpea involved in biotic stress

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ARCHANA SINGH

NATIONAL INSTITUTE FOR PLANT GENOME RESEARCH
NEW DELHI-110067, INDIA

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NATIONAL INSTITUTE FOR PLANT GENOME RESEARCH
Aruna Asaf Ali Marg, New Delhi-110067, India

CERTIFICATE

The research work embodied in this thesis entitled **"Molecular analysis of early-responsive genes of chickpea involved in biotic stress"** has been carried out at the National Institute for Plant Genome Research, New Delhi. The work is original and has not been submitted so far in part or in full for any degree or diploma of any university.

Archana

Archana Singh
(Student)

N. Chakraborty
Director (Officiating)

Praveen Verma

Dr. Praveen Verma
(Supervisor)

.....dedicated to my
parents and all family members

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Contents

Chapter 1: Introduction

Plants represent a valuable nutrient resource for a multitude of potential pathogens and rarely grow without attempted pathogen colonization. Fungi, oomycetes, bacteria, as well as nematodes and insects utilize the photosynthate produced by plants and viruses exploit replication machinery (Dangl and Jones, 2001). The estimated loss caused by pathogens is typically around 10 to 20%. Crop losses due to insect infestation and fungal attack are major parts of the total loss. However plants have developed remarkable strategies to adapt to environmental changes by using preformed and induced mechanisms of defense. Inducible defenses are more energy efficient and durable compared to constitutive defense mechanisms. Therefore, understanding the inducible defense mechanism would be more interesting in the perspective that they can further be extrapolated to the acquisition of resistance in a plant. Appropriate regulation of defense responses is important for plant fitness, as activation of defense responses has deleterious effects on plant growth (Glazebrook, 2005). The molecular mechanisms underlying activation of plant defense responses are exceedingly complex. Responses often begin with gene-for-gene recognition of the pathogen (Dangl and Jones, 2001). Production of certain virulence effectors by the pathogen leads to their recognition by plants that carry corresponding Resistance, or *R* genes.

Most plant disease resistance (R) proteins contain a series of leucine-rich repeats (LRRs), a nucleotide-binding site (NBS), and a putative amino-terminal signaling domain. The LRRs of a wide variety of proteins from many organisms serve as protein interaction platforms, and as regulatory modules of protein activation. Genetically, the LRRs of plant R proteins are determinants of response specificity, and their action can lead to plant cell death in the form of the familiar hypersensitive response (HR). A total of 149 R genes are potentially expressed in the *Arabidopsis* genome, and plant cells must deal with the difficult task of assembling many of the proteins encoded by these genes into functional signaling complexes. Eukaryotic cells utilize several strategies to deal with this problem. First, proteins are spatially restricted to their sub-cellular site of function, thus improving the probability that they will interact with their proper partners. Second, these interactions are architecturally organized to avoid inappropriate signaling events and to maintain the fidelity and efficiency of the response when it is initiated. Recent results provide new insights into how the signaling potential of R proteins might be created, managed and held in check until specific stimulation following infection. Nevertheless, the roles of the R protein partners in these regulatory events that have been defined to date are unclear.

Recognition results in rapid activation of defense responses and consequent limitation of pathogen growth. *R* gene–mediated resistance is usually accompanied by an oxidative burst. It is the rapid production of reactive oxygen species (ROS). This ROS production (Lamb and Dixon, 1997) is required for another component of the response, hypersensitive

cell death (HR) (Greenberg, 2003), a type of programmed cell death thought to limit the access of the pathogen to water and nutrients. *R* gene–mediated resistance is also associated with activation of a salicylic acid (SA)-dependent signaling pathway that leads to expression of certain pathogenesis-related (PR) proteins thought to contribute to resistance. Some other plant defense responses are controlled by mechanisms dependent on ethylene (ET) and/or jasmonates (JA). These responses show considerable overlap with responses to wounding, which are also under ethylene and/or jasmonate control. SA, JA, and ET signaling pathways interact extensively (Schenk *et al.,* 2000; Salzman *et al.,* 2005; Jalali *et al.,* 2006). SA and JA are mutually inhibitory for the expression of many genes. Induced expression of a number of genes requires both ET and JA, whereas expression of other genes requires only one of these signals. There are also cases of negative interaction between ET and JA signaling.

Chickpea (*Cicer arietinum* L.) is an annual, self-pollinating, diploid (2n=2x=16; genome size: 740 Mb) pulse crop that ranks third in world legume production (Food and Agriculture Organization, 2006). Although, Australia is currently the largest exporter of chickpea, India accounts for two-third of world's chickpea production. Under optimum growing conditions, the yield potential of chickpea is approximately 6 t/ha, which is much higher than the current global yield average of 0.8 t/ha. The major constraints in chickpea production are biotic stresses like *Ascochyta* blight, *Fusarium* wilt, pod borer, and abiotic stresses. *Ascochyta* blight caused by *Ascochyta rabiei* (Pass.) L., leads to severe crop losses and is considered to be major factors limiting chickpea production (Nene, 1980). Insect predation also results in severe losses in chickpea production annually. Most of the losses (up to 85%) are caused by the podborer (*Helicoverpa armigera* Hűbner), a polyphagous pest of the developing seeds of several legume species (Giri *et al.,* 1998). Thus, it is intersting to understand the key processes governing the defense mechanisms and pathways of gene induction controlling active defense responses in chickpea upon infection with the blight fungus and infestation by the podborer that would greatly aid to processes directed towards producing cultivars with durable resistance.

The main objective of this work was to isolate and identify defense-related genes involved in resistance against *Ascochyta* blight. Several studies indicated that a large number of plant genes are transcriptionally regulated upon challenge by a pathogen (Maleck *et al.,* 2000; Schenk *et al.,* 2000) but that most of them may be common to both compatible and incompatible interactions (Tao *et al.,* 2003). But focusing on genes strictly involved in the resistance, a comparative account of the expression patterns of genes between resistant and susceptible varieties might lead to understanding the real mechanisms of resistance. For this, using suppression subtractive hybridization (SSH) method will be useful as it is a powerful technique that produces a library of cDNA clones that are (putatively) differentially expressed

in one, tester (resistant), mRNA-population compared to the second, driver (susceptible) population. In order to indicate their role in defense, monitoring the expression patterns of the isolated genes in response to exogenous application of defense regulators will add the information regarding the regulatory mechanism of the resistance against *Ascochyta* infection. It is expected that further characterization of these novel defense-related genes will extend our understanding about defense mechanisms in chickpea and in developing new strategies for crop protection. One of the selected genes showing homology to an LRR domain containing nematode resistance protein, $Hs1^{pro-1}$, was further characterized functionally to show their role in defense.

On the similar lines, identification of defense-related genes and to know the mechanisms of their regulation by transcript profiling was the other objective of this study. Since most studies examining *Helicoverpa*–chickpea interactions have focused on specific gene or protein dynamics (Johnston *et al.,* 1991; Jongsma *et al.,* 1995; Giri *et al.,* 1998; Peng *et al.,* 2005; Srinivasan *et al.,* 2005), the aim of this study was to identify a set of target genes upregulated during mild insect infestation which may contribute to the defense response. In addition to this, investigating the effects of elicitation by mild insect infestation and evaluating by examining signal compound elicitation on induced plant defenses in chickpea by measuring larval feeding behavior was the other objective of this study.

Objectives:

1. Construction of suppression subtractive cDNA library from *Ascochyta* infected chickpea plants and expression analysis during the early stages of infection.

2. Characterization of selected candidate gene involved in defense/resistance.

3. Isolation of *Ascochyta* inducible promoter(s) and its analysis.

4. Construction of subtractive cDNA library from *Helicoverpa*-infested chickpea and to compare gene expression during *Helicoverpa* infestation and mechanical wounding as well as defense regulators.

Chapter 2: Review of Literature

Plants do not have specialized cells to carry out immune functions. Therefore, when challenged by a pathogen or an insect, plant cells undergo reprogramming to prioritize defense over their normal cellular functions. Programmed cell death at the site of invasion is a common plant defense mechanism against biotrophic pathogens and sucking insects, which rely on living host cells to provide nutrients. However, cell death is a prerequisite for the growth of necrotrophs, as these pathogens feed on dead tissue. It is therefore essential that plants activate the appropriate defense response according to the pathogen type. Salicylic acid (SA)-mediated resistance is effective against biotrophs, whereas jasmonic acid (JA)- or ethylene-mediated responses are predominantly against necrotrophs and herbivorous insects (Glazebrook, 2005).The various defense mechanisms containing constitutive physical barriers as well as a battery of inducible defense responses must all be adapted to combat different types of intruders. Intriguingly, some pathogens can induce multiple plant signal molecules and hormones, such as SA and JA. In such cases, crosstalk between these signaling pathways may be the mechanism that allows the plant to prioritize one response over the other (Nürnberger and Lipka, 2005; Abramovitch *et al.,* 2006). Pathogen infection also has profound effects on hormonal pathways involved in plant growth and development. As a virulence strategy, many pathogens have evolved mechanisms to tap into these hormonal signaling networks to interfere with host defense. In response, crosstalk may be used by the host as a direct defense mechanism against pathogen-triggered perturbation of hormone signaling (Robert-Seilaniantz *et al.,* 2007).

2.1 Plant perception systems for pathogen recognition

Plants have evolved multiple defense strategies including both the preformed and inducible defense systems for combating potential pathogens. To successfully infect plants, microbes must first access the plant interior either by directly penetrating the tissue surface, by entering through wounds, or through natural openings such as stomata. Once a pathogen overcomes or bypasses the preformed defense system, it has to face a two-branched innate immunity system, where the central component is non-self recognition (Nürnberger and Lipka, 2005; Chisholm *et al.,* 2006; Jones and Dangl, 2006). The first branch is cultivar-specific, as in the gene-for-gene type of interactions, while the second nonspecifically recognizes the presence of a pathogen by those molecules common to many classes of microbes.

2.1.1 Gene-for-gene recognition

The most effective and efficient way to reduce disease losses in crops is to use resistant plants. Often, the plant disease resistance described is cultivar- or accession specific

and is referred to as the gene-for-gene type of plant-pathogen interactions (Flor, 1971; Keen, 1990; Staskawicz *et al.,* 1995). Variation in host resistance is frequently controlled by the segregation of single *resistance* (*R*) genes, the products of which directly or indirectly interact with specific elicitors encoded by the pathogen *avirulence* (*Avr*) genes (Flor, 1971; Hammond -Kosack and Jones, 1997; Nimchuk *et al.,* 2003). A typical, visible feature of *R-Avr* interactions is the hypersensitive reaction (HR; rapid, localized cell death at the site of an attempted infection), which is accompanied by an oxidative burst and an increased expression of defense-related genes [e.g. *pathogenesis-related* (*PR*) genes] and is thought to restrain pathogen growth and spreading *in planta* (Staskawicz *et al.,* 1995; Dangl *et al.,* 1996; Hammond-Kosack and Jones, 1997; Lamb and Dixon, 1997).

Since isolation of the *Pto* resistance gene of the tomato with a positional cloning strategy, which confers resistance against the *P. syringae* pv. *tomato* bacteria expressing the *Avr* gene *AvrPto* (Martin *et al.,* 1993), many *Avr* gene-specific *R* genes have been isolated and characterized from various species (Dangl and Jones, 2001; Meyers *et al.,* 2003; McDowell, 2004; Meyers *et al.,* 2005). The majority of the R proteins contain a nucleotide binding site (NBS) and leucine-rich repeats (LRR). Such NBS-LRR R proteins have been classified into different groups according to the distinct N-terminal domains of either a coiled-coil (CC) or a TIR domain sharing similarity with the cytoplasmic domain of the *Drosophila* Toll and mammalian interleukin-1 receptor protein (Nimchuk *et al.,* 2003). Of the other LRR-containing R protein structural classes, some *R* genes encode proteins containing kinase or the WRKY domains such as Xa21 and RRS1-R (Song *et al.,* 1995; Deslandes *et al.,* 2003). Interestingly, the *Pto* gene encodes a serine-threonine kinase without the extracellular LRR domain (Loh and Martin, 1995), which is the most common feature of all the R protein classes and thought to mediate protein-protein interactions (Kobe and Kajava, 2001). However, genetic analysis has uncovered that *Pto*-mediated resistance depends on the NBS-LRR Prf protein (Salmeron *et al.,* 1996; Rathjen *et al.,* 1999). The other atypical R protein is RPW8, which contains a putative N-terminal transmembrane domain and a CC domain only (Xiao *et al.,* 2001). The RPW8 functionality requires EDS1, an *R*-gene signaling component (Aarts *et al.,* 1998; Xiao *et al.,* 2001) (Fig. 2.1).

In contrast to the striking degree of similarity in the structural components of the R proteins, most of pathogen-derived Avr proteins show little or no homology to one another and have no functions that are deduced or experimentally defined (Schornack *et al.,* 2006). A direct interaction between the *avr* products and R proteins has been demonstrated in only a few cases (Tang *et al.,* 1996; Jia *et al.,* 2000; Deslandes *et al.,* 2003). As a matter of fact, many Avr proteins have been shown to act as virulence factors that contribute to disease development on the susceptible hosts lacking the corresponding *R* gene (Kjemtrup *et al.,*

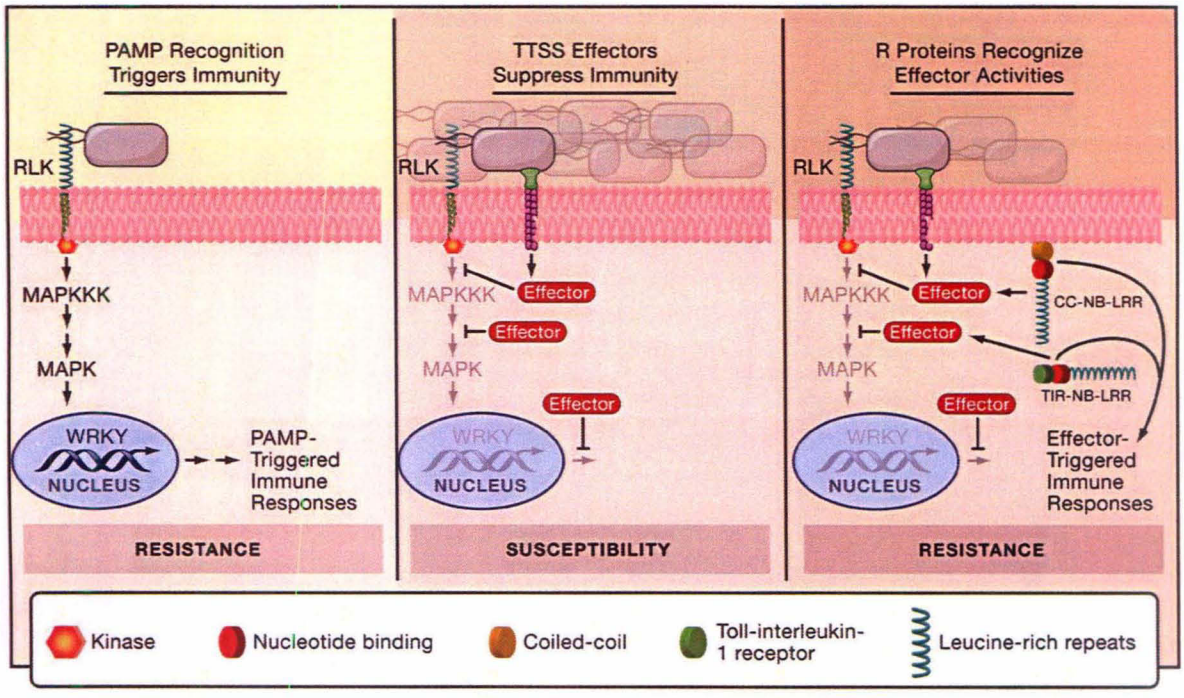

Figure 2.1 Model for the Evolution of Bacterial Resistance in Plants. Left to right, recognition of pathogen-associated molecular patterns (such as bacterial flagellin) by extracellular receptor-like kinases (RLKs) promptly triggers basal immunity, which requires signaling through MAP kinase cascades and transcriptional reprogramming mediated by plant WRKY transcription factors. Pathogenic bacteria use the type III secretion system to deliver effector proteins that target multiple host proteins to suppress basal immune responses, allowing significant accumulation of bacteria in the plant apoplast. Plant resistance proteins (represented by CC-NB-LRR and TIR-NB-LRR; see text) recognize effector activity and restore resistance through effector-triggered immune responses. Limited accumulation of bacteria occurs prior to effective initiation of effector-triggered immune responses.

2000; Anderson *et al.,* 2006). Clearly the simplified ligand-receptor theory for gene-for-gene interaction (Flor, 1971; Gabriel and Rolfe, 1990) does not provide a clear explanation for all types of the *R* controlled disease resistance in plants. To solve this dilemma, Dangl and Jones (2001) have proposed the guard hypothesis that R proteins have evolved to recognize the activities of what is referred to as the multiple Type III effector proteins (Avrs) instead of directly physically matching the pathogen-derived cognate Avr proteins. As a consequence, the R proteins might "guard" a set of key cellular targets of the pathogen effector proteins by detecting physiological changes in the host cells (van der Biezen and Jones, 1998; Dangl and Jones, 2001; Schneider, 2002; Belkhadir *et al.,* 2004). Recent biochemical evidence to support the guard hypothesis centers on the *Arabidopsis* RIN4 protein functioning as a general component of the host defense (Mackey *et al.,* 2002; Axtell and Staskawicz, 2003; Mackey *et al.,* 2003; Nimchuk *et al.,* 2003; Gómez-Gómez, 2004; Chisholm *et al.,* 2006). Two NBS-LRR R proteins, RPM1 and RPS2, have been shown to interact with RIN4 in normal living cells, respectively. The Type III effector proteins AvrRpt2, AvrRpm1 and AvrB are able to target RIN4 upon pathogen infection. The proteolytic activity of AvrPto2 causes RIN4 degradation. Furthermore, loss of RIN4 function confers the constitutive activation of the RPS2-mediated defense responses. These results together indicate that both RPM1 and RPS2 guard the same cellular target RIN4 and monitor the Avr-mediated modifications of RIN4 upon pathogen infection.

2.1.2 Pathogen-associated molecular pattern recognition

In addition to recognizing the pathogen-derived *Avr*-products, recent work has revealed that plants express another defense mechanism against potential pathogens through the receptor mediated recognition of highly conserved microbial structures called pathogen-associated molecular patterns (PAMPs) that often trigger a plant response in a noncultivar-specific manner (Nürnberger and Brunner, 2002; Parker, 2003; Zipfel and Felix, 2005; Robatzek *et al.,* 2006). Such conserved microbial structures including lipopolysaccharides, chitins, cellulose binding elicitor lectins, the necrosis-inducing protein NPP1, flagellin, harpin (hrpZ), the elongation factor Tu, cold-shock proteins, and many others are also classified as general elicitors of plant defense (Asai *et al.,* 2002, Nürnberger and Brunner, 2002; Fellbrich *et al.,* 2002; Parker, 2003; Navarro *et al.,* 2004; Zeidler *et al.,* 2004; Ramonell *et al.,* 2005; Gaulin *et al.,* 2006; Zipfel *et al.,* 2006). Some of these PAMPs are only perceived by a narrow range of plant species, whereas others trigger defense responses in many species. In addition, some plant-derived molecules can also act as general elicitors, such as oligosaccharides and glycopeptides released from attacking phytopathogenic microorganisms (Montesano *et al.,* 2003; Nürnberger, 2004).

Like mammals, plants have evolved plasma-membrane-localized pattern recognition receptors (PRRs) and these function to recognize certain PAMPs (Montesano *et al.,* 2003; Nürnberger and Lipka, 2005; Abramovitch *et al.,* 2006). For example, the *Arabidopsis* genome contains more than 400 receptor-like kinases (RLKs), 235 of which carry a LRR domain and are designated LRR-RLKs. A significant number of these putative transmembrane receptor kinases with an extracellular domain are assumed to be involved in PAMP perception (Montesano *et al.,* 2003; Johnson and Ingram, 2005; Nürnberger and Lipka, 2005). The PRR activation triggers signaling events including the rapid alteration of cytoplasmic Ca^{2+} levels, the generation of ROS and NO, and the activation of post-transcriptionally regulated mitogen-activated protein kinase (MAPK). These signaling events lead to the upregulation of numerous genes encoding transcription factors, hormone-related proteins, RLKs, phosphatases, proteins involved in protein degradation, and defense-related proteins associated with cell-wall reorganization (Asai *et al.,* 2002; Navarro *et al.,* 2004; Nürnberger and Lipka, 2005; Thilmony *et al.,* 2006; Truman *et al.,* 2006; Zipfel *et al.,* 2006). The PAMP-mediated non-self recognition and signal transduction is assumed to activate the first line of inducible defense responses. This defense may eventually stop the attempted invasion of pathogens (Nürnberger and Lipka, 2005; Abramovitch *et al.,* 2006).

To advance our understanding of the PAMP-triggered defense responses, the most studied example is the perception of flagellin flg22, which is a conserved 22 amino acid (aa) peptide of the protein subunit of bacterial flagella, which are required for bacterial motility (Gómez-Gómez and Boller, 2002; Gómez-Gómez, 2004; Zipfel *et al.,* 2004; Zipfel and Felix, 2005). The *Arabidopsis* Flagellin Sensitive2 (FLS2) is a typical LRR-RLK protein consisting of an extracellular LRR domain and an intracellular serine/threonine kinase domain (Gómez-Gómez and Boller, 2002). The *Arabidopsis fls2* mutant is more susceptible than wild-type plants to an infection by the virulent pathogen *P. syringae* pv. *tomato* DC3000, and the treatment of the wild type but not the *fls2* plants with flg22 enhances the wild-type resistance to the same pathogen (Zipfel *et al.,* 2004). In *Arabidopsis* plants, the peptide flg22 activates the MAP kinase cascades and several WRKY transcription factors that function downstream of the flagellin receptor FLS2 (Asai *et al.,* 2002). Evidence to support the hypothesis that the extracellular LRR domain is the binding site that physically interacts with flagellin, a point mutation in one of the LRR of the FLS2 caused a complete absence of specific binding (Bauer *et al.,* 2001). In a very recent report, Chinchilla *et al.,* (2006) showed by chemical cross-linking and immunoprecipitation that the flg22 peptide directly bound the FLS2 protein. Moreover, the heterologous expression of *Arabidopsis FLS2* in tomato cells conferred the specific features characteristic of the flg22 perception in *Arabidopsis* (Chinchilla *et al.,* 2006). Consistent with its role in early pathogen detection, FLS2 was present in leaf epidermal cells

and stomatal guard cells, which are the entry sites for phytopathogens (Robatzek *et al.,* 2006). Notably, recent studies suggest that Type III effectors might suppress the PRR-mediated defense responses. For instance, AvrPto and AvrPtoB, which activate gene-for-gene responses in tomato and tobacco, have been demonstrated to block the early defense signaling activated by different PAMPs such as flg22, HrpZ, and NPP1 (He *et al.,* 2006). This highlights the fact that plant pathogens have acquired the ability to deliver effector proteins to host cells to suppress the PAMP-triggered immunity during the co-evolution of plant-microbe interactions.

2.1.3 Herbivore-associated molecular patterns (HAMPs) and Herbivore Effectors

The term Herbivore-Associated Molecular Pattern (HAMP) is parallel to the widely used term in plant pathology and microbiology, Microbial (or pathogen) - Associated Molecular Pattern (MAMP or PAMP) (Mithofer and Boland, 2008). The terminology for HAMP follows the logic for MAMP; where MAMPS are defined as microbial-derived elicitors produced by the microbe itself or by the host plant during attack that activates defensive responses of the host plant (Mithofer and Boland, 2008). In the current dogma in plant pathology, MAMPs are broadly conserved molecules (e.g. fungal chitin, flagellins, lipopolysaccharides, etc.) associated with a wide range of microbes (Bent and Mackey, 2007). Plants have thus evolved receptors to detect MAMPs resulting in the activation of plant defense pathways (Bent and Mackey, 2007). The literature on fatty acid conjugates (FACs) such as volicitin provides the basis for drawing this comparison. These elicitors have broad taxonomic distribution as recent evidence indicates that they are found not only in Lepidoptera but also in Dipteran and Orthopteran species (Yoshinaga *et al.,* 2007). In the insects they probably perform essential physiological roles. Moreover, a putative receptor for volicitin has been identified in maize. The recently identified inceptins would also fall into the category of HAMPs, as the molecules produced from plant-supplied precursors by proteolytic degradation by the herbivore (Schmelz *et al.,* 2006).

To date, five different classes of insect herbivore-produced elicitors of plant defenses have been chemically identified. Four of these are associated with feeding and the fifth with oviposition. Three of the elicitor types deposited on plant tissues during feeding were isolated and identified from the oral secretions of moth or butterfly larvae. The fourth was found in grasshoppers. The elicitor deposited during oviposition is produced by weevils. Thus, elicitor chemistry has been investigated in a relatively small number of insect species. It is interesting to note that three of the five identified thus far are difunctional fatty acid derivatives with previously unknown structures. The diversity in the chemical structures of these elicitors

suggests that plants must employ a variety of different mechanisms that allows them to detect and initiate defenses against a broad array of insect pests.

The first reported herbivore-produced elicitor was a β-glucosidase, present in the regurgitant of *Pieris brassicae* caterpillars that triggers the same emissions of volatiles in cabbage plants as induced by feeding caterpillars (Mattiaci *et al.,* 1995). Since enzyme activity in the regurgitant is retained when caterpillars are fed a β-glucosidase-free diet, it does not appear to be plant-derived. Presumably, the enzyme acts to cleave sugars coupled to organic compounds that then become more volatile and are released. Thus far, this is the only enzymatic elicitor of plant volatiles found in insects. A distinctly different, low-MW fatty acid derivative, N- (17-hydroxylinolenoyl)-L-glutamine (volicitin), was identified from the regurgitant of beet armyworm caterpillars and induces corn seedlings to release volatile chemical signals (Alborn *et al.,* 1997). Subsequently, other fatty acid–amino acid conjugates (FACs) have been discovered in the regurgitant of several caterpillar species (Alborn *et al.,* 2000; Mori *et al.,* 2003). Common features of the insect-derived FACs discovered thus far are the presence of either L-glutamine or L-glutamic acid linked via an amide bond to linolenic acid, 17-hydroxylinolenic acid or the linoleic acid analogs. While FACs appear to be found in most, but not all, Lepidoptera studied thus far, it is not yet known whether these elicitors of plant volatiles are widespread among other orders of insects. Very recently Yoshinaga *et al.,* (2007) have discovered N-linolenoyl- and N-linoleoylglutamates, as well as hydroxylated FACs and glutamine conjugates in the regurgitant of two closely related cricket species (Orthoptera: Gryllidae) and in *Drosophila* larvae.

There has been very limited work on identifying herbivore effectors, although the ability of herbivores to evade host defenses is becoming better appreciated (Zarate *et al.,* 2007; Musser *et al.,* 2002). We predict that insect herbivores produce a cocktail of effectors that can suppress plant defensive pathways, mimic plant hormones, and/or mask the perception of HAMPs. While the labial saliva of several noctuid species has been shown to suppress direct and indirect plant defenses (Musser *et al.,* 2002; 2006), glucose oxidase (GOX) remains as the one identified salivary constituent contributing to this suppression (Bede *et al.,* 2006; Zong *et al.,* 2004). This enzyme, produced by the labial and mandibular salivary glands, oxidizes β-D-glucose to form gluconic acid and H_2O_2 (Eichenseer *et al.,* 1999). Secretion and synthesis of GOX is highly dependent upon the host plant and diet (Pieffer and Felton, 2005; Babic *et al.,* 2008) indicating that the effects of the enzyme on plant defenses is likely to be context-dependent as described for HAMPs. Recent evidence indicates that in addition to suppressing direct defenses such as the induction of nicotine in tobacco (Bede *et al.,* 2006; Zong and Wang, 2004); saliva (and perhaps GOX) can suppress the induction of volatile, indirect defenses (Delphia *et al.,* 2006; Bede *et al.,* 2006). Using

tobacco and the tobacco budworm *Heliothis virescens*, it was shown that saliva suppressed both quantitative and qualitative changes in OS-elicited plant volatiles (Delphia *et al.*, 2006). Thus, saliva may 'mask' the identity of FACs contained in OS, although the mechanism of action remains unknown.

2.2 Plant disease resistance

Although plants do not have the benefit of a circulating antibody system, the existence of the preformed physical or chemical obstacles and the evolution of the plant immune response have culminated in a highly effective defense system that is able to resist potential attack by potential invaders (Mysore and Ryu, 2004; Chisholm *et al.*, 2006; Jones and Dangl, 2006). The former mechanisms are in place irrespective of whether or not the plant tissue is challenged by microbes, whereas the latter are activated in response to a pathogen attack. Following pathogen or elicitor recognition, systemic signals emanating from the local sites of infection are responsible for the systemic responses.

2.2.1 Nonhost resistance

Nonhost resistance defines the nonspecific resistance against all members of a given pathogen species throughout an entire plant species (Thordal-Christensen, 2003; Mysore and Ryu, 2004). This type of resistance is the most common and durable form of plant resistance to disease causing organisms (Heath, 2000; Thordal-Christensen, 2003; Jones and Takemoto, 2004) and classified into Type I without visible symptoms and Type II related to the HR often resulting from PAMP-induced defense responses (Mysore and Ryu, 2004). A pathogen that cannot cause disease on a nonhost plant is referred to as a nonhost or heterologous pathogen. Nonhost resistance, which is also referred to as heterologous plant-microbe interactions or basic incompatibility, comprises a variety of distinct mechanisms, of which some are preformed and others are inducible (Dangl and Jones, 2001; Thordal-Christensen, 2003; Jones and Takemoto, 2004; Mysore and Ryu, 2004; Chisholm *et al.*, 2006).

The preformed defense mechanisms encompass both the constitutive barriers provided by the epidermis, wax, cuticle, cell wall and the cytoskeleton and the preformed antimicrobial compounds (Heath, 2000; Dangl and Jones, 2001; Dixon, 2001; Mysore and Ryu, 2004; Agrios, 2005; Nürnberger and Lipka, 2005; Halkier and Gershenzon, 2006). A plant epidermis often contains trichomes loaded with defensive metabolites, spines acting as the earliest barriers against pathogens, as well as herbivores (Nürnberger *et al.*, 2004; Nürnberger and Lipka, 2005). The epicuticular wax, a thin layer of hydrophobic constituents, contains highly acidic substances that interfere with an insect herbivore attack (Griffiths *et al.*,

2000). Another efficient barrier is the cell wall composed of cellulose fiber, polysaccharides and proteins (Agrios, 2005). Furthermore, plant actin microfilaments have also been implicated in preventing the ingress of certain fungal pathogens (Mysore and Ryu, 2004). Preformed antimicrobial compounds include a vast array of low-molecular-mass secondary metabolites such as saponins, phenolics, cyclic hydroxamic acids, cyanogenic glycosides, isoflavonoids, sesquiterpenes, and sulfur-containing indole derivatives, and these confer selective reinforcement against a microbial attack (Dixon, 2001; Mysore and Ryu 2004; Nürnberger, 2004). These metabolites and derivatives may be constitutively present in healthy plants (Papadopoulou *et al.,* 1999) or alternatively undergo enzyme-catalyzed transformations in response to a pathogen attack (Halkier and Gershenson, 2006).

In many cases, the preformed structural or chemical barriers effectively halt pathogen colonization or the establishment of infection structures following an attempted attack by nonhost pathogens. However, when nonhost pathogens or their elicitors enter the apoplast of plant cells by bypassing or circumventing constitutive obstacles, the plants immediately initiate a PAMP-induced defense referred to as basal resistance (Mysore and Ryu, 2004; Nürnberger *et al.,* 2004; Nürnberger and Lipka, 2005; Zipfel and Felix, 2005; Melotto *et al.,* 2006). The basal defense responses activated during basic incompatible interactions are often sufficient to restrict the invasion or growth of non host pathogens (Chisholm *et al.,* 2006; da Cunha *et al.,* 2006). The systemic protection against subsequent infection with virulent pathogens can be obtained through infiltration of PAMPs such as HrpZ and flg22 into plants (Nürnberger *et al.,* 2004; Nürnberger and Lipka, 2005), indicating that the PAMP-based recognition events might not only trigger local defense responses, but also potentiate systemic defense responses in the natural environment.

In contrast to the considerable progress made in understanding host resistance, it is genetically ill-defined as to why a particular plant species is typically resistant to potential pathogens that successfully infect other plant species (Heath, 2000; Thordal-Christensen, 2003). Yet, a recent series of mutational analysis revealed that several genes such as *PAD3*, *EDS1* and *NHO1* are required for a nonhost resistance against nonhost pathogens. The nonhost resistance of *Arabidopsis* to the necrotrophic pathogen *Alternaria brassicicola* is compromised in the phytoalexin-deficient mutant *pad3-1* (Thomma *et al.,* 1999b). *PAD3* encodes a putative cytochrome P450 monooxygenase required for the biosynthesis of camalexin (Zhou *et al.,* 1999), demonstrating an important role for the inducible production of the antimicrobial compounds in plant species resistance to one specific necrotrophic fungus. A combination of the loss of actin cytoskeleton function and EDS1 activity resulted in a severe loss of nonhost resistance in *Arabidopsis* against the heterologous fungal pathogen wheat powdery mildew *Blumeria graminis* f. sp. *Tritici* (Yun *et al.,* 2003). The *Arabidopsis*

NHO1, which encodes a glycerol kinase, is required for resistance against the nonhost pathogens *Botrytis cinerea* and *P. syringae* isolates (Kang *et al.,* 2003; Li *et al.,* 2005). Like *eds1*, *nho1* mutation also compromises gene-for-gene resistance mediated by various *R* genes. These observations suggest that nonhost resistance and host resistance might share a common pathway. In addition, nonhost resistance against fungal pathogens is associated with the penetration process (Thordal-Christensen, 2003). The isolation and functional characterization of several *PEN* mutants (Thordal-Christensen, 2003; Nürnberger and Lipka, 2005; Ellis, 2006) provides a mechanistic link between the nonhost and basal penetration resistance at the plant cell wall.

Certain pathogen species or individual strains of a given pathogen species have developed diverse strategies to evade/suppress/subvert early plant defenses conferred by the nonhost defense mechanisms (Abramovitch *et al.,* 2006). For instance, a single amino acid polymorphism of the flg22 peptide was observed in the strains of the black rot pathogen *Xanthomonas campestris* pv. *campestris* (Sun *et al.,* 2006). The eliciting activity of those differential flg22 peptides appears highly correlated with the colonization capability of the individual strains in susceptible *Arabidopsis* plants, indicating that the pathogen tolerance to the partial loss of specific PAMPs may be an important driving force in the coevolution between the plants and pathogens to avoid PRR-mediated detection (Sun *et al.,* 2006). Numerous gram negative bacterial pathogens deliver virulence factors (also referred to as effector proteins) directly into the plant cells via the Type III secretion system (TTSS). The number of identified effector proteins, which suppress the plant basal defense responses, has dramatically expanded over the past two years (Kim *et al.,* 2005; Li *et al.,* 2005; Abramovitch *et al.,* 2006; da Cunha *et al.,* 2006; He *et al.,* 2006). For instance, AvrRpt2 and AvrRpm1 suppress the flagellin-mediated accumulation of callose (Kim *et al.,* 2005). Moreover, a recent screen for the effector proteins that suppress the flg22-induced basal defenses revealed that AvrPto and AvrPtoB strongly inhibit the expression of *FRK1*, which is induced by flg22 treatment, and the activation of AtMPK3 and AtMPK6 downstream of several distinct PRRs (He *et al.,* 2006; Zipfel *et al.,* 2006). Interestingly, mutations in the key residues essential for AvrPtoB, E3 ubiquitin ligase activity do not nullify the capability of AvrPtoB to suppress the basal defenses in *Arabidopsis* (He *et al.,* 2006). This suggests an E3-independent mechanism for basal defense suppression (Wulf *et al.,* 2004; Abramovitch *et al.,* 2006). In addition, some pathogen species may secrete the exoenzymes involved in degrading plant cell walls via the Type II secretion system or produce toxins (Toth and Birch, 2005). Such pathogens render plants susceptibile to disease and are considered homologous pathogens. Furthermore, these plants turn out to be hosts sharing a basic compatibility with a homologous pathogen (Nürnberger and Lipka, 2005). The basal resistance triggered by PAMP in susceptible hosts is

insufficient to stop a pathogen infection. It is believed that a strong, selective pressure on host plants posed by virulent pathogens has ultimately resulted in the coevolution of plant *R* genes. Correspondingly, R proteins directly or indirectly recognize strain- or race-specific effectors and allow for the establishment of a plant cultivar specific disease resistance (Nimchuk *et al.*, 2003; Nürnberger and Lipka, 2005).

2.2.2 Host resistance

Cultivar resistance is restricted to a particular pathogen species and is often referred to as a host resistance, which is tightly associated with the gene-for-gene recognition and accompanied by the HR (Dangl and Jones, 2001; Martin *et al.*, 2003; Nimchuk *et al.*, 2003; Alfano and Collmer, 2004; Mysore and Ryu, 2004). When a plant is resistant, the interaction is then called incompatible, and when a plant is susceptible, the interaction is called compatible. Since *R* genes can be manipulated by plant breeders to raise the resistance in normally susceptible cultivars, the host resistance has been extensively studied for decades. This host resistance consists of the local resistance at the site of infection and the systemic acquired resistance (SAR) in the distal, non inoculated parts of plants following an activation of a local resistance (Ryals *et al.*, 1996). Local resistance has been associated with a number of biochemical and physiological features. These include the rapid induction of the ion fluxes of H^+, K^+, Cl^-, and Ca^{2+} across the plasma membrane, protein phosphorylation or dephosphorylation, oxidative burst, deposition of callose and lignin, biosynthesis of proteins involved in the production of signal molecules such as ET, JA and SA as well as the accumulation of PR proteins and protective secondary metabolites (Dixon *et al.*, 1994; Dangl *et al.*, 1996; Hammond-Kosack and Jones, 1997; Ligterink *et al.*, 1997; Heo *et al.*, 1999; Asai *et al.*, 2002; Cheng *et al.*, 2002). The local HR, the most recognizable form at the site of infection, is often associated with the onset of SAR. SAR has been recognized as a typical response to plant pathogen infection for almost 100 years. Currently, SAR refers to a distinct, integrated set of signal transduction pathways, which is triggered by a local pathogen challenge. This is also associated with the activation of many plant genes that ultimately makes the plant not only locally, but also systemically, more refractory to subsequent infections by a wide variety of unrelated pathogens (Ryals *et al.*, 1996; Durrant and Dong *et al.*, 2004). When the SAR is activated, a normally compatible plant-pathogen interaction can be converted into an incompatible one (Uknes *et al.*, 1992; Mauch-Mani and Slusarenko 1996). Conversely, when the SAR is incapacitated, a normally incompatible interaction becomes compatible (Delaney *et al.*, 1994; Mauch-Mani and Slusarenko, 1996). SAR can be distinguished from other disease-resistance responses by the spectrum of pathogen protection (Ryals *et al.*, 1996). The induction of what is referred to as the SAR-marker genes is tightly

correlated with the onset of the SAR in an uninfected tissue (Métraux *et al.,* 1989; Ward *et al.,* 1991; Uknes *et al.,* 1992). Over the past decade, considerable effort has led to the identificantion of the several components with distinct properties involved in the establishment of SAR (Glazebrook, 2001; Durrant and Dong, 2004; Dong, 2004).

The requirement for SA in SAR was shown using transgenic plants expressing the *NahG* gene (Delaney *et al.,* 1994). This gene encodes a salicylate hydroxylase degrading the SA to catechol, the SA-insensitive *npr1* mutants (Cao *et al.,* 1994; Delaney *et al.,* 1995; Shah *et al.,* 1997), and the SA induction-deficient mutants *sid1* and *sid2* (Nawrath and Métraux 1999; Dewdney *et al.,* 2000; Nawrath *et al.,* 2002). SA was originally thought to be the mobile transducer of SAR (Ryals *et al.,* 1996). However, results obtained from the detachment experiments on *P. syringae* –infected cucumber leaves and the grafting experiments on tobacco plants indicate that SA does not appear to function as the systemically transported signal (Rasmussen *et al.,* 1991; Vernooij *et al.,* 1994). Recently, the genetic characterization of *Arabidopsis defective in induced resistance1-1* (*dir1-1*) suggests that an essential mobile signal during SAR is a lipid-based molecule rather than SA (Maldonado *et al.,* 2002). The *dir1-1* mutant exhibits wild-type local resistance and a normal accumulation of SA in either inoculated (local) or uninoculated (systemic) leaves following pathogen infection but fails to develop SAR and to express the *PR* genes in systemic leaves. Importantly, *dir1-1* is deficient in the mobile signal for the SAR and the *DIR1* gene product is a putative apoplastic lipid transfer protein. These observations suggest that DIR1 may interact with a lipid-based signal molecule and promote long-distance signaling during SAR (Maldonado *et al.,* 2002).

Besides SA, other signal molecules including ET, JA, NO and H_2O_2, which are originated from the local site of the attempted infection, may also be responsible for host resistance (Chen *et al.,* 1993; Penninckx *et al.,* 1996; Shirasu *et al.,* 1997; Alvarez *et al.,* 1998; Dong, 1998; Glazebrook, 2001; Glazebrook, 2005). Indeed, a growing body of evidence suggests that host resistance results from a sophisticated signaling network involving crosstalk among the different signal transduction pathways (Kunkel and Brooks, 2002; Rojo *et al.,* 2003; Bostock, 2005). In addition, the defense pathways involved in the basal resistance and the *R* gene-mediated resistanc are probably linked to each other (Kim *et al.,* 2005; Chisholm *et al.,* 2006; He *et al.,* 2006). A recent breakthrough in understanding the molecular mechanisms behind plant innate immunity is the discovery that the RPM1-interacting protein RIN4 is not only a convergence point for different *R* gene-mediated signaling pathways, but also a regulator of the PRR-mediated signaling (Mackey *et al.,* 2002; Axtel and Staskawicz, 2003; Mackey *et al.,* 2003; Kim *et al.,* 2005; Chisholm *et al.,* 2006).

2.2.3 Induced systemic resistance

In addition to the well-documented SAR, there is a second type of systemic resistance which is referred to as induced systemic resistance (ISR). This ISR is potentiated by some growth promoting rhizobacteria. The best characterized of these rhizobacteria are the strains within several species of fluorescent *Pseudomonas* spp. that do not cause any visible disease symptoms to the plant's root system (van Loon *et al.,* 1998). Although it does not involve the accumulation of the known PR proteins that are characteristic of the SAR in *Arabidopsis*, ISR is effective against a broad range of diseases caused by viruses, bacteria, and fungi (Pieterse *et al.,* 1996; van Loon *et al.,* 1998; Vallad and Goodman, 2004; Leon-Kloosterziel *et al.,* 2005). In contrast to SAR across a wide array of the plant species, the elicitation of the ISR by specific rhizobacterial strains is restricted to certain plant species or genotypes (van Loon *et al.,* 1998; Yan *et al.,* 2002). The onset of ISR does not depend on SA but on ET and JA (Pieterse *et al.,* 1998). Interestingly, NPR1, the central regulatory protein of SAR, is required for developing ISR (Pieterse *et al.,* 1998). Furthermore, ISR and SAR can be activated simultaneously, resulting in an additive level of protection against plant pathogens (van Wees *et al.,* 2000). However, these molecular characterizations are based on a limited number of ISR systems. Other examples of the ISR linked to the production of SA, therefore have more in common with the SAR (Vallad and Goodman, 2004; Madhaiyan *et al.,* 2006).

2.2.4 Wound- and herbivore-elicited resistance

In contrast to a microbial attack, an herbivore attack is often associated with wounding. Because herbivores produce a large number of compounds from oral secretions or oviposition fluids, the perception of herbivore-specific elicitors must be a key event prior to the establishment of the plant wound responses. Although the process of herbivore recognition remains elusive, the wound-induced expression of the defensive compounds is a pronounced feature in both the local damaged tissues and in the undamaged tissues located at distances from the initial site of the attack (Kessler and Baldwin, 2002; Schilmiller and Howe, 2005). These compounds either directly exert toxic or antifeedant effects on herbivores due to their being specifically harmful for their organ systems (nervous, digestive, endocrine, etc.), or the compounds act indirectly by attracting parasitoids and predators of invading herbivores (Kessler and Baldwin, 2002).

Several of the following small molecules have been associated with the induction of local or systemic wound responses: oligogalacturonides, fungal-derived chitosan, systemin, ROS, ET, and JA as well (O'Donnell, 1996; Kessler and Baldwin, 2002; Schilmiller and Howe, 2005) and recent studies demonstrate the involvement of additional plant hormones such as auxin (Park *et al.,* 2006), abscisic acid (Thaler and Bostock, 2004), and methanol (von

Dahl *et al.,* 2006). Since Green and Ryan, (1972) first demonstrated that wounds lead to the systemic accumulation of the defensive proteinase inhibitors (PIs) in the tomato and potato leaves. Since then, disrupting the activity of digestive enzymes in the herbivore midgut, wound-inducible PIs in different plant species have been widely used to elucidate how mobile signals work during the establishment of the systemic responses (Schilmiller and Howe, 2005). Early studies on tomatoes indicate that an 18-aa peptide produced by a proteolytic cleavage of the prosystemin at the wound site called systemin is implicated in the systemic responses (Farmer and Ryan, 1992). Emerging evidence suggests that systemin acts locally to trigger the formation of JA or its derivatives instead of being translocated to the distal tissues (Schilmiller and Howe, 2005). Most likely, JA or related members of the jasmonate family of oxylipins, function as long-distance wound signals to subsequently activate the systemic expression of the PIs (Schilmiller and Howe, 2005). Consistent with this hypothesis, grafting experiments conducted using various mutants defective of JA signaling or JA biosyntheis demonstrated that wound systemic signaling requires both JA biosynthesis at the wound site and JA perception in the remote tissue (Li *et al.,* 2002; Schilmiller and Howe, 2005). Given that the long-distance trafficking of lipid-based signals has been implicated in both the SAR and wound responses (Durrant and Dong, 2004; Schilmiller and Howe, 2005), it is not surprising that analyses of gene expression profiling revealed a substantial overlapping between the wounds and pathogen responses (Durrant *et al.,* 2000; Reymond *et al.,* 2004).

Modern molecular and chemical analytical methods are used to assess the complexity and specificity of plant responses to herbivory that are the consequence of the plant's integration of the plant-derived and herbivore-derived wound signals. One of these methods, cDNA microarrays, has facilitated the large-scale transcriptional characterization of herbivore-specific plant responses to, for example, generalist versus specialist chewing lepidopteran larvae, or piercing versus chewing insects. For example, consumption of *Nicotiana attenuata* by a specialist, *Manduca sexta*, and two generalists, *Heliothis virescens* and *S. exigua*, led to comparable upregulation of genes involved in JA signaling, cell wall modification, and WRKY transcription factors (Voelkel and Baldwin, 2004). Similar results were observed with the specialist *Pieris rapae* and the generalist *S. littoralis* feeding on *Arabidopsis thaliana* (Reymond *et al.,* 2004). However, the transcriptional responses of *N. attenuata* to the two generalists were more similar to each other than to those elicited by the specialist, and this difference was correlated with variation in the FAC composition of the herbivores' regurgitants (Voelkel and Baldwin, 2004). Transcriptional responses of *N. attenuata* to herbivory by the piercing–sucking mirid bug, *Tupiocoris notatus* and *M. sexta* larvae were also similar in terms of secondary metabolism genes but different for photosynthesis-related genes (Voelkel and Baldwin, 2004), while the phloem-feeding aphid,

Myzus persicae, elicits very different transcriptional responses in *Arabidopsis* compared to pathogens and foliar herbivores (De Vos *et al.,* 2005). Thus, while transcript profiles of secondary metabolism genes induced by different herbivores frequently show significant overlap (across host-range and feeding mode), a large part of the transcriptome shows specific differences that could have functional consequences.

The specificity of herbivore-induced volatile organic compound (VOCs) emissions and the resulting attraction of predators and parasitoids are well documented (Pare *et al.,* 2005; Takabayashi *et al.,* 2006; Turlingson and Ton, 2006). By contrast, only few studies have addressed the specific elicitation of direct defense-mediating metabolites and proteins. In *N. attenuata* the similar transcriptional responses elicited by *T. notatus* and *M. sexta* herbivory correlate with a similar profile of induced secondary metabolites and defensive proteins (Kessler and Baldwin, 2004). In *A. thaliana*, however, aliphatic glucosinolates are induced by two aphids, the generalist *Myzus persicae* and the specialist *Brevicoryne brassicae*, and a generalist caterpillar, *S. exigua*, but not by the Brassicaceae specialist *P. rapae* (Mewis *et al.,* 2006); this difference may be correlated with differences in elicitor production by the herbivores (Reymond *et al.,* 2004).

The elicitor composition (inceptins, FACs, insect enzymes) of herbivore salivary secretions plays a crucial role in mediating specific plant responses. This specificity could reflect both a strategy of the plant to adjust its metabolism to a particular herbivore and a strategy of the insect to manipulate plant responses for its own benefit. In either case, specific plant responses should result in differential resistance effects on different herbivores and thus could have a profound influence on the dynamics within the arthropod community associated with the plant. Specificity in herbivore-induced responses could predict the outcome of plant-mediated interactions between different herbivore species feeding on the same host. If different herbivore species induce similar plant responses and these responses mediate resistance, cross-resistance between these herbivores would be expected. Accordingly, *Raphanus sativus* plants damaged by *P. rapae* caterpillars early in the season accumulate indole glucosinolates that make them resistant to a number of subsequent herbivores in different feeding guilds (Agrawal, 1998); the mirid *T. notatus* induces cross-resistance of *N. attenuate* plants to more damaging *Manduca* hornworms (Kessler and Baldwin, 2004); potato plants damaged by *Empoasca fabae* leafhoppers are more resistant to Colorado potato beetles (Lynch *et al.,* 2006); and *Asclepias syriaca* damaged by early stem-feeding weevils, *Rhyssomatus lineaticollis*, are more resistant to a number of leaf chewers, including monarch butterfly larvae, later in the season (Van Zandt and Agrawal, 2004). Such induced cross-resistance occurs not only among above-ground feeding herbivores but, due to systemically induced responses, also between aboveground herbivores and below-ground herbivores

(van Dam *et al.,* 2005). For example, root feeders induced the production of phenolics and glucosinolates in the shoots of *Brassica nigra,* which then reduced the performance of leaf-feeding *P. rapae* on those plants (van Dam *et al.,* 2005).

Induced responses to herbivory can also facilitate choice or performance of another herbivore species, particularly when specialist herbivores inhibit the production of defensive secondary metabolites (Mewis *et al.,* 2006; Musser *et al.,* 2005; Kahl *et al.,* 2000). Such positive plant-mediated interactions between herbivores are more frequently observed with sap-feeding herbivores. Tomato plants damaged by the aphid *Macrosiphum euphorbiae* were preferred for oviposition and supported a better larval growth of *S. exigua* compared to undamaged control and *S. exigua*-damaged plants. The preference was correlated with lower proteinase inhibitor content in aphid-damaged plants compared to *S. exigua*-damaged plants (Rodriguez-Sauna *et al.,* 2005) and likely resulted from differential elicitation of plant signaling by the two herbivore species. Active inhibition of signaling pathways by certain herbivores could have dramatic consequence on the interaction of the plant with other organisms and could alter community structure. For example, artificial silencing of biosynthesis or downstream signaling components of the JA cascade in *N. attenuata* recruits novel generalists to the plant, suggesting that altering of wound-induced JA responses can have a profound effect on herbivore host selection and arthropod community composition (Kessler *et al.,* 2005). Specific inhibition of wound response elicitation may also explain the lack of induced resistance to subsequent herbivore attack in *Solanum dulcamara* plants damaged by *Plagiometriona clavata* tortoise beetles (Viswanathan *et al.,* 2005).

In addition to mediating direct resistance, herbivore induced changes in primary and secondary metabolism can attract natural enemies of herbivores to the plant and thus directly and indirectly influence arthropod community structure. The herbivore-induced production of extra floral nectar and VOCs are the most prominent examples of indirect defenses. The increased production of extra floral nectar is described as an unspecific plant response to herbivory and mechanical damage (Heil *et al.,* 2004; Kost and Heil, 2005) and exposure to VOCs released from damaged neighboring plants (Kost and Heil, 2006) that increases the visitation rate by ants and other nectar-attracted predators. By contrast, the release of herbivore-induced VOCs and the resulting attraction of natural enemies represent a more specific signal (Takabayashi *et al.,* 2006). Once released, VOCs can be used as a signal by any organism able to perceive the compound(s). Therefore, VOC-signaling is not limited to natural enemies but includes herbivores and even neighboring plants. The complex odors emitted from herbivore-attacked plants include at least three different compound classes and differ significantly from those of mechanically wounded plants or between plants damaged by different herbivore species (Kessler and Baldwin, 2002; Takabayashi *et al.,* 2006). Different

levels of signal specificity are required for the attraction of natural enemies. For generalist predators or parasitoids, a rather unspecific signal indicating the presence of a feeding herbivore might be sufficient information. Indeed, the generalist predatory bug *Geocoris pallens* can be attracted by general, single compounds out of the complex odor bouquet of an herbivore-attacked wild tobacco (Kessler and Baldwin, 2001). On the other hand, specialist natural enemies are dependent on finding the exact host species, often in a critical developmental stage. These specialists are able to utilize specific plant-derived VOC signals released in response to attack by their appropriate host (De Moraes *et al.*, 1998; Birkett *et al.*, 2003; van Poecke *et al.*, 2003). Interestingly, herbivorous insects may use the same VOC signals to either accumulate on (Caroll *et al.*, 2006; Landotl *et al.*, 1999) or avoid damaged plants as a food or oviposition site (Kessler and Baldwin, 2001; De Moraes *et al.*, 2001). Moreover, recent studies suggest that VOC emission is not only a signal mediating above-ground multitrophic interactions and also play a role belowground. Root-feeding larvae of *Diabrotica virgifera* leaf beetles elicit the release of bcaryophyllene from maize roots, which attracts entomopathogenic nematodes (Rasmann *et al.*, 2005).

2.3 Defense signaling pathways

In general, from the initial stage of recognition by the plant to the successful confinement or death of the pathogen, the distinct signaling pathways mediated by the small, signaling molecules, such as SA, JA, ethylene (ET), hydrogen peroxide (H_2O_2), nitric oxide (NO), and abscisic acid (ABA), constitute the complex signal transduction network controlling plant defense and thereby endowing the plant with a more sophisticated capacity for the highly complex, multifaceted defense response (Apel and Hirt, 2004; Gfeller and Farmer, 2004; Durrant and Dong, 2004; Mittler *et al.*, 2004; Delledonne, 2005; Lorenzo and Solano, 2005; Torres and Dangl, 2005; van Loon *et al.*, 2006). The relative contribution of such signaling molecules to an inducible defense depends on the particular intruder (Reymond and Farmer, 1998; Glazebrook, 2005). Furthermore, a growing body of evidence regarding cellular signaling transduction and the regulation of expression of defense-related genes suggests that the defense signaling pathways do not function in a linear, independent fashion. Instead, each pathway can influence other pathways through positive or negative regulatory interactions (Kunkel and Brooks, 2002; Rojo *et al.*, 2003; Bostock, 2005). Recent studies demonstrate that, in addition to known defense pathways (signaled by SA, JAs and ET), oxylipins other than JA, and hormones such as brassinosteroids (BL), auxins, gibberellins (GA), cytokinins (CK), and abscisic acid (ABA), play roles in plant responses to pathogen assault. Upon microbial attack, plants modify the relative abundance of these hormones, and the expression of their responsive genes, as an instrument to activate an

efficient defense response allowing plant survival. Importantly, pathogens can counteract this strategy by interfering with these plant hormonal changes and also by producing plant hormones themselves as a component of their invading strategy (Robert-Seilaniantz *et al.,* 2007).

2.3.1 Salicylic acid-mediated defense signaling

The phytohormone salicylic acid (SA) has long been known to play a central role in plant defense signaling. SA levels increase in response to pathogen attack at the site of infection, and this is essential in resistance against various pathogens (Glazebrook, 2005). Moreover, exogenous application of SA protects plants against pathogens and induces the expression of defense-related genes (Van Loon *et al.,* 1997). SA is required also in the establishment of systemic acquired resistance (SAR). SAR is an induced state of resistance that is manifested throughout the plant in response to pathogen-triggered localized necrosis (Métraux *et al.,* 1990; Uknes *et al.,* 1993; Durrant and Dong, 2004). It can last from weeks to even months and is effective against a wide variety of normally virulent pathogens, including viruses, bacteria, fungi, and oomycetes (Thomma *et al.,* 2001; Durrant and Dong, 2004). The induction of SA signaling and SAR is associated with accumulation of such PR proteins as beta-1,3-glucanases, thaumatin-like proteins, chitinases, and PR1, which are thought to contribute to resistance (Van Loon, 1997). Many of the PR proteins have antimicrobial activity *in vitro*, but their roles in the establishment of SAR are unclear. Nevertheless, they serve as molecular markers for the onset of the defense response (Van Loon, 1997; Durrant and Dong, 2004).

SA-mediated defense signaling and SAR are often induced by infection with avirulent pathogens that trigger gene-for-gene resistance and HR, but also in response to necrotizing cell death-causing pathogens (Glazebrook *et al.,* 1997; Durrant and Dong, 2004; Glazebrook, 2005). However, while virulent pathogens do not usually trigger HR, they can induce SA signaling as part of the basal defense response to contain their growth (Glazebrook *et al.,* 1997). SA-dependent defense responses are considered effective mainly against biotrophic pathogens that feed on living tissues, such as the oomycete *P. parasitica*, the fungus *Erysiphe orontii*, and the bacterium *P. syringae* (Glazebrook, 2005). Accordingly, impaired SA production leads to increased susceptibility to various pathogens. For example, SA production is significantly reduced in *sid2* (*SA induction deficient*) plants, resulting in increased susceptibility to both virulent and avirulent forms of *P. syringae* and *P. parasitica* (Nawrath and Métraux, 1999). *SID2* encodes isochorismate synthase (ICS1), and the drastic reduction in the accumulation of SA in the *sid2* mutant indicates that the majority of this hormone in

Arabidopsis is produced via isochorismate (Wildermuth *et al.,* 2001) rather than via the shikimate-phenylalanine pathway, as earlier presumed (Lee *et al.,* 1995).

EDS1 and PHYTOALEXIN-DEFICIENT 4 (PAD4) are important activators of SA signaling (Aarts *et al.,* 1998; Wiemer *et al.,* 2005). These proteins are essential for basal resistance against virulent pathogens, but they are also needed in mediating cultivarspecific resistance activated by R proteins (Feys *et al.,* 2001). For instance, *eds1* mutant has defects in basal defense to virulent isolates of *Erysiphe* sp. and *P. syringae*, and it is also impaired in specific resistance to certain strains of *P. parasitica* (Parker *et al.,* 1996; Glazebrook *et al.,* 1997). EDS1 and PAD4 interact in vivo and are induced by both pathogen infection and SA application, suggesting that they act upstream of SA production (Aarts *et al.,* 1998; Feys *et al.,* 2001). SA also contributes to the expression of both EDS1 and PAD4 as part of a positive feedback loop that seems to be important in defense signal amplification (Feys *et al.,* 2001; Wiemer *et al.,* 2005). Several *R* gene products require the *NDR1* gene in establishing resistance after inoculation with certain avirulent pathogens (Century *et al.,* 1995; Aarts *et al.,* 1998). EDS5, a member of the multidrug and toxin extrusion (MATE) transporter family, is also required for pathogen-induced SA accumulation downstream of EDS1 and PAD4 (Nawrath *et al.,* 2002). In addition, ROS forms an amplification loop with SA; it enhances the SA signal (Shirasu *et al.,* 1997; Durrant and Dong, 2004) and SA then inhibits hydrogen peroxide scavenging enzymes CAT and APX, enhancing ROS accumulation (Durrant and Dong, 2004).The first studies highlighting the importance of SA in defense signaling employed transgenic *Arabidopsis* plants expressing the bacterial SA-degrading enzyme salicylate hydroxylase (*NahG*), which converts SA to catechol (Gaffney *et al.,* 1993; Delaney *et al.,* 1994). *NahG* plants display enhanced susceptibility to several fungal, bacterial, oomycete and viral pathogens, interpreted to result from the lack of SA (Gaffney *et al.,* 1993; Delaney *et al.,* 1994). However, recent studies comparing *NahG* plants with SA-deficient mutants indicate that the observed disease susceptibility phenotype might partly arise from the SA degradation product catechol rather than the lack of SA itself (Heck *et al.,* 2003). Treatment of *NahG* plants with catalase seems to reverse the susceptibility to *P. syringae* pv. *phaseolicola.* This suggests that the accumulation of catechol might trigger increased production of hydrogen peroxide, interfering with the true effects of the lack of SA (Van Wees and Glazebrook, 2003). Mutant screens aimed at finding components involved in SA signal transduction identified multiple alleles of a single gene: *NPR1/NIM1/SAI1* (*Non Expressor PR1,* Cao *et al.,* 1994; *Non-inducible Immunity 1*, Delaney *et al.,* 1995; *SA-insensitive 1*, Shah *et al.,* 1997). *NPR1* encodes an ankyrin repeat-containing protein that plays a central role in SA signal transduction. In mammalian systems, the NPR1 homolog IκB is involved in the repression of immune and inflammatory responses (Cao *et al.,* 1997; Ryals

et al., 1997). *npr1* mutant plants accumulate SA in response to pathogen challenge, but are unable to induce SAR-marker genes. They also display enhanced susceptibility to virulent pathogens and are impaired in some *R* gene-mediated resistance responses (Cao *et al.,* 1994; Delaney *et al.,* 1995; Glazebrook *et al.,* 1996). Overexpression of *NPR1* does not result in constitutive *PR* gene expression, but does enhance resistance to *P. parasitica*, *P. syringae*, and *E. cichoracearum* (Cao *et al.,* 1998; Friedrich *et al.,* 2001). This indicates that NPR1 needs to be activated for SAR induction even if it is expressed at high levels (Cao *et al.,* 1998; Durrant and Dong, 2004). Indeed, in an uninduced state, NPR1 resides in the cytosol as an oligomer. Accumulation of SA induces a redox change, reducing NPR1 to a monomeric, active form that is localized to the nucleus. There it activates the expression of *PR* genes through interaction with TGA transcription factors (Després *et al.,* 2003; Mou *et al.,* 2003). Characterization of various lesion mimic mutants from *Arabidopsis* highlights the role of cell death in the induction of SA-dependent defenses and SAR. Mutants such as *accelerated cell death* (*acd2)* and *lesions stimulating disease resistance* (*lsd1-7)* develop lesions due to light-induced (*acd2*) and spontaneous cell death (Mach *et al.,* 2001; Yao and Greenberg, 2006). The common phenotype of these lesion mimic mutants includes an elevated level of SA, constitutive expression of *PR* genes, and enhanced resistance to virulent pathogens (Durrant and Dong, 2004).

