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GENETIC AND MOLECULAR ANALYSIS OF THE ARABIDOPSIS COPINE I GENE IN PLANT DEFENSE RESPONSES

A Thesis in
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by
Niranjani Jambunathan

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We approve the thesis of Niranjani Jambunathan.

Date of Signature

Dr Timothy W. McNellis
Assistant Professor of Plant Pathology
Thesis Advisor
Co-Chair of Committee

Dr Andrew G. Stephenson
Professor of Biology
Co-Chair of Committee

Dr Sarah M. Assmann
Professor of Biology

Dr Richard J. Cyr
Professor of Biology

Dr Ramesh Raina
Assistant Professor of Biology

Dr Teh-hui Kao
Professor of Biochemistry and Molecular Biology
Chair of the Intercollege Graduate Program in Plant Physiology
Abstract

The copines are a ubiquitous class of calcium-dependent, phospholipid binding proteins of undetermined biological function. Mutation of the Arabidopsis COPINE 1 gene (CPN1) causes a humidity sensitive lesion mimic phenotype with increased resistance to a bacterial and an oomyceteous pathogen, constitutive pathogenesis-related (PR) gene expression, and an accelerated hypersensitive cell death defense response (HR). Low temperature also induces the lesion-mimic phenotype, increased disease resistance and constitutive PR gene expression in the cpn1-1 mutant. CPN1 is a constitutively expressed gene in wild-type plants with increased expression under low humidity and low temperature conditions. Strong expression of CPN1 is observed in wild-type plants when infected with avirulent and virulent pathogens and by salicylic acid treatment. Copine proteins consist of a unique combination of two C2 domains at its N terminus and a VWA domain at the C terminus. Over-expressing VWA domain fragment in wild-type plants resulted in a lesion-mimic phenotype with increased resistance to a bacterial pathogen. The lesion-mimic phenotype could be due to the inhibition of the function of endogenous full length CPN1 by introduced excess VWA domain. The npr1-1 mutation in cpn1-1 mutants did not affect the PR gene expression but delayed the lesion development. These results provide evidence that CPN1 in Arabidopsis may act as a suppressor of cell death and defense responses. These results also show that CPN1 may play a role in the signaling events during plant acclimation to low humidity and low temperature conditions. Taken together, these data suggest that CPN1 may represent a link between plant biotic and abiotic stress responses.
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Abbreviations

Amp: Ampicillin
bp: Base pair
DMSO: Dimethyl sulfoxide
IPTG: Isopropyl β-D-thiogalactoside
Kan: Kanamycin
kDa: kilodaltons
LB: Luria broth
µl: microliters
µg: micrograms
PAGE: Polyacrylamide gel electrophoresis
PCR: Polymerase chain reaction
Rif: Rifampicin
SDS: Sodium dodecyl sulphate
Tet: Tetracycline
T-DNA: Agrobacterium transferred DNA
WT: Wild type
Xgal: 5 bromo-4-chloro-3-indolyl β-D-galactopyranoside
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Chapter 1

Introduction to Plant defense Signaling

Most living organisms have evolved defense systems to fight against invading pathogens. In higher animals, a well-developed circulatory immune system is present for surveillance and destruction of foreign bodies. In plants, a functionally equivalent system exists. During the process of host-pathogen coevolution, plants have developed both preformed and induced defense mechanisms. Plants have existing physical and chemical barriers to ward off pathogen attack. During the battle between plants and pathogens, there are two possible outcomes. One possibility is that the plant succumbs to the pathogen, leading to disease. The other possibility is that the pathogen can be recognized by the plant, when the specific plant disease resistance gene (R) product detects the corresponding avirulence (avr) gene derived signal from the pathogen (Levine et al., 1994; Staskawicz et al., 1995; Hammond-Kosack and Jones, 1996). The recognition of pathogens by the plant leads to a local response involving a localized cell death defense reaction called the hypersensitive response (HR) (Goodman and Novacky, 1994). Activation of local responses triggers generation of systemic-acquired resistance (SAR) leading to increased broad-spectrum immunity to a wide range of pathogens in the whole plant (Ryals et al., 1996).
1.1 Pathogen Avirulence proteins

Pathogen \textit{avr} genes determine the inability of a pathogen to infect a plant carrying corresponding \textit{R} gene. A large number of bacterial \textit{avr} genes have been identified and they are found to be either chromosomal or plasmid borne and most of them share no sequence homology. Phytopathogenic gram-negative bacteria such as \textit{Pseudomonas syringae}, \textit{Erwinia} spp, \textit{Ralstonia solanacearum}, and \textit{Xanthomonas} spp use a Type III secretion system to deliver bacterial proteins including \textit{avr} proteins into the plant cell. The Type III secretion machinery is encoded by a cluster of \textit{hrp} genes (hypersensitive reaction and pathogenecity) localized in 20-25 kb clusters (Alfano and Collmer, 1997). Transient expression of \textit{avr} genes in the plant cell in the absence of pathogen induced \textit{R} gene specific HR (Bonas and Van den Ackerveken, 1997). This implies that the Avr protein is the signal molecule that is transferred into the plant cell via type III secretion system. Avr proteins also play a role as pathogenecity factors in susceptible hosts. Some examples include \textit{avrBs2} from \textit{X.campestris pv. vesicatoria} (Swords et al., 1996), \textit{avrRpm1} from \textit{P. syringae} pv. \textit{maculicola} (Ritter and Dangl ,1995) and \textit{pthA} from \textit{X. citri} (Swarup et al., 1992).

1.2 Plant Resistant genes (R)

Plant defense reactions are triggered by specific gene-for-gene interactions involving the plant \textit{R} gene products with the corresponding \textit{avr} gene products of the pathogen (Flohr, 1971). Plant \textit{R} genes are thought to encode receptors for recognition of ligands encoded by the \textit{avr} genes of the pathogens. Gene-for-gene recognition is
very specific, with each individual $R$ gene conferring recognition and resistance to pathogens bearing a particular $avr$ gene. The $RPS2$ resistance gene in *Arabidopsis* confers resistance in the plant when it interacts with the pathogen carrying $avrRpt2$ avirulence gene (Kunkel et al., 1993). The main class of $R$ gene products in plants contains nucleotide-binding site (NBS) and a carboxy-terminal leucine rich repeats domain (LRR) (Bent et al., 1994). The NBS/LRR class of $R$ proteins are subdivided into members that possess a leucine zipper motif or those that have similarity to the cytoplasmic domains of the *Drosophila* Toll and mammalian interleukin 1 transmembrane receptors in their amino terminal region (Baker et al., 1997). In *Arabidopsis*, $ndr1$ and $eds1$ mutants have been identified that have race specific resistance (Century et al., 1995, Parker et al., 1996). $NDR1$ is required for the LZ-NB-LRR class of $R$ genes and $EDS1$ is essential for TIR-NB-LRR type of $R$ genes (Aarts et al., 1998). Mutational studies of $R$ genes have revealed that NBS-LRR motifs are important for specificity of resistance gene function (Axtell et al., 2001; Tao et al., 2000). In addition, TIR-X (TX) group of proteins that lacks both the NBS and LRR and TIR-NBS (TN) class of proteins that lacks LRRs have been found in a broad range of plants that could be functionally important as plant resistant genes (Meyers et al., 2002).

The other major group of the LRR encoding $R$ genes includes $Cf-9$ and $Cf-2$ $R$ genes that do not contain NBS (Jones et al., 1994). $R$ genes encoding receptor like protein kinases have also been identified, such as $Pto$ in tomato, which confers resistance to bacterial speck disease (Zhou et al., 1997). $Xa21$, a resistance gene in
rice conferring resistance to blast disease has a combination of receptor like protein kinase and an LRR (Song et al., 1995).

Recent molecular data suggest that the plant R proteins may act as guards rather than receptors. Studies with the avirulence proteins AvrRpm1 and AvrB, which are unrelated based on their sequence, have shown that they both induce the HR in RPM1 (R) expressing plants. Both AvrRpm1 and AvrB interact with RIN4. RIN4 acts like a bridge connecting AvrB or AvrRpm1 to RPM1 (Mackey et al., 2002). It is speculated that RPM1 acts as a guard and provides surveillance to RIN4. Similarly, in tomato Prf acts like a guard to protect Pto from AvrPto or AvrPtoB (Kim et al., 2002). Similar evidence has been provided by two other groups on different systems (Kruger et al., 2002; Shao et al., 2002). All these data in plants demonstrate that rather than a gene-for-gene correspondence between avr and R gene product, it is likely that structurally similar proteins of the pathogen can act through a given protein in the plant.

1.3 Early signaling events in defense signal transduction

Pathogen recognition in plants initiates many cellular and systemic signaling processes that activate multi-component defense responses. The early cellular responses include directed movements of organelles and nucleus towards the site of pathogen attack (Freytag et al., 1994; Heath et al., 1997), ion flux, generation of reactive oxygen species (ROS), cell wall appositions and a programmed cell death (PCD).
1.3.1 Role of calcium in defense

Several bacterial and fungal elicitors have been reported to trigger ion fluxes such as H\(^+\), K\(^+\), Cl\(^-\) and Ca\(^{2+}\) across the plasma membrane. In plants, Ca\(^{2+}\) has been found to be an important second messenger in a variety of responses to environmental signals, including pathogens. In parsley cells, an elicitor- responsive calcium channel has been identified and characterized and a transient influx of calcium has been found to occur within minutes after fungal elicitor addition (Zimmermann et al., 1997).

Pharmacological studies have revealed that calcium, serine proteases and protein kinases are important for HR (Sasabe et al., 2000). Several lines of evidence suggest that GTP binding proteins and other protein phosphorylation and dephosphorylation events are involved in transfer of elicitor signals from receptor to calcium channels that activate downstream defense reactions (Schultheiss et al., 2002; Kurosaki et al., 2001). The specific requirement of calcium in HR cell death has been shown by studies with calcium channel blockers (Pennell and Lamb, 1997). Studies on calmodulin isoforms have suggested the dependence of HR on calcium (Heo et al., 1999). Transient elevation of calcium has been found to be necessary for the oxidative burst involving production of reactive oxygen species such as superoxide and hydroxyl free radical. In contrast, studies with soybean cells have established that Ca\(^{2+}\) acts downstream of oxidative burst in the HR in a manner similar to apoptosis in animals (Levine et al., 1996).
1.3.2. Reactive oxygen intermediates (ROIs) in defense

Different ROIs are produced under metabolic and photoactivated processes that lead in various kinds of cell death in plants. During pathogen recognition, ROIs have been found to act as direct toxic agents to pathogens, in initiation of reinforcement of physical barriers, PCD and phytolaexin synthesis. Following pathogen recognition, plants produce a biphasic pattern of oxidative burst (Alvarez et al., 1998). The first peak is non-specific as it is induced both by virulent and avirulent pathogens and occurs within an hour. The second peak occurs four to five hours later, and this sustained burst occurs only with avirulent pathogen treatment (Levine et al., 1994; Baker et al., 1997). Production of ROIs in plants is catalyzed by plasma membrane located NADPH oxidase that releases superoxide radicals that are quickly converted to hydrogen peroxides and oxygen by superoxide dismutase. H$_2$O$_2$ serves multiple roles in plant defense signaling. H$_2$O$_2$ can act as a direct antimicrobial component, help in oxidative cross-linking of cell wall and acts as an intra and inter-cellular signaling molecule (Lamb and Dixon, 1997). ROIs produced during oxidative burst play a central role in development of HR. Addition of antioxidant enzymes or scavengers affects the development of HR (Levine et al., 1994). Transgenic plant with reduced catalase activity (catalyzes decomposition of H$_2$O$_2$) accumulate increased levels of ROIs and develop HR like cell death (Chamnongpol et al., 1996).
1. 3. 3. MAP kinases in defense:

Protein kinases have been found to play a key role in the cell death mediating defense response in plants (Sasabe et al., 2000). Protein kinases with strong homology to mitogen-activated protein kinases (MAP kinases) of yeasts and humans have also been found to be involved in the pathogen-induced signaling processes. In plants, MAPK cascades are activated during physiological, developmental, hormonal processes and in response to environmental stimuli such as cold, touch, pathogens, wounding. The MAP kinase cascade involves MAP kinase kinase kinase (MAPKKK) proteins phosphorylating MAP kinase kinases that in turn phosphorylate MAP kinases. Upon activation, MAPKs are transported to the nucleus where they phosphorylate specific transcription factors. A complete plant MAP kinase cascade in early defense involving MEKKK1, MKK4/MKK5 and MPK3/MPK6 and WRKY22/WRKY29 transcription factors that function downstream of the flagellin receptor FLS2 has been identified (Asai et al., 2002). The role of Arabidopsis edr1 (enhanced disease resistance) mutant encoding a MAPKK kinase and the mpk4 mutant in defense signaling further strengthens the involvement of map kinase signaling cascade in plant defense signaling (Frye et al., 2001; Petersen et al., 2001). A MAP kinase cascade has been speculated to be acting downstream of elicitor-responsive ion channels and upstream or independent of the oxidative burst.

1. 3. 4. Nitric oxide (NO) in defense

NO produced by nitric oxide synthase (NOS) is a key signal molecule in animal immune systems (Lowenstein et al., 1994). In plants, NO accumulates during
incompatible interactions and pharmacological inhibitors of NOS inhibit the establishment of plant disease resistance (Delledonne et al., 1998). A transient increase in NO by injection of mammalian NOS in tobacco plants increases resistance in the plant (Durner et al., 1998). However, a plant gene encoding NOS has not been identified. It is possible that NO could be produced as a byproduct from NO$_2$ accumulation during nitrogen fixation (Durner and Klessig, 1999).

1.3.5. Programmed cell death response

During pathogen induced defense response, a localized cell death defense response occurs that is called the hypersensitive response (HR). This phenomenon was first characterized as hypersensitivity by Stakman (1915) and since then many plant biologists have undertaken extensive research to understand it. Cell death and plant defense have been often observed to be tightly linked.

In *Arabidopsis*, several approaches have been taken to dissect the defense signaling pathways. Since the HR and defense are under positive and negative control, several mutants have been identified that have an altered response to pathogens. Many such mutants are spontaneous lesion-mimic mutants that exhibit improved resistance to pathogens. Based on the appearance of the lesions, they are classified as initiation or propagation class of mutants (Dietrich et al., 1994). In *Arabidopsis*, several lesion mimic mutants that have spontaneous cell death have been characterized (*lsd1*-*lsd7, *acd2*, *acd6*, *cpr6*) (Greenberg and Ausubel, 1993; Dietrich et al., 1994; Greenberg et al., 1994; Weymann et al., 1995; Dietrich et al., 1997; Rate et al., 1999) and some of them have been cloned (*lsd1, acd2*) (Dietrich et al., 1997;
Mutations in the LESION SIMULATING DISEASE I (LSD1) gene have uncontrolled lesion initiation and spread. The LSD1 gene encodes a zinc finger transcription factor that monitors superoxide dependent signals (Dietrich et al., 1997). The Arabidopsis ACCELERATED CELL DEATH 2 gene (ACD2) has been found to encode a red chlorophyll catabolite reductase that suppresses the spread of disease symptoms (Mach et al., 2001).

Although cell death mutants could be powerful tool to study programmed cell death in plants, a variety of signals unrelated to pathogens can also trigger cell death and defense in plants (Mock et al., 1999; Molina et al., 1999). Many of the lesion-mimic mutants in Arabidopsis are dependent on certain environmental conditions such as light, day length, relative- humidity for the phenotype (Morel and Dangl, 1999; Yoshioka et al., 2001). Alterations in metabolic gene(s) have also been observed to trigger cell death and defense response in plants (Molina et al., 1999). These facts make it difficult to determine if genes defined by lesion mimic mutants play a direct role in cell death signaling and control, or represent disruptions of physiology unrelated or indirectly related to defense signaling.

Plant defense responses are sometimes observed in the absence of cell death. Mutant such as dnd1 (defense no death) (Yu et al., 1998) encoding a mutated cyclic nucleotide gated ion channel (Clough et al., 2000) failed to mount HR with Pseudomonas syringae expressing avrRpt2. The cpr1 (Bowling et al., 1994) and cpr6 (Clarke et al., 1998) mutants show constitutive expression of defense genes in the absence of cell death. These mutants suggest the possibility that signaling events that lead in cell death could be a parallel event to defense signaling pathways.
1.4. Systemic acquired resistance: a broad-spectrum resistance response

In addition to the HR, a secondary defense response is triggered in a plant that renders uninfected parts of the plant resistant to a broad range of pathogens. This phenomenon, called systemic acquired resistance (SAR) (Ryals et al., 1996), is associated with increased levels of salicylic acid (SA) in the whole plant (Gaffney et al., 1993). Associated with SAR is the early increased expression of pathogenesis-related (PR) genes that are molecular markers for resistance response (Ryals et al., 1996).

1.4.1. Salicylic acid (SA) and its role in defense

Levels of SA rise in the plant following pathogen attack. Increased SA correlates with the expression of pathogenesis related genes (PR) genes. SA is considered a key signaling molecule in SAR. Several data have suggested a complex role of SA in the activation of HR at primary sites of infection (Alvarez, 2000). In addition, conversion of SA to inactive catechol by salicylate hydroxylase (encoded by bacterial nahG gene) prevents induction of PR genes (Gaffney et al., 1993; Delaney et al., 1994). Loss of SA accumulation by NahG attenuated HR with enlarged lesions at the site of pathogen infection (Gaffney et al., 1993). Arabidopsis mutants such as lsd6 and lsd7 (Weymann et al., 1995) and acd2-2 (Greenberg et al., 1994) that exhibit constitutive expression of PR genes and enhanced resistance to pathogens have high levels of SA. Treatment of plants with SA or its functional analogs also induces SAR.
1.4.2. Pathogenesis related genes

Several plant genes have been identified whose transcript level increases with pathogen infection (Delaney et al., 1994; Ryals et al., 1996; Hammond-Kosack and Jones, 1996). Both HR and disease in plants induce defense-related genes but the magnitude and speed of induction is much greater in incompatible interactions. Several PR genes have been identified and these PR genes are considered to be molecular markers of defense response. PR1, which is strongly induced specifically during plant-pathogen interactions, encodes for a protein with an unknown function. Some other PR proteins in Arabidopsis include PR2 (encodes β-13-glucanase), PR3 and PR4 (for chitinases), PR5 (thauamatin like protein) and PR6 (encodes for proteinase inhibitor) (Van Loon and Van Strein, 1999).

Events occurring after SA accumulation and prior to expression of PR genes are not very clear. Many mutants defective in SAR (SA signal transduction pathway) such as npr1 (Cao et al., 1994), nim1 (Delaney et al., 1995), and sai1 (Shah et al., 1997) have been characterized and found to be key regulators of PR gene expression. The npr1, nim1 and sai1 mutants are allelic and encode a protein with ankyrin repeat (Cao et al., 1997).

1.5. SA independent and JA and ethylene dependent defense signaling

In recent years, jasmonic acid (JA) and ethylene have been found to confer broad- spectrum disease resistance. JA and ethylene act in a pathway antagonistic to SA (Penninckx et al., 1996). Exogenous application of JA causes induction of several plant defense-related proteins such as thionins (Epple et al., 1995), proteinase
inhibitors in tomato (Farmer et al., 1992) and phytoalexin biosynthesis (Blechert et al., 1995). The JA and ethylene mediated pathway has been shown to confer resistance to fungal pathogens *Alternaria* and *Botrytis* (Penninckx et al., 1998). The *coronatine insensitive I (coi1)* (*COI1* encodes a protein with degenerate F-box motif and LRR repeats) and *jasmonate resistant I (jar1)* mutants of Arabidopsis, which fail to respond to JA treatment, have increased susceptibility to *Pythium* and *Alternaria* pathogens (Xie et al., 1994). The *ethylene resistance 1* mutant (*etr1*) and *ethylene insensitive 2* (*ein2*) mutants display enhanced susceptibility to virulent pathogens (Bent et al., 1992). Concomitant activation of both JA and ethylene signaling is required for *PDF1.2* induction (Penninckx et al., 1998).

JA and ethylene have also been shown to be essential for induced systemic resistance (ISR). ISR can be established by root colonizing bacteria *Pseudomonas fluorescens* and confers resistance to virulent *Pseudomonas syringae* pv. tomato (*Pst*) DC3000 (Pieterse et al., 1998). Although JA and SA pathways act in an antagonistic fashion, interactions are observed between them via common regulatory components. ISR functions in a NPR1 dependent pathway where NPR1 is a key regulator of SA dependent SAR (Cao et al., 1998). The *cpr5* (Bowling et al., 1997) and dominant *cpr6* (Clarke et al., 1998) mutants display enhanced expression of *PR* genes and *PDF1.2* further proving some shared regulatory components in the two pathways.
Chapter 2

Screening Arabidopsis mutants for improved resistance to the bacterial pathogen \textit{Pseudomonas syringae pv. tomato}

2.1 Introduction

To study and understand the complexity of defense signaling events, genetic dissection via mutant analysis has been an important tool. Loss of function and gain of function are common strategies applied in understanding any genetic pathway. One of the most effective strategies used to study functional genomics is random insertional mutagenesis. Recently, the huge collection of \textit{Arabidopsis} insertional mutants by various groups has helped in determining the function of various genes. Gene inactivation by T-DNA or transposon mutagenesis and gene activation by activation tagging are common approaches used for insertional mutagenesis.

To study plant-pathogen interactions, Arabidopsis and the bacterial pathogen \textit{Pseudomonas syringae pv. tomato} (\textit{P. s. t.}) serve as an excellent model system. \textit{Arabidopsis thaliana} offers several advantages as a genetic tool and for studies related to pathogen response. Arabidopsis has a simple genome (120Mb), small stature, fast generation time, copious seed production and it is the first plant whose complete genomic sequence is available. Arabidopsis is also a good host to study defense responses as many of the major plant pathogens that cause disease in
important crops also infect Arabidopsis and cause disease (Ausubel et al., 1995). Some of the defense related genes identified in Arabidopsis have homologs in field crops that have similar function(s). *P. syringae* is a major bacterial pathogen and has a wide host range. *P. s.t.* is a major pathogen on tomato and it can infect Arabidopsis causing the bacterial speck disease.

To understand genetic pathways involved in defense, several different mutant screens have been performed. Some of the commonly used screens are to identify plants with defects in pathogen sensing, regulation of HR and disruption in SA accumulation and induction of SAR response. Most of the mutant screens have been performed with loss of function strategy. The major limitation of a loss-of-function screen is that it is difficult to identify the function of those genes that are redundant. To overcome this problem, a gain of function strategy using activation tagging has been widely used. The activation tagging T-DNA vector constitutes four copies of enhancer element from the 35S promoter. The enhancer elements in the T-DNA of activation tagged vector can cause activation of nearby genes resulting in a dominant gain of function when integrated in the plant genome.

The main objective of my research was to identify Arabidopsis mutants with improved resistance and characterize the gene(s) in detail in relation to their role in plant defense response. In order to fulfill my goal, I used a screening strategy for identifying mutants with enhanced resistance to virulent bacterial pathogen *P. s.t.* DC3000.
2.2 Methods

2.2.1 Plant growth conditions

Mutant seeds transgenic for the activation tagged pSKI015 vector (AT)(Fig 2-1) (Weigel et al., 2000) were obtained from the Arabidopsis Stock center (ABRC). Nearly 40,000-50,000 plants representing 10,000 independent Arabidopsis mutant lines were grown in soil (Scotts Redi-earth Plug and seedling mix) (E.C. Geiger, Inc. Harleysville, PA) and irrigated with distilled water under short day conditions (8h light, 16h darkness, 22-25°C, 35-40% RH, and 75-100 µmol m⁻² sec⁻¹ light intensity).

