Molecular, Genetic and Biochemical Characterization of Resistance Protein-Mediated Signaling Against Turnip Crinkle Virus

Rae-Dong Jeong

University of Kentucky, nix0125@hotmail.com

Recommended Citation

Jeong, Rae-Dong, "MOLECULAR, GENETIC AND BIOCHEMICAL CHARACTERIZATION OF RESISTANCE PROTEIN-MEDIATED SIGNALING AGAINST TURNIP CRINKLE VIRUS" (2011). University of Kentucky Doctoral Dissertations. 181.
https://uknowledge.uky.edu/gradschool_diss/181
ABSTRACT OF DISSERTATION

Rae-Dong Jeong

THE GRADUATE SCHOOL
UNIVERSITY OF KENTUCKY
2011
MOLECULAR, GENETIC AND BIOCHEMICAL CHARACTERIZATION OF RESISTANCE PROTEIN-MEDIATED SIGNALING AGAINST TURNIP CRINKLE VIRUS

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By
Rae-Dong Jeong
Lexington, Kentucky

Director: Dr. Pradeep Kachroo, Associate Professor of Plant Pathology
Lexington, Kentucky
2011

Copyright © Rae-Dong Jeong 2011
MOLECULAR, GENETIC AND BIOCHEMICAL CHARACTERIZATION OF RESISTANCE PROTEIN-MEDIATED SIGNALING AGAINST TURNIP CRINKLE VIRUS

Infection of the resistant Arabidopsis ecotype Di-17 with Turnip Crinkle Virus (TCV) elicits hypersensitive response (HR), accompanied by increased expression of defense genes. HR to TCV is conferred by HRT, which encodes a coiled-coil (CC)-nucleotide-binding site (NBS)-leucine-rich repeat (LRR) class of resistance (R) protein. In contrast to HR, resistance requires HRT and a recessive locus designated rrt. Unlike most CC-NBS-LRR R proteins, HRT-mediated resistance is dependent on EDS1 and independent of NDR1. Resistance is also dependent on salicylic acid (SA) pathway and light. A dark treatment, immediately following TCV inoculation, suppresses HR, resistance and activation of a majority of the TCV-induced genes. To determine the genetic, molecular and biochemical basis of light-dependent defense pathway, we studied the role of various photoreceptors in HRT-mediated resistance to TCV, HRT protein levels and its localization. Interestingly, mutation in blue-light photoreceptors led to degradation of HRT via a proteasome-dependent pathway and resulted in susceptibility to TCV. Exogenous application of SA induced transcription of HRT, which restored HRT levels in some, but not all, mutant backgrounds. These results show that different photoreceptors function distinctly in maintaining post-transcriptional stability of HRT. In addition to photoreceptors, HRT also forms a complex with several other proteins, many of which participate in the RNA silencing pathway and are required for HRT-mediated resistance. Together, our results suggest that HRT forms a multi-protein complex and that HRT-mediated signaling involves reconstitution of this complex.
Key words: Turnip Crinkle Virus, Hypersensitive response, Salicylic acid

RAE-DONG JEONG

May 12, 2011
MOLECULAR, GENETIC AND BIOCHEMICAL CHARACTERIZATION OF RESISTANCE PROTEIN-MEDIATED SIGNALING AGAINST TURNIP CRINKLE VIRUS

By

Rae-Dong Jeong

Pradeep Kachroo
Director of Dissertation

Lisa J Vaillancourt
Director of Graduate Studies

May 12, 2011
Date
RULES FOR THE USE OF DISSERTATIONS

Unpublished dissertations submitted for the Doctor’s degree and deposited in the University of Kentucky Library are as a rule open for inspection, but are to be used only with due regard to the rights of the authors. Bibliographical references may be noted, but quotations or summaries of parts may be published only with the permission of the author, and with the usual scholarly acknowledgements.

Extensive copying or publication of the dissertation in whole or in part also requires the consent of the Dean of the Graduate School of the University of Kentucky.

A library that borrows this dissertation for use by its patrons is expected to secure the signature of each other.

Name

Date

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________

________________________________________________________________________
DISSERTATION

Rae-Dong Jeong

The Graduate School
University of Kentucky
2011
MOLECULAR, GENETIC AND BIOCHEMICAL CHARACTERIZATION OF RESISTANCE PROTEIN-MEDIATED SIGNALING AGAINST TURNIP CRINKLE VIRUS

DISTRIBUTION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Agriculture at the University of Kentucky

By

Rae-Dong Jeong

Lexington, Kentucky

Director: Dr. Pradeep Kachroo, Associate Professor of Plant Pathology

Lexington, Kentucky

2011

Copyright © Rae-Dong Jeong 2011
DEDICATED TO MY BELOVED PARENTS
ACKNOWLEDGEMENTS

Although only my name printed on the cover of this thesis, a great many people have contributed to my study. I owe my gratitude to many people who made this thesis possible.

First of all, my deepest gratitude is to my advisor, Dr. Pradeep Kachroo. I have been lucky to have an advisor who gave me positive thinking, enthusiasm, inspiration on my research, and at the same time, the guidance to recover when I was panic. Pradeep taught me how to pursue scientific research and various ways to sort thought complex data set. I wish one day I would like become as passionate an advisor to my students as Pradeep has been to me. I would have been lost without him. I also deeply grateful to Dr. Aardra Kachroo for her wide knowledge, logical scientific thoughts, and discussions that helped me sort out the unsolved problems. I wish to express my warm and sincere thanks to Dr. Ludmila Lapchyk for giving me constant love, encouragement, positive thinking, and invaluable support in the lab as well as my personal life. I can’t forget that forever. Thanks.

I am indebted to all past and current lab members of Drs Kachroo for providing a stimulating and fun environment in which to learn and grow. They inspired me in research and life during the long hours in the lab. In particular, the friendship of Mr. Qing-ming Gao and Dr. Shifeng Zhu is much appreciated and was always a great support in my struggles and frustrations. Thanks. I’m grateful to Dr. Chandra-Shekara for giving me wise advise and patiently introducing me to various projects. I also would like to extend my gratitude to Plant Pathology Department members for their assistance and hospitality.

I also wish to express gratitude to all my committee members, Dr. David Smith, Dr. Said A. Ghabrial, Dr. Guiliang Tang, and outside examiner Dr. Chentao Lin for their
invaluable advice. My special thanks to Dr. Lisa Vaillancourt for providing useful information and advise.

My sincere thanks also go to Dr. Duroy Navarre for salicylic acid estimation, Dr. Said Ghabrial for CMV isolation and CMV coat protein antibody, Dr. Peter Quail for phy mutants, Dr. Anthony Cashmore for cry1-304 and cry2-1 mutants, Dr. Chentao Lin for CRY2 antibody, Dr. John Christie for PHOT1 and PHOT2 antibodies, Dr. Winslow Briggs for phot1 and phot2 mutants, Dr. Gary Whitelam for phyB phyD phyE seed, and Dr. Ken-ichiro Shimazaki for PHOT1 and PHOT2 antibodies, Dr. Michael Goodin for transgenic Nicotiana plant expressing CFP-H2B and pSITE-myc vector, Dr. Jack Morris for TCV CP antibody, and Dr. Jeff Dangl for avrRPT2 and avrRPM1. I also thank Dr. James Carrington for rdr1-1, rdr2-1, rdr6-15, dcl1-7, dcl2-1, dcl3-1, and dcl4-2, Dr. Qu Fu for ago1-11 and drb4-1, Dr. Xuemei Chen for hen1-7, Dr. Zhixin Xie for ago2-1, Dr. Jonathan Clarke for ago3-1, Dr. Steven E. Jacobsen for ago4-1, and Dr. Hailing Jin for ago5-1, ago7-1, ago9-1.

I would like to convey my gratitude to my former advisers, Dr. Jang-Kyung Choi who was the first person to spark my interest in Plant Virology and Dr. Kook-Hyung Kim who introduced me to the world of plant defense to virus. Today I am here thanks to all of them.

Lastly, and the most importantly, I wish to thank my friends, parents, sisters, and parent-in-law for providing a loving environment, their support, encouragement, and sacrifices. I owe my loving thanks to my wife, Eunjung Lee, and my lovely daughter Sarene Jeong. They have lost a lot due to my study. Without their encouragement and understanding it would have been impossible for me to finish this work. I love you.
TABLE OF CONTENTS

Acknowlegements ................................................................. iii
List of tables ........................................................................ vii
List of figures ........................................................................ viii
List of files .............................................................................. x

CHAPTER 1: INTRODUCTION ....................................................... 1

CHAPTER 2: MATERIALS AND METHODS .................................. 3
   Plant growth conditions and genetic analysis ................................ 3
   Generation of transgenic plants .................................................. 3
   Arabidopsis transformation ....................................................... 4
   Sequencing ............................................................................. 4
   Chemical treatment ................................................................. 4
   Trypan-blue staining ............................................................... 5
   Bacterial transformation .......................................................... 5
   Pathogen infection .................................................................. 6
      Turnip Crinkle Virus ............................................................ 6
      Pseudomonas syringe Pv. tomato ........................................... 6
      Cucumber Mosaic Virus ....................................................... 7
   DNA extraction ....................................................................... 7
   RNA extraction, Reverse Transcriptase- Polymerase Chain Reaction and Northern analysis ................................................................. 7
   Synthesis of probe and hybridization ....................................... 8
   Enzyme linked immuno-sorbent assay ...................................... 9
   Extraction and quantification of salicylic acid (SA) and salicylic acid β-glucoside (SAG) ................................................................. 9
   Membrane fractionations, protein extraction, and immunoblot analysis ................................................................. 10
   Agrobacterium-mediated transient expression .......................... 11
   Confocal scanning microscopy and Bimolecular fluorescence assays ................................................................. 11

CHAPTER 3: HRT-mediated hypersensitive response and resistance to Turnip Crinkle Virus does not require the function of TIP, the presumed guardee protein ............... 19
   A mutation in TIP does not abolish HR or resistance to TCV ........... 21
   TIP is not required for SA-mediated induction of HRT .................. 23
   A mutation in TIP promotes replication of TCV and CMV ............ 24
   Discussion .............................................................................. 25

CHAPTER 4: Cryptome 2 and phototrophin 2 regulate resistance protein-mediated viral defense by negatively regulating an E3 ubiquitin ligase ...................... 39
   HRT is a plasma membrane localized protein that is degraded in the Dark ....... 42
   Mutation in blue-light photoreceptors compromises HRT-mediated resistance 43
   CRY2 and PHOT2 are required for posttranscriptional stability of HRT .......... 44
   Overexpression of HRT increases HRT levels in phot2 but not in cry2 background ................................................................. 45
   Blue-light treatment causes degradation of HRT ......................... 46
HRT interacts with COP1 and is degraded in a 26S proteasome dependent manner ........................................................47
Mutations in blue-light photoreceptors do not alter resistance to bacterial pathogen ........................................................................................................49
Discussion ........................................................................................................50

CHAPTER 5: Multiprotein complex containing RNA silencing components mediate resistance signaling against Turnip crinkle virus ........................................71
RDR6 and DCL4 are required for HRT-mediated resistance but not HR ..........74
RDR6 associates with the HRT-interacting CRT1 ..............................................75
The DCL4-interacting DRB4 associates with HRT and regulates its stability...76
CRT1 interacts with DRB4 and is required for the stability of HRT ...........77
Viral avirulence effector, coat protein, dissociates the HRT-DRB4 complex...78
HEN1 is not required for HRT-mediated signaling ........................................79
AGO1 interacts with DRB4 and is part of the HRT complex ......................80
DRB4 is required for RPS2- and RPM1-mediated resistance to bacterial pathogens ........................................................................................................82
Discussion ........................................................................................................82

Appendix-A List of abbreviations ..................................................................115
References .......................................................................................................117
Vita ....................................................................................................................127
LIST OF TABLES