2.3.1.1 Nature of the systemic signal

SA has long been recognized as essential to the establishment of SAR; it accumulates in infected tissues in concert with the induction of *PR* genes and resistance (Malamy *et al.,* 1990; Métraux *et al.,* 1990; Uknes *et al.,* 1993; Durrant and Dong, 2004). SA was originally proposed as the putative signaling molecule mediating the induction of SAR based on the results obtained with cucumber (*Cucumis sativus*) (Métraux *et al.,* 1990) and tobacco (*Nicotiana tabacum*) (Malamy *et al.,* 1990; Malamy and Klessig, 1992). Using *Arabidopsis* plants, Shulaev and coworkers (1995) showed that ^{18}O-labeled SA is transported from pathogen-inoculated leaves of tobacco to systemic, non-inoculated leaves, indicating that SA itself is the signal. SA was also suggested to be converted to volatile methyl salicylate (MeSA), which could induce resistance not only in distal tissues of the infected plant but also in neighboring plants (Shulaev *et al.,* 1997).

Evidence arguing against SA as the mobile signal also exists. When a scion from wild type tobacco was grafted to a pathogen-inoculated rootstock of a plant expressing the SA hydroxylase *NahG* gene, and hence, unable to accumulate SA, the SAR signal was still transmitted to the wild-type plant (Vernooij *et al.,* 1994). However, the authors showed that SA was needed in receiving the SAR signal since *NahG* scions grafted to wild-type rootstock

were unable to establish SAR after the inoculation of the rootstock (Vernooij *et al.,* 1994). Also, detachment of leaves from *P. syringae*-infected plants before SA levels rose did not block SAR development (Rasmussen *et al.,* 1991). In addition, high SA concentrations have been detected in other plant species, such as potato and rice, under non-inducing conditions (Silverman *et al.,* 1995). Recent work suggests that the mobile SAR signal may be a lipid-based molecule. *DIR1* encodes a putative apoplastic lipid transfer protein, and *dir1-1* (*defective in induced resistance 1-1*) plants exhibit wild-type local resistance to virulent *P. syringae*, but fail to develop SAR in systemic, un-inoculated tissues (Maldonado *et al.,* 2002). The phloem sap from *dir1* is deficient in the mobile signal, but the plants were able to establish SAR in response to sap of wild-type plants. This indicates that DIR1 might function in the transmission of the signal (Maldonado *et al.,* 2002). Tobacco SA-Binding protein 2 (SABP2) is also a lipase (Du and Klessig, 1997; Kumar and Klessig, 2003), and silencing of this gene diminished both local resistance and SAR (Kumar and Klessig, 2003). In addition, both EDS1 and PAD4 have homology to lipase-like proteins (Wiemer *et al.,* 2005).

2.3.2 Jasmonic acid-mediated defense signaling

Certain oxygenated fatty acids, oxylipins, have key roles as regulators of different plant responses (Farmer *et al.,* 2003). Interestingly, these lipid-derived molecules have biological activities that resemble some of the roles of well-known mediators in animals, most notably, prostaglandins, which are involved in inflammatory responses (Thomma *et al.,* 2004). Jasmonates, especially phytohormone jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), regulate developmental processes, including embryogenesis, pollen and seed development, and root growth (Farmer *et al.,* 2003; Liechti *et al.,* 2006). Moreover, JAs also mediate resistance to insects, microbial pathogens, and abiotic stress responses to wounding and ozone (Reymond and Farmer, 1998; Overmyer *et al.,* 2000). However, while JA is a terminal product of the octadecanoid pathway, it is not the only one with biological activity. Recent studies suggest that a cyclopentenone precursor of JA, 12-oxo-phytodienoic acid (OPDA), can also induce defense gene expression (Farmer *et al.,* 2003). *Arabidopsis* mutants impaired in the synthesis (*fad3/7/8*) or perception (*coi1*) of JA exhibit enhanced susceptibility to a variety of pathogens, including the fungi *Alternaria brassicicola*, *Botrytis cinerea*, and *Pythium* sp., and the bacterium *E. carotovora* (Thomma *et al.,* 1998, 2001) These pathogens have a common virulence strategy; they kill plant cells to obtain nutrients. Although JA responses are generally considered effective in defense against necrotrophic pathogens (Turner *et al.,* 2002; Farmer *et al.,* 2003), in some cases JA seems to contribute to plant resistance against biotrophs as well. For example, *Arabidopsis constitutive expression of vsp1* (*cev1*) mutant exhibits constitutive JA signaling and enhanced defenses against fungus

E. cichoracearum and bacterium *P. syringae* pv. *maculicola* (Ellis *et al.,* 2002). JA can be metabolized by a variety of routes, including methylation to MeJA and conjugation to amino acids (Liechti *et al.,* 2006). A recent study demonstrates that Jasmonic Acid Resistant 1 (JAR1) is a JA-amino acid synthetase conjugating JA to isoleucine (Ile) (Staswick and Tiryaki, 2004). *jar1* plants exhibit decreased sensitivity to exogenous JA, are susceptible to certain pathogens, and are unable to exhibit rhizobacteria-induced ISR (Pieterse *et al.,* 1998; Staswick *et al.,* 1998). They also have altered response to ozone (Overmyer *et al.,* 2000). However, *jar1* plants are not male sterile, suggesting that the activity of JAR1 is required for optimal JA signaling in some but not all responses in *Arabidopsis* (Staswick and Tiryaki, 2004).

The perception and subsequent signal transduction of JA remain unclear. A receptor for JA has not yet been characterized (Liechti *et al.,* 2006). However, a central element of the JA signaling pathway seems to be the COI1 (Coronatine Insensitive 1) protein (Feys *et al.,* 1994). *coi1* mutants of *Arabidopsis* are male-sterile, fail to express JA-regulated genes, and are susceptible to pathogens (Thomma *et al.,* 1998). COI1 is an F-box protein that forms an active SCFCOI1 complex, which together with the COP9 signalosome (CSN) plays an essential role in JA signaling (Xu *et al.,* 2002). This machinery functions *in vivo* as an ubiquitin ligase complex that removes repression from JA-responsive defense genes. It is thought to target regulatory proteins, including transcriptional repressors, to ubiquitin-proteasome-mediated protein-degradation (Xu *et al.,* 2002; Feng *et al.,* 2003). Feng *et al.,* (2003) demonstrated that, like the *coi1* mutant, plants with reduced CSN function exhibit a JA-insensitive root elongation phenotype and an absence of specific JA-induced gene expression. Interestingly, the recently characterized auxin receptor TIR1 is an F-box protein that, like COI1, forms an ubiquitin protein ligase SCFTIR complex (Dharmasiri *et al.,* 2005). Thus, it is tempting to speculate that, similarly to TIR1, COI1 could act as a receptor for JA. The production of JA eventually leads to the induction of many genes, including *Vegetative Storage Protein* (*VSP)* and *Thionin 2.1* (*THI2.1*), used as markers for JA-dependent defense responses (Berger *et al.,* 1995; Epple *et al.,* 1995; Penninckx *et al.,* 1998; Devoto and Turner 2003). Moreover, transcription of genes that regulate JA synthesis, e.g. *DAD1, LOX2, AOS,* and *OPR3*, is induced by JA (Devoto and Turner, 2003). Some defense-related genes, such as *Plant Defensin 1.2* (*PDF1.2*), Hevein like Protein (*HEL*), and *Basic Chitinase* (*CHIB*), are induced cooperatively by JA and ET in *Arabidopsis* (Penninckx *et al.,* 1998). Conserved MYC transcription factors are involved in JA signaling in both *Arabidopsis* and tomato (Lorenzo *et al.,* 2004). *Jasmonate Insensitive 1* (*JIN1*) encodes AtMYC2, a nuclear-localized basic helix-loop-helix-leucine zipper transcription factor whose expression is rapidly upregulated by JA in a COI1–dependent manner (Lorenzo *et al.,* 2004). AtMYC2 seems to

differentially regulate the expression of two groups of JA-induced genes. Mutation in this locus prevents the activation of *VSP*, which is involved in JA-mediated plant responses to insects, herbivores, and mechanical damage. At the same time, the expression of JA-induced genes involved in pathogen defense is enhanced, and accordingly, *jin1/AtMYC2* mutant plants show enhanced resistance to the necrotrophic fungi *B. cinerea* and *Plectosphaerella cucumerina* (Lorenzo *et al.,* 2004).

2.3.2.1 Jasmonic acid in systemic signaling

Plants have evolved to respond with sophisticated mechanisms to attack by herbivores and certain pathogens that rapidly destroy plant tissues. Wounding induces the expression of defensive foliar compounds that have toxic effects on the invader. In addition, plants under attack can also emit volatile substances that act indirectly by attracting predators of the herbivore (Schilmiller and Howe, 2005). Importantly, signaling originating from the initial wound site induces systemic resistance in undamaged leaves located considerable distances away and protects the plant against a broad spectrum of future attackers (Howe, 2004). Wound response has most been studied in tomato and other *Solanaceae* species, where it results in both local and systemic expression of defensive proteinase inhibitors (PIs) that act by blocking digestive proteases in the herbivore gut (Pearce *et al.,* 1991). Many structurally different molecules play regulatory roles in wound signaling and PI induction. These include cell wall-derived oligogalacturonides (OGAs), the oligopeptide systemin, and molecules with hormonal activity such as JA, ET, and ABA (León *et al.,* 2001).

Gaps still exist in understanding the transmission of the systemic wound signal. The early events acting upstream of the octadecanoid pathway that couple tissue damage to the production of a primary wound signal are unknown. Nor is it clear how the wound response is transmitted from local to systemic tissues. JA with its volatile derivative MeJA and the oligopeptide systemin are considered central in mediating the long-distance signal (Bostock, 2005; Schilmiller and Howe, 2005). Recent studies suggest a central role for JAs; using different mutants of tomato, Li *et al.,* (2002) demonstrated that mutations affecting either JA biosynthesis or JA signaling abolish the systemic expression of PI genes. Moreover, the requirement of JA biosynthesis at the site of wounding and the ability to perceive JA at remote tissues was shown in grafting experiments conducted with various tomato mutants (Li *et al.,* 2002, 2005). Possible gene products involved in the transport of JA have not been characterized to date. Alternatively, JA could regulate the production of the actual signal (Li *et al.,* 2005; Schilmiller and Howe, 2005). Wounding induces the production of systemin, which regulates the activation of over defensive genes in response to herbivore and pathogen attack (Pearce *et al.,* 1991; Ryan 2000). This 18-amino acid peptide is derived by proteolytic

cleavage from a larger, 200-aa precursor protein called prosystemin (Ryan and Moura, 2002). Systemin released from the primary wound site promotes *PI* gene expression and contributes to the long distance defense response by activating and amplifying JA production in vascular tissues (Schilmiller and Howe, 2005). Systemin binds to SR160, a cell-surface receptor homologous to brassinolide receptor BRI1 from *Arabidopsis* (Scheer *et al.,* 2002). Interestingly, the existence and function of systemin or a related peptide have thus far been documented only in *Solanaceae* species (Ryan and Moura, 2002).

2.3.3 Ethylene-mediated defense signaling

Ethylene (ET) is a gaseous plant hormone involved in various physiological processes, including seed germination, organ senescence, leaf abscission, fruit ripening, and morphological responses of organs (Bleecker and Kende, 2000). ET also regulates plant responses to abiotic stresses, including those induced by flooding or drought, and to biotic stresses, such as pathogen attack (Penninckx *et al.,* 1998; O'Donnell *et al.,* 2003). The production of ET is one of the earliest plant responses to pathogens. Diverse viral, bacterial, and fungal microbes trigger accumulation of ET, leading to induction of defense genes, such as basic *PR-1*, basic *β-1,3-Glucanase*, and *CHIB*, which can also be induced by ET-independent pathways (Thomma *et al.,* 1998). ET contributes to resistance in some interactions but can promote disease development in others (Thomma *et al.,* 1998, 1999; Hoffman *et al.,* 1999; Norman-Setterblad *et al.,* 2000). *Arabidopsis ethylene-insensitive 2 (ein2)* plants display enhanced susceptibility to *B. cinerea* and *E. carotovora* (Thomma *et al.,* 1999; Norman-Setterblad *et al.,* 2000). On the other hand, infection of *ein2* with virulent *P. syringae* and *X. campestris* resulted in reduced disease symptoms (Bent *et al.,* 1992). Insensitivity to ET has also been shown to reduce foliar disease development in tomato (Lund *et al.,* 1998). The *never ripe* (*Nr*) tomato mutant impaired in ET perception displayed decreased disease symptoms in comparison with the wild-type after inoculations with *Pseudomonas, Xanthomonas*, and *Fusarium* pathogens (Lund *et al.,* 1998). ET-insensitive tobacco was susceptible to the fungus *Pythium sylvaticum*, which normally is not pathogenic to this species (Knoester *et al.,* 1998). The inability of ET response mutant *etr1* (*ethylene-resistant 1)* to develop pathogen resistance in response to nonpathogenic rhizobacteria demonstrated the requirement of ET in the establishment of ISR (Pieterse *et al.,* 1998).

Characterization of ET-response mutants in *Arabidopsis* has identified components of the ET signal transduction pathway. One class of mutations, exemplified by *etr1*, led to the identification of ET receptors (Bleecker, 1999). CTR1, acts directly downstream of the ET receptors, is similar to the mitogen-activated protein kinase kinase kinases (MAPKKKs). This suggests that this signaling pathway might contain a MAP kinase cascade, but providing

evidence supporting this possibility has proven tricky (Ecker, 2004). A role for MPK6 was thought to exist in ET signaling (Ouaked *et al.,* 2003), although recent evidence indicates that it instead functions as a key regulator of stress-responsive ET biosynthesis (Liu and Zhang, 2004). EIN2 is a transmembrane protein required for ET signaling. While the role of this gene remains unclear, genetic studies locate it between CTR1 and EIN3 (Bleecker and Kende, 2000). Some of the mutations affecting ET signal transduction have identified transcription factors such as the ERF1 protein. It is induced by the EIN3/EIL transcription factors, indicating that ethylene signaling involves a transcriptional cascade (Solano *et al.,* 1998). ERF1 belongs to a family of ET response element binding factor (ERF) proteins (also known as ethylene response element binding proteins, EREBPs) that are transcription factors unique to plants (Fujimoto *et al.,* 2000). ERFs bind to a GCC box found in the promoters of several pathogenesis-related genes, including *β-1,3-glucanase*, *CHIB*, and *PDF1.2* (Wang *et al.,* 2002).

2.3.4 Modulation of ABA content and signaling during plant–pathogen interactions

A number of recent publications have described altered ABA levels during the interaction of plants with invading pathogens. Distinct actions of this hormone depend upon the infection stage and the specific host pathogen interaction. At a pre-invasion phase, plants enhanced resistance to application of *P. syringae* pv. tomato DC3000 by inducing stomatal closure and restricting pathogen entry (Melotto *et al.,* 2006). That the ABA-deficient mutant aba3-1 was more susceptible to *P. syringae* pv. tomato DC3000 when it was sprayed-inoculated onto the leaf surface, suggests that ABA biosynthesis is required for stomatal closure in response to this bacterium (Melotto *et al.,* 2006). In contrast to a role in pre-apoplastic resistance, de Torres-Zabala *et al.,* (2007) showed that bacterial effectors delivered into plant cells enhanced susceptibility by increasing ABA production and activating of ABA-responsive genes. In these studies ABA enhanced bacterial growth by attenuating basal defense, and ABA induction and suppression of basal defense transcripts could be mimicked by the in planta expression of the bacterial effector avrPtoB (de Torres-Zabala *et al.,* 2007). ABA may thus have different actions at different infection steps, favoring resistance during pre-invasion and susceptibility at later stages of colonization. Increased ABA production and activation of ABA-responsive genes has also been measured in plants responding to necrotrophs. Again, opposing effects of ABA on resistance have been reported. ABA antagonizes resistance to *F. oxysporum* (Anderson *et al.,* 2004), *B. cinerea* (Abuqamar *et al.,* 2006) and *Plectosphaerella cucumerina* (Hernandez-Blanco *et al.,* 2007). Exogenous application of ABA reduced JA-activated or ET-activated transcription and pathogen resistance, whereas expression of JA responsive genes and defense were enhanced in ABA

deficient mutants. Moreover, a negative role of ABA on resistance to *B. cinerea* was supported by results showing that production of abscisic acid by *B. cinerea* itself may be involved in pathogenesis (Siewers *et al.,* 2004). In contrast to an antagonistic effect, ABA has been recently shown to act as a positive regulator of plant defense against the necrotrophic pathogens *Phytium irregular* and *Alternaria brassicicola* (Ton *et al.,* 2008). Transcriptome analyses showed the contribution of ABA as an important regulator of plant defense against the oomycete necrotrophic pathogen *P. irregulare*, resistance to which is primarily through the JA-dependent pathway. In these studies ABA levels and expression of ABA-responsive genes increased after infection, and ABA-deficient or ABA-insensitive mutants were more susceptibility to *P. irregulare* and *A. brassicicola.* Interestingly, analysis with the aba2-12 biosynthetic mutant indicated that ABA is required for JA accumulation and JA-dependent defense gene activation after *P. irregulare* infection, suggesting that ABA preceded JA action and activated defense by inducing JA biosynthesis. Corroborating a positive role of ABA in plant defense against *A. brassicicola*, Ton *et al.,* (2008) showed that treatment with ABA enhanced resistance to this pathogen and that infection with an aggressive strain of *A. brassicicola* downregulated ABA accumulation to enhance pathogenicity. Accordingly, the aba1 allelic mutant npq2 was more susceptible to *A. brassicicola* compared to wild type plants (Ton *et al.,* 2008). The action of ABA in inducing resistance is in part exerted through priming the deposition of callose (Ton *et al.,* 2008) a negative regulator of the SA-defense pathway that facilitates the activation of the JA-dependent defense pathway (Nishimura *et al.,* 2003). ABA also contributes to plant resistance by inducing expression of specific defense genes as in *Arabidopsis– Pythium irregulare* interaction (Adie *et al.,* 2007). Similarly, resistance to the vascular wilting inducing bacterium *R. solanacearum*, by mutation in CESA4/IRX5, CESA7/IRX3 or CESA8/IRX1 genes, which alter secondary cell wall formation, was linked to activation of specific ABA responsive genes (Hernandez-Blanco *et al.,* 2007). Disruption of the secondary cell wall in these mutants causes structural and functional alterations in xylem vessels that affected water balance and increased ABA synthesis (Turner and Somerville, 1997). Enhanced susceptibility of ABA mutants, abi1-1, abi2-1 and aba1-6, to *R. solanacearum* supports a direct role of ABA in resistance to this pathogen (Hernandez-Blanco *et al.,* 2007). As with mechanical wounding and structural cell wall alteration (Huckelhoven, 2007) the cellular damage provoked during infection by necrotrophs might generate a water stress, in which the production of ABA is a prominent mechanism. Necrotrophic damage can thus lead to the simultaneous activation of several interacting signaling pathways that aim to control these biotic and abiotic stresses. It is possible that as infection by distinct necrotrophs might damage the cell wall differentially; the activation of defenses and their interaction with other stress signaling pathways might differ depending upon pathogen infection strategies and their endogenous suite of cell wall

degrading enzymes. This may partly explain the seemingly divergent actions of ABA in plant–necrotroph interactions.

2.3.5 Role of nitric oxide in defense signaling

Nitric oxide (NO) was first identified as an important messenger in animal cells (Mayer and Hemmens, 1997). However, it is becoming increasingly clear that it has diverse signaling functions in plants as well (Wendehenne *et al.,* 2004; Mur *et al.,* 2006). Besides developmental regulation and promotion of germination, NO is an important mediator in plant defense signaling (Wendehenne *et al.,* 2004; Delledonne, 2005). In animals, the NO burst is a hallmark of innate immunity response, and also in *Arabidopsis* recognition of bacterial LPS induces a rapid burst of NO (Zeidler *et al.,* 2004). LPS from animal and plant pathogens were shown to induce NO synthase *AtNOS1* as well as activate several defense genes (Zeidler *et al.,* 2004). Zeidler *et al.,* (2004) also demonstrated the essential role of NO in basal resistance; *AtNOS1* mutants were more susceptible to virulent *P. syringae* pv. *tomato* than wild-type plants. Besides contributing to the local and systemic induction of defense genes, NO can also trigger cell death, and thus, it has been suggested to play an important role as an intercellular signal contributing to spread of HR (Zeidler *et al.,* 2004).

2.3.6 Role of reactive oxygen species in defense signaling

In plants, normal, unstressed photosynthetic and respiratory metabolism taking place in chloroplasts and mitochondria results in endogenous generation of such ROS as superoxide radical (O2-.), hydroxyl radical (OH-), and hydrogen peroxide (H_2O_2) (Grene, 2002). ROS is also generated by cytoplasmic, membrane-bound, or exocellular enzymes involved in redox reactions (Wojtaszek, 1997). To avoid potential damage, plant cells contain several enzymatic and non-enzymatic antioxidant scavenging systems that take care of ROS detoxification. These include ascorbate peroxidases (APXs), superoxide dismutases (SODs) and catalases (CATs) as well as such antioxidants as ascorbic acid and glutathione (Noctor and Foyer, 1998; Mittler, 2002). Under unstressed conditions, the formation and scavenging of ROS are in balance. However, several forms of biotic and abiotic stress, such as pathogen invasion, excess light energy, dehydration, and low temperature, increase the generation of ROS. This can result in cellular damage, manifested in inactivation of enzymes or cell death, if the amount of ROS generated exceeds the capacity of the scavenging systems (Foyer, 1994; Grene, 2002).

Although potentially damaging, ROS has been shown to promote plant resistance to pathogens in several ways. During defense responses, ROS is produced by plasma membrane-

bound NADPH oxidases and cell wall-bound peroxidases and amine oxidases in the apoplast (Mahalingam and Fedoroff, 2003). One of the earliest pathogen-induced defense responses is the oxidative burst, a rapid and transient production of large amounts of ROS at the site of attempted invasion (Wojtaszek, 1997). A likely source for this apoplastic superoxide generation is a NADPH oxidase homologous to that of activated mammalian phagocytes and neutrophils (gp91phox) (Overmyer *et al.,* 2003). *AtRBOHD* and *AtRBOHF* genes encoding NADPH oxidase in *Arabidopsis* are required for full ROS generation during bacterial and fungal challenge (Torres *et al.,* 2002). Hydrogen peroxide is also produced in vitro by some peroxidase isoforms at an alkaline pH. Since the apoplast is alkaline following pathogen recognition, peroxidases have been suggested to contribute to the oxidative burst (Wojtaszek, 1997; Grene, 2002). The accumulation of extracellular hydrogen peroxide induced by pathogen challenge has been proposed to crosslink the cell wall proteins, thus strengthening the wall (Neill *et al.,* 2001). The oxidative burst can be directly harmful to invading pathogens but it also contributes to cell death: ROS generated via the oxidative burst play a central role in the development of host cell death during the HR reaction (Lamb and Dixon, 1997). Importantly, ROS is thought to have potential for being a signal in plant defense responses (Neill *et al.,* 2001). Hydrogen peroxide is a relatively stable form of ROS and has the ability to diffuse across membranes and reach locations far from the site of its original generation (Wojtaszek, 1997). Increased ROS generation enhances the accumulation of SA as well as the transcripts of *PR* genes (Maleck and Dietrich 1999). Furthermore, SA has been shown to have inhibitory effects on CAT and APX activities, which may lead to accumulation of hydrogen peroxide, free radicals, and other ROS (Chen *et al.,* 1993; Durner and Klessig, 1995). SA has also been suggested to potentiate the production of NADPH oxidase dependent O_2^- via a positive feedback loop (Van Camp *et al.,* 1998).

Photo-produced hydrogen peroxide and other ROS in the cell also participate in controlling biotic and abiotic stress responses (Karpinski *et al.,* 2003), and recently, mechanisms for plant defense against pathogens were linked to the light-sensing network. For example, induction of *PR1* by SA and its functional analogs was found to correlate strictly with the activity of the signaling pathway controlled by PHYA and PHYB photoreceptors (Genoud *et al.,* 2002). Moreover, the growth of avirulent *P. syringae* pv. *tomato* was enhanced in *Arabidopsis phyA* and *phyB* mutants (Genoud *et al.,* 2002). Plant responses to pathogens seem to share common elements with responses to excess light (Karpinski *et al.,* 2003). A rapid increase in ROS concentration, depletion of antioxidant pools, chlorosis and necrosis of leaves, local and systemic defense responses, and induction of defense gene expression are markers of both responses (Karpinski *et al.,* 2003). However, while the ROS burst during pathogen infection is considered to originate mainly from cytoplasmic NADPH

oxidase, during excess light stress ROS is produced in the chloroplast and peroxisome (Karpinski *et al.,* 2003). High light also induces the accumulation of SA, a central hormone in pathogen defense; Karpinski and coworkers (2003) demonstrated that high-light-acclimated plants had several-fold greater foliar SA than plants cultivated in low light.

2.4 Crosstalk between Signaling Pathways

Plants respond to a variety of abiotic and biotic stimuli from the environment. Following perception of stress, several signal transduction pathways are switched on, resulting in physiological and molecular changes in the plant. When pathways operating in defense signaling are investigated, they are sometimes considered as independent units in order to simplify the interpretation. However, it would be naive to think that signal transduction is mediated through isolated, linear pathways. Defense pathways influence each other through a network of regulatory interactions, and thus, plant responses to various stress stimuli are a result of this complex interplay (Kunkel and Brooks, 2002; Bostock, 2005) (Fig. 2.2).

The term crosstalk is often used when discussing interactions in defense signaling. However, a good definition of what constitutes crosstalk does not exist, and differing opinions have been introduced concerning when it is appropriate to use this term to describe plant defense signaling (Mundy *et al.,* 2006). Uncertainty results partly because not all the components operating in the defense pathways are known. Nevertheless, crosstalk is usually described as including a network of signal interactions in which functional outcomes can be positive, negative, or neutral (Bostock, 2005). In addition to different biotic stress signaling pathways, also biotic and abiotic pathways can "crosstalk". This is exemplified by the effect of ABA on pathogen defense (Mauch-Mani and Mauch, 2005) discussed earlier. In conclusion, in most cases, ABA seems to have a negative effect on SA or JA signaling, impeding pathogen defense. However, on some occasions, ABA can have a positive impact on pathogen resistance; it enhances the accumulation of callose, and thus, increases resistance to certain necrotrophic pathogens (Ton and Mauch-Mani, 2004).

Several studies have described crosstalk among SA, JA, and ET signaling pathways (Kunkel and Brooks, 2003; Bostock, 2005). SA and JA signaling interact on many levels, and in most cases, this relationship seems to be mutually antagonistic (Kunkel and Brooks, 2002). SA can inhibit the synthesis of JA and prevent the accumulation of PIs in response to JA, wounding, systemin, and oligosaccharides (Doares *et al.,* 1995). SA and its functional analogs have also been shown to prevent the expression of JA-dependent defense genes on several occasions (Kunkel and Brooks, 2002). Moreover, Petersen *et al.,* (2000) demonstrated that MAP Kinase 4 (MPK4) regulates negative crosstalk between JA and SA in the activation of

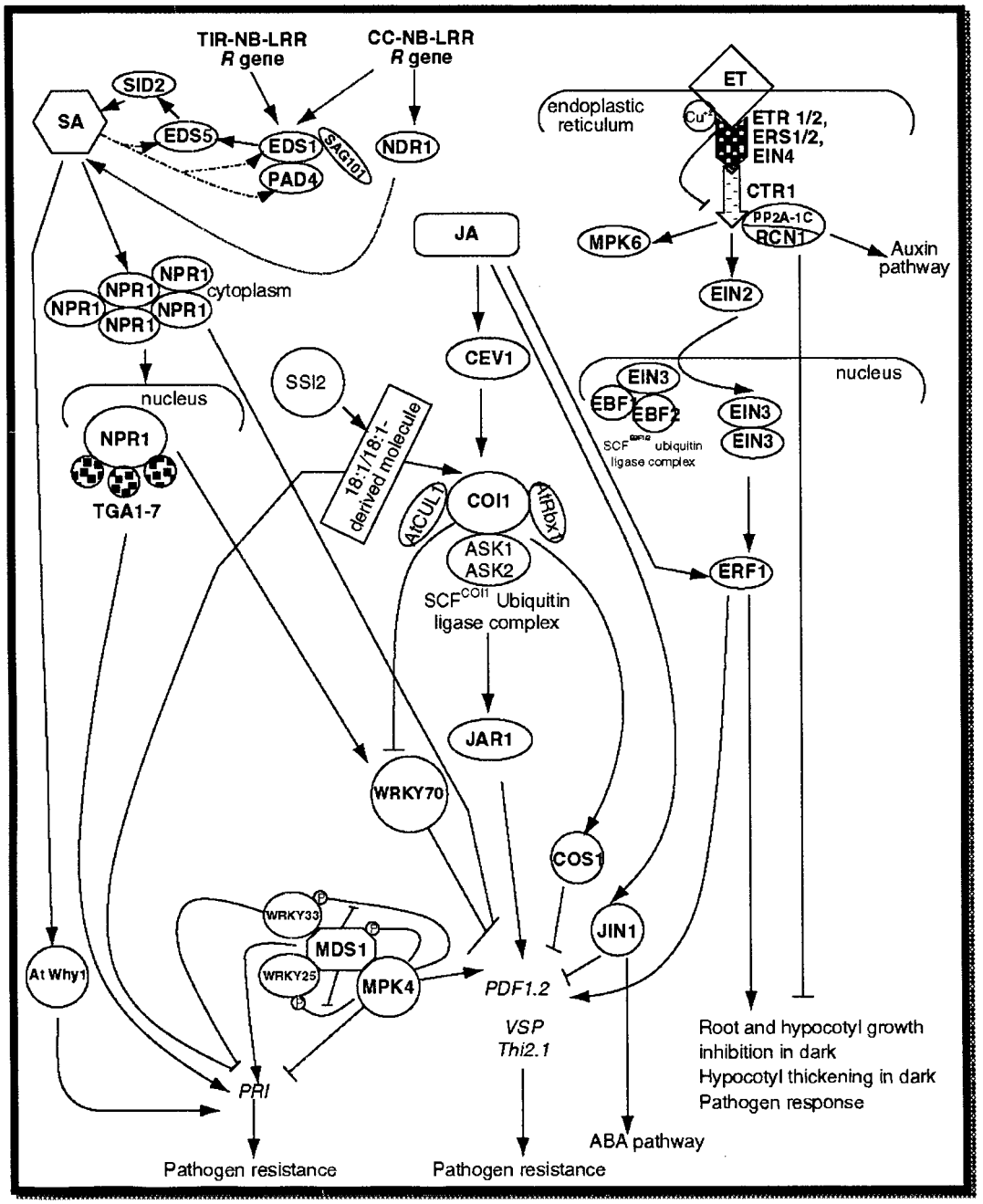

Figure 2.2 The figure describes the major components of the SA-, JA- and ET-mediated plant defense pathways. The defense signaling is initiated upon interaction between R and AVR proteins. Arrows indicate activation, while bars indicate repression of downstream events. Phosphorylation is indicated by an encircled P and copper ion is indicated as an encircled Cu^{+2}. Components known to interact physically are shown in contact with each other. Dotted line from NDR1 (nitrous oxide) to SA indicates partial enhancement of SA levels *via NDR1. Dotted line from CC-NB-LRR type R gene to* EDS1 indicates uncommon signaling event. Dash and dotted line indicates feedback regulation.

defenses. Gene induction triggered by JA is blocked in *mpk4* mutants, indicating the importance of this gene for mediation of the JA signal. Simultaneously, this mutant constitutively expresses SA-regulated defense genes, probably as a result of the elevated SA levels. This suggests that a MAP kinase cascade involving MPK4 represses SA biosynthesis and promotes either JA perception or response (Petersen *et al.,* 2000). A node of convergence between SA and JA signaling seems to be the plant-specific transcription factor WRKY70 (Li *et al.,* 2004). Plants overexpressing *WRKY70* showed decreased JA- but enhanced SA-dependent defense activation, hence improving resistance to *E. carotovora* and *P. syringae* (Li *et al.,* 2004). This indicates that WRKY70 integrates defense signals, and thus, affects pathway activation (Li *et al.,* 2004).

Some evidence also supports synergism between SA and JA defenses. Simultaneous activation of both SAR and rhizobacteria-triggered ISR resulted in an additive effect on induced protection against *P. syringae* (Van Wees *et al.,* 2000). Moreover, ROS has been shown to stimulate accumulation of SA and induction of SAR. At the same time, SA induces the production of ROS such as hydrogen peroxide and NO (Van Camp *et al.,* 1998). This synergism is thought to promote such defense responses as HR and killing of the pathogen.

Reported crosstalk between JA and ET signaling is mostly positive. Transcription factors AtMYC2/JIN1 and ERF1 are important regulators of these interactions in *Arabidopsis* (Lorenzo *et al.,* 2004). The expression of ERF1 (and its target genes) is synergistically activated by ET and JA, and ERF1 integrates these signals into the activation of plant defenses (Lorenzo *et al.,* 2003). ET seems to mediate the interaction between MAPK and CDPK, both of which are triggered by abiotic and biotic stress responses in *Arabidopsis*. Ludwig *et al.,* (2005) demonstrated that CDPK signaling triggers high ET levels, leading to inhibition of stimulus-dependent MAPK activation. In rare cases, JA and ET have the opposite effects; in tobacco nicotine biosynthesis, a direct defense against some herbivores is stimulated by JA and inhibited by ET (Shoji *et al.,* 2000). The above-mentioned examples, especially the interaction between SA and JA signaling, demonstrate how plants can fine-tune their defense responses to different pathogens through crosstalk.

2.5 Role of other hormones during Plant-Pathogen Interaction

Plant hormones are normally associated with growth regulation and meristem activation or repression. However, hormones have a broad effect on plant physiology even in differentiated tissues. Often, when a plant encounters a stress, a pause in growth is observed. Therefore it is not surprising that mutants affected in pathways related to development displaying an altered pathogen response. SA, JA and ET are well known to play crucial roles in plant disease and pest resistance. However, the roles of other hormones such as abscisic

acid (ABA), auxin, gibberellin (GA), cytokinin (CK) and brassinosteroid (BL) in plant defence are less well known. Upon microbial attack, plants modify the relative abundance of these hormones, and the expression of their responsive genes, as an instrument to activate an efficient defense response allowing plant survival. Importantly, pathogens can counteract this strategy by interfering with these plant hormonal changes and also by producing plant hormones themselves as a component of their invading strategy. Much progress has been made in understanding plant hormone signaling and plant disease resistance. However, these studies have mostly proceeded independently of each other, and there is limited knowledge regarding interactions between plant hormone-mediated signalling and responses to various pathogens. Important growth and developmental processes are executed through signaling pathways governed by hormones such as BL, auxins, CK and GA. In addition, there is increasing evidence that regulation of these signaling pathways helps determine the outcome of a plant– pathogen interaction (Fig. 2.3).

2.5 Changes in growth promoting hormones in plants during pathogen infection

2.5.1 Auxins

Auxin is an important plant hormone that affects almost all aspects of plant growth and development. Perturbing auxin homeostasis appears to be a common virulence mechanism, as many pathogens can synthesize auxin-like molecules. Loss of the ability to synthesize auxin-like molecules rendered these pathogens less virulent (Robert-Seilaniantz *et al.*, 2007). Pathogens may also directly impact auxin biosynthesis of the host. Overexpression of the *P. syringae* effector protein AvrRpt2 in plants resulted in morphological phenotypes that are usually associated with modified auxin homeostasis (Chen *et al.*, 2007). Indeed, AvrRpt2 overexpression promoted the biosynthesis of auxin and activated auxin-responsive gene expression. Furthermore, exogenous application of synthetic auxin to plants impaired in auxin signaling exhibited enhanced resistance (Chen *et al.*, 2007; Navorro *et al.*, 2006). These data strongly indicate that auxin is involved in promoting pathogenesis. Auxin, a growth-promoting hormone would be beneficial to biotrophic pathogens that feed on living cells. Perhaps the best example is *Agrobacterium tumefaciens*, which uses auxin and other hormones to induce cell growth and division. This leads to the formation of galls that are "feeding factories", providing the bacterium with a carbon and nitrogen source. An alternative mechanism by which auxin promotes virulence may be by suppressing host defense. Treatment of plants with synthetic auxin was recently demonstrated to repress SA-induced defense gene expression. Therefore, auxin may also promote biotroph invasion by suppression of SA-mediated defenses.

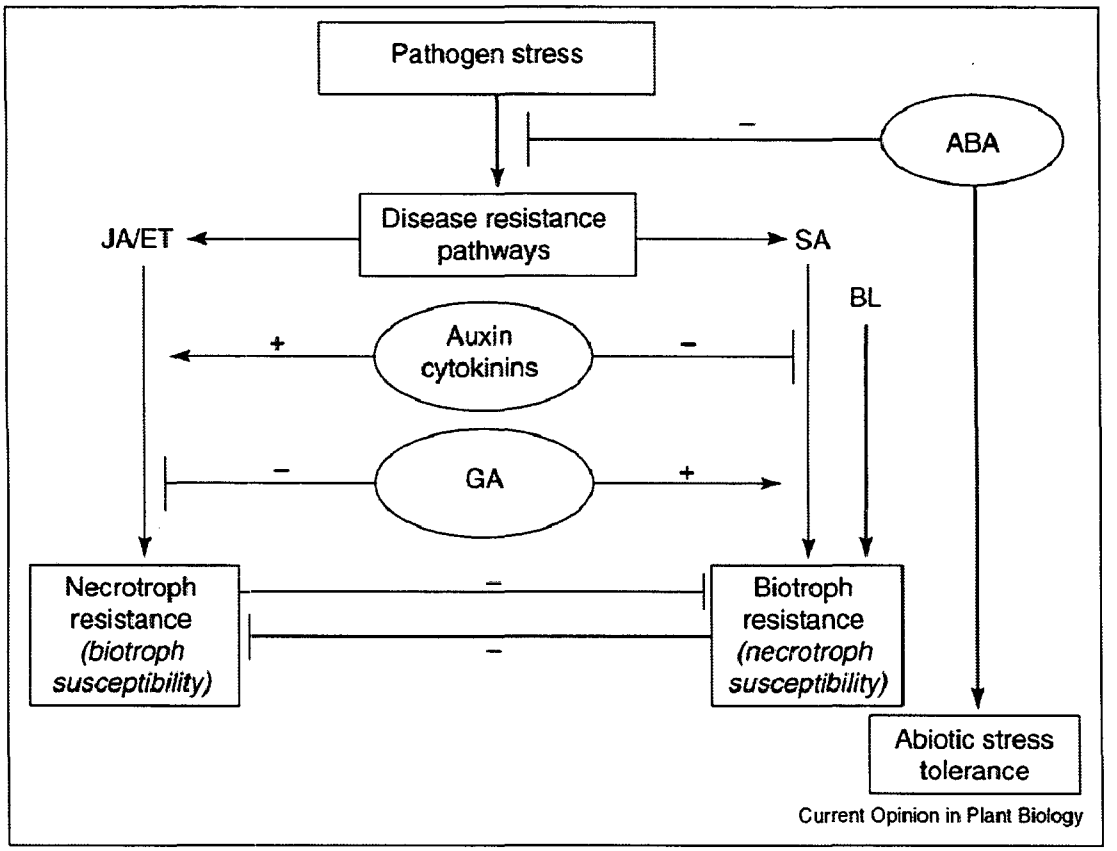

Figure 2.3. Model indicating probable interactions between hormone-signalling pathways during plant–pathogen interactions. (ABA: abscisic acid; JA: jasmonic acid; ET: ethylene; SA: salicylic acid; GA: gibberellic acid; BL: brassinolides.)

2.5.2 Brassinosteroids (BLs)

BLs are essential hormones for plant growth and development. Genetic and molecular analyses have defined key components of the BL signaling pathway, including a cell surface leucine repeat-like kinase receptor BRI1, and a receptor kinase protein (BAK1) that associates with BRI1 to transduce the BL signal across the plasma membrane. In addition to controlling plant development, three independent studies have demonstrated a BL-independent role of BAK1 as a regulator of disease resistance (Kemmerling *et al.,* 2007; Chinchilla *et al.,* 2007). BAK1 was required for controlling cell death, production of ROS and restriction of biotrophic and necrotrophic infections (Kemmerling *et al.,* 2007). He *et al.,* (2007) demonstrated the role of BAK1 in controlling cell death during normal growth. Remarkably, BAK1 interacts with FLS2, a well-characterized pattern recognition receptor, inducing basal resistance upon interaction with the bacterial MAMP flagellin (Chinchilla *et al.,* 2007). Plants carrying bak1 mutations show normal flagellin binding but reduced activation of flagellin-triggered immune responses. Moreover, responses to other MAMPs, such as INF1, CSP22, and EF-tu, which are not recognized by FLS2, were also BAK1 interacts with other pathogen-recognition receptors to activate basal defense. That BAK1 interacts with different receptors to regulate basal defense, cell death and plant growth, demonstrates its role as a key cellular component for the activation of essential plant processes. Moreover, as gene expression profiles following application of either BLs or the active flagellin peptide flg22 showed no apparent overlap (Zipfel *et al.,* 2004), BAK1 probably functions as an adaptor protein in multiple signaling pathways. BAK1 function represents a fascinating example of crosstalk between defense and hormonal pathways regulating plant development.

2.5.3 Gibberllins and Cytokinins

The role of cytokinin in plant defence displays some similarities to that of auxin. Both compounds are produced by biotrophic pathogens. Classically, production of 'green islands' on rust-infected cereals is believed to be associated with retardation of senescence by cytokinins (Angra-Sharma, R and Sharma D K, 1999; Walters and McRoberts, 2006). Further, both compounds are associated with the suppression of the HR (Murphy *et al.,* 1997). Interestingly, in some examples cytokinins has also been reported to induce cell death (Carimi *et al.,* 2003). Similar to what has been observed for auxin, the plant response to the hormone is dependent on the dose (Blatt and Thiel, 1994).

GA seems to have an opposite effect on plant defence. GA promotes plant growth by including the degradation of the DELLA proteins, which are negative regulators of plant growth (Harberd, 2003). Recent studies demonstrated that loss-of-function mutations in

DELLA proteins render the plant more resistant to *Pst*DC3000 through potentiating the SA-dependent defense pathway (Robert-Seilaniantz, 2007). By contrast, the same set of mutants is hyper-susceptible to the necrotrophic pathogen *A. brassicicola*. This suggests that DELLAs promote resistance to necrotrophs and susceptibility to biotrophs, partly by modulating the balance between SA-mediated and JA/ET-mediated defense signaling pathways.

Knowledge of the role of GA and CK in plant–pathogen interactions is limited. Results showing that some pathogens produce these phytohormones as part of their invading strategies (Walters and McRoberts, 2000) indicate that, like for other growth hormones, the GAs and CKs signaling pathways are potential pathogenicity targets. The outer capsid protein P2 of the Rice dwarf virus interacts with ent-kaurene oxidase, an enzyme with a key role in plant gibberellin biosynthesis. The expression of ent-kaurene oxidase and the level of endogenous GA1 were lower during infection and rice plants had a dwarf phenotype, which was restored by exogenous application of GAs (Zhu *et al.*, 2005). CKs were also implicated in the infection of the Brassicae family with *Plasmodiophora brassicae*, a biotrophic pathogen causing an aberrant root phenotype. In addition to producing CKs, this pathogen downregulated the degradation of plant cytokinins and induced the expression of CK receptors (Siemens *et al.*, 2006).

Chapter 3: Materials and Methods

3.1 Materials

3.1.1 Plant materials

Cicer arietinum: Pusa 362 seeds were procured from Dr. N.S.Yadav, Dept. of Genetics, IARI, New Delhi, India.
FLIP84-92C(2) and PI 359075(1) seeds were gifted by Fred J. Muehlbauer, Washington State University, USA.

Nicotiana tabacum Xanthi: Wild type plants were available in the laboratory

3.1.2 Fungal material

Ascochyta rabiei isolates; *Pythium* spp.; and *Alternaria alternata* were gifted by Dr. K.D. Srivastava and Dr. Birendra Singh and, Department of Plant Pathology, IARI, New Delhi.

3.1.3 Insect

Different larval stages of *Helicoverpa armigera* were procured from Department of Zoology, Delhi University, Delhi.

3.1.4 Bacterial strains used

Strain	Genotype
Escherichia coli DH5α	Φ8dlacZ Δ M15, recA1, endA1, gyr A96, thi-1, hsd17 supE44, relA1,deoR, (LacZYA-argF)U19
Agrobacterium tumefaciens (GV3101)	carry pMP90 Ti-plasmid with gentamicin selection and rifampicin chromosomal selection
Sacharomyces cerevisae (AH109)	*MATa, trp-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1$_{UAS}$-GAL1$_{TATA}$-HIS3, GAL2$_{UAS}$-GAL2$_{TATA}$-ADE2, URA3:: MEL1$_{UAS}$-MEL1$_{TATA}$-lacZ, MEL1*

3.1.5 Plasmid vectors used

Strain	Source	Purpose
pDrive U/A vector	Qiagen	PCR product cloning.
Advantage™ Vector	Clontech	PCR product cloning.
pGEM-TEasy vector	Promega	PCR product cloning.
pBI101.2 vector	Clontech	Binary vector with GUS for promoter activity studies.
pBI121 vector	Clontech	Binary vector with GUS for overexpression studies.
pCAMBIA1303	CSIRO	Binary vector with GUS and GFP for subcellular localization
pGBKT$_7$	Clontech	Yeast two hybrid
pGADT$_7$	Clontech	Yeast two hybrid

3.1.6 Chemicals and Materials used

Type	Material	Source
Molecular weight Markers	λ-*Hin*dIII Digested DNA ladder	TaKaRa
	100 bp DNA ladder	TaKaRa
	1 Kb DNA ladder	Fermentas
	Prestained protein ladder	Fermentas
X-ray film	Hyperfilm^{TM} MP	Amersham , Kodak
Nylon Membrane	Hybond N^+	Amersham
Antibiotics	Ampicillin, Kanamycin, Cefatoxime, Rifampicin	Sigma
Radioisotopes	α^{32}P dCTP, [γP^{32}]ATP	Amersham, BARC, Perkin Elmer
Disposable filters	PVDF 0.45 μm filter unit	Millipore
Enzymes	Commonly used restriction enzymes	NEB
	Taq DNA Polymerase	Clontech, Bangalore Genei
	T4 DNA Ligase	Fermentas, NEB
	RNase	BioBasic, Amersham
Dyes	Ethidium Bromide, Xylene cyanol Methylene Blue, Coomasie Brilliant Blue	Amersham
Culture media components	Tryptone, Yeast Extract, Agar, MS salts, BAP, NAA, PDA	Difco, Pronadisa, Himedia
Locally available chemicals	Isopropanol, iso-amyl alcohol, $CaCl_2$, NaCl, NaOH, Glucose, Methanol, $MgCl_2$, KOH, Potassium acetate, Chloroform, Glycerol, Acetic acid, NaH_2PO_4, Na_2HPO_4, $MgSO_4$, HCl, H_2SO_4, Glycine, KCl, Sucrose, Pot. Dichromate, Sodium hypochlorite, Mercuric chloride, tri-Sodium citrate, Formaldehyde.	Qualigens, HiMedia and Merck
Foreign chemicals	RNaseZap, DEPC, HEPES, IPTG, MOPS, Sephadex G-50, EDTA, CTAB, Acrylamide, Bis-Acrylamide, TEMED, Triton-X-100, X-gal, X-gluc, MUG, 4-MU	Amersham, Sigma, Ambion, BBI

Table 3.1.7 : Oligonucleotides used in the present study

M13For	5′ CGCCAGGGTTTTCCCAGTCACGAC 3′	Sequencing and colony PCR
M13Rev	5′ AGCGGATAACAATTTCACACAGGA 3′	Sequencing and colony PCR
T7Pro	5' CTAATACGACTCACTATAGGG 3′	Colony PCR
SP6Pro	5′ CATTTAGGTGACACTATAGAAT 3′	Colony PCR
AAP	5′ GCCAGGCGTCGACTAGTACGGGIIGGGIIGGGIIG 3′	5′ RACE
AUAP5' RACE	5′ GGCCAGGCGTCGACTAGTAC 3′	5′ RACE
GSP1	5'CGGATCCAACGAGTCACCGCCATCAC 3'	5′ RACE
GSP2	5'CCATACCCACCGTCGACACCTGC	5'RACE
CDS III/3′ PCR Primer	5' ATTCTAGAGGCCGAGGCGGCCGACATG $d(T)_{30}N_{-1}N$ 3′ (N=A,G,C, or T; N_{-1}=A,G, OR C)	Full-length cDNA
GUS Seq	5′ TCACGGGTTGGGGTTTCTA 3′	For Sequencing
NOS Ter	5′ CACACAGGAAACAGCTATGACC 3′	For sequencing
AP1	5′ GTAATACGACTCACTATAGGGC 3 ′	Genome walking kit
AP2	5′ ACTATAGGGCACGCGTGGT 3′	Genome walking kit
Y131F	5' GGCCATGGTTGATTTACATTGG 3'	Yeast two hybrid Cloning
Y131R	5' TTCTGCAGCACCCATTTATTATCATTATCC 3'	Yeast two hybrid Cloning
FOEG131	5'CATGCCATGGCATGCATGGTTGATTTACATTGG3'	Cloning in pCAMBIA1303
ROEG131	5'GAAGATCTTCCAAGAAACGTCTTCGCAGCATCC3'	Cloning in pCAMBIA1303
FPBIM131	5'GGGGTACCCCACCAAATGGTTGATTTACATTGG3'	Cloning in pBI121M
RPBIM131	5' GCGTCGACGTCAGAAACGTCTTCGCAGCATC 3'	Cloning in pBI121M
GW1	5' CAGAAGCTACAGCGAAACTAG 3'	Promoter isolation
5131Δ1	5' GCGTCGACTTTGAGTTTGTCAA 3'	Promoter analysis
5131Δ2	5' GCGTCGACTATGAATAAACTT 3'	Promoter analysis
5131Δ3	5' GCGTCGACCCGGGCTGGTAAAAG 3'	Promoter analysis
5131Δ4	5'GCGTCGACTGTGCCGCTCCACC 3'	Promoter analysis
5131Δ5	5' GCGTCGACTCGTTCTCTTCATGC 3'	Promoter analysis
5131ΔR	5' GCAGATCTAAGTAGTAAGATGAAAAG 3'	Promoter analysis

AAP Abridged Anchor Primer

AUAP Abridged Universal Amplification Primer

AP Adaptor Primer

GSP Gene Specific Primer

Methods

3.2.1General sterilization procedures used

All the glassware, tissue culture tools and culture media were sterilized by autoclaving at 121.6°C under 15 lb psi pressures for 15 minutes. The antibiotics and other heat labile components were filter sterilized with dispensable syringe driven PVDF filter unit of 0.22μm and 0.45μm pore size (Millex™, Millipore, USA).

3.2.2 Nutrient media

Composition of LB and YEB medium

LB medium	10 g/l Tryptone 5 g/l Yeast Extract 10 g/l NaCl Adjust pH to 7.0 with NaOH
YEB	10 g/l Beef Extract 2 g/l Yeast Extract 5 g/l Peptone 5 g/l Sucrose 2 mM $MgSO_4$ Adjust pH to 7.0 with NaOH

Composition of Yeast Media

YPD-Medium + adenine	20 g/l Tryptone / Peptone 10-20 g Agar (for plates only) 10 g/l Select yeast extract 20 g/l Glucose 20 mg/l Adenine Adjust pH to 5.8 with HCl
SD-Medium	6.7 g Yeast nitrogen base without amino acids 10-20 g Agar (for plates only) 850 ml H_2O Adjust pH to 5.8 with KOH, Autoclave 100 ml of 10x Drop-in Solution 5-30 mM 3-Amino-1,2,4 Triazole (3-AT) was added when necessary

Composition of MS medium (Murashige and Skoog, 1962)

Potassium nitrate	1900 mg/l
Ammonium nitrate	1650 mg/l
Calcium chloride.$2H_2O$	440 mg/l
Potassium dihydrogen phosphate	170 mg/l
Manganese sulphate. H_2O	16.89 mg/l
Boric acid	6.20 mg/l
Potassium iodide	0.83 mg/l
Sodium molybdate anhydrous	0.21 mg/l
Zinc sulphate. $7H_2O$	8.60 mg/l
Copper sulphate. $5H_2O$	0.025 mg/l
Cobalt chloride. $6H_2O$	0.025 mg/l
Ferrous sulphate.$7H_2O$	27.80 mg/l
EDTA disodium salt.$2H_2O$	37.30 mg/l
Myo-inositol	100.00 mg/l
Thiamine hydrochloride	10.00 mg/l
Pyridoxine hydrochloride	1.00 mg/l
Nicotinic acid	1.00 mg/l
Sucrose	3%

Adjusted pH to 5.8 with 2M NaOH; 0.8% Difco Bacto Agar

3.2.3 Plant growth conditions, maintenance and fungal/chemical treatment/Insect infestation/Mechanical wounding procedures

3.2.3.1 Plant growth conditions

All the Chickpea (*Cicer arietinum* L.) varieties used were grown under similar conditions. Seeds were soaked overnight in tap water and sown in soil (3-4 seeds/ pot) in green house with 16/8 h light/dark cycle at 22-25°C, 50-60% relative humidity (RH) and watered regularly during cultivation.

3.2.3.2 Fungal growth conditions

Ascochyta rabiei isolates were routinely grown on sterilized potato dextrose agar (PDA) media (supplemented with crushed chickpea seed) slants in culture tubes and plates at

room temperature and 12 hours photoperiod. The strains were routinely sub-cultured for their maintenance. The fungus is passed through the plant in order to maintain its virulence where upon the plants were infected with the fungus and once the disease symptoms become visible, the infected samples were inoculated on PDA to facilitate fungus growth. The culture is subsequently sub-cultured before using it for fresh infection.

3.2.3.3 Fungal inoculum preparation and inoculation

For spore collection, PDA tubes with fungus grown on the media were filled with sterile tap water and left for 10 min. The surface was rubbed with a sterile loop to suspend the spores in water. The suspension was filtered through muslin cloth. The concentration of spores was determined using haemocytometer and dilutions were made in sterilized tap water to obtain $0.5x10^5$ spores/ ml. Inoculum was sprayed on 3 weeks old chickpea plants until the leaves were completely covered with the suspension. To maintain high humidity conditions, pots were covered with a transparent plastic sheet. The control plants were sprayed with sterile tap water and grown under similar conditions. Following inoculation, samples were harvested after required time intervals, immediately frozen in liquid nitrogen and stored at -80°C. The control samples were also harvested. To rule out any kind of discrepancy on account of variation in infection the samples were randomly collected in triplicates and mixed. RNA/protein were later isolated from randomly mixed samples.

3.2.3.4 Treatment of signalling molecules and wounding

Aerial parts of 3-weeks old chickpea (Pusa 362) plants, grown in pots were dipped for 30 seconds in the solution of required chemical. The concentrations used were 100µM jasmonic acid (JA), 5mM salicylic acid (SA), 100µM Hydrogen peroxide (H_2O_2), 100µM abscisic acid (ABA). The control plants were dipped in sterile MQ water. The wounding treatment was done by cutting half leaf with scissors from upper and lower part of the plant/ with the help of forceps. The samples were collected after appropriate time intervals.

3.2.3.5 Insect Infestation and Mechanical damage

Larvae of *Helicoverpa armigera* were reared in the laboratory at 25°C and 65-70% relative humidity (RH) on a 14/10h light/dark cycle. The larvae were fed on an artificial diet as described by Armes *et al.*, 1992. The freshly molted fifth-instar larvae were starved overnight before releasing them on the plants. Insect infestation was achieved by the release of fifth-instar *Helicoverpa armigera* larvae on 4 week old chickpea plants (one larva per plant) and allowed to feed for 3-4 h at 25 ± 2°C until ~15-20% of the leaf area was

consumed. Larvae were then removed, and the entire shoot was harvested and stored at -80°C after quick freezing in liquid nitrogen. To mimic insect infestation, leaves were wounded with a punch machine (hole diameter = 4.5 mm) until ~15-20% of leaf area was removed, maintaining time span (3-4 h; continuous wounding with intervals of 1h) and physical conditions (at 25 ± 2°C; 65-70% RH) similar to those of insect feeding. Plants were subsequently harvested.

3.2.3.6 Stay/dispersal experiment

Chickpea plants were subjected to MeJA, SA and ET treatments and wounded mechanically as described previously. For elicitation by insects, plants were infested with newly molted fifth-instar larvae for 3 h until ~15-20% tissue was consumed. After treatment, plants were incubated for 3 h in individual enclosures. The first-instar larvae were removed from the stock culture on wet filter paper and placed at the bottom of round glass Petridishes for 15 min. The treatment satiated the larvae with water and achieved identical physiological conditions. Twenty first-instar larvae (20 larvae=1 replicate) were separately released on each of the treated or control plants. In order to trap straying first-instar larvae, a white sheet coated with odorless glue was placed under treated and control plants in the center of a circular arena (10 inches in diameter). Double sided tape was fixed on the inner margin of the arena before larvae release. Six hours after initial release, the number of trapped larvae was recorded. The experimental procedure included five replications. Water-treated plants served as the control for the above-mentioned experiments. Dispersal percentage was calculated based on the number of larvae dispersed from the plant surface and the total number of larvae released. Five independent experimental data sets were analyzed statistically using analysis of variance (ANOVA; Tukey's Test; Sigma Stat 2.0; Jandel Scientific Software, 1995; Jandel corporation, San Rafael CA).

3.2.3.7 Feeding bioassays

Each freshly molted fifth-instar larva was individually released on control / treated plants (50 plants for each control / treatment), and covered with wire mesh to restrict movement. The initial weight of larva (IWL) was recorded before release and the final weight of larva (FWL) noted after 24 h of feeding. The relative body weight gain of the larvae was calculated as the difference between IWL and FWL. For conducting bioassays with excised plant tissues, equal amount of freshly excised control/treated plant tissues were weighed separately, which gave the initial weight of the tissues (IWT) and transferred into the numbered Petridishes (9 cm X 3 cm). The neonate fifth-instar larvae (50 larvae for each control/treatment) were weighed individually which gave the initial weight of the larvae

(IWL). Larva was released individually into the numbered Petridishes containing the control/treated plant tissues (2000 mg). Same amount of plant tissues were kept in Petridishes without larvae under the same conditions to estimate the loss of moisture for calculating the corrected final weight of consumed tissues. All the Petridishes were kept inside the BOD incubator maintaining the same temperature and humidity as mentioned earlier. Larvae were allowed to feed for 24 h after which larvae were taken out and weighed individually which gave their final weight (FWL). The relative body weight gain of the larvae was calculated as the difference between IWL and FWL. The unconsumed plant tissues were also weighed separately which gave their final weights (FWT). Amount of tissue consumed was calculated by subtracting the corrected FWT from IWT. The data obtained from five independent experiments conducted both on live and excised plants were analyzed statistically using ANOVA (Tukey's Test).