2.2.2. Screening with the virulent bacterial pathogen Pseudomonas syringae pv. tomato

Five-week-old T-DNA mutagenized plants were dipped in virulent bacterial inoculum of Pseudomonas syringae pv. tomato of 7x10⁸ bacterial cells/ml concentration with 0.0025% Silwet L-77 (Lehle seeds. Round Rock, Texas). Wild-type Col-0 plants of the same age were also dipped with the same inoculum. Bacterial inoculum was prepared in 10mM MgCl₂ solution and bacterial concentration was determined using spectrophotometer (1.0 OD₆₀₀ =1x 10⁹ bacterial cells/ml). Plants dipped with bacterial inoculum were placed in high humidity conditions (= 80- 90% RH) by covering them with domes to facilitate bacterial infection. Treated plants were scored for bacterial speck symptoms (water soaked lesions and chlorosis) four days after treatment. Mutant plants that exhibited improved resistance when compared to wild-type plants were selected and allowed to self and set seeds.
Nearly 30-40 progeny of each of the putative mutants were grown in short day conditions. Each line of putative mutant was tested with three different concentrations of bacterial inoculum (1x $10^8$, 3x$10^8$, 5x$10^8$ bacterial cells/ml). Col-0 plants grown under similar conditions were also treated with the three concentrations of the inoculum. The treated plants were kept under high humidity conditions and scored for bacterial speck disease symptoms.

Figure 2-1: Activation tagging vector pSKI015 (Weigel et al., 2000)

The activation tagged T-DNA vector consists of four copies of enhancers of 35S promoter at its right border (RB). The T-DNA bears the BAR gene as a marker for plant selection for herbicide resistance, pBstKS+ that confers bacteria selection for ampicillin resistance. Some of the restriction enzymes that cut the T-DNA are marked. The complete sequence of pSKI015 is available in GenBank (accession no AF187951).
2.2.3. Disease assessment in putative mutants

Based on the level of resistance exhibited with three different concentrations of bacterial inoculum tested, the putative mutants were grouped into three different categories.

Group I: Highly resistant mutants: The putative mutants performed better than wild-type plants under all three bacterial concentrations tested.

Group II: Intermediate resistance: The mutants that performed better than WT plants with treatments with bacterial inoculum of $1 \times 10^8$, $3 \times 10^8$ bacterial cells/ml. However, under high concentrations of $5 \times 10^8$ bacterial cells/ml the mutant lines were as susceptible as wild-type plants.

Group III: Weakly resistant mutant: Those mutants that performed better than wild-type plants only under lowest bacterial concentration treatment ($1 \times 10^8$ bacterial cells/ml).

Escapes: Putative mutants did not exhibit improved resistance in the next generation as compared to WT plants under all the bacterial concentrations tested.

2.2.4. Southern analyses of selected mutants

Genomic DNA was extracted from some of the putative mutants using urea extraction method (Shure et al., 1983). 5µg of genomic DNA was used for over-night restriction digestion at 37°C. Restriction enzymes such as EcoR I, Hind III, Kpn I, Xho I, Pst I, BamH I (New England Biolabs, Beverly, MA) that cut the T-DNA once
were used for digestion. The digested genomic DNA was run on a 1% agarose gel and transferred to a nylon membrane using the traditional alkali transfer method (Hybond N+, Amersham Pharmacia, biotech). Chemiluminiscent probes were generated using the bluescript (pBSKS+) region of the vector and 35S enhancer element. Conditions for hybridization and detection of signals were done as per the Phototope-Star Chemiluminescent Detection Kit manufacturer’s instructions (New England Biolabs).

### 2.3 Results

We conducted a screen to identify *Arabidopsis* mutant lines that exhibited increased resistance to virulent bacterial pathogen *P.s.t* DC3000. Activation tagged seeds were obtained from ABRC that were in at least the third generation after transformation. Hence, the seeds could potentially result in recessive mutants as well as dominant activation tagged mutants.

#### 2.3.1 Mutant frequency:

Nearly 40,000-50,000 plants representing 10,000 independent tagged lines were screened for enhanced resistance. Out of this screen, 150 plants showed improved resistance from first generation of screening. Of the 150 putative mutants initially identified in the screen, only 22 showed increased resistance than the wild-type plants in the second generation. The rest of the putative mutants turned out to be escapes.
2.3.2 Classes of mutants with improved resistance

The 22 mutant with a heritable disease resistance phenotype displayed different levels of resistance to \textit{P.s.t.} when compared to wild-type plants. Based on their level of their resistance, they were grouped into three different classes (Fig 2-2). The strongest mutants were group I mutants that exhibited hardly any disease symptoms when tested with high bacterial inoculum concentration of $5 \times 10^8$ bacterial cells/ml. At this inoculum level, group I mutants displayed some amount of chlorosis and water soaked lesion symptom especially in the older leaves, whereas wild-type plants had completely collapsed due to severe chlorosis and water soaked lesions. Only three mutant lines belonged to the Group I category. Interestingly, two of these mutants displayed lesion-mimic phenotype and the other mutant had short stature with dark colored leaves.

Some of the putative mutants displayed intermediate resistance. These mutant lines collapsed when screened with a very high bacterial inoculum level. These mutants showed lesser disease symptoms compared to wild-type plants when screened at lower bacterial inoculum of $3 \times 10^8$ bacterial cells/ml and $1 \times 10^8$ bacterial cells/ml. Six plants exhibited this pattern. All the six lines looked like wild-type plants.

Nearly 13 of the putative mutants were weakly resistant mutants. These mutant lines were only slightly more resistant than wild-type plants when screened with very low bacterial inoculum level ($1 \times 10^8$ bacterial cells/ml). An increase in humidity level in the growth chamber or a slight increase in bacterial inoculum
concentration made these mutant plants as susceptible as wild-type plants. All 13 mutant lines had normal growth and flowering like that of a wild-type plant. The majority of the putative mutants turned out to be escapes, as they did not exhibit any increased resistance to \textit{P.s.t} DC3000.

### 2.3.3 T-DNA insertions in screened mutants

Southern analyses were performed with genomic DNA of 12 of the mutants digested with enzymes that cut the vector at unique sites. Two different DNA probes were used for verification of number of T-DNA insertions in the mutant. The number of T-DNA insertions in these mutants ranged between 1 and 7 (Figure 2-3). Initially I started my work with two mutants with a single locus of T-DNA insertion. One of the mutants was a very weak resistant mutant and the other belonged to Group I (a highly resistant mutant). Later my work focused on the highly resistant mutant from my screen.

![Wild-type, Group I, Group II, Group III](image)

**Figure 2-2: Disease symptoms in wild-type and different groups of mutant after screening with virulent \textit{P.s.t} DC3000**

Wild-type plant exhibits severe disease symptoms of bacterial speck disease with water soaked lesions and chlorosis. Group I mutants (3 mutants) are highly resistant to \textit{P.s.t} DC3000 and exhibit yellowing only in older leaves at high bacterial inoculum concentration. Group II mutants (6 mutants) are intermediate mutants with moderate resistance and Group III mutants (13 mutants) are weak resistant mutants.
2.3 Discussion

The screen for mutants with increased resistance to virulent \emph{P.s.t.} DC3000 bacteria was very successful. Nearly 22 mutant lines from a population of nearly 10,000 independent lines showed enhanced resistance than wild-type to virulent \emph{P.s.t.} DC3000 pathogen. This accounted for 0.22% mutation frequency. The high
frequency of mutants from the screen could possibly indicate that both dominant and recessive mutants were obtained from the screen. The dominant mutation could be due to over-expression of a gene due to insertion of AT vector in the promoter region of a gene. The loss of function could be a result of insertion of the AT vector in the gene.

One of the interesting features of the screen was that the putative mutants displayed a wide range of resistance. Although the mutants were broadly classified into three groups, each mutant had a different strength of resistance. Defense signaling pathways consist of a complex network of various signaling events. The wide range of resistance observed in the mutants could be explained if the genes encoded by them act in a particular subset of pathway or regulate various defense-signaling events. A weak resistant mutant may allow us to study a downstream gene that may be involved in turning on a particular subset of defense. A strong mutant could help us to understand an upstream gene that may up-regulate various events in defense. It is also possible that over-expression or lack of expression of a particular gene(s) could indirectly affect the turn on of defense.

Although several potential enhanced disease resistant mutants were obtained from the screen, a large number of escapes also were picked during the first stage of the screening process. Disease symptoms in plants are controlled by many factors such as humidity, temperature conditions, inoculum concentration, plant age and planting density. Any slight alteration in these conditions could have affected the disease scoring procedure. Moreover, the mutant screening was done by dipping the plants in bacterial inoculum. By this procedure, smaller stature plants hidden
underneath bigger plants may not have been exposed to bacteria as much. These factors may explain the high rate of escapes in the first stage of the mutant screen.

Several strains of virulent bacteria are used for screening for mutants with enhanced resistance. *Pseudomonas syringae maculicola* (*P.s.m*) is the most popular strain of virulent bacteria used as it is less virulent than *P.s.t* DC3000. Using *P.s.m* can increase the chance of finding low resistant mutants. Since finding low resistant mutants can also increase the chance of adding more escapes, we decided to choose *P.s.t* DC3000 strain in order to identify mutants with strong disease resistance phenotypes.
Chapter 3

A Humidity-Sensitive Arabidopsis Copine Mutant Exhibits Precocious Cell Death and Increased Disease Resistance.

Niranjani Jambunathan, Jennifer M. Siani and Timothy W. McNellis
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A Humidity-Sensitive Arabidopsis Copine Mutant Exhibits Precocious Cell Death and Increased Disease Resistance

Niranjani Jambunathan, Jennifer M. Siani, and Timothy W. McNellis

Department of Plant Pathology, 212 Buckhout Laboratory, Pennsylvania State University, University Park, Pennsylvania 16802

Intercollege Graduate Program in Plant Physiology, Pennsylvania State University, University Park, Pennsylvania 16802

The copines are a newly identified class of calcium-dependent, phospholipid binding proteins that are present in a wide range of organisms, including Paramecium, plants, Caenorhabditis elegans, mouse, and human. However, the biological functions of the copines are unknown. Here, we describe a humidity-sensitive copine mutant in Arabidopsis. Under nonpermissive, low-humidity conditions, the cpn1-1 mutant displayed aberrant regulation of cell death that included a lesion mimic phenotype and an accelerated hypersensitive response (HR). However, the HR in cpn1-1 showed no increase in sensitivity to low pathogen titers. Low-humidity-grown cpn1-1 mutants also exhibited morphological abnormalities, increased resistance to virulent strains of Pseudomonas syringae and Peronospora parasitica, and constitutive expression of pathogenesis-related (PR) genes. Growth of cpn1-1 under permissive, high-humidity conditions abolished the increased disease resistance, lesion mimic, and morphological mutant phenotypes but only partially alleviated the accelerated HR and constitutive PR gene expression phenotypes. The disease resistance phenotype of cpn1-1 suggests that the CPN1 gene regulates defense responses. Alternatively, the primary function of CPN1 may be the regulation of plant responses to low humidity, and the effect of the cpn1-1 mutation on disease resistance may be indirect.

INTRODUCTION

The copines are a highly conserved class of proteins found in a wide variety of organisms, including ciliates, plants, Caenorhabditis elegans, mouse, and human (Creutz et al., 1998). The copines are defined as a protein class by their unique combination of two C2 domains in the N-terminal region and an A domain in the C-terminal region. C2 domains are present in a large number of proteins, including protein kinase C (Azzi et al., 1992), phospholipase C (Essen et al., 1996), synaptotagmin (Brose et al., 1995), Doc2 (Duncan et al., 2000), rabphilin (Wang et al., 2000), and Munc13 (Brose et al., 1995). The C2 domain appears to confer Ca$^{2+}$-dependent phospholipid binding activity to proteins, and mammalian copine I has Ca$^{2+}$-dependent phospholipid binding activity (Creutz et al., 1998). The A domain may be involved in extracellular protein–protein interactions, as in the case of the von Willebrand factor (Williams et al., 1999). However, available biochemical evidence indicates that copines are intracellular proteins (Creutz et al., 1998; Tomsig and Creutz, 2000). Finally, biochemical analysis of human copine III indicates that it has an intrinsic kinase activity that may be novel, because no homology with known kinase catalytic domains exists in copine III (Caudell et al., 2000). Together, these biochemical data raise the intriguing possibility that copines represent a new group of Ca$^{2+}$-dependent signal transduction proteins that might be involved in membrane trafficking. However, the biological functions of the copines are unknown at present.

Calcium is an important second messenger involved in a variety of plant responses to stimuli, including pathogens, drought, light, hormones, touch, cold, heat, and oxidative stress (Reddy, 2001). In plant disease resistance responses, Ca$^{2+}$ acts as a signaling molecule, communicating primary recognition events to multiple downstream responses (Grant et al., 2000) such as phytoalexin production, induction of defense genes (Zimmermann et al., 1997), and the hypersensitive response (HR) (Xu and Heath, 1998). In parsley cells, an elicitor-responsive calcium channel has been identified and characterized (Zimmermann et al., 1997), and a transient influx of Ca$^{2+}$ and H$^+$ and an efflux of K$^+$ and Cl$^-$ occur within minutes of fungal elicitor addition (Hahlbrock et al., 1995; Yang et al., 1997). Pharmacological studies with tobacco BY2 cells revealed that the HR requires serine proteases, calcium, and protein kinases (Sasabe et al., 2000).

The HR is a programmed cell death response: it is an active process involving transcription and translation, is under genetic control, and has some similarities to mammalian...
Figure 1. Humidity-Dependent Developmental and Lesion Mimic Phenotypes of cpn1-1.

The four columns of images, from left to right, represent Col-0 wild type grown in LH, cpn1-1 grown in LH, Col-0 wild type grown in HH, and cpn1-1 grown in HH, respectively. All plants were grown in SD light conditions.
apoptosis (Mittler et al., 1997; del Pozo and Lam, 1998; Maleck and Lawton, 1998). Because of the strong association of the HR with plant disease resistance, the HR is postulated to play an important role in resistance to at least some kinds of pathogens and may serve to localize a pathogen (Goodman and Novacky, 1994). A number of mutants have been identified in Arabidopsis that exhibit precocious cell death, such as the lesion mimic mutants lsd1-1/lsd7, acd2, acd6, and cpr6 (Greenberg and Ausubel, 1993; Dietrich et al., 1994, 1997; Greenberg et al., 1994; Weymarin et al., 1995; Rate et al., 1999). Many lesion mimic mutants are dependent on certain environmental conditions such as light, daylength, and RH for lesion development (Dietrich et al., 1994; Chamnongpol et al., 1996; Morel and Dangl, 1999; Yoshioka et al., 2001). Interestingly, the DND1 gene, which eliminates the HR and can cause a microscopic lesion phenotype when mutated, encodes a putative Ca\(^{2+}\) channel, providing another potential link between Ca\(^{2+}\) and cell death (Clough et al., 2000).

Many lesion mimic mutants exhibit a state of increased disease resistance called systemic acquired resistance (SAR) and show high, constitutive levels of pathogenesis-related (PR) gene expression (Dietrich et al., 1994). It is possible that lesion mimic mutants represent genes that regulate the HR. However, disruptions of cellular physiology apparently unrelated to disease defense also can trigger cell death and SAR (Mock et al., 1999; Molina et al., 1999). This fact can make it difficult to determine whether or not genes defined by lesion mimic mutants play a direct role in cell death signaling and control. In addition, many Arabidopsis mutants (cpr1, cpr5, edr1, mpk4) have been identified that show constitutive expression of defense-related genes in the absence of cell death (Bowling et al., 1994, 1997; Clarke et al., 1998; Frye et al., 2001; Petersen et al., 2000). This indicates that although the HR can trigger systemic PR gene expression and SAR, cell death is not always required for PR gene expression and SAR to occur.

Here, we describe a humidity-sensitive Arabidopsis mutant that exhibited precocious cell death, increased resistance to virulent bacterial and oomyceteous pathogens, and morphological abnormalities. On the basis of molecular analyses and complementation studies, we demonstrate that the phenotype of the mutant is caused by a mutation in a copine gene, CPN1.

**RESULTS**

**cpn1-1 Is a Humidity-Sensitive Lesion Mimic Mutant**

The cpn1-1 mutant was identified from a population of Arabidopsis mutagenized by T-DNA insertion (Weigel et al., 2000). The cpn1-1 mutant exhibited clear humidity-dependent developmental and growth abnormalities, as shown in Figure 1. cpn1-1 plants grown in low-humidity (LH; 35 to 45% RH), short-day (SD; 8-hr-light/16-hr-dark) growth conditions were smaller than their wild-type Columbia (Col-0) counterparts and had a more compact growth habit (Figures 1A and 1B). The leaves of LH-grown cpn1-1 mutants were curled, thicker than wild-type leaves, somewhat irregular in shape with short petioles, and occasionally had indentations at the margins. When cpn1-1 mutants were grown in high-humidity (HH; 75 to 85% RH), SD conditions, these developmental defects were absent and the cpn1-1 plants were morphologically indistinguishable from wild-type plants (Figures 1C and 1D). The phenotype was dependent on the age of the plants: cpn1-1 seedlings were indistinguishable from wild-type seedlings for the first 1 to 2 weeks of growth in LH, SD conditions; the developmental defects manifested after that time. When grown under LH, long-day (LD; 16-hr-light/8-hr-dark) conditions, cpn1-1 mutants were much smaller than Col-0 wild-type plants and had dramatically reduced floral apical dominance, shorter bolts, fewer flowers, and reduced seed yield (data not shown). Growth in HH, LD conditions rescued the cpn1-1 mutant, although the mutant was still slightly smaller than the wild type under these conditions (data not shown).

The leaves of LH-grown cpn1-1 plants displayed small necrotic lesions (Figure 1F). The largest of these lesions usually occurred at or near the margins of the leaf. No lesions were observed in HH-grown cpn1-1 plants (Figure 1H). Lesions began to appear on the third set of true leaves after 2 weeks of growth in LH, SD conditions. These lesions occurred in cpn1-1 plants that had not been inoculated with a pathogen, and they occurred consistently in all cpn1-1 plants grown in LH. However, it was not clear whether or not these lesions were truly spontaneous, because it was not feasible to grow cpn1-1 mutants under axenic, LH conditions and monitor lesion development.

**Figure 1.** (continued).

(A) to (D) Four-week-old whole plants. Bar in (A) = 1 cm for (A) to (D).

(E) to (H) Close-up images of leaves. Arrowhead in (F) denotes a lesion. Bar in (E) = 0.5 cm for (E) to (H).

(I) to (L) Sectors of leaves stained with the vital stain trypan blue. Bar in (I) = 200 μm for (I) to (L).

(M) to (P) UV autofluorescence images of leaf sectors. Bar in (M) = 100 μm for (M) to (P).

(Q) to (T) Leaf sectors stained with aniline blue to detect callose accumulation. The scale is the same as in (M) to (P).

(U) to (X) Scanning electron micrographs of the abaxial surfaces of leaves. Bar in (U) = 50 μm for (U) to (X).
The cpn1-1 lesions stained with trypan blue, indicating the presence of dead cells (Figure 1J). In addition, trypan blue staining revealed numerous microscopic lesions consisting of clusters of a few dead cells throughout LH-grown cpn1-1 leaves (Figure 1J and data not shown). No lesions or dead cells were observed in LH- or HH-grown Col-0 wild-type leaves or in HH-grown cpn1-1 leaves (Figures 1I, 1K, and 1L).

The leaves of LH-grown cpn1-1 plants showed accumulation of autofluorescent phenolic compounds in the areas near the lesions and in scattered patches throughout the leaf (Figure 1N). Autofluorescent phenolic compounds were not observed in LH- or HH-grown Col-0 plant leaves or in HH-grown cpn1-1 plant leaves (Figures 1M, 1O, and 1P). LH-grown cpn1-1 leaves also showed substantial accumulation of callose, as determined by aniline blue staining (Figure 1R), whereas LH- and HH-grown Col-0 wild-type plant leaves and HH-grown cpn1-1 plant leaves did not (Figures 1Q, 1S, and 1T). The accumulation of autofluorescent phenolic compounds and callose has been observed in a number of lesion mimic mutants and is a hallmark of plant stress, including pathogen stress (Bowling et al., 1994; Dietrich et al., 1994).

Because the cpn1-1 mutant was smaller than the Col-0 wild type when grown in LH, we speculated that the cell size might be reduced in cpn1-1. Light microscopic analysis of LH-grown cpn1-1 mutant leaves indicated that the cell size was reduced (Figure 1J and data not shown). In contrast, however, scanning electron microscopy of LH-grown cpn1-1 and Col-0 wild-type abaxial leaf epidermal cells showed no dramatic difference in cell size (Figures 1U and 1V). These observations imply that the mesophyll cells may be smaller in LH-grown cpn1-1 than in LH-grown wild type, whereas the abaxial epidermal cells in LH-grown cpn1-1 may be normal in size but fewer in number. The borders separating cells appeared somewhat thicker in LH-grown cpn1-1 leaves compared with LH-grown Col-0 wild-type leaves, possibly indicating the presence of thicker cell walls in LH-grown cpn1-1 plants. Abaxial epidermal cell size in HH-grown Col-0 wild-type and cpn1-1 mutant plant leaves was similar and larger than that observed in LH-grown wild-type and cpn1-1 plants (Figures 1W and 1X).

cpn1-1 Has an Accelerated Hypersensitive Cell Death Response

The development of necrotic lesions in cpn1-1 plants implies that the cpn1-1 mutation causes aberrant regulation of cell death. To investigate this possibility further, the time of HR onset after inoculation with avirulent Pseudomonas syringae pv. maculicola (avrRpt2) (P.s.m. [avrRpt2]) was compared in cpn1-1 and Col-0 wild-type plants. The macroscopic HR can be induced by infiltrating a high concentration (10^8 colony-forming units [cfu/mL]) of avirulent bacteria into the intercellular spaces of the mesophyll of resistant plant leaves (Klement et al., 1963). The HR is observed as a massive collapse of infiltrated tissue within ~12 to 36 hr after infiltration and involves the loss of membrane integrity and electrolyte leakage (Goodman, 1968, 1972).

Col-0 wild-type plants possess the RPS2 disease resistance gene and are able to recognize and resist P. syringae bacteria bearing the corresponding avrRpt2 avirulence gene through gene-for-gene–mediated disease resistance (Kunkel et al., 1993). cpn1-1 was isolated from the Col-0 genetic background and appeared to have a functional RPS2 gene (see below). cpn1-1 and Col-0 wild-type leaves were infiltrated with high concentrations of P.s.m. (avrRpt2) bacteria, and the appearance of the HR was monitored over time. Interestingly, both LH- and HH-grown cpn1-1 plants exhibited a faster HR than Col-0 wild-type plants grown under the same conditions, as measured by both the onset of macroscopic leaf collapse and electrolyte leakage, as shown in Figure 2.

LH-grown cpn1-1 plants had significantly more HR responses than LH-grown Col-0 wild-type plants at 12, 16, 20, and 24 hr after inoculation, whereas HH-grown cpn1-1 plants had significantly more HR responses than HH-grown Col-0 wild-type plants at 16 and 20 hr after infiltration (Figure 2A). By 30 hr after infiltration, all cpn1-1 and Col-0 wild-type leaves had collapsed. Significant electrolyte leakage could be detected from LH-grown cpn1-1 tissues infiltrated with P.s.m. (avrRpt2) within 4 to 8 hr after inoculation, whereas similar leakage from LH-grown Col-0 wild-type tissues did not occur until 16 to 20 hr after inoculation (Figure 2B). The peak of electrolyte leakage occurred at 12 hr after inoculation in LH-grown cpn1-1, whereas in LH-grown Col-0, the peak was observed at 24 hr after inoculation. In HH-grown cpn1-1, an increase in ion leakage was observed at 4 hr after inoculation, whereas a similar increase in electrolyte leakage in Col-0 wild type was not observed until 24 hr after inoculation. Some of the electrolyte leakage from HH-grown Col-0 wild-type and cpn1-1 leaves may have been attributable to tissue damage from the infiltration, because the HH-grown leaf tissues were very tender and easily damaged. Together, these results indicated that not all aspects of the cpn1-1 mutant phenotype were humidity sensitive.

Some lesion mimic mutants have a “hair trigger” HR, meaning that they will develop a macroscopic HR at lower bacterial inoculum concentrations than do wild-type plants (Dietrich et al., 1994). cpn1-1 was tested for a hair trigger HR phenotype by determining the threshold concentration of P.s.m. (avrRpt2) that could trigger a macroscopic HR in cpn1-1 compared with Col-0 wild-type plants. The minimum concentration of P.s.m. (avrRpt2) that was sufficient to evoke a macroscopic HR was 5 × 10^7 cfu/mL in LH- and HH-grown Col-0 wild-type and cpn1-1 plants (data not shown). Therefore, although cpn1-1 plants had a faster HR than Col-0 wild-type plants, they did not appear to have increased sensitivity to low titers of P.s.m. (avrRpt2).
Figure 2. Accelerated HR in *cpn1-1*.