Table 2.1, Seed materials used in the study ..........................................................13
Table 2.2, List of primers used in this study..........................................................15
Table 3.1, Segregation of resistance in Di-17 x Col-0 and Di-17 x tip plants ..........28
Table 4.1, Epistatic analysis of F2 population derived from crosses between Di-17 and
various wild-type or photoreceptor mutants...........................................................51
Table 5.1, Epistatic analysis of F2 population derived from crossed between Di-17 and
various wild-type or RNA silencing component mutants......................................85
LIST OF FIGURES

Figure 3.1. Isolation of a knock-out (KO) mutation in TIP ........................................29
Figure 3.2. Morphological and molecular phenotypes in HRT tip plants .................30
Figure 3.3. HR formation, HRT transcript levels, TCV resistance and systemic spread of
TCV in HRT tip plants .................................................................................................32
Figure 3.4. Molecular analysis of HRT ssi2 tip plants .............................................34
Figure 3.5. Basal resistance in tip plants .....................................................................36
Figure 3.6. Phylogenetic tree of 46 NAC proteins that showed homology to TIP .......38
Figure 4.1. Characterization of transgenic plants Di-17 and Col-0 lines expressing HRT-
FLAG transgene ........................................................................................................52
Figure 4.2. Morphological phenotype, systemic spread and HRT expression levels in
dark-treated plants ....................................................................................................53
Figure 4.3. HRT-FLAG protein is degraded in dark ..................................................54
Figure 4.4. Mutations in blue-light photoreceptors compromise HRT-mediated resistance
.......................................................................................................................................56
Figure 4.5. Systemic spread HRT expression, HR formation, and levels of SA and SAG
........................................................................................................................................58
Figure 4.6. HRT-FLAG protein and transcript levels, PR-1 expression, HR and resistance
to TCV in plants pretreated with BTH ......................................................................60
Figure 4.7. Blue-light causes degradation of HRT-FLAG and overexpression of HRT is
unable to increase HRT-FLAG levels in cry2 plants ..............................................63
Figure 4.8. HRT interacts with COP1 and is degraded via 26S proteasome pathway ...65
Figure 4.9. Localization of TCV-CP and bimolecular fluorescence complementation
assays showing interaction between COP1-HRT and PHOT2-COP1 ........68
Figure 4.10. RPS2- and RPS4-mediated resistance are not dependent on blue-light
photoreceptors ..............................................................................................................70
Figure 5.1. HRT-mediated resistance is dependent on RDR6 and DCL4 .................87
Figure 5.2. Morphological and biochemical phenotypes of HRT rdr and HRT dcl plants
........................................................................................................................................89
Figure 5.3. CRT1 interacts with RDR6. (A) Confocal micrographs showing BiFC for
indicated proteins ..............................................................................................................91

Figure 5.4. HRT does not interact with DCL4. (A) Coimmunoprecipitation of CRT1-MYC and RDR6-MYC with HRT-FLAG .................................................93

Figure 5.5. HRT-mediated resistance is dependent on DRB4 ........................................94

Figure 5.6. HRT drb4 plants accumulate normal levels of SA and HRT transcript ......96

Figure 5.7. CRT1 and DRB4 form a complex with HRT and negatively regulate COP1..
....................................................................................................................................97

Figure 5.8. DRB4 and CRT1 self interact with each other .............................................99

Figure 5.9. DRB4 and CRT1 interact with COP1 .........................................................100

Figure 5.10. CP dissociates HRT-DRB4 complex and relocalizes to nucleus in presence

of CRT1 and DRB4 .........................................................................................................101

Figure 5.11. CP does not dissociates DCL4-DRB4 and AGO1-DRB4 complex.............103

Figure 5.12. HEN1 is not required for TCV-induced HR or PR-1 expression ..........106

Figure 5.13. AGO1 interacts with HRT via DRB4 .....................................................107

Figure 5.14. CP facilitates interaction between HRT and AGO1...............................108

Figure 5.15. Typical morphological phenotypes of TCV inoculated Di-17, HRT ago and

Col-0 plants. Plants were photographed at 14 dpi ......................................................112

Figure 5.16. DRB4 is required for RPS2- and RPM1-mediated resistance..............113
LIST OF FILES

Rae-Dong_ Jeong_2011.pdf..........................................................141.5 megabytes
CHAPTER 1
INTRODUCTION

The Arabidopsis-TCV pathosystem

Turnip Crinkle Virus (TCV) is a small icosahedral virus belonging to the carmovirus group. Its 4 kb genome consists of a single-stranded, positive-sense RNA and encodes five open reading frames. Most Arabidopsis ecotypes are susceptible to TCV; TCV spreads systemically in susceptible plants, which is associated with a crinkled leaf and drooping bolt appearance (Dempsey et al., 1997; Kachroo et al., 2000). A resistant line, designated Di-17, was isolated from the Dijon (Di) ecotype (Dempsey et al., 1997). Following TCV infection, Di-17 plants develop hypersensitive response (HR), express several defense genes, and accumulate salicylic acid (SA) (Dempsey et al., 1997; Kachroo et al., 2000). HR to TCV is governed by a dominant gene, HRT, which encodes a protein with homology to coiled coil (CC)-nucleotide binding site (NBS)-leucine rich repeat (LRR) class of resistance (R) proteins. Plants lacking HRT fail to develop HR after TCV infection and do not induce defense gene expression or SA accumulation. However, HRT alone is not sufficient to confer TCV resistance, since all F1 plants and ~75% of HR-developing F2 plants derived from a cross between resistant (Di-17) and susceptible (Columbia; Col-0) ecotypes succumb to disease (Kachroo et al., 2000). Furthermore, ~90% of transgenic Col-0 plants expressing the HRT transgene are susceptible to TCV even though these plants develop HR upon TCV inoculation (Cooley et al., 2000). Subsequent studies showed that the recessive allele of a second, as yet unidentified, locus designated rrt (regulates resistance to TCV), is also required for resistance (Kachroo et al., 2000; Chandra-Shekara et al., 2004). Thus, Arabidopsis-TCV system is one of the few tractable plant-virus systems in which resistance is conferred via pathogen-induced defense responses and where HR and resistance can be studied as two distinct phenotypes. The Arabidopsis-TCV system also provides many other advantages for studying incompatible plant-viral interactions. Arabidopsis is a useful model host, which can be manipulated at the genetic and molecular levels using routine techniques.
Similarly, TCV has been characterized extensively using molecular, biochemical and structural tools.

This study was undertaken to decipher molecular and biochemical processes underlying HRT-mediated signaling. Previous work from our laboratory showed that SA pathway and light plays a critical role in HRT-mediated resistance (Chandra-Shekara et al., 2004; Chandra-Shekara et al., 2006; Kachroo et al., 2000). Here, I first evaluated role of TCV-coat protein interacting protein in HR and resistance to TCV (Chapter 3). Role of light was studied by assaying contribution of various photoreceptors in HRT-mediated signaling (Chapter 4). Lastly, I studied role of RNA silencing components in HRT-mediated signaling (Chapter 5). Together, results obtained here suggest a complex network of interaction between components of SA pathway, light photoreceptors and RNA silencing pathways, which regulate HRT-mediated resistance to TCV.
CHAPTER 2

MATERIALS AND METHODS

Plant growth conditions and genetic analysis
The seeds were sown on soil and subjected to overnight cold treatment to achieve synchronized germination. The plants were grown in MTPS 144 (Conviron, Winnigen, MN, Canada) walk-in chamber at 22°C, 65% relative humidity and 14 h photoperiod. The photon flux density (PFD) of the day period was 106.9 µmoles m\(^{-2}\)s\(^{-1}\) (measured using a digital light meter, Phytotronic Inc, MO). Fluence rates were measured using LI-1400 data logger fitted with remote quantum sensor (LiCOR, Lincoln, NE). Light spectra was measured using multi spectroradiometer (EPP 2000-VIS-200, StellarNet Inc, FL). After viral inoculations, the plants were transferred to a Conviron MTR30 reach-in chamber maintained at 22°C, 65% relative humidity and 14h photoperiod. Dark treatments were carried out in a similar chamber maintained at 22°C, 65% relative humidity (0 µmoles m\(^{-2}\)s\(^{-1}\)). For blue-light treatment, light was filtered through a blue Roscolene filter and the spectra of the filtered light was measured using spectroradiometer. Genotypes used in this study are listed in Table 2.1. All crosses were performed by emasculating the flowers of recipient genotype and pollinating with the pollen from the donor. The wild-type (wt) and mutant alleles were identified by PCR, Cleaved amplified polymorphic sequences (CAPS) (Konieczny and Ausubel, 1993), or derived (d)-CAPS (Neff et al., 1998) analysis. The primers used for genotyping are listed in Table 2.2.

Generation of transgenic plants
The HRT-FLAG construct was created by ligating the 5’ 2355 bp fragment of the HRT coding region with a 3’ 375 bp fragment fused to the FLAG epitope (DYKDDDDK). The full length HRT-FLAG coding region was cloned into pBluescript II KS\(^{+}\), sequenced and recloned downstream of the HRT promoter in the binary vector pBAR1. For the HRT-GFP construct, the FLAG epitope was replaced with GFP. The pBAR-HRT FLAG and pBAR-HRT-GFP constructs were electroporated into Agrobacterium (MP90) and transformed into Di-17, Col-0, HRT cry2, and HRT phot2.
**Arabidopsis transformation**

*Agrobacterium tumefaciens* strain MP90 was cultured overnight in 500 ml LB and centrifuged for 20 min at 6,000 rpm to pellet cells. The pellet was dissolved in transformation solution (one liter contained 2.15 g Murashige and Skoog (MS) basal salt mixture, 30 g sucrose (3%), 0.5 mL of Silwett-L-77, sterile, and the solution was adjusted to a pH 5.7 by 1 M KOH). The transformation solution was added to square containers and plants were immersed (pot upside-down) into the solution. Two pots/containers were placed inside a desiccator and infiltrated under vacuum. After 5-7 min, the pots were removed and the plants were rinsed gently under tap water. The transformed plants were placed under a dome for 12-24 h after which the plants were allowed to seed.

**Sequencing**

The sequencing reaction was carried out in 10 µL total volume containing 50 µg of PCR- or gel-purified-DNA (Qiagen, CA, USA), 1 µL of 5 µM primer and 1 µL of BigDye Terminator V3.1 (Applied Biosystems, CA, USA). The reaction product was precipitated, washed with 70% alcohol and air-dried before submitting to sequencing facility at Advanced Genetic Technologies Center (AGTC), Department of Plant Pathology, University of Kentucky.

**Chemical treatments**

Three-week-old plants were sprayed or subirrigated with a solution of 500 µM SA (Sigma, USA) or 100 µM BTH (CIBA-GEIGY Ltd, USA). Control plants were treated with water and two days after treatment, three leaves per plant were inoculated with TCV.

For 26S proteosome- and plant protease-inhibitor experiments, 100 µM MG132 (Sigma-Aldrich) or a mixture of plant protease inhibitors (Sigma-Aldrich) were dissolved in 0.1% Dimethyl sulfoxide (DMSO) and infiltrated into the abaxial surface of Arabidopsis leaves using a needle-less syringae. The protease inhibitor cocktail contained pepstain A, leupeptin, bestatin, 4-(2-aminoethyl) benzenesulfonyl fluoride, 4-guanidino and 1,10-
phenanthroline. Dexamethasone (Sigma) in methanol was used at a concentration of 0.1 to 20 µM.

**Trypan-blue staining**

The leaves were vacuum-infiltrated with trypan-blue stain prepared in 10 mL acidic phenol, 10 mL glycerol, and 20 mL sterile water with 10 mg of trypan blue. The samples were placed in a heated water bath (90°C) for 2 min and incubated at room temperature for 2-12 h. The samples were destained using chloral hydrate (25 g/10 mL sterile water; Sigma), mounted on slide and observed for cell death under compound microscope. The samples were photographed using AxioCam camera (Zeiss, Germany) and images were analyzed using Openlab 3.5.2 (Improvision) software.