3.2.4 Cloning of DNA fragments

3.2.4.1 Polymerase Chain Reaction (PCR)

Specific DNA fragments were amplified using the polymerase chain reaction (PCR) (Mullis and Faloona, 1987). The reaction started with the denaturation of two strands of a DNA template. The 5′ complementary strands of the denatured DNA was recognized and hybridized with specific primers (annealing). A Taq-polymerase enzyme catalyzes elongation of a newly synthesized chain and the complementary polymerization of nucleotides to the free 3′-OH group of the primer. Repeating the previous steps (denaturation, annealing and elongation) for x cycles (usually from 25 to 35) will exponentially enrich the reaction with the primer-flanked DNA sequence. In some cases a suitable synthetic restriction sites were incorporated to the 5′-end of the primer for cloning purposes. The PCR reaction was carried out in a 20 µl reaction volume with the following constituents: 10-50 ng template DNA, 2 µl of 10 pmole sense primer, 10 pmole antisense primer, 0.2 mM dNTPs, 2 µl of 10x Taq-buffer, 2 U Taq-polymerase and H_2O up to 20 µl. The amplification reaction was done in a PCR thermocycler using the following program:

	Initial denaturation	2 minutes 94 °C
	Denaturation	1 minute 94 °C
25-35 x Cycle	Annealing	30 seconds 50-65 °C
	Elongation	1 minute/kb 72 °C
	Final elongation	10 minutes 72 °C

3.2.4.2 Reverse Transcription PCR (RT-PCR)

The reverse transcription PCR (RT-PCR) is a technique used for mRNA detection and quantification. The technique consists of two parts: the synthesis of cDNA from RNA by reverse transcription and the amplification of a specific cDNA by PCR. The RT-PCR reaction was conducted using the ABI First Strand cDNA Synthesis kit following the manufacturer's instructions. In brief, a reaction mixture containing 1µg isolated total RNA, 0.2 µg/µl random primers/Oligo dT and RNase free H_2O up to 11 µl was prepared. The reaction was incubated for 10 minutes at 70 °C then chilled on ice. To this mixture 4 µl of 5x Reaction Buffer, 20 units of RNase inhibitor and 2 µl of 10 mM dNTPs were added and the reaction was incubated for 5 minutes at 25 °C. To the mixture, 1 µl of Reverse Transcriptase enzyme (200u/µl) was added and the mixture was incubated at 25 °C for 10 minutes followed by 42 °C for 60 minutes. Finally, the reaction was heated at 70 °C for 10 minutes. A PCR reaction using the gene specific primers was carried out using 2 µl of the synthesized cDNA reaction.

3.2.4.3 Cloning of PCR Products

The DNA molecule amplified using the Taq- Polymerase is characterized by the presence of additional deoxyadenosine nucleotides (dA) at the 3′-end of the PCR product, which is due to the terminal deoxy-nucleotidiltransferase activity nature of the Taq-polymerase enzyme. PCR product with the 3′-dA overhangs can be used to clone a vector having a complementary 3′-deoxytimidine (dT). For this purpose the pGEM®-T vector system kit (Promega)/pDrive (Qiagen) was used. The ligation reaction was performed following the manufacturer's instructions.

3.2.4.4 Separation of DNA on Agarose Gels

DNA samples were mixed with 1/10 volume of 10x DNA loading buffer and then separated on horizontal agarose gels (10 x 7 x 0.3 cm) containing 1x TAE buffer. The gel was prepared by dissolving Agarose in 1x TAE and the concentration of the gel ranged between 1-2% depending on the size of the expected DNA fragment, shorter the fragment higher agarose concentration. Electric current of 3 V/cm was used for 1-2 hours to run the gel, and the gel was ended depending on the distance between the migrated bands of the dyes present in the DNA loading buffer. Ethidium bromide solution (0.1% w/v) was used to satin the DNA fragments. The DNA detection was done under UV light. Before exposure to the UV light, the gel was rinsed briefly in H_2O to reduce background staining. In a gel-documentation station, gels were visualized on a UV-transilluminator and documented. The sizes and amount of the DNA fragments were determined using DNA standards.

3.2.4.5 Elution of DNA from agarose gel

The PCR product was fractionated on 1% agarose/EtBr gel. The band was cut by using sterile blade and collected in a 1.5 ml sterile micro-centrifuge tube. The gel elution was performed by using MinElute gel extraction kit (Qiagen, Germany). The elution was done according to the manufacturers instructions with minor modifications. Three volumes (one volume of gel, 100 mg ~ 100 µl) of buffer QG was added to the eppendorf containing the gel slice and incubated at 40°C for 30 min to dissolve the agarose. After the gel slice has dissolved completely, one gel volume of isopropanol was added and mixed by inverting the tubes 4-5 times. This sample was loaded into the MinElute column which was kept on a 2ml collection tube and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and the column was again placed in the same collection tube. Further, 500 µl of QG buffer was loaded to the column and centrifuged at 13,000 rpm for 1min. The flow-through was discarded and column was again placed in the same collection tube. To wash the column, 750 µl buffer PE was loaded into the column and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and column was again placed in the same collection tube and centrifuged for an additional 1 min to remove the residual ethanol. The MinElute column was then placed in clean 1.5 ml micro-centrifuge tube. To elute the DNA, 10 µl of elution buffer (100mM TrisCl, pH 8.0) or sterile nuclease free water was loaded directly on the matrix. The column was left as such for 5 min and then centrifuged at 13,000 rpm for 2 min. DNA was obtained as flow through. The eluted DNA was stored at -20°C.

3.2.4.6 Purification of PCR products

The PCR product was purified by using MinElute™ PCR purification kit (Qiagen, Germany). Purification was done according to manufacturer's instructions with minor modifications. Five volumes of PB buffer was added to one volume of the PCR reaction product and mixed. The mixture was then applied to the MinElute column which was kept in 2 ml collection tube and centrifuged at 13,000 rpm for 1 min to bind the DNA to the membrane, flow-through was discarded and column was again placed in same collection tube. To wash the column, 750 µl PE buffer was applied to the column and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and column was again placed in the same collection tube and centrifuged for an additional 1 min to remove the residual ethanol. Now, the MinElute column was placed in a clean 1.5 ml microcentrifuge tube. To elute the DNA, 10 µl of elution buffer or sterile nuclease free water was loaded directly on the matrix. The column was left for 5 min and then centrifuged at 13,000 rpm for 2 min. The DNA was obtained as flow-through. The eluted DNA was stored at -20°C.

3.2.4.7 Restriction Digestion of DNA Molecules

The restriction enzymes of endonucleases type II were used to digest a double stranded DNA molecule for analytical and cloning purposes. The enzymes cut the DNA either as 5′ or 3′ "sticky" overhangs or as blunt ends. The digestion reactions were incubated in a buffer system optimized for the used enzyme and in the case of double digestion a universal buffer system was used. The activity of the restriction enzymes was estimated in units (U), where 1 U stands for the amount of enzyme cutting completely at optimal conditions 1 µg of λ DNA for 60 minutes. The minimal amount of enzyme necessary for each restriction was determined according to the following formula:

$$U_{min} = \cdot \frac{[\text{bp } (\lambda) \,.\, \text{recognition sites (DNA)}}{[\text{Recognition sites } (\lambda) \,.\, \text{bp (DNA)}]}$$

Where bp (λ) = 48500

3.2.4.8 Ligation of DNA Fragments

The conventional cloning of a DNA fragment into a selected plasmid was performed using the T4-DNA ligase enzyme, which is able to catalyze the formation of a phosphodiesther chemical bond between free 5′-phosphate and 3′-OH groups of double-stranded DNA fragments and vectors. The donor DNA fragment (10x accesses to the vector) was incubated with the vector DNA, 2 µl of ligation buffer and 1 µl of T4-DNA ligase for 16 hours at room temperature.

3.2.4.9 Preparation of Competent Bacterial Cells

For cloning purpose, *E. coli* DH5α bacterial strain were made competent by the below given methods and used for transformation.

Calcium Chloride Method

The $CaCl_2$ method was adopted from Sambrook and Russell (2001) with some minor modifications. From the overnight grown pre-culture of bacterial cells, 1ml of inoculum was used to inoculate 100 ml LB medium in a culture flask. This culture was grown at 37°C with vigorous shaking (200-250 rpm) to an A_{600} of 0.3-0.4. The culture was chilled on ice for 15-20 min, transferred to 50 ml Oak-ridge tubes and centrifuged at 5000 rpm for 5 min at 4°C in Sorvall® RC5C plus centrifuge (Kendro Lab., USA) with SA-600 rotor. The pellet in each tube was gently suspended in 0.5 volumes (of original culture) of ice-cold 100mM $CaCl_2$ by gently swirling the tubes and incubated on ice for 30 min. The cells were collected by

centrifugation as above and resuspended in 0.1 volumes ice-cold 100mM $CaCl_2$ by gently swirling the tube.

Preparation of ultra-competent bacterial cells

The competent cells were prepared as described by Inoue *et al.,* (1990) with few modifications. From the frozen culture, DH5α bacterial cells were streaked on LB agar plate and were grown overnight at 37°C. Approximately 5-10 large colonies were inoculated in 200 ml SOB media (Appendix A) with a sterile loop and grown at 22°C with vigorous shaking at 200-250 rpm till the OD_{600} reaches to 0.45. The culture flask was removed from the incubator and placed on ice for 10 min. The culture was transferred to sterile Oakridge centrifuge tubes, 50 ml each, and centrifuged at 2500x g for 10 min at 4°C. The pellet obtained was resuspended in 16 ml of ice-cold HTB (Appendix B), incubated on ice for 10 min and centrifuged at 2500x g for 10 min at 4°C. The pellet obtained was gently resuspended in 4 ml of HTB and DMSO was added to a final concentration of 7% with gentle swirling. Cells were kept on ice bath for 10 min. One hundred microlitres of the cell suspension was dispensed in 1.5 ml micro-centrifuge tubes and snap-frozen in liquid nitrogen. The frozen competent cells were stored at - 80°C for future use.

3.2.4.10 Transformation

Competent *E. coli* cells were transformed according to the standard protocol given by Hanahan, (1983). A vial of competent cells, stored at - 80°C was carefully thawed on ice avoiding any temperature shock. The ligated product or plasmid was directly added to 100 µl competent cell suspension, mixed by gentle tapping and subsequently kept on ice for 30 min. All the steps of transformation were carried out in laminar hood under sterile conditions. The cells were then given a heat shock at 42°C for 90 sec and quick chilled on ice for 5 min. This is followed by addition of 0.9 ml of LB and the cells were allowed to grow at 37°C for 45 min with gentle shaking. The transformed competent cells were plated on LB plate containing appropriate antibiotic. Blue-white selection if needed was carried out by plating the cells on X-gal/IPTG plate. The plates were then incubated at 37°C overnight.

3.2.4.11 Confirmation for the presence of insert

The presence of the insert in the clone was confirmed by the colony PCR by using either gene specific primers or primers compatible with cloning vector. Individual colonies were picked from overnight grown plate and mixed in 20 µl sterile water in a 0.5 ml micro-centrifuge tubes. The cells were lysed by boiling for 2 min and centrifuged at 13,000 rpm for

30 sec. Eight microlitre of the supernatant was taken as template for PCR. The master mix was prepared according to the number of the PCR reactions and distributed in thin-walled PCR tubes. Number of PCR cycles and cycling conditions were adjusted according to the Tm of primers used for amplification.

3.2.4.12 Alkaline lysis midiprep of plasmid DNA

A single colony of bacterial cell containing the desired clone was inoculated to the 100ml of LB medium containing the appropriate antibiotic and allowed to grow overnight at 37°C. The bacterial cells were harvested by centrifugation at 5000 rpm for 10 min at 4°C. The pellet was resuspended in 5ml of ice cold solution I (50mM glucose, 10mM EDTA, 25mM TrisCl). Then 5 ml of freshly prepared solution II (0.2N NaOH,1%SDS) was added and mixed gently by inversion, and incubated for 5 min at room temperature followed by addition of 5ml of ice cold solution III (3M potassium acetate, pH 4.8) and the mixture was incubated on ice for 15 min. This mixture was then centrifuged at 14,000 rpm for 30 min at 4°C and the supernatant was transferred to a fresh Oakridge tube. The supernatant was subjected to RNase treatment 20 µg/ml at 37°C for 45 min. The supernatant was extracted twice with phenol: chloroform: isoamyl alcohol (25:24:1) and followed by separation of upper aqueous phase containing the plasmid in a fresh Oakridge tube. Equal volume of isopropanol was added to precipitate the DNA by centrifugation at 10,000 rpm for 30 min at room temperature. This was followed by washing with 70% alcohol. The pellet was dried at 37°C and dissolved in 100 µl of sterile water.

3.2.4.13 Purification of Plasmid by PEG Precipitation for Sequencing

Eight microlitre of 4M NaCl and 40 µl of 13% polyethylene glycol (PEG 8000) was added to the plasmid dissolved in 32 µl sterile water and the mixture was incubated on ice for 30 min. DNA was pelleted by spinning at 12,000x g for 20 min at 4°C. The pellet obtained was washed twice with 70% alcohol, dried and dissolved in sterile water. Visual quantification of DNA was done and 150 ng of plasmid in 2 µl was used for automated sequencing with 96 capillary based DNA analyzer (Hitachi and ABI PRISM, Applied Biosystems)

3.3 Gene expression analysis by Northern Hybridization

Before starting RNA work, mortar, pestle, glassware, spatula and other required materials were baked at 180°C for 5-6 hrs. Gel electrophoresis assembly and other plastic wares were treated with 3% H_2O_2 overnight.

3.3.1 Isolation of RNA from Chickpea

Total RNA was isolated from Chickpea with TRIZOL Reagent according to the protocol provided by the manufacturer (Invitrogen, USA) with few modifications. About 0.8g plants tissue was crushed to fine powder with mortar and pestle in liquid nitrogen without letting it to thaw. The powdered material was transferred to a 2 ml eppendorf tube, immediately 1 ml TRIZOL Reagent was added to the tube and it was vigorously shaked in order to homogenize the sample quickly. The homogenized samples were incubated for 15 min at room temperature for complete dissociation of nucleoprotein complexes. Two hundred microlitre of chloroform was added per ml of TRIZOL reagent used and tube was vigorously shaked for 30 sec with tube capped tightly, incubated at RT for 10 min and centrifuged at 12,000x g for 15 min at 4°C. Following centrifugation, the upper aqueous phase was aliquoted into three tubes (kept on ice) equally without disturbing the lower whitish layer. The RNA from the aqueous layer was precipitated by mixing with 0.7 volumes of isopropyl alcohol in each tube, according to the volume of supernatant aliquoted in each tube earlier, incubated for 10 min at RT and centrifuged at 12,000x g for 10 min at 4°C. The supernatant was discarded by inverting the tubes on tissue paper and RNA pellet was washed two times with 75% ethanol by dislodging the pellet from the surface of tube with vigorous shaking and centrifuging at 7,500x g for 5 min at 4°C. At the end of the procedure, RNA pellet was briefly dried for 10 min and dissolved in adequate volume of DEPC-treated water or for long term storage, the ethanol washed pellet was left in 75% ethanol and kept at -80°C.

3.3.2 RNA quantification

The water dissolved RNA was incubated at 55°C for 10 min and quickly chilled on ice. After brief centrifugation, it was collected at the bottom of tube and tapped gently to mix. Two microlitre of the RNA was diluted 500 times by adding 1 ml of DEPC-treated water and mixed thoroughly. The O.D of this diluted RNA was taken at 260 nm spectrophotometer (U-2010, HITACHI) against DEPC-treated water as blank. Concentration of the RNA was calculated according to the following formula-

$$\text{RNA conc. } (\mu g / \mu l) = \frac{O.D_{260} \times 40 \times \text{Dilution factor}}{1000}$$

Purity of the RNA was checked by taking O.D at 230, 260, and 280 nm wavelengths. The RNA was indicated as pure if the ratio of O.D (260/280) is 1.7-2.0 (<1.7 is typically protein contamination and O.D, 260/230 is >2.0 ;<2.0 is due to guanidinium isothiocyanate).

3.3.3 Isolation of mRNA

Messenger RNA has a tail of approximately 200 adenylates in length which gets shortened to 40-65 adenylates during aging of mRNA. The presence of poly-A tail in mRNA is exploited to separate them from non polyadenylated RNAs (rRNA and tRNA). The method relies on the base pairing between the poly-A residues at the 3' ends of the mRNA species and the biotin labeled oligo-$(dT)_{20}$ probe which is bound to avidin or streptavidin solid support. Lysis Buffer was added to total RNA, mixed and incubated at 65°C for two minutes. In the meantime magnetic particles were resuspended thoroughly and the required volume of streptavidin magnetic particles was aliquoted in a fresh tube. The magnetic particles were then separated from storage buffer using the magnetic separator (Amersham, U.K). The supernatant was discarded and the tube was removed from the magnetic separator. Lysis buffer was added to resuspend the particles and the beads were again separated under magnetic field and the supernatant was removed. This procedure was repeated thrice to wash the beads. The biotin labeled oligo-$(dT)_{20}$ probe was added to the sample (Total RNA) and mixed properly. This mixture was added to the washed streptavidin magnetic particles and incubated for 5 min at 37°C. The magnetic particles were then separated by magnetic separator. The particles were then washed thrice with washing buffer. In the final step of washing the buffer was removed completely. The mRNA was eluted by adding the redistilled water (supplied in the kit) to the magnetic particles and was incubated at 65°C for 2 min. Finally, the magnetic particles were separated from the fluid. The supernatant containing mRNA was then transferred to a fresh RNase- free tube. The mRNA so obtained was quantified spectrophotometrically.

3.3.4 Denaturing formaldehyde gel for RNA electrophoresis

Total RNA was run in 1.2 % denaturing formaldehyde gel. For preparation of gel, 1.2 g agarose was added to 64 ml DEPC treated water and boiled for 1.5 min. Once the temperature comes down to 60°C, 16.4 ml formaldehyde and 20 ml 5X MOPS buffer was added. The contents were mixed by swirling. Formaldehyde is harmful for eyes, hence adequate precautions were taken. The molten gel was poured in casting tray with combs already fitted into it. Meanwhile, RNA samples were prepared by mixing eight microgram of total RNA and RNA loading dye (1 ml contains 500 µl formamide, 166 µl formaldehyde, 200 µl 5X MOPS and 134 µl DEPC water) in 1:3 (v/v) ratio. The samples were heat denatured at 65-67°C for 10 min and immediately chilled. The samples were run at 20-30 Volts for 5-6 hours in 1X MOPS buffer.

3.3.5 Transfer of total RNA on Nylon Membrane

The gel was rinsed with DEPC treated water for 30 min to remove formaldehyde and it was equilibrated with 20X SSC for 30 min. The RNA was transferred to Hybond–N^+ Nylon membrane (Amersham, UK) by vertical capillary action using 20X SSC for 16 h. After that the RNA was cross-linked to the nylon membrane in UV crosslinker (Stratagene, USA) at 1200kJ/cm^2 and this RNA cross-linked membrane was treated with 5% glacial acetic acid for 15 min. To check the RNA transfer on the membrane, it was stained with 0.04% methylene blue (Solution prepared in 0.5 M Na-acetate, pH 5.2. Excess of the stain on the membrane was removed by washing with sterile MQ water. Image of ribosomal RNA was captured on Fluor-S™ MultiImager (Bio-Rad, USA) at highest resolution available to show equal loading of RNA. The hybridized nylon membrane was wrapped in a saran wrap to avoid it from drying.

3.3.6 Radioactive probe preparation, purification and hybridization

For probe preparation radiolabel was used, hence all steps were performed in radioactive room taking adequate safety measures. In a hybridization incubator, the RNA cross-linked nylon membranes were incubated at 60°C with 10 ml of pre-hybridization solution (0.5M Phosphate buffer, pH 7.2, 7% SDS, and 1mM EDTA, pH 8.0) in hybridization bottles for 4 hrs. In the meantime the probe was prepared using random primers labeling NEBlot® kit (NEB Inc., U.K). For probe preparation, in 1.5 ml micro-centrifuge tube 50 ng of DNA (fragment to be used as probe) was taken in final volume of 10 µl. The dsDNA was denatured for 5 min in boiling water bath and quickly chilled on ice. For 50 µl reaction, the following components were added in the order- 26 µl of MQ H_20, 5.0 µl of 10X labeling Buffer, 2.0 µl of dATP, 2.0 µl of dGTP, 2.0 µl of dTTP, 2.0 µl of radioactive α^{32}P-dCTP (3000 Ci/mmole, Amersham Biosciences) and 5 units of Klenow polymerase enzyme. The final mixture was incubated at 37°C for one hour in water bath. For purification of free radioactive dNTPs from the mixture, Sephadex G-50 column was prepared as described. One ml fresh disposable syringe sterile TE (pH 8.0) was packed at the bottom with the glasswool. This column was packed with sephadex G-50 (soaked in TE, pH 8.0) up to appropriate volume by centrifugation in a 15 ml falcon tube and was equilibrated thrice with TE, pH 8.0. Prior to purification it was centrifuged again, to remove excess TE, at 2,300 rpm for 4 min. The volume of the reaction mix was made upto 100 µl with TE, pH 8.0. The reaction mix was loaded on the packed column and centrifuged at 2,300 rpm for 3-5 min. Purified probe was collected as flowthrough in a decapped eppendorf and transfered to fresh eppendorf. It was subsequently denatured for 5 min in boiling water bath and quick chilled for 5 min. After a

brief spin, the probe was added directly to the pre-hybridization solution kept in hybridization bottle. The probe was left for hybridization for 14-16 hr at 60°C in hybridization incubator.

3.3.7 Washing and Autoradiography

Filters (Hybridized nylon membrane) were washed thrice for 5 min at room temperature in low stringency solution (2X SSC and 1% SDS). Filters were then checked for the count by the radiation monitor. This was followed by washing at 60°C in medium stringency washing solution (0.4X SSC and 0.1% SDS) for 10 minutes or more depending upon the background count. The filters were then wrapped in saran wrap to avoid drying and the X-ray film was exposed to the membrane in the Hypercassette™ (Amersham Pharmacia biotech, U.K) for the time period depending upon the signal intensity. Subsequently, the X-ray film was developed using Developer and Fixer solutions (Kodak Affiliate Products, India). The autoradiograms obtained were scanned in Fluor-S™ MultiImager (Bio-Rad, USA).

3.4 Construction of subtractive cDNA library

Subtractive hybridization is a powerful technique that enables researchers to compare two populations of mRNA and obtain clones of the genes that are expressed in one population but not in the other. Although there are several different methods, the basic theory behind subtraction is simple. First, both mRNA populations are converted into cDNA. The cDNA that contains specific (differentially expressed) transcripts is referred as "tester," and the reference cDNA as "driver." Tester and driver cDNAs are hybridized, and the hybrids are then removed. Consequently, the remaining unhybridized cDNAs represent genes that are expressed in the tester, but are absent from the driver mRNA. Clontech's PCR-Select™ cDNA Subtraction is a unique method based on selective amplification of differentially expressed sequences, which overcomes technical limitations of traditional subtraction methods (Diatchenko *et al.,* 1996; Gurskaya *et al.,* 1996). The overview of the different steps is given in Fig. 3.1.

3.4.1 Isolation of high quality total RNA and mRNA

Total RNA from 24 hrs *Ascochyta rabiei* infected and water sprayed control chickpea plants was isolated using Trizol method. Spectrophotometric estimation quantity and quality of total RNA was done as described previously. RNA was run on 1.2% formaldehyde denaturing agarose gel. Afetr the run was completed; the gel was treated with water for 2-3 hrs to remove formaldehyde and subsequently stained in the EtBr solution to visualize RNA

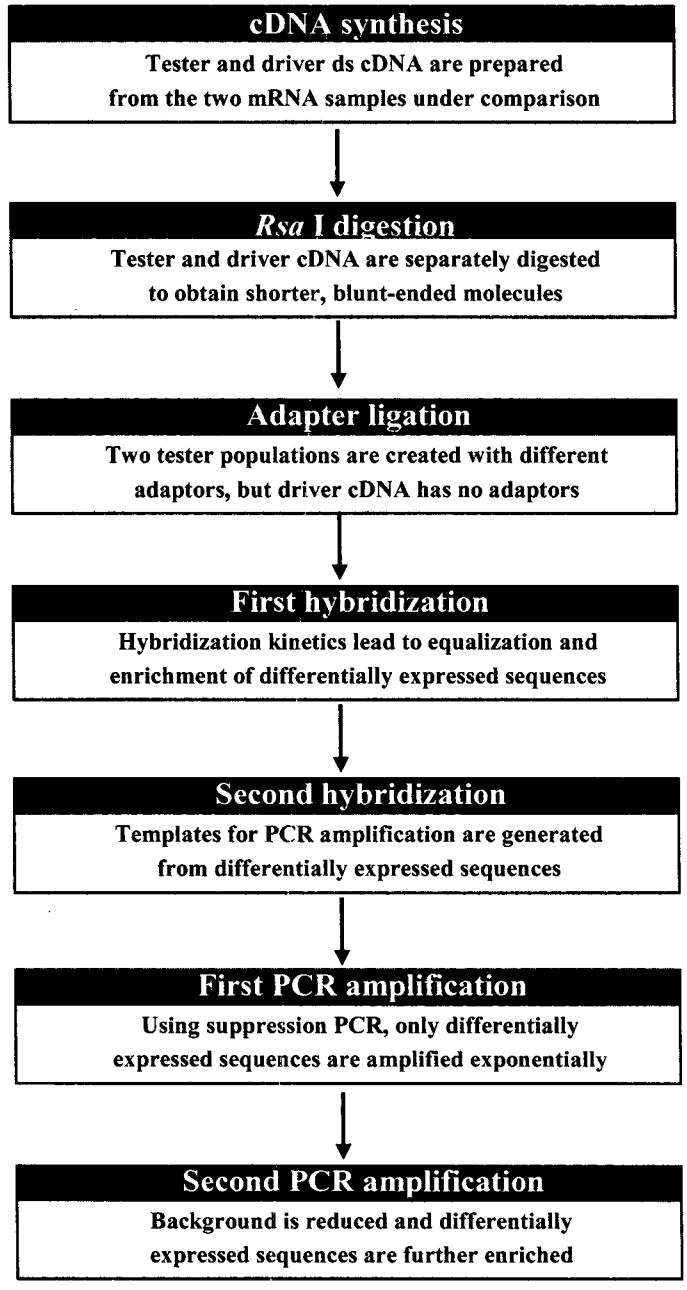

Figure 3.1. Overview of the Clontech PCR-Select™ procedure. The cDNA in which specific transcripts are to be found is called "tester" and the reference cDNA is called "driver."

under UV. High quality of total RNA was ensured by visualizing the intensities of 28S and 18S ribosomal RNA. For intact and good quality RNA 28S:18S ribosomal RNAs should be in 2:1 ratio. Messenger RNA was isolated using oligo-(dT) tagged magnetic beads (Roche Diagnostics GmbH, Germany). Spectrophotometric estimation of isolated mRNA at OD_{260} was done. At least 2.0 µg of mRNA per reaction was used; use of less than 2.0 µg of mRNA may result in loss of rare transcripts during subtraction.

3.4.2 First strand cDNA synthesis

First strand cDNA synthesis was performed using 2.0 µg of mRNA from tester and driver samples. One microlitre of first strand cDNA synthesis primer was added to 2.0 µg of mRNA in microcentrifuge tube and total volume was made to 5.0 µl. The contents were mixed and spun briefly. After incubation in thermal cycler at 70°C for 2 min the tubes were cooled on ice and briefly centrifuged. To this, following components were added to; 2.0 µl 5X First-Strand Buffer, 1 µl dNTP mix (10mM each), 1 µl sterile H_2O and 1 µl AMV Reverse Transcriptase (20 units/µl). After mixing and brief spin, the tubes were incubated at 42°C in air incubator for 1.5 hrs. Tubes were then placed on ice to terminate the reaction.

3.4.3 Second-strand synthesis

The following components were added to the first-strand synthesis reaction tubes (containing 10 µl); 48.4 µl sterile water, 16.0 µl 5X Second-Strand Buffer, 1.6 µl dNTP mix (10mM) and 4.0 µl 20X Second-Strand Enzyme Cocktail. The total volume of the reaction was made upto 80 µl. The contents were mixed properly and incubated at 16°C (water bath or thermal cycler) for 2 hr. Two microlitres (6 units) of T4 DNA Polymerase was added and mixed thoroughly. Tubes were incubated at 16°C for 30 min in a thermal cycler. Four microlitres of 20X EDTA/Glycogen mix was added to terminate the second-strand synthesis reaction. Further, 100 µl of phenol: chloroform: Isoamyl alcohol (25:24:1) mix was added. The contents were vortexed thoroughly, and centrifuged at 14,000 rpm for 10 min at room temperature. The top aqueous layer was transferred to a sterile 0.5 ml microcentrifuge tube and the interphase and lower phases were discarded. Hundred microlitre of chloroform: isoamyl alcohol (24:1) was again added to the aqueous layer, vortexed and centrifuged to obtain upper aqueous layer into a separate tube. Forty microlitres of 4M NH_4OAc and 300 µl of 95% ethanol was added and immediately preceded with precipitation. The contents were vortexed thoroughly and centrifuged at 14,000 rpm for 20 min at room temperature. The supernatant was removed carefully and 500 µl of 80% ethanol was added. The tubes were centrifuged at 14,000 rpm for 10 min and the supernatant was removed. The pellet was air dried for about 10 min to evaporate residual ethanol and dissolved in 50 µl of sterile H_2O and

0.0 hl of this was transferred to a fresh micro-centrifuge tube. This sample was stored at –20°C until it was estimated later for Kpn I digestion.

3.4.4 Kpn I Digestion

Kpnl digestion was performed with each experimental double stranded testet and trivet cDNAs to generate shorter, blunt-ended double stranded fragments which are optimal for subtraction and necessary for adabtor ligation. Following reagents were added: 2.2 hl double stranded cDNA, 2.0 hl 10X Kpn I restriction buffer and 1.2 hl Kpn I (10 units/hl). Contents were mixed by vortexing and centrifuging briefly. The reaction was carried out at 37°C for 1.2 hr. Five microlitres of the Kpn I digested mixture was set aside to analyze the efficiency of Kpn I digestion. To terminate the reaction, 2.2 hl of 250 X0 EDTA\Glycogen mix was added. Then 20 hl of phenol: chloroform: Isoamyl alcohol mix in 25:24:1 ratio was added, vortexed thoroughly and centrifuged at 14,000 rpm for 10 min to separate the aqueous phase. The extraction was repeated twice and the top aqueous layer was transferred to fresh 0.2-ml tube. To this aqueous layer 25 hl of 7.5 M NH_4OAc and 2.5 hl of 95% ethanol was added and immediately processed with precipitation. The mixture was thoroughly vortexed and centrifuged at 14,000 rpm for 20 min at room temperature. The supernatant was carefully removed and gently the pellet was overlaid with 200 hl of 80% ethanol. The tubes were centrifuged at 14,000 rpm for 2 min and carefully the supernatant was removed. Pellets were air dried for 2–10 min and dissolved in 2.2 hl of H_2O and stored at –20°C. This 2.2 hl sample of Kpn I digested cDNAs served as our experimental trivet cDNA and testet cDNA. The Kpn I digested cDNA was compared with undigested cDNA on 1.0% agarose\EtBr gel to check the efficiency of Kpn I digestion.

3.4.5 Adabtor Ligation to testet cDNA

One microlitre of each Kpn I digested testet cDNA was diluted with 2 hl of sterile H_2O. Ligation mix was prepared by combining the following reagents in two seperate 0.2 ml micro-centrifuge tubes: 3 hl sterile H_2O, 2 hl 2X Ligation Buffer, 1 hl T4 DNA ligase (400 units/hl) and 2 hl of diluted testet cDNA. To one of the two tubes Adabtor1 and to the other Adabtor 2R were added. Contents were pipetted in and out to mix them thoroughly. To prepare unsubtracted testet control 2 hl from each tube was taken in a fresh tube and mixed. This, after ligation, would act as unsubtracted testet control. Tubes were centrifuged briefly and incubated at 16°C overnight. Ligation reaction was terminated by adding 1.0 hl of EDTA\Glycogen mix. Samples were incubated at 75°C for 2 min and briefly spun to inactivate ligase. Adabtor-ligated testet cDNAs and unsubtracted testet controls were thus

obtained. One microlitre unsubtracted tester control was diluted with 1 ml of sterile H_2O. These samples were later used for PCR. Till then, the samples were stored at -20°C.

3.4.6 First Hybridization

In the following procedure, excess of driver cDNA was added to each tester cDNA, and the samples were heat denatured and allowed to anneal. The 4X Hybridization buffer was kept at 37°C for 10 min to ensure that no precipitate remains. First hybridization reactions were then set. The first reaction mixture contained 1.0 µl 4X Hybridization Buffer, 1.5 µl Adaptor 2R-ligated Tester cDNA and 1.5 µl *Rsa* I digested driver cDNA. The second reaction mixture contained 1.0 µl 4X Hybridization Buffer, 1.5 µl Adaptor 1-ligated Tester cDNA and 1.5 µl *Rsa* I digested driver cDNA. Samples were overlaid with mineral oil and centrifuged briefly and subsequently, incubated in a thermal cycler at 98°C for 1.5 min and then at 68°C for 8 hr. These two hybridized samples were designated as first hybridization sample N1 and N2. Immediately, the second hybridization was performed.

3.4.7 Second Hybridization

The two samples N1and N2 from the first hybridization were mixed together, and fresh denatured driver cDNA was added to further enrich for differentially expressed sequences. New hybrid molecules formed consist of differentially expressed cDNAs with different adaptors on each end. The primary hybridization samples were not denatured at this stage and the entire procedure was performed while the samples were still in thermal cycler at 68°C. Following components were added for second hybridization; one microlitre driver cDNA, 1 µl 4X Hybridization Buffer and 2 µl Sterile H_2O in 0.5 ml PCR tube. From this sample mixture 1 µl was transferred to a 0.5 microcentrifuge tube, overlaid with 1 drop of mineral oil and this tube was incubated in thermal cycler at 98°C for 1.5 min. The freshly denatured driver cDNA was removed from the thermal cycler. Strictly following this step the driver was mixed with the first hybridization samples N1 and N2 simultaneously, which ensured that the two hybridization samples mix together only in the presence of freshly denatured driver. For this, following protocol was followed. The Micropipette was set at 15 µl. Pipette tip was gently touched to the mineral oil and sample interface of the tube containing hybridization sample N2. The entire sample was carefully drawn halfway into the pipette tip without caring if a small amount of mineral oil is transferred with the sample. Pipette tip was removed from the tube, and a small amount of air was drawn into the tip, creating a slight air space below the droplet of sample. The pipette tip then contained hybridization sample N2 and the driver cDNA separated by a small pocket of air. The entire mixture was transferred to the tube containing hybridization sample N1. Samples were mixed

by pipetting up and down, briefly centrifuged if necessary and incubated at 68°C overnight. Added 200 µl of dilution buffer to the tube and mixed by pipetting. Heated in a thermal cycler at 68°C for 7 min and stored at –20°C.

3.4.8 PCR Amplification

Differentially expressed cDNAs were selectively amplified using the reactions described in this section. Prior to thermal cycling, missing strands of the adaptors were filled in by a brief incubation at 75°C. This created the binding site for PCR Primer 1. In the first amplification, only ds cDNAs with different adaptor sequences on each end were exponentially amplified. The second, nested PCR was performed to further reduce the background and to enrich for differentially expressed sequences. We performed two PCR reactions one with subtracted tester 1 cDNA and the other with unsubtracted tester control. All PCRs were done using a PTC-200 Thermal cycler (MJ Research). The enzyme used was *Taq* DNA polymerase mix and a hot start PCR was performed. PCR reaction was prepared as follows

Sterile H_2O	19.5 µl
10X PCR reaction buffer	2.5 µl
dNTP mix (10 mM)	0.5 µl
PCR Primer 1 (10 µM)	1.0 µl
50X Advantage cDNA Polymerase Mix	0.5 µl
Total volume	24.0 µl

PCR conditions:

Denaturation	94°C for 30 sec	27 cycles
Annealing of primers	66°C for 30 sec	
Primer extension	72°C for 1.5 min	

Eight microlitres of this PCR product was analyzed on 2 % agarose/EtBr gel.

3.4.9 Secondary PCR

Three microlitre of each primary PCR mixture was diluted with 27 µl of H_2O. One microlitre of this diluted primary PCR product was used for secondary PCR.

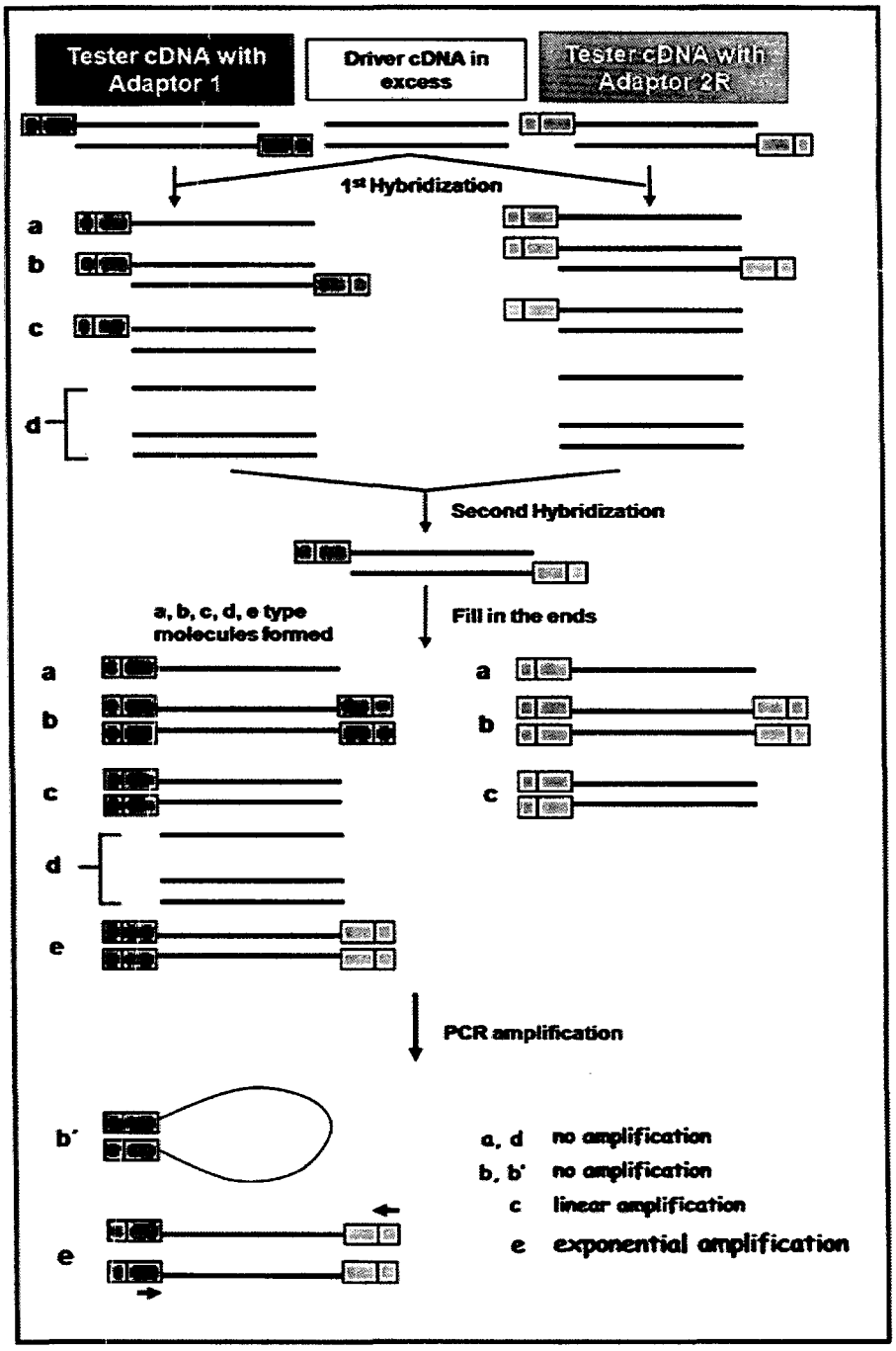

Figure 3.2. Diagrammatic representation of the strategy employed for construction of subtracted library by suppression PCR method. The two different adaptor ligated "Tester cDNAs" were separately subjected to "First Hybridization" with excess of "Driver cDNA" at 68°C for 8h resulting into type a, b, c and d molecules. "Second hybridization" was done by mixing the two different "First hybridization" components at 60°C in the presence of excess of driver cDNA. Apart from a, b, c and d type molecules "e" type molecules were also formed. The "e" type molecules represented differential genes, which amplified in the presence of adaptor specific 1 and 2R primers. Type a, b and c molecules could amplify either linearly or not at all due to lack of adaptor sequence. Type b molecules could not amplify at all due to "*suppression-PCR effect*".

Sterile H_2O	18.5 µl
10X PCR reaction buffer	2.5 µl
Nested PCR primer 1 (10 µM)	1.0 µl
Nested PCR primer 2R (10 µM)	1.0 µl
dNTP mix (10 mM)	0.5 µl
50X Advantage cDNA Polymerase Mix	0.5 µl
Total volume	24.0 µl

Contents were mixed well by vortexing, and briefly centrifuged and overlaid with 1 drop of mineral oil and immediately commenced for thermal cycling as mentioned:

Denaturation	94°C for 30 sec	10-12 cycles
Annealing of primers	68°C for 30 sec	
Primer extension	72°C for 1.5 min	

Eight microlitres of PCR product was analyzed on a 2.0% agarose/EtBr gel and the remaining PCR product was stored at -20°C. This PCR product was enriched for differentially expressed cDNAs (Fig. 3.2).

3.4.10 Cloning of amplified differential cDNA

These cDNAs were directly ligated to pDrive U/A cloning vector (Qiagen, Germany) and transformed to *E.coli* DH5α competent cells, plated and positive clones were patched and specific clone number was given to each of the positive clone. Clones were sequenced, analyzed and cataloged.

3.5 Dot Blot/ Macroarray

Individual clones of the subtracted cDNA library were amplified, purified, and denatured by adding an equal volume of 0.6 M sodium hydroxide. Equal volumes of each denatured PCR product (about 100 ng) were spotted on Hybond™ N membranes (Amersham Pharmacia Biotech, NJ, USA) using a 96 well dot-blot apparatus (BIO-RAD Laboratories, CA, USA).). In addition, PCR products of chickpea actin cDNA (Accession No. AJ012685) using primers (5'-GGTAACATTGTCTTGAGTGG-3' and 5'-CCAGATCCGTAACAATACAC-3') and neomycin phosphotransferase (NPTII) gene from the binary vector pBI121 (Accession No. AF485783.1) using primers

(5'-TGCTCGACGTTGTCACTGAAG-3' and 5'-GTCAAGAAGGCGATAGAAGGC-3') were respectively spotted as an internal control and negative control. The membranes were neutralized with neutralization buffer (0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl) for 3 min, washed with 2X SSC, and immobilized with UV cross-linker (Stratagene, La Jolla, CA, USA).

Probes were prepared for DNA array hybridization by first-strand reverse transcription (Powerscript^{TM} RT, BD Biosciences, CA, USA) with 1μg mRNAs isolated from different samples and labeled with α^{32}P-dCTP (10 μCi/μl; 3,000 Ci/mmol). Radio-labeled cDNAs were purified by Sephadex G-50 (Amersham Pharmacia Biotech, NJ, USA), suspended in pre-hybridization buffer (7% SDS, 0.3 M Sodium phosphate pH 7.4, 1 mM EDTA) and hybridized at 60°C for overnight. The membranes were then washed three times with washing buffer (1X SSC, 1% SDS, 20 min each at 60°C). Autoradiographs were scanned employing a FSMI (Fluor-S-Multiimager, CA, Bio-Rad, USA) to acquire images and signal intensities analyzed by subtracting background noise. Actin cDNA was used as the internal control whose subtracted volume value was used for comparison with the control values. Differential screening and expression pattern data were generated as means (±SD) of the three independent experiments to ensure biological and technical replications. A paired Student's *t*-test on $\log_2$-transformed data was applied to determine if statistical differences between expression ratios of each treatment and control pair were evident. Genes significantly different from controls in any of the treatments were selected and presented in Table 1. The following two criteria were chosen to demarcate differentially expressing genes based on previous report (Major and Constabel, 2006): (1) a greater than two fold induction level; and (2) a $P < 0.05$ level of significance as determined by a *t*-test for three independent experiments. Expression profiles of stress inducible cDNAs were also analyzed by clustering performed using SOTA (Self organizing tree algorithm) by TIGR Multiple Experiment Viewer version 3.0 using complete linkage (available at http://www.tigr.org/software/tm4/menu/TM4).

3.6 Full-length gene isolation

3.6.1 5′-RACE

Using the 5′ RACE System Version 2.0 (Invitrogen, USA) cDNA end of the genes were amplified according to the manufacturer's instructions with minor modifications according to the experimental need.

3.6.1.1 First Strand cDNA Synthesis

In a thin walled 0.2 ml PCR tube, 2.5 pmoles of GSP1, 2.5 µg of RNA (preferably from the time period of the sample where the clone has high mRNA transcript level) were added and volume was made up to 15.5 µl with DEPC-treated sterile water. The mixture was incubated for 10 min at 70°C in thermal cycler to denature the RNA and immediately chilled on ice for 1 min. After brief centrifugation, following components were added in the order given- 2.5 µl of 10X PCR buffer, 2.5 µl of 25mM $MgCl_2$, 1 µl of 10mM dNTP mix, 2.5 µl of 0.1M DTT and mixed gently. After quick spin, this mixture was incubated at 42°C for 1 min then 1 µl of SUPERSCRIPT™ II Reverse Transcriptase was added and incubated at 42°C for 50 min. This reaction was terminated by incubating at 70°C for 15 min and after a brief spin 1 µl of RNase H was added and incubated for 30 min at 37°C to remove the RNA. The mixture was collected by brief centrifugation and kept on ice. This reaction mixture can be stored at -20°C.

3.6.1.2 Purification of cDNA

The binding solution (6M NaI) was equilibrated to RT while in a 1.5 ml tube 1ml of sterile water was heated to 65°C for later use. To the first strand reaction, 120 µl of binding solution was mixed and this mixture was transferred to a GLASSMAX spin cartridge. After centrifuging column at 13,000x g for 20 seconds the flow through (saved until recovery of the cDNA was confirmed) was transferred to a microcentrifuge tube and the column was put back into the collection tube. This column was then washed with 0.4 ml of cold (4°C) 1X wash buffer four times by centrifuging at 13,000x g for 20 seconds. Then the column was washed two times with 400 µl of cold (4°C) 70% ethanol. After removing the final 70% ethanol wash from the tube, the column was centrifuged at 13,000x g for 1 min. In a fresh tube the single stranded cDNA was recovered by adding 50 µl of preheated water at 65°C and centrifuging at 13,000x g for 20 seconds.

3.6.1.3 *TdT* tailing of cDNA

In a 0.2 ml tube, following components were added and mixed gently- 6.5 µl of DEPC-treated water, 5.0 µl of 5X tailing buffer, 2.5 µl of 2mM dCTP and 10 µl of earlier GLASSMAX purified cDNA (The cDNA can be used in variable amounts according to the relative amount of RNA transcript of the desired gene). This mixture was incubated at 94°C for 2-3 min and quickly chilled on ice. After collecting the mixture by brief centrifugation at the bottom of tube 1 µl of *TdT* was added gently and the tube was incubated for 10 min at

37°C. To heat inactivate the *TdT*, reaction mixture was heated at 65°C for 10 min. After brief centrifugation the tube was kept at 4°C.

3.6.1.4 PCR of dC-tailed cDNA

In a 0.2 ml thin walled PCR tube, following components were added; 34 µl of sterilized distilled water, 5 µl of 10X PCR buffer, 1 µl of 10mM dNTP mix, 2 µl of 10 µM Nested GSP2, 2 µl of 10 µM Abridged Anchor Primer, 5 µl of dC-tailed cDNA and 1 µl of 50X Titanium *Taq* DNA Polymerase. PCR of 30-35 cycles was performed with following conditions.

<table>
<tr><td colspan="3">94°C for 2 min</td></tr>
<tr><td>Denaturation</td><td>94°C for 0.5-1 min</td><td rowspan="3">30-35 cycles</td></tr>
<tr><td>Annealing of primers</td><td>55°C for 0.5-1 min</td></tr>
<tr><td>Primer extension</td><td>72°C for 1-2 min</td></tr>
<tr><td>Final extension</td><td colspan="2">72°C for 5-7 min</td></tr>
<tr><td>Indefinite hold</td><td colspan="2">4°C, until samples were removed</td></tr>
</table>

Eight microlitres of 5′ RACE product was analyzed on 1% EtBr /agarose gel. To confirm the validity of specific amplification nested amplification was done.

3.6.1.5 Nested Amplification

After diluting the primary PCR product 100 times, nested amplification of the primary PCR product was performed using the nested primer and AUAP (Abridged Universal amplification primer) or UAP (Universal Amplification Primer) primers and keeping the PCR conditions same as in the primary PCR. The PCR products were eluted from the 0.8% agarose gel after electrophoresis and cloned into pDrive U/A Cloning vector and sequenced with M13 sequencing primers.

3.7 Southern Hybridization

3.7.1 Isolation of Genomic DNA from Chickpea

Genomic DNA was isolated as described by Poresbski *et al.*, 1997. Approximately 10 ml of preheated extraction buffer was added to 1g of finely powdered plant tissue and the suspension incubated at 65°C for 30 min. The lysate was centrifuged at 12000 rpm for 20 min

at room temperature. An equal volume of a 24:1 solution of Chloroform: isoamyl alcohol was added and mixed properly. The aqueous phase was separated by centrifugation at 12000 rpm for 5 min at room temperature. The extraction step was repeated till a clear interphase was obtained following which DNA was precipitated by addition of 2 volumes of 100% ethanol to the aqueous phase. The pellet obtained by centrifugation was air dried and resuspended in TE. RNase treatment and further steps of DNA precipitation was as described previously.

Genomic DNA was isolated as mentioned by Murray and Thompson, 1980 with some modifications. Five gm tissue was frozen in liquid nitrogen, crushed using pestle and mortar, transferred to fresh oakridge tube and 5-8 ml extraction buffer (2% CTAB, 1.4M NaCl, 20mM EDTA, pH 8.0, 100mM Tris-HCl, pH 8.0, 100mM β-ME) was added to the ground tissue. Subsequently, the tubes were transferred to 60^0C and left for 1 hr. To this, 5-8 ml of chloroform: isoamyl alcohol (24:1) was added and mixed gently for 2-3 hrs followed by centrifugation at 10,000x g for 10 min at room temperature. The upper aqueous phase was transferred to another vial, and once again DNA was extracted with 5-8 ml of chloroform: isoamyl alcohol (24:1). To the final aqueous phase 0.6 volume of isopropanol was added for precipitating the genomic DNA which was then spooled out. The genomic DNA was then washed thrice with 70% ethanol, dried in vacuum, dissolved in TE containing 10 mg/ml RNase and incubated at 37^0C for 30 min. This was followed by extraction with phenol: chloroform: isoamyl alcohol (25:24:1) and the aqueous phase was transferred to a fresh tube. Thereafter the genomic DNA was precipitated by adding equal volume of isopropanol. The pellet was obtained by centrifugation at 10,000x g for 20 min at 4^0C and washed with 70% ethanol, air dried and dissolved in TE.

3.7.2 Spectrophotometeric estimation of DNA

The quality and quantity of nucleic acid was determined by measuring the absorbance at 260 nm and 280 nm. The amount was calculated as: 1.0 A_{260} = 50μg/ml for DNA and 1.0A_{260} = 40μg/ml for RNA. The purity of nucleic acid was determined by calculating the ratio A_{260}/A_{280} for each sample.

3.7.3 Digestion of genomic DNA

Digestion of 20μg of genomic DNA was performed overnight with selected restriction enzymes. The digested DNA was precipitated by adding $1/10^{th}$ of its volume of 3 M sodium acetate, pH 5.2 and twice the volume of ice cold absolute alcohol. The sample was mixed thoroughly and left at 4°C O/N. Subsequently, they were centrifuged at 10,000 rpm for

10 min. The pelleted DNA was washed with 70% alcohol twice, air dried and dissolved in requisite volume of sterile water.

3.7.4 Gel Electrophoresis

Twelve microgram of genomic DNA digested with specific restriction enzymes was loaded on 0.7% agarose gel along with λ-*Hind* III digested DNA MW marker (TaKaRa Bio Inc., Japan) and electrophoresed at constant voltage (40 volts) for 12-16 hr. After electrophoresis the gel was stained with 0.5 μg/ml EtBr in 1X TAE buffer for 20 min and photographed under UV. Holes were punctured through the gel on various bands of DNA marker so that later size of the obtained bands can be estimated. The unwanted parts of the gel were trimmed with a razor blade. The top left hand corner of the gel was cut to serve as orientation mark during the succeeding operations.

3.7.5 Transfer of DNA to Nylon Membrane

The transfer of DNA from agarose gel to nylon membrane was achieved by the capillary transfer method as described by Sambrook and Russell (2001). The gel was then transferred to a dish containing 0.2N HCl and agitated gently on a rotary platform for 20 min. This acid depurination step brings about partial hydrolysis of the DNA before alkaline denaturation and helps in the transfer of large DNA fragments. The DNA was denatured by soaking the gel in several volumes of denaturing solution (1.5M NaCl and 0.5N NaOH) for 45 min with constant gentle agitation. The gel was rinsed briefly in sterile MQ water and then neutralized by soaking in neutralizing solution (1.5M NaCl and 1M TrisCl, pH 7.6) for 45 min with constant gentle agitation. While the gel was in neutralizing solution, a solid support larger and wider than the gel was placed inside a tray and transfer buffer (10X SSC) was added to the tray to reach about half the height of solid support. Three, 3mm sheets were soaked in the transfer buffer and were kept onto solid support one after another so that they hang on either side of the support. Air bubbles, if any, were removed with a glass rod. Without touching the nylon membrane with bare hands, Nylon membrane and three pieces of 3mm whatman sheets were cut to the size of the gel. One corner of the nylon membrane was trimmed with scissors to help later in directional alignment. The nylon membrane was floated on the surface of water and then immersed in transfer buffer for at least 5 min. The inverted gel on a glass plate was placed on the support so that it was centered on 3mm papers. The gel was surrounded but not covered, with saran wrap from all the four sides to prevent ascending of liquid directly from the reservoir to filter papers placed on top of the gel. The wet nylon membrane was placed on top of the gel and the cut corners were aligned. The air bubbles between the membrane and the gel were removed by sliding the glass rod over the membrane. Three pieces of 3mm whatman paper were soaked in 2X SSC and

placed on top of the nylon membrane. Air bubbles were smoothed out by a glass rod. A stack of blotting papers (about 2 inches high) just smaller than 3 mm whatman paper was kept on them. A glass plate was placed over the stack and a 500 g weight was put on it. The transfer of DNA was allowed to proceed for about 16 hr. The wet blotting papers were replaced periodically by fresh ones. After the transfer, the arranged transfer assembly was dismantled. The positions of marker slots on the nylon membrane were marked with a soft lead pencil and the membrane was lifted from the gel using forceps. The nylon membrane (with the DNA transferred side facing upwards) was placed on a 3mm whatman paper soaked in 2X SSC and the DNA was cross-linked in UV crosslinker at12,000 J/ cm^2 (Stratagene).

3.7.6 Radioactive labeling of DNA to be used as probe

Radioactive labelling of the DNA fragment to be used as probe was prepared using the random primers labeling NEBlot® kit (NEB Inc., U.K).The method followed was same as written above in material method for Northern analysis. By this method the DNA was usually labeled to specific activity of 10^8 to 10^9 cpm/μg.

3.8. Western blotting

3.8.1. Total protein extraction

The tissue (100mg) was frozen in liquid nitrogen and grinded in 300μl of grinding buffer (400mM sucrose, 50mm Tris pH 7.5, 10% glycerol, 2.5mMEDTA) with the help of mortar and pestle.Then PMSF was added (0.5μl for every 100μl of grinding buffer). The protein extract was transferred to fresh eppendorf tube and centrifuged at 5000rpm for 5min. to pellet down the debris.The supernatant was then transferred to fresh tube and an aliquot of 5μl was taken out in a separate tube for the estimation of protein by Bradford assay. To the rest of the protein extract, appropriate volume of 4X sample buffer (200mM Tris pH 6.8, 400mM DTT, 4% SDS, 0.025 Bromophenol blue, 20%glycerol) (grinding buffer / 3 = volume of 4X sample buffer).

3.8.2 SDS-PAGE

SDS-PAGE was performed as described by Laemmli (1970). In SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE), proteins are separated largely on the basis of polypeptide length. The electrophoresis of the protein was done using a discontinuous buffer system, in which a non-restrictive large pore gel, called a stacking gel, is layered on top of a separating gel called a resolving gel (Laemmli, 1970). The recipe for the resolving gel was consisting of: 10-12% (w/v) acrylamide/bisacrylamide (19:1), 400 mM Tris-HCl pH 8.8, 0.1% (w/v) SDS,

0.1% (w/v) TEMED and 0.1% (w/v) ammonium persulfate. The stacking gel was consisting of: 4% (w/v) acrylamide/bisacrylamide (37.5:1), 125 mM Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.2% (w/v) TEMED and 0.1% (w/v) ammonium persulfate. The protein samples were denatured by adding 1/5 volume of 5X Laemmli buffer (50mMTris HCl pH 6.8, 100mM DTT, 2%SDS, 0.1% bromophenol blue, 10% glycerol) and boiling for 10min in water bath and then run on the gel. The denatured protein extract samples were boiled at 95 °C for 5 minutes then cooled on ice and loaded into the gel. The native extracted protein samples were mixed with 10 µl of protein loading buffer (in some cases the ß-ME was omitted) and denatured at 95 °C for 5 minutes, cooled on ice and then loaded on the gel. The electrophoresis was performed at 15-20 mA in 1x SDS-PAGE running buffer until the bromphenolblue band run out of the gel. 6 µl prestained protein ladder was loaded on each gel for the estimation of the size of the separated proteins. Initially the proteins were run at low voltage so that the proteins get stacked and when the proteins enter the separating gel then the voltage was increased.When the run is over the gels were stained with Coomassie brilliant blue R-250 (CBB-0.2%, 50% methanol, 10% acetic acid and destained as described by Laemmeli).

3.8.3 Blotting

Western blotting was performed using the Super signal west pico chemilumiescent substrate kit and following the instructions as described in user's manual provide by Pierce. The samples were then run on SDS-PAGE gel and transferred to C++ extra nitrocellulose membrane at 100 mAmps for 5hrs in transfer buffer (Tris 7.56g, Glycine 47g, 20% methanol in 2.5 litres) in (Bio Rad apparartus).The membrane was stained with Ponceau to confirm the protein transfer and then washed with sterile milliQ water.The membrane was then kept for 1hr in 20ml blocking buffer with 0.05% Tween 20 at room temperature with shaking. The blocking reagent was removed and the primary Ab diluted (1:5000) in 15ml blocking buffer with 0.05% Tween 20 was added and incubated for 1hr with shaking at room temperature.The membrane was then washed with 10ml of wash buffer (25mM Tris pH7.2, 0.15M NaCl) for 5 times.The secondary Ab conjugated with HRP diluted (1:30,000) in 15ml blocking buffer with 0.05% Tween 20, was then added and incubated for 1hr with shaking at room temperature. The membrane was washed with 10ml of wash buffer 5times at room temperature. The working solution of substrate was made by mixing 1:1 peroxide solution and luminol/ enhancer solution and the blot was incubated in that working solution for 5min.The blot was then removed from the working solution and covered with saran wrap in cassette and exposed to X-ray film for 60 sec.

3. 9 Promoter isolation by Genome Walking

The promoter was isolated using Universal Genomewalker™ Kit (Clontech, USA). From this kit a pool of uncloned, adaptor-ligated genomic DNA fragments were obtained, which were later used for isolation of gene specific promoter. Basically five steps were performed to make genomic library.

3.9.1 Determination of quantity and purity of genomic DNA

High quality genomic DNA was isolated from chickpea using the protocol given in section 3.9.1. The quality of genomic DNA was checked running it on agarose/EtBr gel along with control genomic DNA on the gel. 0.1 µg of each genomic DNA was loaded. The DNA obtain was intact as no smear was observed.