The macroscopic HR was first observed at ~12 hr after inoculation. Fifty to 100 leaves of each type were inoculated for each experiment. For details, see Methods.

(A) Proportions of inoculated Col-0 wild-type and *cpn1-1* leaves exhibiting partial and complete collapse over time after inoculation. Asterisks indicate percentages of leaves with partial or complete HR collapse in *cpn1-1* plants that are significantly higher than Col-0 wild-type plants grown under the same humidity conditions, at the same time during the experiment, using Student’s *t* test. A leaf was scored as showing complete HR when the leaf had collapsed totally; a leaf was scored as showing partial HR when from 10 to 90% of the infiltrated leaf had collapsed. Leaves showing <10% collapse were not scored as having an HR.

(B) Electrolyte leakage from inoculated LH-grown Col-0 wild-type and *cpn1-1* leaves over time after infiltration with *P. syringae* (avrRpt2) or 10 mM MgCl\(_2\) (control).

(C) Electrolyte leakage from inoculated HH-grown Col-0 wild-type and *cpn1-1* leaves over time after infiltration with *P. syringae* (avrRpt2) or 10 mM MgCl\(_2\) (control).

WT, wild type.

Bars in (B) and (C) indicate standard errors.
**cpn1-1 Has Normal Stomatal Conductance**

The sensitivity of *cpn1-1* to low RH led us to speculate that *cpn1-1* might be defective in responding to low humidity. For example, if *cpn1-1* were defective in controlling water loss in low humidity, this might trigger stress, cell death, and other physiological problems. However, the stomatal conductance of LH-grown *cpn1-1* and Col-0 wild-type plants was similar, as shown in Figure 3. This finding suggests that *cpn1-1* is not defective in the regulation of stomatal conductance. Also, *cpn1-1* did not appear to be particularly sensitive to drought stress, because it did not have a wilty phenotype or a lower tolerance than Col-0 wild type for low soil water potential (data not shown).

**cpn1-1 Plants Exhibit Strong, Humidity-Dependent Resistance to Virulent *P. syringae***

Many lesion mimic mutants have increased resistance to virulent pathogens. LH-grown *cpn1-1* had dramatically increased resistance to virulent *P. syringae pv tomato (P.s.t.)* bacteria compared with Col-0 wild-type plants, as shown in Figure 4. Four days after dip inoculation with $5 \times 10^8$ cfu/mL *P.s.t.*, wild-type plants showed severe disease symptoms typical of bacterial speck disease, including massive tissue collapse, formation of water-soaked lesions, and chlorosis (Figure 4A). In contrast, the *cpn1-1* mutant showed virtually no symptoms of the disease after inoculation (Figure 4B). The leaf tissues of inoculated *cpn1-1* mutant plants remained green and healthy, although symptoms occasionally occurred on older leaves, especially when the bacterial inoculum was $>10^9$ cfu/mL (Figure 4B). HH-grown *cpn1-1* plants did not have increased resistance to *P.s.t.* compared with Col-0 wild-type plants, as measured by visible symptoms (Figures 4C and 4D).

The ability of virulent *P. syringae* bacteria to grow within LH-grown *cpn1-1* mutant leaf tissue was sharply restricted compared with that observed in LH-grown Col-0 wild-type tissue, as shown in Figure 5A. When Col-0 wild-type leaves were infiltrated with $10^6$ cfu/mL virulent *P.s.t.*, the bacterial population increased by several orders of magnitude over the course of 4 days. In contrast, when the leaves of LH-grown *cpn1-1* mutant plants were infiltrated with virulent *P.s.t.*, the bacteria were able to multiply only to a much lower level, approximately two or three orders of magnitude less than that observed in wild-type plants. The restriction of virulent *P.s.t.* multiplication in *cpn1-1* mutant leaf tissues was consistent with the dramatic reduction of bacterial speck disease symptoms observed in inoculated LH-grown *cpn1-1* mutant plants. Virulent *P.s.t.* grew to similar levels in HH-grown Col-0 wild-type and *cpn1-1* mutant plant leaves after the plants were dip inoculated with a *P.s.t.* bacterial suspension of $2 \times 10^7$ cfu/mL (Figure 5B). These results were consistent with the strong bacterial speck disease symptoms observed in inoculated HH-grown Col-0 wild-type and *cpn1-1* mutant plants and indicated that the increased resistance of *cpn1-1* to virulent *P.s.t.* was a humidity-sensi-
Humidity-Sensitive Lesion Mimic 2231
tive phenotype. Growth curves determined by infiltration inoculation or dip inoculation produced similar results (data not shown).

To gauge the relative level of resistance of LH-grown cpn1-1 plants to virulent *P. s.t.*, the degree of restriction of virulent *P. s.t.* growth in cpn1-1 was compared with the restriction of avirulent *P. s.t.* growth in Col-0 wild-type plants (Figure 5A). Growth of avirulent *P. s.t.* (*avrRpt2*) was sharply restricted in both Col-0 and cpn1-1 plants grown under LH conditions. The degree of restriction of virulent *P. s.t.* growth in LH-grown cpn1-1 plants was similar to the restriction of avirulent *P. s.t.* (*avrRpt2*) bacterial growth in LH-grown Col-0 wild-type plants. This indicates that the level of resistance of LH-grown cpn1-1 plants to virulent *P. s.t.* was similar to the level of resistance of LH-grown Col-0 wild-type plants to avirulent *P. s.t.* (*avrRpt2*) bacteria. Interestingly, the growth of avirulent *P. s.t.* (*avrRpt2*) bacteria in LH-grown cpn1-1 was moderately but significantly lower than the growth of virulent *P. s.t.* in LH-grown cpn1-1 plants, suggesting that there may be a slight additive effect of the *cpn1-1* mutation and *RPS2*-mediated gene-for-gene resistance responses. The growth of avirulent *P. s.t.* (*avrRpt2*) bacteria was restricted in both Col-0 and cpn1-1 plants grown under HH conditions (Figure 5B). This indicates that *RPS2*-mediated gene-for-gene recognition and resistance was intact in *cpn1-1* and that disease resistance mechanisms functioned normally in plants grown under high RH.

*cpn1-1* Plants Constitutively Express Defense-Related Genes

Because *cpn1-1* mutant plants had increased resistance to *P. s.t.* and also had a lesion mimic phenotype, we reasoned that LH-grown *cpn1-1* plants might be exhibiting SAR. Therefore, the expression of defense-related genes was investigated in *cpn1-1* plants. LH-grown *cpn1-1* plants showed high-level accumulation of *PR1*, *PR2*, and *PR5* transcripts, whereas these transcripts were not detectable in LH-grown Col-0 wild-type plants, as shown in Figure 6 (Uknes et al., 1992). This result suggests that the *cpn1-1* mutant was undergoing constitutive SAR in LH. Interestingly, there was moderate accumulation of *PR2* transcript and very low but detectable accumulation of *PR5* transcript in HH-grown *cpn1-1* plants. In contrast, transcripts were not detected for any of these genes in HH-grown Col-0 wild-type. This finding indicates that growth in HH did not completely abolish the effects of the *cpn1-1* mutation on *PR* gene expression.

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**Figure 5.** Humidity-Dependent Inhibition of Bacterial Growth in *cpn1-1*.

(A) Growth of virulent *P. s.t.* (DC3000) and avirulent *P. s.t.* (*avrRpt2*) bacteria in LH-grown Col-0 wild-type (WT) and *cpn1-1* plants. Plants were inoculated with the bacterial strains (10⁵ cfu/mL) by syringe infiltration of the leaf mesophyll. For details, see Methods.

(B) Growth of virulent *P. s.t.* (DC3000) and avirulent *P. s.t.* (*avrRpt2*) bacteria in HH-grown Col-0 wild-type (WT) and *cpn1-1* plants. Plants were inoculated with the bacterial strains (2 × 10⁷ cfu/mL) by dipping, as described in Methods. This accounts for the higher initial bacterial count in (B) compared with that in (A). Bars in (A) and (B) indicate standard errors.

The increased resistance to virulent *P. s.t.* and the constitutive *PR* gene expression observed in *cpn1-1* implied that
The plant cell was undergoing salicylic acid–dependent SAR in LH. This suggested that the mutant also might be resistant to other pathogens that are controlled by salicylic acid–dependent SAR, such as the oomyceteous pathogen *Peronospora parasitica* (Bowling et al., 1994; Nawrath and Métraux, 1999). Two-week-old LH-grown *cpn1-1* plants had dramatically increased resistance to virulent *P. parasitica* (Ahco 2) compared with LH-grown Col-0 wild-type plants, as shown in Figure 7. Virulent *P. parasitica* was able to sporulate on LH-grown Col-0 wild-type plants, but virtually no sporulation was observed on LH-grown *cpn1-1* plants. This finding indicated that LH-grown *cpn1-1* had improved resistance to both virulent *P. syringae* and virulent *P. parasitica*. This supports the interpretation that LH-grown *cpn1-1* was undergoing SAR.

**Genetic Analysis of *cpn1-1***

The *cpn1-1* mutation was isolated in the homozygous state. The original homozygous *cpn1-1/cpn1-1* mutant was outcrossed to Col-0 wild type (*CPN1/CPN1*). The *CPN1/cpn1-1* F1 plants were allowed to self-pollinate, and the segregation of the *cpn1-1* phenotype was monitored in the F2 generation. The *cpn1-1* phenotype segregated in a mendelian fashion as a single, recessive locus, as indicated in Table 1. The *cpn1-1* mutant phenotype was assessed in the F2 generation based on plant morphology, lesion development, and resistance to virulent *P.s.t*. The mutant phenotypes associated with *cpn1-1* were observed to cosegregate in all 103 F2 *cpn1-1/cpn1-1* individuals examined. This result suggested that the *cpn1-1* mutant phenotype was most likely to be caused by a mutation in one gene or possibly by mutations in two or more very tightly linked genes.

**Identification of the *CPN1* Gene**

Genetic analysis indicated that the *cpn1-1* mutation likely was T-DNA tagged. The T-DNA contained the *bar* gene conferring resistance to the herbicide ammonium glufosinate as a dominant trait (D’Halluin et al., 1992; Weigel et al., 2000). Glufosinate resistance in the F2 generation of the *CPN1/CPN1 × cpn1-1/cpn1-1* cross-segregated in a 3:1 ratio of herbicide-resistant to herbicide-sensitive plants in a group of more than 500 F2 individuals (data not shown). This finding indicated the presence of a single T-DNA integration locus.
Table 1. Genetic Segregation Analysis of the cpn1-1 Mutation

<table>
<thead>
<tr>
<th>Female × Male</th>
<th>Generation</th>
<th>No. of Plants</th>
<th>Strong Mutant Phenotype</th>
<th>None</th>
<th>χ² (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col-0 × cpn1-1/cpn1-1</td>
<td>F1</td>
<td>4</td>
<td></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Col-0 × cpn1-1/cpn1-1</td>
<td>F2</td>
<td>394</td>
<td>103&lt;sup&gt;a&lt;/sup&gt;</td>
<td>291</td>
<td>0.27 (0.7)</td>
</tr>
</tbody>
</table>

<sup>a</sup>The lesion mimic, disease resistance, and leaf curling phenotypes segregated together as a recessive trait.

The complete cDNA sequence of the CPN1 gene was determined by 5' and 3' rapid amplification of cDNA ends (RACE) PCR. The 5' and 3' RACE PCR amplified single bands corresponding to the CPN1 gene transcript (Figure 8B). This indicated that the CPN1 gene produced a single transcript species. The 5' and 3' RACE PCR products were cloned and sequenced to obtain the full-length CPN1 cDNA sequence (Figure 8C). The splicing of the CPN1 cDNA was exactly as predicted by the annotation of the Arabidopsis genome database. The transcription start site was 82 bp upstream of the ATG, and the cDNA had a 192-bp 3' untranslated region after the TAG stop codon. The CPN1 gene was expressed in leaf tissue of both LH- and HH-grown Col-0 wild-type plants (Figure 8D). As expected, no complete CPN1 transcript was detected in the cpn1-1 mutant leaves (Figure 8D).

The predicted protein product of the CPN1 gene is composed of 578 amino acids, has a molecular mass of 63.1 kD, and has homology with copine proteins (Creutz et al., 1998). The putative CPN1 protein, like other copines, has two C2 domains in the N-terminal portion of the protein and an A domain in the C-terminal region of the protein. In CPN1, the two C2 domains are composed of amino acids 55 to 188 and 199 to 283; the A domain is composed of amino acids 341 to 560 (Figure 8C). The CPN1 protein has a high degree of homology with other copines in Arabidopsis and other species, as indicated in Table 2.

To prove that the disruption of the CPN1 gene was responsible for the mutant phenotype in cpn1-1 mutant plants, the cpn1-1 mutant was complemented with a wild-type copy of the CPN1 gene. An 8-kb genomic DNA fragment containing the CPN1 gene coding regions plus 3.4 kb of upstream and 1.3 kb of downstream DNA was introduced into the genome of cpn1-1/cpn1-1 mutant plants by Agrobacterium-mediated dip transformation (Clough and Bent, 1998). The 8-kb CPN1 genomic fragment extended partly into the neighboring open reading frames on either side of CPN1. cpn1-1/cpn1-1 plants transformed with the 8-kb CPN1 genomic fragment had a wild-type phenotype, indicating that the T-DNA insertion into the CPN1 gene is responsible for the mutant phenotype of cpn1-1 (data not shown).

**DISCUSSION**

The biological functions of genes defined by lesion mimic mutations are difficult to determine with certainty. It is likely that at least some lesion mimic mutants represent defects in genes that directly regulate the HR defense response. However, it is also likely that many lesion mimic mutants represent perturbations of plant cell physiology unrelated to disease defense responses. For example, physiological alterations caused by overexpression of genes such as pyruvate decarboxylase in potato and a bacterial proton pump in tobacco can trigger lesion formation and increased disease resistance, although the overexpressed genes do not appear to have any direct role in disease resistance responses (Mittler et al., 1995; Tadege et al., 1998). In addition, salicylic acid appears to potentiate cell death triggered by free radicals, implying that plants with increased salicylic acid might be more sensitive to the generation of free radicals by stresses (Mazel and Levine, 2001). The complexity of cell death signaling in plants makes it difficult to determine the specific function of the CPN1 protein. Nevertheless, speculation on the function of CPN1 can be made based on the results presented here and biochemical analyses of related copines, assuming that CPN1 has similar biochemical
characteristics. One possibility is that CPN1 plays a direct role in defense cell death signaling and has indirect effects on LH adaptation. Alternatively, CPN1 may have a direct function in LH adaptation and may have indirect effects on plant defense signaling.

**CPN1 as a Regulator of Defense and Cell Death**

The improved disease resistance and accelerated HR phenotypes of *cpn1-1* imply that CPN1 may play a direct role in regulating defense responses, including the HR. Because...
the cpn1-1 mutation is recessive and probably eliminates CPN1 gene function, CPN1 could function as a repressor of cell death. An interesting possibility is that a pathogen-triggered Ca\(^{2+}\) flux may activate the repressive function of CPN1, possibly by triggering the localization of CPN1 to a membrane where it could exert a repressive influence on cell death signal transduction. This might seem counterintuitive at first, but genetic evidence suggests that the HR is under negative control as well as positive control to prevent uncontrolled cell death and lesion expansion after HR initiation (Dietrich et al., 1994). The accelerated HR in cpn1-1 plants supports a role for CPN1 as a negative regulator of the avirulent pathogen-induced HR defense response.

However, our data indicate that CPN1 also is required for the repression of cell death and PR gene expression in the absence of a pathogen infection. In particular, CPN1 is essential for the repression of cell death in LH growth conditions. It is possible that some aspect of LH signaling or LH adaptation, such as a Ca\(^{2+}\) flux, has the potential to trigger the HR. CPN1 may have a genetically nonredundant role in preventing LH-potentiated cell death from occurring. In plants, increases in intracellular calcium have been observed in response to a wide range of biotic and abiotic stimuli, including osmotic stress, cold, touch, and drought (for reviews, see Knight, 2000; Reddy, 2001). Although the calcium signatures differ for each stimulus, the calcium molecule appears to be a key shared component of signal transduction pathways controlling responses to various environmental stimuli (for reviews, see McAinsh and Hetherington, 1998; Bowler and Fluhr, 2000). CPN1 may play a role in defining Ca\(^{2+}\) signaling specificity by preventing cell death and other defense responses from being triggered by Ca\(^{2+}\) fluxes that are not induced by pathogens. According to this model, cell death occurs in the cpn1-1 mutant in LH because there is no CPN1 protein present to prevent LH signaling events from triggering cell death.

CPN1 also may play a direct role in suppressing PR gene expression in the absence of pathogen attack. Low levels of PR gene expression were observed in HH-grown cpn1-1, indicating a partial derepression of activation of PR gene expression in cpn1-1 mutants even in the absence of cell death and SAR. Thus, PR gene expression in cpn1-1 may occur by different mechanisms in LH and HH: in LH-grown cpn1-1, cell death occurs, triggering SAR and high-level PR gene expression; in HH-grown cpn1-1, low-level PR gene expression occurs because the repressive influence of CPN1 on PR gene expression is absent.

### Table 2. Homology of CPN1 with Other Copines

<table>
<thead>
<tr>
<th>Source</th>
<th>Protein Accession Number</th>
<th>Amino Acid Identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabidopsis</td>
<td>CAB87919</td>
<td>80.7</td>
</tr>
<tr>
<td></td>
<td>AAB70417</td>
<td>53.2</td>
</tr>
<tr>
<td></td>
<td>CAB87781</td>
<td>34.7</td>
</tr>
<tr>
<td>Human</td>
<td>AAC46074</td>
<td>41.4</td>
</tr>
<tr>
<td></td>
<td>AAC15920</td>
<td>40.4</td>
</tr>
<tr>
<td>Mouse</td>
<td>NP.034077</td>
<td>38.1</td>
</tr>
<tr>
<td>C. elegans</td>
<td>AAC71103</td>
<td>29.8</td>
</tr>
<tr>
<td>Paramecium</td>
<td>AAC15919</td>
<td>31.4</td>
</tr>
</tbody>
</table>

### CPN1 as a Regulator of LH Adaptation

A low air moisture level coupled with adequate soil water potential results in high rates of water uptake in the roots and transpiration in the leaves (Mohr and Schopfer, 1995). Under these conditions, the plant must regulate cellular ion balance by reducing transpiration, controlling ion uptake, extruding ions, and sequestering ions in subcellular compartments such as the vacuole. In cpn1-1, the humidity-sensitive phenotype is unlikely to be attributable simply to increased or uncontrolled water loss, because stomatal conductance in cpn1-1 was normal compared with that in Col-0 wild-type plants. Instead, CPN1 may be a key player in LH adaptation.

It is possible that cpn1-1 is defective in controlling ion balance in LH or is defective in responding to altered ion balance in LH. As a Ca\(^{2+}\)-dependent phospholipid binding protein, CPN1 could regulate ion transport at a membrane in response to Ca\(^{2+}\) fluxes, for instance. Defective ion homeostasis in cpn1-1 could trigger cell death by a number of mechanisms in LH, including hyperaccumulation of essential metals or oxidative stress (Wang et al., 1996; Briat and Lebrun, 1999; Hagemeyer, 1999). For example, manganese toxicity causes leaf crinkle and lesion formation in cotton, possibly as a result of increased production of reactive oxygen species (Foy et al., 1978). Ionic imbalances in LH-grown cpn1-1 could trigger cell death and lesion formation, which in turn could trigger SAR. Thus, the increased disease resistance phenotype of cpn1-1 might be an indirect effect of the mutation. It is interesting, however, that HH-grown cpn1-1 plants also exhibited an accelerated HR phenotype and very low-level expression of PR genes. This suggests that CPN1 might be necessary to control cellular homeostasis even in HH conditions, although the defect of the cpn1-1 mutant is much more pronounced in LH.

### Conclusion

Plants must integrate signals from a wide range of biotic and abiotic sources to optimize their developmental pattern and physiology for their environment. The growing interconnectedness of plant signaling pathways reflects the integrated nature of plant responses to the environment. The presence of common regulatory components during signal transduction responses to abiotic and biotic stresses suggests that cellular signaling is a web, with proteins receiving...
inputs from multiple partners and pathways (Bent, 2001). Slight perturbations in a particular signaling pathway could easily deregulate other signaling events in the plant as a result of interconnections between them. The phenomenon of cross-tolerance may reflect this kind of signaling interconnection (for review, see Bowler and Fluhr, 2000). Our results suggest that the CPN1 gene may represent an important component of the plant’s ability to monitor and respond to its environment. The CPN1 protein may act as a Ca2+-sensitive modulator of plant responses to environmental signals, including humidity and pathogens.

METHODS

Plant Growth Conditions and Mutant Screening

Arabidopsis thaliana plants were grown in soil (Scotts Redi-Earth Plug and Seedling Mix [E.C. Geiger, Inc., Harleysville, PA]) and watered with distilled water. For low-humidity (LH) growth, plants were maintained at 24°C under either short-day (SD) or long-day (LD) conditions with 75 to 100 µmol·m−2·sec−1 light intensity and 35 to 45% relative humidity. For high-humidity (HH) growth, plants were maintained at 24°C under either SD or LD conditions with 55 to 65 µmol·m−2·sec−1 light intensity and 75 to 85% RH.

Pools of activation-tagged Arabidopsis seed (Columbia [Col-0] ecotype) (Weigel et al., 2000) were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). We screened this population for mutants with improved resistance to the virulent Pseudomonas syringae pv tomato (P. s. t.) strain DC3000 (Cuppels, 1986). The cpn1-1 mutant was identified from among ~10,000 independent T-DNA lines screened for mutants with increased resistance to P. s. t. For mutant screening, 4-week-old plants grown under LH, SD conditions were screened for increased resistance to P. s. t. For mutant screening, 4-week-old plants grown under LH, SD conditions were screened for increased resistance to P. s. t. by dipping the plants into a bacterial suspension of 5 × 106 colony-forming units [cfu/mL] in 10 mM MgCl2 with 0.00025% (v/v) Silwet L-77 surfactant (Lehle Seeds, Round Rock, TX). After inoculation, plants were maintained at 24°C under SD conditions with 55 to 65 µmol·m−2·sec−1 light intensity and 75 to 95% RH for the development of bacterial speck disease symptoms. Bacterial speck disease symptoms were assessed 4 days after inoculation.

Bacterial Strains and Growth Conditions

For mutant screening, virulent P. s. t. strain DC3000 bacteria were grown on Pseudomonas Agar F (PA) (Sigma) supplemented with 100 µg/mL rifampicin (Sigma) at 28°C. For growth curves, P. s. t. strain DC3000 bacteria were used carrying either the plasmid pVSP61 (empty vector) or pV288 (bearing avrRpt2) and were grown on PA supplemented with 100 µg/mL rifampicin and 25 µg/mL kanamycin (Sigma) (Whalen et al., 1991). For hypersensitive response (HR) assays, P. syringae pv maculicola (P. m.) strain 4326) bacteria were used bearing either plasmid pVPS61 or pV288 and were grown on PA supplemented with 100 µg/mL rifampicin and 25 µg/mL kanamycin. The P. m. (4326) strain was used for the HR assays because it is less virulent on Arabidopsis than P. s. t. and allows for a clearer distinction between the HR and disease symptoms.

Bacterial Growth Curves and Peronospora parasitica Inoculations

In planta bacterial growth assays were performed by inoculating 5-week-old plants by either infiltration or dipping. For infiltration inoculations, leaves were inoculated with a suspension of 1010 cfu/mL bacteria in 10 mM MgSO4 for dip inoculations, plants were inoculated with a suspension of 2 × 109 cfu/mL bacteria in 10 mM MgCl2 plus 0.00025% (v/v) Silwet L-77. Plants were maintained in SD, 55 to 65 µmol·m−2·sec−1 light intensity, 75 to 90% RH conditions for the duration of the experiment after inoculation. Bacterial populations were assessed at 0, 1, 2, and 4 days after inoculation. Three samples were taken for each data point. For each sample, three whole leaves were harvested, weighed, and ground in 1 mL of 10 mM MgCl2. Bacteria per unit tissue weight was determined by serial dilution plating essentially as described by Whalen et al. (1991), except that bacteria were plated on PA medium supplemented with 100 µg/mL rifampicin and 25 µg/mL kanamycin. Each growth curve experiment was replicated three times. Statistical analyses were performed using Student’s t test of the differences between means of log-transformed data.