**Bacterial transformation**

Heat-shock and electrophoresis method were used for bacterial transformation. For heat-shock transformation, a single isolated colony of DH5α cells (Invitrogen) was grown overnight in 5 mL LB broth at 37°C. A 1% inoculum from overnight grown culture was transferred into 100 mL LB broth, grown to an OD of 0.5 (A600) and chilled on ice for 15 min. The cells were centrifuged at 3000 rpm for 10 min at 4°C, and the pellet was suspended in 50 mL ice-cold Tfb I buffer containing 30 mM Potassium acetate pH 5.8, 100 mM RbCl2, 10 mM CaCl2 and 15% glycerol. After 30 min incubation on ice, the cells were again centrifuged at 3000 rpm for 10 min and the pellet was resuspended in 5 mL of ice-cold Tfb II buffer (10 mM MOPS pH 6.5, 75 mM CaCl2 10 mM RbCl2, 15% glycerol). After 15 min incubation on ice, these cells were dispensed as 100 mL aliquots in 1.5 mL microfuge tubes and stored at -80°C till further use. For transformation ~50 ng of DNA was mixed with 100 µL of competent cells, incubated on ice for 30 min, followed by heat shock at 42°C for 90 Sec. The cells were chilled on ice for 5 min, mixed with 1 mL of LB broth and incubated at 37°C for 1 h.

For electroporation transformation, a single isolated colony of DH5α, MP90, or LBA4404 was grown overnight in 5 mL LB broth at 37°C or 29°C. A 1% inoculum from overnight grown culture was transferred into 100 mL LB broth, grown to an OD of 0.5
(A$_{600}$) and chilled on ice for 15 min. The cells were centrifuged at 3000 rpm for 10 min at 4°C, and the pellet was suspended in cold autoclaved 8.0% glycerol water. These cells were dispensed as 20 µL aliquots in 1.5 mL microfuge tubes and stored at -80°C till further use. For transformation ~50 ng of DNA was mixed with 20 µL of competent cells, placed in pre-cooled cuvet and given a pulse at 2500 volts (12.5 kV/cm). The suspension was transferred to a 1.5 mL microfuge tube containing LB broth tube and incubated for 1 hr at 37°C. The transformed cells were plated on LB-agar plates containing appropriate antibiotic and incubated overnight at 29°C or 37°C.

**Pathogen infection**

**Turnip Crinkle Virus (TCV):**
The 6 µg of linearized pT7TCV66 (Sma I) was used to synthesized TCV RNA using T7 RNA polymerase (NEB, USA), 5x transcription buffer (Promega, USA), 0.1M DTT, rNTPs (10mM each), RNase inhibitor (Promega, USA), and Diethyl pyrocarbonate (DEPC) treated water. After incubation at 37°C for 1h, TCV RNA was purified with phenol: chloroform: isoamyl alcohol (25:24:1) and precipitated with isopropanol. Viral RNAs were resuspended in DEPC water and used for viral infections.

**Pseudomonas syringe** *Pv. tomato*:
The bacterial strain DC3000 derivatives containing pVSP61 (empty vector), *avrRpt2*, *avrRps4* or *avrRpm1* were grown overnight in King’s B medium containing rifampicin (Sigma, USA). The bacterial cells were harvested, washed and suspended in10 mM MgCl$_2$. The cells were diluted to a final density of 10$^5$ to 10$^7$/mL (A$_{600}$) and used for infiltration. The bacterial suspension was injected into the abaxial surface of the leaf using a needle-less syringe. Three leaf discs from the inoculated leaves were collected at 0, 3 or 6 dpi. The leaf discs were homogenized in 10 mM MgCl$_2$, diluted 10$^3$ or 10$^4$ fold and plated on King’s B medium. The plates were incubated at 29°C and colonies were counted using Colony counter (Fisher Scientific, USA).
Cucumber Mosaic Virus (CMV):
50-100 µg of CMV-infected tobacco leaves was homogenized with 100 mL of 0.1M ammonium citrate buffer (pH 6.5) containing 10 mM EDTA and 0.1% (v/v) 2-mercaptoethanol and 100 mL of chloroform. The homogenate was centrifuged at 6,000 rpm for 10 min (Thermo, Sorvall) and the supernatant was centrifuged at 40,000 rpm for 2 hr at 4 °C (Beckman, L8-55M 30K rotor). The pellet was resuspended in 10 mL of 0.1M ammonium citrate buffer (pH 6.5) containing 10 mM EDTA. After incubation at 4 °C for 1 h, the suspension was centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was added to 0.5 mL of 20% Triton X-100, mixed gently, and centrifuged at 40,000 rpm for 2 h at 4 °C. The pellet was resuspended with 5 mM sodium borate (pH 9.0) buffer containing 0.5 mM EDTA. The purified CMV (100 µg) was used for plant inoculations.

**DNA extraction**
Small scale DNA extraction was carried out from a single Arabidopsis leaf. Leaf sample was frozen in liquid nitrogen and ground using disposable pestle (Fisher Scientific, USA). The extract was suspended in 150 µL of DNA extraction buffer containing 200 mM Tris, 25 mM EDTA, 1% SDS and 250 mM NaCl. The homogenate was mixed with 75 µL of phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged for 10 min at high speed. The supernatant was precipitated with 100 µL of isopropanol and centrifuged immediately for 10 min at high speed. The DNA pellet was air dried and suspended in 40-75 µL of DEPC-water or Tris:EDTA (10:1, pH 8.0).

**RNA extraction, Reverse Transcriptase-Polymerase Chain Reaction and Northern analysis**
RNA extraction was carried out using Trizol reagent (Invitrogen). Two or three Arabidopsis leaves were frozen in liquid nitrogen, ground using disposable pestles and suspended in 1 mL of Trizol. To this 200 µL of chloroform was added and the samples were centrifuged at high speed for 15 min. The supernatant was precipitated with 0.5 mL of isopropanol. The RNA precipitate was washed with 75% alcohol, air dried and suspended in 15-20 µL of DEPC treated water. The RNA was quantified
spectrophotometrically ($A_{260}$) and 7 µg of total RNA was electrophoresed on 1.5% agarose gel containing 3% formaldehyde and 1X MPOS. MOPS buffer was prepared by mixing 4.18 g MOPS, 680 mg NaOAc, 37 mg EDTA in 1 L sterile water and adjusted to pH 7.0. Before loading, RNA was mixed with with 39 µg/mL ethidium bromide, 0.39X MOPS, 13.7% formaldehyde and 39% formamide, denatured at 65°C for 15 min, chilled on ice for 15 min and mixed with 2µL of RNA loading dye (50% glycerol, 1mM EDTA, 0.4% bromophenol blue and 0.4% xylene cyanol).

For cDNA synthesis, total RNA was denatured at 65°C and annealed to oligo dT$_{17}$. The reaction mixture was supplemented with 1 µL reverse transcriptase (200U/µL, Invitrogen, USA), 1 µL RNAase inhibitor (40U/µL), 0.5 mM dNTPs and 10 mM DTT and incubated at 42°C for 1 h. The reaction was stopped by incubating the tubes at 75°C for 15 min and subsequently used for RT-PCR.

For northern analysis, RNA was transferred onto Hybond-NX (GE Healthcare) nylon membrane. After overnight wet-transfer, RNA was fixed under UV for 0.9 min in a CL-1000 ultraviolet Cross-linker (UVP). The membrane was washed in 2xSSC, dried at 65°C and used for hybridization. The membrane was hybridized in sodium phosphate buffer (pH 7.0) containing sheared salmon sperm DNA (100 µg/mL), 7% SDS and 1.25 mM EDTA.

**Synthesis of probe and hybridization**

DNA fragments were labeled using DNA polymerase I Klenow fragment. DNA fragments used for labeling were PCR- or gel-purified (Qiagen, USA), denatured and mixed with Klenow enzyme (NEB, 2000U/mL), hexanucleotide primers, dATP, dGTP, dTTP, BSA and 50 µCi α-32P-dCTP (Perkin Elmer, USA). The reaction was incubated at 37°C for 1 h and purified using MicroSpin G-50 sephadex column (GE Healthcare). The labeled DNA was denatured using one-tenth volume of 2N NaOH, neutralized with 1M Tris pH 7.5 and added to the hybridization buffer. Hybridization was routinely carried out overnight. The hybridized membrane was washed once at room temperature with 2xSSC, 0.5% SDS, twice at 65°C with 2xSSC, 0.5% SDS and once at 65°C with 1xSSC,
0.1% SDS solutions. The membrane was exposed using a Storage Phosphor Screen (Amersham Biosciences) and scanned on a Typhoon 9400 Variable Mode Imager (GE Healthcare). The signal intensity was quantified using ImageQuant TL V2005 software.

**Enzyme linked immuno-sorbent assay (ELISA)**

Total protein was extracted in buffer containing 50 mM Tris (pH 8.0), 1 mM EDTA, 12 mM β-mercaptoethanol and 10 µg ml⁻¹ phenylmethylsulfonyl fluoride (PMSF). Proteins were fractionated on a 10-12% SDS-PAGE to confirm the quality. The extracted total protein was added in coating buffer (1.59 g of Na₂CO₃, 2.93 g of NaHCO₃ and 0.2 g of NaN₃ to 1 liter) to microtiter plates and then plates were incubated for 1 h at 37°C. The plates were washed with PBS-Tween (0.1 %) (120g of NaCl, 3 g of KH₂PO₄, 17.3g of Na₂HPO₄, 3g of KCl, and 3g of NaN₃ for 3 liters of 5x PBS)-Tween three times of at least three minutes per wash. 100 µl of blocker (0.5g of BSA in 100 mL PBS) was added in the plates and incubated for 1 h at 37°C. After incubation, plates were washed three times with PBS-Tween. 100 µl antiserum diluted 1:5,000 in 0.5% BSA in PBS containing 0.5% BSA, was added and plates were incubated for 1 h at 37°C. After incubation, plates were washed three times with PBS-Tween followed by addition of goat anti-rabbit-alkaline phosphatase conjugate antibody diluted 1:1,000 in PBS containing 0.5% BSA. The plates were incubated for 1 h at 37°C. After the incubation, plates were washed three times with PBS-Tween followed by addition of 200 µl of substrate (Sigma) and the plates were were read at 405 nm in a plate reader at appropriate intervals.

**Extraction and quantification of salicylic acid (SA) and salicylic acid β-glucoside (SAG)**

SA and SAG were extracted from ~300 mg of leaves using anisic acid as internal standard. Samples were analyzed on an Agilent 1100 (Agilent Technologies) with diode-array detector and fluorescence-array detector detection, using a Novapak C18 column (Waters, Milford). Sample extraction and analysis was carried out in collaboration with Dr. Duroy Navarre’s laboratory at USDA-ARS, Prosser, Washington.


Membrane fractionations, protein extraction and immunoblot analysis

For total protein, plant tissues were grounded with liquid-nitrogen and protein extraction buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 5 mM DTT, 0.5% Triton-X-100, and 1 X protease inhibitor cocktail. Protein concentration was determined by using Bio-Rad protein assay kit.

For membrane fractionation, tissues were ground in liquid nitrogen and suspended in extraction buffer (50 mM Tris-MES, pH 8.0, 0.5 M sucrose, 1 mM MgCl₂, 10 mM EDTA, 10 mM EGTA, 10 mM ascorbic acid, 5 mM DTT, protease inhibitor cocktail (Sigma-Aldrich, USA). All fractionation steps were carried out at 4 °C. The total extract (T) was centrifuged at 10,000 x g for 10 min. The supernant (S) was centrifuged again at 125,000 x g (45,000 rpm) for 1 h to remove any insoluble material. The pellet (membrane fraction) was resuspended in a detergent free buffer (5 mM potassium phosphate, pH 7.8, 2 mM DTT, 1x protease inhibitor cocktail).