3.9.2 Digestion of genomic DNA

In four different 1.5 ml sterile tubes, four digestion reactions were set up using the enzymes *Dra*I, *Eco*RV, *Pvu*II and *Stu*I. All these enzymes produce blunt ends. In each reaction following components were combined:

Genomic DNA (0.1 µg/µl)	25 µl
Restriction enzyme	8 µl
Restriction enzyme buffer	10 µl
Deionized H_2O	57 µl
Total volume	100 µl

Mixed gently and incubated at 37^{0}C for 2 hr. The tubes were tapped gently and again kept for 16-18 hr. From each tube 5 µl reaction mix was checked for digestion on 0.5% agarose/EtBr.

3.9.3 Purification of DNA

To each of the reaction tube, an equal amount (95 µl) of phenol was added and slowly vortexed for 10 sec. After brief spin aqueous layer was transferred to a new tube and again the above step was repeated to isolate protein contamination. After second extraction, 2 volumes (190 µl) of ice cold 95% ethanol, 1/10 volumes(9.5 µl) of 3M NaOAc, and 20 µg of glycogen was added and vortexed slowly for 10 sec. To pellet the digested DNA, the tubes were centrifuged at 15,000 rpm for 10 min and the supernatant was decanted. The pellets obtained were washed with in 100 µl of ice cold 80% ethanol and centrifuged at 15,000rpm

for 5 min. The supernatant was decanted, pellet was air dried and dissolved in 20 µl of TE (10/0.1, pH 7.5). After a slow speed vortex for 5 sec, 1 µl of the Digested DNA quality and quantity was checked on a 0.5% agarose/ EtBr gel.

3.9.4 Ligation of Genomic DNA to Genome-walker adaptors

For ligation, 4 µl of each digested and purified DNA was taken in 0.5 ml tubes and to each of the four tubes was added the following- 1.9 µl Genomewalker adaptor (25µM), 1.6 µl 10 Ligation buffer and 0.5 T4 DNA ligase (6 units/µl).

In order to find out cis-acting element organization of CaAr131 genome walking was performed using the method described in Clontech genome walking kit. A pair of primers was designed and the PCR was performed. The PCR product was cloned in pGEMT and has been given for sequencing. The sequence was analyzed on PLACE signal scan search and PLANTCARE search program

3.10 Functional analysis of 5' upstream region of *CaAr131* in tobacco

3.10.1 Cloning of different deletions of 5' upstream region of *CaAr131* in pBI101.2 vector

To characterize the promoter further and to determine the regulatory properties of *CaAr131* promoter, four deletions of its upstream region has been cloned into the binary vector pBI101.2 (Jefferosn *et al.*, 1987) as a transcriptional fusion in front of a promoterless β-glucuronidase (GUS) gene. The resulting construct has been used to transform the tobacco by the method given by Gelvin *et al.*1987.

3.10.2 Transformation of *Agrobacterium*

Recombinant plasmids constructed in pBI101.2 and pBI121 were transferred into *Agrobacterium* by the freeze-thaw method. For the preparation of competent cells, *Agrobacterium tumefacians* (LBA4404) was grown in 50 ml YEB medium at 28°C with vigorous shaking until the OD_{600} reached 0.5. The culture was chilled on ice and centrifuged at 3000 X g for 5 min at 4°C. The pellet was resuspended in 1 ml of ice cold $CaCl_2$ (20 mM) and 0.1 ml aliquots were dispensed in pre- chilled eppendorf tubes and stored at -80°C.

Transformation of *Agrobacterium* with various plasmid constructs was done by mixing 1 µg of DNA with competent cells followed by immediate freezing in liquid nitrogen. Subsequently cells were thawed by incubating the eppendorf tube at 37°C for 5 min. Thereafter 1 ml of YEB medium was added to the tube and incubated at 28°C for 1 h. Cells

were spread on a YEB agar plate supplemented with 50 μg/ml kanamycin and 25 μg/ml rifampicin and incubated at 28°C. Transformed colonies that appeared after 1-2 days were analyzed either by PCR or by colony hybridization and the positive colonies were confirmed by restriction digestion of the purified recombinant plasmid.

3.10.3 Agrobacterium mediated leaf disc transformation.

One positive colony of *Agrobacterium tumefacians* from each construct was used to transform tobacco by leaf disc method (Horsch *et al.,* 1985) and *Arabidopsis* by vacuum infiltration method (Clough and Bent, 1998). A single colony was inoculated into 30 ml of liquid YEM medium with kanamycin plus rifampicin and grown for one day 28°C. One ml of this bacterial culture was inoculated into fresh medium and grown overnight. Uniformed sized, healthy, young tobacco leaves were harvested and leaf squares (1 cm^2) were prepared. The leaf squares were then immersed in *Agrobacterium* culture containing various constructs (1:10 dilution of overnight grown culture in liquid MS medium for 5 min. Leaf squares were taken out, blot dried and placed upside down on theMS104 (MS supplemented 1 mg NAA, 1 mg BAP) culture plates. The leaf explants were cultured for 2-3 days on medium without antibiotics.

3.10.4 Selection and Plant Regeneration

Immediately after co-cultivation, explants were transferred to MS medium with BAP and NAA containing 50 μg/ml kanamycin and 250 μg/ml cefataxime. After 3-4 weeks, shoots with a defined stem were removed from explants and placed on MS rooting medium. As soon as the roots started appearing, the plantlets were removed from culture jars and rinsed in water and planted in pots containing vermiculite. After 7-10 days of hardening the plants were transferred to soil and grown under green house condition to allow production of seeds.

3.10.5 Seed Plating in Culture Medium

Seeds (80 mg) of *Tobacco* were surface sterilized with 2 ml of sterilization solution [30% v/v bleach (100% bleach contain 5% hypochlorite) with 1 μl/ml of 20% Triton X- 100] and gently mixed by inversion for 20 min. The seeds were allowed to settle for 1 or 2 min before decanting the bleach. The tubes with seeds was filled with sterile water and mixed. Washing of seeds was repeated several times till the bleach smell disappeared. The seeds were finally resuspended in required volume of sterile water and then plated on GM medium containing kanamycin. Sterile water was allowed to evaporate in laminar flow hood and plates transferred to the culture room. The seedlings, which survived on kanamycin, were transferred to soil pots.

3.10.6 GUS histochemical assay

Gus activity staining was performed to screen the putative positive transgenic plants for verification of expression of *gus* gene (Jefferson, 1987).This histochemical assay was carried out in intact tissues (organ or whole seedlings or free hand cut sections).The tissue from the control and transgenic plants were submerged in a fixative buffer in microtiter plates kept on ice (2% formaldehyde, 50mM sodium phosphate buffer pH 7.0, 0.05 % Triton X-100), and vacuum infilterated for 4-5 min. The fixation buffer was removed and tissue was washed twice with 50mM phosphate buffer pH 7.0.Then the tissue was stained by adding 600µl of X-gluc buffer (1.5mM X-gluc , 50mMphosphate buffer pH7.0,0.1% Triton X-100) and vacuum infilterated for 10min.The sample tissue with X-gluc buffer was kept at 37°C overnight in darkness. Then X-gluc buffer was removed and the tissue was kept in 70% ethanol to remove the chlorophyll.

Chapter 4: Differential transcript profiling in the Ascochyta-resistant and -susceptible chickpea lines

4.1 Introduction

Plants are constantly under attack by pathogens, which can potentially cause significant crop losses. Upon pathogen attack, plants activate an array of inducible defense mechanisms. Following detection of a pathogen, the hypersensitive response is a rapid and efficient plant resistance mechanism leading to cell death at the site of infection (Heath, 2000). Among the rapid defense mechanisms triggered in plants are productions of reactive oxygen species (ROS), expression of defense related proteins (PR-proteins), cell wall reinforcement, synthesis of antimicrobial compounds and enzyme inhibitors (Glazebrook, 2005, Dangl and Jones, 2001). Evidences suggest that these defense responses result from a highly complex and interconnected networks of signal transduction pathways. Therefore, the signaling pathways leading to active defense in the host could differ depending on the lifestyle of the pathogen. A model which classifies pathogens into necrotrophs and biotrophs proposes that the lifestyle of a pathogen might be a predictor of whether the pathogen will be affected by the jasmonate response or not (McDowell and Dangl, 2000). In support of this model, it has been demonstrated in *Arabidopsis* that the salicylic acid-dependent pathway is required for defense against *Peronospora parasitica* and *Erysiphe orontii*, two biotrophic fungi. In contrast, a jasmonate/ ethylene-dependent pathway has been shown to be effective against *Botrytis cinerea*, via the functional ethylene insensitive (*EIN2*) gene (Thomma *et al.*, 1999a), whereas a jasmonic acid (JA)-dependent but ethylene-independent pathway, in parallel with a pathway leading to camalexin production, provides resistance against *B. cinerea* and *A. brassicicola* (necrotrophic fungal pathogens).

4.1.1 *Ascochyta* blight in chickpea

Among many diseases that affect chickpea, *Ascochyta* blight (AB) is most devastating, causing up to 100% yield losses in severely affected fields. The disease initially appears in small areas within the affected field and spreads rapidly when cool and wet conditions (15-25°C and >150 mm rainfall) prevail (Kaiser, 1973, 1992). Intermittent rain splashes facilitate dispersal of conidiospores. Expanding necrotic areas appear as disease symptoms on stems which depending upon pathotype aggressiveness and cultivar susceptibility, often girdle the stem. This may result in stem breakage and, as a consequence, in heavy yield loss (Akem, 1999). Symptoms of AB can develop on all aerial parts of a plant. Seed borne infection leads to brown lesions at the stem base of emerged seedlings. Subsequently, the lesions enlarge in size and girdle the stem causing its breakage and death of the plant. Plants are attacked at any growth stage, depending on the inoculum availability. The presence of a teleomorph in the *A.rabiei* life cycle (Fig. 4.1) contributes to variability within the pathogen population, which may generate new combinations of virulence genes and thus

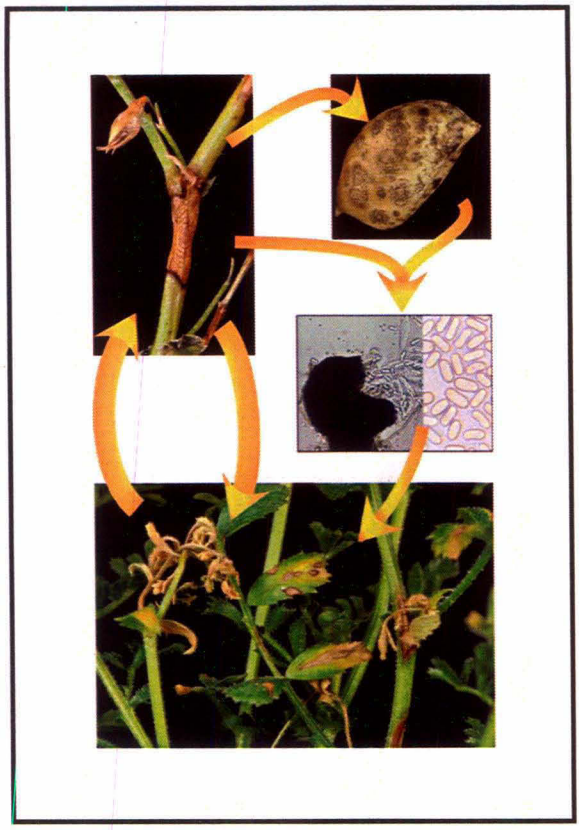

Figure 4.1. Disease Cycle of Blight of Chickpea caused by Ascochyta.
The pathogen overwinters in seed or infected crop debris (top right) and spores produced in spring and winter infect young plants. Many cycles of spore production and infection can occur during the growing season.

the development of new pathotypes. The teleomorph also help in long term survival of the pathogen, but this stage has never been observed on newly infected plants.

4.1.2 Genetic Basis of *Ascochyta* Resistance in Chickpea

Various mechanisms explaining blight resistance in chickpea have been proposed (Ahmed *et al.*, 1952; Dey and Singh 1993; Tekeoglu *et al.*, 2002). The first genetic analysis of blight resistance in chickpea concluded that there were two dominant genes conferring blight resistance (Ahmed *et al.*, 1952). However, using an F2 population from the same parental lines it was shown that blight resistance was conferred by one dominant gene (Ahmed *et al.*, 1952). A dominant genetic mechanism for blight resistance had been supported by many other reports (Vir *et al.*, 1975; Singh and Reddy 1983). However, using a population of F2-derived F3 families it was reported that blight resistance was conferred by two recessive genes acting additively (Kusmenoglu, 1990). Later the recessive genetic nature of blight resistance, was confirmed using three recombinant inbred line (RIL) populations that were derived from crosses of resistant and susceptible lines (Tekeoglu *et al.*, 2000). Quantitative genetic studies conducted using RAPD and ISSR markers, identified three Quantitative Trait Loci (QTLs) for blight resistance (Santra *et al.*, 2000). Further studies added Sequence-Tagged Microsatellite Site (STMS) markers to the same linkage map and confirmed the two earlier identified QTLs for blight resistance (Santra *et al.*, 2000; Tekeoglu *et al.*, 2002). It was postulated that the two earlier identified QTLs were associated with the two recessive genes for blight resistance (Kusmenoglu, 1990).

Although the quantitative nature of blight resistance in chickpea was revealed as mentioned earlier (Kusmenoglu, 1990; Tekeoglu *et al.*, 2000; Flandez- Galvez *et al.*, 2003), genetic roles of the genes in pathotype-dependent blight resistance and the dominance and recessiveness of the genes could not be elucidated. This was because of dramatic changes in resistance patterns of the population depending on the pathogenic and the environmental conditions. Since, pathogenicity is a critical factor in determining the blight resistance, efforts were made to determine the pathogenicity of various ascochyta pathotypes (Jamil *et al.*, 2000). For the first time, genomic regions for pathotype-specific blight resistance in chickpea were identified, although the genetic information was insufficient to elucidate the mechanism (Udupa and Baum, 2003). This was due to unreliability of pathotype-dependent resistance patterns of the mapping population. Recently, the blight resistance pattern of the RILs derived from the cross of PI 359075(1) (susceptible to both pathotypes I and II of *A. rabiei*) × FLIP-84-92 C (2) (resistant to both pathotypes) was studied (Cho *et al.*, 2004). They found it to vary significantly, depending upon the pathogenicity of *A. rabiei* which in turn was determined by relative humidity and the pathotypes. To conclude, complete immunity of

chickpea *to A. rabiei* could not be demonstrated even in highly resistant chickpea lines and pathotypes with greater virulence always had potential to cause disease regardless of the level of resistance. In that aspect, blight resistance in each RIL seemed to be genetically predetermined at a certain level that could be overcome by pathotypes with sufficient virulence (Cho *et al.,* 2004).

4.1.3 Role of Secondary metabolites in Chickpea-*Ascochyta* Interaction

Apart from a quest to strike upon a gene conferring resistance against *Ascochyta,* researchers had also been interested in knowing the role played by phytoalexins in defense. It was suggested that pterocarpan (maackiain) and phytoalexins (medicarpin) might play an important role in chickpea fungal resistance. This was based on the detection of phenolic compounds, phytoalexin biosynthesis intermediates and specific inducible enzymes which constitute components of secondary metabolism in legumes following pathogenic attack (Kessmann and Barz, 1986). Moreover, it has been observed that pathogenic strains of *A. rabiei* can efficiently degrade chickpea phytoalexins to overcome the plant defense mechanisms suggesting their significance with respect to disease resistance. It produces phytotoxins (solanapyrones A, B and C, and cytochalasin D) to destroy chickpea defense system. These compounds seem to play a critical role in causing the disease (Alam *et al.,* 1989; Hohl *et al.,* 1991; Latif *et al.,* 1993). Thus there appear to be two different models of chickpea resistance to *A. rabiei,* the production of phytoalexins and the neutralisation of fungal toxins. Till date, the genetic and molecular basis of chickpea blight resistance is not well known. This as mentioned earlier also reflects the paucity of genetic resistance in available commercial cultivars.

4.1.4 Molecular basis of Chickpea-*Ascochyta* interaction

Many differentially expressed genes in *Ascochyta rabiei*-inoculated chickpea plants and elicited cell cultures, which were arranged into five groups, namely defense related pathways, signal transduction pathways, regulation of gene expression, catabolic pathways and primary metabolism have been isolated (Ichinose *et al.,* 2000). The cDNA clones encoding rab type and rac type small GTP-binding proteins were isolated from Ascochyta rabiei-inoculated chickpea leaves and the elicitor-treated cell cultures. Rac type transcript showed enhanced expression in inoculated leaves indicating correlation with the defense response (Ichinose *et al.,* 2000). Differential screening in chickpea plants infected with *Ascochyta rabiei* has lead to the identification of cDNAs coding for two glycine-rich proteins (GRPs), GRP1 and GRP2. The glycine-rich-repeats of these proteins are known to be implicated in cell wall fortification by oxidative cross-linking (Cornels *et al.,* 2000). Its has

been found that during fungal infection of chickpea plants expression of *PR-5a* and *PR-5b* genes proceeds much faster in an *A. rabiei* resistant cultivar than in susceptible one (Hanselle and Barz., 2001). It has been reported that copper amine oxidase (*CuAO*) plays an important role in chickpea for protection against *Ascochyta rabiei.* This was demonstrated by its in vivo inhibition by 2-bromoethylamine after inoculation of resistant variety with *Ascochyta rabiei.* This resulted in extensive cell damage in sclerenchyma and cortical parenchyma tissues (Rea *et al.,* 2002). Quantitative methods for the analysis of expression profiles have the capacity to improve the overall understanding of the coordinated defence response at a molecular level, as illustrated by the successful application of cDNA microarray analysis to study the defence response of tomato (Gibly *et al* ., 2004), cassava (Lopez *et al* ., 2005) and soybean (Moy *et al* ., 2004). Recently, Coram and Pang (2005a) reported the characterization of a set of chickpea unigenes and a smallscale cDNA microarray study of the chickpea response to *A. rabiei* infection (Coram and Pang, 2005b).

4.1.5 Involvement of Defense regulators during Chickpea-*Ascochyta* Interaction

Recent studies of signalling events inducing local and systemic defence responses in plants have led to the identification of salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) as key regulators of these pathways (Schenk *et al.* 2000; Salzman *et al.* 2005; Jalali *et al.* 2006). Subsequently, Cho and Muehlbauer (2004) studied the response of their selected genes to treatment with SA and methyl jasmonate (MeJA), but found no correlation to the fungal responses. In addition, the authors found that *Ascochyta* blight resistance in RILs generated from the cross of a resistant and susceptible line did not cosegregate with the expression of the genes induced either by *Ascochyta* blight inoculation or the signal chemicals. As a result, the authors proposed that fungal resistance in chickpea may be controlled by constitutive or unknown resistance mechanisms independent of SA- or JA-mediated signaling. Coram and Pang (2007) used the same microarray previously used for the *Ascochyta* blight study to profile potential changes after treatment with SA, MeJA and an ethylene precursor, aminocyclopropane carboxylic acid (ACC). They determined that genotypes resistant to Ascochyta blight displayed a far greater range of defence-related gene inductions with all treatments compared with controls and the susceptible genotype. This indicated that genes within the conserved SA, MeJA and ethylene-type pathways were also likely to be involved in the defence response against *Ascochyta* blight. Furthermore, there was evidence for the involvement of resistance mechanisms other than SA, MeJA and ACC.

4.2 Results

4.2.1 Construction of subtracted cDNA library and dfferential screening

We screened for genes that are either differentially expressed at the basal level or shows differential expression upon *Ascochyta* infection in the resistant and susceptible varieties of chickpea using the PCR-based method Suppression Subtractive Hybridization (SSH) (Diatchenko *et al.,* 1996). SSH is a powerful technique that produces a library of cDNA clones that are (putatively) differentially expressed in one, tester (resistant), mRNA-population compared to a second, susceptible, population (PCR amplification. Three hours post-inoculation (p.i.) and basal level of expression were chosen for the screen because it is immediate early and constitutive in the disease process. Although PCR-select cDNA subtraction is a powerful tool for identifying differentially expressed genes, subtractive products may contain some cDNAs that are common to or have similar levels in both tissue types. To avoid analysis of false positive clones and to provide further data on relative expression level of the cloned cDNAs, we performed a further screen using a cDNA macroarray [Fig. 4.3 (A) and (B)]. Therefore, all the transformants obtained by SSH were subjected for differential screening and sequencing. After screening for differential expression either at the basal level or upon *Ascochyta* infection sequencing, 84 unique genes were identified by BLASTx analysis. Similarity search of these clones resulted in identification of transcripts not previously reported to be induced during *Ascochyta* infection and some functionally unknown transcripts. In addition to this, some transcripts already known to be responsive to necrotrophic fungus in other plants were also obtained which appears to support the validity of the subtracted cDNA library.

4.2.2 Identification of the Differentially Expressed Genes

The identified genes might play a variety of functions during resistance response during fungal attack. All the identified genes were grouped into seven functional classes based on their respective roles in defense or resistance: Defense-related, Gene/protein expression, Signal transduction, Abiotic stress, Miscellaneous, Unknown. (Fig.4.2 (B) and Table 4.1). The major functional category corresponded to genes involved in defense, secondary compound synthesis and cell wall fortification and was classified as defense-related (22%). Gene transcription and translation (14%) was found to be one of the most important classes of genes, reflecting the immediate early response of chickpea transcripts in the gene expression and regulation. In addition, another category comprised of genes involved in signaling (5%) and a significant fraction of genes were involved in abiotic stress (5%). The genes for which no known function could be assigned were grouped under the unknown

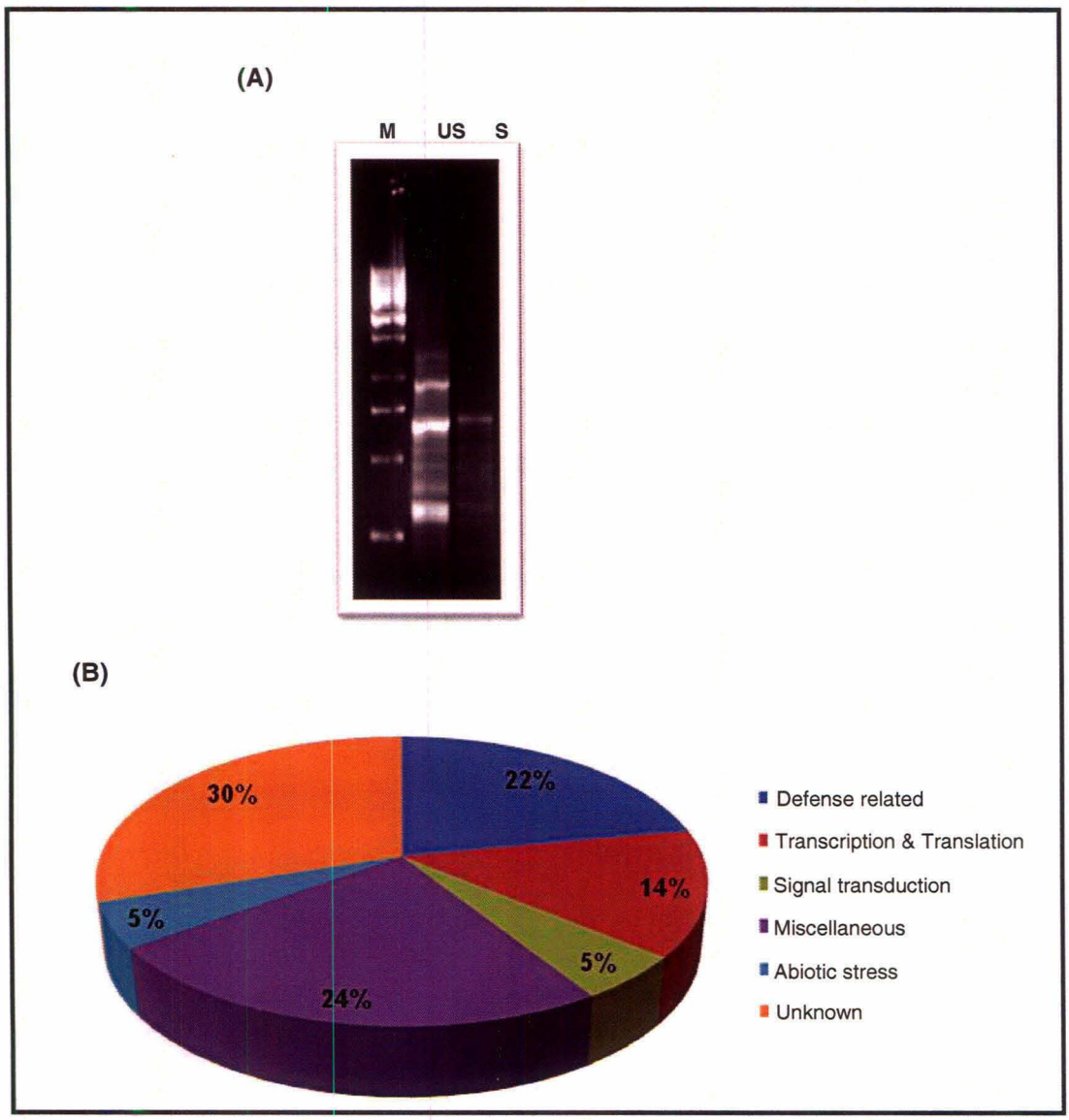

Figure 4.2. (A)1% EtBr-agarose gel showing the smear of amplified subtracted cDNAs. (M; 1 Kb ladder.US; "unsubtracted cDNA", S; "subtracted cDNA" obtained after primary and secomdary amplifications). **(B)Functional cataloging of *Ascochyta*-responsive genes.** The identified *Ascochyta*-responsive genes were assigned a putative function based on their homology and functionally categorized as presented in the pie-chart.

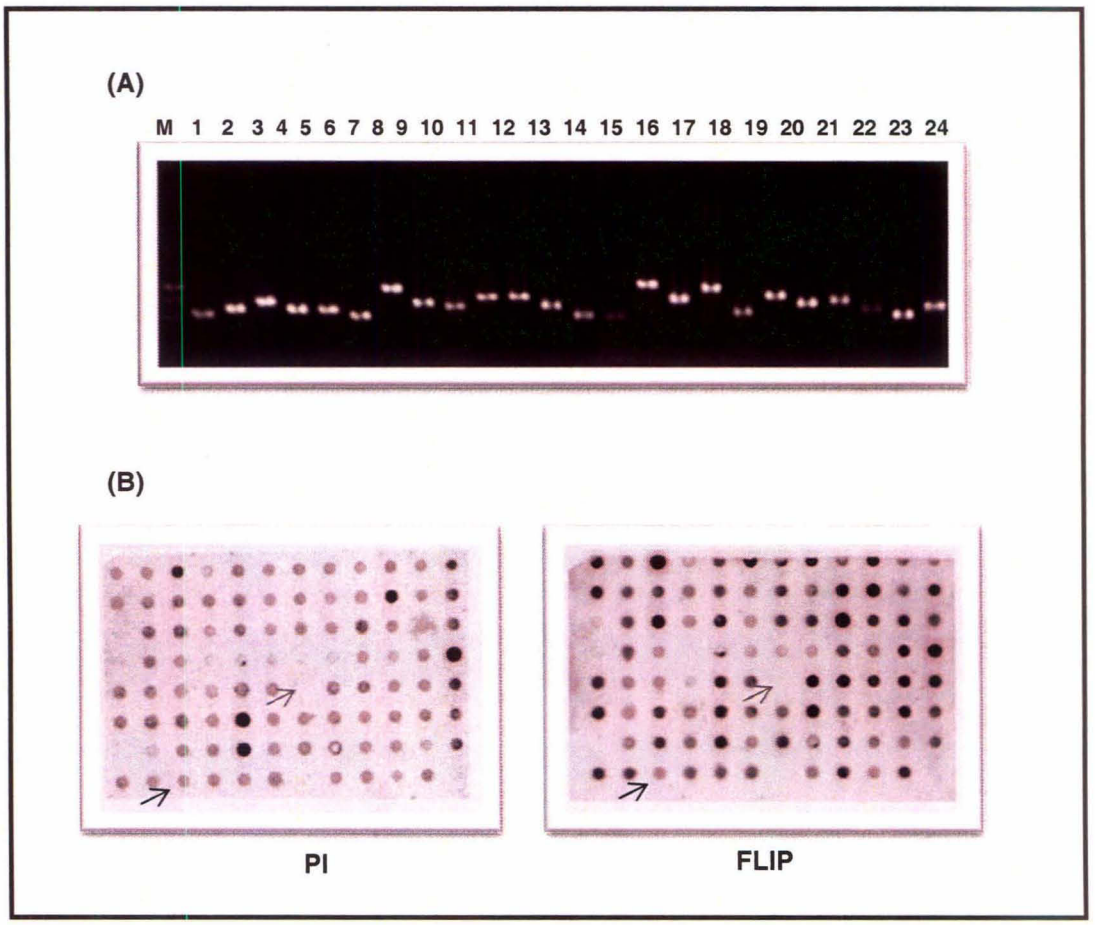

Figure 4.3. (A). 1% EtBr stained gel showing PCR amplification of the positive clones. (B) Dot blot showing fold induction of the transcripts obtained from subtraction library. Positive clones were PCR amplified using M13 sequencing primers. Approximately 100 ng of visually quantified PCR product was blotted using 96 well Dot Blot apparatus. A replica of the blot was also made. The two blots were respectively hybridized with radiolabelled first strand cDNA probe prepared from 1µg mRNA from PI (susceptible) and FLIP (resistant) lines. The black arrows indicate the positive control and the grey arrows indicate the negative control in the blots.

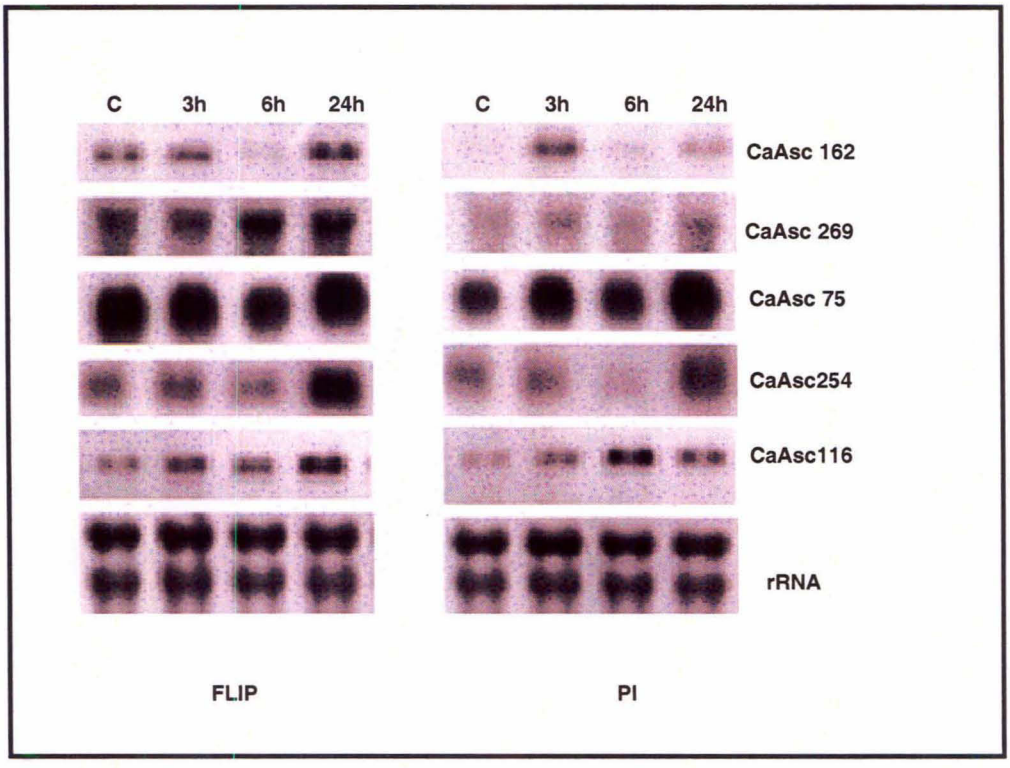

Figure 4.4. Differential transcript profiles during *Ascochyta* infection in the resistant (FLIP) and susceptible (PI) varieties of chickpea. RNA gel-blot analysis of a few selected genes. rRNA of the same blot was used as the loading control.

category that accounted for 30% of the *Ascochyta* responsive transcriptome and 24% of genes were classified as miscellaneous.

4.2.3 Differential transcript level of the identified genes in resistant and susceptible varieties of chickpea

All the unique genes isolated by SSH strategy were used to study their comparative transcript levels (by DNA macroarray and northern blots, Fig. 4.4 and table 4.1) at the basal level in the resistant germplasm line and susceptible germplasm line. To demarcate differentially expressing genes a greater than two fold induction level was chosen based on previous report (Major and Constabel, 2006). Among these, 33 subtracted cDNAs showed differential expression levels significantly between resistant and susceptible lines, suggesting that these genes are expressed constitutively in the resistant germplasm line but not in the susceptible line and therefore indicating their role in blight resistance by constitutive resistance mechanism. Out of these 35, five genes are defense related: one PR protein (PR-10), two LRR domain containing protein, one gene involved in synthesis of secondary metabolites (Phenylalanine ammonia lyase) and one gene playing role in cell wall fortification (proline rich protein). Genes having functions in gene regulation (bZIP transcription factor, AT-rich element binding factor, translation initiation factor SUI I) and signal transduction (putative ADP ATP carrier protein) also showed differential basal transcript level among resistant and susceptible lines. Eleven genes which show differential expression are of unknown function thirteen fall under miscellaneous category (e.g. glyceraldehyde-3-phosphate dehydrogenase, microtubule binding (APG8H), putative vacuolar ATPase subunit H protein mRNA, putative NADH-dependent hydroxypyruvate reductase mRNA). One gene showing homology with chaperonin 60 expressed constitutively in the resistant line but not in the susceptible line. Although these genes do not show constitutive expression in the susceptible lines but they do show induction in their mRNA level upon *Ascochyta* infection. There are few genes which show similar expression pattern in both resistant and susceptible lines (e.g. putative zinc dependent protease, DJ-1 family protein/protease-related, Metallothionein-like protein, Cytokinin regulated Kinase, Phosphoglucan water kinase, ERD15 protein mRNA, Syringolide induced protein, heavy-metal-associated domain-containing protein and some unknown genes). As they have similar expression patterns in the two germplasm, we predict their role in basal defense against Ascochyta blight.

4.2.4 Comparative Expression Analysis in response to JA and SA

A preliminary data using reverse northern showed that among the two defense regulators, JA altered more transcripts than SA (Table I). Out of 84 genes, 51 were

upregulated by MeJA (60.71%); and 20 by SA (23.8%). Fourteen genes showed mRNA increases in both the treatments, including few well known defense-related genes (e.g. two LRR domain containing proteins, PR-10, proline rich protein, WRKY 20) and some genes from miscellaneous class and others from unknown class. There are 37 genes which show induction exclusively by JA and seven genes by SA alone. These results suggest that both JA and SA are associated in this interaction either individually at different stages of infection or they are involved in the signaling pathway crosstalks. There are some genes (22) which neither show induction by JA nor by SA significantly. We have selected only one time point to study the expression pattern i.e. 6h of treatments. Therefore it is possible that the genes which do not show induction by either of the signaling compounds in this study, might be getting induced at some other time points or these genes could be regulated by some other defense regulators. To confirm the expression data, a subset of five selected genes was analyzed by northern blots. The results demonstrated congruence between both methods, with the exception of a few minor differences (Fig. 4.5). However, we need to confirm these data by using three independent macroarray experiments.

4.3 Discussion

The aim of this work was to elucidate the responses of chickpea to the necrotrophic fungus *Ascochyta rabiei*, using transcript profiling approach. On the basis of previous reports (Cho and Muehlbauer, 2004) we chose two lines of chickpea, a partially resistant line FLIP84-92C (2), and a susceptible line PI359075 (1). Use of the SSH technology followed by differential hybridization screening resulted in the identification of 35 ESTs that are preferentially expressed constitutively in the resistant line but not in the susceptible variety. These results further strengthen the quantitative disease resistance conferred by multiple genes and constitutive resistance mechanism in blight resistance in chickpea. Previous report by Cho *et al.*, (2005) also predicted and then proved the constitutive resistance mechanism by examining constitutive transcript levels in the resistant and susceptible germplasm lines and found that a cDNA-AFLP fragment homologous to *Arabidopsis* flavanone 3-hydroxylase (F3H) showed higher constitutive expression in the resistant lines than the susceptible lines which is congruent with our studies. They also showed that F3H is located at the end of linkage group 5, which is one of the linkage group associated with blight resistance. Therefore, from our point of view, the most interesting genes identified in this analysis were those showing significant effects of both genotype and infection. Genes that showed only a genotype effect may have reflected the genetic background, whereas genes exhibiting only a treatment effect may have reflected the overall plant response to infection. It is therefore conceivable that genes showing significant effects of both genotype and treatment might play

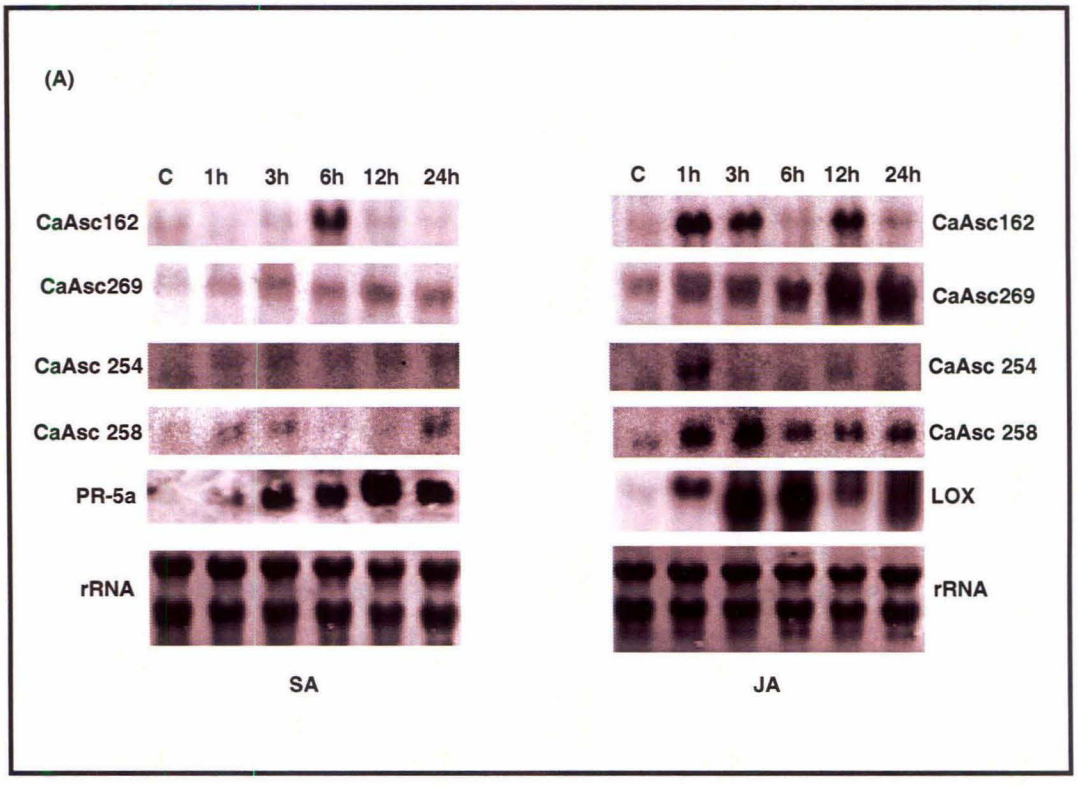

Figure 4.5. Contribution of Jasmonic Acid (JA) and Salicylic Acid (SA) to *Ascochyta*-related gene expression. (**A**) RNA gel-blot analysis of a few selected genes after JA and SA treatments. The lipoxygenase (*LOX*) gene was used as a marker for JA treatment and PR-5a for SA treatment. rRNA of the same blot was used as the loading control.

a role in the mediation of defense mechanisms rather than simply respond to the pathogen. In this context, many genes were identified which are predicted to be involved in resistance. As for example, two LRR-domain containing protein (polygalacturonase inhibiting protein and $Hs1^{pro-1}$ protein), PAL (phenyl ammonia lyase), bZip transcription factor, AT-rich element binding factor 1 (ATF1), and an esterase/lipase/ thioesterase have been identified. Lipases are hydrolytic enzymes that break down triacylglycerol into fatty acids and glycerol. *PGIP*s (one of the LRR-domain containing protein identified in this study) are ubiquitous plant cell-wall proteins that are directed against fungal polygalacturonases (PGs) which are important pathogenicity factors. The inhibiting activity of *PGIP*s directly reduces the aggressive potential of PGs. *PGIP*s fall into the category of resistant genes with LRR domain and kinase domain also together with *Cf-9* of tomato and *Xa21* of rice. Another LRR-domain containing protein found in this study presents strong homology with $Hs1^{pro-1}$ protein encoded by a nematode resistance gene isolated in sugarbeet (Cai *et al.,* 1997). Interestingly, phytoalexins, an end product of the phenylpropanoid pathway, was also found to be involved in the resistance of chickpea against blight disease. We found the higher expression of PAL (a component of phenylpropanoid pathway) gene in the resistant germplasm line as compared to the susceptible line. Esterase/lipase/ thioesterases are hydrolytic enzymes that break down triacylglycerol into fatty acids and glycerol. The *A. thaliana* genes enhanced disease susceptibility 1 (*EDS1*) and phytoalexin-deficient 4 protein (*PAD4*), which encode lipase-like proteins, were found to be required for expression of multiple defense responses and basal plant disease resistance (Falk *et al.,* 1999; Jirage *et al.,* 1999; Feys *et al.,* 2001). PR-10 and proline-rich proteins also showed differential expression between resistant and susceptible lines. In chickpea, the H_2O_2 from an elicitor-induced oxidative burst has been shown to control directly the insolubilization of a proline-rich protein in cell walls and be induced in response to a fungal pathogen (Coram and Pang, 2006), and thus the proline-rich protein in this study may be induced by the oxidative burst and be effective in limiting penetration during an incompatible interaction. One of the ESTs showing homology to Peroxiredoxin Q also showed differential behavior among the two lines. Peroxiredoxins are ubiquitous thioredoxin- or glutaredoxin-dependent peroxidases, the function of which is to destroy peroxides. The expression of this protein and of type II peroxiredoxin is modified in response to an infection by two races of *Melampsora larici-populina*, the causative agent of the poplar rust. In the case of a hypersensitive response, the peroxiredoxin expression increased, whereas it decreased during a compatible interaction (Rouhier *et al.,* 2004).

In this study, few more ESTs were identified that do not show differential behavior at the constitutive level between the resistant and susceptible lines but shows induction upon Ascochyta infection. Based on their transcription pattern they may be classified as general

defense-related genes. We obtained two proteinases, two wound-induced proteins, glutamate decarboxylase, matallothionein, PR-5a which were categorized as defense-related. We predict their function in basal defense as they show induction even in the susceptible lines and do not show genotype specificity. Proteases are known to be involved in senescence and programmed cell death but they play crucial roles in biotic stresses (Grudkowska and Zagdanska, 2004). Metallothionins are involved in metal homeostasis and detoxification and form a component of basal defense. PR-5 and *nsLTP* also showed induction upon *Ascochyta* infection. These are well known pathogen-related proteins and they possess antifungal activities (Anand *et al.*, 2004).

Some of the regulatory proteins and transcription factors are known to play important roles in disease signaling by controlling the transcriptional activity of defense-associated genes. Here, we observed differential constitutive expression of two of the transcription factors in the resistant line and not in the susceptible line. There is a report describing the fact that ATF1 acts as a complete transcription activator and indicates that there is significant effect of ATF1 on the activation of chalcone synthase (CHS) promoter in *Pisum sativum*. G box binding (GBF) factors are also involved in the activation of CHS promoters and they are involved in plant defense through JA pathway (Boter *et al.*, 2004). CHS is a key speed-limiting enzyme in the phenylpropanoid pathway which plays an important role in plant defense response against pathogens. In the CHS1 promoter, there is an AT-rich element which is required for the maximal elicitor-mediated activation. bZIP transcription factors also play crucial role in plant defese. The promoter of the *Arabidopsis PR-1* gene contains a binding site for TGA-bZIP factors (the sequence TGACG) that serves as a positive *cis*-acting element for SA induction.

Among the ESTs categorized as playing role in signal transduction, putative ADP ATP carrier protein showed differential behavior between the two lines. But others including MAPK 3, cytokinin regulated kinase and phosphoglucan water kinase do not show any difference in the constitutive expression between the two germplasms. Among the MAPKs previously studied, AtMPK4 (Gupta *et al.*, 1998) and AtMPK6 (Ulm *et al.*, 2002) play a role in plant defense responses. AtMPK6 appears to be implicated in the activation of both local disease resistance, regulated by specific *R* genes, and basal resistance (Menke *et al.*, 2004). Moreover, a number of different pathogenic stimuli activate AtMPK6 (Nhüse *et al.*, 2000), and SA inducible protein kinase (SIPK) in tobacco (orthologous to MPK6 in *A. thaliana*).

Studies have demonstrated that there is an overlap between stress and defense pathways indicating an extensive cross-talk between the plant defenses and wounding/stress pathways. Many cDNA clones show homology with genes which are known to be involved in abiotic stress as ERD15 protein mRNA, Syringolide induced protein mRNA, Chaperonin 60

and heavy-metal-associated domain-containing protein. Chaperonin 60 among them show differential constitutive expression in the resistant line and not in the susceptible line indicating its role in constitutive resistance mechanism against blight disease. Few cDNAs involved in the photosynthetic pathway and glycometabolism have also been identified as Chl a/b binding protein. A number of clones isolated showed homology with unknown proteins and some others of miscellaneous functions whose direct role in defense is not known. These induced transcripts are proposed to play crucial role during *Ascochyta* infection as they either exhibit differential constitutive expression or get induced by Blight disease. Moreover, they also show induction upon exogenous application of signaling compounds. Further study is required to understand whether and how they function during *Ascochyta*-resistance response.

When we compared the response of these isolated genes on exogenous application of JA and SA, involvement of both the defense regulators was identified. There is more number of genes (37) which get induced by JA. SA induces 22 genes and 14 genes are commonly induced by SA and JA both. Ascochyta rabiei is a necrotrophic fungus, so JA-mediated signaling is expected. But during the early phase of infection HR can be seen (Singh, 2005) which accounts for SA-mediated signaling. Synergistic induction by both the defense regulators suggests a crosstalk among them and it has been reported previously (Coram and Pang, 2007). Since some of the genes showed induction by none of them involvement of some other defense regulators such as ethylene and some phytohormones is implicated. These observations indicate that although JA and SA are partially involved in the signaling cascade, they are not responsible for mediating the entire response that may lead to resistance. These results are in congruence with previous studies (Coram and Pang, 2007 and Cho and Muehlbauer, 2004). The necrotrophic nature of the Ascochyta may contribute to the involvement of unknown mechanism, and some defense responses may occur constitutively or without the need of known signaling pathways.

In conclusion, genes showing higher constitutive expression in the blight resistant germplasm line, FLIP84-92C (2) compared to the blight susceptible line, PI359075 (1) and genes showing induction upon *Ascochyta* infection were identified. Genes with constitutive higher expression in the resistant lines are predicted to be directly involved in the resistance and genes showing no difference between resistant and susceptible line but showing induction after *Ascochyta* infection, are predicted to be involved in basal defense. Higher accumulation of some of the transcripts at the basal level indicates that plants are already prepared for resisting against the fungus. Involvement of both JA and SA together with some other unknown factors is implicated in the resistance mechanism against *Ascochyta*. However, detailed expression analysis is further needed to confirm the macroarray data (with more number of genes as positive control to minimize variation). These results provided novel

insights to the molecular control of chickpea cellular processes, which may assist the understanding the chickpea defense mechanisms and allow enhanced development of disease resistant cultivars.

Table 4.1: Genes differentially expressed in response to *Ascochyta* infection, SA and JA

GENE	Clone no.	FC/PIC	F Asc	PI Asc	SA	JA
Defense-related						
Wound-inducible P450 hydroxylase	CaAr41	1.42	1.28	1.25	1.00	1.39
mRNA for hin 1 gene	CaAr150	1.35	1.49	1.40	2.90	1.39
Putative zinc dependent protease	CaAr273	1.40	1.60	2.53	2.69	1.58
Xyloglucan endo-1,4-beta-D-glucanase	CaAr273	1.59	1.05	1.77	0.75	2.74
Peroxiredoxin (2-Cys PRx gene)	CaAsc59	2.03	1.05	1.56	1.42	1.87
Esterase/lipase/thioesterase family protein	CaAsc62	1.72	1.70	1.48	3.45	1.28
mRNA for PR-10-1	CaAsc75	2.15	1.88	2.73	3.86	2.11
Glutamate decarboxylase-like protein	CaAsc76	1.93	1.31	1.53	1.63	1.78
DJ-1 family protein/protease-related	CaAsc254	1.38	2.11	2.48	1.32	2.05
Wound-responsive protein-related mRNA	CaAsc259	1.45	1.42	3.70	1.45	3.56
Disease resistance protein-related/LRR protein/PGIP	CaAsc269	2.17	1.26	3.47	2.62	2.47
GPRP mRNA	CaAsc376	1.82	1.29	2.79	1.37	2.89
Putative $Hs1^{pro1}$-like protein	CaAsc162	2.58	0.88	2.89	2.15	3.11
Metallothionein-like protein	CaAsc315	1.33	1.30	1.43	1.66	2.03
Proline rich protein	CaAsc74	2.56	1.31	2.87	4.83	2.99
Phenylalanine ammonia-lyase	CaAsc116	2.55	1.37	1.66	1.42	1.15
Thaumatin-like protein PR-5a	CaAsc180	1.45	0.66	3.68	2.44	1.84
mRNA for glycine-rich protein 2	CaAsc536	1.93	1.27	3.31	1.91	3.24
mRNA for lipid transfer protein	CaAsc336	1.72	1.28	3.12	1.29	3.41
Transcription and translation						
G-box element binding protein (GBF)	CaAr19	1.71	1.15	1.37	2.00	2.32
WRKY20 protein	CaAr101	1.28	1.24	1.19	2.28	4.14
WRKY43 mRNA	CaAr128	0.97	1.04	0.90	2.48	1.55
bZIP transcription factor	CaAsc7	2.33	1.29	2.92	1.70	3.05

AT-rich element binding factor 1 (ATF1) mRNA	CaAsc109	2.59	1.18	1.90	1.73	1.71
Large subunit 26s ribosomal RNA gene	CaAr220	1.01	1.51	2.90	1.22	1.21
5s ribosomal RNA gene	CaAsc46	1.58	0.95	1.45	1.54	2.89
mRNA for ribosomal protein L23	CaAsc69	2.81	1.01	3.98	1.67	2.96
Translation initiation factor SUI 1	CaAsc82	2.52	1.34	1.69	2.01	1.30
ribosomal protein (CL22) mRNA	CaAsc212	1.70	1.22	2.15	1.63	3.26
ribosomal protein S27 a	CaAsc223	1.59	1.38	3.30	1.23	2.49
60s ribosomal protein L17-like protein mRNA	CaAsc706	3.70	1.15	3.69	1.82	2.64
Signal transduction						
Cytokinin regulated Kinase	CaAr344	1.12	1.50	1.44	2.39	2.38
Putative ADP ATP carrier protein	CaAsc78	2.04	1.36	2.51	1.02	0.96
Phosphoglucan water kinase	CaAsc210	1.29	1.13	1.52	0.75	0.66
MAPK 3	CaAr37	1.85	2.22	3.41	1.07	2.00
Miscellaneous						
pentameric polyubiquitin (ubi4) mRNA	CaAr15	1.73	1.25	1.64	0.35	5.24
mRNA for polyubiquitin	CaAr130	0.99	1.37	1.15	0.95	1.56
ubiquitin mRNA	CaAr196	1.13	1.13	1.19	1.99	2.67
glyceraldehyde-3-phosphate dehydrogenase	CaAsc10	3.17	1.01	3.09	1.30	1.50
mRNA for uiquitin-like protein	CaAsc16	1.32	1.71	4.03	3.67	1.68
microtubule binding (APG8H)	CaAsc44	2.34	1.12	2.56	2.50	3.44
Rubisco activase (Rca) mRNA	CaAsc45	1.07	1.02	2.77	4.50	6.92
10 kDa photosystem II polypeptide	CaAsc48	2.58	1.12	2.59	1.58	2.29
Putative vacuolar ATPase subunit H protein mRNA	CaAsc88	2.34	1.32	3.35	1.43	2.95
Putative NADH-dependent hydroxypyruvate reductase mRNA	CaAsc96	2.73	1.16	2.62	1.55	2.36
mRNA for phosphoribosyl pyrophosphate synthase	CaAsc172	1.01	1.84	4.39	0.19	1.89
TMS membrane family protein/tumour differentially expressed	CaAsc237	2.20	1.29	2.91	2.26	2.54
Putative plastid triose phosphate translocator mRNA	CaAsc279	2.44	1.12	3.68	1.53	2.66
Putative vacuolar ATPase subunit H protein mRNA	CaAsc294	2.34	1.32	3.35	1.43	2.95
auxin-regulated protein IAA8	CaAsc306	0.88	1.24	2.39	0.81	1.98
chloroplast ribulose-1,5-bisphosphate carboxylase	CaAsc436	2.65	1.07	4.07	1.25	2.79

VHA2 mRNA for p-type H+-ATPase	CaAsc441	1.85	1.24	2.46	1.45	1.91
transposon Mutator-like	CaAsc620	3.04	1.14	4.55	1.89	2.99
mRNA for chloroplast rp121	CaAsc694	3.55	1.03	4.91	1.45	1.90
chlorophyll a/b binding protein	CaAsc91	1.44	1.65	3.10	1.42	1.72
Abiotic stress						
ERD15 protein mRNA	CaAr50	1.22	1.51	1.21	1.20	1.38
Syringolide induced protein	CaAr131	1.04	1.25	1.24	1.59	1.45
heavy-metal-associted domain-containing protein	CaAr163	1.00	1.31	1.49	1.30	1.27
mRNA Chaperonin 60	CaAsc66	2.03	1.42	3.13	1.55	1.69
Unknown						
Unknown Chloroplast protein	CaAr18	1.55	0.97	1.23	2.20	2.61
Unknown (Clone 75 microsatellite)	CaAsc518	1.90	1.49	3.00	1.31	2.15
Unknown	CaAr220	1.28	1.45	2.25	0.22	1.21
Unknown	CaAr243	1.61	1.08	1.38	0.93	2.14
Unknown	CaAr276	1.20	1	2.04	2.46	2.55
Unknown	CaAsc238	1.90	1.39	2.39	2.46	2.55
Unknown	CaAsc60	2.50	1.26	2.42	1.41	2.06
Unknown	CaAsc80	2.23	1.46	2.02	0.96	1.62
Unknown	CaAsc89	3.12	1.07	3.19	1.47	2.38
Unknown	CaAsc95	3.09	1.11	2.75	1.69	2.77
Unknown	CaAsc101	3.28	1.05	2.79	1.14	1.78
Unknown	CaAsc106	1.41	1.02	3.21	0.85	4.01
Unknown	CaAsc147	2.23	1.36	2.03	2.17	5.13
Unknown	CaAsc171	1.49	0.86	4.00	0.49	1.94
Unknown	CaAsc192	1.83	1.28	2.98	0.59	1.69
Unknown	CaAsc195	2.91	1.18	2.53	1.67	2.51
Unknown	CaAsc234	2.43	1.25	3.41	1.19	2.29
Unknown	CaAsc244	2.80	1.20	2.43	1.53	2.40
Unknown	CaAsc271	1.93	1.62	3.70	1.09	2.68
Unknown	CaAsc273	1.63	1.15	2.32	1.72	1.98

Unknown	CaAsc306	2.17	1.25	2.51	1.11	2.03
Unknown	CaAsc343	2.79	0.97	4.87	1.8	3.25
Unknown	CaAsc401	2.79	0.97	4.87	1.80	3.25
Unknown	CaAsc466	1.65	1.16	1.68	3.59	3.56
Unknown	CaAsc519	1.94	1.24	2.08	0.69	1.77

cDNA clones obtained from subtractive library are listed in **Table 4.1**. BLASTX searches were conducted to determine homologous genes and the putative function of the cDNA fragments. Ratios of signal intensity were determined by cDNA macroarray hybridization as described in the "Materials and methods". Shown for each gene are the expression ratios (average of two independent experiments) in resistant and susceptible lines of chickpea at the basal level (FC/PI C) and in response to *Ascochyta* (F Asc and PI Asc). Expression ratios (average of two independent experiments) of the transcripts in response to Jasmonic acid (JA), and Salicylic Acid (SA) were also presented. The transcripts are listed according to their probable functions. Values highlighted if expression ratios more than two fold.

Chapter 5: Characterization of an LRR-domain containing protein, CaAr131

5.1 Introduction

Plants have evolved highly specific mechanisms to resist pathogens. The most studied of these involves deployment of resistance (R) proteins, which in most cases are effective against specific races of pathogens carrying corresponding avirulence (*Avr*) genes ("gene-for-gene" interactions, Flor, 1971). The disease resistance genes (*R*) are the specificity determinants of the plant immune response. This simple but sophisticated immune system involves an allele specific genetic interaction between a host R gene and a pathogen avirulence gene (*avr*). When this genetic interaction takes place, a defense response is triggered. This response is characterized by rapid calcium and ion fluxes, an extracellular oxidative burst, transcriptional reprogramming within and around the infection sites and, in most cases, a localized programmed cell death, which is termed the hypersensitive response (HR) (Greenberg and Vinatzer, 2003; Nimchuk *et al.,* 2000). It is thought that the sum of these events leads to a halt in pathogen growth. In the absence of specific recognition, a basal defense response also occurs, which is apparently driven by pathogen-associated molecular patterns (PAMPS) such as flagellin and lipopolysaccharides (LPS) (Gomez-Gomez and Boller, 2002). The basal defense response overlaps significantly with R-protein-mediated defense, but is temporally slower and of lower amplitude. Basal defense does not prohibit pathogen colonization but does limit the extent of its spread (Glazebrook *et al.,* 1997). Thus, R-protein action apparently accelerates and amplifies innate basal defense responses.

5.1.1 Diversity of Plant R genes

Many (*R*) and avirulence genes have been identified in recent years from a wide range of plant species, such as *Arabidopsis*, flax (*Linum usitatissimum*), tomato (*Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*), sugar beet (*Beta vulgaris*), apple (*Malus domestica*), rice (*Oryza sativa*), barley (*Hordeum vulgare*), and maize (*Zea mays*). Their structural and functional comparisons have been well documented, revealing several different classes (Hammond-Kosack and Jones, 1997; Hammond- Kosack and Parker, 2003; To¨ r *et al.,* 2003). The largest group of R genes encodes cytoplasmically localized proteins that contain a central nucleotide binding (NB) site and a carboxyl Leucine-rich repeat (LRR) domain (NB-LRR genes). Nucleotide binding motifs share sequence similarities with the NB regions of apoptosis regulators such as CED4 from *Caenorhabditis elegans* and Apaf-1 from humans (Dangl and Jones, 2001). This would suggest that R protein activity may require, at least in part, the activity associated with ATP binding and/or hydrolysis (Tameling *et al.,* 2002). The LRR is typically 20–30 amino acids in length and these motifs have been identified in proteins ranging from viruses to eukaryotes. These proteins participate in a range of processes from development to disease resistance. Collectively, LRRs appear to be

involved in formation of protein-protein interactions. This group can be further subdivided into two major subclasses: those having an amino terminal coiled-coil (CC) domain (CC-NB-LRR) and those containing an amino-terminal domain resembling the cytoplasmic signaling domain of the Toll and Interleukin-1 (TIR) transmembrane receptors (TIR-NBLRR). The CC-NB-LRR subclass includes examples such as the *Arabidopsis RPS2* (Mindrinos *et al.,* 1994) and *RPM1* (Grant *et al.,* 1995) genes conferring bacterial resistance, *RPP13* (Bittner-Eddy *et al.,* 2000) and *RPP8* (McDowell *et al.,* 1998) conferring downy mildew (*Peronospora parasitica*) resistance, and HRT (Cooley *et al.,* 2000) conferring viral resistance from the same locus as *RPP8*. The TIR-NB-LRR subclass includes genes such as the tobacco *N* (Whitham *et al.,* 1994) gene for viral resistance, the flax *L6* (Lawrence *et al.,* 1995) gene for rust resistance, and the *Arabidopsis RPP5* (Parker *et al.,* 1997) and *RPP1* (Botella *et al.,* 1998) genes for downy mildew resistance. Sequencing of the complete genome of *Arabidopsis* has revealed approximately 149 NB-LRR genes (Meyers *et al.,* 2003).