Resistance to P. parasitica (Ahco 2) was tested in 18-day-old plants. The infected plants were scored after 7 days. More than 100 leaves from 20 plants were chosen at random, and the number of conidiophores per leaf was counted. Statistical significance was measured by Student’s t test.

Histochemistry and Microscopy

Leaf samples were taken from 3-week-old plants and stained for dead cells using trypan blue (125 µg/mL) (Vogel and Somerville, 2000). Samples for autofluorescence and callose examination were prepared as described by Dietrich et al. (1994). For scanning electron microscopy, leaves were fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, rinsed in 0.1 M cacodylate buffer, pH 7.4, and then postfixed with OsO4. Samples were dehydrated by a series of increasing concentrations of ethanol, and critical point drying was performed using CO2. Samples were then sputter coated with a gold-palladium mixture and viewed in a scanning electron microscope at a 15° angle.

HR Tests

Plants grown under LH and HH conditions were inoculated with the P. s. m. strains described above at a concentration of 104 cfu/mL in 10 mM MgCl2. Fifty to 100 leaves were infiltrated for each plant-bacterial combination per experiment. Infiltrated leaves were scored for HR development as 0 for no HR, 1 for partial HR, and 2 for complete HR at 4, 8, 12, 16, 20, 24, and 30 hr after inoculation. Statistical analysis was performed using Student’s t test. This experiment was repeated three times with similar results.

Electrolyte Leakage Assay

Electrolyte leakage was measured essentially as described by Goodman (1968). LH- and HH-grown cpn1-1 and Col-0 wild-type plants were inoculated with P. s. m. strains as described for the HR tests, and control plants of both genotypes were inoculated with 10 mM MgCl2. Measurements were performed at 0, 4, 8, 12, 16, 20, 24, and 30 hr after inoculation. For each sample at each time point, five 0.07-cm2 leaf
discs were excised from infiltrated leaves using a number 1 cork borer and immersed in 3 ml of distilled water and agitated gently in a shaker for 8 min at 28°C. The conductivity of the solution was measured using a Traceable Conductivity Resistivity TDS Salinity Concentration meter (Control Company, Friendswood, TX). Three independent samples were taken at each time point for each plant–inoculum combination.

**DNA Gel Blotting and Plasmid Rescue**

Genomic DNA was isolated using a modification of the method of Shure et al. (1983) as described previously (McNellis et al., 1998). DNA gel blot analysis was performed as described previously (McNellis et al., 1998). DNA gel blot analyses of genomic DNA of the cpn1-1 mutant were performed using enzymes that cut the pSKI015 T-DNA once, including BamHI, Spel, EcoRI, and HindIII (New England Biolabs, Beverly, MA). The pBluescript KS+ (Stratagene) plasmid was used as a probe for the pSKI015 T-DNA. Probe labeling and detection were performed using the Phoito-Stop-Star Chemiluminescent Detection Kit (New England Biolabs) according to the manufacturer’s instructions. DNA gel blotting of cpn1-1 DNA cut with BamHI revealed 8- and 10-kb fragments hybridizing to pBluescript. Plasmid rescue was done by digestion of 5 ug of cpn1-1 genomic DNA with 40 units of BamHI, self-ligation using T4 DNA polymerase (New England Biolabs), and transformation into E. coli XL-2 Blue. Escherichia coli cells (Stratagene) according to the manufacturer’s instructions, except that Luria-Bertani medium supplemented with 100 µg/ml ampicillin (Sigma) was used to select transformants. Both the 8- and 10-kb fragments were rescued as plasmids. A partial sequence of the plant DNA flanking the T-DNA left borders in the rescued clones was obtained by cycle sequencing at the Nucleic Acid Facility at Pennsylvania State University using the T7 primer. The DNA sequence flanked by the T-DNA insert was identified by BLAST search. The exact location of the T-DNA insert was determined by sequencing of the rescued plasmids using the primers 5′-GGA-ACCTCAATTCCCGTGC3′ (forward) and 5′-ACCTAAAATGAC-CATAAATGG3′ (reverse).

**Reverse Transcriptase–Mediated PCR**

First-strand cDNA synthesis was performed using 5 µg of total RNA and the RETROscript kit (Ambion). As a positive control, PCR was performed to amplify the cDNA of a constitutively active actin gene using primers 5′-GTTGGGATGACAGAGAGGA3′ (forward) and 5′-GACCCACCCGATCCAGACT-3′ (reverse) (annealing at 58°C). cDNA synthesized from the total RNA of cpn1 and Col-0 wild-type plants was tested for copine expression by PCR using the primers 5′-TCTAGATCCGGAAGGATCTTGGCAT-3′ (forward) and 5′-ATCATGACT-TCTACACCGTGC3′ (reverse) (annealing at 58°C), which produce a 467-bp product.

**Genetic Complementation of cpn1-1**

An 8-kb BamHI fragment from Transformation-competent artificial chromosome (TAC) clone K22G18 (Liu et al., 1999) consisting of the copine gene coding regions plus upstream and downstream sequences was cloned into the BamHI site of the pCLD0451 binary vector to create pCLD0451-CPN1 (Bent et al., 1994). pCLD0451-CPN1 was introduced into Agrobacterium tumefaciens strain GV3101 by heat shock transformation, and transformants were selected on Luria-Bertani medium supplemented with 50 µg/ml gentamycin, 3 µg/ml tetracycline, and 25 µg/ml kanamycin (Sigma). cpn1-1 mutant plants were transformed with pCLD0451-CPN1 using the floral dip method (Clough and Bent, 1998). Transformants were selected on GM medium (1 × Murashige and Skoog [1962] salts, pH 5.7 [Gibco BRL], 1 × B5 vitamins, solidified with 0.8% phytagar [Gibco BRL]) supplemented with 50 µg/ml kanamycin. Transformants were
grown in LH, SD conditions and observed to have a wild-type phenotype.

CPN1 Sequence Analysis

The complete sequence of the CPN1 cDNA, including the 5’ and 3’ untranslated regions, was determined by performing 5’ and 3’ rapid amplification of cDNA ends (RACE) reactions using the SMART RACE cDNA kit (Clontech, Palo Alto, CA) and cloning the resulting PCR products using the Original TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The 5’ RACE gene-specific primer was 5’-GGGATTCGGAGCTCGTCC-3’. The 3’ RACE gene-specific primer was 5’-TCCTG-GAGCTCGTCC-3’. The domains of the CPN1 protein were identified using Pfam and Prosite Profile Scan databases (http://hits.isb-sib.ch/cgi-bin/hits_motifscan). Pairwise alignment of copine sequences was performed using the SIM Alignment Tool (www.expasy.ch/tools/sim-prot.html).

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REFERENCES


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Chapter 4

The Arabidopsis COPINE 1 Gene is Pathogen Regulated

Niranjani Jambunathan and Timothy W. McNellis
Department of Plant Pathology, 212 Buckhout Laboratory, Pennsylvania State University, University Park, PA 16802, and Intercollege Graduate Program in Plant Physiology, Pennsylvania State University, University Park, PA 16802.
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1To whom correspondence should be addressed. E-mail mcnellis@psu.edu; fax 814-863-7217.
Abstract
The copines are a widely distributed class of calcium-dependent, phospholipid binding proteins of undetermined biological function. Mutation of the Arabidopsis COPINE 1 gene (CPN1) causes a humidity-sensitive lesion mimic phenotype with increased resistance to a bacterial and an oomyceteous pathogen, constitutive pathogenesis-related (PR) gene expression, and an accelerated hypersensitive cell death defense response (HR). Here, we show that the disease resistance phenotype of the cpn1-1 mutant was also temperature sensitive; demonstrate increased CPN1 gene transcript accumulation in wild-type plants under low humidity conditions; and present a detailed analysis of CPN1 gene transcript accumulation in response to bacterial pathogens. In wild-type plants, CPN1 transcript accumulation was rapidly, locally, and transiently induced by both avirulent and virulent strains of Pseudomonas syringae pv. tomato (P.s.t.) bacteria. However, the induction of CPN1 transcript accumulation by avirulent bacteria was much faster and stronger than that induced by virulent bacteria. Bacterial induction of CPN1 transcript accumulation was dependent on a functional type III bacterial protein secretion system. In planta expression of the avrRpt2 avirulence gene was sufficient to trigger rapid CPN1 transcript accumulation. CPN1 transcript accumulation was induced by salicylic acid (SA) treatment but was not observed during lesion formation in the lesion mimic mutants lsd1 and lsd5. These results provide clear evidence for the involvement of CPN1 in plant disease resistance responses and indicate that CPN1 may represent a link between plant disease resistance and plant adaptation to low humidity and low temperature conditions.
4.1. Introduction

The copines are a class of highly conserved proteins present in organisms ranging from protozoans to complex forms such as mouse, human and higher plants (Creutz et al., 1998). These proteins are named copine (the French feminine noun meaning “friend”) due to their tight association with lipid membranes (Creutz et al., 1998). The identifying feature of copine proteins is the unique combination of two protein kinase C conserved 2 (C2) domains in the N-terminal region and a von Willebrand A (VWA) domain in the C-terminal region. The C2 domain is a widely distributed protein motif that often has Ca$^{2+}$-dependent membrane lipid binding activity (Xu et al., 1997). C2 domain-containing proteins include protein kinase C (Azzi et al., 1992), phospholipase C (Essen et al., 1996), synaptotagmin (Brose et al., 1995), and rabphilin (Wang et al., 2000). The VWA domain is another widely distributed protein motif that is involved in mediating protein-protein interactions in a range of extracellular and intracellular proteins (Whittaker and Hynes, 2002).

Although several biochemical studies of copines have revealed that they have a calcium-dependent phospholipid binding activity (Creutz et al., 1998; Tomsig and Creutz, 2000), no specific biological functions for any copines have yet been defined.

Previously, we conducted a genetic screen to identify Arabidopsis mutants with increased resistance to virulent P.s.t bacteria (Jambunathan et al., 2001). One of the mutants identified from our screen was the cpn1-l mutant, a recessive, T-DNA insertion mutant of the CPN1 gene, which encodes a copine-like protein (Genbank Accession numbers: AY045764 AND AY045765). The cpn1-l mutant exhibits a
strict humidity-dependent lesion mimic phenotype: *cpn1-1* plants grown under low humidity conditions (LH; 35-45% RH) are small in size and have curled leaves, minute lesions at the leaf margins, dramatically increased resistance to virulent *P.s.t* and *Peronospora parasitica* strains, and display constitutive PR gene expression. In contrast, *cpn1-1* plants grown under high humidity (HH; 75-85% RH) conditions are morphologically indistinguishable from wild-type plants and no longer exhibit increased resistance to virulent *P.s.t.* bacteria. However, both LH- and HH-grown *cpn1-1* mutant plants have an accelerated HR compared to wild-type plants following avirulent bacterial inoculation. We hypothesized that the *CPN1* gene product could act as a mediator of plant adaptation to low humidity or alternatively as a suppressor of defense-related cell death and defense responses (Jambunathan et al., 2001). Hua et al. (2001) isolated mutants with T-DNA insertions at the same locus, characterized the mutants as temperature-sensitive dwarf plants, and named the corresponding gene *BONZAI 1 (BON1).* The *bon1* mutants exhibited a dwarf phenotype at low temperature conditions (22°C) and were restored a wild-type phenotype at 28°C. Hua et al. speculated that the *BON1* gene product could be a regulator of growth homeostasis under low temperature conditions.

Environmental conditions such as light intensity, day length, relative humidity and temperature play key roles in the growth and development of most plants. The ability of the plant to adapt to environmental stress conditions is essential for normal plant development. For example, loss of plant adaptation to low temperature in mutants such as *asculis1, asculis3* and *asculis4* in Arabidopsis leads to a defect in leaf expansion and stem elongation (Akamatsu et al., 1999; Tsukaya et al., 1993. One of
the major ways that plants respond to the environment is at the level of gene transcription. In many instances, genes involved in controlling the plant response to a particular stress are induced at the transcriptional level in response to that particular stress stimulus. Members of the cold responsive transcription factor family CBF/DREB1 are induced within 15 min of cold treatment (Gilmour et al., 1998; Liu et al., 1998), and transgenic plants over-expressing CBF/DREB1 transcription factors have accumulation of solutes with cryoprotective activities and increased freezing tolerance (Gilmour et al., 2000). Several genes known to be key regulators of plant defense responses are transcriptionally regulated by pathogen infection. Important plant defense signaling genes such as NON-RACE-SPECIFIC DEFENSE RESPONSE 1 (NDR1) (Century et al., 1997), ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) (Falk et al., 1999), EDS5 (Nawrath et al., 2002), and PHYTOALEXIN DEFICIENT 4 (PAD4) (Jirage et al., 1999) are rapidly induced by pathogen inoculation. NDR1 and EDS5 are induced within two hours after inoculation, with peak transcript levels observed at eight hours after infection.

These genes are part of a complex signaling network that allows the plant to recognize and defend itself against pathogens and deal with other environmental stresses. Plant interactions with phytopathogens may lead to the development of either disease or resistance in the plant. In the case of resistance, the plant is able to recognize quickly the presence of the pathogen and mount appropriate defense responses. In contrast, during disease development, pathogen recognition by the plant is delayed or non-existent, and the defense responses are slower, less pronounced, and much less effective. Some of the early events following pathogen recognition by the
plant include an inward flux of Ca\textsuperscript{2+} and H\textsuperscript{+} and an outward flux of K\textsuperscript{+} and Cl\textsuperscript{-} ions (Hahlbrock et al., 1996; Levine et al., 1996; Schaller and Oecking, 1999; Yang et al., 1997); the activation of a plasma membrane-associated NADPH-oxidase complex leading to production of reactive oxygen intermediates (ROI) (Bolwell et al., 1999); and the initiation of the HR (Klement et al., 1993). These early events in turn lead to a state of increased disease resistance in the whole plant known as systemic acquired resistance (SAR), which is marked by high levels of PR gene expression and elevated levels of salicylic acid (SA) (Malamy et al., 1990; Uknes et al., 1992).

Mutational analysis has lead to the identification of a number of genes that participate in plant defense signaling. A number of Arabidopsis mutants have been identified which lack the ability to express effective defense responses, including ndr1, eds5 and npr1. NDR1, a small membrane associated protein, is involved in coiled coil-nucleotide-leucine rich repeat (CC- NB- LRR) class R protein signal transduction (Aarts et al., 1998; Century et al., 1995; Century et al., 1997). EDS5, a membrane protein with homology to multidrug and toxin extrusion (MATE) transporters, is important for the accumulation of salicylic acid (SA) during defense (Nawrath et al., 2002). Downstream of SA signaling, NPR1 is a novel protein with ankyrin repeats that is important in the induction of pathogenesis-related genes such as PRI, PR2 and PR5 (Cao et al., 1994, 1997).

At the other extreme are the lesion mimic mutants, which display spontaneous cell death and often develop SAR. Lesion mimic mutants in Arabidopsis include lsd1- lsd7, acd2, acd6, cpr6, and cpr22 (Dietrich et al., 1994, 1997; Greenberg and Ausubel, 1993; Greenberg et al., 1994; Rate et al., 1999; Weymann et al., 1995;
Lesion formation in many lesion mimic mutants is dependent on environmental factors, such as light, daylength, and relative humidity (Chamnongpol et al., 1996; Dietrich et al., 1994, Jambunathan et al., 2001, Yoshioka et al., 2001). At least some of these lesion mimic mutants may represent suppressors of plant defenses, including the HR. However, it is possible that many lesion mimic mutants may represent gene that are not necessarily directly involved in plant defense, because perturbations of cellular physiology apparently unrelated to disease resistance can result in cell death and SAR (Mock et al., 1999; Molina et al., 1999). In addition, number of mutants that exhibit constitutive SAR in the absence of spontaneous cell death have also been identified, such as *cpr1*, *edr1*, and *mpk4* (Bowling et al., 1994, 1997; Clarke et al., 1998; Frye et al., 2001; Petersen et al., 2000).

In this report, we build on our previous results by performing further characterization of the *cpn1-1* mutant phenotype and analyzing the expression pattern of the *CPN1* gene in wild-type plants in response to temperature, humidity, and pathogen stimuli. Our results indicate that the *cpn1-1* lesion-mimic phenotype is dependent on both temperature and humidity and that the expression of the *CPN1* gene is induced by low humidity, low temperature, and pathogen stimulus. Because pathogen-derived signals appeared to be the most effective inducers of *CPN1* gene expression, we performed a comprehensive analysis of bacterial pathogen-induced *CPN1* gene expression patterns. These results strongly support the involvement of *CPN1* in plant disease resistance responses and are consistent with the potential role of CPN1 as a suppressor of plant defense responses.
4.2 Methods

All experiments described were replicated independently at least 2-4 times with similar results.

4.2.1. Plant Growth Conditions

All plants were grown in a soil-less potting mix (Scotts Redi-earth Plug and Seedling Mix (E.C. Geiger, Inc. Harleysville, PA) and irrigated with distilled water. Plants grown for *cpn1-1* mutant phenotypic analysis and for analysis of humidity and temperature dependence of *CPN1* transcript accumulation were grown under SD conditions with a light intensity of 75-100 \( \mu \text{mol m}^{-2} \text{sec}^{-1} \), while temperature and humidity parameters were varied as described in the Results. Plants used for bacterial induction of *CPN1* gene expression analysis, including WT, *ndr1-1*, *eds5-1*, *npr1-2*, and DEX::*avrRpt2* plants, were grown under LT, LH, SD conditions, 35-40% RH, 21-22°C and 50-65 \( \mu \text{mol m}^{-2} \text{sec}^{-1} \) light intensity. *lsl1* plants were grown under LT, LH, SD conditions and 50-65 \( \mu \text{mol m}^{-2} \text{sec}^{-1} \) light intensity for 4 weeks and then moved to LT, LH, LD conditions and 75-100 \( \mu \text{mol m}^{-2} \text{sec}^{-1} \) light intensity. *lsl5* mutant plants were grown under LT, LH, LD and 75-100 \( \mu \text{mol m}^{-2} \text{sec}^{-1} \) light intensity conditions for 3 weeks and then moved to LT, LH, SD conditions and 50-65 \( \mu \text{mol m}^{-2} \text{sec}^{-1} \) light intensity.

4.2.2. In Planta Bacterial Growth Analyses

These assays were performed by infiltration inoculation as described previously, except that bacterial populations were assayed at 0 and 3 days after
inoculation. The bacterial strains used were the same as described previously (Jambunathan et al., 2001).

4.2.3. Bacterial Induction of CPNI Expression

The *P.s.t.* DC3000 and *P.s.t.* DC3000 (*avrRpt2*) bacterial strains were the same as used previously. *P.s.t.* DC3000 (*avrRpm1*) bacteria carried the plasmid pVARM (Kunkel et al., 1993). The *hrcU* mutant carries a Tn3gus transposon insertion into the *hrcU* gene (Mudgett and Staskawicz et al., 1999). All the strains were grown at 28°C on Pseudomonas Agar F (PA, Sigma, St. Louis, MO) media supplemented with 100 µg/ml rifampicin and 25 µg/ml kanamycin (Sigma). The *Pseudomonas fluorescens* strain was grown at 28°C on PA supplemented with 20µg/ml Nalidixic acid (Sigma). Leaves of five-week-old plants were syringe-inoculated with bacteria suspended in 10mM MgCl₂ at a concentration of 1x 10⁶ cfu/ml. Following inoculation, the plants were maintained under SD, LH, LT conditions and 65 µmol m⁻² sec⁻¹ light intensity. DEX treatments were performed by syringe infiltration of dexamethasone (Sigma) as described previously (McNellis et al., 1998).

4.2.4. SA Treatments

Five-week-old plants were sprayed to the point of runoff with 1mM SA (Sigma) in water with 0.00025% Silwet77 surfactant (Lehle Seeds, Round Rock, TX). Control plants were sprayed with water containing 0.00025% Silwet77. The plants
were left covered with a dome for 4 h to maintain high humidity, after which the
dome was removed. Tissues were harvested 24h after treatment.

**4.2.5. RNA isolation and RNA gel blot analyses**

Leaf tissue was collected from treated plants and flash frozen in liquid
nitrogen. The permissive condition RNA sample for the *lsd1* mutant was obtained
from leaf tissue collected from four-week-old *lsd1* mutant plants grown under
continuous SD conditions. The non-permissive condition RNA sample for the *lsd1*
mutant was obtained from *lsd1* mutant plants grown under SD conditions four weeks
and then moved to LD conditions for 48 hours, at which time spreading lesions were
observed. The permissive condition RNA samples for the *lsd5* mutant were obtained
from leaf tissues collected from four-week-old *lsd5* mutant plants grown under
continuous LD conditions. The non-permissive condition RNA samples for the *lsd5*
mutant were obtained from *lsd5* mutant plants grown under LD conditions for four
weeks and then moved to SD conditions for 48 hours, at which time lesion formation
was evident. RNA extractions and RNA gel blotting was performed as described
previously, except that 15 micrograms of total RNA were loaded in each gel lane in
all gel blot experiments (Jambunathan et al., 2001). The *PR1, PR2,* and *PR5* gene
probes were the same as described previously (Jambunathan et al., 2001). The *CPN1*
gene-specific probe consisted of a 738 bp fragment comprising corresponding to
amino acids 332-578 of the predicted CPN1 protein. The probe was generated by
PCR amplification using a *CPN1* cDNA clone plasmid as the template and the
following primers: forward primer, 5’-TCTAGAGTACTTGGCATCTGGA-3’; reverse primer, 5’-GAATTCTCATGGAGGAATCGGTTTCAT-3’; annealing at 55°C. The primers contain engineered XbaI and EcoRI restriction sites, highlighted in italics; *CPN1*-homologous sequences are in Roman. The PCR amplified product was cloned into the pCR®-Blunt vector using the Zero Blunt® PCR cloning kit according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). The cloned product was released using XbaI and EcoRI restriction enzymes (New England Biolabs, Beverly, MA). The released product was run on agarose gel, extracted and purified using Gel extraction kit (Qiagen, Valencia, CA). About 25-50 ng of the cleaned product was used for probe labeling with dCTP 32P using the Redi-prime labeling kit (Amersham-Biotech, Piscataway, NJ) according to the manufacturer’s instructions. The labeled probe was cleaned using Performa™ spin columns (Edge Biosystems, Gaithersburg, MD) according to the manufacturer’s instructions. Northern hybridization was performed at 42°C using Ultrahyb hybridization buffer as recommended by the manufacturers (Ambion, Austin, TX). Blot washing and exposure was performed as described previously (McNellis et al., 1998).

### 4.3. Results

#### 4.3.1. The *cpn1-1* Lesion Mimic, Increased Disease Resistance, and *PR* Gene Expression Phenotypes are Temperature Sensitive

We initially identified the *cpn1-1* mutant as a humidity-sensitive lesion mimic mutant (Jambunathan et al., 2001). However, mutations in the *BON1* gene, which corresponds to *CPN1*, were identified as temperature-dependent dwarf mutants that
had a mutant phenotype when grown at 22°C or lower (Hua et al., 2001). We therefore decided to determine whether the *cpn1-1* lesion mimic phenotype was temperature-sensitive as well as humidity-sensitive. *cpn1-1* plants grown at low temperature (LT; 21+/-0.5°C) under HH, short-day conditions (SD, 8 hours light/16 hours darkness) developed minute lesions at the leaf margins after 2-3 weeks of growth as shown in Figure 1B. Columbia-0 (Col-0) ecotype wild-type (WT) plants grown under same conditions did not display any lesions (Figure 4-1A). For the first 1-2 weeks of growth under LT, HH conditions, the *cpn1-1* plants were indistinguishable from the WT plants. With the onset of lesion development after the second week, *cpn1-1* plants appeared stunted compared to WT plants. The lesions appeared consistently in all *cpn1-1* plants in the absence of any pathogen inoculation. However, the lesion- mimic phenotype of *cpn1-1* under LT, HH conditions did not appear to be as strong as the phenotype of *cpn1-1* grown in LH conditions. The leaves of LT, HH -grown *cpn1-1* plants did not display epinastic curling as severe as that observed for the leaves of *cpn1-1* grown under LH, high temperature (HT; 24.5+/-0.5°C) conditions (Jambunathan et al., 2001, and data not shown). *cpn1-1* plants grown under HH, HT conditions were morphologically indistinguishable from the WT plants, with normal leaves and no lesions or dwarfing evident (Jambunathan et al., 2001, and data not shown).