For Ponceau-S staining, PVDF membranes were incubated in Ponceau-S solution (40% methanol (v/v), 15% acetic acid (v/v), 0.25% Ponceau-S). The membranes were destained using deionized water.

For Co-immunoprecipitation, after Agrobacterium-mediated transient expression for 48 h, *Nicotiana benthamiana* leaves (approximately 0.3 g) were harvested and ground to a powder in liquid nitrogen. Ground tissues were resuspended in 2-3 mL of IP buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Nonidet P-40, 5 mM dithithreitol, and 1 x protease inhibitor cocktail). The crude lysates were then centrifuged at 13,000 rpm for 15 min at 4 °C. After centrifugation, 1 mL of supernatant was incubated overnight with 30 µl of anti-FLAG M2 affinity gel (Sigma) for overnight. After incubation, the pellet was washed three times with 1 mL of IP buffer and the pellet was resuspended in 3x SDS-PAGE loading buffer. The samples were boiled for 5 min prior to loading on a SDS-PAGE gel.

For preparation of high-purity plasma membranes, the complete plasma membrane purification was carried out at 4 °C. 10 g of Arabidopsis leaves were ground using a pre-
cooled mortar and pestle in 10 mL of extraction buffer (0.5 M sucrose, 50 mM HEPES-KOH, pH 7.5, 5 mM ascorbic acid, 1 mM DTT, and 0.6 % (w/v) (vinylpyrrolidone). The homogenate was filtered (240-μm nylon cloth) and most of the chloroplasts and mitochondria were pelleted at 13,000 rpm for 10 min. A microsomal pellet was obtained after centrifugation of the supernatant at 45,000 rpm for 30 min. This pellet is suspended to a total volume 10 mL in 0.33 M sucrose, 3 mM KCl, 5 mM potassium phosphate, pH 7.8. This suspension was added to the 27.0-g phase mixture (11.16 g of 20% (w/w) dextran T-500, 5.58 g of 40% (w/w) polyethylene glycol 3350, 3.05 g of sucrose, 0.675 ml of 0.2 M potassium phosphate, pH 7.8, 0.041 mL of 2 M KCl, and added water to a final weight of 27.00 g) to give a 36.0-g phase system with a final composition of 6.2 % (w/w) polyethylene glycol 3350, 0.33 M sucrose, 3 mM KCl, 5 mM potassium phosphate, pH 7.8. The phase system was thoroughly mixed by 20-30 inversions of the tube and phase settling was facilitated by centrifugation in a swinging bucket centrifuge at 3,000 rpm for 5 min. The upper phase (U1) was carefully transferred to a new tube and extracted again with lower phase as above to obtain U1. In the same manner, the lower phase (L0) was reextracted with fresh upper phase to yield L1. Phase U1 (PM) and L1 (endosome) were diluted with 2x and 10x microsomal buffer, respectively, and then centrifuged at 45,000 rpm and 4 °C for 1 h to pellet the membrane. The supernant was removed and membrane pellets were boiled in 300 μl of 3 x SDS-PAGE sample buffer prior to gel electrophoresis.

Agrobacterium-mediated transient expression

For transient expression, Agrobacterium was grown overnight at 28 °C on Luria-Bertani broth containing appropriate antibiotics. Cells were resuspended in induction media (10 mM MES, pH 5.6, 10 mM MgCl2, and 150 μM acetosyringone) and incubated at room temperature for 3 h prior to infiltration into N. benthamiana leaves.

Confocal scanning microscopy and Bimolecular fluorescence assays

For confocal imaging, samples were scanned on an Olympus FV 1000 microscope (Olympus America) equipped with a water immersion PLAPO60XWLSM 2 (NA 1.0) objective and lasers spanning the spectral range of 405-633 nm. Green fluorescence
protein (GFP) was excited using 488 nm laser line. For Bimolecular fluorescence (BiFC) assays, the various proteins were fused to the N/C-terminal halves of E-yellow fluorescence protein (YFP) (nEYFP/cEYFP) using the pSITE-3CA-EYFP vectors. The various construct were introduced in *A. tumefaciens* C58C1 strain. Agrobacterium strains carrying various proteins were infiltrated into CFP-H2B tagged *N. benthamina* transgenic plants expressing nuclear localized CFP or wild-type *N. benthamiana* plants. For single protein GFP or Red fluorescence protein (RFP) localization, the various proteins were fused into pSITE-3CA-GFP or pSITE-3CA-RFP vectors and these constructs were introduced in *A. tumefaciens* C58C1. Agrobacterium strains carrying various proteins were introduced into wild-type or GFP-tagged ER or RFP-tagged endoplasmic reticulum (ER) transgenic plants. 48 h later, water-mounted sections of leaf tissues were examined by confocal microscopy. CFP and YFP overlay images or GFP and RFP overlay images (40X magnification) were acquired at a scan rate of 10 ms/pixel. Olympus FLUOVIEW 1.5 was used to control the microscope, image acquisition and the export of TIFF files.
Table 2.1. Seed materials used in the study.