The second group contains the cytoplasmic Ser/Thr kinase and has been represented by *Pto* (Martin *et al.,* 1993), which confers resistance to the bacterial pathogen Pseudomonas syringae pv tomato. The third group of *R* genes encodes the receptor-like kinases (RLKs). The characteristic features of these proteins are an extracellular LRR domain with a single transmembrane spanning region and a cytoplasmic kinase domain. This group contains the rice *Xa21* gene (Song *et al.,* 1995), which confers resistance to bacterial pathogen *Xanthomonas oryzae* pv oryzae. Receptor-like proteins (RLPs) comprise the next group of *R* genes. These are similar to RLK genes in that they encode extracellular LRRs and a C-terminal membrane anchor but lack the cytoplasmic kinase domain. Members of this group include the tomato *Cf-2, Cf-4, Cf-5*, and *Cf-9* genes conferring resistance to the fungal pathogen *Cladosporium fulvum* (Jones *et al.,* 1994; Dixon *et al.,* 1996), the tomato *Ve* genes for Verticillium resistance (Kawchuk *et al.,* 2001), and the apple *HcrVf2* gene for resistance to *Venturia inequalis* (Belfanti *et al.,* 2004). There are also some unexpected structures as well. Two genes encode, in addition to a TIR-NB-LRR structure, a WRKY domain that is likely to confer DNA-binding capacity. WRKY proteins are plant-specific zinc-finger transcription factors that are transcriptionally activated during some plant defence responses (Eulgem *et al.,* 2000). In addition, one TIR-NB-LRR gene has been annotated to carry not only a WRKY, but also a protein kinase domain.

5.1.2 Interaction between R and avr gene products

Plants have evolved the ability to recognize and respond to particular pathogen molecules, leading to rapid activation of defense responses. Prior to the advent of molecular genetics, this phenomenon was observed as an interaction between pathogens carrying single

dominant genes (avirulence genes) that caused them to be recognized by plant hosts carrying single dominant resistance (*R*) genes, leading to the "gene-for-gene" nomenclature. Pathogens that are recognized in this way and that therefore fail to cause disease are called avirulent pathogens, the host is called resistant, and the interaction is called incompatible. In the absence of gene-for-gene recognition, due to absence of the avirulence gene in the pathogen and/or of the *R* gene in the host, the pathogen is virulent, the host is susceptible, and the interaction is compatible.

5.1.3 Gene for Gene Model

The simplest mechanistic explanation of the genetic interaction between *R* and *avr* genes is that the latter encode or generate 'specific ligands' that interact physically with a 'receptor' that is encoded by the corresponding plant *R* gene. However experimental data that support this model are rare (Jia *et al.,* 2000: Deslandes *et al.,* 2003). In fact, many avirulence proteins are actually required for maximal virulence on susceptible hosts that lack the corresponding *R* gene (Kjemtrup *et al.,* 2000); hence, they are actually virulence factors that contribute to disease. One plausible generality is that *R* proteins have evolved to recognize the functions of pathogen virulence factors. If this is true, then R proteins appear not to have evolved to recognize Avr proteins directly, as predicted by receptor–ligand models, but rather to recognize the action of virulence factors as they modify or perturb host cellular targets. This model of indirect recognition has been termed 'the guard hypothesis', as it hypothesizes that R proteins have a surveillance role in cellular homeostasis (Dangl and Jones, 2001).

5.1.4 Guard Hypothesis

The guard model suggests the following plausible cellular scenarios. First, R proteins are likely to be part of a multiprotein complex that should include proteins that are targeted by pathogen virulence factors. Second, the Avr proteins, presumably acting as virulence factors, specifically target one or more host proteins. These targets are probable partners of R proteins. Third, the perturbation of these cellular targets of pathogen virulence factors may or may not be required for virulence. Fourth, in either case, target perturbation leads to R-protein activation. Fifth, R proteins either constitutively bind to their partner(s) and then dissociate after modification of the complex by the type-III effector or form a new interaction with a cellular target that leads to activation.

5.1.5 Indirect Mechanism of interaction

Contrary to the predicted models, it is known that bacterial effector recognition and signaling has likely evolved as an indirect mechanism. Although many *R* genes and their corresponding pathogen effectors have been cloned, direct binding between them has rarely

been demonstrated. This seemingly limited repertoire of plant resistance receptors begs the question of how an effector-triggered immune response in plants coordinates resistance to a broad range of pathogens and their corresponding effectors. The majority of characterized bacterial effectors possesses enzyme activity (Chisholm *et al.,* 2006) and modifies plant proteins. Evidence is emerging that the enzymatic functions of multiple effectors target the same host proteins. Rather than develop receptors for every possible effector, host plants have evolved mechanisms to monitor common host targets. By monitoring for perturbations, R proteins indirectly detect the enzymatic activity of multiple effectors (Van der Biezen and Jones, 1998).

Molecular evidence for indirect pathogen recognition has come from work studying resistance responses in Arabidopsis plants following infection with *P. syringae* expressing the effector AvrPphB, a cysteine protease (Shao *et al.,* 2003). The activity of AvrPphB is indirectly detected by the R protein RPS5. This work demonstrated that perception and subsequent resistance signaling is initiated not by the direct perception and association that perception and subsequent resistance signaling is initiated not by the direct perception and association of R protein-effector molecule pairing but by an indirect mechanism. During infection, AvrPphB cleaves the host protein PBS1. AvrPphB cleavage of PBS1 is then perceived by the R protein RPS5, which in turn activates resistance signaling. Additional studies have also revealed similar, indirect mechanisms for resistance signaling (Axtell and Staskawicz, 2003; Mackey *et al.,* 2002, 2003). The best characterized example of the activation of resistance by way of monitoring bacterial effector activity is that of the Arabidopsis protein RIN4. RIN4 is monitored by at least two R proteins, RPM1 and RPS2. RPM1 and RPS2 have each been shown to physically associate with RIN4 in planta (Axtell and Staskawicz, 2003; Mackey *et al.,* 2002). The Arabidopsis protein RPM1 recognizes two unrelated P. syringae effector proteins, AvrRpm1 and AvrB (Bisgrove *et al.,* 1994). Interestingly, the soybean RPG1 R protein recognizes AvrB but not AvrRpm1. Although RPM1 and RPG1 are both NB-LRR proteins, they show limited sequence homology, suggesting that they evolved independently to detect AvrB (Ashfield *et al.,* 2004). When AvrRpm1 or AvrB is delivered to the plant cell, RIN4 is hyperphosphorylated, which in turn leads to the activation of RPM1-mediated resistance. Thus, although RPM1 resistance is activated in the presence of either AvrB or AvrRpm1, it is activated through an indirect mechanism (i.e., detection of the modified state of RIN4). It has recently been shown that AvrRpm1 inhibits PAMP-triggered defense responses, presumably through its modification of RIN4 and other host targets (Kim *et al.,* 2005b).

Although there is evidence in support of the indirect recognition model for bacterial effector recognition, plants may employ alternate detection mechanisms for other pathogens.

It is still unclear whether fungal and oomycete pathogens are perceived directly or indirectly by host R genes. One example of direct recognition of a fungal effector is that of AvrPita, which is recognized by the rice resistance gene *Pi-ta*. AvrPita has been shown to directly bind to Pi-ta by yeast two-hybrid and in vitro binding assays (Jia *et al.*, 2000). There is also a well-characterized example of indirect recognition in fungal pathogenesis. The tomato Cf-2 R protein recognizes the *Cladocporium fulvum* effector Avr2. During infection, Avr2 binds to and inhibits the secreted tomato protease Rcr3, which in turn is responsible for Cf-2 activation (Rooney *et al.*, 2005). Whether R proteins recognize most pathogen effectors directly or indirectly is a question that remains to be elucidated. While indirect mechanisms of pathogen recognition permit the detection of multiple unrelated effectors by a single R protein, a direct interaction between pathogen effectors and R proteins would allow for the detection of structurally conserved effector molecules. Direct detection would only be efficient against multiple effectors containing common structural motifs. Therefore, indirect recognition likely evolved following direct recognition as a means to detect emerging effector diversity.

5.1.6 Post-recognition R protein signaling

The receptors i.e. the R proteins act as regulatory signal transduction switches and are activated upon direct or indirect perception of non-self structures. Recent findings indicate that nucleo-cytoplasmic partitioning and nuclear activity is critical for the function of several plant immune sensors, thereby linking receptor function to transcriptional reprogramming of host cells for pathogen defense. How plant NB-LRR proteins activate immune responses following recognition of pathogen-derived effectors has been a major question since the molecular isolation of the founding members of this protein family (Bent *et al.*, 1994). Recent findings suggest that members of the CC- and TIR-type receptor families function in the nucleus. Allelic barley MLA CC-type receptors recognize isolate-specific effectors of the grass powdery mildew fungus, *Blumeria graminis* f sp hordei (Ridout *et al.*, 2006). Fractionation of cell extracts using transgenic plants that express native levels of epitope-tagged MLA as well as visualization of a fluorochrome-marked MLA in living epidermal cells localized the majority of the receptor to the soluble cytoplasmic fraction and approximately 5% to the nucleus (Shen *et al.*, 2007). Perturbation of nucleocytoplasmic MLA10 partitioning by expression of a receptor fusion protein containing a nuclear export signal (NES), which enhances nuclear export over import, abrogated MLA10-specified disease resistance (Shen *et al.*, 2007). Similarly, adding a NES to the tobacco TIR-type N receptor, which conditions immunity against the tobacco mosaic virus (TMV) upon recognition of the p50 TMV replicase, impaired both N nuclear accumulation and TMV disease resistance (Burch-Smith *et al.*, 2007). Nuclear action of MLA and N was unexpected,

because both proteins lack a canonical nuclear localization signal (NLS). Unlike this, the Arabidopsis TIR-type RPS4 protein, conditioning immunity to *Pseudomonas syringae* strains expressing avrRps4 (Gassmann *et al.,* 1999), contains a bipartite NLS, and this targeting signal is required for both nuclear import and disease resistance. Similar to barley MLA, less than 10% of total cellular RPS4 was found in Arabidopsis nuclei preparations, whereas the bulk of the receptor associates with endosomes. Re-inspection of all 71 annotated Arabidopsis TNL and 54 CNL subfamily members (Meyers *et al.,* 2003) reveal a widespread potential for nuclear localization of other R proteins; using the 51 TNL and 39 CNL protein models contain predicted monopartite or bipartite NLSs. Given the fact that in yeast 43% of known nuclear proteins enter the nucleus without discernible NLSs (Lange *et al.,* 2007), the utilization of NLS dependent and seemingly NLS-independent nuclear import pathways for plant R proteins is not surprising.

Transcriptional reprogramming of plant cells upon pathogen attack is extensive, affecting between 3 and 12% of the 24,000 tested Arabidopsis genes upon fungal or bacterial challenge, respectively (Nishimura *et al.,* 2003; Thilmony *et al.,* 2006). How the perception of non-self structures by PRRs and R proteins leads to transcriptional activation of defense-response genes has been a long-standing question. In this context, nuclear activities of barley MLA, tobacco N, and Arabidopsis RPS4 reveal novel insights. Quantitative fluorescence lifetime imaging of fluorochrome-tagged receptor was employed to visualize in vivo in nuclei an effector-dependent physical association between the MLA10 receptor and two WRKY transcription factors (HvWRKY1 and HvWRKY2 TFs (Shen *et al.,* 2007), suggesting that the transcription factors (TFs) serve as immediate downstream targets of the activated receptor. This protein– protein association is mediated by the invariant N-terminal CC domain of allelic MLA receptors. Because the polymorphic C-terminal LRR region of MLA has been shown to determine recognition specificity (Shen *et al.,* 2003), it is possible that this region senses, directly or indirectly, the presence of powdery mildew effectors, while the N-terminal CC of the activated receptor acts as a signal relay moiety to the WRKY TFs. Accordingly, different structural modules at opposite ends of the receptor might account for sensory and signal transmission sub-functions. Whilst it remains to be seen whether MLA and RPS4 proteins detect the corresponding effectors in the cytoplasm and/or nucleus, the cytoplasmic pool of tobacco N appears to detect the TMV p50 viral effector. When the p50 effector was fused to the NES, thereby depleting the nuclear p50 pool and enforcing cytoplasmic localization, plant cells retained the ability to trigger N mediated disease resistance (Burch-Smith *et al.,* 2007).

A similar regulatory logic might thelp to explain previous in planta experiments with autoactive forms of the flax TIR-NB-LRR protein L6 (Howles *et al.,* 2005). Wild-type L6 confers typical race-specific immunity associated with localized cell death to strains of the

flax rust fungus that carry the cognate avirulence gene, designated *AvrL567*. In recovered transgenic plants expressing autoactive L6 defense-related gene expression is chronically activated without signs of cell death. However, when the transgenic plants were challenged with flax rust isolates that are virulent on wild-type L6 plants, effective immunity was observed that was accompanied by an L6-like cell death response. Thus, while autoactive L6 alone is unable to drive plant cells into suicide, MAMPs released during fungal attack might trigger cell death-associated immune responses because of the simultaneous presence of autoactive L6.

Unlike direct links between MLA or N receptor function and the transcriptional machinery, nuclear RPS4 activity requires EDS1, a protein of unknown biochemical function(s) that lacks known chromatin- or DNA-binding domains and resides in both cytoplasmic and nuclear compartments (Wirthmueller *et al.,* 2007). RPS4-triggered immunity, but not nucleo-cytoplasmic partitioning or receptor stability, is abolished in an eds1 null mutant background. Together with an almost complete breakdown of RPS4/EDS1-dependent activation/repression of approximately 130 defense- related genes in eds1 plants (Bartsch *et al.,* 2006; Wirthmueller *et al.,* 2007), this suggests that EDS1 acts as intermediary positive signal transducer between the receptor and defense gene expression.

The Arabidopsis genome contains another *R* gene homolog, in which an N-terminal WRKY DNA-binding domain is fused to a TIR-NB-LRR protein. This deduced protein contains an additional C-terminal kinase domain. Although no biological function has been assigned to the WRKY-TIR-NB-LRR-kinase to date, it is of note that the Populus trichocarpa genome contains 40 NB-LRR gene models, not present in Arabidopsis, which carry an N-terminal BEAF and DREF DNA-binding finger (BED) DNA-binding zinc-finger domain (Aravind, 2000; Tuskan *et al.,* 2006). This domain is also present at the N-terminus of the rice Xa1 NB-LRR R protein to *Xanthomonas oryzae*; (Yoshimura *et al.,* 1998). Thus, it is possible that a subgroup of plant immune receptors has acquired direct DNA-binding capacity by domain co-option involving WRKY or BED domains.

It remains to be seen how many plant NB-LRR proteins function in the nucleus. The widespread occurrence of NLSs in Arabidopsis TIR- and CC-type receptor subfamilies is an indication that their nuclear location might not be an exception. Direct targeting of the transcriptional machinery by NB-LRR proteins as in the case of MLA receptors implies a short signaling pathway that may not depend on authentic signalling components. This could explain why mutational approaches in plants have failed so far in identifying signaling mutants that exclusively compromise NB-LRR receptor function. Derepression of MAMP-triggered immune responses through MLA receptor interference with WRKY repressors is likely to be only one of several potential convergence points between MAMP- and R protein-

triggered signalling pathways. Convergence points could also be generated by MAMP-triggered and MAP kinase-dependent R protein phosphorylation, in turn modulating effector triggered receptor activity and/or nucleo-cytoplasmic receptor partitioning. In this context, nuclear translocation of a plant MAP kinase upon treatment of cell cultures with an oomycete-derived MAMP deserves special note (Ligterink *et al*, 1997). If nuclear action of R proteins is a widespread phenomenon, one would expect that evolution favored diverse interception points with the transcriptional machinery to avoid a loss by pathogens. Thus, whether different nuclear immune sensors target the same, different, or overlapping chromatin sites and how this translates into spatio-temporal changes of defense gene expression patterns could become a focus of future experimentation.

5.2 Results:

5.2.1 Isolation and Sequence Analysis of *CaAr131* gene

Several *Ascochyta*-induced cDNA fragments from *Ascochyta* resistant chickpea line, FLIP84-92 C (2), were isolated by SSH. Sequence annotation using BLASTx of one of the clones revealed significant homology with Hs1 $^{pro-1}$, an LRR domain containing protein of sugarbeet (Cai *et al.,* 1997). This clone was truncated and the full-length *CaAr131* was isolated by 5' Random Amplification of cDNA ends (RACE). The amplified fragments were cloned and sequenced. The full length clone was obtained which showed an open reading frame of 1.3 Kb (Fig. 5.1). The deduced amino acid sequence showed that this protein contain a total of 458 amino acid residue (obtained from http://tw.expasy.org/tools/#translate) with a predicted molecular mass of 52 kD and isoelectric point of 6.42 (http://tw.expasy.org/tools/tagident.html). CaAr131 contains three potential o-(β)-glycosilation sites (at 13th, 23rd and 152nd amino acid) and putative 6 phosphorylation sites (one tyrosine, one threonine and four serine) with scores ≥ 9 (at amino acid 23rd, 25th, 28th, 160th, 312th and 316th) obtained (Fig. 5.3 A and B) from NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/). This gene was isolated from many other plants like *Glycine max*, *Pisum sativum*, *Hordeum vulgare*, *Oryza sativa* and *Arabidopsis*. The deduced amino acid sequences were aligned using CLUSTALW program which highlights an N-terminal extension compared to the Hs1 $^{pro-1}$ protein from sugarbeet. The chickpea homolog of Hs1^{Pro-1}, possess an imperfect LRR but no nucleotide binding site or kinase domain (Fig. 5.2). However, Hs1 $^{pro-1}$ protein from sugarbeet was reported to have a transmembrane domain (Cai *et al.,* 1997), although no evidence was given in support. The corresponding transmembrane domain region in CaAr131 protein does show several differences and therefore presence of any transmemebrane domain has not been predicted (http://www.ch.embnet.org/software/TMPRED_form.html). We also examined the

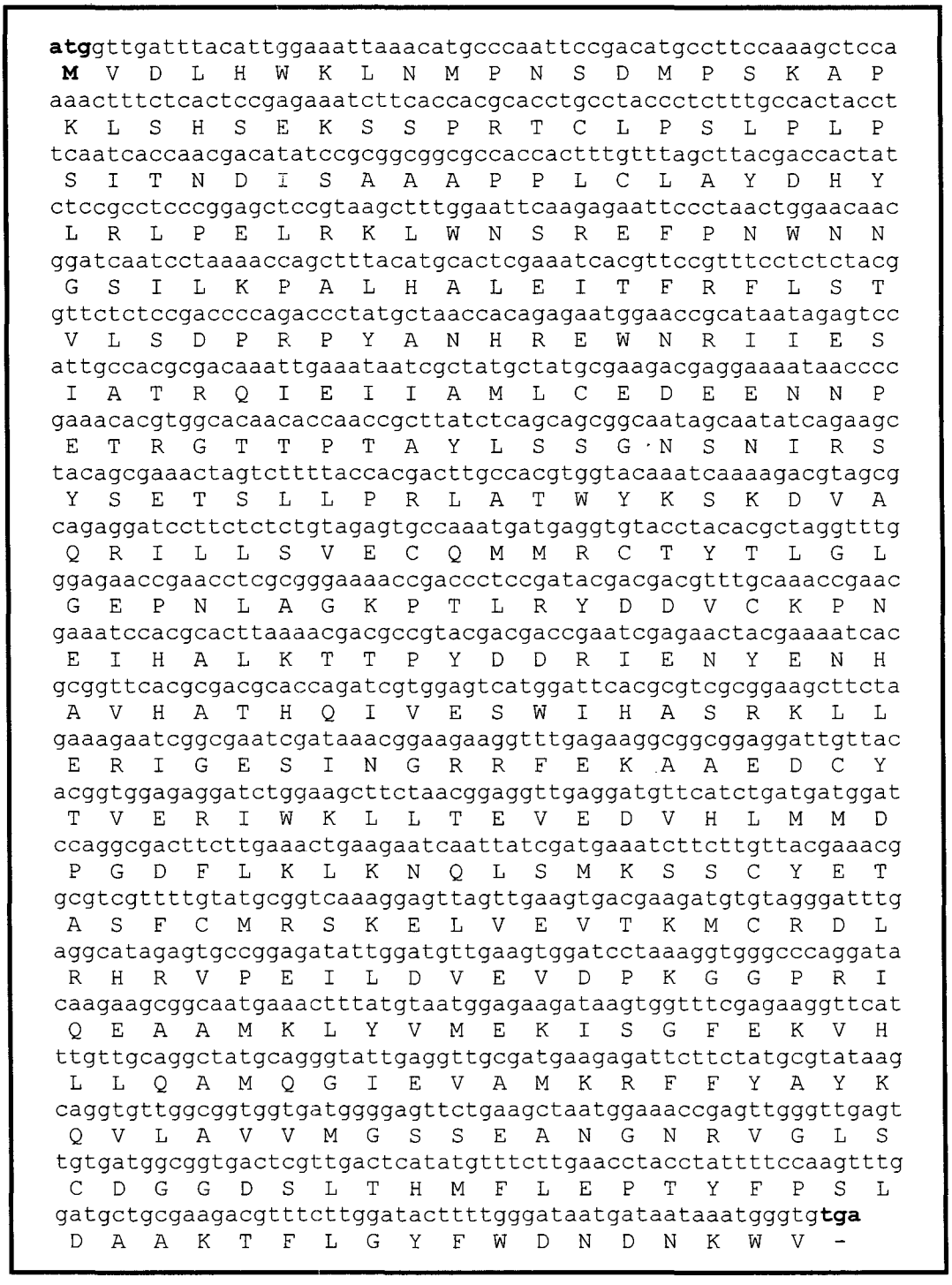

Figure 5.1. DNA and encoded amino acid sequence of the Hs1^{Pro-1}homolog in chickpea, *CaAr131*.

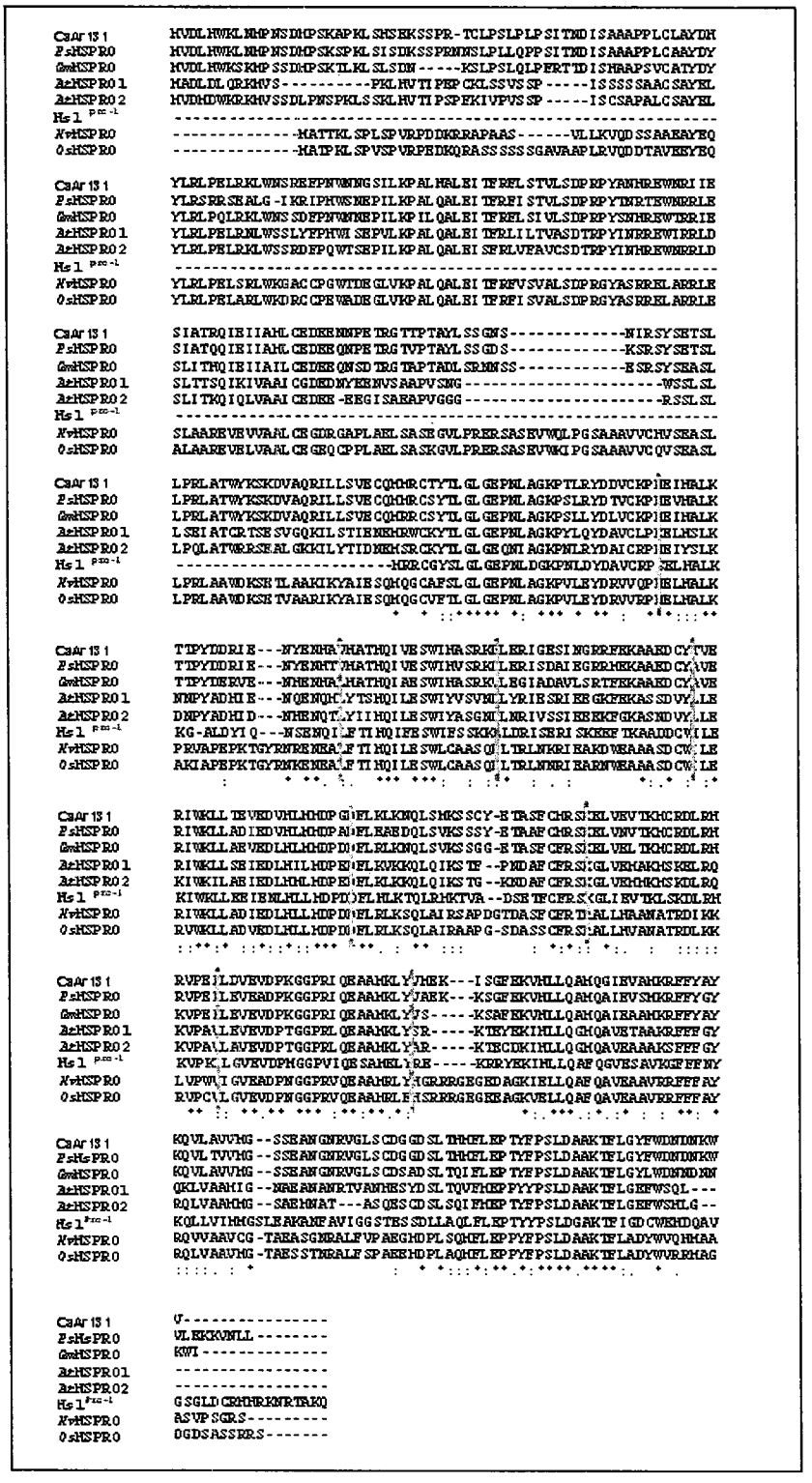

Figure 5.2 Alignment of the CaAr131 protein and the plant Hs1^{Pro-1} -related proteins available in the database. Alignments were realized using the Clustal W2 program and the Hs1^{Pro-1} protein from sugarbeet and Hs1^{Pro-1} -like proteins from *Glycine max, Pisum sativum, Arabidopsis, Oryza sativa* and *Hordeum vulgare.* The imperfect LRR repeats (delimited by blue vertical lines) defined in the Hs1^{Pro-1} protein are positioned.

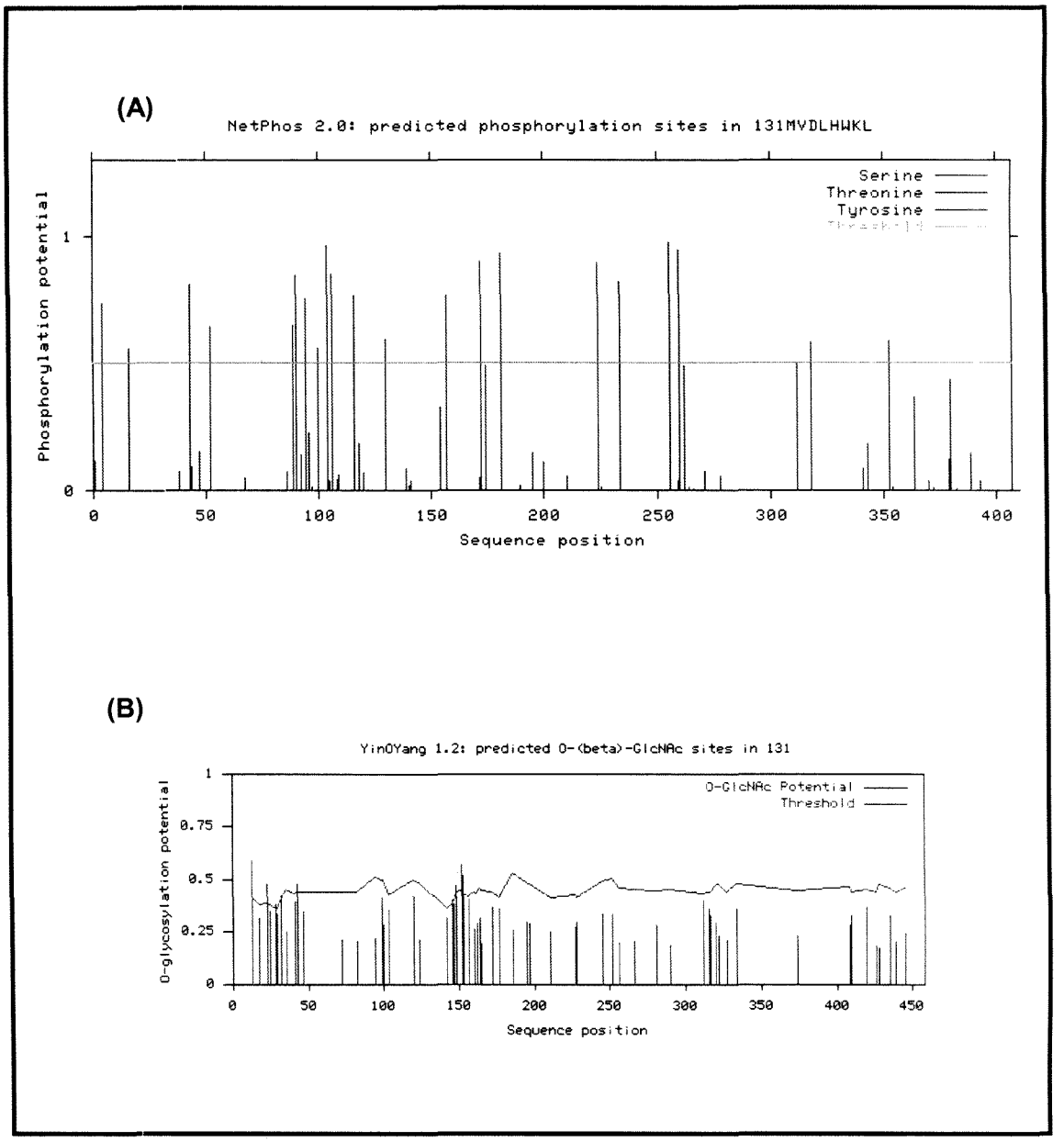

Figure 5.3 (A) Predicted sites of phosphorylation present in CaAr131 protein. A total of six phosphorylation sites (score>9) were predicted. **(B) Presence of O-glycosylation and sites in CaAr131 protein.** There are three potential O-glycosylation sites in CaAr131.

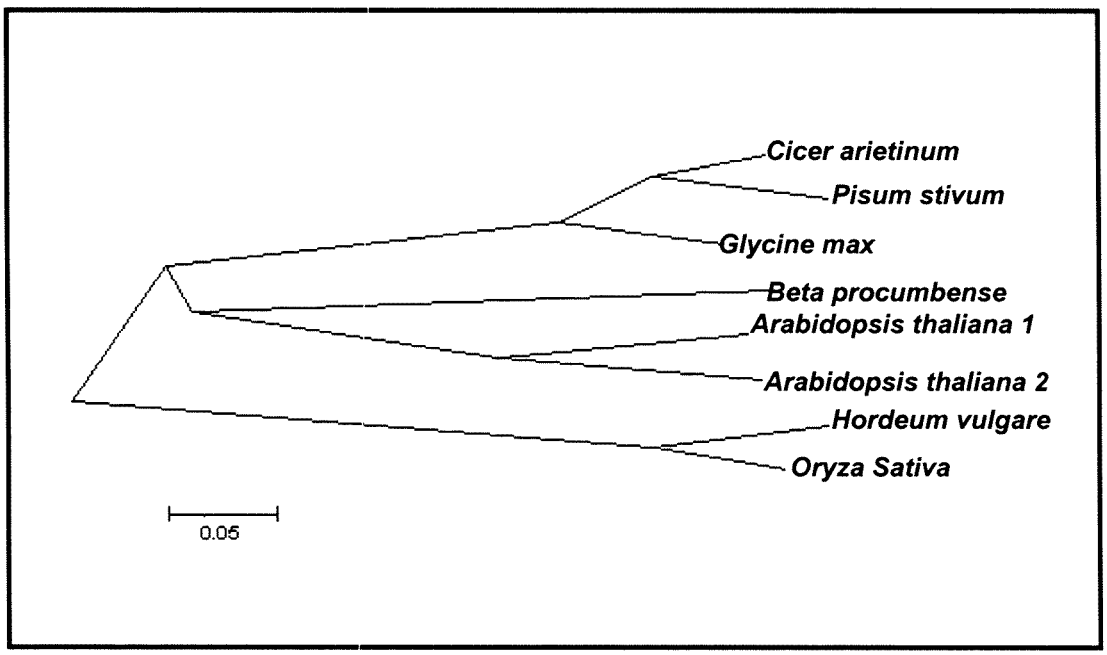

Figure 5.4. Phylogenetic tree showing relationship between CaAr131 and other well-studied Hs1^{Pro-1} family proteins.

phylogenetic relationship between CaAr131 and other Hs1 $^{pro-1}$ homologs from other plants (Fig. 5.4). A rooted phylogenetic tree was constructed using neighbor joining phylogeny (by using Mega2 program; http://www.megasoftware.net/). A phylogenetic analyses clusters showed CaAr131, PsHs1 $^{pro-1}$, GmHs1 $^{pro-1}$, BpHs1 $^{pro-1}$ and two of the *Arabidopsis* homologs (At1Hs1 $^{pro-1}$ and At2Hs1 $^{pro-1}$) in the same clade consistent with the sequence analysis. The next related clade contains OsHs1 $^{pro-1}$ and HvHs1^{pro-1}, the two homolog from monocot plants. The BpHs1^{pro-1} and GmHs1^{pro-1} are implicated in resistance against nematode (Cai *et al.*, 1997; McClean *et al.*, 2007). The Arabidopsis homologs (At1Hs1^{pro-1} and At2Hs1^{pro-1}) are known to interact with AKINβγ protein which is implicated in metabolic responses to nutritional and environmental stresses (Gisset *et al.*, 2006). However their exact biological functions are not yet determined.

5.2.2 Genomic Southern Analysis of *CaAr131* gene

In order to determine the presence of *CaAr131* gene in chickpea and its copy number, Southern analysis was carried out using genomic DNA isolated from different varieties of chickpea. Approximately 10 µg of chickpea DNA from different varieties was digested with *Hind*III, and *Eco*RI, restriction enzymes. The full length cDNA of *CaAr131* was hybridized, under high stringency conditions, to a DNA blot of chickpea genomic DNA digested with different restriction enzymes (Fig. 5.5). A single prominent hybridizing band was observed for *CaAr131* consistent with the single copy gene in the genome when the genomic DNA was digested with *Hind* III and *Eco* RI enzymes (Fig. 5.5). One interesting observation needs the special attention that in the blot where genomic DNA was digested with *Eco* RI, the bands were of different size in different varieties of chickpea including the resistant and susceptible germplasm lines. Therefore it can be used further as a marker for different lines of chickpea.

5.2.3 Expression patterns of *CaAr131* in response to *Ascochyta* infection

The transcript levels of *CaAr131* gene were analyzed in the chickpea resistant and susceptible lines inoculated with *Ascochyta rabiei*. The *CaAr131* gene was differentially induced in both resistant and susceptible lines of chickpea (Fig. 5.6). Most importantly, the basal level of the transcript was found to be significantly more in the resistant line [FLIP-84-92 C(2)] as compared to the susceptible line [Pusa-362]. Specific upregulation of CaAr131 by *Ascochyta* infection was also shown at the protein level in the chickpea plants (Fig. 5.7). Although, a biphasic induction of *CaAr131* was observed at the transcript level, a continuous induction of CaAr131 protein was observed during the entire cycle of *Ascochyta* infection, suggesting that the regulation of CaAr131 might vary at the transcript and protein levels (Fig. 5.7).

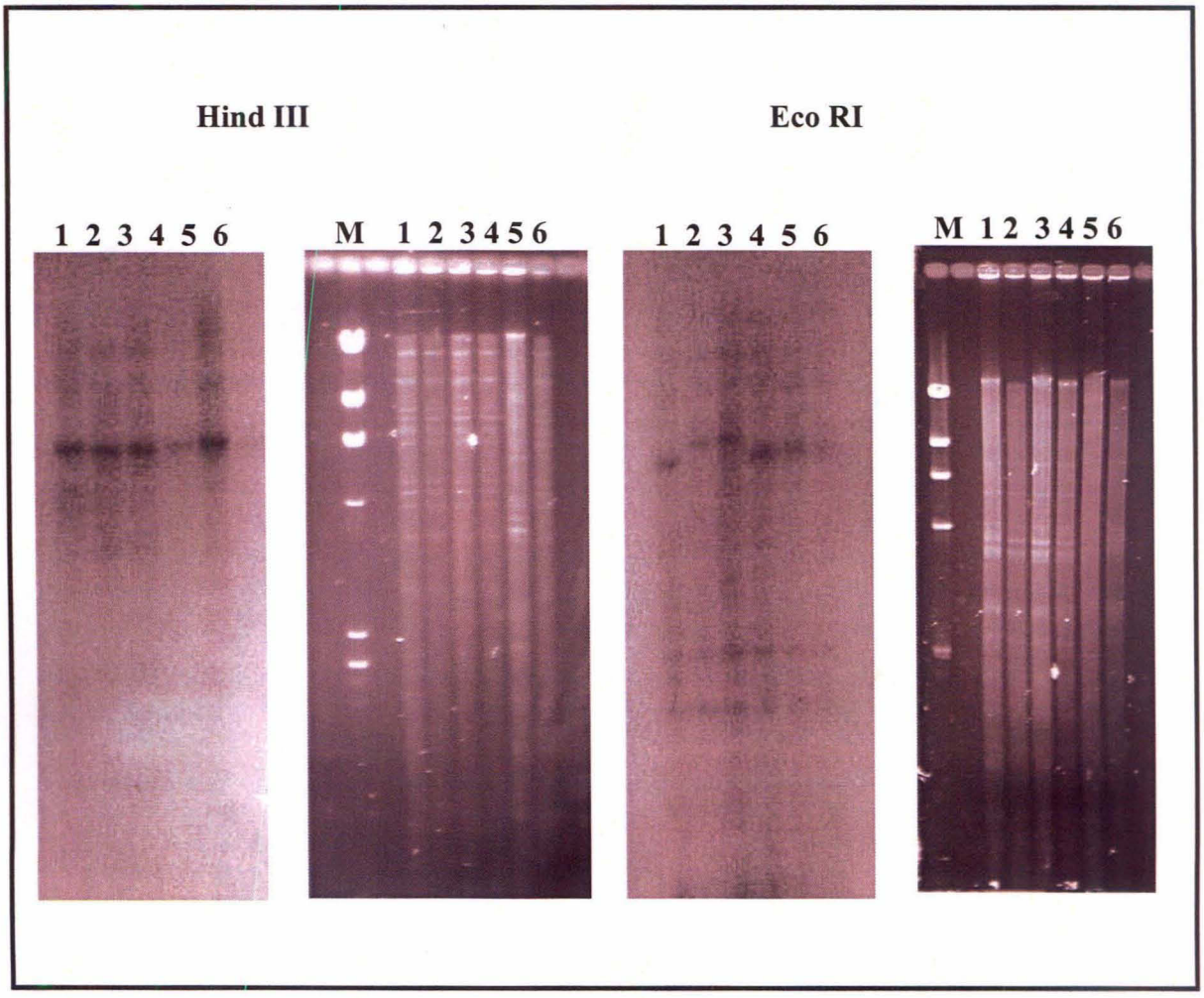

Figure 5.5. DNA blot analysis of *CaAr131* in different varieties of chickpea. An ethidium bromide stained 0.8% agarose gel showing complete digestion of genomic DNA with *Hind* III and *Eco* RI. The λ *Hind* III marker was run along with the digested DNA. Autoradiogram showing Southern by using complete cDNA sequence of *CaAr131* as a probe. M : λ Hind III fragment ;1. PI359075 2; FLIP8492C (2);12004 4; 11879 5; 4475 6; Pusa- 362.

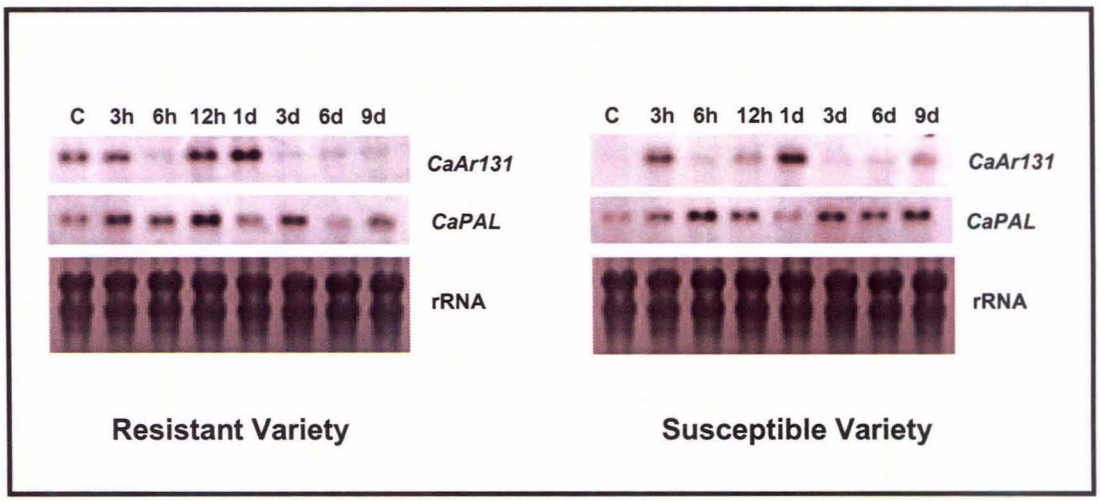

Figure 5.6. Northern blot showing transcript levels of *CaAr131* at basal level and in response to *Ascochyta* infection in resistant and susceptible varieties of chickpea. Ten microgram of total RNA was blotted and hybridized with *CaAr131* probe. The filter was deprobed and rehybridized with *CaPAL* for comparison. The rRNA on the membrane was visualized by staining with methylene blue as an equal loading control (lower panel).

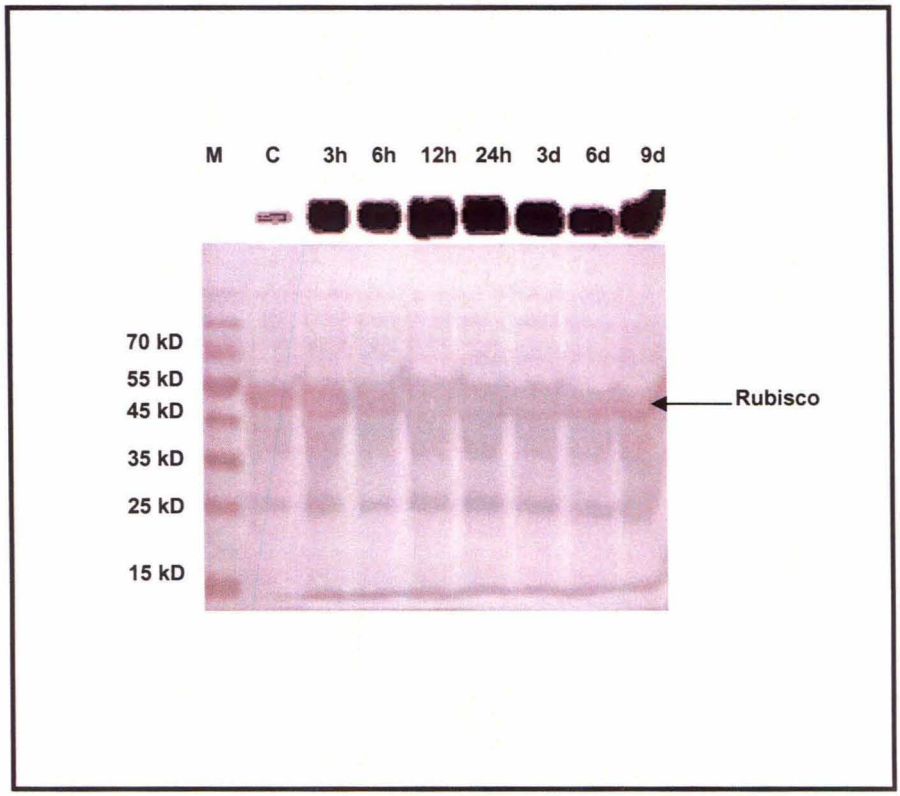

Figure 5.7. Immunoblot analysis of *CaAr131* protein during Ascochyta infection. Twenty five µg of total protein isolated from chickpea were loaded. The total protein on the membrane was visualized by ponceau staining.

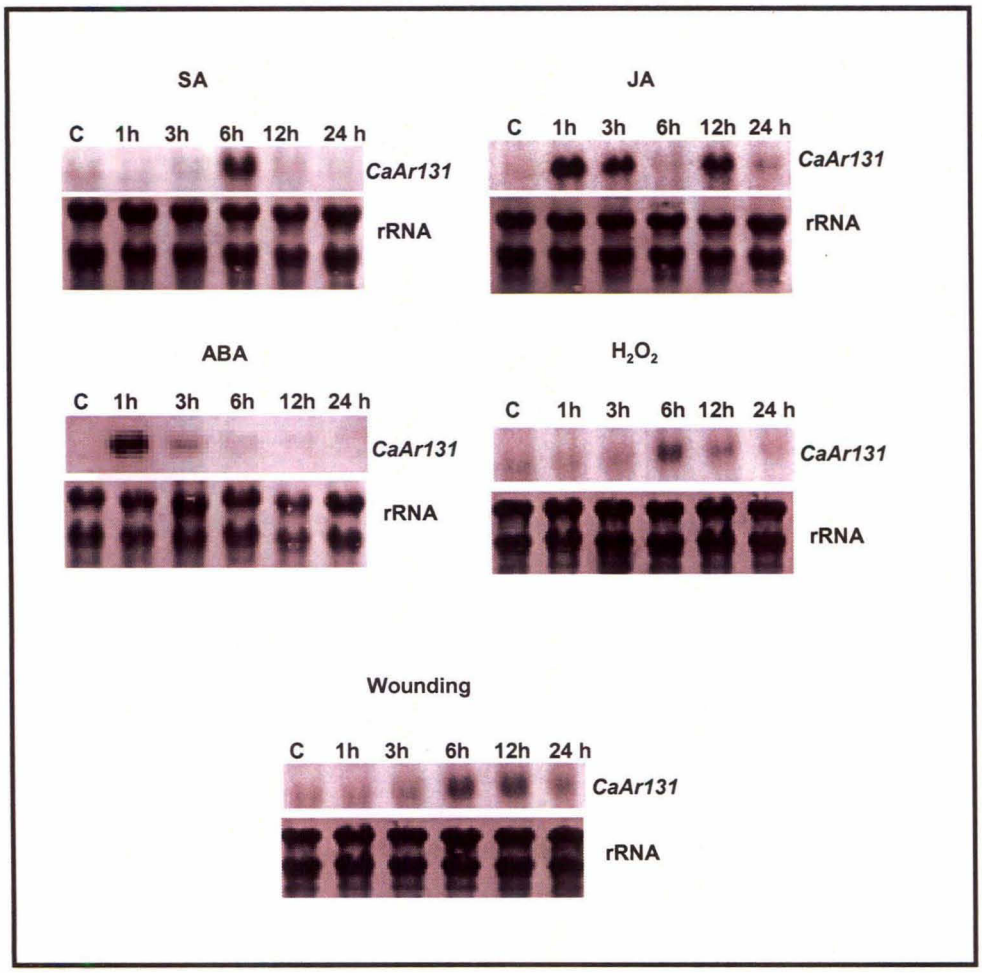

Figure 5.8. The expression of the *CaAr131* gene in the chickpea plants exposed to various Defense Regulators. Total RNA (10µg) from chickpea plants at various time points after treatment was loaded into each lane. The *CaAr131* cDNA were used as probes. Methylene blue stained rRNA are shown as loading control.

5.2.4 *CaAr131* gene expression in response to defense/stress-related signaling molecules and wounding

Signaling molecules including ABA, JA and SA may accumulate in plants upon pathogen infection, which mediate defense responses through involvement in signal transduction pathways (Glazebrook, 2001). Crosstalk among them may modulate the expression of biotic and abiotic stress-responsive genes. Since, the *CaAr131* transcript level gets modulated on *Ascochyta* infection, it was imperative to look for modulation of its transcript level in response to defense-related signaling molecules like Salicylic Acid (SA), Jasmonic Acid (JA) and Abscisic Acid (ABA). The three weeks old chickpea seedlings were sprayed with 5 mM SA, 100 µM JA, and 100 µM ABA respectively. The samples were harvested after 1h, 3h, 6h, 12h and 24h and total RNA was isolated. The control samples were sprayed with sterile distilled H_2O. Northern blot analysis was performed to study the expression patterns of *CaAr131* gene on treatment with various defense regulators including SA, JA, and ABA. The *CaAr131* transcripts were shown to increase significantly 6h after SA treatment, but decreased thereafter. Treatment with ABA resulted in minor increase in *CaAr131*transcription (Fig.5.8). In the JA treated chickpea plants, *CaAr131* accumulated at high levels at 1h, 3h and 12h and then began to decline.

Hydrogen peroxide (H_2O_2) has been shown to function, not only as a local signal for HR, but also as a diffusible signal for the induction of defense genes in the adjacent cells (Alvarez *et al.*, 1998). We noted a very faint band upon H_2O_2 treatment (100 µM). As a response to mechanical wounding, we noted an elevation in the induction of *CaAr131* transcription after 6h of treatment (Fig. 5.8).

5.2.5 *in vivo* subcellular localization of *CaAr131 protein*

To characterize the subcellular localization of the *CaAr131* protein, we generated the CaAr131-green fluorescent protein fusion protein construct in the pCAMBIA1303 vector, which harbors the strong CAMV35S promoter to drive the gene expression (Fig. 5.9). The construct was used to transform tobacco and stable transformants generated. The subcellular localization of CaAr131::GFP fusion protein was evaluated in the transgenic tobacco root. The confocal microscopic images detected expression of CaAr131::GFP fusion protein in the cytoplasm. The root cells of the transgenic tobacco transformed with vector alone exhibited fluorescence throughout the cell (Fig. 5.10).

5.2.6 Overexpression of *CaAr131* in transgenic tobacco

The complete ORF of *CaAr131* was cloned in plant expression vector pBI121M (Fig. 5.11) and transferred to *Agrobaterium* strain LBA4404. Leaf discs from axenically

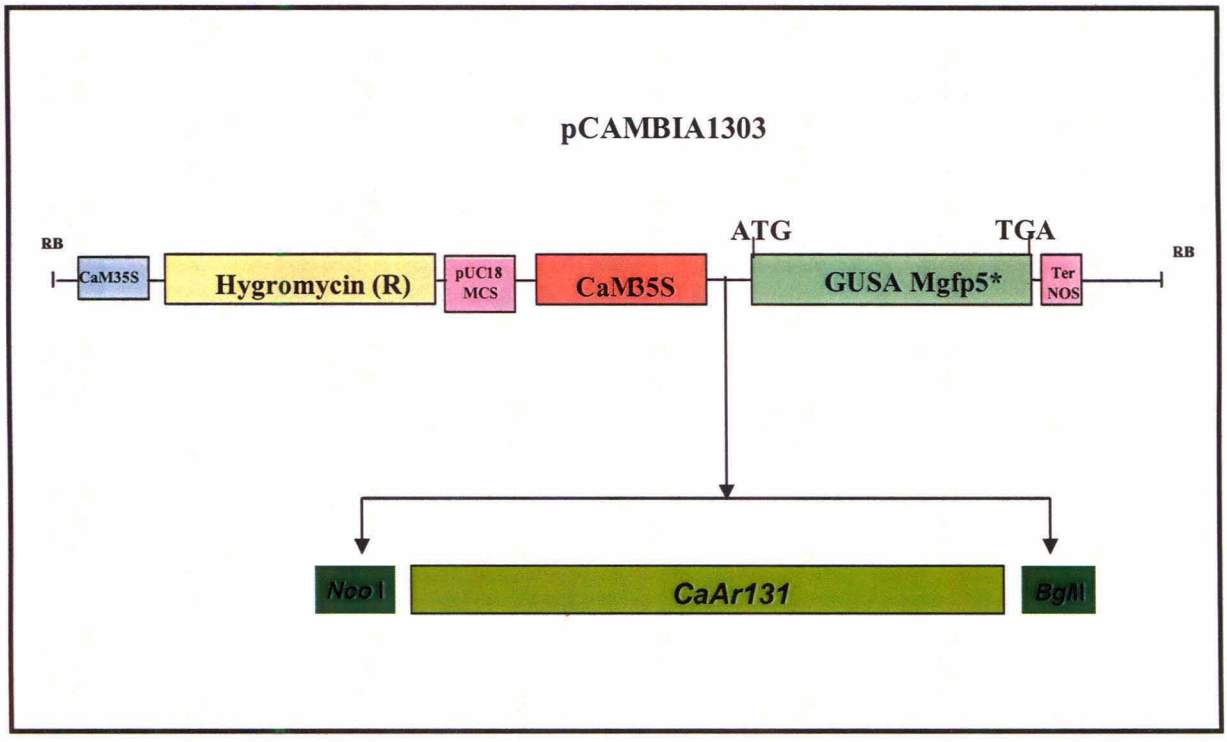

Figure 5.9. The schematic diagram of cloning strategy of CAM35S::CaAr131 in the binary vector pCAMBIA1303 for localization.

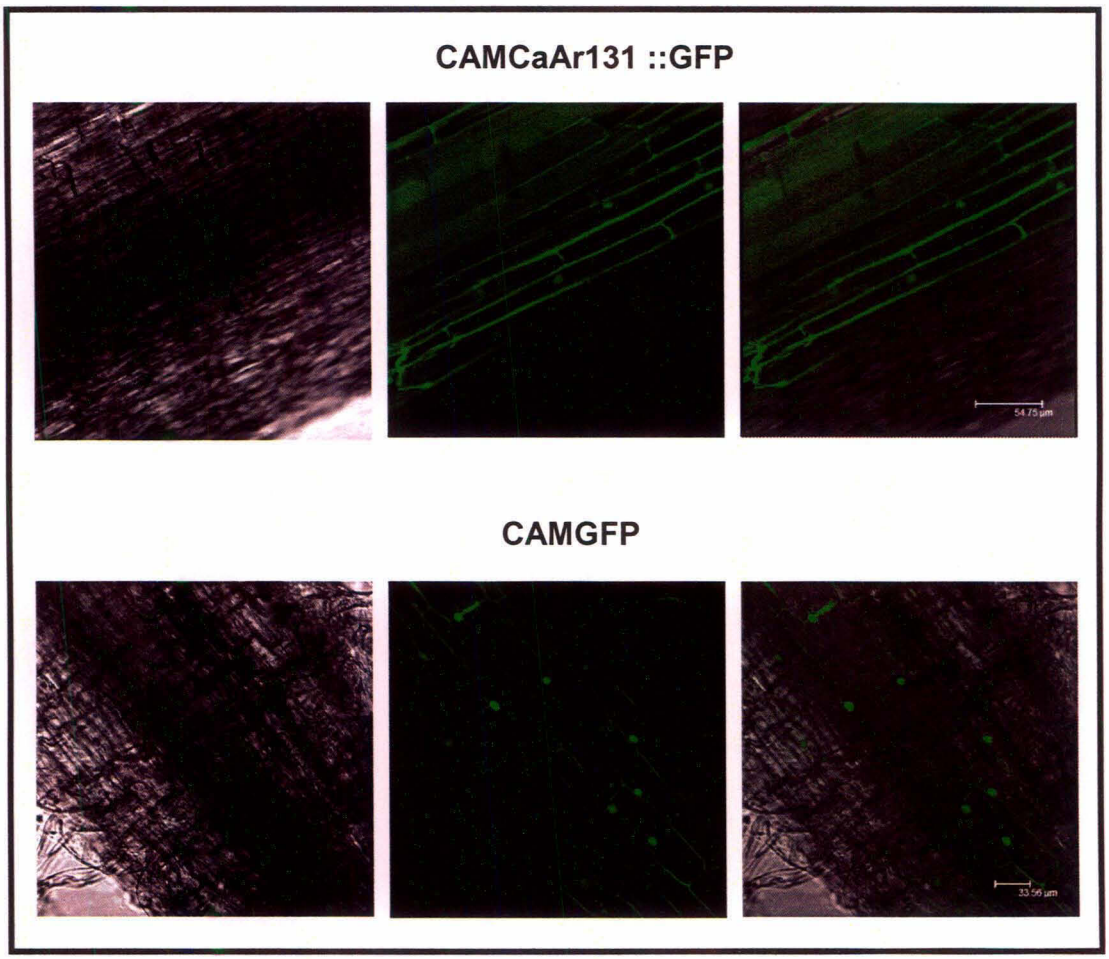

Figure 5.10. Subcellular localization of theCAM*CaHs1*$^{Pro-1}$ ::GFP protein in tobacco root cells. Tobacco transformants expressing only vector (CAMGFP) and CAMCaAr131 ::GFP fusion proteins were examined under a confocal laser scanning microscope (Leica) equipped with filters (excitation filter, 450–490 nm; emission filter, 520 nm).

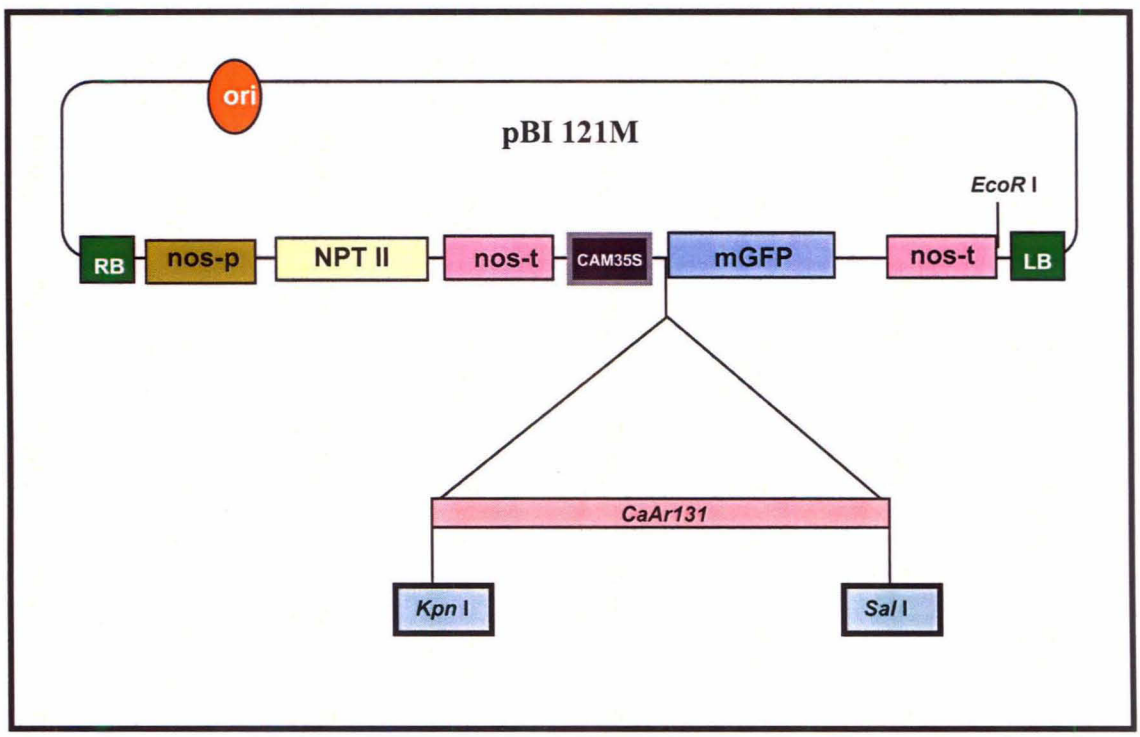

Figure 5.11. The schematic diagram of cloning strategy of CAM35S::CaAr131 in the binary vector pBI121M for overexpression.