Since *cpn1-1* mutant plants grown under LH conditions had increased resistance to *P.s.t.* (Jambunathan et al., 2001), we reasoned that LT-grown *cpn1-1* might also have increased resistance to *P.s.t.* bacteria. Growth of virulent *P.s.t.* strain DC3000 (*P.s.t.* DC3000) bacteria was monitored in leaves of LT-grown *cpn1-1* and
WT plants on day 0 and day 3 after infiltration with the bacteria. The growth of virulent bacteria was reduced by more than 10-fold in LT-grown *cpn1-1* mutants when compared to LT-grown WT plants (Figure 4-1C). In addition, bacterial speck disease symptoms were very weak in LT-grown *cpn1-1* plants compared to WT plants (data not shown). The restriction of virulent bacterial growth in LT-grown *cpn1-1* was similar to the restriction of the growth of avirulent *P.s.t.* DC3000 bacteria carrying the *avrRpt2* avirulence gene (*P.s.t.* DC3000 (*avrRpt2*) in WT plants (Figure 1C). This indicates that the level of resistance to virulent *P.s.t.* bacteria observed in LT-grown *cpn1-1* mutant plants was as strong as gene-for-gene resistance in WT plants to avirulent *P.s.t.* DC3000 (*avrRpt2*) bacteria mediated by the *RPS2* disease resistance gene (Bent et al., 1994; Mindrinos et al., 1994; Whalen et al., 1991). The growth of avirulent *P.s.t.* DC3000 (*avrRpt2*) bacteria was even more strongly restricted in LT-grown *cpn1-1* plants compared to the WT (Figure 4-1C). Since the *cpn1-1* mutant has a functional *RPS2* disease resistance gene (Jambunathan et al., 2001), this indicates that the *cpn1-1* mutation has a partially additive effect with the function of *RPS2* in mediating resistance to *P.s.t.* DC3000 (*avrRpt2*) bacteria.

In previous work (Jambunathan et al., 2001), we observed that LH-grown *cpn1-1* plants accumulate high levels of *PR* gene transcripts. Since LT conditions also triggered the lesion-mimic and increased disease resistance phenotypes of *cpn1-1*, we also examined *PR* gene transcript accumulation in LT-grown *cpn1-1* plants. In the absence of any pathogen inoculation, *PR1*, *PR2* and *PR5* gene transcripts accumulated to high levels in LT-grown *cpn1-1* plants (Figure 4-1D). LT- and LH-grown *cpn1-1* plants showed similar patterns of *PR1*, *PR2*, and *PR5* transcript
accumulation (Figure 1D). Very low levels of PR1, PR2 and PR5 gene transcript accumulation were seen in HH, HT-grown cpn1-1 plants (Figure 4-1D). Taken together, these results indicate that the lesion mimic, increased disease resistance, and PR gene expression phenotypes of cpn1-1 are both humidity and temperature sensitive.

4.3.2. Humidity and Temperature-Dependent CPNI Transcript Accumulation

Hua et al, (2001) observed that the BON1 (CPNI) transcript level in WT plants is regulated by temperature conditions. Higher levels of BON1 expression were observed when plants were moved from higher temperature to lower temperature. Since both LH and LT conditions triggered a lesion-mimic phenotype in cpn1-1, we tested whether both these conditions influenced the accumulation of CPNI transcript in WT plants. WT plants grown under LH, HT or HH, LT conditions showed increased accumulation of CPNI transcript when compared to WT plants grown under HH, HT conditions, as shown in Figure 4-2. In cpn1-1 plants, there was no detectable CPNI transcript under any of the conditions tested (Figure 4-2). Overall, the CPNI gene appeared to be expressed at low levels, and the CPNI transcript was difficult to detect.

4.3.3. The CPNI Transcript Accumulates in Response to both Virulent and Avirulent Bacterial Inoculation
The lesion mimic and increased disease resistance phenotypes of the *cpn1-1* mutant suggest that the *CPN1* gene may play a role in plant pathogen defense signal transduction, possibly as a repressor of cell death and other defense functions (Jambunathan et al., 2001). Therefore, we speculated that *CPN1* gene transcript accumulation might be pathogen-regulated. *CPN1* transcript accumulation was monitored in LT, LH-grown WT plants inoculated with virulent and avirulent strains of *P.s.t.* *CPN1* transcript accumulation was monitored both in the inoculated leaves and in distal, uninoculated leaves in order to test for systemic induction of *CPN1* transcript accumulation. In the inoculated leaves, *CPN1* transcript accumulation was strongly induced at 24 hours after infiltration with virulent *P.s.t.* DC3000 bacteria and avirulent strains of *P.s.t.* DC3000 carrying either the *avrRpt2* or the *avrRpm1* avirulence gene, as shown in Figure 4-3. In the distal, uninoculated tissues, no induction of *CPN1* was detected at 24 hours after inoculation with virulent *P.s.t.* DC3000 bacteria and avirulent *P.s.t.* DC3000 (*avrRpt2*) bacteria (Figure 4-3). However, a very slight induction of *CPN1* transcript accumulation in distal tissues was detected 24 hours after inoculation with avirulent *P.s.t.* DC3000 (*avrRpm1*). Mock inoculation did not induce *CPN1* transcript accumulation, indicating that bacteria were required for the induction to occur. It should be noted that the induction of *CPN1* transcript accumulation by *P.s.t.* DC3000 bacteria was dramatically higher than that caused by LT and LH, since the plants used in these experiments were maintained in LH, LT conditions.

In order to determine the kinetics of *CPN1* transcript accumulation in response to bacterial inoculation, a time course experiment was performed. The level
of *CPN1* transcript accumulation in inoculated leaves of WT plants at various times after infiltration with avirulent *P.s.t. DC3000 (avrRpt2)* bacteria was assayed, as shown in Figure 4A. Avirulent *P.s.t DC3000 (avrRpt2)* bacteria triggered increased *CPN1* transcript accumulation as early as four hours after inoculation and reached a peak at six hours after inoculation. The levels of *CPN1* transcript then slowly decreased until 36 hours after inoculation. By 48 hours after inoculation, *CPN1* transcript levels dropped to the basal level. The plants used for this experiment were grown and maintained in LT, LH conditions, so the induction of *CPN1* transcript accumulation by *P.s.t. DC3000 (avrRpt2)* bacterial inoculation was substantially higher than that induced by LT or LH. Prolonged autoradiographic exposure of the RNA gel blot in this experiment allowed detection of *CPN1* transcript in all lanes, but resulted in the overexposure of the induced time points. In order to gauge the rapidity of *CPN1* transcript accumulation relative to a known pathogen-inducible gene, we compared the induction kinetics of *CPN1* transcript accumulation to that of *PR1*. The onset of *CPN1* transcript accumulation occurred five hours earlier than the onset of *PR1* expression after inoculation with *P.s.t. DC3000 (avrRpt2)* (Figure 4-4A). *PR1* transcript accumulation reached a peak at 36 hours after inoculation and then started to decrease, but remained elevated until the end of the time course at 72 hours after inoculation. These results indicated that *CPN1* transcript accumulated rapidly and transiently following inoculation with avirulent *P.s.t. DC3000 (avrRpt2)* bacteria.

The kinetics of *CPN1* transcript accumulation in response to inoculation with virulent *P.s.t. DC3000* bacteria was also determined (Figure 4-4B). The kinetics of *CPN1* transcript accumulation in leaves inoculated with virulent *P.s.t. DC3000* was different
and slower than that observed following inoculation with avirulent *P.s.t.* DC3000 (*avrRpt2*). For these experiments, plants were grown and maintained in LH, LT conditions. Induction of *CPN1* transcript accumulation was observed at 24 hours after inoculation, with peak levels occurring at 36 hours post inoculation. By 48 hours after inoculation, *CPN1* transcript accumulation decreased nearly to the basal level. Severe bacterial speck disease symptoms developed by 72 - 96 hours after inoculation. By way of comparison, the kinetics of accumulation of the *PR1* gene transcript was monitored in leaves inoculated with virulent *P.s.t.* DC3000. The *PR1* gene transcript became detectable at 24 hours post inoculation and reached a peak at 36 hours after inoculation. The kinetics of *CPN1* gene transcript accumulation following virulent *P.s.t.* DC3000 inoculation was similar to that of *PR1*, except that the *PR1* transcript remained at elevated levels until the end of the time course at 72 hours post inoculation, whereas *CPN1* transcript returned to basal levels at 48 hours post inoculation.

4.3.4. A Functional Bacterial Type III Protein Secretion System is Required for Bacterial Induction of *CPN1* Transcript Accumulation

Many plant and animal pathogenic bacteria, including *P.s.t.*, use the Type III protein secretion pathway to deliver some of their proteins into the host cell during pathogenesis (Reviewed by Hueck, 1998). In the case of *P. syringae*, the Type III protein secretion system is required both for virulence on compatible host plants and for the elicitation of the HR on incompatible host plants (Alfano and Collmer, 1997; He et al., 1993). The bacterial genes required for pathogenicity in susceptible plants
and HR in resistant plants have been defined as *hrp* (for hypersensitive response and pathogenicity) genes (Bonas et al., 1991). *Hrp* genes that are highly structurally conserved across bacterial species are called *hrc* (for hypersensitive response and conserved) (Bogdanove et al., 1996). A mutation in any of the *hrp* genes disables the type III protein secretion system of the bacteria and renders them unable to cause disease or elicit an HR. To test whether induction of *CPN1* transcript accumulation by inoculation with *P.s.t* strains depended on the Type III protein secretion system, we monitored *CPN1* transcript accumulation in WT plants inoculated with bacterial strains that are non-pathogenic and/or are defective in Type III secretion, as shown in Figure 4-5. No induction of *CPN1* transcript accumulation above basal levels was observed in leaves of WT plants 24 hours after infiltration with a *hrcU*-mutant strain of *P.s.t* DC3000 (*P.s.t. DC3000 (hrcU)*), which is defective in Type III protein secretion and is also unable to elicit defense responses, including the HR, in incompatible host plants (Mudgett and Staskawicz, 1999). Also, no induction of *CPN1* transcript accumulation above basal levels was observed in leaves of WT plants 24 hours after infiltration with non-pathogenic *Pseudomonas fluorescens* (*P. fluorescens*) bacteria. As expected, virulent *P.s.t* DC3000 and avirulent *P.s.t* DC3000 (*avrRpt2*) bacteria, which have an intact Type III protein secretion system, were able to induce *CPN1* transcript accumulation in inoculated leaves at 24 hours after infiltration (Figure 4-5). For comparison, we also examined the induction of *PRI1* gene transcript accumulation under the same inoculation conditions. *PRI1* gene transcript accumulated to high levels in leaves inoculated with avirulent *P.s.t* DC3000 (*avrRpt2*) bacteria and to low levels in leaves inoculated with virulent *P.s.t.*
DC3000 bacteria at 24 hours after inoculation. Taken together, these data indicate that \textit{CPN1} transcript accumulation was induced specifically by pathogenic bacteria and that \textit{CPN1} induction by bacteria requires a functional bacterial Type III protein secretion system.

4.3.5. \textit{In Planta} Expression of an Avirulence Gene is Sufficient to Induce \textit{CPN1} Transcript Accumulation

Although \textit{CPN1} transcript accumulation occurred in response to both virulent and avirulent bacterial inoculation, the induction was fastest and strongest following avirulent \textit{P.s.t.} DC3000 (\textit{avrRpt2}) bacterial inoculation. This suggests that the stronger and more rapid induction of \textit{CPN1} transcript accumulation by \textit{P.s.t.} DC3000 (\textit{avrRpt2}) was due to gene-for-gene recognition of the \textit{avrRpt2} determinant by the corresponding \textit{RPS2} \textit{R} gene product in the host (Leister et al., 1996). To determine whether \textit{RPS2}-mediated recognition of AvrRpt2 was sufficient for induction of \textit{CPN1} transcript accumulation, we tested whether glucocorticoid-inducible expression of \textit{avrRpt2} in transgenic plants was sufficient to induce \textit{CPN1} transcript accumulation. For this experiment, we used stable transgenic Arabidopsis lines bearing a glucocorticoid-inducible \textit{avrRpt2} gene in either the Col-0 WT genetic background having a functional \textit{RPS2} gene or in the \textit{rps2-101C} mutant genetic background, which does not have a functional \textit{RPS2} gene. Infiltration of leaves of these transgenic plants with dexamethasone (DEX), a strong, synthetic glucocorticoid, induces the expression of the \textit{avrRpt2} transgene. In the \textit{RPS2} (WT) genetic background, glucocorticoid-induced \textit{avrRpt2} expression triggers hypersensitive cell death within 12 - 24 hours due to \textit{RPS2}-mediated gene-for-gene recognition of the
avrRpt2 avirulence determinant. In the rps2-101C mutant, no hypersensitive cell death occurs because gene-for-gene recognition of the avrRpt2 avirulence determinant does not take place.

Transgenic plants with the glucocorticoid-inducible avrRpt2 gene in the RPS2 genetic background exhibited strong induction of CPN1 transcript accumulation as early as three hours after DEX infiltration, as shown in Figure 4-6. The levels of CPN1 transcript decreased slowly after a peak at three hours post infiltration until 12 hours post infiltration, at which point the leaves showed near complete collapse due to hypersensitive cell death. Transgenic plants with the glucocorticoid-inducible avrRpt2 transgene in the rps2-101C mutant genetic background did not exhibit induction of CPN1 transcript accumulation following DEX infiltration during the time frame tested (Figure 4-6). These results show that in planta expression of the avrRpt2 avirulence gene was sufficient to stimulate CPN1 transcript accumulation, and that this effect depended on the presence of a functional RPS2 gene. This result indicates that RPS2-mediated gene-for-gene recognition of the avrRpt2-derived avirulence determinant is sufficient to trigger CPN1 transcript accumulation.

4.3.6. Salicylic Acid Stimulates CPN1 Transcript Accumulation

SA is a key chemical inducer of plant defense responses and is required for the development of SAR (Gaffney et al., 1993). Biochemical and genetic data suggests that SA can potentiate defense responses by promoting cell death (Weymann et al., 1995). SA treatment of plants can also induce SAR and the expression of PR genes (Ward et al., 1991). To test whether SA treatment can induce the accumulation
of the *CPN1* gene transcript, WT Arabidopsis plants were sprayed with 1mM SA and the accumulation of *CPN1* transcript was monitored 24 hours later. As shown in Figure 4-7, SA treatment induced dramatic accumulation of *CPN1* transcript relative to the water-treated control plants at 24 hours after treatment. High level accumulation of *PRI* transcript was also observed in SA-treated plants but not in the water-treated controls, as expected. These results indicate that accumulation of *CPN1* gene transcript can be induced by SA.

### 4.3.7. *CPN1* Transcript Accumulation in Other Lesion Mimic Mutants

Conditional lesion mimic mutants such as *lsd1* and *lsd5* are sensitive to day-length conditions. *lsd1* maintains a non-lesion phenotype under permissive, SD conditions. Spreading cell death in the *lsd1* mutant can be initiated by shifting the plants from SD to long day (16 hours light / 8 hours dark, LD) conditions (Jabs et al., 1996). *lsd5*, another conditional mutant, initiates spreading cell death under SD conditions, but can maintain a non-lesion mimic phenotype under permissive, LD conditions (Morel and Dangl, 1999). We speculated that the day length-induced cell death in *lsd1* and *lsd5* might trigger *CPN1* transcript accumulation. However, as shown in Figure 4-8, no induction of *CPN1* transcript accumulation was observed in *lsd1* or *lsd5* mutant plants grown under permissive light conditions or after the mutants were shifted to non-permissive (lesion-inducing) conditions. In contrast, *PRI* transcript accumulation was strongly induced in both mutants when grown under conditions that favored lesion formation (Figure 4-8). The *lsd1* mutant plants showed substantial *PRI* transcript accumulation even under permissive, SD conditions.
4.4. Discussion

The results presented here are significant in that they provide clear evidence that a copine gene has a biological function in plant defense: the rapid, specific, and tightly regulated accumulation of \textit{CPN1} gene transcript in response to pathogen signals implies a direct role for \textit{CPN1} in plant defense. These observations also make it unlikely that the effects of the \textit{cpn1-1} mutation on plant defense responses are simply due to perturbations of plant cell homeostasis unrelated to plant defense signaling.

Particularly strong evidence for a role for \textit{CPN1} in plant defense comes from the observation that \textit{CPN1} gene transcript accumulation was responsive to gene-for-gene mediated defense signaling. The relatively rapid and high level of \textit{CPN1} transcript accumulation following inoculation with avirulent \textit{P.s.t. (avrRpt2)} bacteria as compared to that observed with virulent \textit{P.s.t.} bacteria (Figure 4-4) indicated that \textit{RPS2}-mediated recognition of the \textit{avrRpt2} signal triggered the rapid accumulation of \textit{CPN1} transcript. It is not unusual for pathogen-induced genes to be induced by both virulent and avirulent pathogens, although induction by avirulent pathogens is generally much stronger and more rapid than that by virulent pathogens, as observed with both \textit{CPN1} and \textit{PRI} (Figure 4-4). Additional evidence for the induction of \textit{CPN1} transcript accumulation via gene-for-gene mediated pathogen recognition came from the induction of \textit{CPN1} transcript accumulation by glucocorticoid-inducible
expression of *avrRpt2* in transgenic plants having a functional *RPS2* gene (Figure 4-6). The fact that *avrRpt2* expression *in planta* can specifically trigger *CPN1* transcript accumulation, in the absence of any pathogen inoculation, and that this induction requires the presence of a functional *RPS2* disease resistance gene, indicates that *CPN1* transcript accumulation is responsive to gene-for-gene mediated signaling.

The dependence of bacterial induction of *CPN1* transcript accumulation on a functional Type III protein secretion system also supports a specific role for the *CPN1* gene product in plant responses to pathogens (Figure 4-5). The lack of *CPN1* transcript accumulation following inoculation with *P. fluorescens*, a non-pathogenic strain related to *P. syringae*, implies that *CPN1* transcript accumulation is specifically triggered by pathogenic bacteria. The lack of *CPN1* transcript accumulation following inoculation with the *P.s.t. DC3000* (*hrcU*) mutant, which is defective in Type III protein secretion, indicated that a functional Type III protein secretion system is specifically required for induction of *CPN1* transcript accumulation by *P.s.t. DC3000* bacteria (Mudgett and Staskawicz, 1999). This also supports the conclusion that pathogenic bacteria specifically stimulate *CPN1* transcript accumulation, since the *P.s.t. DC3000* (*hrcU*) mutant is non-pathogenic.

Our results also suggest that *CPN1* may encode an early component of plant defense signaling: induction of *CPN1* transcript accumulation was observed very early following avirulent pathogen inoculation, within four hours after inoculation, which was substantially faster than *PR1* gene transcript accumulation (Figure 4-4). The transient nature of *CPN1* transcript accumulation implies that the role of the
CPN1 gene product in defense may be restricted to early steps. The local rather than systemic induction of CPN1 transcript accumulation is also consistent with a role for CPN1 as a suppressor of hypersensitive cell death, since in that case CPN1 activity would presumably be needed at the site of infection rather than systemically.

It is interesting to note that although SA treatment could induce CPN1 transcript accumulation in WT plants, the induction of cell death in the lesion mimic mutants lsd1 and lsd5 did not stimulate CPN1 expression, even though, in lsd1 at least, runaway cell death requires SA accumulation (Aviv et al., 2002). This could indicate that CPN1 is not involved in the cell death signaling pathways defined by lsd1 and lsd5. Or perhaps induction of CPN1 expression in the mutants occurred in a transient manner that was missed at the time point tested. Alternatively, if CPN1 is a necessary repressor of the HR, then the lack of expression of CPN1 in the lsd1 and lsd5 mutants may represent part of the defect of these mutants, and the lack of CPN1 transcript accumulation might actually contribute to the lesion mimic phenotype of these mutants.

Taken together, the phenotypic analyses of the cpn1-1 mutant and the gene expression patterns of the CPN1 gene suggest that the CPN1 gene product may function as a negative regulator of plant defense responses, including the HR. Indeed, the strong, rapid, and specific activation of CPN1 gene transcript accumulation in response to pathogen inoculation implies that plant defense functions may represent the primary role of CPN1. However, the temperature and humidity related aspects of the cpn1-1 mutant phenotype and the activation of CPN1 transcript accumulation by these same environmental parameters adds another level of complexity to the
biological role of CPN1. Apparently, CPN1 plays a non-redundant role as a suppressor of potentially cell death-inducing effects of LT and LH environmental conditions.

Humidity and temperature play important roles in plant disease development (Agrios, 1997), and overlaps between environmental and pathogen signaling are not unusual in plants. In the Arabidopsis lesion mimic mutants *lsd6* and *cpr22*, high humidity has been found to suppress the SA-dependent spontaneous lesion phenotype (Weymann et al., 1995; Yoshioka et al., 2001). Plants treated with avirulent pathogen and grown under high humidity conditions have been found to have a delayed HR with reduced SA levels (Hammond-Kosack and Jones, 1996). Although the mode of action of high humidity in modifying plant defense responses is not clear, these observations suggest that high humidity has the potential to suppress the HR and SA-dependent defenses in plants. Similarly, a range of factors including low temperature, low humidity, hyper-osmolarity, wounding, and harpin elicitors have been found to activate rapidly ATMPK4 and ATMPK6 in *Arabidopsis* (Desikan et al., 2001; Ichimura et al., 2000). In addition, phenotypic analysis of the *mpk4* mutant has revealed that ATMPK4 may serve as a negative regulator of SAR (Petersen et al., 2000). Also, the *EDS5* gene is activated by UV light and pathogens (Nawrath et al., 2002). These findings implicate a connection between abiotic and biotic stress signaling and may provide a molecular basis for the phenomenon of cross-tolerance in plants, in which a plant subjected to one stress, such as UV light or ozone, for example, can become more resistant to pathogens (Bowler and Fluhr, 2000; Sharma et al., 1996; Yalpani et al., 1994).
But what could account for the apparent involvement of CPN1 in plant responses to both biotic and abiotic stimuli? It is possible that the answer could be related to Ca$^{2+}$. Ca$^{2+}$ is a ubiquitous second messenger that is involved in plant responses to diverse stimuli, such as drought, touch, cold, heat, and oxidative stress (Knight, 2000; Reddy, 2001). Ca$^{2+}$ fluxes are involved in defense signaling in plants (Grant et al., 2000; Zimmermann et al., 1997). Since Ca$^{2+}$ is such a non-specific signaling molecule that is involved in many different types of signaling pathways, the specificity of Ca$^{2+}$ signaling must be accomplished by the timing, duration, and location of Ca$^{2+}$ fluxes (Bowler and Fluhr, 2000; McAinsh and Hetherington, 1998). It is possible that CPN1, as a Ca$^{2+}$-dependent membrane-associated protein, is involved in determining the specificity of Ca$^{2+}$ signaling and preventing inappropriate defense responses to LT and LH conditions. The mechanism of action of the CPN1 protein is unknown, but it has been hypothesized that copines may function by recruiting proteins with which they interact via their VWA domain to a membrane location (Tomsig et al., 2003).
A B

Day0 (vir) Day0 (avr) Day3 (vir) Day3 (avr)
Days after bacteria inoculation

C

Log CFU/g of tissue

Day0 (vir) Day0 (avr) Day3 (vir) Day3 (avr)
Days after bacteria inoculation

Col-O cpn1-1

D

LH, HT HH, HT HH, LT
M W M W M W

PR1 PR2 PR5 rRNA
Figure 4-1. Temperature-Dependent Lesion Mimic and Increased Disease Resistance Phenotypes of *cpn1-1*.