<table>
<thead>
<tr>
<th>Sl No.</th>
<th>Mutants and transgenic seeds</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Columbia-0 (Col-0)</td>
<td>Kachroo et al. (2003)</td>
</tr>
<tr>
<td>2</td>
<td>Dijon (Di-17)</td>
<td>Kachroo et al. (2000)</td>
</tr>
<tr>
<td>3</td>
<td>Ler (Landsberg erecta)</td>
<td>Kachroo et al. (2003)</td>
</tr>
<tr>
<td>4</td>
<td>ssi2</td>
<td>Kachroo et al. (2001)</td>
</tr>
<tr>
<td>5</td>
<td>HRT ssi2</td>
<td>Chandra-Shekara et al. (2004)</td>
</tr>
<tr>
<td>6</td>
<td>tip</td>
<td>Jeong et al. (2008)</td>
</tr>
<tr>
<td>7</td>
<td>HRT tip</td>
<td>Jeong et al. (2008)</td>
</tr>
<tr>
<td>8</td>
<td>HRT ssi2 tip</td>
<td>Jeong et al. (2008)</td>
</tr>
<tr>
<td>9</td>
<td>phyA-201</td>
<td>Nagatani et al. (1993)</td>
</tr>
<tr>
<td>10</td>
<td>phyB-1</td>
<td>McCormac et al. (1993)</td>
</tr>
<tr>
<td>11</td>
<td>phyC-1</td>
<td>Franklin et al. (2003)</td>
</tr>
<tr>
<td>12</td>
<td>phyD-1</td>
<td>Aukerman et al. (1997)</td>
</tr>
<tr>
<td>13</td>
<td>phyE-1</td>
<td>Devlin et al. (1998)</td>
</tr>
<tr>
<td>14</td>
<td>cry1-304</td>
<td>Lin et al. (1996)</td>
</tr>
<tr>
<td>15</td>
<td>cry2-1</td>
<td>Guo et al. (1997)</td>
</tr>
<tr>
<td>16</td>
<td>phot1-5</td>
<td>Huala et al. (1997)</td>
</tr>
<tr>
<td>17</td>
<td>phot2-1</td>
<td>Jarillo et al. (2001)</td>
</tr>
<tr>
<td>18</td>
<td>HRT phyA-201</td>
<td>Jeong et al. (2010)</td>
</tr>
<tr>
<td>19</td>
<td>HRT phyB-1</td>
<td>Jeong et al. (2010)</td>
</tr>
<tr>
<td>20</td>
<td>HRT phyC-1</td>
<td>Jeong et al. (2010)</td>
</tr>
<tr>
<td>21</td>
<td>HRT phyD-1</td>
<td>Jeong et al. (2010)</td>
</tr>
<tr>
<td>22</td>
<td>HRT phyE-1</td>
<td>Jeong et al. (2010)</td>
</tr>
<tr>
<td>23</td>
<td>HRT cry1-304</td>
<td>Jeong et al. (2010)</td>
</tr>
<tr>
<td>24</td>
<td>HRT cry2-1</td>
<td>Jeong et al. (2010)</td>
</tr>
<tr>
<td>25</td>
<td>HRT phot1-5</td>
<td>Jeong et al. (2010)</td>
</tr>
<tr>
<td>26</td>
<td>HRT phot2-1</td>
<td>Jeong et al. (2010)</td>
</tr>
<tr>
<td>27</td>
<td>HRT phyA phyB</td>
<td>Jeong et al. (2010)</td>
</tr>
<tr>
<td>28</td>
<td>HRT cry1 cry2</td>
<td>Jeong et al. (2010)</td>
</tr>
<tr>
<td>29</td>
<td>HRT phot1 phot2</td>
<td>Jeong et al. (2010)</td>
</tr>
<tr>
<td>30</td>
<td>HRT-FLAG (Di-17)</td>
<td>Jeong et al. (2010)</td>
</tr>
<tr>
<td>31</td>
<td>HRT-FLAG (Col-0)</td>
<td>Jeong et al. (2010)</td>
</tr>
<tr>
<td>32</td>
<td>HRT-FLAG cry1</td>
<td>Jeong et al. (2010)</td>
</tr>
<tr>
<td>33</td>
<td>HRT-FLAG cry2</td>
<td>Jeong et al. (2010)</td>
</tr>
<tr>
<td>34</td>
<td>HRT-FLAG phot1</td>
<td>Jeong et al. (2010)</td>
</tr>
<tr>
<td>35</td>
<td>HRT-FLAG phot2</td>
<td>Jeong et al. (2010)</td>
</tr>
<tr>
<td>36</td>
<td>rdr1-1</td>
<td>Xie et al. (2004)</td>
</tr>
<tr>
<td>37</td>
<td>rdr2-1</td>
<td>Xie et al. (2004)</td>
</tr>
<tr>
<td>38</td>
<td>rdr6-15</td>
<td>Xie et al. (2004)</td>
</tr>
<tr>
<td>39</td>
<td>dcl1-7</td>
<td>Xie et al. (2003)</td>
</tr>
<tr>
<td>40</td>
<td>dcl2-1</td>
<td>Xie et al. (2004)</td>
</tr>
<tr>
<td>41</td>
<td>dcl3-1</td>
<td>Xie et al. (2004)</td>
</tr>
<tr>
<td>42</td>
<td>dcl4-2</td>
<td>Xie et al. (2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>43</td>
<td><em>ago1-11</em></td>
<td>Haas et al. (2008)</td>
</tr>
<tr>
<td>44</td>
<td><em>ago2-1</em></td>
<td>Lobbes et al. (2006)</td>
</tr>
<tr>
<td>45</td>
<td><em>ago3-1</em></td>
<td>Lobbes et al. (2006)</td>
</tr>
<tr>
<td>46</td>
<td><em>ago4-1</em></td>
<td>Zilberman et al. (2004)</td>
</tr>
<tr>
<td>47</td>
<td><em>ago5-1</em></td>
<td>Katiyar-Agarwal et al. (2007)</td>
</tr>
<tr>
<td>48</td>
<td><em>ago7-1</em></td>
<td>Katiyar-Agarwal et al. (2007)</td>
</tr>
<tr>
<td>49</td>
<td><em>ago9-1</em></td>
<td>Katiyar-Agarwal et al. (2007)</td>
</tr>
<tr>
<td>50</td>
<td><em>drb4-1</em></td>
<td>Qu et al. (2008)</td>
</tr>
<tr>
<td>51</td>
<td><em>hen1-7</em></td>
<td>Park et al. (2002)</td>
</tr>
<tr>
<td>52</td>
<td><strong>HRT-rdr1-1</strong></td>
<td>Present work</td>
</tr>
<tr>
<td>53</td>
<td><strong>HRT-rdr2-1</strong></td>
<td>Present work</td>
</tr>
<tr>
<td>54</td>
<td><strong>HRT-rdr6-15</strong></td>
<td>Present work</td>
</tr>
<tr>
<td>55</td>
<td><strong>HRT-dcl1-7</strong></td>
<td>Present work</td>
</tr>
<tr>
<td>56</td>
<td><strong>HRT-dcl2-1</strong></td>
<td>Present work</td>
</tr>
<tr>
<td>57</td>
<td><strong>HRT-dcl3-1</strong></td>
<td>Present work</td>
</tr>
<tr>
<td>58</td>
<td><strong>HRT-dcl4-2</strong></td>
<td>Present work</td>
</tr>
<tr>
<td>59</td>
<td><strong>HRT-ago1-11</strong></td>
<td>Present work</td>
</tr>
<tr>
<td>60</td>
<td><strong>HRT-ago2-1</strong></td>
<td>Present work</td>
</tr>
<tr>
<td>61</td>
<td><strong>HRT-ago3-1</strong></td>
<td>Present work</td>
</tr>
<tr>
<td>62</td>
<td><strong>HRT-ago4-1</strong></td>
<td>Present work</td>
</tr>
<tr>
<td>63</td>
<td><strong>HRT-ago5-1</strong></td>
<td>Present work</td>
</tr>
<tr>
<td>64</td>
<td><strong>HRT-ago7-1</strong></td>
<td>Present work</td>
</tr>
<tr>
<td>65</td>
<td><strong>HRT-ago9-1</strong></td>
<td>Present work</td>
</tr>
<tr>
<td>66</td>
<td><strong>HRT-FLAG-rdr6-15</strong></td>
<td>Present work</td>
</tr>
<tr>
<td>67</td>
<td><strong>HRT-FLAG-dcl4-2</strong></td>
<td>Present work</td>
</tr>
<tr>
<td>Name</td>
<td>Primer</td>
<td>Purpose (enzyme)</td>
</tr>
<tr>
<td>------</td>
<td>--------</td>
<td>-----------------</td>
</tr>
<tr>
<td><strong>HRT</strong></td>
<td>AAT GCA GAG TTT AGG GAT ACA ACA CGT ATC CAA AAG TCT TCC TCC TTA</td>
<td>RT-PCR</td>
</tr>
<tr>
<td><strong>tip</strong></td>
<td>GGT CCA AAG GAC AAA AGA AGA G CGA ATT CTC AAA GTC TCA CGC</td>
<td>Genotyping</td>
</tr>
<tr>
<td><strong>TIP</strong></td>
<td>ATG AAA GAA GAC ATG GAA GTA CTA TC CTC TTT TGT CCT TTT GCC C</td>
<td>RT-PCR</td>
</tr>
<tr>
<td><strong>ssi2</strong></td>
<td>TTG GTG GGG GAC ATC ACA GAA GA AAG TAG GAC TAG CAC CTG TTT CAT CC</td>
<td>dCAPS (NsiI)</td>
</tr>
<tr>
<td><strong>HRT</strong></td>
<td>CAT TTC CCT ACC TCT AAT GGA TGA ATG ACT TTT GTG AAG CAG CCT CTA</td>
<td>CAPS (Alu I)</td>
</tr>
<tr>
<td><strong>phyA</strong></td>
<td>GAA GTG TTG ACT GCT TCC ACG AGT TAG CAA GAT CAA GAC AAC GCC</td>
<td>CAPS (Hinf I)</td>
</tr>
<tr>
<td><strong>phyB</strong></td>
<td>GTC AAG GTT CTT GTT TAA GC TCT TTT ATC TGG ACT TCA CT</td>
<td>dCAPS (Acc I)</td>
</tr>
<tr>
<td><strong>phyC</strong></td>
<td>ACT CAT GGA GAG AGG AAC ATT G ATC AGC GTT CTT TAA GGC AAC</td>
<td>Genotyping</td>
</tr>
<tr>
<td><strong>phyD</strong></td>
<td>GTC GTC ACA CCA GCG CTG CAG AAC TGT TTT TGG ACT CGT TTC CGC CAC</td>
<td>CAPS (Alu I)</td>
</tr>
<tr>
<td><strong>phyE</strong></td>
<td>GTC ACT TGC CGA TGA GAT TG CTC CAA AGA CTT CAC CGG G</td>
<td>CAPS (Hinf I)</td>
</tr>
<tr>
<td><strong>cry1-304</strong></td>
<td>GGT AGG GTT TCT AGG TGG TGG CTC GGT GGA AGA AGA GAC TCA GGG</td>
<td>Genotyping</td>
</tr>
<tr>
<td><strong>cry2-1</strong></td>
<td>GGT AGG GTT TCT AGG TGG TGG CTC GGG GGA AGA AGA GGA GAC TCA GGG</td>
<td>Genotyping</td>
</tr>
<tr>
<td><strong>phot1-5</strong></td>
<td>GAT AGT TTC TGG GAG CTC ACG GAA GAT CTG TTC CAA CGA GTT CCA CTA G</td>
<td>Genotyping</td>
</tr>
<tr>
<td><strong>phot2-1</strong></td>
<td>GCT CAC AAT TTT AGG TGA AAG CTA GTA TAC ACG AAG TAC AGG ATC</td>
<td>CAPS (Sau3AI)</td>
</tr>
<tr>
<td><strong>HRT</strong></td>
<td>TCT AAA GCC AAA GAA GAG AAC TTA GCT ATC CAA AGA GAA CTT</td>
<td>q-RT-PCR</td>
</tr>
<tr>
<td><strong>Actin</strong></td>
<td>CAC TGT GCC AAT CTA CGA GGG TT ACA ATTT TCC CGC TCT GCT GTT GTG</td>
<td>q-RT-PCR</td>
</tr>
<tr>
<td><strong>β-tubulin</strong></td>
<td>CGT GGA TCA CAG CAA TAC AGA GCC CCT CCT GCA CTT CCA CTT CGT CTT C</td>
<td>RT-PCR</td>
</tr>
<tr>
<td><strong>HRT attB</strong></td>
<td>AAA AAG CAG GCT TAA TGG CTG AAG CAT TTG TGT CTG AGA AAG CTT GGG TAC TAC TGG TCA CAG TTG ATA AAT</td>
<td>Gateway cloning</td>
</tr>
<tr>
<td>Gene</td>
<td>attB</td>
<td>Sequence</td>
</tr>
<tr>
<td>------</td>
<td>------</td>
<td>----------</td>
</tr>
<tr>
<td><strong>CRY2</strong></td>
<td></td>
<td>AAA AAG CAG GCT TAA TGA AGA TGG SACAA TAA AAG CTA T AGA AAG CTG GGT ATC ATT TGC AAC CAT TTT TTC CCA</td>
</tr>
<tr>
<td><strong>PHOT2</strong></td>
<td></td>
<td>AAA AAG CAG GCT TAA TGG AGA GGC CAA GAG CCC CTC C AGA AAG CTG GGT ATT AGA AGA GGT CAA TGT CCA AGT</td>
</tr>
<tr>
<td><strong>TCV</strong></td>
<td><strong>CP</strong>&lt;br&gt;<strong>attB</strong></td>
<td>AAA AAG CAG GCT TAA TGG AAA ATG ATC CTA GAG TCC G AGA AAG CTG GGT ACT AAA TTC TGA GTG CTT GCA ATT</td>
</tr>
<tr>
<td><strong>CIB1</strong></td>
<td><strong>attB</strong></td>
<td>AAA AAG CAG GCT TAA TGA ATG GAG CTA TAG GAG GTG A AGA AAG CTG GGT ATC AAA ATG ATC AAT TGC CAT AGA G</td>
</tr>
<tr>
<td><strong>COP1</strong></td>
<td><strong>attB</strong></td>
<td>AAA AAG CAG GCT TAA TGG AAG ATG TTT CGA CGG ATC C AGA AAG CTG GGT ATC ACG CAG CTA CCA GAA CTT</td>
</tr>
<tr>
<td><strong>CRT1</strong></td>
<td><strong>attB</strong></td>
<td>AAA AAG CAG GCT TAA TGG CGA AAA ATT ACA CAG TCG CC AGA AAG CTG GGT ACT AAA CTC TTA AAT TGC CAT AGA G</td>
</tr>
<tr>
<td><strong>rdr1-1</strong></td>
<td></td>
<td>TTG GAA GCT CAC GAG GCT CTT CCA ATC AAT CTC CTA ACG CCA</td>
</tr>
<tr>
<td><strong>rdr2-1</strong></td>
<td></td>
<td>TCT CTC TTC TCA AAG CAA GCT TTT GGT CCA TAT TTC AGC TGC</td>
</tr>
<tr>
<td><strong>rdr6-15</strong></td>
<td></td>
<td>GAA GAT GAA GTA GGA ATT GTT TGG CC GAC ACG GGG TGA AGA AGC CAG CTG</td>
</tr>
<tr>
<td><strong>dcl1-7</strong></td>
<td></td>
<td>TGA GAC ACA GTA TCA TTA GAA TGG GAA TCA AAT TGG CAG CAC CAT CGC</td>
</tr>
<tr>
<td><strong>dcl2-1</strong></td>
<td></td>
<td>GGT TAT AGG TTT GGA TTG CAT GCA GAT TTG GAT GCA GGG TCA AAT CTG</td>
</tr>
<tr>
<td><strong>dcl3-1</strong></td>
<td></td>
<td>GAG AGA TTT TGC ATT GCC TTG ATA GTG GAG GGG TTG TTT TCG GTT AAA</td>
</tr>
<tr>
<td><strong>dcl4-2</strong></td>
<td></td>
<td>GTT TCG TGC AAG AGG GAT TTT GCC G GAC TTG GTA GAA TCC TGT GGG G</td>
</tr>
<tr>
<td>Gene</td>
<td>Sequence</td>
<td>Genotyping</td>
</tr>
<tr>
<td>--------</td>
<td>----------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td>ago1-1</td>
<td>TAG GCA GGA GCT CAT TCA GG CGG ATG GCA TCA AGT TCA TA</td>
<td>CAPS (Bsr I)</td>
</tr>
<tr>
<td>ago2-1</td>
<td>TGT GGA AGA GGA ATT GAT TGG AGC ACC AAT GAA CAT GAC CTC</td>
<td>Genotyping (T-DNA insertion)</td>
</tr>
<tr>
<td>ago3-1</td>
<td>CGA TAG TCC CGA CTG ACT CTG AAA CAG AGA GAC AGT GGA CGC</td>
<td>Genotyping (T-DNA insertion)</td>
</tr>
<tr>
<td>ago4-1</td>
<td>TGG CTT GGC TAA CTA CGT ACG CAC AAA AAG TCA CAA ACC CAG</td>
<td>Genotyping (T-DNA insertion)</td>
</tr>
<tr>
<td>ago5-1</td>
<td>GGA TTG TCT CTC AGT GTT GCC TGA GCA TTT GCA ACT GAT CAG</td>
<td>Genotyping (T-DNA insertion)</td>
</tr>
<tr>
<td>ago7-1</td>
<td>GTA TTC TGG AGG CAG AGG AGC CTC CTC TTT TTC TTT TGC ACC</td>
<td>Genotyping (T-DNA insertion)</td>
</tr>
<tr>
<td>ago9-1</td>
<td>ATG AGT GGC CAT TGT CTT GAG TTT CCT TTT TGC TTG TGG ATG</td>
<td>Genotyping (T-DNA insertion)</td>
</tr>
<tr>
<td>drb4-1</td>
<td>AGG CGA TTC TCT TCG AAT TTC TTG TAG GCA ACA TCA ATT CCC</td>
<td>Genotyping (T-DNA insertion)</td>
</tr>
<tr>
<td>hen1-7</td>
<td>AGC AAT TCC TCA AAA AGG TCC TCG TGC ATT CCG AGA TTT TAC</td>
<td>Genotyping (T-DNA insertion)</td>
</tr>
<tr>
<td>crt1</td>
<td>GCA GTT TTA CTG ATC CGT AAC ATT GTG GAG CGT GAG TGA GAG</td>
<td>Genotyping (T-DNA insertion)</td>
</tr>
<tr>
<td>CRT1</td>
<td>AAA AAG CAG GCT TAA TGG CGA AAA ATT ACA CAG TCG CC AGA AAG CTG GGT ACT AAA CTT GTT GCA TCT CCT TCT T</td>
<td>Gateway cloning</td>
</tr>
<tr>
<td>RDR6</td>
<td>AAA AAG CAG GCT TAA TGG GGT CAG AGG GAA ATA TGA A AGA AAG CTG GGT ATT AGA GAC GCT GAG CAA GAA ACT</td>
<td>Gateway cloning</td>
</tr>
<tr>
<td>DCL4</td>
<td>AAA AAG CAG GCT TAA TGC GTG ACG AAG TTG ACT TGA G AGA AAG CTG GGT ATC AGC AAA GGA ATC CAG AAT GCT</td>
<td>Gateway cloning</td>
</tr>
<tr>
<td>SGS3</td>
<td>AAA AAG CAG GCT TAA TGA GTT CTA GGG CTG GTC CAA TG AGA AAG CTG GGT ATC AAT CAT CTT CAT TGT</td>
<td>Gateway cloning</td>
</tr>
<tr>
<td>Gene</td>
<td>Sequence</td>
<td>Technology</td>
</tr>
<tr>
<td>-------</td>
<td>----------</td>
<td>------------------</td>
</tr>
<tr>
<td><strong>DRB4</strong></td>
<td>AAA AAG CAG GCT TAA TGG ATC ATG TAT ACA AAG GTC AA AAG AAG CTG GGT ATT ATG GCT TCA CAA GAC GAT AGG C</td>
<td>Gateway cloning</td>
</tr>
<tr>
<td><strong>AGO1</strong></td>
<td>AAA AAG CAG GCT TAA TGG TGA GAA AGA GAA GAA CGG AT AGA AAG CTG GGT ATC AGC AGT AGA ACA TGA CAC G</td>
<td>Gateway cloning</td>
</tr>
<tr>
<td><strong>CMV-2b</strong></td>
<td>AAA AAG CAG GCT TAA TGG AAT TGA ACG TAG GTG CAA TG AGA AAG CTG GGT ATC AGC AGT AGA ACA TGA CAC G</td>
<td>Gateway cloning</td>
</tr>
<tr>
<td><strong>CC (HRT)</strong></td>
<td>AAA AAG CAG GCT TAA TGG CTG AAG CAT TTG TGT CGT TTG AGA AAG CTG GGT ACT AAT CGC TTT CAG AAC TGT CAG GAT A</td>
<td>Gateway cloning</td>
</tr>
<tr>
<td><strong>NBS (HRT)</strong></td>
<td>AAA AAG CAG GCT TAA TGC AGA GTT TAG GGA TAC AAC AA AGA AAG CTG ACT AAA TTT GAA GGA AGT TCT CTT CTT T</td>
<td>Gateway cloning</td>
</tr>
<tr>
<td><strong>LRR (HRT)</strong></td>
<td>AAA AAG CAG GCT TAA TGA GAG AAG CAT TTT TAT CTA AA AGA AAG CTG GGT ACT GGT CAC AGT TGA TAA ATT G</td>
<td>Gateway cloning</td>
</tr>
<tr>
<td><strong>TCV CP R6A</strong></td>
<td>AAA AAG CAG GCT TAA TGG AAA ATG ATC CT AGA AAG CTG GGT ACT AAA TTC TGA GTG CTT GCA ATT</td>
<td>Gateway cloning</td>
</tr>
<tr>
<td><strong>TCV CP R8A</strong></td>
<td>AAA AAG CAG GCT TAA TGG AAA ATG ATC CTA GAG TC AGA AAG CTG GGT ACT AAA TTC TGA GTG CTT GCA ATT</td>
<td>Gateway cloning</td>
</tr>
<tr>
<td><strong>RPM1</strong></td>
<td>AAA AAG CAG GCT TAA TGG ATT TCA TC AGA AAG CTG GGT ATC AAT TTG GAA CA</td>
<td>Gateway cloning</td>
</tr>
<tr>
<td><strong>RPS2</strong></td>
<td>AAA AAG CAG GCT TAA TGG ATC TCA TG AGA AAG CTG GGT ATC AAT TTG GAA CA</td>
<td>Gateway cloning</td>
</tr>
</tbody>
</table>