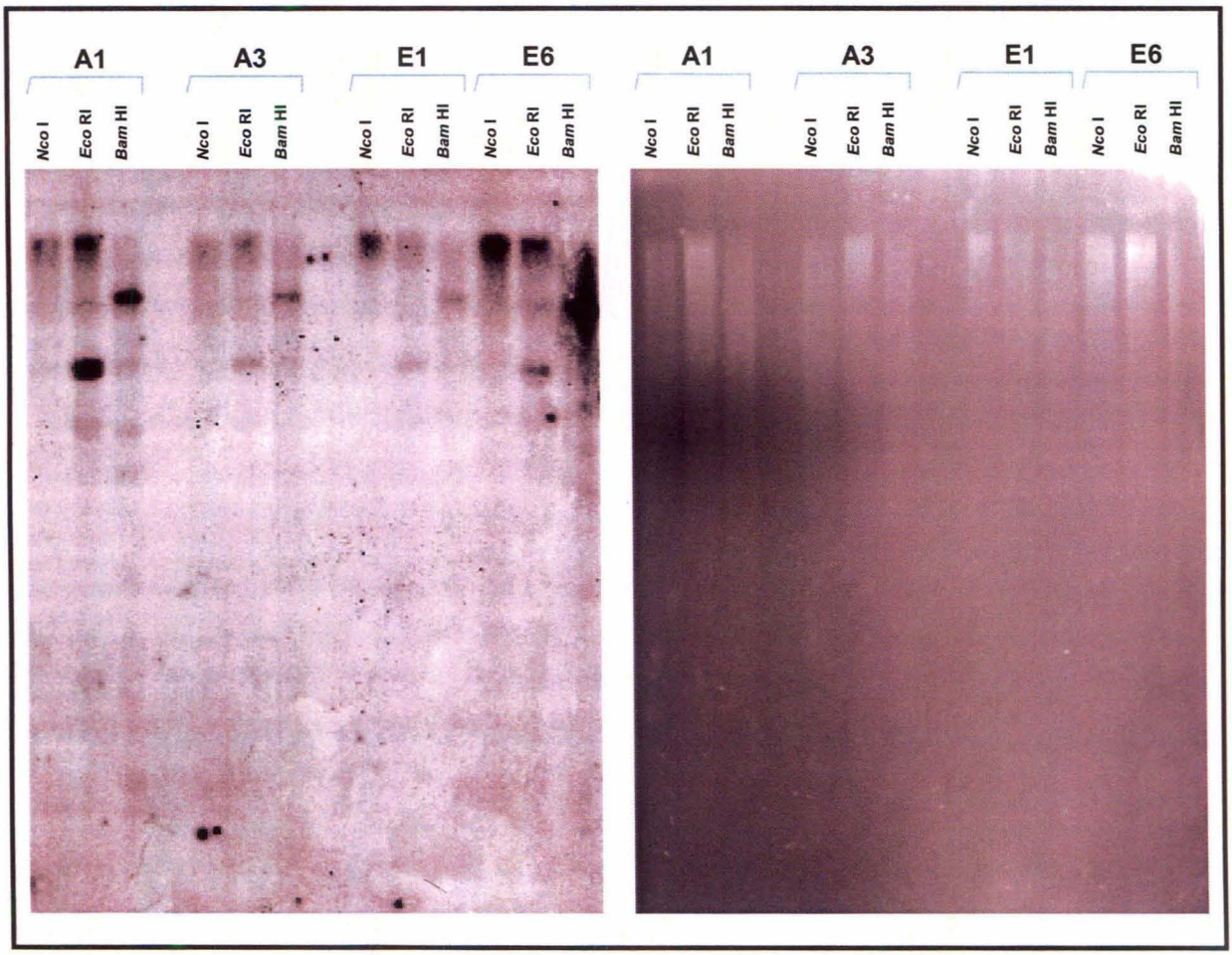

Figure 5.12. DNA blot analysis of *CaAr131* in four independent transgenic lines of tobacco. An ethidium bromide stained 0.8% agarose gel showing complete digestion of genomic DNA with *Nco*I, *Eco* RI and *Bam* HI. The λ *Hind* III marker was run along with the digested DNA. Autoradiogram showing Southern by using complete cDNA sequence of *CaAr131* as a probe.

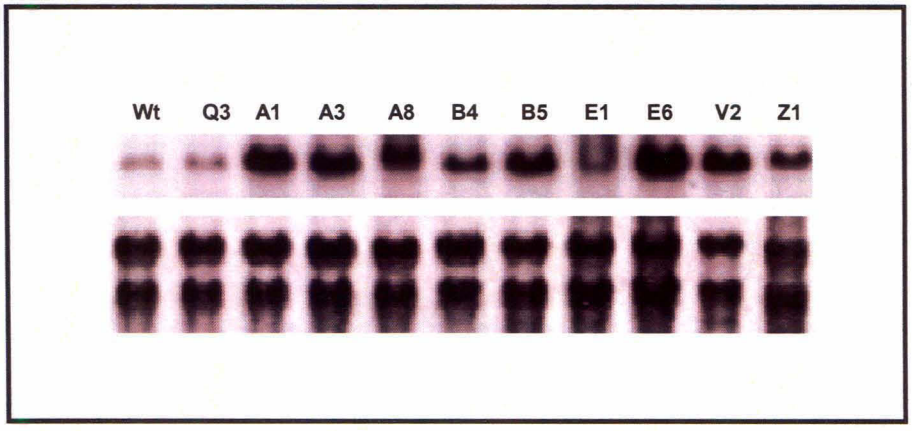

Figure 5.13. The expression of the *CaAr131* gene in the transgenic lines of tobacco. Total RNA (10µg) from transgenic tobacco was loaded into each lane. The *CaAr131* cDNA were used as probes. Methylene blue stained rRNA are shown as loading control.

grown tobacco plants were used as explants for transformation. Leaf disc has been infiltrated with *Agrobacterium* strain LBA4404 harbouring the pBI121M::*CaAr131* plasmid. Following co-cultivation explants were selected on MS medium containing BAP 1μg and NAA 0.1 μg containing kanamycin and regeneration was initiated three weeks later. The fully-grown plantlets were transferred on to rooting medium with a basal Murashige-Skoog (MS) medium. The plants were subsequently transferred to vermiculite in pots for hardening and finally transferred to green house. For control experiments the pBI121M vector were also used to transform tobacco.

5.2.7 Confirmations of putative transgenic lines

Putative transgenic lines selected on kanamycin were confirmed by PCR using gene specific primers and genomic DNA. Four of the lines were also confirmed by southern blot analysis using *CaAr131* cDNA as a probe (Fig. 5.12). Northern blot analysis was performed to check overexpression of *CaAr131* at transcript levels (Fig. 5.13). The plants are presently growing in the green house. Further analysis of these transgenic plants is presently going on at the time of submission of the thesis.

5.2.8 Isolation of 5'-upstream region of *CaAr131* and analysis of tissue-specific expression of the *CaAr131* promoter-*β*-glucuronidase Reporter gene

In order to isolate the 5'-upstream region, a genomic library from chickpea [FLIP_84-92C (2)] was constructed using Universal Genome Walker Kit (Clontech). Nested primers were designed according to the manual of the kit. The amplified product (Fig. 5.14) was cloned and sequenced. The sequence was analyzed on PLACE signal scan search (http://www.dna.affrc.go.jp/PLACE/signalscan.html) and PLANTCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) search programs, which revealed the presence of AT-rich sequence, wound-responsive motif, TCA-element (known for SA response), AP-2 like motif and many light responsive elements. Few more elements present include WBOXATNPR1, GT-1motif and most importantly MYB-like recognition sites (Fig. 5.15). The presence of these *cis*-acting elements function during *CaAr131* gene expression is a matter of conjecture.

To determine the regulation of *CaAr131* gene expression, the full length 5' upstream region and its four deletions were cloned into the binary vector pBI101.2 (Jefferosn *et al.*, 1987) as a transcriptional fusion in front of a promoterless β-glucuronidase (GUS) gene (Fig. 5.16). The resulting construct has been used to transform the tobacco by the method given by Gelvin *et al.*, 1987 and Kanamycin resistant transgenic plants were analyzed. To confirm the

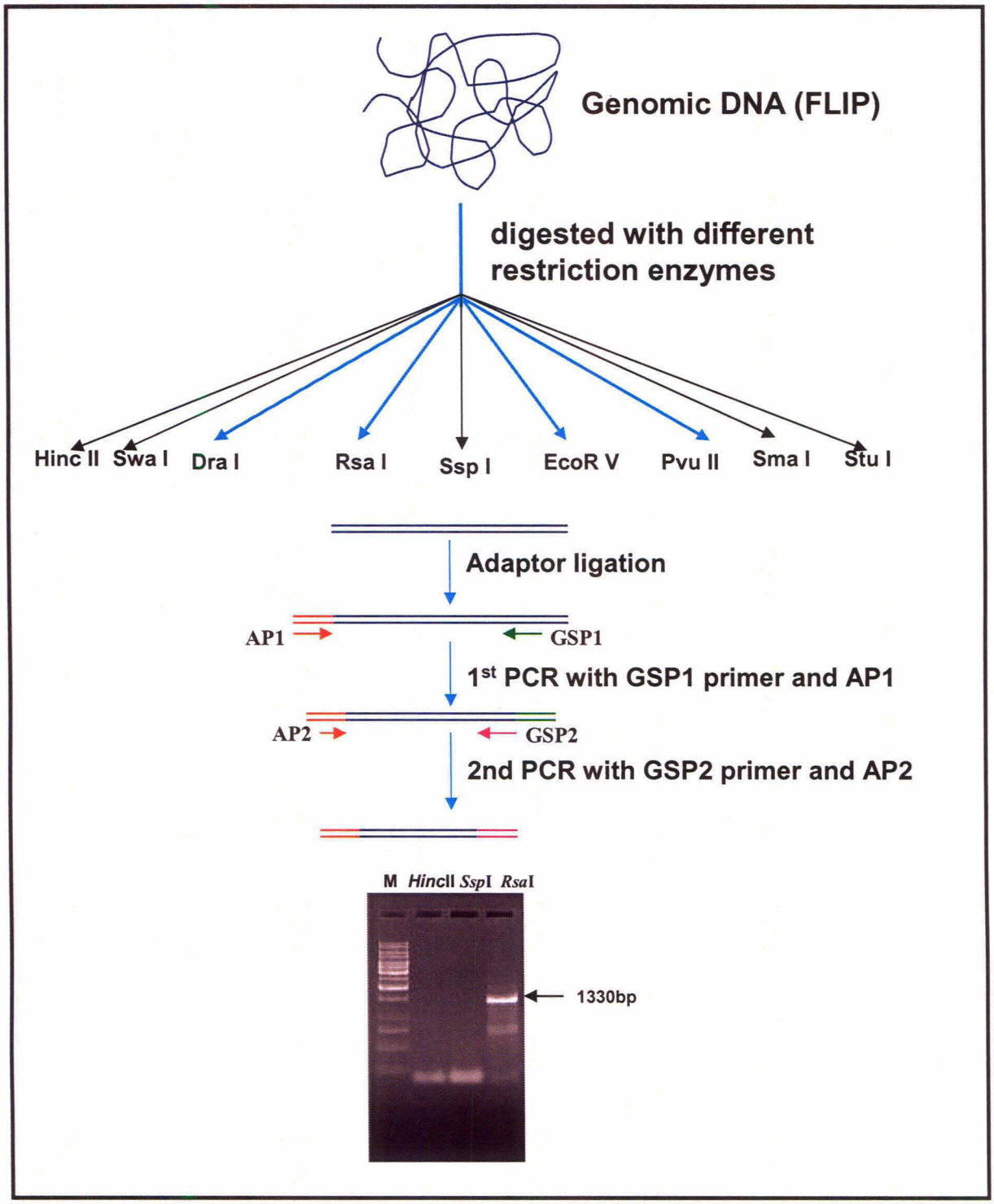

Figure 5.14. Isolation of 5'-upstream sequence of *CaAr131* gene. The flow chart for Genome walking and the secondary PCR of genome walking with *Hinc* II, *Ssp*I & *Rsa*I chickpea library using AP2 & GW1 (gene specific) primers.

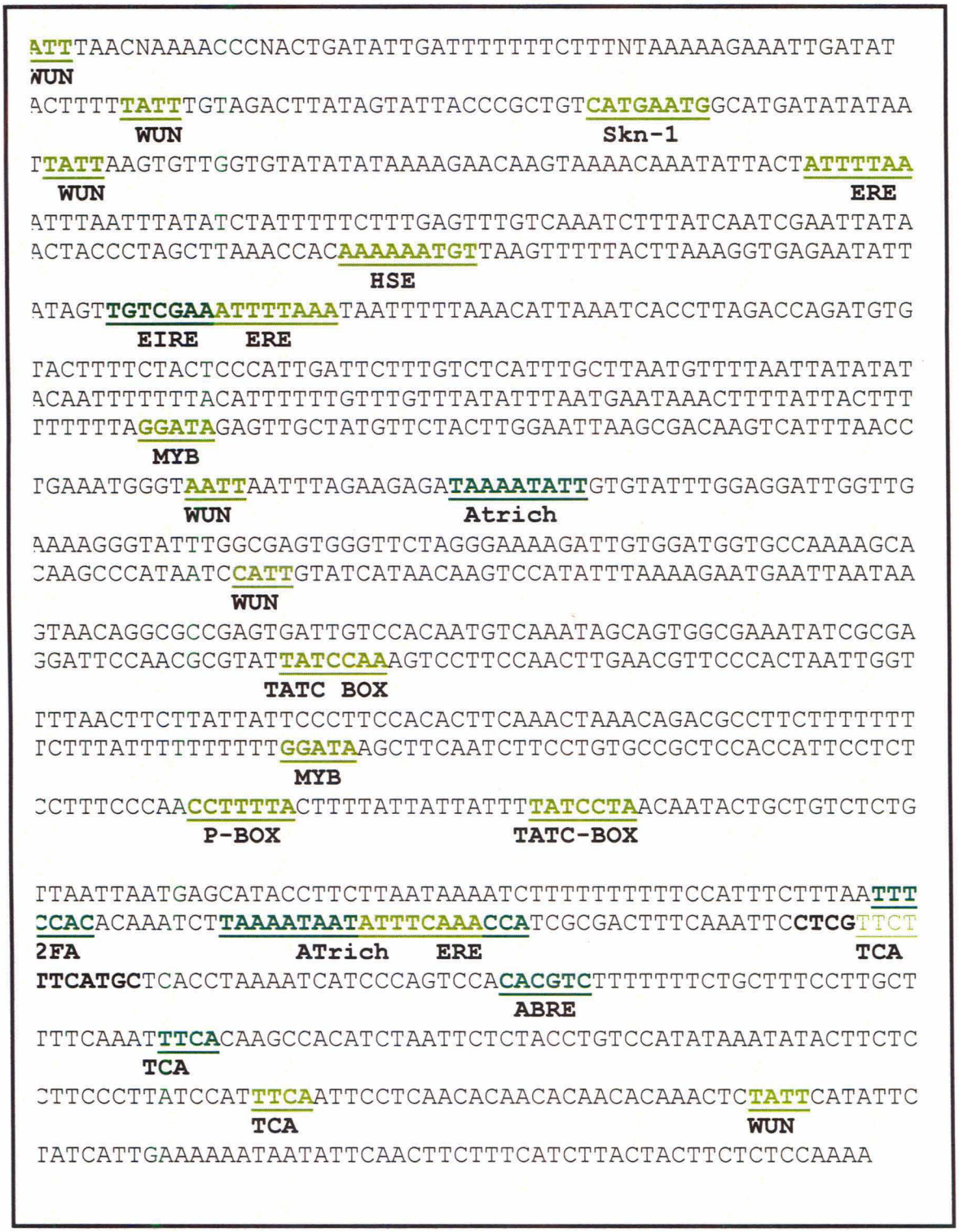

Figure 5.15. Nucleotide sequence of the promoter region of the *CaAr131* gene. The important predicted *cis*-acting elements are shown as green letters and are underlined. (WUN: wound induction motif; TCA: SA responsive; ABRE: ABA responsive; P Box: GA responsive; EIRE: Elicitor responsive ; ERE: ethylene responsive; HSE: temperature responsive.

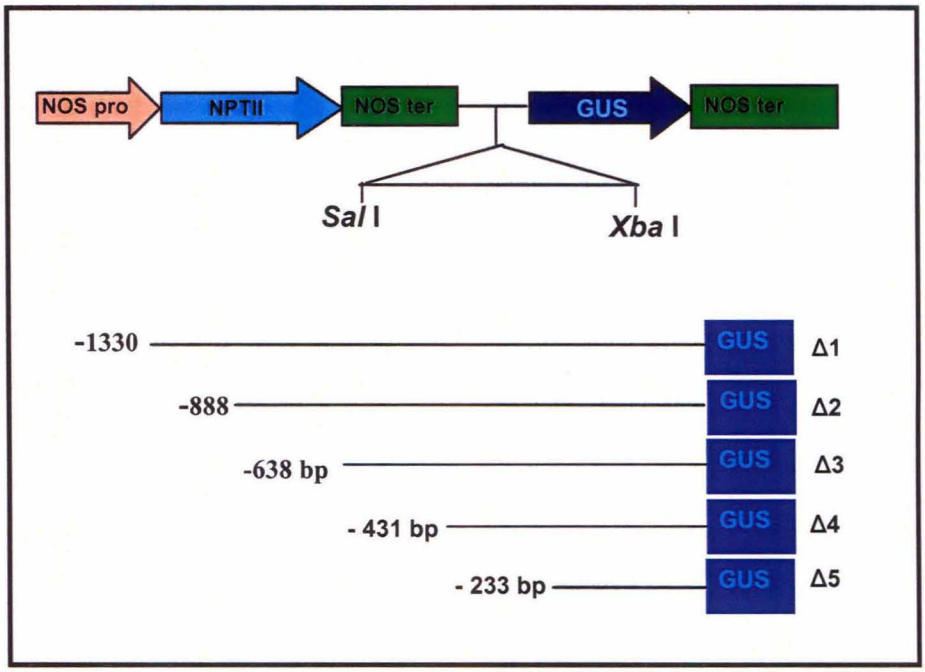

Figure 5.16. Effects of internal deletions on the tissue specific expression of the *CaAr131* promoter. Diagrammatic representation of cloning of 1330 bp *CaAr131* promoter sequence in *Sal* I and *Xba* I sites of pBI101.2 vector. The promoter is fused upstream to GUS gene.

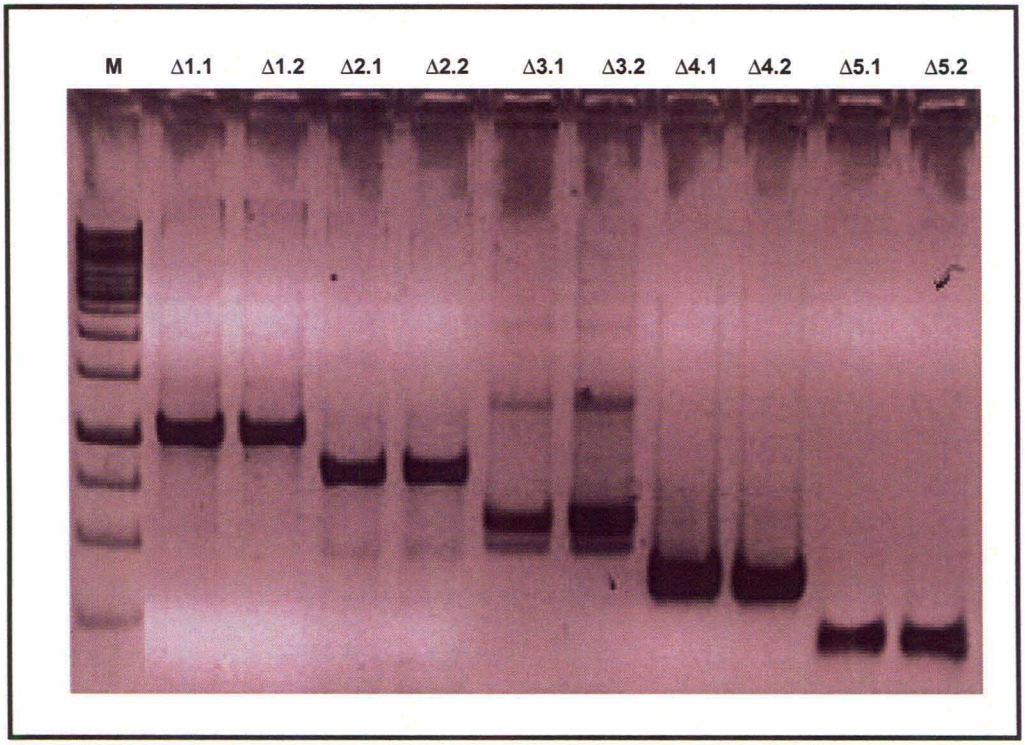

Figure 5.17. 1% Agarose gel showing amplification of *CaAr131* promoter in *CaAr131*-promoter::GUS fusion containing tobacco transgenic lines. PCR amplification of five deletion constructs of promoter of *CaAr131* using GUS Reverse and gene specific primers in transgenic lines of tobacco.

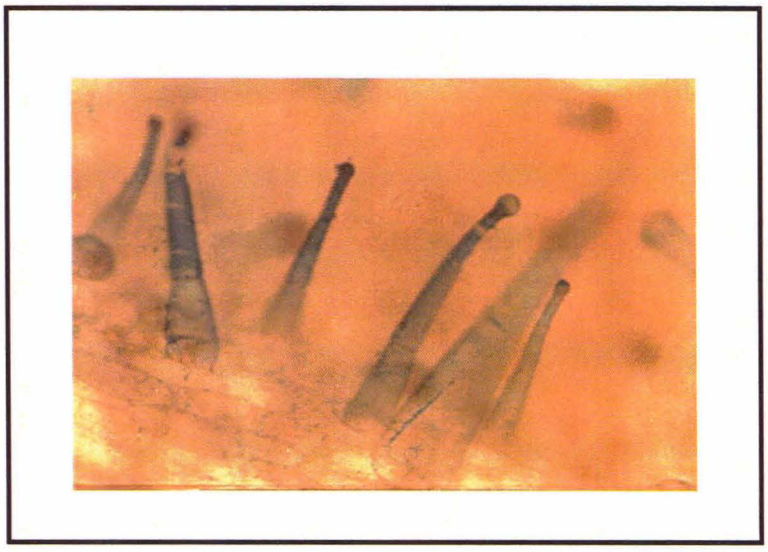

Figure 5.18. Histochemical localization of GUS activity in *CaAr131::GUS* transgenic tobacco. The positive transgenic lines were investigated for tissue specific expression by histochemical GUS analysis and it was observed that maximum GUS expression was shown in the trichomes of leaf and stem.

insertion of the constructs, genomic PCR was performed using gene specific forward primers and vector specific GUSR primer (Fig. 5.17) taking genomic DNA from different transgenic lines as a template. The confirmed lines were used for further analysis. The leaves of transgenic tobacco plants were stained with 5-bromo-4-chloro-3-indolyl- β-glucuronide (X-gluc) and the strongest GUS-staining was found to be present in the trichomes of the tobacco leaves (Fig. 5.18).

5.3 Discussion

Plants recognize attempted pathogen infection and activate defense responses to limit and activate defense responses to limit damage caused by disease with the mechanisms of recognition and response depending on the nature of the pathogen encountered. Current molecular models of pathogen response signaling have been derived largely from studies that center on plant interactions with biotrophic pathogens. In general, plants recognize pathogens through race-specific effectors or general elicitors referred to as pathogen-associated molecular patterns (PAMPS). Direct or indirect recognition of effectors by plant R proteins triggers a cascade of defense responses culminating in the hypersensitive responses culminating in the hypersensitive response (HR) and resistance to biotrophic pathogens. However, for necrotrophic pathogens, mechanisms of recognition and response are poorly understood. Till date, no known race specific effectors or corresponding R proteins described for necrotrophs-plant interactions, and HR may enhance infection by necrotrophic pathogens (Govrin and Levine, 2000). Recently, an R protein was implicated in susceptibility to a necrotrophic pathogen (Lorang *et al.,* 2007). FLS2-mediated MAPK activation regulates defense gene transcription and resistance to *P. syringae* and *Botrytis*, a necrotrophic fungus (Asai *et al.,* 2002). Thus, signal transduction components may be shared among defense responses induced by effectors from biotrophic and necrptrophic pathogens. PAMP-triggered basal responses are triggered by diverse pathogens, including necrotrophs, but neither the pattern recognition receptors nor the elicitors are characterized in many plant-necrotroph interactions.

In this context, we have chosen a potentially important and relatively uncharacterized member of the resistant gene homolog, a leucine rich repeat (*LRR*) protein closely related to $Hs1^{Pro-1}$ resistant gene that confer resistance to the beet cyst nematode, a major pest in the cultivation of sugarbeet (Cai *et al.,* 1997). The soybean homolog of $Hs1^{Pro-1}$ resistant gene when overexpressed confers resistance in soybean against soybean cyst nematode (McLean *et al.,* 2007). Its role in chickpea-*Ascochyta* interaction remains unstudied. There are evidences which suggest the quantitative nature of resistance mechanism against *Ascochyta* (Cho *et al.,* 2005). This quantitative resistance is conferred by multiple

genes and therefore, it would be interesting to elucidate individual genetic factors determining disease resistance.

5.3.1 Important Structural motifs of CaAr131

CaAr131 carries several sequence motifs that may contribute to its structure and function. The most important conserved domain present in CaAr131 is the LRR-flanking domains, which is a hallmark of most of the resistance (R) proteins. LRR motifs have been shown to mediate protein-protein and receptor-ligand interactions and also known to determine the specificity (Dangl and Jones, 2001). Parts of the LRR motif in plant R proteins may also participate in relaying downstream signaling through interactions with effector proteins (Shen *et al.,* 2007).The LRR domain present in CaAr131 is an imperfect LRR motif, not the typical one. Therefore, it would be interesting to study whether the imperfect LRR does participate in protein function. In addition, CaAr131 also contains few putative phosphorylation sites and O-glycosylation sites. All these motifs present in the deduced amino acid sequence indicates its major role in specific recognition of the pathogen and in relaying downstream signaling for initiation of defense related pathways (van der Hoorn *et al.,* 2001).

5.3.2 Role of *CaAr131* in constitutive resistance mechanism

To compare the kinetics of *CaAr131* transcript levels after *Ascochyta* infection in the susceptible [PI359075 (1)] and resistant [FLIP-84-92C (2)] chickpea germplasm lines, both the varieties were grown under similar conditions and were inoculated at the same time with *Ascochyta* spore suspension. To investigate the temporal relationships between the expression and development of disease resistance after pathogen inoculation, we monitored its transcript levels at various time points which revealed that the transcript was detected at the basal level itself and it reaches its maximum level at 24 h of infection implying its role in pre-existing and inducible surveillance systems in the resistant variety. In contrast to the resistant line, the constitutive nature of expression was not observed in the susceptible line. However, the transcript level starts increasing as early as 3h in both resistant and susceptible lines, suggesting its role in early response during infection. In addition, the biphasic expression profile of *CaAr131* gene was observed, which is typically observed in plant responses to pathogen infection (Alignan *et al.,* 2006), could correspond to the recognition of a specific fungal elicitor by the plant (3 h) and a delayed response (12h–72 h) to pathogen infection. Similar biphasic expression pattern was observed also for *CaPAL* (phenyl ammonia lyase from chickpea) gene of chickpea which was taken as a marker gene for *Ascochyta* infection. The bi-phasic production of apoplastic ROS (Reactive Oxygen species), which is the

oxidative burst during the incompatible interactions, is a central feature in successfully recognizing plant pathogens (Lamb and Dixon, 1997). Therefore, biphasic induction of *CaPAL* and *CaAr131* might also be correlated with biphasic induction of ROS.

5.3.3 Differenential expression of *CaAr131* gene in response to defense/stress -related signaling molecules and wounding

Signaling molecules such as SA, JA, and ABA are known to be involved in the regulation of defense responses in the host plant and are known to be involved in the signal transduction pathways which mediate defense responses (Zeevaart and Creelman, 1998; Glazebrook, 2001). Cross-talk between these signal compounds may modulate the expression of abiotic and biotic stress responsive genes in plants. Therefore we have investigated the effect of signaling molecules on the transcript level of *CaAr131.* The results showed upregulation of transcript by most of the signaling molecules, indicating its role in broad spectrum resistance. We also looked for *CaAr131* gene expression in response to wounding and it showed increase in transcript level also by wounding. Wounding and JA are correlated, thus a common expression patterns were obtained. The pattern observed indicates its role in the synergistic interaction of SA and JA during defense. Elevated levels of SA, along with H_2O_2, serve to activate local PR gene expression and also function as systemic signals in the activation of SAR in the systemic leaves (Devadas *et al.,* 2002) and serve as signals for ROS. Transcripts of *CaAr131* gene accumulated after treatment with hydrogen peroxide, thereby suggesting that the *CaAr131*gene shows activation by ROS signals.

5.3.4 Cytoplasmic localization of CaAr131

Although presence of transmembrane domain was predicted for $Hs1^{Pro-1}$ protein in sugar beet (Cai *et al.,* 1997), the corresponding regions in the two *Arabidopsis* $Hs1^{Pro-1}$ proteins present several differences. The Munich Information Center for Protein Sequences (MIPS) Arabidopsis database does not predict the presence of any transmembrane domain in these proteins (Gissot *et al.,* 2006). Similarly, CaAr131 also exhibit several differences and no transmembrane domain was predicted. Therefore, we looked for its subcellular localization in transgenic tobacco root harboring CaAr131:: GFP and it was observed that the maximum GFP was observed in the cytoplasm. This result was supported by the previous study by Gissot *et al.,* 2006, where they describe the existence of interactions in the cytosol between AKINβγ and two $Hs1^{Pro-1}$ proteins which is possible only when the protein would be localized in the cytoplasm.

5.3.5 Trichome specific expression and pathogen inducible nature of *CaAr131* promoter

Trichomes are specialized cells that produce secretions that are thought to provide a first line of defense against pests and pathogens. Trichomes are specialized unicellular or multicellular structures derived from the epidermal cell layer. Unicellular non-glandular trichomes, such as those present in *A. thaliana*, are not able to produce or secrete phytochemicals but may function as defensive physical structures against herbivores (Eisner *et al.,* 1998), as sinks for toxic heavy metals and xenobiotics (Gutie´rrez-Alcala´ *et al.,* 2000; Domı´nguez-Solı´s *et al.,* 2004), and in regulating water absorption (Werker, 2000).

Multicellular trichomes can be found in many different species and often form glands that secrete phytochemical compounds (e.g. organic acids, polysaccharides, terpenes, or salt) as well as secondary compounds such as those produced in trichome exudates (e.g. terpenoids, flavonoids, and phenylpropanoids) (Duke *et al.,* 2000). Glandular trichomes show various forms and can be unicellular or multicellular and morphological distinction can be observed between the apical and the basal part of the glands (Werker, 2000). Glandular secretory trichomes have potential biotechnological applications as a result of the great variety of phytochemical molecules produced. Many of these molecules have significant commercial application in the production of flavours and fragrances, such as vanillin and benzaldehyde, the pharmaceutical industry, such as artemisinin (Mahlberg and Kim, 1992; Li *et al.,* 2002), and in host defence or plant– plant allelopathy (Werker, 2000). Extensive references about secreted molecules from plant trichomes are available (Callow, 2000; Wagner *et al.,* 2004).

The 5' upstream region directs tissue-specific GUS expression in the trichomes. A trichome-specific promoter has been isolated from cotton (LTP3 gene promoter) and from tobacco (CYP71D16 gene promoter) previously (Liu *et al.,* 2000; Wang *et al.,* 2004a). These promoters were able to direct GUS expression, the former in non-glandular and the latter in glandular trichomes (Liu *et al.,* 2000; Wang *et al.,* 2002). The CYP71D16 promoter was also successfully used to suppress cembratrieneols in trichome exudates and to reduce aphid infection in tobacco (Wang *et al.,* 2004a). These reports further confirm the defensive nature of trichomes against biotic stress. The common cis-acting element which is present in all these promoters including the promoter of *CaAr131*, are the MYB binding sites (GGATA, CAGTTG and GTTAGGAA) and the essential role of the MYB binding in trichome expression, localized to the RDL1 promoter region, has previously been described (Wang *et al.,* 2004b). MYB binding sites are present in sugarbeet *Hs1* $^{pro-1}$ promoter (Thurau *et al.,* 2003).

The MYB gene family represents one of the largest regulatory factor families in plants, and one of the important functions for MYB factors is to control development and

determination of cell fate and identity. In *Arabidopsis*, the *GB1* governs leaf trichome formation and the MIXTA gene from *Antirrhinum majus*, when ectopically expressed in tobacco, can promote trichome differentiation. Also members of several transcription factor families have been implicated in defense gene regulation; MYB is one of them. Therefore, we predict that the trichome-specific expression of *CaAr131* promoter could be regulated by one of the MYB transcription factors. This promoter has promise for use in molecular farming and for enhancing trichome-based pest/disease resistance in plants with trichomes.

Chapter 6: Helicoverpa-induced responses in Chickpea

6.1 Introduction

A broad range of interactions occur between plants and insects as they have co-evolved for millions of years. Some of the interactions can be detrimental and plants may lose substantial proportions of their biomass on insect attack. Since loss of biomass is costly, they have developed an array of defense genes to protect themselves against insects. However, insects counter responses are also highly adaptive and able to evade natural plant defenses. Plants have developed a multitude of defense mechanisms for dealing with insect attack including constitutive preformed physical and chemical barriers, reducing their access. But these barriers can be breached with little efforts by much clever attackers. Therefore plants possess another set of defenses i.e. inducible defenses, a more energy efficient and durable defense system (Agrawal, 1998; Karban and Baldwin, 1997; Thomma *et al.,* 1998; Kessler and Baldwin, 2002). Induced defenses operate via both direct and indirect modes. Defense-related protein expression, reinforcement of the cell wall, biosynthesis of secondary compounds, and production of reactive oxygen species are examples of direct induced defenses. Volatile organic compounds provide indirect defense by attracting enemies of the attacker (Pare and Tumlinson, 1997; Kessler and Baldwin, 2002). Complex crosstalk networks have been uncovered which serve to recruit various signal pathways in defense induction regulation (Walling, 2000; Rojo *et al.,* 2003). While methyl jasmonate (MeJA) signaling plays a primary role in chewing insect defense (McConn *et al.,* 1997; Reymond *et al.,* 2004; De Vos *et al.,* 2006), ethylene mediated expression is also involved (Stotz *et al.,* 2000; Kessler and Baldwin, 2002; von Dahl *et al.,* 2007). In addition, salicylic acid (SA) is an important plant-produced signal. During biotrophic pathogen interactions, SA activates plant defense responses against pathogen attack (McDowell and Dangl, 2000; Glazebrook, 2005).

6.1.1 Molecular Basis of Insect-Plant Interaction

Large-scale transcriptional changes accompany insect-induced resistance, and herbivore-specific cues orchestrate the responses (Kessler and Baldwin, 2002). Transcript pattern changes in response to herbivory have been generated in many plant species including *Arabidopsis thaliana* (Reymond *et al.,* 2000; Reymond *et al.,* 2004; Kempema *et al.,* 2007), *Nicotiana attenuata* (Hermsmeier *et al.* 2001; Hui *et al.,* 2003), *Citrus sinensis* (Mozuruk *et al.,* 2006), *Picea sitchensis* (Ralph *et al.* 2006b) and poplar (Ralph *et al.,* 2006a; Major and Constabel, 2006). These studies have provided insights into the molecular basis of insect-plant interactions, but little information regarding cultivated crops are available. Moreover, recent studies reveal that differential gene expression is dependent on the type of attacker and in some cases species specific (Zarate *et al.,* 2007). For example, insect-inducible genes identified in *N. attenuata* had little sequence homology with upregulated genes in

Arabidopsis (Korth, 2003). Moreover, attack from the same lepidopteran herbivore resulted in species-specific transcriptional responses in two species of solanaceous host plants (Schmidt *et al.,* 2005).

6.1.2 Differences between Mechanical Damage and Insect infestation

Plants distinguish between mechanical damage and herbivory. Insect attacks on plants results in wounding, but a plant's molecular response to mechanical damage differs (Korth andDixon, 1997; Reymond *et al.,* 2000). Several different types of elicitors, including fatty acid conjugates (e.g. Volicitin, Alborn *et al.,* 1997) and enzymes (glucose oxidase, Felton and Eichenseer, 1999; β-glucosidase, Mattiacci *et al.,* 1995; and alkaline phosphatase, Funk, 2001), are present in the oral secretions and regurgitant of herbivores (Parè and Tumlinson, 1999), which may contribute to the differential response.

6.1.3 Involvement of Defense Regulators during Insect-Plant Interaction

Recruitment of the signal pathways (jasmonate, salicylate and ethylene, mainly; De Vos *et al.,* 2005) in the regulation of the induced genes imparting role in defense against insect attack also varies depending on the host and the mode of insect feeding i.e. whether the insect is a generalist or a specialist or may be due to involvement of some other factors. The quantity and timing of the production of SA, MeJA and ET varies widely depending on the type of attacking insect and once a signal is induced, it results in the activation of a specific set of genes eventually which account for the defense response. MeJA among them are the most crucially important regulators of induced defense responses against insect attack (De Vos *et al.,* 2006). While MeJA signaling is considered to have a primary role in defense against chewing insects, ET is involved as well often, in opposition to JA (Mewis *et al.,* 2006). In addition to jasmonate and ethylene, salicylate is also known to be important plant-produced signals that can activate plant defense response against herbivory (Van Poecke *et al.,* 2003). How the induced defense response gained by plants by prior application of signaling compounds and vaccination affects the growth and feeding behavior of herbivores remains unexplored except for widely studied insect plant interactions (*N.attenuata-M.sexta* interaction, Voelckel and Baldwin, 2004; *Raphanus sativus*, Brassicaceae-*Pieris rapae*, Agrawal, 2000).

6.1.4 Induced Plant Defense

Plants, like animals, alter their induced defenses in response to prior experiences (Baldwin and Schmelz, 1996). Induced plant defense in response to herbivory is a common phenomenon present in plants and enable them to survive better by reducing subsequent

insect attack (Karban and Baldwin, 1997). A mild insect infestation induces an adaptive mechanism by which the quality of plants as a food source reduces which can reduce insect preference and its performance on induced plant compared to that on uninduced plant (Agrawal, 1998). Induced resistance to subsequent attacks is due to plant changes in molecular and biochemical composition, which subsequently modify metabolic processes involved in the adaptive response. Pretreatment with MeJA induced a substantial resistance in *Nicotiana attenuata* which could decrease growth and development of the specialist herbivore, *Manduca sexta* (van Dam *et al.,* 2000). Prior attack by sap-feeding mirids results in vaccination of the plants against subsequent attacks by chewing hornworms (Voelckel and Baldwin, 2004). Vaccination against subsequent attack results due to changes in transcriptional status of a number of genes which modifies the metabolic processes for the adaptive response.Limited reports describe the effect of induced defense on the growth and feeding behavior of herbivores (Agrawal, 2000; Voelckel and Baldwin, 2004). Therefore, further exploration in other plant systems is warranted.

6.1.5 Chickpea-*Helicoverpa* Interaction

Chickpea (*Cicer arietinum* L.) is an important legume crop due to its role in the human diet and use in animal feed. One of the major threats to its successful production is the generalist herbivore, *Helicoverpa armigera*, which damages the aerial parts of the plant, including leaves and pods. First remedy for this damage came as frequent use of insecticides which resulted in the development of considerable level of resistance to insecticides and proved to be an environmental hazard. As a next step towards defending herbivory, plant proteinase inhibitors were discovered which are known to affect the growth of herbivores and may function as defensive agents against insects which use proteinases to digest their food proteins (Johnston *et al.* 1991; Jongsma *et al.* 1995). The induction of proteinase inhibitors in response to chewing by *H. armigera* has been reported but the HGPs (Gut proteinases) are able to destroy the protease inhibitors (Giri *et al.,* 1998). Thus it can be concluded that induction of proteinase inhibitors is not enough to provide sufficient resistance against *H. armigera* and it was recognized that exploring plant resistance may be the most effective and economic option for pest management. A preliminary study on differential defense responses induced during plant communication with *Helicoverpa armigera* resulted in upregulation of defense related genes PR1, BGL2, and PAL genes in tobacco and tomato but it was significantly higher in the case of tomato as compared to tobacco (Peng *et al.* 2005). Since most studies examining *Helicoverpa*-chickpea interactions have focused on specific gene or protein dynamics (Johnston *et al.* 1991; Jongsma *et al.* 1995; Giri *et al.,* 1998; Peng *et al.,*

2005; Srinivasan *et al.,* 2005), examining large scale transcriptional analysis would broaden the scope.

6.2 Results

6.2.1 Validation of *Helicoverpa* and wound-inducible responses by differential expression of Lipoxygenase (LOX) gene

Jasmonates are known to be an important signaling component of plant defense and LOX gene play significant role in MeJA biosynthesis, therefore in order to check the validity of insect infestation and wounding experiments, we have monitored the changes in the transcript level of LOX gene during *Helicoverpa* infestation and mechanical damage before proceeding for SSH. Comparing the transcript pattern of LOX gene in response to infestation and mechanical damage, it was observed that mRNA level of LOX steadily increased during caterpillar feeding over the 24 h time course, but during mechanical damage its increase in transcript level is slow and less abundant (Fig. 6.1). The increased transcript level of LOX indicates the higher concentration of JA that can actively regulate defense gene expression and elicit resistance in plants against insect feeding (Reymond *et al.,* 2004).

6.2.2 Isolation and Identification of differentially Expressed genes

In order to decipher genes upregulated during mild infestation by *Helicoverpa* which may lead to defense, a forward subtractive cDNA library was constructed using suppression subtractive hybridization (SSH) strategy. As a result, 715 recombinant colonies were obtained which were subjected for differential screening and sequencing. After screening for induction during insect infestation (Fig. 6.3) and sequencing, 63 unique genes were identified by BLASTx analysis which included transcripts not previously reported to be induced during insect attack and some functionally unknown transcripts. In addition to this, some transcripts already known to be responsive to insect attack in other plants were also obtained which appears to support the validity of the subtracted cDNA library. To gain insights into the function of differentially expressed genes, we categorized them into eight classes based on their putative roles during *Helicoverpa*-infestation (Table 6.1; Fig. 6.2). The major functional category corresponded to genes involved in defense, secondary compound synthesis and cell wall fortification and was classified as defense-related (29%). In addition, another category comprised of genes involved in signaling and gene regulation (10%) and a significant fraction of genes were involved in detoxification (8%). Genes were also found to play a role in protein synthesis (6%), abiotic stress (6%), photosynthesis or energy metabolism (6%) and a major fraction (13%) are listed as miscellaneous. Genes, whose function were not ascertained (22%)

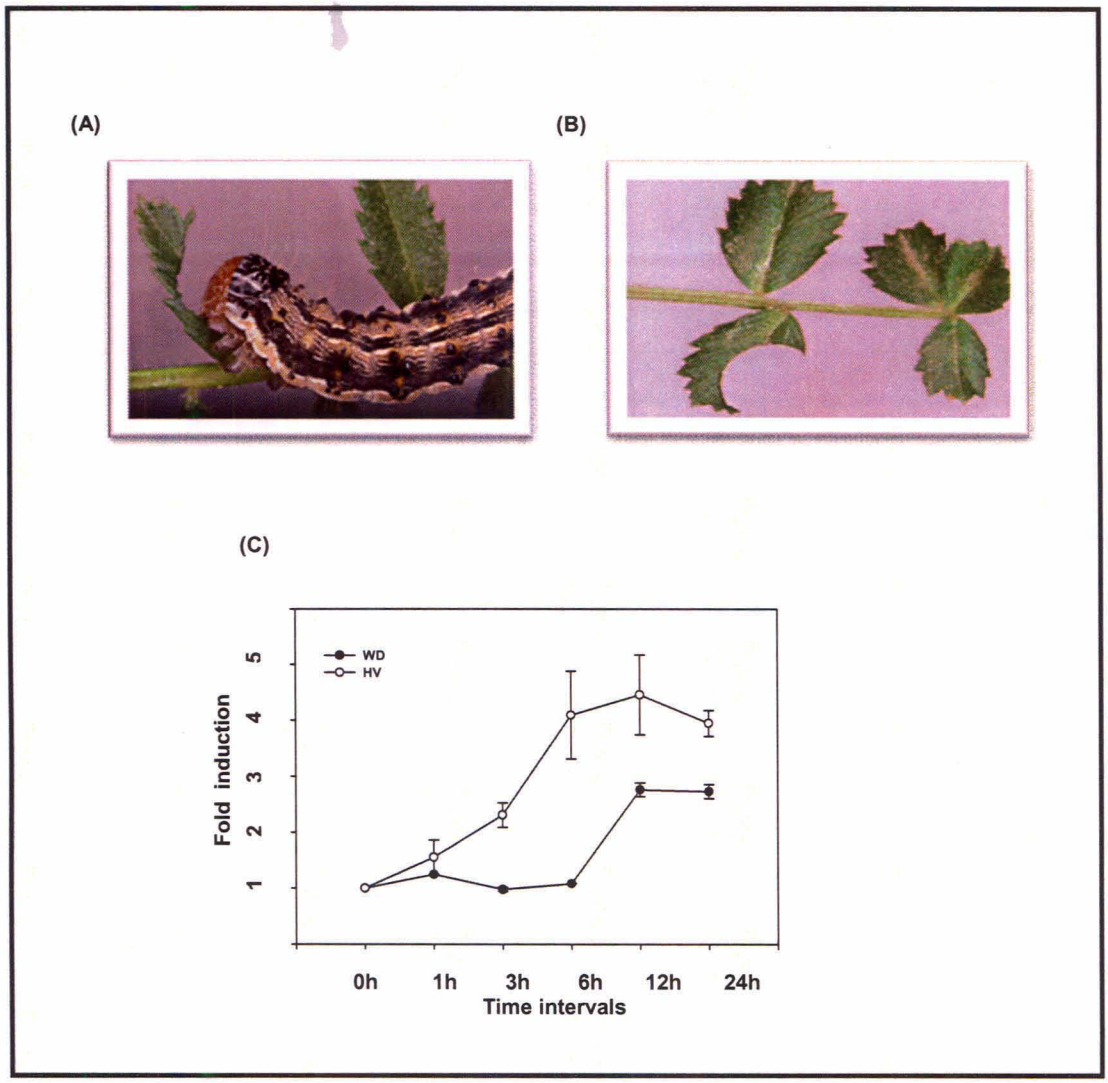

Figure 6.1. *Helicoverpa*-infested and mechanically wounded young leaves of chickpea under greenhouse conditions. (A)Representative photograph of leaf damage inflicted by caterpillar feeding. **(B)**Chickpea leaves wounded by punch. **(C)**Differential transcript patterns of chickpea Lipoxygenase (LOX) geneduring *Helicoverpa* infestation and mechanical damage. The graph shows a comparative analysis of the relative intensity of mRNA levels at various time points.

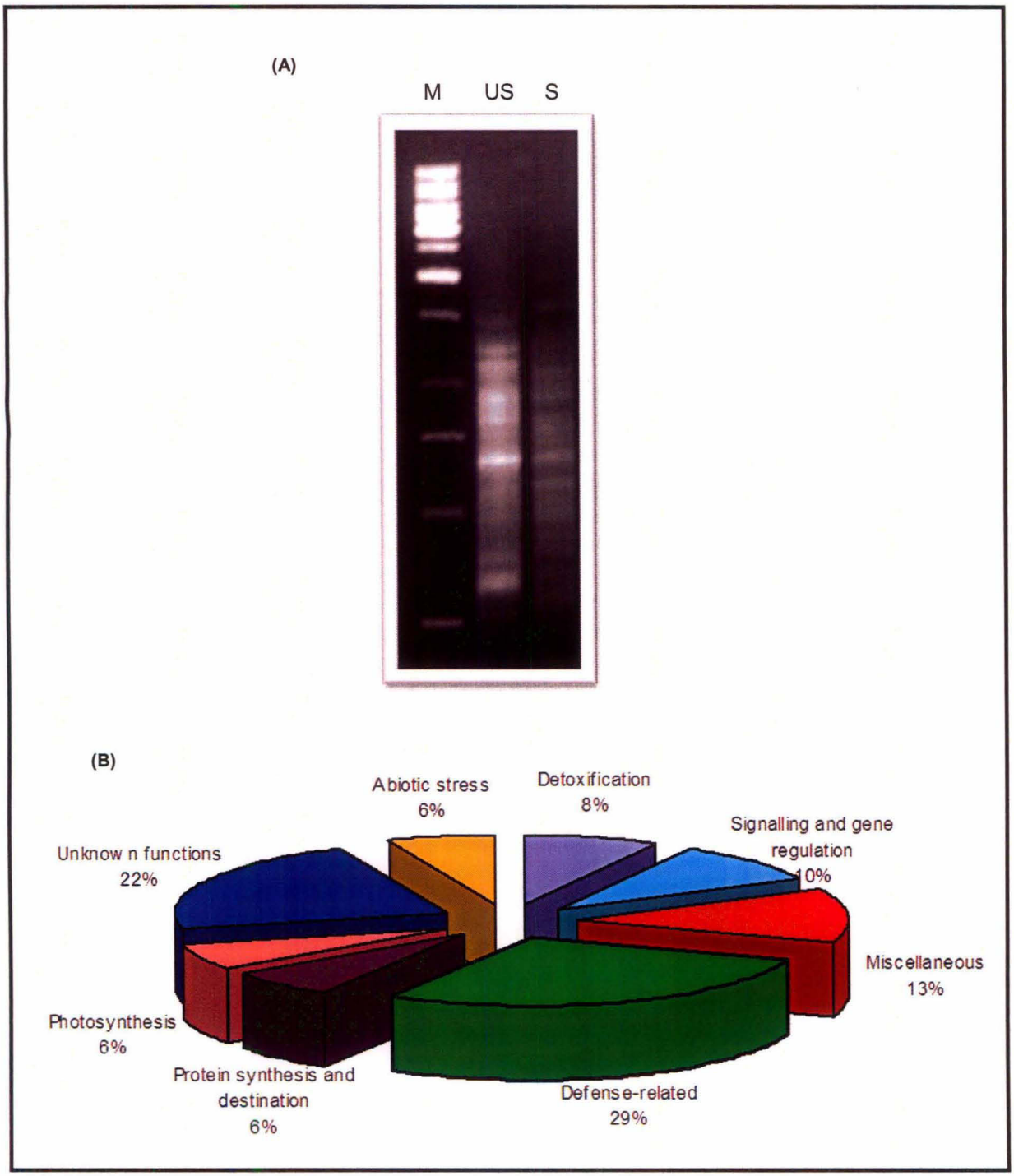

Figure 6.2 (A) 1% EtBr-agarose gel showing the smear of amplified subtracted cDNAs. M; 1 Kb ladder. US; "unsubtracted cDNA", S; "subtracted cDNA" obtained after primary and secondary amplifications. **(B) Functional cataloging of *Helicoverpa*-responsive genes.** The identified *Helicoverpa*-responsive genes were assigned a putative function based on their homology and functionally categorized as presented in the pie-chart.

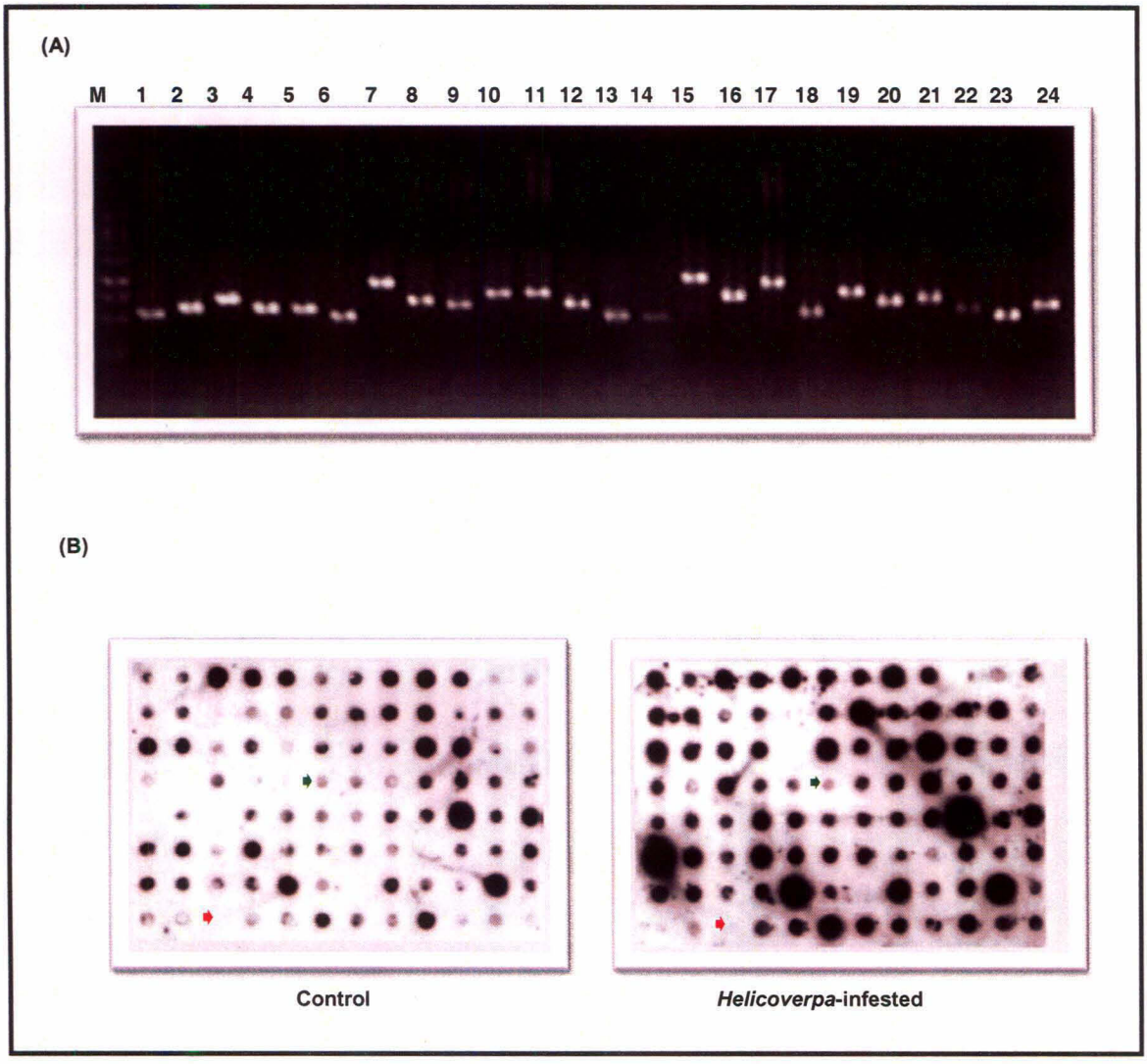

Figure 6.3. 1% EtBr stained gel showing PCR amplification of the positive clones. (B) Dot blot showing fold induction of the transcripts obtained from subtraction library. Positive clones were PCR amplified using M13 sequencing primers. Approximately 100 ng of visually quantified PCR product was blotted using 96 well Dot Blot apparatus. A replica of the blot was also made. The two blots were respectively hybridized with radiolabelled first strand cDNA probe prepared from 1μg mRNA from Control and *Helicoverpa*-infested samples. The dots indicated by green arrows are actin (+ve control) and the dots indicated by red arrows are npt II gene (-ve control).

were categorized as "unknown functions", and considered to be *Helicoverpa*-responsive. This is also to be mentioned that for some of the genes the functional categorization might be arbitrary and there may be some overlaps.

6.2.3 Cluster analysis revealed distinct responses to *Helicoverpa* infestation, mechanical damage, MeJA, ET and SA

In order to achieve a comprehensive overview of expression profile of genes that were co-expressed during insect infestation, mechanical damage and treatments of signaling compounds, SOTA clustering was performed. The expression ratios obtained by macroarray were log2 transformed in order to reduce the noise level. The analysis yielded 11 clusters and the clusters with n>10 were selected to study the expression patterns for functionally similar genes (Fig. 6.4). Maximum number of genes were grouped into cluster 11 and comprised of genes which showed very high expression level during *Helicoverpa*-infestation, MeJA and ET treatments (Fig. 6.5). In contrast to this, expression of the genes in this cluster was less during mechanical damage and SA treatment. This group was found to be enriched in genes involved in defense, abiotic stress, protein synthesis and destination and genes of unknown functions. Another major group, cluster 4, consisted of defense-related genes and genes playing role in signaling and gene regulation and detoxification as well. The genes in this cluster showed similar expression patterns during *Helicoverpa*-infestation, MeJA and ET treatments but their expression was almost nil during mechanical damage. In cluster 1, genes showing higher expression during *Helicoverpa*-infestation and no induction by SA were placed. Almost all functional categories are represented in this cluster. The miscellaneous class and genes with unknown functions showed no clear clustering and were present in almost all the clusters which maybe due to heterogeneous composition of these categories. Characterization of these genes can provide valuable insight in understanding chickpea-*Helicoverpa* interaction better.

6.2.4 Different transcript signatures for *Helicoverpa* feeding and mechanical wounding

Of 63 unique genes selected for further analysis, the transcripts of 46 genes were upregulated upon *Helicoverpa* infestation but wounding altered transcript levels of only 8 genes above the cut-off value (as described in material and methods) on comparing with control. For the genes whose mRNA levels were co-induced during both types of stress, the transcript levels were higher on *Helicoverpa* infestation (Table 1). *Helicoverpa* infestation and wounding pair expression ratios were compared, and revealed 29 gene ratios were significantly different and are presented as "volcano plots" (Jin *et al.*, 2001) in (Fig. 6.6B) . A subset of five genes was analyzed by northern blot to validate the macroarray dataset (Fig.

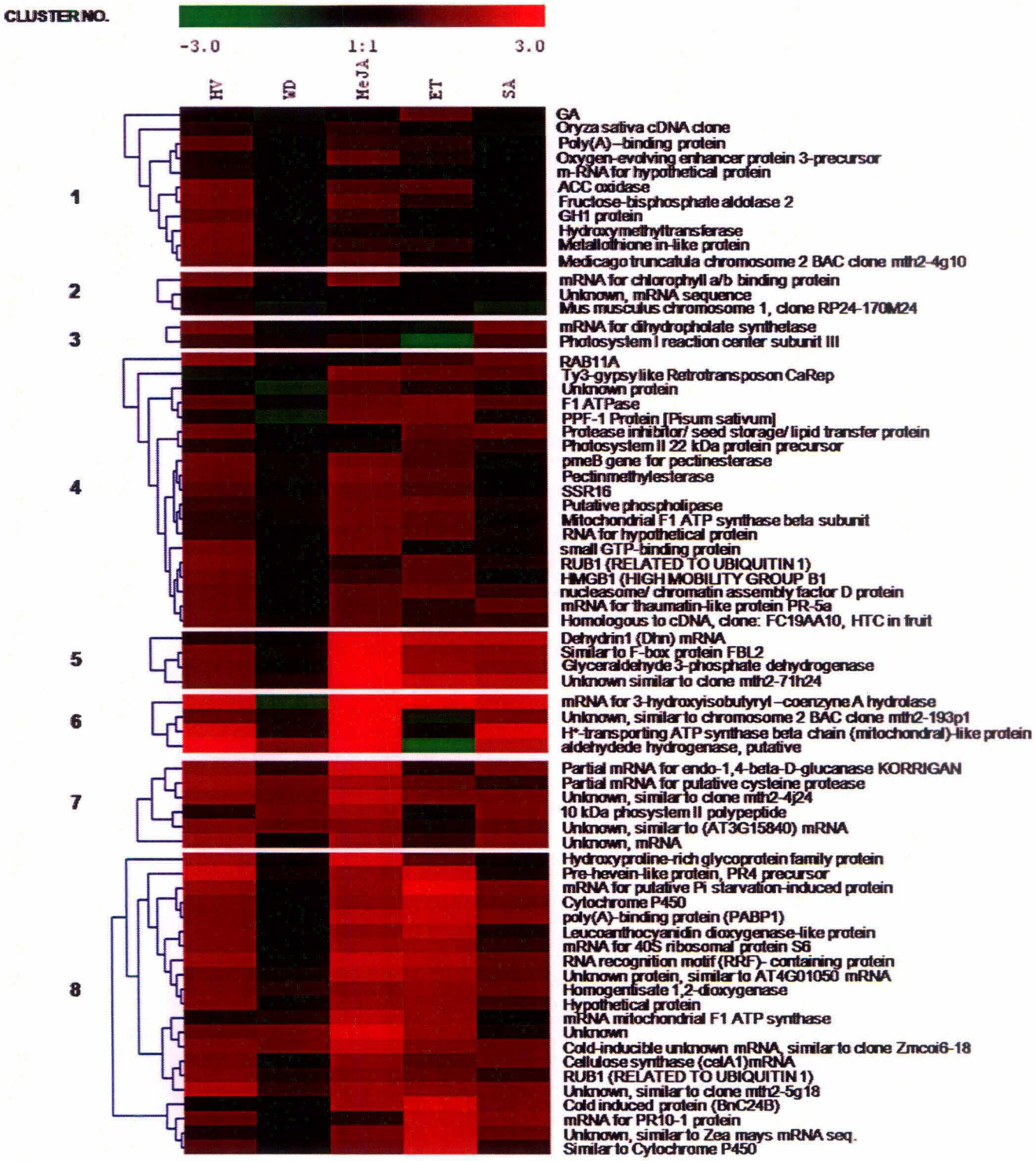

Figure 6.4. Clustering Analysis of expression profiles of *Helicoverpa*-responsive genes. SOTA cluster tree of selected genes are shown to illustrate differential induction patterns after *Helicoverpa* infestation (HV), mechanical damage (WD), Methyl Jasmonate (MeJA), Ethephon (ET), and Salicylic Acid (SA) treatments. Each gene is represented by a single row of colored boxes, and a single column represents each treatment.