(A) Five-week-old WT plant grown under LT, HH, SD conditions. 
(B) Five-week-old *cpn1-1* mutant plant grown under LT, HH, SD conditions. Arrows indicate lesions at the leaf margins. 
(C) Growth of virulent *P.s.t. DC3000* bacteria and avirulent *P.s.t DC3000 (avrRpt2)* bacteria in WT and *cpn1-1* plants grown under LT, HH, SD conditions. Plant leaves were infiltrated with a bacterial suspension at a concentration of $1 \times 10^5$ cfu/mL. Bacterial populations were monitored on day 0 and day 3 post inoculation. Bars represent standard deviation. 
(D) *PR* gene transcript accumulation in SD-grown WT (W) and *cpn1-1* (M) plants grown under three different humidity and temperature conditions. RNA gel blot analyses of *PR1*, *PR2* and *PR5* transcript levels in *cpn1-1* and WT plants grown under three different environmental conditions: LH, HT; HH, HT; and HH, LT. rRNA, 28S rRNA stained with methylene blue to show relative amount of RNA in each lane.
Figure 4-2. Effects of Temperature and Humidity on CPN1 Transcript Accumulation.

RNA gel blot analysis of CPN1 transcript accumulation in WT (Col-0) and cpn1-1 mutant plants grown under three different environmental conditions: LH, HT; HH, HT, and HH, LT. Five-week-old, SD-grown plants were used. rRNA, 28S rRNA stained with methylene blue to show the relative amount of RNA in each lane. For details, see the Methods.
Figure 4-3. \textit{CPNI} Transcript Accumulation Following Bacterial Inoculation.

RNA gel blot analysis of \textit{CPNI} transcript accumulation in WT plants following bacterial inoculation. Five-week-old WT plants were either mock inoculated with 10mM MgCl\textsubscript{2} or inoculated with 1 x 10\textsuperscript{5} cfu/mL of one of the following bacterial strains by syringe infiltration of four fully-expanded leaves: \textit{P.s.t.} DC3000; \textit{P.s.t.} DC3000 (\textit{avrRpt2}); or \textit{P.s.t.} DC3000 (\textit{avrRpm1}). \textit{CPNI} transcript accumulation was monitored at 0 and 24 hours post inoculation in the inoculated leaves as well as at 24 hours post inoculation in uninoculated, distal leaves. 24D, RNA sample from uninoculated, distal leaves 24 hours post inoculation. rRNA, 28S rRNA stained with methylene blue to show relative amount of RNA in each lane.
Figure 4-4. Kinetics of CPN1 Transcript Accumulation Following Bacterial Inoculation.

(A) RNA gel blot analysis of the kinetics of CPN1 transcript accumulation following inoculation with avirulent *P.s.t* DC3000 (*avrRpt2*) bacteria. Leaves of five-week-old WT plants were syringe-inoculated with 1 \times 10^5 cfu/mL of *P.s.t*. DC3000 (*avrRpt2*) bacteria and RNA samples were extracted from inoculated leaves at 0, 2, 4, 6, 9, 12, 24, 36, 48, 72 hours post inoculation. *PR1*, the same blot re-probed with a *PR1* gene-specific probe. Mock, RNA samples from leaves inoculated with 10mM MgCl₂ at 0 and 24 hours post inoculation. rRNA, 28S rRNA stained with methylene blue to show relative amounts of RNA in each lane.

(B) RNA gel blot analysis of the kinetics of CPN1 transcript accumulation following inoculation with virulent *P.s.t*. DC3000 bacteria. This experiment was performed in a manner identical to that described for panel (A).
Figure 4-5. Specific Induction of \textit{CPN1} Transcript Accumulation by Pathogenic Bacteria.

RNA gel blot analysis of \textit{CPN1} transcript accumulation following inoculation with pathogenic and non-pathogenic bacterial strains. Leaves of five-week-old LH-, LT-grown WT plants were either mock inoculated with 10mM MgCl$_2$ or inoculated with 1 $\times$ 10$^5$ cfu/mL of one of the following bacterial strains by syringe infiltration: virulent \textit{P.s.t.} DC3000; avirulent \textit{P.s.t.} DC3000 (\textit{avrRpt2}); non-pathogenic \textit{P. fluorescens}; or non-pathogenic mutant \textit{P.s.t.} DC3000 (\textit{hrcU}). RNA samples were extracted from inoculated leaves at 0 and 24 hours post inoculation. \textit{PR1}, the same blot re-probed with a \textit{PR1} gene-specific probe. rRNA, 28S rRNA visualized with methylene blue to show relative amounts of RNA in each lane.
Figure 4-6. *CPN1* Transcript Accumulation in Response to *In Planta* Expression of *avrRpt2*.

RNA gel blot analysis of *CPN1* transcript accumulation in transgenic plants bearing a glucocorticoid-inducible *avrRpt2* avirulence gene over time following glucocorticoid treatment. DEX::*avrRpt2* (Col-0), the transgenic line bearing the glucocorticoid-inducible *avrRpt2* gene in the Col-0 WT genetic background; DEX::*avrRpt2* (rps2), the glucocorticoid-inducible *avrRpt2* gene in the *rps2-101C* mutant genetic background. Leaves of both plant lines were infiltrated with either 30 µM dexamethasone in 0.1% ethanol (DEX) or 0.1% ethanol (EtOH) as a control. RNA samples were extracted from DEX-infiltrated leaves at 0, 3, 6, and 12 hours post infiltration, and from EtOH-infiltrated leaves at 0 and 12 hours post inoculation. rRNA, 28S rRNA visualized by methylene blue staining to show relative amounts of RNA in each lane.
Figure 4-7. Salicylic Acid Induction of CPN1 Transcript Accumulation.

RNA gel blot analysis of CPN1 gene transcript accumulation in five-week-old WT plants 24 hours after treatment with either 1 mM salicylic acid or water. PRI, the same blot re-probed with a PRI gene-specific probe. rRNA, 28S rRNA visualized with methylene blue to show relative amounts of RNA in each lane.
Figure 4-8. *CPN1* Transcript Accumulation in Other Lesion Mimic Mutants.

RNA gel blot analysis of *CPN1* transcript accumulation in *lsd1* and *lsd5* mutant plants. *CPN1* transcript accumulation was monitored in four-week-old *lsd1* plants grown continuously in permissive short SD conditions and 48 hours after transfer from SD conditions to non-permissive, LD conditions, at which point spreading lesions were observed. Similarly, *CPN1* transcript accumulation was monitored in three-week-old *lsd5* mutant plants grown continuously under permissive LD conditions and 48h after transfer to non-permissive, SD conditions that triggered cell death and visible lesion formation. RNA samples from WT plants 0 and 12 hours after inoculation with 1 x 10^5 cfu/mL of *P.s.t. (avrRpt2)* served as a positive control. *PR1*, the same blot re-probed with a *PR1* gene-specific probe. rRNA, 28S rRNA visualized with methylene blue to show relative amounts of RNA in each lane.
Chapter 5

Structure-Function Analysis of the Arabidopsis COPINE I (CPN1) Protein in Plant Defense Responses

5.1 Introduction

Copines (CPNs) are ubiquitous proteins present in many organisms from Paramecium to complex forms such as mouse, plants and humans. These proteins exhibit a Ca$^{2+}$ dependent phospholipid binding property. These proteins were first identified in Paramecium tetraurelia by their binding to phospholipid membranes in presence of Ca$^{2+}$ (Creutz et al., 1998). Copine proteins have a unique combination of two C2 domains at the N terminus region and a von Willebrand A (VWA) domain in the C terminus end. Arabidopsis Copine I (CPN1) is composed of two C2 domains of amino acids 55 to 188 and 199 to 283, and the VWA domain is composed of amino acids 341-560 (Jambunathan et al., 2001). The C2 domain exhibits Ca$^{2+}$-dependent binding to phospholipids and is present in proteins such as protein kinase C, phospholipase C, synaptotagmin, rabphilin and Munc13. The VWA domain is a well-studied domain observed in many extracellular proteins and some intracellular proteins. They are found to be involved in cell adhesion, integrin receptors and in
extracellular matrix domains (Whittaker and Hynes, 2002). The VWA domain is similar to protein-binding A domain of integrin proteins and are believed to be involved in protein-protein interactions. The VWA domain of copines as like the integrins has a divalent cation-dependent ligand-binding site and can bind to Mn\(^{2+}\) and can be displaced by Mg\(^{2+}\) or Ca\(^{2+}\) (Tomsig and Creutz, 2000).

Biochemical studies of mammalian and plant copine have revealed that copines retain their Ca\(^{2+}\) dependent phospholipid binding property after purification (Tomsig and Creutz, 2000; Hua et al., 2001). Mammalian CPNs bind to phosphatidylserine and not to phosphatidylcholine vesicles and they show specificity mainly to negatively charged phospholipids. Studies on aggregation of purified copine from bovine spleen by mass spectrometry/liquid chromatography show that copine I exists as monomer in absence of Ca\(^{2+}\) and addition of millimolar Ca\(^{2+}\) forms multimolecular aggregates (Tomsig and Creutz, 2000). Studies with mammalian Copine III have shown that the protein has kinase activity. Kinase assays have shown that in addition to phosphorylation of myelin basic protein, copines can also undergo autophosphorylation (Caudell et al., 2000).

Studies with Arabidopsis copine by our group and by Hua et al. (2001) have been the first to define the potential biological functions of copines. Based on our studies, we have speculated that Arabidopsis CPN1 could be a repressor of cell death and defense responses and also play a role in acclimation of plants to low humidity or low temperature conditions (Jambunathan et al., 2001; Jambunathan and McNellis, 2003). An Arabidopsis Copine I mutant (termed Bonzai I) was identified from another screen for altered growth under low temperature conditions by Hua et al.
Based on their studies they speculated that BON1 could be acting as a catalyst of membrane fusion or as a preserver of membrane function at low temperature conditions.

Our initial studies with the Arabidopsis *cpn1-1* mutant and *CPN1* gene expression studies have shown that CPN1 in Arabidopsis has an important role in plant defense response. The key to understanding any protein will be to elucidate the functional role of its core domains. The functions of C2 domain and VWA domain have been speculated based on function of proteins that have them. Based on the information available on the domains, possible roles of CPN1 can be hypothesized. Structure-function analyses of CPN1 would provide information on how the domains in CPN1 work to accomplish the functions implied by the mutant phenotype and the gene expression analyses.

Since our data have shown that Arabidopsis CPN1 may act as a repressor of defense in plants, we hypothesized that over-expressing CPN1 in wild-type plants will make them more susceptible to pathogens. In addition, we hypothesized that over-expressing a particular domain of copine in wild-type plants would result in a phenotype related to plant defense. If the truncated CPN1 protein is directly involved in defense then we would expect the transgenic plants to have increased susceptibility response to pathogens. Alternatively, if the truncated CPN1 protein competes with the basal CPN1, then we might expect a phenotype similar to *cpn1-1* with improved resistance. Three gain of function approaches were used by over-expressing full copine gene, over-expressing the two C2 domains and over-expressing the VWA domain in wild type plants to study effects on plant defense responses.
5.2 Materials and Methods

5.2.1 Vector construction

All the CPN1 fragments generated for vector construction were obtained from cDNA synthesized from 2 µg of total RNA from wild-type Col-0 plants as per the manufacturer’s instructions (Retroscript PCR kit, Ambion, TX). The primers and PCR conditions used for generating different fragments of CPN1 are listed below. All the primers were designed with XbaI restriction sites at their 5' end and EcoRI restriction sites at their 3' end for maintaining the orientation of the inserts in the 35S-2 cloning vector (Figure 5-1).

5.2.1.1 PCR primers and conditions for obtaining full length CPN1 product

Forward 5'- TCTAGAATTATGGGGAAATTGTGCTCGGAT-3'
Reverse 5'-GAATTCTCATGGAGGAATCGGTTTCAT-3'

1µl of the first strand cDNA was used as a template for PCR reaction with final dNTP and primer concentration of 0.25 mM and 2.5 µM. Advantage Taq polymerase (Clonetech) was used as per the manufacturers instructions. The following PCR conditions were used, 95°C for 30 seconds denaturation, 57 °C for 45 seconds annealing, and 72°C for 2 minutes extension for 35 cycles. The start codon (ATG) is marked in the forward primer. The estimated product size was 1800 bp.
5.2.1.2. PCR primers and conditions for obtaining C2 domains of CPN1 product

Forward 5'- TCTAGAATT\textit{ATG}GGGAATTGTGCTCCGAT-3'
Reverse 5'-GAATTCTCAATGAAGTTTTTCTAAGTCTGA-3'

The ingredient for PCR reaction was used as mentioned above. The start codon (ATG) is marked in the forward primer. The following PCR conditions were used: 94 °C for 30 secs, 60 °C for 45 secs and 1 min at 72 °C for 30 cycles. The primers cover 1-283 amino acids of CPN1 and result in a PCR product of 849 bp.

5.2.1.3. PCR primers and conditions for obtaining VWA domain of CPN1 product

Forward 5'- TCTAGAATT\textit{ATG}GGGAATCTAGAGTACTTGGCATCTGGA-3'
Reverse 5'-GAATTCTCATGGAGGAATCGGTTTCAT-3'

With PCR ingredients as mentioned above, the following conditions were used: 94 °C for 30 secs, 62 °C for 45 secs and 1 min at 72 °C for 30 cycles. The start codon (ATG) is marked in the forward primer. The estimated product size was 900 bp. The forward primer is designed with XbaI enzyme overhang and the first three codons of CPN1 from the start codon and sequences of ten amino acids upstream of VWA domain. The primer combination covers three codons in the N terminus and 331-578 amino acids of CPN1.

All the three amplified products were cloned in pCR blunt vector (Stratagene) and transformation of the ligated product was performed as per the manufacturer’s instructions. The cloned product was sequenced to proofread the sequence using
M13 forward and M13 reverse primers for products of C2 domains and VWA domain (Nucleic Acid facility, Penn State University). For full length CPN1 product, along with the M13 forward and M13 reverse primers, two internal primers of CPN1, COP8 and COP3, were used.

- **M13 forward primer**
  
  5'- GTTTTCCCAGTCACGAC-3'

- **M13 reverse primer**
  
  5'-CAGGAAACAGCTATGAC-3'

- **COP8 primer**
  
  5' GCGTTGATCACTGGACCCCAAGGG-3'

- **COP3 primer**
  
  5'-GGAACTCCAAATTCCGTGTC-3'

After verification of the sequence integrity, the full-length CPN1 and its domain products were excised by digesting the plasmid with XbaI and EcoRI enzyme (New England Biolabs) and ligating into 35S-2 vector (Figure 5-1) (http://www.pgreen.ac.uk/a_hom_fr.htm) digested with the same set of enzymes. The ligated product was transformed in *E.coli* cells. Plasmid was extracted from the cloned cells and the cassette containing 35S promoter driving the insert was released using EcoRV enzyme and ligated to the binary vector 0229 (Figure 5-1) (http://www.pgreen.ac.uk/a_hom_fr.htm) cut with EcoRV enzyme (New England Biolabs). The transformation plasmids were labeled as pGreen 0229 35S CPN1FL, pGreen 0229 35S C2 and pGreen 0229 35S VWA. The cloned 35S driven fragment in binary vector 0229 (Figure 5-2) was introduced into Agrobacterium strain GV3101 containing the compatible helper plasmid pSoup (Figure 5-1) http://www.pgreen.ac.uk/a_hom_fr.htm having the replicase gene (*Rep A*) using the heat shock method. The transformed Agrobacterium was selected on LB +Gen^50^+Kan^25^+Tet^3^ and confirmed by plasmid extraction and digestion.
5.3. Plant transformation and selecting transgenics

Arabidopsis plants of the Col-0 ecotype were grown in long day conditions (16h light, 8h darkness). Initiation of bolting started when the plants were 3-week old and plants were dipped with the Agrobacterium carrying the plasmids pGreen 0229-35S-CPN1FL, pGreen 0229-35S-C2 and pGreen 0229-35S-VWA using the method described by Clough and Bent, 1998. The plants were dipped with Agrobacterium culture three times at weekly intervals and allowed to set seeds. Seeds collected from the dipped plants were grown under short day conditions and seedlings were sprayed with 0.25% (v/v) of Finale herbicide with 0.00025% (v/v) Silvet 77 for selection of transgenics (the binary vector contains the bar gene for herbicide resistance). Plants that survived after two consecutive herbicide sprays were selected and designated as the T1 transgenic generation. The T1 generation plants were allowed to self and set seeds (T2 generation). Nearly 100 plants of each T2 line were grown under SD conditions and screened again by herbicide selection. Genetic segregation was monitored to determine the possible number of loci of insertion. The T2 plants were monitored for any visible phenotype and allowed to self and set seeds (T3). The T3 generation lines were tested for homozygosity by their resistance to herbicide. The homozygous T3 lines were monitored for visible phenotype related to defense response.
Figure 5-1: Vectors used for construction of over-expressor lines of CPN1
(http://www.pgreen.ac.uk/a_hom_fr.htm)

A: The 35S-2 vector with the 35S cauliflower mosaic virus promoter. The XbaI and
EcoRI sites were used for cloning the CPN1 full-length, VWA domain, and C2
domain inserts. The 35S promoter driving the insert was released using EcoRV
enzyme.

B: The pGreen 0229 binary vector that can replicate in \textit{E.coli} and Agrobacterium.
The 35S promoter driving the insert was cloned in the EcoRV site of 0229. The
binary vector has bialaphos resistance gene as plant selection marker that confers
resistance to herbicide.

C: pSoup vector is the helper plasmid transformed into Agrobacterium along with the
binary vector. The compatible plasmid pSoup carries the Replicase gene (RepA) and
its origin of replication. The vector has a tetracycline gene as a selection marker.
A

CPN1 cDNA

0229 –35S-full length

CPN1

5033 bp

pGreen-0229 vector

Xba I

35S promoter

EcoR I

LB

EcoR V

RB

EcoR V

B

Two C2 domains of CPN1

0229 –35S-C2 domains of CPN1

5386 bp

pGreen-0229 vector

Xba I

35S promoter

EcoR I

LB

EcoR V

RB

EcoR V
**Figure 5-2: Vectors constructed to generate Arabidopsis CPN1 over-expressor transgenic lines**

**A:** pGreen 0229-35S-CPN1FL: Vector constructed for constitutive over-expression of full-length copine I gene of Arabidopsis.

**B:** pGreen 0229-35S-C2: Vector constructed to generate constitutive over-expression of two C2 domains of Arabidopsis CPN1 gene.

**C:** pGreen 0229-35S-VWA: Vector generated for over-expression of A domain of Arabidopsis CPN1 gene.

**D:** pGreen 0229-35S: Control vector constructed to generate control transgenic lines harboring the 35S promoter and the binary vector without any CPN1 insert.
5.2.3.1 Cloning of fragment into expression vector

To generate CPN1 antibodies, CPN1 protein was expressed in *E. coli*. For CPN1 protein expression in *E. coli*, full length CPN1 fragment was obtained from cDNA synthesized from 2 µg of Arabidopsis total RNA. The following primers were used with EcoRI restriction site at its forward primer and XbaI site at its reverse primer for in frame cloning into pMAL-C2X expression vector (New England Biolabs).

Forward primer: 5'-GAATTCATGGGGGAATTGTTGCTCCGAT-3'
Reverse primer: 5'-TCTAGATCATGGAGGAATCGGTTT-3'

The PCR reaction composition and conditions used are the same as described in 5.2.2.1. 0.5µg of PCR fragment was digested with EcoRI and XbaI and cloned into pMAL vector (C-2X) digested with the same set of enzymes. TB1 competent cells (New England Biolabs) were used for transformation. The transformed colonies were screened on LB +Amp\textsuperscript{100} plates. Colonies were selected and stabbed onto a master LB amp plate and another plate containing 80 µg/ml Xgal and 0.1 mM IPTG. The white colonies were confirmed for insert by performing plasmid miniprep (Qiagen) and digesting with EcoRI and XbaI enzymes.

5.2.3.1 Induction of CPN1 protein

The induction of protein was performed by growing a colony from LB amp plate in a 5 ml culture in LB amp broth to 2 X 10\textsuperscript{8} cells/ml (OD 600=0.5). 1 ml of the sample was withdrawn and was pelleted and resuspended in 50 µl of protein SDS-PAGE Sample buffer. 0.3 mM of IPTG was added to the remaining culture and
sample was incubated at 37 °C with good aeration for 2 hours. 0.5ml of sample was withdrawn and centrifuged for 2-3 minutes and supernatant was discarded. The cells were then suspended in 100 µl of SDS-PAGE buffer. This sample would provide the induced protein of size of 105.6 kDa (CPN1 of 63.1 kDa and Maltose binding protein (MBP) of 42.5kDa). After confirmation of induction of protein on a SDS-PAGE gel, the whole procedure was scaled up to 1 liter of LB broth with glucose and ampicillin. The induced cells were harvested by centrifugation at 4000g for 20 minutes and cells were suspended in column buffer and frozen overnight at –20 °C. The frozen sample was thawed in cold water and sonicated in short pulses for 15 seconds or less. The release of the protein was monitored by the change of color of Bradford’s dye (Sigma, St Louis, MO).

Composition of the buffers used:
SDS sample buffer: 5% SDS, 10% 2-mercaptoethanol, 0.02 M sodium phosphate, pH 7.0, 20% glycerol.
Column buffer: 20mM Tris-HCl, 200mM NaCl, 1mM EDTA

5.2.3.3 Affinity chromatography for purification of fusion protein

15 ml of Amylose resin (New England Biolabs) was poured in a 50 ml syringe plugged with glass wool and washed with nearly eight volumes of column buffer. The induced protein crude extract was poured at the rate of 1ml/minute. The column was washed with 12 volumes of column buffer. The fusion protein was eluted with 40 mls of column buffer +10mM maltose with fractions collected for every 3ml. The
fractions containing the protein were tested by Bradford protein assay (Sigma, St Louis, MO). The protein containing fractions were pooled and concentrated using Amicon centricron concentrator (Millipore).

5.2.3.4 Cleavage of the CPN1+MBP fusion protein

A pilot experiment was conducted by mixing 1 mg of fusion protein with 1 µl of Factor Xa protease at room temperature for 3 hours. The cleaved fragment was verified by running on a SDS-PAGE gel. The experiment was scaled up for large-scale digestion.

5.2.3.5. Sepharose™ ion exchange chromatography for separating CPN1 protein

Sepharose™ was washed in 20 ml of 10 mM Tris-HCl, 25 mM NaCl, pH 8.0 buffer and the resin was poured in 1x10 cm column. The column was washed several times with the same buffer. The cleaved fusion protein mixture was loaded onto the column. A gradient of 25 mM NaCl to 500 mM NaCl in 20mM Tris-HCl was started and 1 ml fractions were collected. The fractions obtained were tested for protein by Bradford dye.
5.2.4. Raising antibodies against CPN1 and western blot analysis

The antibody for the fusion protein was generated in Pocono Rabbit Farm and Laboratory, PA 18325. Nearly 25-50 µg of purified antigen was injected subcutaneously using Complete Freund’s Adjuvant onto two rabbits every four weeks. Bleeds from the animals were collected on day 63, day 84 and day 120. The antisera obtained from the inoculated rabbits did not have CPN1 antibodies.

5.2.5. Growth analyses of virulent and avirulent bacteria

The growth of virulent and avirulent bacteria was monitored at day 0 and day 3 in the transgenic lines over-expressing either full-length CPN1 or C2 domains of CPN1 or VWA domain of CPN1 as described in Jambunathan et al., 2001.

5.2.6: Northern blot analyses

The RNA gel blot analyses were performed with 10 µg of total RNA (Jambunathan et al., 2001). The PCR product obtained for C2 domains and VWA domain were used as probes and labeled as described in Jambunathan and McNellis, 2003.
5.2.7: Reverse-transcriptase PCR

Reverse transcriptase PCR was conducted using the primers for CPN1 and for actin as described in Jambunathan et al., 2001.