Copyright © Rae-Dong Jeong 2011
CHAPTER 3

HRT-mediated hypersensitive response and resistance to Turnip Crinkle Virus does not required the function of TIP, the presumed guardee protein.

Plants have evolved some highly specific mechanisms to resist pathogens. The most studied of these involve the deployment of resistance (R) proteins, which generally impart protection against specific races of pathogens carrying corresponding avirulence (Avr) genes (“gene-for-gene” interactions, Flor, 1971). R proteins are believed to function as direct or indirect receptors for the appropriate Avr proteins. The tomato Pto and the rice Pi-ta proteins were shown to interact with their cognate Avr proteins, AvrPto and Avr-Pita, respectively in a yeast two-hybrid screen (Scofield et al., 1996; Tang et al., 1996; Jia et al., 2000), and the Arabidopsis RPS2 protein formed an in vivo complex with AvrRpt2 (Leister and Katagiri, 2000). However, other R/Avr protein pairs have not yielded a detectable interaction (Nimchuk et al., 2000). Thus, it was suggested that R proteins “guard” other plant proteins that are targets of Avr proteins (Van der Biezen and Jones, 1998; reviewed in Innes, 2004). Supporting this “guard” model, RIN4 was shown to physically interact with both the nucleotide binding (NB)-leucine rich repeat (LRR) protein RPM1, and its avirulence factors AvrB and AvrRPM1 (Mackey et al., 2002). RIN4 also interacts with RPS2 and its avirulence factor AvrRpt2 (Axtell and Staskawicz, 2003; Mackey et al., 2003). Thus, it was proposed that RIN4 is guarded by at least two different R proteins; modifications of RIN4 brought about by the various Avr proteins would lead to activation of the respective R proteins. Similarly, cleavage of protein kinase PBS1 by AvrPphB activates the R protein RPS5 (Shao et al., 2003). In addition, Avr2 inhibits the tomato Rcr3 protease required for Cf-2-mediated resistance (Rooney et al., 2005). These data strongly support an indirect mechanism of pathogen recognition by

---

* The results shown in this chapter were published in the following journal article:


19
a majority of NB-LRR proteins.

Downstream of the recognition event, the signals activated by various Arabidopsis R proteins appear to converge into a small number of pathways (Parker et al., 1996). The pathway activated by TIR-NBS-LRR proteins generally requires the *EDS1* gene (Parker et al., 1996), while that activated by most CC-NBS-LRR proteins requires the *NDRI* gene (Century et al., 1995). However, several CC-NBS-LRR *R* genes, including *RPP8*, *RPP13-Nd*, and *HRT*, as well as *RPP7*, signal resistance via a pathway(s) that is independent of *NDRI* (Bittner-Eddy and Beynon et al., 2001; Chandra-Shekara et al., 2004; McDowell et al., 2000). Strikingly, the CC-NBS-LRR gene *HRT*, which confers HR and resistance to TCV, is dependent on *EDS1* (Chandra-Shekara et al., 2004).

Resistance to TCV is also dependent on *EDS5*, *PAD4* and *SID2* genes of the SA pathway but does not require *NPR1*, *RAR1* and *SGT1* (Chandra-Shekara et al., 2004). Resistance to TCV is also independent of jasmonic acid (JA)- or ethylene-derived defense pathways (Kachroo et al., 2000). Exogenous application of SA or the SA analog benzo (1,2,3) thiadiazole-7-carbothioic acid (BTH) restores resistance to TCV in SA-deficient Di-17 plants containing the *eds1*, *eds5* or *sid2* mutations. However, exogenous application of SA/BTH does not confer enhanced resistance in plants lacking *HRT* or *PAD4* (Chandra-Shekara et al., 2004). In contrast to resistance, TCV-induced HR and *PR-1* gene expression are not affected by mutations in the SA pathway, suggesting that these phenotypes are independent of SA.

In addition to HRT, resistance to TCV requires a recessive allele at a second locus, designated *rrt* (regulates resistance to TCV) (Chandra-Shekara et al., 2004; Chandra-Shekara et al., 2006; Kachroo et al., 2000). However, exogenous application or increased endogenous levels of SA in *HRT* containing plants can overcome this requirement for *rrt* (Chandra-Shekara et al., 2004; Chandra-Shekera et al., 2006). Transgenic overexpression of *HRT* can also overcome a requirement for *rrt* but is not associated with an increase in endogenous SA (Cooley et al., 2000; Chandra-Shekara et al., 2004).

HR to TCV is initiated upon direct or indirect interaction between HRT and viral coat...
protein (CP) (Cooley et al., 2000). The TCV CP, which acts as an avirulence factor (Zhao et al., 2000), also physically associates with a protein belonging to the NAC family of transcription activators, designated TIP (TCV-interacting protein) in yeast two-hybrid assays (Ren et al., 2000; Ren et al., 2005). Furthermore, transient co-expression of TIP and CP in tobacco cells has shown that TCV CP prevents nuclear localization of TIP (Ren et al., 2005). These observations have prompted the suggestion that HRT guards TIP and is activated when CP retains TIP in the cytosolic compartment (Ren et al., 2005; Soosar et al., 2005).

Hence, I have analyzed the requirement of TIP for basal resistance to TCV and CMV and for HRT-mediated HR and resistance to TCV. I show that TIP is required for basal resistance to CMV but not for HRT-mediated HR and resistance to TCV. Furthermore, absence of TIP does not affect the SA-mediated induction of HRT or alter any of the phenotypes associated with overexpression of HRT. These results suggest that the interaction between CP and TIP does not govern downstream signaling leading to HR formation and resistance.

A mutation in TIP does not abolish HR or resistance to TCV

To study the role of TIP in HRT-mediated signaling, the SALK insertional database was examined for lines that carry T-DNA insertion within TIP. One line was obtained where the T-DNA was inserted 15 bases upstream of the translational start site (Figure 3.1A). Plants homozygous for the T-DNA insertion were obtained and analyzed for TIP expression. At least five different homozygous lines were analyzed and none of these showed any detectable expression of TIP (Figure 3.1B). The TIP knock out (KO) (tip) plants exhibited normal morphology (Figure 3.1C), which was consistent with basal level expression of PR-1 and absence of microscopic cell death in tip plants (Figure 3.1D and data not shown).

Since retention of TIP in the cytosol has been proposed to induce HRT-mediated signaling (Ren et al., 2005), we analyzed the role of TIP in an HRT background. TCV resistant Di-17 (Dijon ecotype) was crossed with wild-type (wt) Columbia ecotype (Col-
0) or tip (Col-0) plants. The F2 progeny derived from these crosses were genotyped for the presence of HRT and tip. The HRT tip plants were morphologically similar to wt plants (Figure 3.2A), did not show any visible or microscopic cell death (Figure 3.2B), and showed basal expression of various defense genes (Figure 3.2C). Upon TCV-inoculation, HRT tip plants developed HR within 3 days post inoculation (dpi), and the size and distribution of these lesions were similar to those in Di-17 or HRT TIP plants (Figure 3.2A&B). The HR-forming leaves from HRT tip plants also expressed increased levels of PR-1, PR-2, PR-5 and GST1 genes, similar to those in Di-17 or HRT TIP leaves (Figure 3.2C).

The role of TIP in HRT-dependent HR formation was further evaluated in planta by analyzing F1 progeny derived out of a cross between Di-17 or HRT tip with plants overexpressing the TCV CP (35S-CP; Cooley et al., 2000). Previously, it was demonstrated that TCV CP acts as the avirulence factor and plants expressing both CP and HRT show in premature death at the seedling stage (Cooley et al., 2000). Sixteen F1 seeds, derived from Di-17 x 35S-CP or HRT tip x 35S-CP crosses each, were sown in soil and all the F1’s showed stunted morphology and premature death at the seedling stage (Figure 3.2D). Together, these data suggest that a KO mutation in TIP does not impede HR formation or defense gene induction in response to TCV.