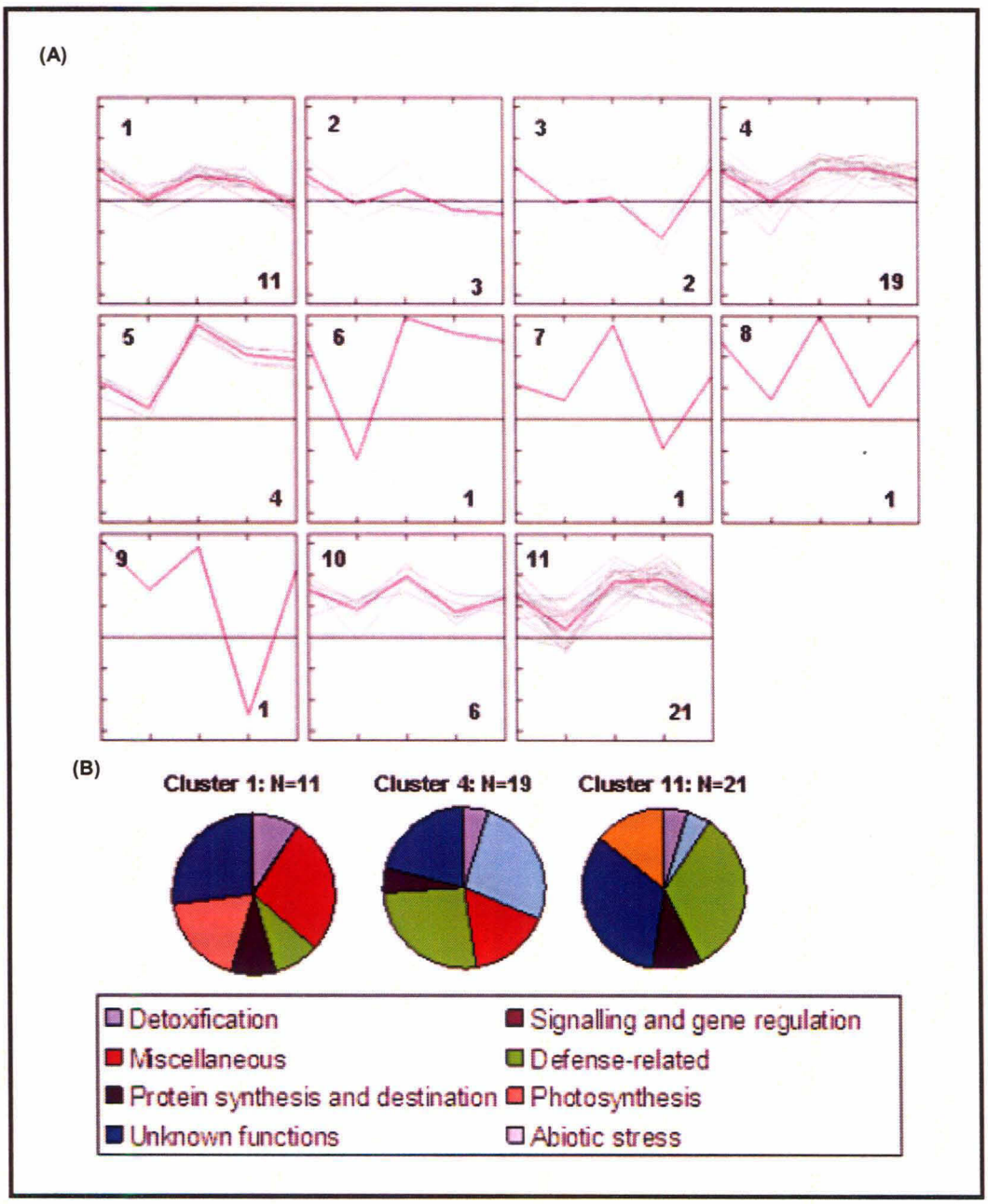

Figure 6.5. (A)The differentially expressed 63 unique genes were clustered into 11 clusters based on their expression profiles. The expression profile of each individual gene in the cluster is depicted by grey lines, while the mean expression profile is marked in pink for each cluster. The number of genes in each cluster is given in the right lower corner and the cluster number in the left upper corner. **(B)Functional cataloging of the genes present in different clusters.** The classified *Helicoverpa*-responsive genes were categorized based on their function and presented in the pie-chart. (Clusters with N>10 were taken into consideration).

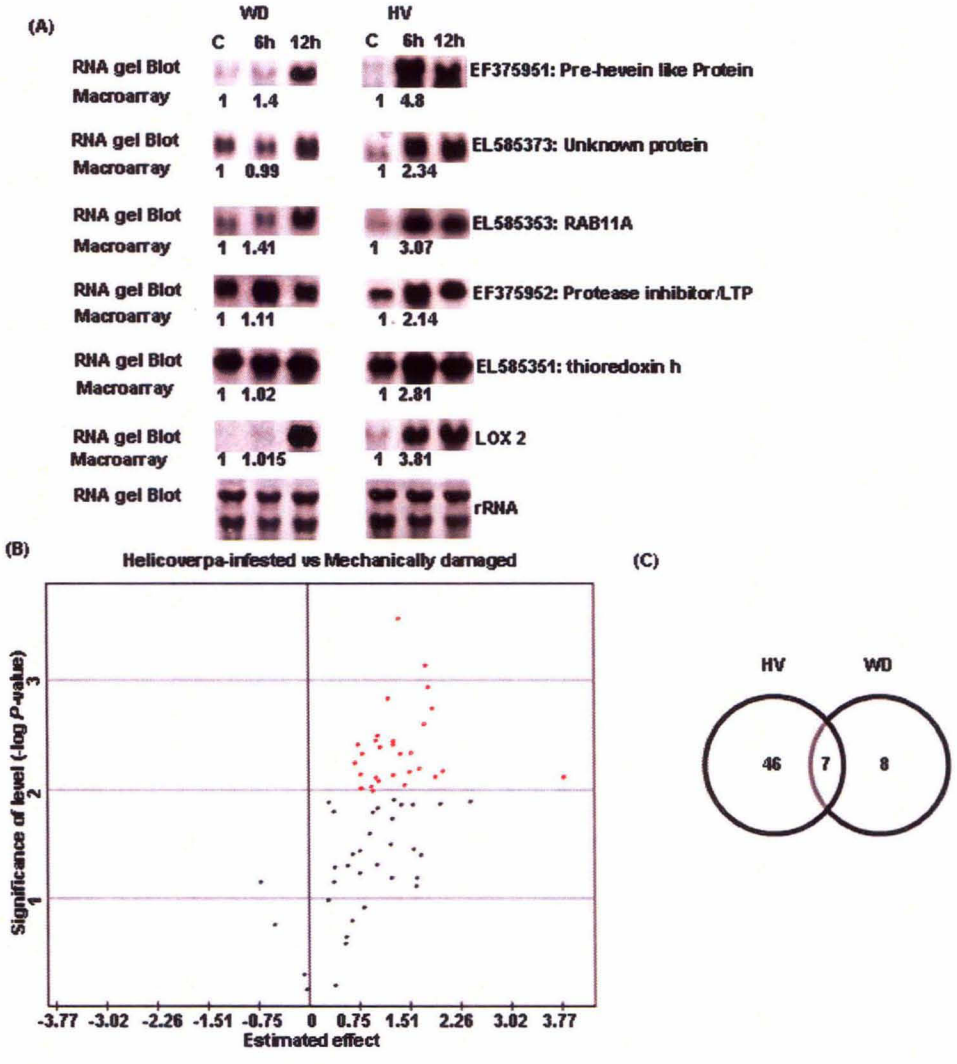

Figure 6.6. Differential transcript profiles during *Helicoverpa*-infestation and mechanical damage. (A) RNA gel-blot analysis of a few selected genes and the corresponding cDNA macroarray data. The lipoxygenase (LOX) gene was used as a marker for insect attack and wounding. rRNA of the same blot was used as the loading control. **(B)** Volcano plot of significance comparing gene expression in response to *Helicoverpa* infestation(HV) and wounding (WD) as measured by macroarray analysis. The plot shows differences in transcript abundance between *Helicoverpa* infestation and mechanically damaged plants. Significance is indicated as the negative $\log_{10}$-transformed *P*-values from a *t*-test calculation. Each of the 63 genes selected for analysis was plotted as a point. The horizontal line corresponds to the significance scale. Red circles represent genes that exhibited differences ($p < 0.05$) in expression during *Helicoverpa* infestation against wounding. **(C)** The Venn diagram presents the number of genes that differed in macroarray analyses of *Helicoverpa*-infested and mechanically damaged samples versus control. The numbers in circles indicate the genes having differential accumulations. The numbers in the common area represent genes with similar pattern of accumulation.

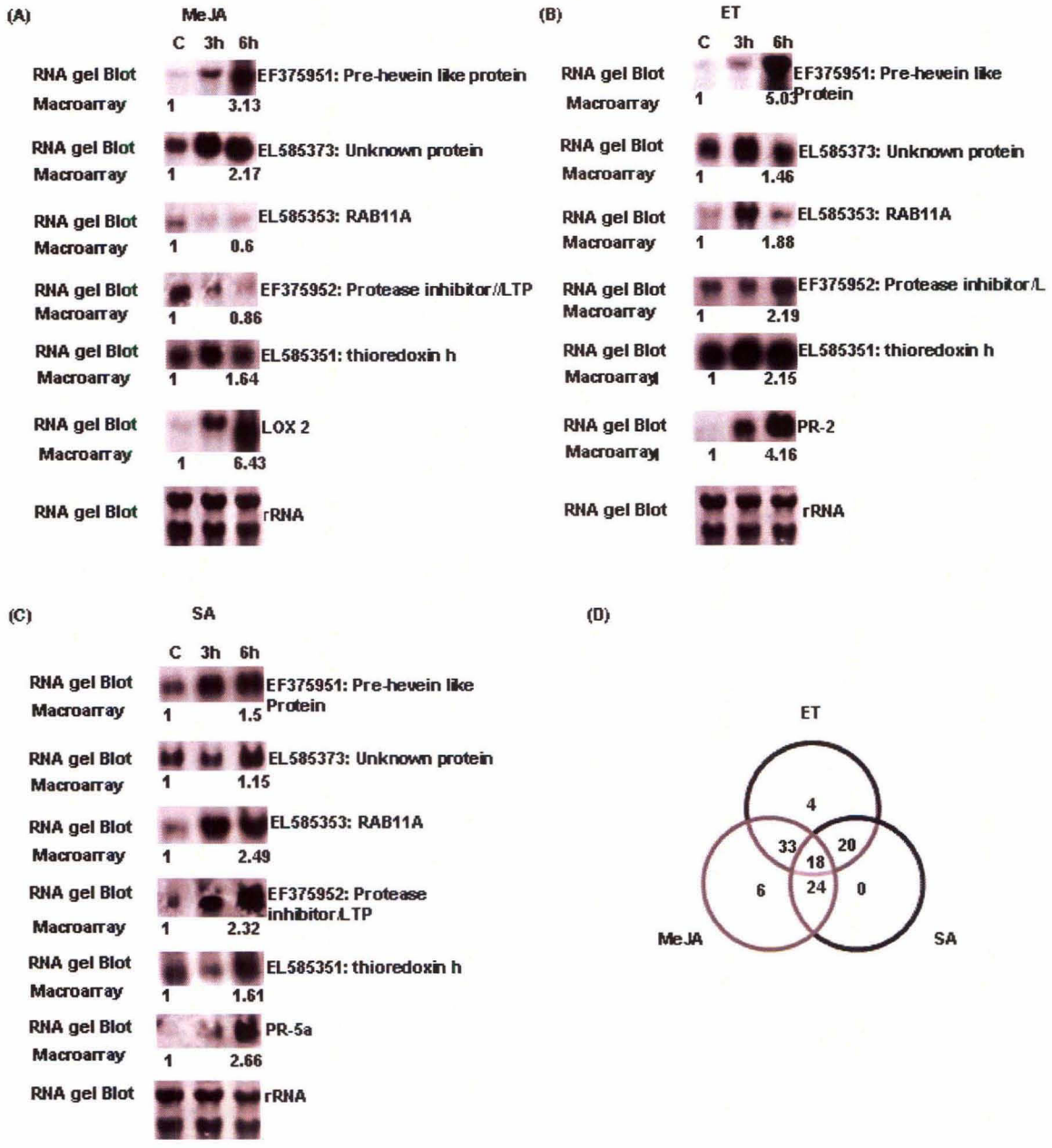

Figure 6.7 Contribution of Methyl Jasmonate (MeJA), Ethephon (ET) and Salicylic Acid (SA) to *Helicoverpa*-inducible gene expression. RNA gel-blot analysis of a few selected genes after (A) MeJA; (B) ET and (C) SA treatments and the corresponding cDNA macroarray data. The lipoxygenase (LOX) gene was used as a marker for MeJA treatment; PR-2 for ethylene treatment and PR-5a for SA treatment. rRNA of the same blot was used as the loading control. **(D)** The Venn diagram presents the number of genes that differed in macroarray analyses of signaling molecules treated samples versus control, for one or more of the three signaling compounds. The numbers in circles indicate the genes having differential accumulations. The numbers in the common area represent genes with similar pattern of accumulation.

6.6A). In general, the results of RNA gel-blot were consistent with the macroarray expression data analysis, with few differences between the two methods.

6.2.5 *Helicoverpa*-responsive genes are differentially regulated by MeJA, ET and SA

Among the three defense regulators, MeJA altered more transcripts than SA and ET (Table 6.1; Fig. 6.7). Out of 63 genes, 47 were upregulated by MeJA (74.6%); 39 by ET (61.9%); and 27 by SA (42.85%). Eighteen genes showed mRNA increases in all three treatments, including three well known defense-related genes (e.g., cellulose synthase, hydroxyisobutryl-coenzyme A hydrolase, homogentisate 1, 2-dioxygenase) and two abiotic stress related genes (e.g., dehydrin 1, cold-induced protein). Since none of these genes showed upregulation exclusively by SA, its association in this interaction was either less pronounced or it was involved in the signaling pathway crosstalks. To confirm the expression data, the same subset of five selected genes was analyzed by northern blots. The results demonstrated congruence between both the methods, with the exception of a few minor differences (Fig. 6.6).

6.2.6 Elicited chickpea plants could defend effectively during subsequent infestation by *Helicoverpa*

To indicate induced plant defense in chickpea, stay/dispersal tests were performed which showed that the percentage dispersal from control plants was significantly lower than ET- and MeJA-treated and pre-infested plants. The mean proportion of dispersed larvae from control plants was 5 ± 3.5 (mean ± SD), compared with 35 ± 7.9 for ET-treated plants. The dispersal percentage for MeJA-treated and pre-infested plants was 30 ± 7.9 and 19 ± 4.18, respectively. No significant difference was found between the dispersal percentage of SA-treated, wounded and control plants (14 ± 4.18 and 12 ± 5.7) (Fig. 6.8).

The effects of induced plant defense were tested by feeding larvae on elicited plants under no choice conditions. The results were consistent with the previous experiment. The lowest mean body mass change was observed for larvae feeding on ET-treated plants (41.73 mg ± 1.97), followed by pre-infested plants (50.73 mg ± 1.31). The body mass increment of larvae fed on MeJA-treated plants (59.65 mg ± 2.01) and wounded plants (53.14 mg ± 2.77) were significantly different from control plants. In contrast to other treatments, the average body mass change of larvae fed on SA-treated plants (72.10 mg ± 1.89) was not significantly different from control plants (Fig. 6.9). Similarly in the experiment where tissue consumed was calculated, which allowed us to correlate between amount of tissue consumed and relative weight gain of the larvae, it was observed that the lowest tissue was consumed for

ET-treated (292.6 ± 6.6), followed by pre-infested tissues (349 ± 12.6) The amounts of tissue consumed by the larvae feeding on MeJA-treated tissue (354 ± 8.3) and wounded tissue (360 ± 11.8) were significantly different from control plants. But the consumption was not significantly different when SA-treated and control tissues were compared (Fig. 6.9). The results of relative weight gain of the larvae were similar to the previous experiment, suggesting that the reduced weight gain of the larvae feeding on ET- and MeJA-treated, mechanically wounded and pre-infested plant tissue are because of less consumption of the treated tissue as compared to control.

6.3 Discussion

6.3.1 The potential role of elicited transcripts in induced plant defense

Among the genes likely to be directly involved in defense, we found PR proteins (PR-10 and PR-5), hevein-like protein and LTP/protease inhibitor in the subtractive library. Secondary metabolites such as phytoalexins, radical scavengers and structural barriers serve a vital role in pathogen and insect defense. We identified several genes potentially involved in secondary metabolite synthesis, including leucoanthocyanidin dioxygenase, dihydrofolate synthetase, homogentisate 1, 2-dioxygenase, cytochrome P450 and hydroxymethyltransferase. Evidence suggested homogentisate 1, 2-dioxygenase was involved in phenylpropanoid and lignin biosynthesis (Raes *et al.,* 2003). Furthermore, during amino acid metabolism, hydroxymethyltransferase was shown to be upregulated in response to elicitation of insect oral secretions (Giri *et al.,* 2006). Endo-1, 4-beta-D-glucanase, cellulose synthase, and pectinmethylesterase encoding proteins that function in cell wall fortification were also upregulated. During induced defense response, an increased accumulation of secondary metabolites, cell-wall reinforcing enzymes and defensin proteins with toxic, antidigestive and antinutritive activity has repeatedly been associated with diverse plant-insect interactions which reduce the palatability of the subsequent attackers and serve as a defensive tool for the plants (Kessler and Baldwin, 2002; 2004).

Genes potentially involved in protection of cells from oxidative stress were upregulated on insect attack namely thioredoxin h, metallothionein-like protein and *RUB 1.* Thioredoxins are a group of small proteins functioning in the regulation of redox status of the cell during oxidative stress (Gelhaye *et al.,* 2004). The precise role of metallothionein is not clear, but dual role has been assigned to this protein, detoxification of metal ions released during protein breakdown and serving as a metal chelator and to function as metal binding proteins for storage or transport into developing organs (Giritch *et al.,* 1998). The involvement of ubiquitin-proteasome-dependent proteolysis during insect feeding is reflected by the upregulation of RUB1 and F Box protein, which are associated with ubiquitination

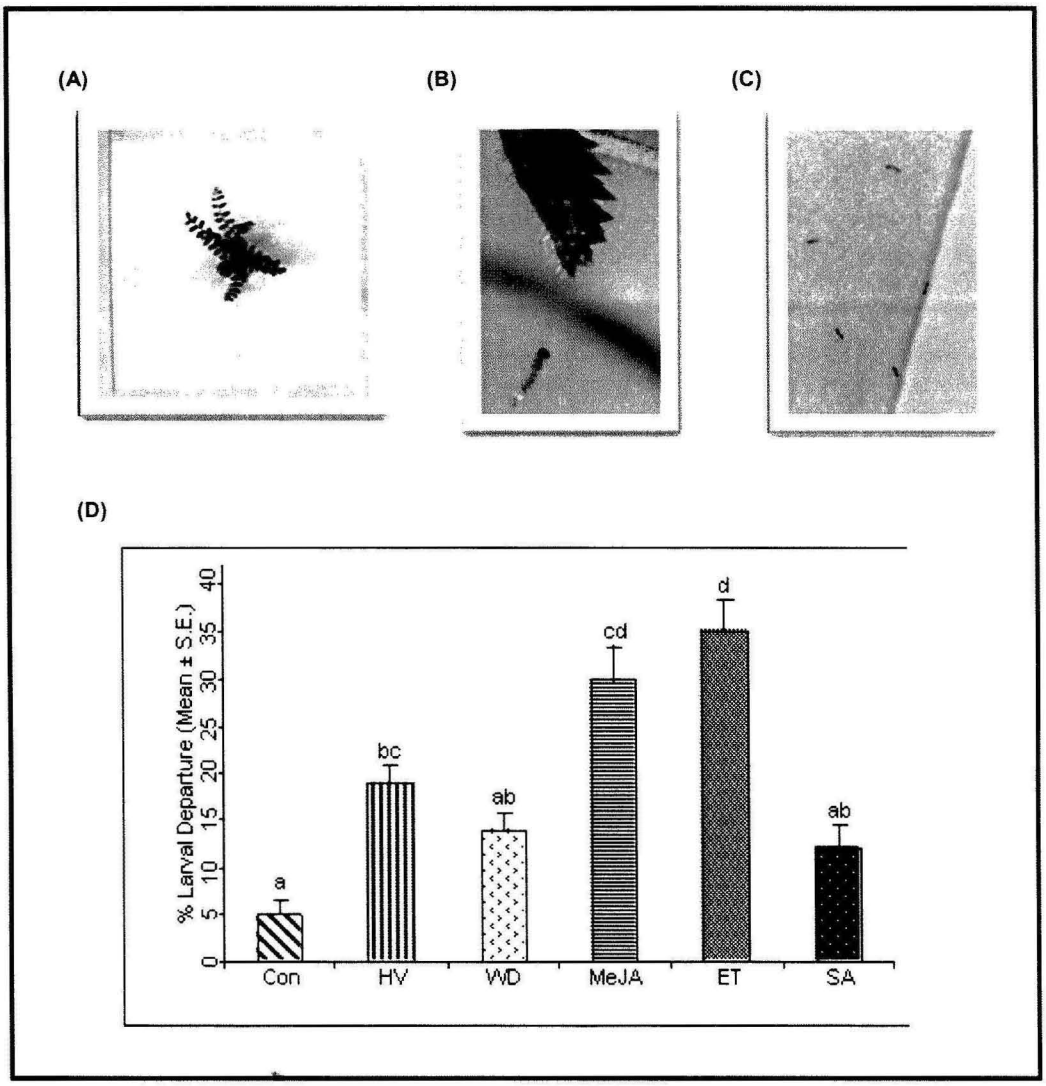

Figure 6.8. Behavioral response of the 1st instar larvae of *H. armigera* control versus treated plants (Con, Control; HV, *Helicoverpa* infestation; WD, Wounding; MeJA, Methyl Jasmonate; ET, Ethephon; SA, Salicylic Acid). (A) A photograph to show the set up designed to study dispersal behavior of 1st instar larvae. **(B)** A representative photograph of the dispersing 1st instar larvae. **(C)** A representative photograph of the dispersed 1st instar larva and arrested with odorless glue on white sheet. **(D)** Comparison of the mean (±S.E.) dispersal percentage of first-instar larvae from the control and treated plants. Means superscripted by the letters in lowercase of various treatments are significantl different (Turkey's Test, $p<0.001$).

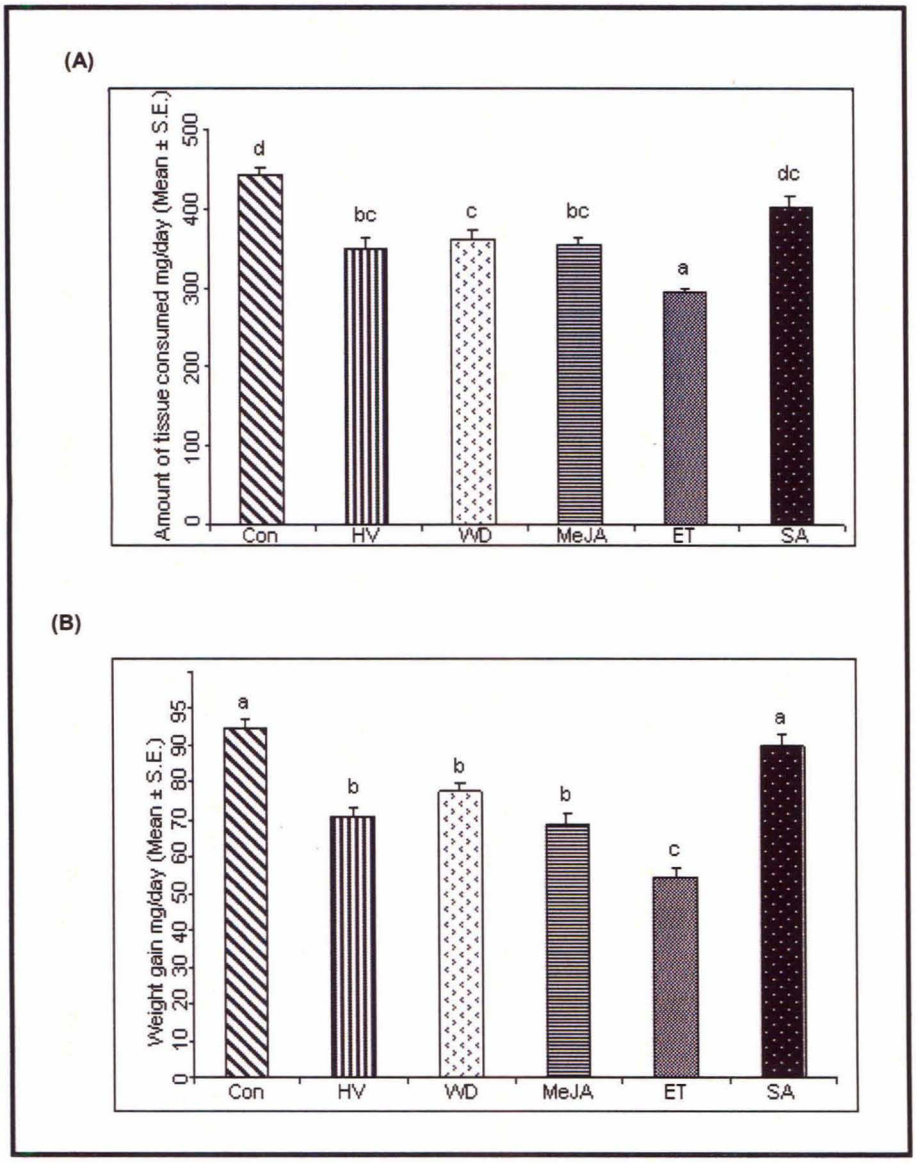

Figure 6.9. Correlation between amounts of treated/control plant tissue consumed and weight gain by *Helicoverpa* (Con, Control; HV, *Helicoverpa* infestation; WD, Wounding; MeJA, Methyl Jasmonate; ET, Ethephon; SA, Salicylic Acid). (A)Comparison of the mean (±S.E.) amounts of plant tissue (control and treated) consumed by *Helicoverpa*.**(B)** Comparison of the mean (±S.E.) weight gain of the larvae fed on the control and treated plant tissues. (Values in the graph represent the amount of tissue consumed (A) and fresh weight gain mg/d (mean ±S.E.) of 5th instar larvae of *Helicoverpa* (B) fed on plants exposed to different treatments. Means of various treatments superscripted by different lowercase letters are significantly different (Tukey's Test, $p < 0.001$).

cascade. The exact role of F Box protein has not been implicated in herbivory but a regulatory role for ubiquitin-dependent proteolysis during senescence has been assigned to this protein (Gepstein *et al.,* 2003). An F box protein, SON1, has been implicated in regulation of induced defense response independent of SA (Kim and Delaney, 2002). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) also shows six fold inductions during insect infestation. This gene is previously reported to be upregulated by herbivoral attack in native tobacco (Giri *et al.,* 2006). GAPDH mainly play role in catalytic function of glycolysis, but it may be a part of reactive oxygen species signaling during herbivory. Two of the clones homologous to GTP-binding proteins and ATPase were also induced on *Helicoverpa*-infestation. GTP-binding proteins are known to regulate many cellular responses including signal transduction, cytoskeletal organization and vesicle trafficking (Haizel *et al.,* 1995). Ran-A1, a GTP-binding protein has previously been reported to be induced on insect attack in *Nicotiana attenuata* (Hui *et al.,* 2003). ATPases are reported to be upregulated in poplar on insect attack and the function assigned to them may be actively transporting a range of ions like H^+, Ca^{2+}, Na^+, etc. into or out of the vacuoles or cells to support many biological functions (Ralph *et al.,* 2006). Aphid feeding could induce the expression of H^+ ATPase in a resistant plant indicating its role in defense (Thompson *et al.,* 2006).

Ethylene and MeJA were induced in response to insect herbivory and wounding in several plant species and therefore considered key regulators in plant defense mechanisms (Arimura *et al.,* 2000; Winz and Baldwin, 2001; De Vos *et al.,* 2005). In addition, ET and MeJA mediate upregulation of defense-related genes such as protease inhibitors (O'Donnell *et al.,* 1996), defensin (Penninckx *et al.,* 1998) and PR proteins (Díaz *et al.,* 2002). In the present study, *Helicoverpa* infestation induced a gene likely involved in ethylene biosynthesis (ACC oxidase), suggesting increased ethylene biosynthesis following insect attack. Furthermore, recent studies reported that ACC oxidase (Ralph *et al.,* 2006a; Ralph *et al.,* 2006b; von Dahl *et al.,* 2007) were induced in plant-insect interactions. The induced expression of ACC oxidase indicates the pronounced accumulation of ET in the process which may contribute to induced plant defense by regulating expression of defense-related genes or proteins that may affect the infesting larvae. One of the gene regulated by auxin (GH1) were also induced by *Helicoverpa* infestation, suggesting involvement of this phytohormone during the response. Jang *et al.* (2003) reported the induction of auxin-induced protein and response factors during the Hessian fly larval attack on wheat-rye plants. Moreover, ethylene and auxin are determined regulators of the octadecanoid pathway (Walling, 2000), suggesting its involvement during herbivory. We identified a group of genes in this study whose direct or indirect roles in insect defense were not previously known, including HMGB1 (High Mobility Group B1), Pi starvation-induced protein, GH1 protein

(auxin-induced), cold-induced protein *BnC24B*, *PPF-1*, *RAB11A* and among several others. Furthermore, some of these genes were involved in other types of stress, such as abiotic stress. Other genes identified in our study were upregulated due to a stress response or the facilitation of transcriptional and translational changes during stress. In addition, we propose the genes with unknown function to be defense-related genes as most of them get induced on application of defense regulators.

6.3.2 Different transcript signatures in response to *Helicoverpa*-infestation and mechanical damage

The macroarray analysis demonstrates significant induction of transcription as a key feature of the response of chickpea plants to *Helicoverpa* feeding. Most of the genes isolated in this study were novel and induced by *Helicoverpa* but only 16 genes were induced by mechanical damage. The genes differentially induced during *Helicoverpa* infestation were likely related to insect specific elicitors present during infestation but absent during wounding (McCloud and Baldwin, 1997; Korth and Dixon, 1997). Previous reports demonstrated similar differential gene responses during mechanical damage and insect-infestation (Reymond *et al.*, 2000; Schittko *et al.*, 2001; Reymond *et al.*, 2004). Many defense-related genes were placed in this category, including pre-hevein-like protein, LTP/protease inhibitor, PR-10, cysteine protease, and hydrolase, among others. Pre-hevein-like protein is reported to be upregulated by insect infestation but not by mere mechanical damage (Reymond *et al.*, 2000). These results further strengthen the fact that plants distinguish between mechanical damage and insect infestation and insect-elicited transcriptional changes differed from mechanical damage (Reymond and Farmer, 1998; Zhu-S *et al.*, 2005). LOX gene served as a marker for wounding and insect infestation (Hui *et al.*, 2003; Reymond *et al.*, 2004).

6.3.3 Contribution of Defense Regulators during chickpea-*Helicoverpa* interaction

The results of the transcript profiles revealed maximum number of genes getting upregulated by MeJA and ET but SA could induce relatively less number of genes. Another indication that the JA and ET pathways are involved in plant-insect interaction is the finding that few genes participating in biosynthesis of JA (LOX) and ET (ACC oxidase) were induced on *Helicoverpa*-infestation (Table 6.1). LOX (Hui *et al.*, 2003; Reymond *et al.*, 2004) and ACC oxidase (Ralph *et al.*, 2006) have been reported to be induced in other plant-insect interactions studied recently. It is also reported that antisense LOX expression increases herbivore performance by decreasing defense responses in *N. attenuata*. A mutant *fad3-2fad7-2fad8* of *Arabidopsis* deficient in linolenic acid and is unable to synthesize jasmonic acid has been found to be susceptible to insect infestation (Mc Conn *et al.*, 1997).

Many genes showed common induction by both MeJA and ET. Three plant defense genes (PDF1.2, PR1b and Osmotin) were identified in *Arabidopsis* and induced synergistically by MeJA and ET (Pennickx *et al.,* 1998, Kessler and Baldwin, 2002). In insect- and MeJA-induced responses, studies have shown that a large proportion of genes are commonly induced by both the responses (Reymond *et al.,* 2004; Bodenhausen and Reymond, 2007). The participation of SA in regulation of *Helicoverpa*-inducible gene expression proposed to be less significant (as no gene was induced exclusively by SA, Table 1), but marker genes for SA like PR-5 shows induction during *Helicoverpa*-infestation.

6.3.4 Pre-infested chickpea plants could defend well on subsequent infestation by *H. armigera*

Plants induce a defense strategy in response to insect attack which may be adaptive under certain circumstances. During induced defense response, an increased accumulation of secondary metabolites and defensin proteins like proteinase inhibitors with toxic, antidigestive and antinutritive activity has repeatedly been associated with diverse plant-insect interactions which reduce the palatability of the subsequent attackers (Kessler and Baldwin, 2004). In the present study, we found reduced growth rates of *Helicoverpa* larvae feeding on pre-infested chickpea plants when compared with control, suggesting induced plants had relatively higher fitness than uninduced plants (Agrawal, 1998). Similar results were obtained with dispersal behavior test where pre-infested plants could not attract larvae for feeding rather they were dispersing from the pre-infested plants in search of new food source and the dispersal percentage was higher for both first instar and fifth instar larvae. Higher defense status was maintained by induced plants (with mild insect infestation) than by uninduced plants, which may be attributed to either the induction of defense related anti-nutritive and anti-digestive proteins (Kessler and Baldwin, 2002; 2004) or to the much early events occurring before gene expression like detection of defense regulators. Gene activation and subsequent metabolic changes can be detected even after approximately 1 h of infestation (Maffei *et al.,* 2007) though it might take few more hours to be in effect to cause induced defense. Moreover events occurring before gene expression (such as pronounced accumulation of signaling compounds) can affect growth and feeding behavior of the larvae. There is evidence which suggest that *H. zea* can intercept the plant defense signals elicited by its own feeding activity and can detect plant signal molecules and the allelochemical end products (Li *et al.,* 2002). Therefore, we can say that even if the toxic concentrations of anti-feedant compounds may not be available in the induced plants, *H. armigera* could detect a higher defense status by tasting the signals.

6.3.5 MeJA and ET-induced responses does affect larval preference for feeding and their performance

Contribution of MeJA and ET-induced defense responses was depicted by the behavioral experiments performed. Interestingly, we observed that maximum larval dispersal occurred from ET elicited plants in both first instar and fifth instar larval test, indicating the ET-mediated signaling during insect-inducible responses. Percentage larval dispersal was also quite high from MeJA-elicited plants, but not in case of SA-elicited plants. In a similar study, significantly less aphid infestation was observed on MeJA-pretreated sorghum seedlings suggesting the effectiveness of plant defense elicited by MeJA against aphid invasion (Zhu-S *et al.,* 2004). Similarly, when we looked for larval performance feeding on elicited plants under no choice conditions; it was observed that larvae grew better on SA-elicited plants than on ET/MeJA-elicited plants. This result was in consistent with the previously reported evidence suggesting that exogenous application of SA on cotton plant could not affect the growth of *Helicoverpa zea* (Bi *et al.,* 1997). The direct role of ET in induced defense response correlating with differential gene expression observed in this study has not been reported previously. We observed larval growth getting affected by MeJA and ET-elicited plants which corresponded with the previous report concluding that JA elicitation of *N. attenuata* conferred dramatic induced resistance in both field (Baldwin, 1998) as well as laboratory (van Dam *et al.,* 2000) trials with *M. sexta* larvae by increasing its production of secondary metabolites after elicitation with MeJA which diminished the plant's palatability (Kessler and Baldwin, 2004) for the feeding insects. In both the experiments performed, effect of ET-elicited induced response was also effective together with MeJA, indicating that ET-mediated defense response pathway could be more active in this specific insect-plant interaction or a synergistic pathway could be predicted. MeJA induced ethylene production is reported to be responsible for defense response in a conifer (Hudgins and Franceschi, 2004) and few plant defense genes are known to be induced synergistically by MeJA and ET in *Arabidopsis* (Pennickx *et al.,* 1998), citing examples for synergistic effects of MeJA and ET.

The negative effect on plant acceptance on phytohormone-treated plants may be attributed to both elicited defense response and the direct influence of the phytohormone on the insect's behavior. Significantly less aphid infestation was observed previously on MeJA-treated plants (Ellis *et al.,* 2002), suggesting effective plant defense elicited by MeJA. Involvement of JA and ET increases due to chewing insects was shown by induction of modest but significant increases in ET production and a clear increase in JA production (De Vos *et al.,* 2005; Leitner *et al.,* 2005). The differential behavior of larvae on pre-infested plants may be attributed to the pronounced accumulation of signaling compounds (MeJA and ET) and allelochemicals, which detract the larvae.

Table 6.1: Genes differentially expressed in response to HV, WD, SA, MeJA and ET

Gene	Accession	HV	WD	SA	MeJA	ET
Defense-related						
Pre-hevein-like protein PR-4 precursor (*Pisum sativum)*	EF375951	**4.84±0.83**	1.40±0.09	1.50±0.51	**3.13±0.72**	**5.03±1.15**
Protease inhibitor/seed storage/LTP family protein (*A. thaliana*)	EF375952	**2.14±0.38**	1.11±0.01	**2.32±0.13**	0.86±0.16	**2.19±0.31**
Thaumatin-like protein PR-5a (*Cicer arietinum*)	CAR010502	**2.19±0.88**	1.08±0.32	**2.01±0.66**	**2.14±1.05**	1.66±0.82
PR10-1 protein (*Medicago truncatula*)	EL585361	**2.10±0.78**	1.09±0.32	**2.66±0.48**	1.68±0.30	**4.66±0.84**
Putative cysteine protease, *plp* gene (*Pisum sativum*)	EL585377	**3.71±1.93**	**2.19±0.02**	1.74±0.67	**3.72±1.44**	**2.04±0.79**
Putative phospholipase (*Arabidopsis thaliana*)	EL585367	1.82±0.71	1.42±0.01	1.52±0.41	**2.57±0.69**	**2.43±0.65**
Endo-1,4-beta-D-glucanase KORRIGAN (*kor-1* gene) (*Pisum sativum)*	EL585369	**3.05±0.74**	1.70±0.21	**2.53±0.40**	**5.00±0.79**	1.56±0.24
Hydroxyproline-rich glycoprotein family protein (*Arabidopsis thaliana*)	EL585371	**3.68±1.64**	0.73±0.01	1.31±0.04	**5.52±0.17**	**2.08±0.06**
Cellulose synthase (*celA1*) (*Gossypium hirsutum*)	EL585372	**2.43±1.12**	1.22±0.28	**2.20±0.13**	**3.06±0.18**	**2.09±0.12**
Pectinmethylesterase (*Vigna radiata*)	EL585378	**2.39±1.01**	1.39±0.19	1.09±0.56	**3.01±1.55**	**2.19±1.12**
Pectinesterase, *pmeB* gene (*Pisum sativum*)	EL585357	**2.28±0.83**	1.44±0.04	1.39±0.15	**2.61±0.28**	1.81±0.19
3-Hydroxyisobutyryl-coenzyme A hydrolase (*Arabidopsis thaliana*)	EL585370	**5.21±1.81**	0.47±0.57	**5.79±3.52**	**9.41±5.72**	**6.63±4.03**
Homogentisate 1,2-dioxygenase [*Lycopersicon esculentum*]	EL585364	**2.34±0.70**	1.71±0.26	**2.20±0.03**	**2.92±0.05**	**3.06±0.08**
Leucoanthocyanidin dioxygenase-like protein (*Arabidopsis thaliana*)	EL585374	1.59±0.53	1.13±0.16	1.67±0.04	**2.94±0.07**	**4.36±0.07**
***Arabidopsis* thaliana mRNA for dihydrofolate synthetase (*dhfs/fpgs3* gene)**	EL585376	1.48±0.63	0.61±0.11	0.38±0.05	0.69±0.10	0.64±0.10
Aldehyde dehydrogenase, putative (*ALDH*) (*Arabidopsis thaliana*)	EL585382	**8.57±3.27**	**2.92±0.58**	**4.59±3.76**	**7.39±6.05**	0.01±0.01
Hydroxymethyltransferase (*Arabidopsis thaliana*)	EL585358	**2.68±1.22**	0.93±0.12	1.08±0.51	1.57±0.74	1.36±0.64
Cytochrome P450	CAB50768	**2.67±0.96**	1.89±0.22	**2.24±0.03**	**3.14±0.04**	**4.43±0.06**

Detoxification/Oxidative Stress/Senescence						
Metallothionein-like protein (*Pisum sativum*)	CAA65008	**2.56±1.03**	1.17±0.08	1.01±0.12	1.75±0.21	1.66±0.20
Thioredoxin h (*Pisum sativum*)	EL585351	**2.81±1.05**	1.02±0.02	1.61±0.55	1.64±1.25	**2.15±0.74**
***RUB1* (Related to ubiquitin 1) (*Arabidopsis thaliana*)**	EL585368	**2.65±1.10**	1.61±0.03	1.72±0.55	**2.79±0.90**	**2.15±0.69**
Similar to F-box protein, *FBL2* (*Homo sapiens*)	EL585381	**2.66±0.73**	1.34±0.38	**3.22±1.12**	**6.75±2.36**	**3.63±1.27**
Glyceraldehyde 3-phosphate dehydrogenase (*Lycopersicon esculentum*)	EF375948	**2.26±0.64**	1.23±0.16	**3.03±0.45**	**7.56±1.13**	**3.48±0.52**
Transcriptional Regulation/Signal Transduction						
***HMGB1* (HIGH MOBILITY GROUP B 1) (*Arabidopsis thaliana*)**	EL585365	**2.43±1.07**	0.98±0.09	1.30±0.17	1.94±0.26	**2.04±0.27**
Nucleasome/chromatin assembly factor D protein *NFD101* (*Zea mays*)	EL585384	**2.31±0.92**	0.83±0.13	1.81±0.12	**2.14±0.15**	**2.60±0.18**
RNA recognition motif (RRM)-containing protein (*Arabidopsis thaliana*)	EL585383	**3.29±1.44**	1.22±0.12	**2.04±0.25**	**4.61±0.57**	**3.99±0.50**
Small GTP-binding protein (*sra1*) (*Glycine max*)	EL585380	**2.55±0.83**	0.90±0.07	1.45±0.39	**2.85±0.77**	1.34±0.36
***RAB11A* (*Lotus corniculatus*)**	EL585353	**3.29±0.39**	1.22±0,09	**2.04±0.12**	0.61±0.21	1.88±0.34
***F1 ATPase* (*Pisum sativum*)**	EL585385	**2.18±0.71**	0.62±0.28	**2.06±0.27**	**2.48±0.32**	**3.53±0.46**
Abiotic Stress						
Cold induced protein (*BnC24B*)(*Brassica napus*)	EL585348	0.99±0.25	0.84±0.05	**2.39±0.69**	**3.70±1.10**	**5.55±1.62**
Cold-inducible unknown mRNA (*Zea mays*)	EL585360	**2.87±1.28**	**2.20±0.38**	**2.16±0.04**	**4.93±0.09**	**2.76±0.05**
Putative Pi starvation-induced protein (*Cicer arietinum*)	CAA07232	**3.43±0.90**	0.98±0.24	**2.45±1.19**	**3.37±1.64**	**6.01±2.93**
Dehydrin 1 (*Dhn*) (*Picea abies*)	EL593260	1.52±0.04	1.02±0.11	**4.48±0.35**	**9.07±0.72**	**4.99±0.39**
Protein synthesis and destination						
SSR16 (*Arabidopsis thaliana*)	**EL585375**	**1.97±0.79**	**1.24±0.15**	**1.06±0.15**	**2.52±0.37**	**1.92±0.28**
Poly (A)-binding protein (*PABP1*) (*Nicotiana tabacum*)	EL585354	**2.55±0.42**	0.88±0.12	**2.77±0.28**	**4.35±0.45**	**4.22±0.43**
Poly (A)-binding protein (*Cucumis sativus*)	EL585349	1.79±0.83	0.90±0.02	0.93±0.28	1.82±0.55	1.72±0.52

40S ribosomal protein S6 (*Cicer arietinum*)	AJ010227	**2.51±0.33**	0.86±0.16	1.72±0.18	**3.59±0.30**	**3.76±0.40**
Photosynthesis/Energy						
Chlorophyll a/b binding protein (*Cicer arietinum*)	CAR131044	**2.49±1.09**	1.04±0.06	0.98±0.06	**2.37±0.16**	0.70±0.04
10 kDa photosystem II polypeptide (*Trifolium pretense*)	EF375955	1.53±0.21	**2.40±0.18**	1.65±0.65	1.90±0.95	1.32±0.56
Fructose-bisphosphate aldolase 2, chloroplast precursor (*Pisum sativum*)	EL585379	**2.59±0.94**	1.06±0.23	0.88±0.26	1.88±0.57	1.74±0.52
Oxygen-evolving enhancer protein 3 precursor (*Pisum sativum*)	EL585359	1.48±0.58	0.94±0.09	0.94±0.36	**2.18±0.85**	1.69±0.66
Miscellaneous						
***GA* (*Pisum sativum*)**	EF375953	0.95±0.26	0.60±0.03	0.76±0.21	1.16±0.31	**2.05±0.56**
1-Aminocyclopropane-1-carboxylate oxidase (*EFE*) (*Pisum sativum*)	EL585350	**2.74±0.59**	1.37±0.53	0.85±0.36	1.92±0.82	**2.06±0.88**
GH1 protein (*GH1*) (*Glycine max*)	EL585366	**2.06±0.76**	1.16±0.12	0.82±0.21	1.80±0.47	1.10±0.28
PPF-1 protein (*Pisum sativum*)	EL585355	1.50±0.46	0.43±0.04	1.54±0.12	**2.62±0.21**	**2.79±0.22**
Ty3-gypsy like Retrotransposon, *CaRep* (*Cicer arietinum*)	AJ411814	0.72±0.11	1.04±0.06	**2.07±0.05**	**2.94±0.08**	**2.25±0.06**
Mitochondrial F1 ATP synthase beta subunit (*A. thaliana*)	EL585393	1.63±0.59	1.41±0.08	1.45±0.25	**2.91±0.51**	**2.33±0.40**
F1 ATP synthase beta (*Arabidopsis thaliana*)	EL585352	1.45±0.38	1.61±0.20	1.24±0.07	**4.16±0.24**	**3.09±0.18**
H+transporting ATP synthase beta chain like protein (*A. thaliana*)	EL585363	**5.18±1.55**	1.51±0.34	**6.28±0.51**	**10.27±0.84**	**1.33±0.10**
Unknown functions						
Homologous to clone FC19AA10, HTC in fruit (*Solanum lycopersicum*)	EL585389	**2.22±0.72**	1.24±0.12	1.70±0.39	**2.02±0.47**	1.77±0.40
Unknown protein (*Zea mays*)	EF375947	1.49±0.09	0.76±0.01	1.99±0.16	**2.21±0.17**	**4.65±0.37**
Unknown protein (*Oryza sativa*)	EF375949	1.18±0.13	0.41±0.01	1.38±0.59	**2.03±0.87**	1.68±0.72
Unknown gene, genomic DNA, chromosome 1, (*Lotus corniculatus*)	EF375956	**2.21±0.61**	**2.35±0.49**	1.50±0.08	**6.38±0.37**	**3.89±0.23**
Unknown mRNA sequence (*Lycopersicon esculentum*)	EF375957	1.33±0.44	1.12±0.26	0.74±0.13	0.98±0.18	0.78±0.14
Unknown gene homologous to clone mth2-71h24 (*Medicago truncatula*)	EL585392	**2.60±1.17**	1.56±0.23	**4.48±0.35**	**9.07±0.72**	**4.99±0.39**
Unknown protein, AT4G01050 mRNA (*Arabidopsis thaliana*)	EF593261	**2.34±0.39**	1.55±0.08	**2.01±0.52**	**4.29±1.12**	**3.69±0.96**

Unknown, homologous to clone mth2-4j24 (*Medicago truncatula*)	EL585395	**3.06±1.27**	**2.16±0.22**	**2.49±0.52**	**4.72±1.00**	**2.67±0.56**
Hypothetical protein (*Plantago major*)	EL585394	**2.61±1.07**	1.45±0.41	1.82±0.61	**3.21±1.08**	**3.03±1.02**
Homologous to unknown clone mth2-193p1 (*Medicago truncatula*)	EL585391	**2.11±0.65**	1.46±0.57	**2.61±0.38**	**8.32±1.23**	0.50±0.07
Unknown protein (AT3G15840) mRNA (*Arabidopsis thaliana*)	EL585387	**2.66±1.24**	**2.01±0.20**	**2.07±0.02**	**2.70±0.03**	1.67±0.02
Hypothetical protein (*Arabidopsis thaliana*)	EL585388	1.56±0.44	1.07±0.11	1.57±0.10	**2.58±0.17**	**2.28±0.15**
Unknown homologous to clone mth2-5g18 (*Medicago truncatula*)	EL585390	**4.03±1.74**	1.79±0.12	**2.32±0.34**	**3.86±0.56**	**2.90±0.42**
Unknown mRNA (*Pisum sativum*)	EL585386	**3.50±1.40**	0.92±0.06	**2.96±0.18**	**3.76±0.23**	1.77±0.11

cDNA Sequences of all unigenes listed in **Table 6.1** have been submitted to the GenBank database and the assigned Accession nos. were mentioned. BLASTX searches were conducted to determine homologous genes and the putative function of the cDNA fragments. Ratios of signal intensity were determined by cDNA macroarray hybridization as described in the "Materials and methods". Shown for each gene are the expression ratios with Standard Deviations (SD) in response to *Helicoverpa*-infestation (HV) and wounding (WD) above background of the control samples. Expression ratios of the transcripts in response to Methyl Jasmonate (MeJA), Ethylene (ET) and Salicylic Acid (SA) were also presented with SDs. The transcripts are listed according to their probable functions. Values highlighted in bold if expression ratios more than two fold. Genes in bold if showing differential expression ratios between *Helicoverpa*–infested and mechanically damaged plants ($P<0.05$).

Chapter 7: Summary

Plants are sessile organisms that are exposed to a constant barrage of environmental stresses which impact on growth, development and reproduction. Important traits such as yield and the resistance to biotic stress depend on internal physiological programs and their regulation by signal transduction pathways. Plants are the major source of food and biomaterials worldwide but their production is severely compromised by pathogens that cause disease and reduce yield and quality. Therefore, understanding how plants defend themselves against pathogens and herbivores, and how that may be manipulated, is therefore of critical importance for successful and sustainable agriculture.

Chickpea (*Cicer arietinum* L.), is a major source of high quality protein. Among temperate pulses, it is the most tolerant crop to heat and drought and is suitable for production in low fertility soils (Pande et al. 2005). Despite its economic importance, chickpea productivity has been low because of yield losses due to devastating foliar and soil-borne fungal diseases like *Ascochyta* Blight (AB), Fusarium wilt and Botrytis Grey mould, and insect pests like pod borer. Among these, AB caused by the ascomycete fungus *Ascochyta rabiei* (Pass.) Labrousse (teleomorph *Didymella rabiei* (Kovachevski) v. Arx) is the most important biotic constraint for chickpea production (Nene and Reddy 1987; Gaur and Singh 1996). The main objective of this work was to identify resistance-related genes by following one of the transcript profiling strategies. To understand quantitative disease resistance conferred by multiple genes, individual genetic factors determining disease resistance need to be elucidated. We decided to enrich genes that may contribute to constitutive resistance mechanism or by other defense system by screening for genes showing constitutively different expression levels between resistant and susceptible lines using transcript profiling. Once identified, the next objective would be to functionally characterize one of the selected genes. In addition, we also identified a set of genes that show significant induction upon *Helicoverpa* infestation, the other major factor that causes severe crop losses. These genes may also serve as 'candidate genes' for transformation and crop improvement in future.

Identification of differentially expressed genes among *Ascochyta* resistant and susceptible lines of chickpea and their analysis

To isolate chickpea genes involved in the resistance to the *Ascochyta* blight fungi, we used the suppression subtractive hybridization (SSH) method to generate cDNA libraries enriched in sequences expressed in chickpea plants during the early stages of infection and we also studied the differential behavior of their expression at basal level in resistant and susceptible germplasm lines. For focusing on genes strictly involved in the resistance, cDNA from resistant plants were subtracted with cDNA from the susceptible plants. As a result,

genes showing higher constitutive expression in the blight resistant germplasm line, FLIP84-92 C(2) compared to the blight susceptible line, PI359075 (1) and genes showing induction upon *Ascochyta* infection were identified. Genes with constitutive higher expression in the resistant lines are predicted to be directly involved in the resistance and genes showing no difference between resistant and susceptible line but showing induction after *Ascochyta* infection are predicted to be involved in basal defense. Higher accumulation of some of the transcripts at the basal level indicates that plants are already prepared for resisting against the fungus. In order to indicate their role in defense we also monitored the expression patterns of the isolated genes in response to exogenous application defense regulators. Involvement of both JA and SA together with some other unknown factors is implicated in the resistance mechanism against *Ascochyta*. These results provided novel insights to the molecular control of chickpea cellular processes, which may assist the understanding the chickpea defense mechanisms and allow enhanced development of disease resistant cultivars.

Isolation and characterization of *CaAr131* from chickpea

A chickpea cDNA fragment, inducible by the *Ascochyta* blight and that shows high homology with $Hs1^{Pro1}$ resistant gene was chosen for further characterization. Further its corresponding full-length cDNA clone was isolated from a chickpea cDNA library using *CaAr131* truncated primer as a probe. The full length clone obtained was completely sequenced and analyzed. The *CaAr131* cDNA is 1.3 kb long and encodes a predicted protein of 458 amino acid with an estimated mass of 52 kD. The *CaAr131* contain an imperfect LRR domain, phosphorylation sites and o-glycosylation sites. The phylogenetic analysis based on sequence alignment suggests that CaAr131 has a strong homology with $Hs1^{Pro1}$ gene of beet root which confers resistance against a nematode. DNA gel blot hybridization strongly suggests that the chickpea genome contains a single copy of the gene. To further assess the expression pattern of *CaAr131* during fungal infection and various defense regulators, RNA gel blot analysis was performed which indicated that various plant defense regulators and osmotic stress conditions induce *CaAr131* expression.To further understand the mechanisms of regulation of *CaAr131* the functional characterization of its promoter was performed by isolation of 5'-upstream region and generation of deletion constructs and their analysis. To examine the spatial and temporal as well as tissue specific expression of this gene, its 5'-upstream sequences fusion construct (5'upstream of *CaAr131*::GUS) was generated and used to transform tobacco. The trichomes of transgenic tobacco plant showed strongest GUS activity.

To further demonstrate its functionality in plant defense we need to analyze its over-expression in transgenic plants and look for its response to pathogen infection.

Isolation, Identification and analysis of differentially expressed chickpea genes upon *Helicoverpa* infestation

Another major threat to chickpea successful production is the generalist herbivore, *Helicoverpa armigera*, which damages the aerial parts of the plant, including leaves and pods. Since most studies examining *Helicoverpa*-chickpea interactions have focused on specific gene or protein dynamics (Johnston *et al.* 1991; Jongsma *et al.* 1995; Giri *et al.,* 1998; Peng *et al.,* 2005; Srinivasan *et al.,* 2005), our aim was to identify target genes upregulated during mild insect infestation which may contribute to the defense response. To isolate *Helicoverpa*-induced genes, a subtractive cDNA library was constructed from chickpea seedlings under *Helicoverpa* mild infestation using SSH. In addition to known defense genes, we identified a number of genes and presumed biochemical functions that have not been previously associated with defense responses against insects. Using macroarray, we profiled and compared transcript patterns elicited by both herbivore and mechanical wounding. Comparative expression patterns on exogenous applications of various signaling compounds were obtained to evaluate the dynamics of regulatory pathways. In addition to investigating the effects of elicitation by mild insect infestation, induced plant defenses in chickpea were evaluated by examining signal compound elicitation on larval feeding behavior. In conclusion, this study shows that *Helicoverpa* attack triggers changes in transcript levels that are distinct from mechanical damage and are controlled mainly by MeJA and ET. Directly or indirectly, the majority of the genes identified as being *Helicoverpa*-activated, may have a significant effect on insects performance as it was depicted that elicitation with mild insect infestation, MeJA and ET affected larval feeding behavior. We expect that further functional characterization of these novel *Helicoverpa*-responsive genes which are regulated by MeJA and ET will extend our understanding about defense responses against insects and to develop new strategies for crop protection. Therefore, the results of this study advance the understanding of non-model plant-insect interactions on a broader scale.

Overall, this study isolated and characterized numerous defense related genes and their regulatory mechanisms that may be important in defense against various pests and pathogens, as well as other cellular functions. The findings of the present analysis can provide novel insights to the molecular control of chickpea cellular processes, which may assist the understanding of chickpea defense mechanisms and allow enhanced development of resistant cultivars. Further functional characterization of the novel *Ascochyta*- and *Helicoverpa*-induced genes will extend our understanding about defense responses against the two important biotic factors which limits chickpea production and in developing new strategies for crop protection. The work embodied in this thesis would help improve our understanding of molecular mechanisms involved in resistance/defense in chickpea. In future,

development of *Ascochyta* and *Helicoverpa* resistant/tolerant chickpea varieties would also reduce the cost of disease control. Furthermore, the harmful impact on the environment incurred by the extensive use of antifungal chemicals and pesticides could be avoided by successful development of these varieties.

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RESEARCH PAPER

Differential transcript accumulation in *Cicer arietinum* L. in response to a chewing insect *Helicoverpa armigera* and defence regulators correlate with reduced insect performance

Archana Singh[1,*], Indrakant Kumar Singh[2,*] and Praveen Kumar Verma[1,†]

[1] *National Institute of Plant Genome Research, Aruna Asaf Ali Marg, JNU Campus, New Delhi 110 067, India*

[2] *Centre of Advanced Study in Zoology, Department of Zoology, University of Delhi, Delhi 110 007, India*

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Abstract

Monitoring transcriptional reorganization triggered in response to a particular stress is an essential first step for the functional analysis of genes involved in the process. To characterize *Cicer arietinum* L. defence responses against *Helicoverpa armigera* feeding, transcript patterns elicited by both herbivore and mechanical wounding were profiled and compared, and the application of defence regulators was assessed. A combination of approaches was employed to develop transcript profiles, including suppression subtractive hybridization (SSH), macroarray, northern blot, and cluster analysis. Of the 63 unique genes isolated, 29 genes expressed differentially when *Helicoverpa* feeding and wounding responses were compared. Comparative macroarray analyses revealed that most of the *Helicoverpa*-induced transcripts were methyl jasmonate (MeJA) and ethylene (ET) regulated. The effects of mild insect infestation and the exogenous application of signalling compounds on larval feeding behaviour were also monitored. Bioassays were performed to measure dispersal percentage and growth of larvae on elicited plants. Larvae released on elicited plants had decreased larval performance, demonstrating the central role of induced plant defence against herbivory. Similarly, wounding and exogenous application of MeJA and ET also affected larval growth and feeding behaviour. Our results demonstrated that *Helicoverpa* attack up-regulated large transcriptional changes and induced chickpea defence responses. Therefore, the results of this study advance the understanding of non-model plant–insect interactions on a broader scale.

Key words: Chickpea, ET, *Helicoverpa*, induced plant defence, MeJA, SA, SSH.

Introduction

Plants respond to both pathogen and herbivore attack by constitutive and induced defence mechanisms (Karban and Baldwin, 1997; Thomma *et al.*, 1998; Kessler and Baldwin, 2002). The advantage of induced defence depends on the type of attacker and the subsequent cost of defence. Induced defences operate via both direct and indirect modes. Defence-related protein expression, reinforcement of the cell wall, biosynthesis of secondary compounds, and production of reactive oxygen species are examples of direct induced defences. Volatile organic compounds provide indirect defence by attracting enemies of the attacker (Paré and Tumlinson, 1997; Kessler and Baldwin, 2002). Complex cross-talk networks have been uncovered which serve to recruit various signal pathways in the regulation of defence induction (Walling, 2000; Rojo *et al.*, 2003). While methyl jasmonate (MeJA) signalling plays a primary role in chewing insect defence (McConn *et al.*, 1997; Reymond *et al.*, 2004; De Vos *et al.*, 2006), ethylene-mediated expression is also involved (Stotz *et al.*, 2000; Kessler and Baldwin, 2002; von Dahl *et al.*, 2007). In addition, salicylic acid (SA) is

* These authors contributed equally to the paper.
† To whom correspondence should be addressed. E-mail: praveenverma@india.com

an important plant-produced signal. During biotrophic pathogen interactions, SA activates plant defence responses against pathogen attack (McDowell and Dangl, 2000; Glazebrook, 2005).