5.2 Results

5.3.1. CPN1 protein expression in E. coli and Western blot analysis

A 105.6 kDa MBP-CPN1 fusion protein was expressed in the bacterial crude extract. (Figure 5-3). The fusion protein was purified using an amylose column. The purified protein was cleaved using Factor Xa, producing a 63.1 kDa CPN1 protein and a 42.5 kDa MBP. Cleavage of the fusion protein could be detected on a SDS-PAGE gel when small amounts of protein were used. Scaling up of the cleavage of the fusion protein and then the purification process caused instability of the protein. Purification by gradient ion exchange column did not yield any protein fractions. Since the fusion protein was stable, antibodies were generated with the fusion protein. The generated antisera obtained from two different rabbits failed to cross-react with plant CPN1 protein or bacterial expressed CPN1. Different dilutions of primary and secondary antibodies were tried but no specific signal was obtained from plant protein extract.
5.3.2 Over-expression of the full length CPN1 protein in wild-type plants

Seeds generated from dipped pGreen 0229-35S-CPN1-FL were selected for transgenic-35S-full length CPN1 plants. Nearly 10,000 seedlings were screened in the T1 generation and only seven plants were resistant to herbicide selection marker. This indicated a very poor transformation efficiency as Arabidopsis (Col-0) has a transformation frequency of nearly 1%. The seven transgenic lines were allowed to self and set seed, and homozygous lines were selected in the T3 generation based on uniform resistance to herbicide. Five homozygous lines (designated F1, F8, F13, F15, F17) were compared with control vector plants (35-6) for over-expression of CPN1.
Northern analyses of these transgenic lines did not show any signal with 10 µg of total RNA (data not shown). Membranes were not allowed extended exposure for detecting any signals (Technical note: Arabidopsis CPN1 is extremely low expressed gene in WT plants; prolonged exposure of the blots are required to detect any signal in an uninoculated plant).

Reverse-transcriptase PCR, being a more sensitive method, was performed to detect the CPN1 transcript and constitutively active actin gene expression in the transgenic lines compared to vector control plants (Figure 5-4). PCR was performed with same quantity of cDNA for all the samples. PCR reactions were stopped at various cycles (15, 20 and 25 cycles) to detect the differences between the expression patterns in these lines. Actin gene expression was detected in all the lines strongly after 20 cycles of PCR amplification. CPN1 could be detected only after 25 cycles of PCR reaction in F1, F8, F13 and F15 plants. No detection of CPN1 was observed in F17 plants after 25 PCR cycles. In control plants, CPN1 could not be detected after 25 cycles since CPN1 is a very low expressed gene and required nearly 30-35 PCR cycles to be detected. These data suggested that the transgenic lines generated were weakly over-expressing CPN1. The over-expression of CPN1 protein could not be detected due to the poor cross reactivity of CPN1 antibodies.

5.3.3 Phenotypic description of 35S:CPN1 plants

The weakly over-expressing CPN1 plants were grown under SD conditions (8 hours light/16 hours darkness) along with the vector control plants and wild-type plants. The plants were monitored in the T2 and T3 generation for any visible
phenotype related to defense responses. All seven over-expressing lines looked like the control and Col-0 plants grown under same conditions (Figure 5-5). Time of flowering and seed set were also normal in these plants.

5.3.3 Growth of virulent and avirulent bacteria in 35S:CPN1 lines

Since our studies with cpn1-1 mutant and CPN1 gene expression have indicated that CPN1 could be acting as a repressor of defense in plants, we expected that CPN1 over-expressing lines might show an increased susceptibility to pathogens. We monitored the growth of both virulent (P.s.t DC3000) and avirulent bacteria (P.s.t (avrRpt2)) in these lines compared to the vector control plants and wild-type Col-0 plants (Figure 5-6). The growth of virulent bacteria in these over-expressor lines was comparable with that of the control plants. Growth of avirulent bacteria was restricted in all lines due to the gene for gene resistance in the plants. The level of restriction of avirulent bacteria was similar in control plants and the over-expressor lines.

5.3.4. Over-expression of C2 domains in wild-type plants

Nearly 2000 seeds from plants dipped with Agrobacterium carrying pGreen 0229-35S-C2 vector were germinated in short day conditions. The seedlings were sprayed with 0.25% finale with 0.00025% Silwet 77 for selecting transgenic plants that constitutively over-expressed the two C2 domains of CPN1. Nearly 25 T1 lines were selected based on screening for selection marker (resistance to herbicide). All
the lines were selfed and T2 plants were tested again for segregation for Finale resistance. Homozygous lines were selected in T3 generation. The over-expression of truncated transcript of \textit{CPN1} C2 domains was confirmed by performing a northern analysis using total RNA from these lines (Figure 5-7).

The transgenic lines generated had different levels of expression of the C2 domain fragment mRNA. Lines such as C2-13, C2-14, C2-18 and C2-24 strongly expressed the introduced C2 domain mRNA of \textit{CPN1} (Figure 5-7). Lines such as C2-1 and C2-6 weakly expressed the introduced fragment. Interestingly line C2-25 did not show any expression of the C2 domains mRNA but expressed \textit{PR1} strongly (Figure 5-7). Again, the over-expression in these lines could be confirmed only at the transcript level and not at the protein level.

5.3.5. Phenotypic description of C2 domain over-expressing lines

All the transgenic lines except (C2-25) looked like wild-type and vector control plants grown under same conditions (Figure 5-8). Flowering and seed set were also normal like the control plants. The phenotype of the C2-25 line resembled the \textit{cpn1-1} mutant phenotype. Homozygous lines of C2-25 grown under SD conditions had smaller leaves with marginal cell death like \textit{cpn1-1} plants and had stunted bolts under LD conditions. It is possible that C2-25 line had cosuppressed the \textit{CPN1} gene, resulting in a \textit{cpn1-1} mutant phenocopy.
5.3.6. Growth of virulent and avirulent bacteria in C2 domain over-expressing lines

Growth of virulent (\textit{P.s.t.} DC3000) and avirulent (\textit{P.s.t} \textit{avrRpt2}) bacteria was monitored in some of the C2 domain over-expressing lines compared to wild-type and vector control plants (Figure 5-9). Virulent bacterial growth was monitored on day 0 and day 3. Virulent bacteria could multiply and grow well in wild-type Col-0 and vector control plants. Growth of virulent bacteria in some of the C2 domain over-expressing lines (C2-6, C2-14, C2-17 and C2-24) was comparable with the control lines. These data suggested that these C2- domain over-expressing lines were neither more susceptible nor more resistant to virulent \textit{P.s.t.} DC3000 (Figure 5-9).

Avirulent bacterial growth was monitored to verify whether gene-for-gene resistance is intact in these plants. All the C2 over-expressing lines and Col-0 and vector control plants limited the avirulent bacterial growth and this indicated that gene-for-gene resistance is active in all the plants tested (Figure 5-9).

These data suggest that over-expression of C2 domain transcript in wild-type plants does not have an impact on the plant defense or disease responses.

5.3.7. Phenotypic description of VWA domain over-expressing lines

VWA domain-over-expressing lines were monitored both in T2 and T3 generation grown under SD conditions. The T2 lines looked like wild-type plants until the plants were four-week-old. After five weeks, very minute lesions started to
develop in the newly formed leaves in most of the T2 lines (Figure 5-10). Sometimes the lesions were observed in the leaf margins as in *cpnl-1*, but some lines showed scattered lesions (Figure 5-10). The lesions were very faint and they usually appeared when the plants were 5 week old. The lesion formation in these plants also was dependent on the humidity and temperature conditions. A slight increase in humidity in the growth chambers delayed the lesion formation to six-weeks (data not shown).

The T2 lines were allowed to self and homozygous lines were selected in the T3 generation. The T3 plants were monitored for emergence of lesions. The lesion formation phenotype was lost in the T3 plants. The T2 and T3 lines were grown together to monitor for lesion formation under favorable conditions. Under favorable conditions, only T2 lines exhibited lesion formation.

5.3.8. Expression of VWA domain in the over-expressing lines

Since the VWA domain over-expressing lines exhibited the lesion forming phenotype only in the T2 generation and not in the T3 generation, the over-expression of VWA domain was monitored in the T2 plants and in the homozygous T3 lines (Figure 5-11). Strong expression of the VWA domain mRNA was observed in the T2 lines of VWA domain over-expressors. No signal could be detected in the vector control and Col-0 plants as expected due to the lack of introduced VWA domain. Interestingly, most homozygous lines of T3 generation did not show any expression of VWA domain (Figure 5-11). This indicated that there was loss of expression of VWA domain in most or all of the over-expressor lines. The loss of expression in the
T3 generation correlates with the loss of lesion formation in the T3 generation. In the T2 generation, there is over-expression of VWA domain that results in lesion formation whereas in the T3 generation lines, there is loss of expression and hence loss in lesion phenotype.

5.3.9. VWA domain over expressor suppressed growth of virulent and avirulent P.s.t

VWA domain over-expressor lines exhibited enhanced resistance to virulent P.s.t DC3000 bacteria (Figure 5-12). Plants were sprayed with very high inoculum of bacterial culture of $3 \times 10^8$ bacterial cells/ml. The T2 generation plants of VWA domain overexpressor exhibited improved resistance when compared to control plants.

Bacterial growth was monitored in the T2 generation plants on day 0 and day 3 (Figure 5-12). The growth analyses were performed using six-week-old plants with visible lesions in the VWA-T2 lines. The five VWA-T2 lines tested had different intensity and patterns of lesion. These five VWA-T2 lines were compared with Col-0 and vector control plants. Virulent bacteria could grow and multiply well in Col-0 and vector control plants (35-6) that were grown along with the VWA-T2 lines. Growth of the virulent bacteria was restricted 10-100 fold in all of the five -T2 lines tested. Although the level of restriction of growth of virulent bacteria varied, all five lines showed significantly reduced growth of bacteria. These data suggested that all these lines exhibited different levels of defense response. The lesion formation and
restriction of virulent bacterial growth in these VWA-T2 lines strongly suggested that these plants exhibited defense response.

Growth of avirulent bacteria was monitored in these lines to confirm the activation of gene-for-gene resistance in these plants. All the five VWA-T2 lines tested exhibited restriction of avirulent bacteria growth like the Col-0 and vector control plants (35-6). This suggested that gene-for-gene resistance is maintained in all the VWA-T2 lines.

**5.4. Discussion**

To understand the structure-function relationship of Arabidopsis CPN1 in plant defense response, a gain of function strategy was applied. Plants were generated that over-expressed mRNAs of C2 domains of CPN1 and VWA domain of CPN1. Plants over-expressing C2 domain behaved like wild-type plants. Interestingly, plants over-expressing VWA domain exhibited resistance to virulent *P.s.t* DC3000 pathogen.

Our studies with the *cpnl-1* mutant and the expression profile of *CPNL* in response to pathogen treatments indicated that CPN1 could be potentially acting like a repressor of defense in plants. We expected that over-expressing CPN1 would result in increased susceptibility to pathogens and would have slower HR response. Although plants over-expressing full length of CPN1 were generated, these plants exhibited a very weak increase in expression of *CPNL*. The expression of CPN1 observed in these lines did not approach the *CPNL* level observed in WT plants.
following avirulent bacterial inoculation, hence indicated an ineffective level of over-expression. All these lines behaved like wild-type plants and neither conferred improved resistance nor susceptibility response. Based on these data, it is impossible to rule out the possibility that CPN1 may not be involved as a repressor of defense. The plants generated should have over-expressed CPN1 at least to the level seen in induced WT plants to make any conclusions. However, the transgenic plants exhibited only a slight increase in CPN1 expression. It is possible that this slight increase in expression does not significantly alter the plant physiology or its response to pathogens. Hence, based on the available data it is difficult to conclude anything.

In addition, it is possible that constitutive high level expression of CPN1 production may be lethal to plants. The poor transformation efficiency obtained for these plants may also indicate that strong expression of CPN1 might have resulted in seed lethality resulting in loss of viability. Alternatively, it is possible that the vector used might have carried a mutation that resulted in low expression of the gene. The results from this experiment are inconclusive. In the future, it will be a good idea to study the CPN1 gene by over-expressing it in an inducible system. This approach might be used to overcome the possible problem of lethality of CPN1 overexpression.

The C2 domains of CPN1 are known for their phospholipid membrane binding property. Transgenic plants that over-expressed mRNAs of C2 domain constitutively in wild-type plants did not exhibit any property linked to defense or disease response. Assuming that accumulation in mRNAs of C2 domain also resulted in accumulation of protein we can speculate regarding the following possibilities. It is possible that the function of C2 domains of CPN1 is limited to binding of CPN1
protein to the membranes. If availability of C2 domain binding sites on the membrane is not a limiting factor, the over-expressed C2 domain will bind to the membrane without competing with endogenous CPN1. Hence, the transgenic plants over expressing C2 domains may function normally without change in phenotype. Based on our previous studies, we had speculated that calcium ion fluxes that occur during early defense signaling pathway may involve CPN1. It is possible that changes in calcium flux activate the C2 domains of CPN1 to bind to membranes and the VWA domain may be the actual component interacting with proteins that play a role in defense response. Hence, over-expressing C2 domains in wild type plants would have just allowed this truncated protein to bind to plasma membrane during calcium flux and not interfere with defense signaling pathway.

If the membrane binding space of C2 domain had been a limitation, then we would have expected C2 domain to compete with endogenous CPN1 resulting in a phenotype. It is possible that the C2 domain (a truncated CPN1 protein) would have been unstable and lacked the ability to bind to membrane. The inability of C2 domain to bind to membranes would have prevented it from competing with endogenous CPN1 and /or membrane binding proteins.

Since the over-expressor lines were tested only at the transcript level, it is unknown whether the truncated C2 domains of CPN1 were stable or active in these plants.

The VWA domain of copines are believed to be involved in protein-protein interactions. The VWA domain is a widely distributed protein domain that is mainly
observed in many extracellular proteins. Copines, which are intracellular proteins, also possess VWA domain (Whittaker and Hynes, 2002). The existence of VWA domain in CPNs indicate that they may play an important role in interacting with other proteins during different signaling events. Over-expression of VWA domain in wild-type plants resulted in a lesion mimic phenotype with increased resistance to *P.s.t* DC3000 like the *cpn1-1* mutant. The timing and pattern of lesions in the VWA domain over-expressor lines did not exactly phenocopy *cpn1-1*. But the lesion formations in these lines was dependent on humidity and temperature conditions. This dominant-negative phenotype in these plants suggests that the VWA domain in CPN1 play an important role in defense signaling functions of CPN1.

The lesion mimic phenotype in the A domain over expressor lines could be due to the excess VWA domain competing with the endogenous full length CPN1 for interactions with target proteins during defense response signaling, thus preventing the full length CPN1 protein from performing its function normally. This kind of competitive inhibition property of VWA domain may act in a dose dependent manner. The variations in the phenotypes of different lines of VWA domain over-expressors may be due to its dose dependent inhibition property. Interestingly, recent biochemical studies on mammalian copine VWA domain have shown that the VWA domain fragment of human Copine 1 protein can prevent full length copine I from recruiting target proteins in a dose dependent manner (Tomsig et al., 2003).

Calcium is a well-known secondary messenger in many biotic and abiotic stress responses (Bowler and Fluhr, 2000). Copines including Arabidopsis CPN1 have been shown to have a calcium dependent membrane binding property. The
ability of copines to associate with membranes following a calcium flux may be important in regulating the signal processes that occur at the membrane surfaces. Based on our studies with *cpn1-1* mutant and *CPN1* expression analysis, we have found that Arabidopsis CPN1 could be playing multiple roles. Under different conditions, the VWA domain of CPN1 may be interacting with different kinds of proteins and regulating different pathways.

It will be interesting to identify the proteins that interact with CPN1 VWA domain during defense response. Studies on copine interacting proteins would further clarify the role of Arabidopsis CPN1 in plant defense response. The mammalian VWA domain of copine protein has been shown to interact with a diverse group of proteins such as map kinase, calcium-binding proteins, phosphatase, ubiquitin conjugating enzymes, collagen and transcription modulators (Tomsig et al., 2003). All the interacting proteins identified are intracellular proteins and are known to be important players in different signaling pathways. It is possible that the VWA domain of Arabidopsis CPN1 might interact with multiple proteins or protein complexes and that would give us clues about the various functions of CPN1 in Arabidopsis.
Figure 5-4: Reverse Transcriptase PCR to detect *CPN1* expression in transgenic CPN1 full-length over-expressor lines.

CPN1 expression in five different T3 generation transgenic lines of Full-length CPN1 over-expressors (F1, F8, F13, F15, F18) compared with vector control line. PCR reactions were stopped at 15, 20 and 25 cycles to determine the relative level of *CPN1* expression compared to the constitutive active actin gene.
Figure 5-5: Five-week-old transgenic full-length-CPN1 over-expressor line grown under short day conditions.

The full-length-CPN1 over-expressor lines did not exhibit any unique phenotype. All the lines looked normal like the vector control and Col-0 plants.
Figure 5-6: Virulent and avirulent bacterial growth in transgenic full-length-CPN1 over-expressor lines

A: Growth analysis of virulent *P.s.t* DC3000 bacteria in full length CPN1 over-expressor lines compared to vector control and Col-0 plants. The bacterial growth in the FL- CPN1 over-expressor lines is comparable to control plants.

B: Growth analysis of avirulent *P.s.t* (*avrRpt2*) bacteria in full-length CPN1 over-expressor lines compared to vector control and Col-0 plants. Bacterial growth is restricted in all the lines due to gene-for-gene resistance.
Figure 5-7: RNA gel blot analysis to detect expression of C2 domain mRNA in transgenic Arabidopsis C2 domain (CPN1) over-expressor lines

Lane 1: WT (wild-type, Col-0), lane 2: C1 (vector control, 35-6), lane 3: C2 (vector control, 35-10), lane 4-10: 7 lines of C2 domain overexpressors (C2-1, C2-6, C2-13, C2-16, C2-18, C2-24, C2-25). The expression of C2 domain confirmed in the transgenic lines. The membrane reprobed with *PRI* to detect induction of defense related gene. The 28S rRNA serves as a loading control.
Figure 5-8: Five week old C2 domain (CPN1) overexpressor lines grown under short day conditions

The C2 domain over-expressor lines did not exhibit any unique phenotype. All the lines looked normal like the vector control and Col-0 plants.
Figure 5-9: Virulent and avirulent bacteria growth in C2 domain over-expressor lines

A: Growth of virulent *P.s.t.* DC3000 in C2 domain over-expressor lines compared to vector control and Col-0 plants. No significant difference was observed in the growth of DC3000 bacteria in control plants Vs the four C2 domain overexpressing lines (C2-6, C2-14, C2-17, C2-24).

B: Growth of avirulent *P.s.t.* (*avrRpt2*) in C2 domain overexpressing lines compared with the growth in vector control and Col-0 plants. Restriction of avirulent bacterial growth is observed in all the plants tested.
Figure 5-10: Six-week-old VWA domain over-expressor line (A31) grown under short day conditions

A: Six-week-old vector control and A31 plant. VWA domain over-expressor line (A31) develops very minute lesions on the younger leaves. The lesions are observed either at the leaf margins or they are scattered.

B: Closeup view of the lesions observed in A31 plants. The lesions are marked with arrows. Both marginal and scattered lesions are found in A31 plants.
Figure 5-11: RNA gel blot analysis to confirm the expression of VWA domain mRNA in T2 and T3 generation of VWA domain overexpressor lines.

Six VWA domain overexpressor lines (1: A7, 2: A28, 3: A31, 4: A32, 5: A36, 6: A37) in the T2 and T3 generations are compared for the expression of VWA domain. Most of the lines do not show the expression of VWA domain in the homozygous state in the T3 generation. C represents vector control plants, WT represents Col-0 plants. The loading of RNA is represented by methylene blue staining of 28S rRNA.
Figure 5-12: VWA domain over-expressor lines are resistant to *P. syringae* DC3000

A: VWA domain over-expressor lines exhibit improved resistance to virulent DC3000. Both A32 and A36 lines of T2 generation showed very little disease symptoms in the new leaves. The older leaves exhibit disease symptoms like the control plants.

B: Growth of virulent bacteria *P. syringae* DC3000 in VWA domain over-expressor lines. Virulent bacterial growth was significantly restricted in all the five A domain-overexpressor lines. The restriction of bacterial growth was 10-100 fold when compared to the growth in control plants (35-6: vector control, Col-0).

C: Growth of avirulent bacteria *P. syringae* (*avrRpt2*) in VWA domain over-expressor lines. Similar levels of restriction of avirulent bacteria was observed in all the lines tested.
Chapter 6

Role of *NPR1* in disease resistance, defense gene expression and cell death in the *cpn1-1* mutant

6.1 Introduction

Plants have developed effective ways to resist potential pathogens. Systemic acquired resistance (SAR) is an induced resistance in plants that provides generalized resistance to a broad spectrum of virulent pathogens (Ryals et al., 1996). SAR is often induced by the recognition by plant *R* gene products of the corresponding avirulence product of the pathogen (Hammond-Kosack and Jones, 1996). SAR can also be induced in plants by exogenous application of salicylic acid (SA) and 2,6-dichloroisonicotinic acid (INA) and benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH), which are molecular chemical analogs of SA (Malamy et al., 1990; Gaffney et al., 1993; Delaney et al., 1994). SAR involves the coordinated accumulation of transcripts of several pathogenesis related (*PR*) genes (Ward et al., 1991).

SA and the NPR1/NIM1 protein are the two molecules that have been proven important for establishment of SAR. SA accumulation has been shown to be required
for SAR (Malamy et al., 1990). Blocking of SA accumulation in nahG transgenic plant (encodes for salicylate hydroxylase that converts SA into inactive catechol) results in an increased susceptibility to a broad range of bacterial and Oomyceteous pathogens (Ryals et al., 1996).

Arabidopsis NPR1/NIM1 is the only well known component that acts downstream of SA that controls the establishment of SAR in plants (Cao et al., 1994, 1997). Arabidopsis npr1/nim1 mutants were isolated from four independent mutant screens for mutants that lack the ability to express SAR after SAR inducing treatments (Cao et al., 1994; Delaney et al., 1994; Glazebrook et al., 1996; Shah et al., 1997). The npr1/nim1 mutant exhibits an increased susceptibility to several virulent and avirulent pathogens (Cao et al., 1994; Delaney et al., 1995).

NPR1 was cloned using map based cloning strategy and found to encode for a novel protein with ankyrin repeats (Cao et al., 1997) known for mediating protein-protein interactions (Segdwick and Smerdon, 1999). Other well-studied ankyrin repeat containing proteins are transcription-regulating proteins such as NF-κB precursor p105 and the inhibitor I-κB. In mammalian systems, NF-κB complex is nuclear-localized following bacterial and viral pathogen stimuli. Anti-inflammatory drugs such as aspirin block the degradation of NF-κB inhibitor I-κB causing retention of the transcription factor in the cytosol (Kopp et al., 1994). It is interesting to note that ankyrin repeat proteins work in defense responses observed in plant and mammalian systems.

In Arabidopsis, over-expression of NPR1 conferred a broad-spectrum resistance in the plants (Cao et al., 1998). NPR1 over-expressing plants did not have
an increase in the basal expression of PR genes indicating that NPR1 is normally inactive in the absence of induction by pathogens or SA treatments. Studies with the yeast two-hybrid system have revealed that NPR1 protein interacts with several TGA family of basic leucine zipper transcription factors (Després et al., 2000; Zhou et al., 2000; Chern et al., 2001). Interaction of NPR1 with TGA2 enhanced the binding of TGA2 to several motifs present in the PR-1 gene (Després et al., 2000; Zhou et al., 2000).

The cpn1-1 mutant exhibits enhanced resistance to several pathogens and constitutive expression of several PR genes (PR1, PR2 and PR5) that are indicators of constitutive SAR in cpn1-1. To understand and determine whether the SAR in cpn1-1 mutant requires an NPR1 dependent defense signaling pathway, we sought a genetic approach by creating double mutants of cpn1-1npr1-1.

6.2 Methods

6.2.1 Construction of the cpn1-1npr1-1 double mutant

The cpn1-1npr1-1 double mutant was created using the pollen from cpn1-1 plant and fertilizing the flowers of npr1-1 mutant. The success of the cross was determined by selecting for resistance to Finale herbicide encoded in the T-DNA in the cpn1-1 mutant and the loss of the cpn1-1 mutant phenotype. The cpn1-1 mutant bearing the T-DNA is homozygous and contains the bar gene encoding resistance to
glufosinate. The F1 plants were allowed to self-pollinate and set seeds. The F2 seeds were screened in MS media containing 100μM SA plates. Homozygous npr1-1/npr1-1 mutant plants bleach when grown in high concentration of SA. The F2 plants that bleached in MS +SA plates were rescued and transferred to soil. The selected F2 plants were then tested for Finale resistance after 1-2 weeks of growth and monitored for cpnl-1 like phenotype. The screened F2 plants were allowed to self-pollinate and set seeds. The F3 progeny were confirmed again for homozygosity for npr1-1 and cpnl-1 mutant alleles by screening for MS+SA and for cpnl-1 like phenotype and finale resistance. To further confirm the npr1-1 mutant allele in the F3 lines, PCR analysis was performed with the DNA of the F3 plants (see below).