To determine if TIP was required for resistance to TCV, segregation of resistant plants in a TCV-inoculated F2 population derived from Di-17 x Col-0 and Di-17 x tip crosses was studied (Table 3.1). Unlike HR, which cosegregates with HRT, resistance to TCV is dependent upon the presence of least one copy of HRT and a recessive locus, rrt (Kachroo et al., 2000; Chandra-Shekara et al., 2004). Only HRT-containing plants showed resistance to TCV and ~25% of F2 plants from both crosses developed resistance (Table 3.1). Furthermore, ~25% of the HRT tip plants were resistant to TCV. This was further confirmed by RNA gel blot analysis; HRT tip plants scored as susceptible showed presence of viral transcript in the systemic tissues (Figure 3.2E). Further, the susceptible HRT tip plants accumulated similar amounts of viral transcript in the systemic tissues as the Col-0 plants and showed the typical stunting and crinkling phenotype (Figure 3.2F).
By comparison, the *HRT tip* plants scored as resistant did not show any viral transcript in the systemic tissues and developed normal bolts (Figures 3.2E and F). Together, these data suggest that the null mutation in *tip* does not impair resistance to TCV.

**TIP is not required for SA-mediated induction of HRT**

Exogenous application of SA/BTH upregulates *HRT* expression and this increase in expression suppresses HR and enhances resistance to TCV in plants containing the *RRT* allele (Chandra-Shekara et al., 2004). Next, I evaluated abilities of *HRT tip* plants to induce SA-mediated *HRT* expression, suppress HR due to overexpression of *HRT* and induce *HRT*-derived resistance in response to exogenous application of BTH. As expected, HR to TCV was suppressed in Di-17 plants pretreated with BTH two days prior to inoculation (Figure 3.3A). This in turn correlated with increased levels of *HRT* transcript in the BTH-treated Di-17 plants (Figure 3.4B). Similar to the Di-17 plants, exogenous application of BTH on *HRT tip* plants also suppressed HR and increased *HRT* expression (Figure 3.3A&B).

Next, evaluation was made by whether the exogenous application of BTH altered susceptibility to TCV in *HRT RRT tip* plants. As expected, water-treated *HRT RRT tip* plants were susceptible and accumulated viral transcripts in their systemic tissues (Figure 3.3C&D). In comparison, pretreatment with BTH induced resistance in ~65% of *HRT RRT tip* plants (Figure 3.3C), and this was comparable to the BTH-induced resistance in *HRT RRT* or *HRT RRT sid2* plants (Chandra-Shekara et al., 2004). Exogenous application of BTH on *TIP* or *tip* plants did not have any effect in the absence of *HRT*. Together, these results suggest that the SA-triggered increase in *HRT* expression, suppression of HR, and enhanced resistance are independent of TIP.

The above results were further confirmed by mobilizing the *tip* mutation into the *HRT ssi2* background, which contains the *RRT* allele and high endogenous SA. Previously, it was shown that the increased SA in the mutant *ssi2* background leads to increased *HRT* expression and confers resistance to TCV in an *RRT*-independent manner (Chandra-Shekara et al., 2004). The *HRT ssi2 tip* plants showed stunted morphology.
shown) and constitutively expressed the PR-I gene, similar to the HRT ssi2 plants (Figure 3.4A). Unlike Di-17 and HRT tip plants, both HRT ssi2 and HRT ssi2 tip plants expressed high levels of HRT (Figure 3.4B), which also correlated with enhanced resistance in these plants; ~95-98% of HRT ssi2 and HRT ssi2 tip plants were resistant to TCV (Figures 3.4C&D). Absence of ssi2 or HRT led to pronounced susceptibility. Analysis of viral transcripts detected high levels of TCV in the systemic tissues of susceptible genotypes (HRT tip, ssi2, ssi2 tip and Col-0) but not in the resistant plants (Di-17, HRT ssi2 or HRT ssi2 tip) (Figure 3.4E). Together, these results suggested that TIP was neither required for the ssi2-induced increase in HRT expression nor ssi2-conferred resistance in the HRT RRT background.

A mutation in TIP promotes replication of TCV and CMV
Earlier reports showing interaction between CP and TIP (Ren et al., 2000; Ren et al., 2005), and the above result that a null mutation in TIP does not impair HRT-mediated HR or resistance to TCV, suggested the possibility that TIP might serve as a host factor that regulates replication and/or movement of TCV in susceptible ecotypes like Col-0, and thus affect basal resistance. Therefore, TCV replication, as determined by CP levels, were assessed at 1, 2 and 3 dpi in inoculated leaves of TIP versus tip plants. There were no significant differences in the levels of CP accumulation at 1 or 2 dpi (Figure 3.5A). However, by 3 dpi tip plants consistently accumulated slightly increased levels of CP (Figure 3.5A) and its corresponding transcript (data not shown). To assess if TIP plays a role in the systemic movement of TCV or appearance of disease symptoms, transcript levels of CP (Figure 3.5B) and symptoms (Figure 3.5C) were analyzed in systemic tissues at 7 and 14 dpi. No differences were seen between wt and mutant plants. Therefore, TIP does not appear to play a significant role in symptom development to TCV.

To determine whether TIP functions in basal resistance to an unrelated viral pathogen, TIP and tip plants were inoculated with CMV and assayed for CMV-CP levels in the inoculated leaves at 1, 2 and 3 dpi. Disease symptoms were also recorded. Strikingly, significantly higher levels of CMV-CP were detected in tip versus TIP leaves throughout
the time-course (Figure 3.5D). This correlated with enhanced disease symptoms; in comparison to TIP, tip plants showed severe stunting and drooping of the bolts (Figure 3.5E). These data suggested that TIP is required for basal resistance to CMV.

To determine if TIP functions in basal- or R gene-mediated resistance to a bacterial pathogen, TIP and tip plants were inoculated with virulent or avirulent (containing avrRpt2) Pseudomonas syringae pv. tomato (Pst). No difference was detected between TIP versus tip in their resistance status to either virulent or avirulent Pst (Figures 3.5F & G). Together, these data suggest that TIP is not required for basal- or RPS2-mediated-resistance against Pseudomonas.

Discussion
In this study, the role of TIP in HRT-mediated HR and resistance to TCV was evaluated. Earlier work carried out by Ren et al. (2005) showed that GFP-TIP was retained in the cytosol when co-infiltrated with TCV CP. This, together with the result that TCV CP binds specifically to TIP, led to the assumption that the uneven distribution of TIP in the presence of CP signals the activation of HRT-mediated defense signaling (Ren et al., 2005). An alternate possibility is that HRT-mediated signaling was activated upon loss or degradation of TIP. Precedence for this is provided by the result that Pseudomonas syringae avirulence protein, avrRpt2, induces degradation of the host protein RIN4 and loss of RIN4 appears to activate RPS2-mediated resistance against P. syringae (Axtell and Staskawicz, 2003; Mackey et al., 2003). Moreover, deletion of RIN4 is lethal in plants containing functional RPS2 but has no apparent phenotype in plants lacking RPS2 (Axtell and Staskawicz, 2003; Mackey et al., 2003). A similar possibility for the activation of HRT-mediated signaling can be ruled out, since HRT tip plants are wt-like and show basal expression of defense genes. Absence of detectable TIP transcript in tip mutant plants suggests that the KO mutation leads to a null phenotype. The normal HR and resistance in HRT tip plants suggest that these defense responses do not require a functional TIP. We therefore conclude that neither the binding between TIP and CP nor the cellular distribution of TIP contribute to HRT-mediated HR or resistance to TCV.
The possible role of TIP suggested by Ren et al., (2000, 2005) also relied upon the observation that mutations in CP that prevented their interaction with TIP also abolished HR to TCV. A likely explanation for breakdown of resistance and HR seen upon inoculation of CP variants, which cannot bind TIP, is that HRT is unable to perceive the altered forms of CP, which results in failure to activate HR and escape of the virus to the systemic tissues.

TIP is a member of the NAC family of proteins, which are involved in diverse physiological responses ranging from development of plant embryos and flowers (Aida et al., 1997; Aida et al., 1999), lateral root development (Xie et al., 2000), cell division (Kim et al., 2006), defense (Xie et al., 1999; Collinge and Boller, 2001) and abiotic stress responses (Fujita et al., 2004; Tran et al., 2004). The Arabidopsis genome contains 105 NAC-like transcription factors (Ooka et al., 2003; Olsen et al., 2005), which show varying levels of homology to TIP. It is therefore conceivable that TIP may fulfill redundant functions and the loss of TIP could be compensated for by other TIP-like NAC proteins. Amino acid comparison and phylogenetic analysis of TIP to other members of the NAC family showed highest similarity to AtNTL6 (NAC62, Figure 3.6A).

Furthermore, only AtNTL6 showed homology (~60% identity) to the C-terminal domain of TIP that was shown to interact with CP (Ren et al., 2005). However, two-hybrid analysis did not detect any interaction between the full length CP and full length or C-terminal 150 amino acids from AtNTL6 (data not shown), suggesting that AtNTL6 is unlikely to compensate for the loss of TIP.

A subclass of the NAC family of transcription factors contains a transmembrane domain at the C-termini and are thereby membrane-associated (Kim et al., 2007). The membrane-associated NAC proteins are usually expressed as dormant precursors, which upon controlled proteolytic cleavage are released from the membranes and enter into the nucleus to activate transcription of target genes. In a recent study conducted by Kim et al. (2007), the authors showed that overexpression of AtNTL6 (NAC62), which shows highest similarity to TIP, produced a phenotype only if expressed as a C-terminal truncated protein. The C-terminal truncation removed the transmembrane domain of
AtNTL6, generating the active form of the transcription factor, which was now able to localize to the nucleus and initiate transcription. These results were further substantiated by immuno-localization studies, which showed that AtNTL6 localizes to the membranes and could be processed to a smaller molecular weight protein (Kim et al., 2007).

Structural analysis of TIP using the ARAMENNON membrane protein database predicts a strong α-helical transmembrane domain at the C-terminal region (Figure 3.6B). A comparison of the amino acid sequence of the transmembrane domains of TIP and AtNTL6 showed ~80% identity (Figure 3.6B) suggesting that, similar to AtNTL6, TIP may also localize to the membranes. However, the database prediction does not concur with the result that a GFP-TIP fusion protein localizes to the nucleus of tobacco cells (Ren et al., 2005). The altered localization of TIP in these experiments could either be the result of overexpression or misfolding due to the presence of GFP.

The putative membrane localization of TIP along with the fact that positive-sense viral replicase complexes are assembled on intracellular membranes (Alhquist, 2006), support the possibility that TIP might play a role in CMV and/or TCV replication. Indeed, CMV replication proteins are known to localize to the vacuolar membranes (Cillo et al., 2002). However, the location of the TCV replicase is as yet unknown. Increased accumulation of TCV- and CMV-CPs in the inoculated leaves of tip plants further support a role for TCV in CMV/TCV replication. Other members of NAC family transcription factors are also known to modulate the replication of wheat dwarf geminivirus (Xie et al., 1999) and Tomato Leaf Curl Virus (Selth et al., 2004). However, since viruses use different intercellular membranes for replication, the relationship between the localization of TIP and viral replication remains unclear. Further examination of the requirement for TIP in CMV resistance could help elucidate the precise role of TIP in plant viral defense.
Table 3.1. Segregation of resistance in Di-17 x Col-0 and Di-17 x *tip* plants.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Total number of plants analyzed</th>
<th>Genotype</th>
<th>Number of plants obtained</th>
<th>HR</th>
<th>R</th>
<th>S</th>
<th>(\chi^2)</th>
<th>(p^f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Di-17 x Col-0</td>
<td>148</td>
<td><em>HRT/-</em></td>
<td>97</td>
<td>+</td>
<td>22</td>
<td>75</td>
<td>0.15</td>
<td>0.69</td>
</tr>
<tr>
<td>Di-17 x <em>tip</em></td>
<td>394</td>
<td><em>HRT/- tip/ tip</em></td>
<td>47</td>
<td>+</td>
<td>9</td>
<td>23</td>
<td>0.85</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>HRT/- TIP/-</em></td>
<td>183</td>
<td>+</td>
<td>40</td>
<td>143</td>
<td>0.96</td>
<td>0.32</td>
</tr>
</tbody>
</table>

*a* The pollen-accepting plant is indicated first and the pollen donor second.