Plants, like animals, alter their induced defences in response to prior experiences (Baldwin and Schmelz, 1996). A mild insect infestation promotes an adaptive mechanism resulting in deterioration of plant quality as a food source; this reduces insect preference and performance on an induced plant compared with that on an uninduced plant (Agrawal, 1998; Voelckel and Baldwin, 2004). Induced resistance to subsequent attacks is due to plant changes in molecular and biochemical composition, which subsequently modify metabolic processes involved in the adaptive response. Limited reports describe the effect of induced defence on the growth and feeding behaviour of herbivores (Agrawal, 2000; Voelckel and Baldwin, 2004). Therefore, further exploration in other plant systems is warranted.

Plants distinguish between mechanical damage and herbivory. Insect attacks on plants results in wounding, but a plant's molecular response to mechanical damage differs (Korth and Dixon, 1997; Reymond *et al.*, 2000). Several different types of elicitors, including fatty acid conjugates (volicitin; Alborn *et al.*, 1997) and enzymes (glucose oxidase, Felton and Eichenseer; 1999; β-glucosidase; Mattiacci *et al.*, 1995; and alkaline phosphatase; Funk, 2001), are present in the oral secretions and regurgitant of herbivores (Paré and Tumlinson, 1999), which may contribute to the differential response.

Large-scale transcriptional changes accompany insect-induced resistance, and herbivore-specific cues orchestrate the responses (Kessler and Baldwin, 2002). Transcript pattern changes in response to herbivory have been generated in many plant species including *Arabidopsis thaliana* (Reymond *et al.*, 2000, 2004; Kempema *et al.*, 2007), *Nicotiana attenuata* (Hermsmeier *et al.*, 2001; Hui *et al.*, 2003), *Citrus sinensis* (Mozuruk *et al.*, 2006), *Picea sitchensis* (Ralph *et al.*, 2006*b*), and poplar (Ralph *et al.*, 2006*a*; Major and Constabel, 2006). These studies have provided insights into the molecular basis of insect–plant interactions, but little information regarding cultivated crops are available. Moreover, recent studies reveal that differential gene expression is dependent on the type of attacker and in some cases species specific (Zarate *et al.*, 2007). For example, insect-inducible genes identified in *N. attenuata* had little sequence homology with up-regulated genes in *Arabidopsis* (Korth, 2003). Moreover, attack from the same lepidopteran herbivore resulted in species-specific transcriptional responses in two species of solanaceous host plants (Schmidt *et al.*, 2005). Therefore, studying each insect–plant interaction is required.

Chickpea (*Cicer arietinum* L.) is an important legume crop due to its role in the human diet and use in animal feed. One of the major threats to its successful production is the generalist herbivore, *Helicoverpa armigera*, which damages the aerial parts of the plant, including leaves and pods. Since most studies examining *Helicoverpa*–chickpea interactions have focused on specific gene or protein dynamics (Johnston *et al.*, 1991; Jongsma *et al.*, 1995; Giri *et al.*, 1998; Peng *et al.*, 2005; Srinivasan *et al.*, 2005), our aim was to identify target genes up-regulated during mild insect infestation which may contribute to the defence response. To isolate *Helicoverpa*-induced genes, a subtractive cDNA library was constructed from chickpea seedlings under *Helicoverpa* mild infestation using SSH. In addition to known defence genes, a number of genes and their presumed biochemical functions, that have not been previously associated with defence responses against insects, were identified. Using macroarray, transcript patterns elicited by both herbivore and mechanical wounding were profiled and compared. Comparative expression patterns on exogenous applications of various signalling compounds were obtained to evaluate the dynamics of regulatory pathways. In addition to investigating the effects of elicitation by mild insect infestation, induced plant defences in chickpea were evaluated by examining signal compound elicitation on larval feeding behaviour.

Materials and methods

Plant and insect growth conditions

Chickpea seeds (*C. arietinum L.*: Pusa-362) procured from the Indian Agricultural Research Institute, New Delhi, India, were sown in pots containing autoclaved potting soil mixture (peat compost and vermiculite; 1:1 v/v). Plants were grown for 4 weeks in a greenhouse with 16/8 h light/dark cycle at 22–25 °C, 50–60% relative humidity (RH), and watered regularly during cultivation.

Larvae of *H. armigera* were reared in the laboratory at 25 °C and 65–70% relative humidity (RH) on a 14/10 h light/dark cycle. The larvae were fed on an artificial diet as described by Armes *et al.* (1992). The freshly moulted fifth-instar larvae were starved overnight before releasing them on the plants.

Plant treatments

Insect infestation was achieved by the release of fifth-instar *H. armigera* larvae on 4-week-old chickpea plants (one larva per plant) and allowed to feed for 3–4 h at 25±2 °C until ~15–20% of the leaf area was consumed. Larvae were then removed, and the entire shoot was harvested and stored at –80 °C after quick freezing in liquid nitrogen. To mimic insect infestation, leaves were wounded with a punch machine (hole diameter=4.5 mm) until ~15–20% of leaf area was removed, maintaining time span (3–4 h; continuous wounding with intervals of 1 h) and physical conditions (at 25±2 °C; 65–70% RH) similar to those of insect feeding. Plants were subsequently harvested. For treatments involving exogenous signalling molecules, equal volumes of aqueous solutions of MeJA (100 μM; Aldrich, St Louis, MO, USA), SA (5 mM; Sigma, St Louis, MO, USA), and ethephon (50 μM, 2-chloroethanephosphonic acid, Sigma, St Louis, MO, USA) were sprayed onto chickpea plants according to published procedures (Stotz *et al.*, 2000). Each plant received not more than 500 μl of the aqueous solutions of the signalling compounds. The plants were then

maintained in individual enclosures under the same conditions and harvested at different time points. In order to verify the effect of treatments, mRNA levels of marker genes namely PR-5 (Thomma *et al.*, 1998), LOX (Stotz *et al.*, 2000), and β 1,3-glucanase (Felix and Meins, 1987) for SA, MeJA, and ET treatments, respectively, were checked by northern hybridization.

Isolation of RNA and construction of subtracted cDNA library

Total RNA was prepared following treatment using 1 g of tissue (pooled from 20 plants grown at the same time under similar physical conditions) with TRIzol® Reagent (Invitrogen® Life Technologies, Rockville, MD, USA). Poly A^+ RNA was purified using an mRNA isolation kit (Roche Applied Science, Manheim, Germany) according to the manufacturer's protocol. A forward suppression subtractive hybridization (SSH) was performed using PCR-Select™ cDNA Subtraction Kit (BD Biosciences, Palo Alto, CA, USA) following the manufacturer's protocol. The enriched differentially expressed cDNAs were cloned into the pDrive Cloning Vector (Qiagen, Germany). In order to confirm differential expression of the individual clones during mild infestation by *Helicoverpa*, differential screening was performed with macroarray using subtracted cDNA probes, and unsubtracted probes respectively. The differentially expressed clones were then selected for sequencing. The recombinant plasmids were sequenced via Big Dye Terminator™ kit version 3.0 (Applied Biosystems, Foster City, CA, USA) and examined with the 3700 ABI Prizm 96 capillary sequence analyser. All sequences were screened for homology in GenBank database using BLASTx (http://www.ncbi.nlm.nih.gov/BLASTX). Sequences were submitted to GenBank and the assigned accession numbers are provided in Table 1.

cDNA macroarray and data analysis

Individual clones of the subtracted cDNA library were amplified, purified, and denatured by adding an equal volume of 0.6 M sodium hydroxide. Equal volumes of each denatured PCR product (about 100 ng) were spotted on Hybond™ N membranes (Amersham Pharmacia Biotech, NJ, USA) using a 96 well dot-blot apparatus (Bio-Rad Laboratories, CA, USA). In addition, PCR products of chickpea actin cDNA (Accession no. AJ012685) using primers (5′-GGTAACATTGTCTTGAGTGG-3′ and 5′-CCAGATCCGTAACAATACAC-3′) and neomycin phosphotransferase (NPTII) gene from the binary vector pBI121 (Accession no. AF485783.1) using primers (5′-TGCTCGACGTTGTCACTGAAG-3′ and 5′-GTCAAGAAGGCGATAGAAGGC-3′) were respectively spotted as an internal control and a negative control. The membranes were neutralized with neutralization buffer (0.5 M TRIS–HCl, pH 7.4, 1.5 M NaCl) for 3 min, washed with 2× SSC, and immobilized with UV cross-linker (Stratagene, La Jolla, CA, USA).

Probes were prepared for DNA array hybridization by first-strand reverse transcription (Powerscript™ RT, BD Biosciences, CA, USA) with 1 μg mRNAs isolated from different samples and labelled with α^{32}P-dCTP (10 μCi μl^{-1}; 3000 Ci $mmol^{-1}$). Radiolabelled cDNAs were purified by Sephadex G-50 (Amersham Pharmacia Biotech, NJ, USA), suspended in prehybridization buffer (7% SDS, 0.3 M sodium phosphate pH 7.4, 1 mM EDTA) and hybridized at 60 °C overnight. The membranes were then washed three times with washing buffer (1× SSC, 1% SDS, 20 min each at 60 °C). Autoradiographs were scanned employing a FSMI (Fluor-S-Multiimager, CA, Bio-Rad, USA) to acquire images and signal intensities analysed by subtracting background noise. Actin cDNA was used as the internal control whose subtracted volume value was used for comparison with the control values. Differential screening and expression pattern data were generated as means (±SD) of the three independent experiments to ensure biological and technical replications. A paired Student's *t* test on $\log_2$-transformed data was applied to determine if statistical differences between expression ratios of each treatment and control pair were evident. Genes significantly different from controls in any of the treatments were selected and presented in Table 1. The following two criteria were chosen to demarcate differentially expressing genes based on a previous report (Major and Constabel, 2006): (i) a greater than 2-fold induction level; and (ii) a $P < 0.05$ level of significance as determined by a *t* test for three independent experiments. Expression profiles of stress-inducible cDNAs were also analysed by clustering performed using SOTA (self organizing tree algorithm) by TIGR Multiple Experiment Viewer version 3.0 using complete linkage (available at http://www.tigr.org/software/tm4/menu/TM4).

Northern hybridization

Twelve micrograms of total RNA were fractionated in 1.2% agarose gel containing formaldehyde and transferred onto positively charged Hybond™ N membrane (Amersham Biosciences, NJ, USA) according to Sambrook and Russell (2001). Equal loading and lane transfer was verified by membrane staining with methylene blue (0.02%). PCR-amplified individual cDNA fragments (with primers corresponding to adaptor 1 and 2R, provided in the SSH kit) were purified from agarose gel extraction. In addition, LOX2 (Accession no. AJ276265) PR-5 (Accession no. AJ010501), and PR-2 (Accession No. CV793598) were amplified (the primers used for amplification 5′-TGAAGCCAGTGGCCATCGAAT-3′ and 5′-CGAAGGCCGTGTGGGAAGAT-3′; 5′-TGGTGGACTTCAATGCAC-3′ and 5′-GGCATCTCTATATGAGGAGC-3′; and 5′-CGTCTCACGGATCTTTCCGTT-3′ and 5′-GCTATTTGACATCTGCCGTG-3′ primer sets, respectively,) and purified from agarose gel isolation. Probes were labelled with α ^{32}P-dCTP using NEBlot® kit (New England Biolabs, MA, USA) and purified. Northern hybridization was performed and band-intensity was evaluated as described above for cDNA macroarray.

Stay/dispersal experiment

Chickpea plants were subjected to MeJA, SA, and ET treatments and wounded mechanically as described previously. For elicitation by insects, plants were infested with newly moulted fifth-instar larvae for 3 h until ~15–20% tissue was consumed. After treatment, plants were incubated for 3 h in individual enclosures. The first-instar larvae were removed from the stock culture on wet filter paper and placed at the bottom of round glass Petri dishes for 15 min. The treatment satiated the larvae with water and achieved identical physiological conditions. Twenty first-instar larvae (20 larvae=1 replicate) were separately released on each of the treated or control plants. In order to trap straying first-instar larvae, a white sheet coated with odourless glue was placed under treated and control plants in the centre of a circular arena (10 inches in diameter). Double-sided tape was fixed on the inner margin of the arena before larvae release. Six h after initial release, the number of trapped larvae was recorded. The experimental procedure included five replications. Water-treated plants served as the control for the above-mentioned experiments. Dispersal percentage was calculated based on the number of larvae dispersed from the plant surface and the total number of larvae released. Five independent experimental data sets were analysed statistically using analysis of variance (ANOVA; Tukey's Test; Sigma Stat 2.0; Jandel Scientific Software, 1995; Jandel corporation, San Rafael CA).

Feeding bioassays

Each freshly moulted fifth-instar larva was individually released on control/treated plants (50 plants for each control/treatment), and covered with wire mesh to restrict movement. The initial weight of larva (IWL) was recorded before release and the final weight of larva (FWL) noted after 24 h of feeding. The relative body weight

Table 1. *Genes differentially expressed in response to HV, WD, SA, MeJA, and ET*

cDNA Sequences of all unigenies listed in Table 1 have been submitted to the GenBank database and the assigned Accession nos. were mentioned. BLASTX searches were conducted to determine homologous genes and the putative function of the cDNA fragments. Ratios of signal intensity were determined by cDNA macroarray hybridization as described in the Materials and methods. Shown for each gene are the expression ratios with standard deviations (SD) in response to *Helicoverpa* infestation (HV) and wounding (WD) above background of the control samples. Expression ratios of the transcripts in response to methyl jasmonate (MeJA), ethylene (ET), and salicylic acid (SA) were also presented with SDs. The transcripts are listed according to their probable functions. Values are highlighted in bold if the expression ratios are more than 2-fold. Genes are in bold if showing differential expression ratios between *Helicoverpa* infested and mechanically damaged plants ($P < 0.05$).

Gene	Accession	HV	WD	SA	MeJA	ET
Defence-related						
Pre-hevein-like protein PR-4 precursor (*Pisum sativum*)	EF375951	**4.84±0.83**	1.40±0.09	1.50±0.51	**3.13±0.72**	**5.03±1.15**
Protease inhibitor/seed storage/LTP family protein (*A. thaliana*)	EF375952	**2.14±0.38**	1.11±0.01	**2.32±0.13**	0.86±0.16	**2.19±0.31**
Thaumatin-like protein PR-5a (*Cicer arietinum*)	CAR010502	**2.19±0.88**	1.08±0.32	**2.01±0.66**	**2.14±1.05**	1.66±0.82
PR10-1 protein (*Medicago truncatula*)	EL585361	**2.10±0.78**	1.09±0.32	**2.66±0.48**	1.68±0.30	**4.66±0.84**
Putative cysteine protease, *plp* gene (*Pisum sativum*)	EL585377	**3.71±1.93**	**2.19±0.02**	1.74±0.67	**3.72±1.44**	**2.04±0.79**
Putative phospholipase (*Arabidopsis thaliana*)	EL585367	1.82±0.71	1.42±0.01	1.52±0.41	**2.57±0.69**	**2.43±0.65**
Endo-1,4-β-D-glucanase KORRIGAN (*kor-1* gene) (*Pisum sativum*)	EL585369	**3.05±0.74**	1.70±0.21	**2.53±0.40**	**5.00±0.79**	1.56±0.24
Hydroxyproline-rich glycoprotein family protein (*Arabidopsis thaliana*)	EL585371	**3.68±1.64**	0.73±0.01	1.31±0.04	**5.52±0.17**	**2.08±0.06**
Cellulose synthase (*celA1*) (*Gossypium hirsutum*)	EL585372	**2.43±1.12**	1.22±0.28	**2.20±0.13**	**3.06±0.18**	**2.09±0.12**
Pectinmethylesterase (*Vigna radiata*)	EL585378	**2.39±1.01**	1.39±0.19	1.09±0.56	**3.01±1.55**	**2.19±1.12**
Pectinesterase, *pmeB* gene (*Pisum sativum*)	EL585357	**2.28±0.83**	1.44±0.04	1.39±0.15	**2.61±0.28**	1.81±0.19
3-Hydroxyisobutyryl-coenzyme A hydrolase (*Arabidopsis thaliana*)	EL585370	**5.21±1.81**	0.47±0.57	**5.79±3.52**	**9.41±5.72**	**6.63±4.03**
Homogentisate 1,2-dioxygenase [*Lycopersicon esculentum*]	EL585364	**2.34±0.70**	1.71±0.26	**2.20±0.03**	**2.92±0.05**	**3.06±0.08**
Leucoanthocyanidin dioxygenase-like protein (*Arabidopsis thaliana*)	EL585374	1.59±0.53	1.13±0.16	1.67±0.04	**2.94±0.07**	**4.36±0.07**
***Arabidopsis* thaliana mRNA for dihydrofolate synthetase (*dhfs/fpgs3* gene)**	EL585376	1.48±0.63	0.61±0.11	0.38±0.05	0.69±0.10	0.64±0.10
Aldehyde dehydrogenase, putative (*ALDH*) (*Arabidopsis thaliana*)	EL585382	**8.57±3.27**	**2.92±0.58**	**4.59±3.76**	**7.39±6.05**	0.01±0.01
Hydroxymethyltransferase (*Arabidopsis thaliana*)	EL585358	**2.68±1.22**	0.93±0.12	1.08±0.51	1.57±0.74	1.36±0.64
Cytochrome P450	CAB50768	**2.67±0.96**	1.89±0.22	**2.24±0.03**	**3.14±0.04**	**4.43±0.06**
Detoxification/oxidative stress/senescence						
Metallothionein-like protein (*Pisum sativum*)	CAA65008	**2.56±1.03**	1.17±0.08	1.01±0.12	1.75±0.21	1.66±0.20
Thioredoxin h (*Pisum sativum*)	EL585351	**2.81±1.05**	1.02±0.02	1.61±0.55	1.64±1.25	**2.15±0.74**
***RUB1* (Related to ubiquitin 1) (*Arabidopsis thaliana*)**	EL585368	**2.65±1.10**	1.61±0.03	1.72±0.55	**2.79±0.90**	**2.15±0.69**
Similar to F-box protein, *FBL2* (*Homo sapiens*)	EL585381	**2.66±0.73**	1.34±0.38	**3.22±1.12**	**6.75±2.36**	**3.63±1.27**
Glyceraldehyde 3-phosphate dehydrogenase (*Lycopersicon esculentum*)	EF375948	**2.26±0.64**	1.23±0.16	**3.03±0.45**	**7.56±1.13**	**3.48±0.52**
Transcriptional regulation/signal transduction						
***HMGB1* (High Mobility Group B 1) (*Arabidopsis thaliana*)**	EL585365	**2.43±1.07**	0.98±0.09	1.30±0.17	1.94±0.26	**2.04±0.27**
Nucleasome/chromatin assembly factor D protein *NFD101* (*Zea mays*)	EL585384	**2.31±0.92**	0.83±0.13	1.81±0.12	**2.14±0.15**	**2.60±0.18**
RNA recognition motif (RRM)-containing protein (*Arabidopsis thaliana*)	EL585383	**3.29±1.44**	1.22±0.12	**2.04±0.25**	**4.61±0.57**	**3.99±0.50**
Small GTP-binding protein (*sra1*) (*Glycine max*)	EL585380	**2.55±0.83**	0.90±0.07	1.45±0.39	**2.85±0.77**	1.34±0.36
***RAB11A* (*Lotus corniculatus*)**	EL585353	**3.29±0.39**	1.22±0.09	**2.04±0.12**	0.61±0.21	1.88±0.34
***F1 ATPase* (*Pisum sativum*)**	EL585385	**2.18±0.71**	0.62±0.28	**2.06±0.27**	**2.48±0.32**	**3.53±0.46**
Abiotic stress						
Cold induced protein (*BnC24B*) (*Brassica napus*)	EL585348	0.99±0.25	0.84±0.05	**2.39±0.69**	**3.70±1.10**	**5.55±1.62**
Cold-inducible unknown mRNA (*Zea mays*)	EL585360	**2.87±1.28**	**2.20±0.38**	**2.16±0.04**	**4.93±0.09**	**2.76±0.05**
Putative Pi starvation-induced protein (*Cicer arietinum*)	CAA07232	**3.43±0.90**	0.98±0.24	**2.45±1.19**	**3.37±1.64**	**6.01±2.93**
Dehydrin 1 (*Dhn*) (*Picea abies*)	EL593260	1.52±0.04	1.02±0.11	**4.48±0.35**	**9.07±0.72**	**4.99±0.39**
Protein synthesis and destination						
SSR16 (*Arabidopsis thaliana*)	**EL585375**	**1.97±0.79**	**1.24±0.15**	**1.06±0.15**	**2.52±0.37**	**1.92±0.28**
Poly (A)-binding protein (*PABP1*) (*Nicotiana tabacum*)	EL585354	**2.55±0.42**	0.88±0.12	**2.77±0.28**	**4.35±0.45**	**4.22±0.43**
Poly (A)-binding protein (*Cucumis sativus*)	EL585349	1.79±0.83	0.90±0.02	0.93±0.28	1.82±0.55	1.72±0.52
40S ribosomal protein S6 (*Cicer arietinum*)	AJ010227	**2.51±0.33**	0.86±0.16	1.72±0.18	**3.59±0.30**	**3.76±0.40**
Photosynthesis/energy						
Chlorophyll *a/b* binding protein (*Cicer arietinum*)	CAR131044	**2.49±1.09**	1.04±0.06	0.98±0.06	**2.37±0.16**	0.70±0.04
10 kDa photosystem II polypeptide (*Trifolium pratense*)	EF375955	1.53±0.21	**2.40±0.18**	1.65±0.65	1.90±0.95	1.32±0.56
Fructose-bisphosphate aldolase 2, chloroplast precursor (*Pisum sativum*)	EL585379	**2.59±0.94**	1.06±0.23	0.88±0.26	1.88±0.57	1.74±0.52
Oxygen-evolving enhancer protein 3 precursor (*Pisum sativum*)	EL585359	1.48±0.58	0.94±0.09	0.94±0.36	**2.18±0.85**	1.69±0.66

Continued

Table 1. *Continued*

Gene	Accession	HV	WD	SA	MeJA	ET
Miscellaneous						
GA (*Pisum sativum*)	EF375953	0.95±0.26	0.60±0.03	0.76±0.21	1.16±0.31	**2.05±0.56**
1-Aminocyclopropane-1-carboxylate oxidase (*EFE*) (*Pisum sativum*)	EL585350	**2.74±0.59**	1.37±0.53	0.85±0.36	1.92±0.82	**2.06±0.88**
GH1 protein (*GH1*) (*Glycine max*)	EL585366	**2.06±0.76**	1.16±0.12	0.82±0.21	1.80±0.47	1.10±0.28
PPF-1 protein (*Pisum sativum*)	EL585355	1.50±0.46	0.43±0.04	1.54±0.12	**2.62±0.21**	**2.79±0.22**
Ty3-gypsy like Retrotransposon, *CaRep* (*Cicer arietinum*)	AJ411814	0.72±0.11	1.04±0.06	**2.07±0.05**	**2.94±0.08**	**2.25±0.06**
Mitochondrial F1 ATP synthase β subunit (*A. thaliana*)	EL585393	1.63±0.59	1.41±0.08	1.45±0.25	**2.91±0.51**	**2.33±0.40**
F1 ATP synthase beta (*Arabidopsis thaliana*)	EL585352	1.45±0.38	1.61±0.20	1.24±0.07	**4.16±0.24**	**3.09±0.18**
H+transporting ATP synthase beta chain like protein (*A. thaliana*)	EL585363	**5.18±1.55**	1.51±0.34	**6.28±0.51**	**10.27±0.84**	**1.33±0.10**
Unknown functions						
Homologous to clone FC19AA10, HTC in fruit (*Solanum lycopersicum*)	EL585389	**2.22±0.72**	1.24±0.12	1.70±0.39	**2.02±0.47**	1.77±0.40
Unknown protein (*Zea mays*)	EF375947	1.49±0.09	0.76±0.01	1.99±0.16	**2.21±0.17**	**4.65±0.37**
Unknown protein (*Oryza sativa*)	EF375949	1.18±0.13	0.41±0.01	1.38±0.59	**2.03±0.87**	1.68±0.72
Unknown gene, genomic DNA, chromosome 1, (*Lotus corniculatus*)	EF375956	**2.21±0.61**	**2.35±0.49**	1.50±0.08	**6.38±0.37**	**3.89±0.23**
Unknown mRNA sequence (*Lycopersicon esculentum*)	EF375957	1.33±0.44	1.12±0.26	0.74±0.13	0.98±0.18	0.78±0.14
Unknown gene homologous to clone mth2-71h24 (*Medicago truncatula*)	EL585392	**2.60±1.17**	1.56±0.23	**4.48±0.35**	**9.07±0.72**	**4.99±0.39**
Unknown protein, AT4G01050 mRNA (*Arabidopsis thaliana*)	EF593261	**2.34±0.39**	1.55±0.08	**2.01±0.52**	**4.29±1.12**	**3.69±0.96**
Unknown, homologous to clone mth2-4j24 (*Medicago truncatula*)	EL585395	**3.06±1.27**	**2.16±0.22**	**2.49±0.52**	**4.72±1.00**	**2.67±0.56**
Hypothetical protein (*Plantago major*)	EL585394	**2.61±1.07**	1.45±0.41	1.82±0.61	**3.21±1.08**	**3.03±1.02**
Homologous to unknown clone mth2-193p1 (*Medicago truncatula*)	EL585391	**2.11±0.65**	1.46±0.57	**2.61±0.38**	**8.32±1.23**	0.50±0.07
Unknown protein (AT3G15840) mRNA (*Arabidopsis thaliana*)	EL585387	**2.66±1.24**	**2.01±0.20**	**2.07±0.02**	**2.70±0.03**	1.67±0.02
Hypothetical protein (*Arabidopsis thaliana*)	EL585388	1.56±0.44	1.07±0.11	1.57±0.10	**2.58±0.17**	**2.28±0.15**
Unknown homologous to clone mth2-5g18 (*Medicago truncatula*)	EL585390	**4.03±1.74**	1.79±0.12	**2.32±0.34**	**3.86±0.56**	**2.90±0.42**
Unknown mRNA (*Pisum sativum*)	EL585386	**3.50±1.40**	0.92±0.06	**2.96±0.18**	**3.76±0.23**	1.77±0.11

gain of the larvae was calculated as the difference between IWL and FWL. For conducting bioassays with excised plant tissues, equal amounts of freshly excised control/treated plant tissues were weighed separately, which gave the initial weight of the tissues (IWT) and transferred into the numbered Petri dishes (9 cm×3 cm). The neonate fifth-instar larvae (50 larvae for each control/treatment) were weighed individually which gave the initial weight of the larvae (IWL). Larvae were released individually into the numbered Petri dishes containing the control/treated plant tissues (2000 mg). The same amount of plant tissue was kept in Petri dishes without larvae under the same conditions to estimate the loss of moisture for calculating the corrected final weight of consumed tissues. All the Petri dishes were kept inside the BOD incubator, maintaining the same temperature and humidity as mentioned earlier. Larvae were allowed to feed for 24 h after which larvae were taken out and weighed individually which gave their final weight (FWL). The relative body weight gain of the larvae was calculated as the difference between IWL and FWL. The unconsumed plant tissues were also weighed separately which gave their final weights (FWT). Amount of tissue consumed was calculated by subtracting the corrected FWT from IWT. The data obtained from five independent experiments conducted both on live and excised plants were analysed statistically using ANOVA (Tukey's test).

Results and discussion

Identification of differentially expressed genes

In order to decipher genes up-regulated during mild infestation by *Helicoverpa* which may lead to defence, a forward subtractive cDNA library was constructed using the suppression subtractive hybridization (SSH) strategy. As a result, 715 recombinant colonies were obtained which were subjected to differential screening and sequencing. After screening for induction during insect infestation and sequencing, 63 unique genes were identified by BLASTX analysis which included transcripts not previously reported to be induced during insect attack and some functionally unknown transcripts. In addition to this, some transcripts already known to be responsive to insect attack in other plants were also obtained which appears to support the validity of the subtracted cDNA library. The library served to elucidate transcriptional changes and subsequent differential responses in chickpea triggered by *Helicoverpa* mild infestation.

The potential role of elicited transcripts

To gain insights into the function of differentially expressed genes, they were categorized into eight classes based on their putative roles during *Helicoverpa* infestation (Table 1; Fig. 1). The major functional category corresponded to genes involved in defence, secondary compound synthesis, and cell wall fortification and was classified as defence-related (29%). In addition, another category comprised genes involved in signalling and gene

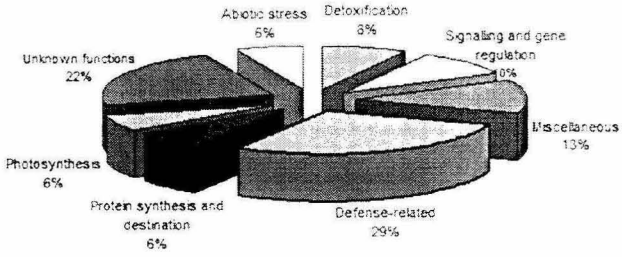

Fig. 1. Functional cataloging of *Helicoverpa*-responsive genes. The identified *Helicoverpa*-responsive genes were assigned a putative function based on their homology and functionally categorized as presented in the pie-chart

regulation (10%) and a significant fraction of genes were involved in detoxification (8%). Genes were also found to play a role in protein synthesis (6%), abiotic stress (6%), photosynthesis or energy metabolism (6%), and a major fraction (13%) are listed as miscellaneous. Genes, whose function were not ascertained (22%) were categorized as 'unknown functions', and considered to be *Helicoverpa*-responsive. This is also to be mentioned that for some of the genes the functional categorization might be arbitrary and there may be some overlaps.

Among the genes likely to be directly involved in defence, PR proteins (PR-10 and PR-5), hevein-like protein, and LTP/protease inhibitor were found in the subtractive library. Secondary metabolites such as phytoalexins, radical scavengers, and structural barriers serve a vital role in pathogen and insect defence. Several genes potentially involved in secondary metabolite synthesis were identified, including leucoanthocyanidin dioxygenase, dihydrofolate synthetase, homogentisate 1,2-dioxygenase, cytochrome P450, and hydroxymethyltransferase. Evidence suggested homogentisate 1,2-dioxygenase was involved in phenylpropanoid and lignin biosynthesis (Raes *et al.*, 2003). Furthermore, hydroxymethyltransferase was shown to be up-regulated in response to elicitation of insect oral secretions (Giri *et al.*, 2006). Endo-1, 4-β-D-glucanase, cellulose synthase, and pectinmethylesterase encoding proteins that function in cell wall fortification were also up-regulated. During the induced defence response, an increased accumulation of secondary metabolites, cell-wall reinforcing enzymes, and defensin proteins with toxic, antidigestive, and antinutritive activity has repeatedly been associated with diverse plant–insect interactions which reduce the palatability of the subsequent attackers and serve as a defensive tool for the plants (Kessler and Baldwin, 2002, 2004).

Genes potentially involved in protection of cells from oxidative stress were up-regulated on insect attack namely thioredoxin h, metallothionein-like protein, and *RUB 1*. Thioredoxins are a group of small proteins functioning in the regulation of redox status of the cell during oxidative stress (Gelhaye *et al.*, 2004). The precise role of metallothionein is not clear, but a dual role has been suggested to this protein: the detoxification of metal ion released during protein breakdown and serving as a metal chelator and to function as metal binding proteins for storage or transport into developing organs (Giritch *et al.*, 2006). The involvement of ubiquitin proteasome-dependent proteolysis during insect feeding is reflected by the up regulation of RUB1 and F Box proteins, which are associated with the ubiquitination cascade. The exact role of F Box protein has not been implicated in herbivory but a regulatory role for ubiquitin-dependent proteolysis during senescence has been assigned to this protein (Gepstein *et al.*, 2003). An F box protein, SON1, has been implicated in the regulation of the induced defence response independent of SA (Kim and Delaney, 2002). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) also shows 6-fold inductions during insect infestation. This gene has previously been reported to be up regulated by herbivoral attack in native tobacco (Giri *et al.*, 2006). GAPDH mainly play role in catalytic conversion of glycolysis, but it may be a part of reactive oxygen species signalling during herbivory. Two of the clones homologous to GTP-binding proteins and ATPase were also induced on *Helicoverpa* infestation. GTP binding proteins are known to regulate many cellular responses including signal transduction, cytoskeletal organization, and vesicle trafficking (Haizel *et al.*, 1995). Ran-A1, a GTP binding protein has previously been reported to be induced on insect attack in *Nicotiana attenuata* (Hui *et al.*, 2003). ATPases are reported to be up-regulated in plant on insect attack and the function assigned to them may be actively transporting a range of ions like H^+, Ca^{++}, Na^+ etc. into or out of the vacuoles or cells to support many biological functions (Ralph *et al.*, 2006*b*). Aphid feeding could induce the expression of H^+ ATPase in a resistant plant indicating its role in defence (Thompson and Goggin, 2006).

Ethylene and MeJA were induced in response to insect herbivory and wounding in several plant species and therefore considered key regulators in plant defence mechanisms (Arimura *et al.*, 2000; Wasternack, 2001; De Vos *et al.*, 2005). In addition, ET and MeJA mediate up-regulation of defence-related genes such as protease inhibitors (O'Donnell *et al.*, 1996), defensin (Penninckx *et al.*, 1998) and PR proteins (Diaz *et al.*, 2002). In the present study, *Helicoverpa* infestation induced a gene probably involved in ethylene biosynthesis (ACC oxidase), suggesting increased ethylene biosynthesis following insect attack. Furthermore, recent studies reported that ACC oxidase (Ralph *et al.*, 2006*a*, *b*; von Dahl *et al.*, 2007) were induced in plant insect interactions. The induced expression of ACC oxidase indicates the pronounced accumulation of ET in the process which may contribute to induced plant defence by regulating expression of defence-related genes or proteins that may

affect the infesting larvae. One of the genes regulated by auxin (GH1) were also induced by *Helicoverpa* infestation, suggesting involvement of this phytohormone during the response. Jang *et al.* (2003) reported the induction of auxin-induced protein and response factors during the Hessian fly larval attack on wheat–rye plants. Moreover, ethylene and auxin are determined regulators of the octadecanoid pathway (Walling, 2000), suggesting a defensive role during herbivory. A group of genes was identified in this study whose direct or indirect roles in insect defence were not previously known, including HMGB1 (High Mobility Group B1), Pi starvation-induced protein, GH1 protein (auxin-induced), cold-induced protein *BnC24B*, *PPF-1*, *RAB11A*, and among several others. Furthermore, some of these genes were involved in other types of stress, such as abiotic stress. Other genes identified in our study were up-regulated due to a stress response or the facilitation of transcriptional and translational changes during stress. In addition, it is proposed that the genes with unknown function are defence-related genes as most of them are induced on the application of defence regulators.

Cluster analysis revealed distinct responses to H. armigera *infestation, mechanical damage, MeJA, ET, and SA*

In order to achieve a comprehensive overview of expression profile of genes that were co-expressed during insect infestation, mechanical damage and treatments of signalling compounds, SOTA clustering was performed. The expression ratios obtained by macroarray were log2 transformed in order to reduce the noise level. The analysis yielded 11 clusters and the clusters with $n > 10$ were selected to study the expression patterns for functionally similar genes (Fig. 2). The maximum number of genes were grouped into cluster 11 which comprised genes which showed a very high expression level during *Helicoverpa* infestation, MeJA and ET treatments (Fig. 2C). In contrast to this, expression of the genes in this cluster was less during mechanical damage and SA treatment. This group was found to be enriched in genes involved in defence, abiotic stress, protein synthesis and destination, and genes of unknown functions. Another major group, cluster 4, consisted of defence-related genes and genes playing a role in signalling and gene regulation and detoxification as well. The genes in this cluster showed similar expression patterns during *Helicoverpa* infestation, MeJA and ET treatments but their expression was almost nil during mechanical damage. In cluster 1, genes showing higher expression during *Helicoverpa* infestation and no induction by SA were placed. Almost all functional categories are represented in this cluster. Detailed information on genes within each cluster can be found in Supplementary Fig. S1 at *JXB* online. The miscellaneous class and genes with unknown functions showed no clear clustering and were present in almost all the clusters, which may be due to the heterogeneous composition of these categories. Characterization of these genes can provide a valuable insight into understanding the chickpea–*Helicoverpa* interaction better.

Different transcript signatures for Helicoverpa *feeding and mechanical wounding*

Of 63 unique genes selected for further analysis, the transcripts of 46 genes were up-regulated upon *Helicoverpa* infestation, but wounding altered transcript levels of only eight genes above the cut-off value (as described in the Materials and methods) compared with the control (Fig. 3C). For the genes whose mRNA levels were co-induced during both types of stress, the transcript levels were higher on *Helicoverpa* infestation (Table 1). *Helicoverpa* infestation and wounding pair expression ratios were compared and revealed 29 gene ratios were significantly different and are presented as 'volcano plots' (Jin *et al.*, 2001) in Fig. 3B. The genes differentially induced during *Helicoverpa* infestation were probably related to insect-specific elicitors present during infestation but absent during wounding (McCloud and Baldwin, 1997; Korth and Dixon, 1997). Previous reports have demonstrated similar differential gene responses during mechanical damage and insect infestation (Reymond *et al.*, 2000; Schittko *et al.*, 2001; Reymond *et al.*, 2004). Many defence-related genes were placed in this category, including pre-hevein-like protein, LTP/protease inhibitor, PR-10, cysteine protease, and hydrolase, among others. Pre-hevein-like protein is reported to be up-regulated by insect infestation but not by mere mechanical damage (Reymond *et al.*, 2000). A subset of five genes was analysed by northern blot to validate the macroarray dataset (Fig. 2A). In general, the results of RNA gel-blot were consistent with the macroarray expression data analysis, with few differences between the two methods. These results further strengthen the fact that plants distinguish between mechanical damage and insect infestation and insect-elicited transcriptional changes differed from mechanical damage (Reymond and Farmer, 1998; Zhu-Salzman *et al.*, 2005). LOX gene served as a marker for wounding and insect infestation (Hui *et al.*, 2003; Reymond *et al.*, 2004).

H. armigera*-responsive genes are differentially regulated by MeJA, ET, and SA*

Among the three defence regulators, MeJA altered more transcripts than SA and ET (Table 1; Fig. 4D). Out of 63 genes, 47 were up-regulated by MeJA (74.6%), 39 by ET (61.9%), and 27 by SA (42.85%). Eighteen genes showed mRNA increases in all three treatments, including three well-known defence-related genes (cellulose synthase,

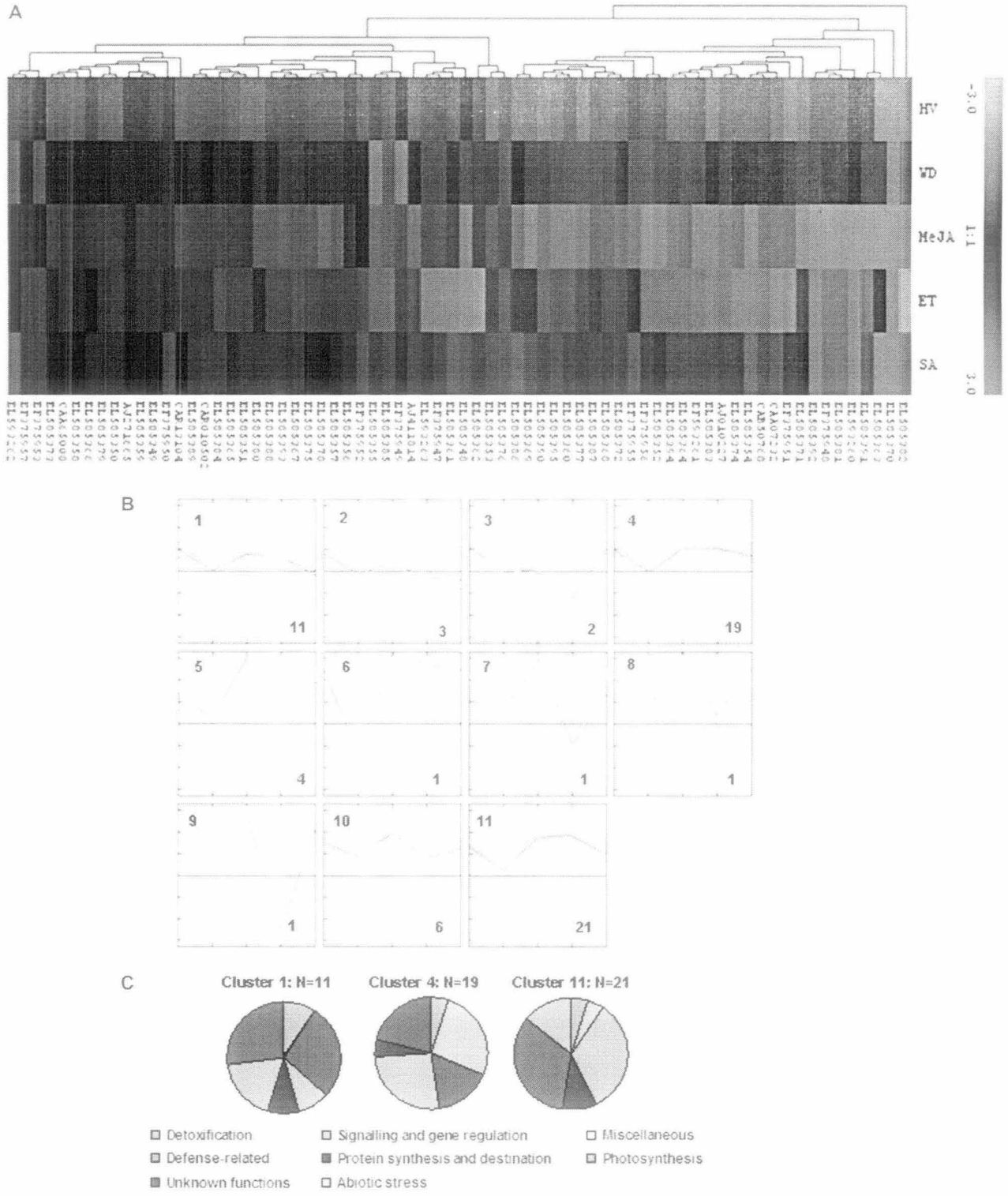

Fig. 2. Clustering analysis of expression profiles of *Helicoverpa*-responsive genes. (A) SOTA cluster tree of selected genes are shown to illustrate differential induction patterns after *Helicoverpa* infestation (HV), mechanical damage (WD), methyl jasmonate (MeJA), ethephon (ET), and salicylic acid (SA) treatments. Each gene is represented by a single row of coloured boxes, and a single column represents each treatment. Accession numbers provided by NCBI are given in the heat map and the corresponding gene names are available in Table 1. (B) The differentially expressed 63 unique

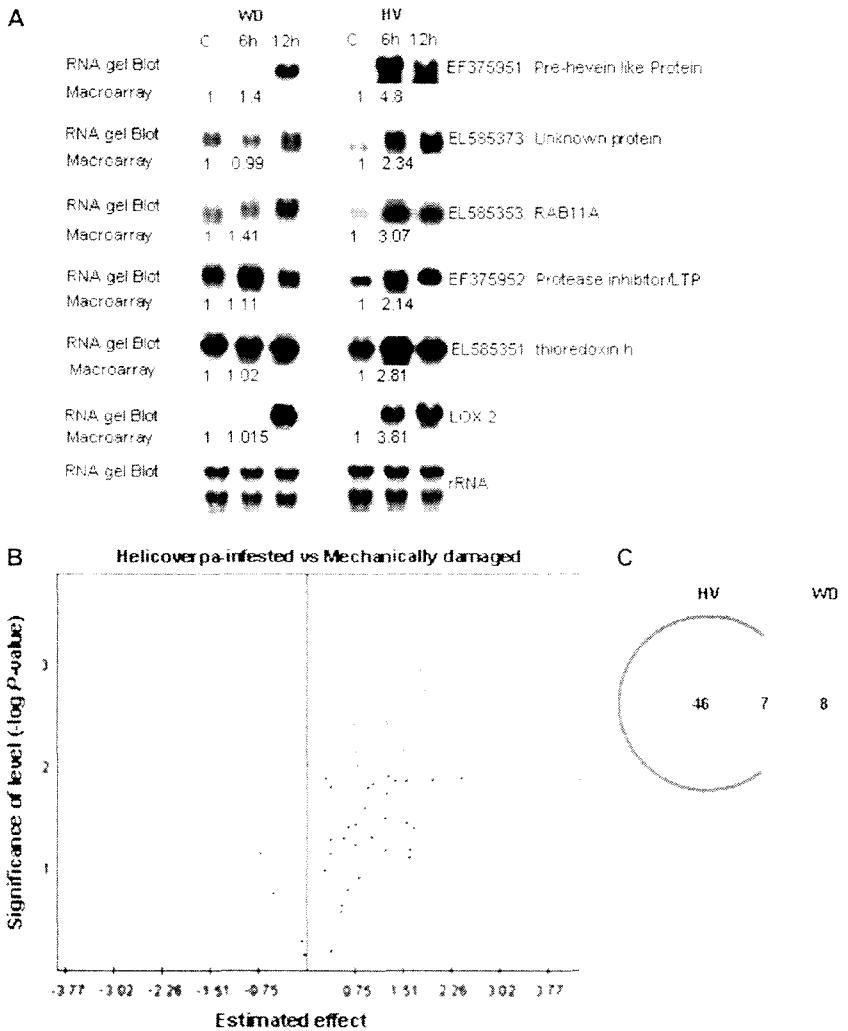

Fig. 3. Differential transcript profiles during *Helicoverpa* infestation and mechanical damage. (A) RNA gel-blot analysis of a few selected genes and the corresponding cDNA macroarray data. The lipoxygenase (LOX) gene was used as a marker for insect attack and wounding. rRNA of the same blot was used as the loading control. (B) Volcano plot of significance comparing gene expression in response to *Helicoverpa* infestation (HV) and wounding (WD) as measured by macroarray analysis. The plot shows differences in transcript abundance between *Helicoverpa* infestation and mechanically damaged plants. Significance is indicated as the negative $\log_{10}$-transformed P-values from a t test calculation. Each of the 63 genes selected for analysis was plotted as a point. The horizontal line corresponds to the significance scale. Red circles represent genes that exhibited differences ($P < 0.05$) in expression during *Helicoverpa* infestation against wounding. (C) The Venn diagram presents the number of genes that differed in macroarray analyses of *Helicoverpa* infested and mechanically damaged samples versus control. The numbers in circles indicate the genes having differential accumulations. The numbers in the common area represent genes with similar pattern of accumulation.

hydroxyisobutyryl-coenzyme A hydrolase, homogentisate 1, 2-dioxygenase) and two abiotic stress related genes (dehydrin 1, cold-induced protein). Since none of these genes showed exclusive up-regulation by SA (Fig. 4D), its association in this interaction was either less pronounced or it was involved in the signalling pathway cross-talks. Three plant defence genes (PDF1.2, PR1b, and Osmotin) were identified in *Arabidopsis* and induced synergistically by MeJA and ET (Xu *et al.*, 1994; Penninkx *et al.*, 1998; Kessler and Baldwin, 2002). In insect- and MeJA-induced

genes were clustered into 11 clusters based on their expression profiles. The expression profile of each individual gene in the cluster is depicted by grey lines, while the mean expression profile is marked in pink for each cluster. The number of genes in each cluster is given in the right lower corner and the cluster number in the left upper corner. (C) Functional cataloging of the genes present in different clusters. The classified *Helicoverpa*-responsive genes were categorized based on their function and presented in the pie-chart. (Clusters with $N > 10$ were taken into consideration.)

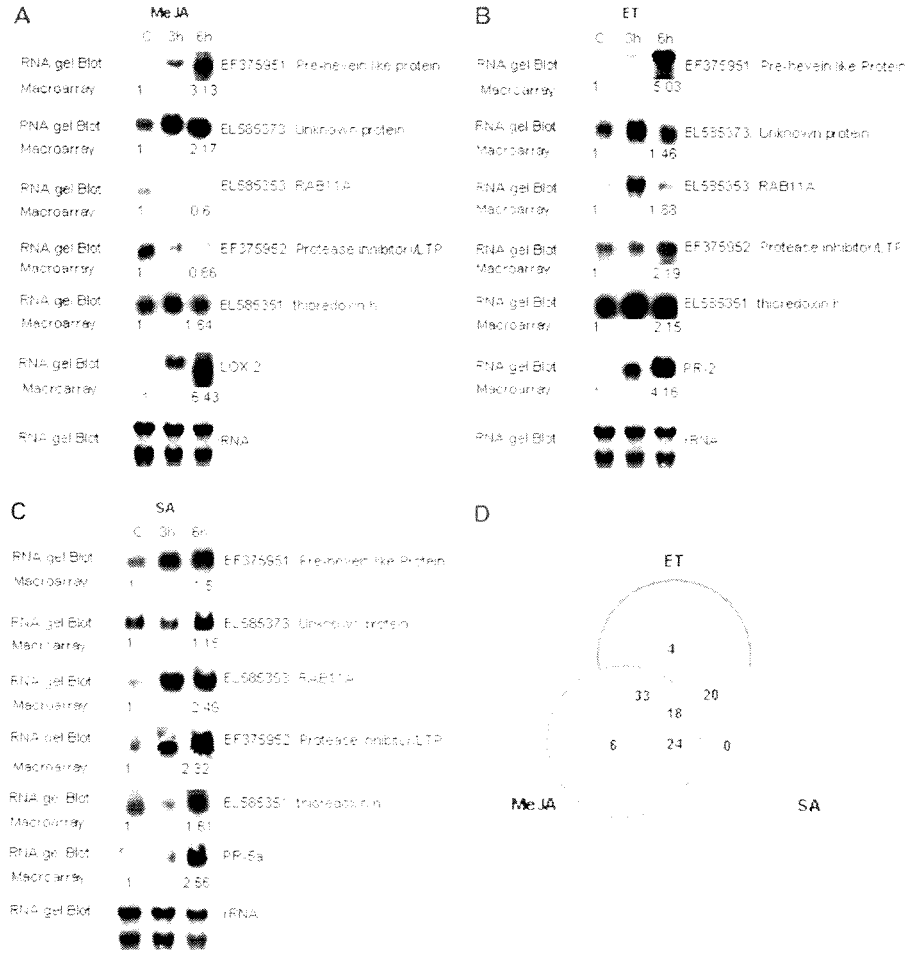

Fig. 4. Contribution of methyl jasmonate (MeJA), ethephon (ET), and salicylic acid (SA) to *Helicoverpa*-inducible gene expression. RNA gel blot analysis of a few selected genes after (A) MeJA, (B) ET, and (C) SA treatments and the corresponding cDNA macroarray data. The lipoxygenase (LOX) gene was used as a marker for MeJA treatment, PR-2 for ethylene treatment, and PR-5a for SA treatment. rRNA of the same blot was used as the loading control. The Venn diagram (D) presents the number of genes that differed in macroarray analyses of signalling molecules treated samples versus control, for one or more of the three signalling compounds. The numbers in circles indicate the genes having differential accumulations. The numbers in the common area represent genes with similar pattern of accumulation.

responses, studies have shown that a large proportion of genes are commonly induced by both the responses (Reymond *et al.*, 2004; Bodenhausen and Reymond, 2007). To confirm the expression data, the same subset of five selected genes was analysed by northern blots. The results demonstrated congruence between both methods, with the exception of a few minor differences (Fig. 4A, B, C).

Elicited chickpea plants could defend effectively during subsequent infestation by H. armigera

To indicate induced plant defence in chickpea, stay/dispersal tests were performed which showed that the percentage dispersal from control plants was significantly lower than ET- and MeJA-treated and pre-infested plants. The mean proportion of dispersed larvae from control plants was 5±3.5 (mean ±SD), compared with 35±7.9 for ET-treated plants. The dispersal percentage for MeJA-treated and pre-infested plants was 30±7.9 and 19±4.18, respectively. No significant difference was found between the dispersal percentage of SA-treated, wounded and control plants (14±4.18 and 12±5.7) (Fig. 5A). The negative effect on plant acceptance on phytohormone-treated plants may be attributed to both the elicited defence response and the direct influence of the phytohormone on the insect's behaviour. Significantly less aphid infestation had been observed previously on MeJA-treated plants (Ellis *et al.*, 2002; Zhu-Salzman *et al.*, 2004), suggesting effective plant defence elicited by MeJA. Involvement of JA and ET increases due to chewing insects was shown by the induction of modest but significant increases in ET production and a clear increase in JA production (De Vos *et al.*, 2005; Leitner *et al.*,

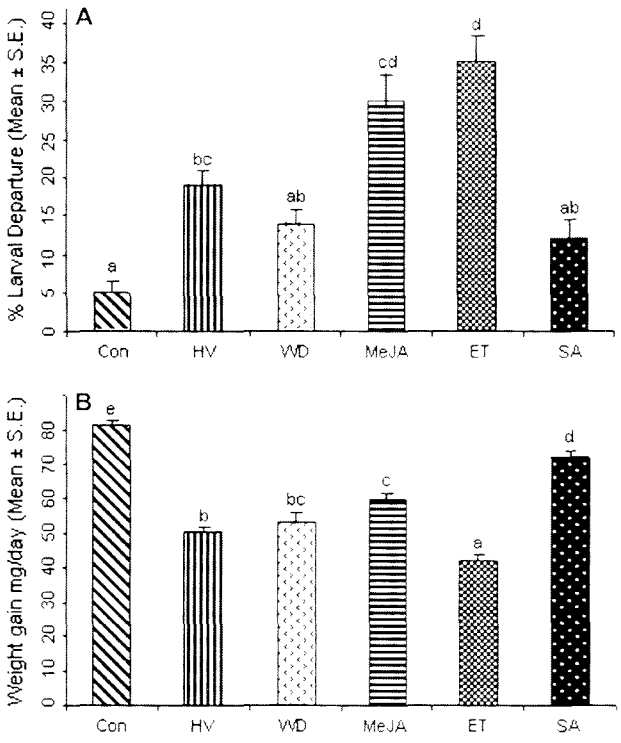

Fig. 5. Elicitor-induced resistance in chickpea plants against *Helicoverpa* infestation (Con: control; HV: *Helicoverpa* infestation; WD: wounding; MeJA: methyl jasmonate; ET: ethephon; SA: salicylic acid). (A) Comparison of the mean (± SE) dispersal percentage of first-instar larvae from the control and treated plants. (B) Comparison of the mean (± SE) weight gain of the larvae fed on the control and treated plants. Values in the graph represent the fresh weight gain mg d^{-1} (mean ± SE) of 5th instar larvae of *Helicoverpa* fed on plants exposed to different treatments. Means of various treatments superscripted by different lowercase letters are significantly different (Tukey's Test, $P < 0.001$).

2005). The differential behaviour of larvae on pre-infested plants may be attributed to the pronounced accumulation of signalling compounds (MeJA and ET) and allelochemicals, which detract the larvae.

The effects of induced plant defence were tested by feeding larvae on elicited plants under no choice conditions. The results were consistent with the previous experiment. The lowest mean body mass change was observed for larvae feeding on ET-treated plants (41.73 ± 1.97 mg), followed by pre-infested plants (50.73 ± 1.31 mg). The body mass increment of larvae fed on MeJA-treated plants (59.65 ± 2.01 mg) and wounded plants (53.14 ± 2.77 mg) were significantly different from control plants. In contrast to other treatments, the average body mass change of larvae fed on SA-treated plants (72.10 ± 1.89 mg) was not significantly different from control plants (Fig. 5B). Similarly, in the experiment where tissue consumed was calculated, which allowed us to correlate between the amount of tissue consumed and relative weight gain of the larvae, it was observed that the lowest tissue was consumed for ET-treated (292.6 ± 6.6), followed by pre-infested tissues (349 ± 12.6). The amounts of tissue consumed by the larvae feeding on MeJA-treated tissue (354 ± 8.3) and wounded tissue (360 ± 11.8) were significantly different from control plants. But the consumption was not significantly different when SA-treated and control tissues were compared (Fig. 6A). The results of relative weight gain of the larvae were similar to the previous experiment (Fig. 6B), suggesting that the reduced weight gain of the larvae feeding on ET- and MeJA-treated, mechanically wounded and pre-infested plant tissue are because of less consumption of the treated tissue as compared to control. The reduced growth of larvae fed on MeJA-elicited plants corresponded with previous reports. For example, JA elicitation of *N. attenuata* conferred induced resistance in both field (Baldwin, 1998) and laboratory (van Dam *et al.*, 2000) trials. *N. attenuata* increased production of secondary metabolites following MeJA elicitation, which diminished the plant's palatability for *Manduca sexta* (Kessler and Baldwin, 2004). In both the experiments performed in this study, the ET-elicited induced response was also effective. MeJA-induced ethylene production is reported to be responsible for defence responses (Dicke and van Poecke, 2002; Hudgins *et al.*, 2004). The results of Bi *et al.* (1997) suggested that exogenous application of SA on cotton plants did not affect the growth of *Helicoverpa zea*, congruent with our study.

Higher defence status was also maintained by induced plants (with mild insect infestation) than by uninduced plants, which may be attributed either to the induction of defence-related anti-nutritive and anti-digestive proteins (Kessler and Baldwin, 2002, 2004) or to the much earlier events occurring before gene expression, such as detection of defence regulators. Gene activation and subsequent metabolic changes can be detected even after approximately 1 h of infestation (Maffei *et al.*, 2007) although it might take few more hours to cause induced defence. Moreover, events occurring before gene expression (such as the pronounced accumulation of signalling compounds) can affect growth and feeding behaviour of the larvae. There is evidence to suggest that *H. zea* can intercept the plant defence signals elicited by its own feeding activity and can detect plant signal molecules and the allelochemical end products (Li *et al.*, 2002). Therefore, we can say that even if the toxic concentrations of anti-feed compounds may not be available in the induced plants, *H. armigera* could detect a higher defence status by tasting the signals.

In conclusion, this study shows that *Helicoverpa* attack triggers changes in transcript levels that are distinct from mechanical damage and are controlled mainly by MeJA and ET. Directly or indirectly, the majority of the genes identified as being *Helicoverpa* activated, may have a significant effect on insects performance, as it was depicted that elicitation with mild insect infestation, MeJA, and ET affected larval feeding behaviour. It is

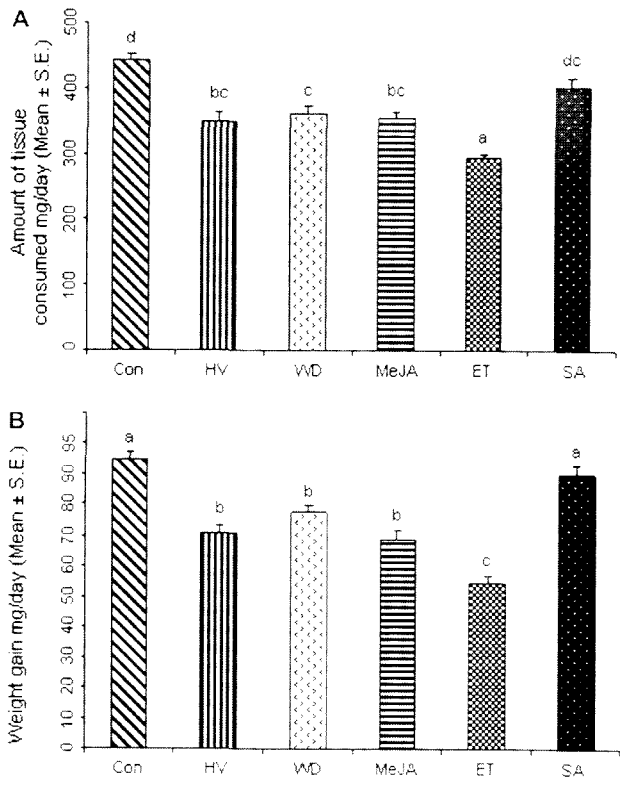

Fig. 6. Correlation between amounts of treated/control plant tissue consumed and weight gained by *Helicoverpa*. (Con, control; HV, *Helicoverpa* infestation; WD, wounding; MeJA, methyl jasmonate; ET, ethephon; SA, salicylic acid). (A) Comparison of the mean (±SE) amounts of plant tissue (control and treated) consumed by *Helicoverpa*. (B) Comparison of the mean (±SE) weight gain of the larvae fed on the control and treated plant tissues. Values in the graph represent the amount of tissue consumed (A) and fresh weight gain mg d^{-1} (mean ± SE) of 5th instar larvae of *Helicoverpa* (B) fed on plants exposed to different treatments. Means of various treatments superscripted by different lowercase letters are significantly different (Tukey's Test, $P < 0.001$).

expected that further functional characterization of these novel *Helicoverpa*-responsive genes which are regulated by MeJA and ET will extend our understanding about defence responses against insects and in developing new strategies for crop protection.

Supplementary data

An online supplementary figure, S1 is available at *JXB* online. This figure provides detailed information on the genes within each cluster which are given in Fig. 2.

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