6.2.2 Sterilization of seeds, preparation of MS media with SA

The F2 and F3 progeny seeds were surface sterilized in 5 mL of isopropanol. (70% v/v) The isopropanol was removed and seeds were rinsed in 5 mL of 50% bleach for 5 minutes. The seeds were vortexed at a low speed to allow complete mixing of seeds with bleach solution. The bleach solution was removed under sterile conditions and sterile dd water was used to wash the bleach. The washing step was done 4-5 times in laminar hood to prevent any contamination.

Murashige and Skoog (MS) media plates were made with 4.64 g of MS salts (Gibco, MD), 10g of phyta-agar in a liter of double-distilled water. The MS media was autoclaved in a liquid cycle for 15 min. The media was allowed to cool and supplemented with B5 vitamins. SA (Sigma, MO) at a concentration of 100 μM
(20mM SA stock concentration) was added to the media and the media was allowed to mix well. The lukewarm media was poured in petri plates in a laminar air-flow hood. Seeds were mixed with sterile 0.1% phytaagar and spread onto the solidified media. The petri plates were wrapped with vent tape and allowed to stratify at 4 °C for 3 days and then transferred to short day conditions (8h light and 16h darkness) at 22 °C, 45-50% RH.

6.2.3  PCR based CAPS markers for analyses of the npr1-1 allele

Genomic DNA was isolated from cpn1-1npr1-1 and npr1-1 mutant plants using modified Shure et al (1983) method. PCR analyses was performed using the primers for npr1-1 allele
Forward 5'- CGAATGTACATAAGGCACTTG-3'
Reverse 5'-AGTTGCTTCTGATGCACTTGC-3'
The PCR conditions used for the reaction were
Template (genomic DNA):  25-30 ng
Final dNTP concentration:    0.25 mM
Final primer concentration:    2.5µM
DMSO:    1X
Taq Buffer:  1X
Tm=  57 °C

The PCR amplified products are digested with NlaIII restriction enzyme for confirmation of npr1-1 allele.
6.2.3 RNA gel blot analyses

RNA gel blot analyses using PR1 and CPN1 as probe is described in Chapter 4.

6.3 Results

6.3.1 cpn1-1npr1-1 double mutant plants

Since the cpn1-1 mutant forms spontaneous cell death that resembles the HR and expresses very strong expression of PR genes, it was assumed that npr1 would be downstream of cpn1-1 in SAR signaling pathway. To confirm this, crosses were performed to generate double mutants of cpn1-1 and npr1-1.

cpn1-1 and npr1 being recessive, confirmation of double mutants was performed in the F2 generation and later. In the F1 generation, the cross of npr1 with cpn1-1 pollen was confirmed by selecting for basta resistance and loss of cpn1-1 phenotype. In the F2 generation, seeds were plated in 100 μM MS +SA plates. Plants that started to bleach were restored and tested for cpn1-1 like phenotype (Figure 6-1). Around 100 F2 plants were tested and 1/16th of them showed the cpn1-1 like phenotype and bleaching affect. The selected F2 plants were allowed to set
seeds and confirmed again for bleaching affect in MS+SA, tested for Finale resistance, \textit{cpn1-1} like phenotype and by PCR analysis.

6.3.2 Phenotypic characterization of \textit{cpn1-1npr1-1} double mutant

\textit{cpn1-1npr1-1} double mutant plants when grown under short day (8h light and 16h darkness) conditions, 45-50\% RH, 22-23°C, 65 Umolm\(^2\)/sec were smaller in size than the \textit{npr1-1} mutant plants but slightly larger than \textit{cpn1-1} plants. The \textit{cpn1-1npr1-1} double mutant plants had normal leaf shape unlike the \textit{cpn1-1} mutant. The development of lesions in \textit{cpn1-1} started at 2 weeks after germination, but in \textit{cpn1-1npr1-1} no visible lesions were seen even at 4 week stage of the plant (Figure 6-2). Very faint lesions developed on the margins of the leaves of \textit{cpn1-1npr1-1} when the plants were five-week-old. The phenotype in \textit{cpn1-1npr1-1} indicate that \textit{NPR1} mutation in \textit{cpn1-1} partially restored the size of the plant, normal leaf pattern and delayed the process of cell death.

\textit{cpn1-1npr1-1} double mutants grown under long day conditions (LD)(16h light and 8h darkness) had a characteristic bleaching of the leaves and stems. \textit{cpn1-1npr1-1} plants that were moved from SD to LD conditions exhibited bleaching of the newly formed leaves and stems (Figure 6-3). The bleaching phenotype resembled \textit{npr1} plants grown on SA (Cao et al., 1994) and this could probably due to high production of SA in these plants under LD conditions. Sometimes the bleaching affect was strong enough to kill the entire plant. The siliques that were formed also
became bleached, resulting in loss of seed set. Very minimal seeds could be collected from these plants and most of them turned out to be not viable. When *cpn1-1npr1-1* plants were allowed to enter flowering stage in SD conditions, even in a period of six-month time, the plants hardly entered flowering stage and set seeds. The *NPR1* mutation in *cpn1-1* suppressed the seeds set condition. The extremely poor seed set created a major drawback to pursue a more detailed study of this double mutant.

### 6.3.3. Expression of PR1 gene in the *cpn1-1npr1-1* double mutant

Expression of *PR-1*, a marker for SAR in plants, was analyzed in the *cpn1-1npr1-1* plants by Northern blot analysis. In the untreated plants, *PR1* was strongly and constitutively expressed in *cpn1-1* plants and in *cpn1-1npr1-1*. Very strong expression of *PR1* was observed in WT plants by 24h with avirulent pathogen treatment and by 48h by virulent pathogen indicating the turn on of defense in plants (Figure 6-4). Some amount of *PR1* gene expression was observed in the *npr1* mutant, suggesting that signaling events for *PR1* expression can occur independent of NPR1 during pathogen treatment. Both avirulent and virulent pathogens strongly induce the expression of *PR1* gene in *cpn1-1npr1-1*. The level of expression in *cpn1-1npr1-1* is as high as observed in *cpn1-1*. This suggest that *PR1* gene expression in *cpn1-1* mutant is independent of NPR1.

### 6.3.4. Expression of CPN1 in the *npr1-1* mutant

Wild-type plants treated with avirulent pathogen strongly express *CPN1* by 24h in the inoculated leaves (Jambunathan and McNellis, 2003). *npr1* mutant plants
inoculated with avirulent pathogen also has a high expression of \textit{CPN1} at 24h after treatment (Figure 6-5). This suggests that either CPN1 is upstream of NPR1 in defense signaling pathway or activation of \textit{CPN1} during pathogen treatment is independent of NPR1 signaling pathway. However, since the time point taken was 24 hours, it does not show whether the gene-for-gene specified spike in \textit{CPN1} expression following avirulent bacterial inoculation depends on NPR1.

### 6.4 Discussion

In this study, we generated \textit{cn1-1npr1-1} double mutants to study the role of NPR1 in the \textit{cn1-1} mutant phenotype. The \textit{cn1-1npr1-1} double mutant exhibited delayed lesion formation, bleaching of stems and siliques under LD conditions but did not affect the \textit{PR} gene expression or loss of resistance to virulent bacterial pathogen \textit{P. s.t} DC3000.

The delayed cell death in \textit{cn1-1npr1-1} plants suggests that NPR1 has a role in promoting HR. Several previous studies have shown that SA and NPR1 can potentiate cell death in plants. In Arabidopsis mutants like \textit{acd2, acd5} and \textit{acd6-1} (Rate and Greenberg, 2001; Greenberg et al., 2000; Rate et al., 1999), NPR1 has been shown to promote cell death. On the other hand, NPR1 over-producing plants (Cao et al., 1998) suppressed cell death during infection of \textit{P. syringae} carrying \textit{avrRpm1} (Rate and Greenberg, 2001). This suggests that NPR1 can play different roles in regulating cell death based on its level of expression. The \textit{cn1-1npr1-1} plants also
exhibited a more normal leaf pattern than *cpn1-1*. The *cpn1-1npr1-1* plants had enlarged leaves when compared to *cpn1-1* but did not completely restore the normal leaf shape as in Col-0 wt *npr1*. The role of NPR1 in alteration of growth based on cellular context and level of signal transduction has been observed earlier (Vanacker, 2001). It is possible that NPR1 may play a role in suppressing cell growth and expansion in *cpn1-1* mutant.

The *cpn1-1npr1-1* plants exhibited a unique phenotype when grown under long day conditions. The *cpn1-1npr1-1* plants developed characteristic bleaching of leaves, stems and siliques. Similar phenomena were observed in *cpr5npr1* and in *acd6npr1* double mutant plants (Bowling et al., 1997; Rate et al., 1999). It is possible that increased day length in *cpn1-1npr1-1* plants resulted in increased production of SA in these plants. The excess SA may require NPR1 to act as a downstream component. Presence of excess SA and absence of NPR1 in *cpn1-1npr1-1*, hence resembles the bleaching phenotype when SA is exogenously provided to *npr1* plants.

The *cpn1-1npr1-1* double mutant exhibited constitutive *PR* gene expression. Some amount of *PR1* gene activation occurs with both avirulent and virulent pathogen treatments in *npr1* plants, indicating the existence of NPR1 independent *PR1* gene activation pathway. Activation of an NPR1 independent pathway has been observed in mutants such as *cpr5* and *cpr6*, which have constitutive *PR* gene expression with resistance to *P. parasitica* but loss of resistance to *P. syringae maculicola* (Bowling et al., 1997; Clarke et al., 1998). Based on our data, the
resistance pathway observed in \textit{cpn1-1} might be different from that observed in \textit{cpr5}
and \textit{cpr6}. Resistance to the bacterial pathogen \textit{P.s.m} and constitutive expression of
\textit{PR} genes was observed in \textit{snc1npr1} double mutant plants. The \textit{snc1} mutation maps
to the \textit{RPP5} \textit{R}-gene cluster (Li et al., 2001). Lesion-mimic Arabidopsis mutants \textit{ssi1}
and \textit{ssi2} confer NPR1 independent expression of \textit{PR} genes and resistance against
bacterial pathogens as observed in \textit{cpn1-1} plants (Shah et al., 1999, 2001). These
data provide evidence for the existence of defense pathway that acts independent of
NPR1 in induction of \textit{PR1} and resistance to bacterial pathogens. That \textit{cpn1-1} may act
in a similar pathway observed in \textit{ssi1}, \textit{ssi2} and/or \textit{snc1}. Further detailed epistasis
analyses of \textit{cpn1-1} with other signaling mutants may clarify the defense-signaling
pathway observed in the \textit{cpn1-1} plant. Based on our results, we can speculate that
\textit{cpn1-1} may have a SA dependent and NPR1 independent defense signaling, as \textit{cpn1-1}
exhibits strong induction of \textit{PR1}, \textit{PR2} and \textit{PR5} genes that are considered as
markers of SAR (Jambunathan et al., 2001) independent of \textit{NPR1} mutation. Since
\textit{CPN1} is strongly expressed soon after pathogen inoculation (Jambunathan and
McNellis, 2003), it is possible that \textit{CPN1} may act in \textit{EDS1} dependent pathway like
\textit{snc1} mutant (Li et al., 2001) that activates SA accumulation but does not use NPR1
as its downstream component during signaling events. Activation of \textit{CPN1} in the
\textit{npr1} mutant by \textit{P.s.t avrRpt2} by 24h can suggest different possibilities. It is possible
that \textit{CPN1} may be acting upstream of NPR1 or independent of NPR1. \textit{CPN1}
expression was looked at 24h after \textit{P.s.t avrRpt2} inoculation and not at 6h when the
peak expression of \textit{CPN1} occurs. \textit{CPN1} transcript accumulation is observed in WT
plants inoculated with virulent pathogen by 24h. Hence, at this time point it is
difficult to distinguish between general non-specific pathogen response and gene-for-
gene responses.

Studies with *cpn1-1npr1-1* gave interesting results about the role of NPR1 in
controlling cell death, phenotype and *PR* gene activation in *cpn1-1* mutant. However,
the main drawback was that bacterial resistance in the *cpn1-1npr1-1* could not be
monitored due to lack of seeds. The poor seed set and poor viability of *cpn1-1npr1-1*
plants caused a major hindrance in repeating the experiments.

To overcome the problem of lack of *cpn1-1npr1-1* seeds, the double mutant
can be selected again from the segregating population of *cpn1cpn1-1Npr1-1npr1* of
F2 generation. Although this may be a cumbersome process, it is good strategy to do
a detailed study on *cpn1-1npr1-1* double mutant plants and to confirm the existing
data and do more experiments.
Figure 6-1: Screening for npr1 allele in MS +SA plates

Seeds of Col-0, cpn1-1, npr1-1 and cpn1-1npr1-1 germinated in MS +0.1mMSA containing plates. npr1 mutant has a characteristic bleaching affect when grown on MS +0.1mM SA plates. Both Col-0 and cpn1-1 mutant plants are able to grow normally in MS +0.1mM SA plates. cpn1-1npr1-1 Double mutants exhibited the characteristic bleaching affect.
Figure 6-2: Four week-old Col-0, npr1, cpn1-1 and cpn1-1npr1-1 plants grown under short day conditions

(A): Both Col-0 and npr1 plants grown under SD conditions have normal growth. cpn1-1 mutant plant is small in size with curled leaves. cpn1-1npr1-1 double mutant plants have normal leaf pattern and they are slightly bigger in size than the cpn1-1 mutant plants. The scale is indicated by a line representing 1 cm length of rosette in plant

(B): Close-up of four-week-old cpn1-1 and cpn1-1npr1-1 leaves. Four-week-old cpn1-1 plants have clear marginal cell death but cpn1-1npr1-1 mutant leaf does not show any lesions at this age. Lesion formation developed in cpn1-1 when the plants are two-week-old. Lesions were observed at the leaf margins. cpn1-1npr1-1 double mutant plants had delayed lesion formation.
Figure 6-3: Onset of bleaching in *cpn1-1npr1-1* double mutant plants

(A): *cpn1-1npr1-1* double mutants when moved to long day conditions, start initiation of bleaching in its leaves and stems. Newly formed leaves are completely bleached and sometimes bleaching progresses to older leaves.

(B): Stunted bolts and bleached siliques in *cpn1-1npr1-1* plants. There was a reduction in apical dominance in *cpn1-1npr1-1* when grown under LD conditions. Multiple stunted bolts were observed. The stems are bleached and the siliques that emerge were bleached with hardly any seed set.
Figure 6-4: Expression of PR-1 gene in cpn1-1npr1-1, cpn1-1, npr1, Col-0 plants in response to pathogen treatment

In both the cpn1-1npr1-1 double mutant and cpn1-1 mutant plants, PR1 was constitutively expressed in the absence of any pathogen. In wild-type plants, PR1 was turned on by 24h by avirulent pathogen and by 48h by virulent pathogen. Slight induction of PR1 was observed in npr1 plants treated with pathogen.
Figure 6-5: Expression of CPN1 in Col-0 and npr1

RNA gel blot analysis of Col-0 and npr1-1 plants treated with avirulent pathogen. Strong expression of CPN1 was observed at 24h in Col-0 and in the npr1 mutant.
Chapter 7

Conclusion, future directions of Copine I studies on plant defense signaling

Copines are unique class of proteins found in a wide range of organisms such as nematodes, Paramecium, mouse, humans and plants. Copines are highly conserved in many eukaryotic phyla but are not present in *Drosophila melanogaster* or in *S. cerevisiae* (Whittaker and Hynes, 2002). The phylogenetic distribution of copines suggests that a single copine in an ancestral organism common to humans, *C.elegans* and *A. thaliana* has expanded to several copies in various species. The highly conserved nature and multiple copies of copines in these organisms strongly suggest that these proteins must be playing an important biological role. Several biochemical studies on mammalian copines suggest that these proteins have a calcium dependent membrane binding property.

From a non-targeted genetic screen in Arabidopsis for mutants with increased resistance to *P.s.t* DC3000, the *cpn1-1* mutant was identified with a mutation in the *COPINE I* gene of Arabidopsis (Jambunathan et al., 2001). Interestingly, another Arabidopsis COPINE I mutant (*bon1* for BONZAI1) was picked up from another screen for altered growth response under low temperature conditions (Hua et al., 2001). Detailed studies by our group and by Hua et al. defined the potential
biological role of copines for the first time. The detailed studies on mutant
classification, CPN1 gene expression profiles, structure-function analyses supports
a role for CPN1 in Arabidopsis as a repressor of cell death and defense response.

Based on the available data, several speculations can be made on mode of
function of Arabidopsis CPN1. Arabidopsis CPN1 has a strong association with
phospholipids and calcium enhances binding of CPN1 to membranes (Hua et al.,
2001). It is possible that CPN1 exists in free state as well as in association with
membranes. The main role of CPN1 in the cell might be to suppress the initiation of
cell death events that might trigger defense in the plant. A pathogen triggered Ca$^{2+}$
flux may activate the repressive role of CPN1 and this could involve the localization
of CPN1 to membrane (Figure 7-1). The membrane bound CPN1 may exert pressure
to suppress the cell death process at the plasma membrane. This would explain our
observations on accelerated cell death in cpn1-1 mutant compared to wild-type plants
after inoculation with avirulent P.s.t. Again, our data indicate that CPN1 is essential
for repressing cell death in the absence of pathogen infection under certain
environmental conditions such as low temperature and low humidity. Thus, this
raises the possibility that CPN1 may be triggered by Ca$^{2+}$ fluxes that may not be
pathogen induced. Increase in intracellular calcium concentrations has been observed
in a wide range of biotic and abiotic stress responses including low temperature and
low humidity (Wood et al., 2000; Bowler and Fluhr, 2000). Although different
signaling events have specific calcium signatures, the calcium molecule seems to be
the point of convergence for different responses to environmental signals (McAinsh
and Hetherington, 1998; Bowler and Fluhr, 2000). Different calcium signatures in the
plant may affect different levels of binding of CPN1 to membrane. CPN1 may serve as a connecting link for biotic and abiotic stress response in repressing cell death and defense responses at the plasma membrane. Under different Ca^{2+} fluxes CPN1 may facilitate the association and fusion of vesicles to membrane to enhance membrane trafficking. The strong fusion of vesicles under pathogen response, low temperature or low humidity conditions may help in preventing cell death and maintain the membrane structure.

Although the exact mechanism of action of CPN1 protein is unknown, it is likely that CPN1 may function by recruiting proteins with its VWA domain. At the plasma membrane CPN1 protein may form a multimeric protein complex by interacting with several proteins that might act together in suppressing cell death initiation. Several plasma membrane associated proteins are believed to act in a heteromultimeric membrane associated protein complex. Recent studies on tomato Cf-9 protein, a plasma membrane associated protein (that confers race-specific resistance to *Cladosporium fulvum*) indicates that it may exist in a multi-protein complex (Rivas et al., 2002). Studies with mammalian copine I has revealed that the VWA domain interacts with a wide range of proteins (Tomsig et al., 2003). Preliminary evidence suggests that the VWA domain of Arabidopsis CPN1 can interact with several different kinds of proteins (J. Sinn and T. W. McNellis, personal communication). This is not surprising, as CPN1 has been observed to play roles in several signaling events. CPN1 under different signaling response may recruit different proteins that may work together in carrying out different functions in the
plants: as a repressor of cell death and defense under pathogen response and under low temperature or low humidity conditions.

CPN1 could be playing a key role under pathogen induced defense signaling response. The strong and early induction of \( CPN1 \) during pathogen induced defense response strongly suggests that CPN1 is an important player. CPN1 could be acting during the early defense signaling involving calcium flux. Being a membrane binding protein, it could be one of the early targets in \( R \) gene mediated signaling. It is possible that it could be directly interacting with a class of \( R \) gene that possess the coiled-coil (CC) domain. Recent studies on mammalian copines have provided strong evidence that mammalian copine I has strong affinity to interact with proteins with CC domain (Tomsig et al., 2003). Preliminary data on CPN1 suggests that some of its interacting proteins possess CC domains (J. Sinn and T. W. McNellis, personal communication). Arabidopsis genes, \( RPW8.1 \) and \( RPW8.2 \) that confer resistance to powdery mildew possess a N-terminal transmembrane domain and a coiled-coil domain (Xiao et al., 2001). Environmental conditions such as low temperature (22°C), humidity (80-85%) and normal light favor cell death in transgenic plants possessing extra copies of \( RPW8 \) (Xiao et al., 2002). The lesion mimic phenotype disappears when temperature/humidity/ light levels are increased. The strong similarity in phenotype in \( cpn1-1 \) and \( RPW8 \) transgenic plants suggest that the extra copies of RPW8 introduced interacts with CPN1 and blocks the function of CPN1. It will be interesting to see whether introducing extra copies of \( CPN1 \) restores normal wild-type phenotype in the transgenic \( RPW8 \) plants. It is possible that during \( \text{avr-R} \) gene product interactions, several \( R \) gene mediated signaling events might occur to
trigger HR and defense responses. CPN1 as a repressor of HR mediated cell death might directly interact with R gene product to inhibit some of its activity.

An association can be proposed between CPN1 signaling and MAP kinase signaling events. ATMPK4 and ATMPK6 have been found to have increased activity under low humidity and low temperature conditions (Ichimura et al., 2001). Both ATMPK4 and ATMPK6 have been found to be rapidly activated with harpin elicitors (Desikan et al., 2001). Studies on Arabidopsis mpk4 mutant have revealed that it may serve as negative regulator of SAR (Petersen et al., 2001). Both low humidity and low temperature have been found to directly play a role in defense in the cpn1-1 mutant. Based on the studies described in this thesis, CPN1 transcript accumulation has been found to be regulated by low humidity, low temperature and by pathogens. Based on the available data, one can propose a link between CPN1 and MAP kinase cascades. Earlier studies on plant-pathogen interaction studies have found that MAP kinase signaling occurs immediately downstream of calcium flux signaling. It is possible that CPN1 could be interacting directly with components of MAP kinase cascade.

It is interesting to speculate on the various mode of action of CPN1 based on available data from my research and others. However, an exact functional assignment for CPN1 is still a complicated issue. My results provide foundations to investigate the function of CPN1 mainly during pathogen-induced defense in plants. Further research on CPN1 is needed to clarify its function in Arabidopsis and whether CPN1 functions specifically in plant defense signaling. It is difficult to say whether this research can be extrapolated to the function of other copines in other organisms.
Nevertheless, these findings are relevant to study the interconnections that exist among different signaling events in plant to maintain their growth and development.
Figure 7-1: A model for the role of CPN1

CPN1 acts as a repressor of cell death both in presence and absence of pathogen signals. Pathogen induced signals trigger a strong calcium flux that may enhance the membrane binding property of CPN1. CPN1 exerts a repressive force at the plasma membrane to prevent the initiation of the cell death process. CPN1 also plays a role in suppressing cell death that may occur during plant adaptation to low temperature or low humidity conditions that may also be mediated through calcium flux.
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VITA
Niranjani Jambunathan

EDUCATION
Ph.D in Plant Physiology, May 2003, The Pennsylvania State University, USA
M.Sc in Biotechnology, June 1997, Tamil Nadu Agricultural University, India
Thesis title: "In vitro microspore culture and biochemical analyses of dihaploids in rice"
B. Sc in Agriculture, May 1995, Tamil Nadu Agricultural University, India

EXPERIENCE
Teaching Assistant at the department of Biology, Penn State University
Junior Research Fellow at SPIC Science Foundation, Chennai, India, 1997-1998
   Project: Mitochondrial DNA analyses for cytoplasmic male sterility in rice

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J. Ben and Helen D. Hill Award (1999) presented by Eberly College of Science, PSU Travel award presented by College of Agricultural Sciences, Penn State University, 2001
Travel award presented by Plant Physiology Program, Penn State University, 2001
Plant Physiology Fellowship (Penn State University): Fall 1998, Fall 2000, Spring 2001
Department of Biotechnology and Jawaharlal Nehru University, India. Fellowship award, 1995-1997
Department of Biotechnology, India. Summer training grant award for research in M.S. Swaminathan Research Foundation
Tamil Nadu Agricultural University Merit Scholarship Award, India. 1991-1995

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