*b* The genotype at *HRT* and various mutant loci was determined by CAPS analysis.

*c* HR, hypersensitive response.

*d* Resistant, no disease symptoms.

*e* Susceptible, disease symptoms include crinkling of leaves and drooping of the bolt.

*f* One degree of freedom.
Figure 3.1. Isolation of a knockout (KO) mutation in TIP. (A) Line diagram showing structure of TIP and the site of T-DNA insertion. Black boxes represent exons and intervening lines represent introns. (B) RT-PCR analysis showing transcript levels of TIP in Di-17, Col-0 and the tip KO plants. (C) Morphological phenotype of four-week-old grown Col-0 and tip plants. (D) Microscopy of trypan blue-stained leaves from tip plants showing absence of any microscopic death in tip plants.
A

<table>
<thead>
<tr>
<th>Mock</th>
<th>TCV</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Leaf Images" /></td>
<td><img src="image2" alt="Leaf Images" /></td>
</tr>
</tbody>
</table>

*HRT* tip  *TIP* (Di-17)  *HRT* tip  *HRT* *TIP*  *TIP* (Col-0)

B

<table>
<thead>
<tr>
<th>Mock</th>
<th>TCV</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image3" alt="Microscopic Images" /></td>
<td><img src="image4" alt="Microscopic Images" /></td>
</tr>
</tbody>
</table>

*HRT* tip  *HRT* tip  *HRT* *TIP*  *TIP* (Col-0)

C

<table>
<thead>
<tr>
<th>HRT tip</th>
<th>TIP (Di-17)</th>
<th>HRT tip</th>
<th>HRT <em>TIP</em></th>
<th>TIP (Col-0)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image5" alt="Gene Expression" /></td>
<td><img src="image6" alt="Gene Expression" /></td>
<td><img src="image7" alt="Gene Expression" /></td>
<td><img src="image8" alt="Gene Expression" /></td>
<td><img src="image9" alt="Gene Expression" /></td>
</tr>
</tbody>
</table>

*PR-1*  *PR-2*  *PR-5*  *GST1*  rRNA

D

<table>
<thead>
<tr>
<th>Di-17 x Col-0</th>
<th>Di-17 x 35S-CP</th>
<th><em>HRT</em> tip x 35S-CP</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image10" alt="Plant Images" /></td>
<td><img src="image11" alt="Plant Images" /></td>
<td><img src="image12" alt="Plant Images" /></td>
</tr>
</tbody>
</table>
Figure 3.2. Morphological and molecular phenotypes in HRT tip plants. (A) Visible HR formation in TCV-inoculated plants at 3 dpi. All mock-inoculated plants showed absence of any visible lesions, similar to HRT tip. (B) Microscopy of trypan blue-stained leaves of mock- or TCV-inoculated plants. The Di-17 and HRT tip plants showed similar extents of cell death. (C) Defense gene expression in indicated genotypes at three days after mock- or TCV-inoculation. All mock-inoculated plants showed basal level expression of defense genes, similar to HRT tip plants. Ethidium bromide staining of rRNA was used as a loading control. (D) Morphological phenotypes of two-week-old F1 plants derived from the indicated crosses. Arrow indicates F1’s that remained stunted and eventually aborted. (E) Systemic spread of TCV to uninoculated tissue in TCV-inoculated F2 plants derived from a Di-17 x tip cross. RNA was extracted from the uninoculated tissues at 18 dpi and analyzed for the presence of the viral CP transcripts. R and S indicate resistance and susceptible phenotypes, respectively. (F) Typical morphological phenotypes of TCV-inoculated Di-17, Col-0 and a resistant and a susceptible HRT tip F2 plant derived from a Di-17 x tip cross. The susceptible plants showed crinkling, stunted bolt development and drooping of bolts. Plants were photographed at 18 dpi.
Figure 3. HR formation, *HRT* transcript levels, TCV resistance and systemic spread of TCV in *HRT* tip plants. (A) Visible HR formation in water- and BTH-treated plants at 3 dpi. (B) RT-PCR analyses showing expression of *HRT* in water- and BTH-treated plants. The level of $\beta$-tubulin was used as an internal control to normalize the amount of cDNA template. (C) Percentage of TCV resistant plants obtained after exogenous application of water or BTH. Resistance was analyzed 3-weeks post-inoculation. The numbers of plants tested are indicated above each bar. The *HRT tip* plants are F3 progeny of a susceptible *HRT tip* F2 plant that was derived from a Di-17 x tip cross. *HRT tip* progeny from three different F3 lines were analyzed and all showed similar results. Asterisk indicates 100% susceptibility. (D) Systemic spread of TCV to uninoculated tissue in TCV-inoculated plants. RNA was extracted from the uninoculated tissues at 14 dpi and analyzed for the presence of the viral *CP* transcripts.
Figure 3.4. Molecular analysis of *HRT ssi2 tip* plants. (A) *PR-1* gene expression in indicated genotypes at three days after mock- or TCV-inoculation. Ethidium bromide staining of rRNA was used as a loading control. (B) RT-PCR analyses showing expression of *HRT* in water- and BTH-treated plants. The level of β-tubulin was used as an internal control to normalize the amount of cDNA template. (C) Percentage of TCV resistant plants. Resistance was analyzed 3-weeks post-inoculation. The numbers of plants tested are indicated above each bar. Asterisk indicates 100% susceptibility. (D) Typical morphological phenotypes of TCV-inoculated plants at 14 dpi. (E) Systemic spread of TCV to uninoculated tissue of mock- or TCV-inoculated plants. RNA was extracted at 14 dpi and analyzed for the presence of the viral *CP* transcripts.
Figure 3. Basal resistance in tip plants. (A) ELISA showing levels of TCV CP in the inoculated leaves at 1-3 dpi. Statistical significance was determined using Students t-test. The difference in the levels of CP at 3 dpi was statistically significant (P=0.02). (B) Systemic spread of TCV to uninoculated tissue in TCV-inoculated plants. RNA was extracted from the uninoculated tissues at 7 and 14 dpi and analyzed for the presence of the viral CP transcripts. (C) Typical morphological phenotypes of TCV-inoculated plants at 14 dpi. (D) ELISA showing levels of CMV CP in the inoculated leaves at 1-3 dpi. Statistical significance was determined using Students t-test. (E) Typical morphological phenotypes of CMV-inoculated plants at 10 dpi. (F) Growth of a virulent isolate of P. syringae in Col-0 and tip plants. (G) Growth of an avirulent isolate of P. syringae containing AvrRPT2 in Col-0 and tip plants.
Figure 3. 6. (A) Phylogenetic tree of 46 NAC proteins that showed homology to TIP. Sequence alignments were carried out using the MegAlign program in the DNASTAR package and the tree was constructed using the program PAUP, version 4b10. (B) Alignment of 100 amino acids at the C-terminal of TIP and NAC62 proteins. Alignment was carried out using MegAlign program in the DNASTAR package. Amino acids in box indicate predicted transmembrane domain.

Copyright © Rae-Dong Jeong 2011
CHAPTER 4

CRYPTOCHROME 2 AND PHOTOTROPHIN 2 REGULATE RESISTANCE PROTEIN-MEDIATED VIRAL DEFENSE BY NEGATIVELY REGULATING AN E3 UBIQUITIN LIGASE

Plants are dependent on light for their survival. The light-absorbing ability of plants is derived from the activities of three known classes of photoreceptors. These include phytochromes (PHY) that detect light in the red/far-red (600-700nm) range, and cryptochromes (CRY) and phototropins (PHOT) that detect light in the blue and UVA (320-500 nm) range (reviewed in Casal, 2000; Chen et al., 2004; Christie, 2007; Gyula et al., 2003; Lin and Shalitin, 2003; Liscum, 2000). Photon-absorption activates the PHY proteins from their physiologically inactive to active far-red absorbing forms. Light also modulates the phosphorylation and nucleo-cytoplasmic translocation of PHY proteins, which is essential for their function in mediating light-responsive physiological changes in plants. CRY photoreceptors are flavoproteins that share sequence similarity to DNA-repair enzymes called photolyases. However, CRY proteins have no DNA-repair activity (Lin and Shalitin, 2003; Cashmore, 2003). CRY proteins were first characterized in Arabidopsis, but are also widely distributed in bacteria and eukaryotes. These proteins usually contain an amino terminal photolyase-related region and a carboxy domain of variable size. Both isoforms of CRY (CRY1 and CRY2) in Arabidopsis undergo blue-light-dependent phosphorylation (Shalitin et al., 2002; 2003) and CRY2, but not CRY1, is degraded in response to blue light (Ahmad et al., 1998; Lin et al., 1998). Both CRY1 and CRY2 interact with COP1 (Constitutively Photomorphogenic 1), a E3 ubiquitin ligase (Wang et al., 2001; Yang et al., 2001). It is thought that blue-light perception by CRY photoreceptors triggers the rapid inactivation of COP1 through their direct protein-

\[\text{The results shown in this chapter were published in the following journal article:}\]


“Copyright (2010), National Academic of Science, U. S. A.”
protein interactions (Wang et al., 2001; Yang et al., 2001), resulting in the abrogation of COP1-mediated degradation of the bZIP transcription factor HY5 (Elongated Hypocotyl 5) and other COP1 substrates (Hardtke et al., 2000). While CRY1 protein shuttles between the cytoplasm and nucleus, the CRY2 protein is mostly present in the nucleus (Yu et al., 2007). Since CRY2 also contributes to anion channel-mediated currents across the plasma membrane (Folta et al., 2001), it is possible that some CRY2 might also be present in the cytosol.

The PHOT proteins are plasma membrane-localized protein kinases that comprise of two light-oxygen-voltage (LOV) domains in the N-terminus and a serine/threonine kinase domain at the C-terminus (reviewed in Christie, 2007; Kimura and Kagawa, 2006). LOV1 and LOV2 are essential for the photoreceptor activity of PHOT proteins. In the dark, LOV2 binds the kinase domain and inhibits its phosphorylation activity. Light inhibits the binding between the kinase domain and LOV2, resulting in the activation of kinase activity (Matsuoka et al., 2005). Although the in planta substrates of PHOT-derived phosphorylation are unknown, both PHOT1 and PHOT2 autophosphorylate to likely promote their own dissociation from the plasma membrane (Sakamoto et al., 2000; Kong et al., 2006; Inoue et al., Wan et al., 2008). Upon blue-light irradiation, PHOT1 moves rapidly to the cytoplasm, while a fraction of PHOT2 moves to the Golgi apparatus. The significance of this relocalization or autophosphorylation remains unclear.

Increasing evidence indicates that light is important for the proper induction of plant defense and for resistance to pathogens (reviewed in Karpinski et al., 2003; Roberts et al., 2006). However, the molecular and biochemical interaction between light and defense signaling pathways remains unclear. Genetic evidence supporting the role of light in defense was provided by studies on mutants that are defective in the perception of light. Mutations in either phyA or phyB compromise the ability to induce localized cell death at the site of pathogen entry (Genoud et al., 2002). This phenomenon, termed the hypersensitive response (HR), is one of the earliest visible manifestations of induced defense signaling and resembles programmed cell death in animals (Shirasu et al., 2000).
In addition to HR development, the *phyA* and *phyB* mutants are also repressed in the salicylic acid (SA)-induced expression of the pathogenesis-related (*PR*) genes. The more severe effect of the *phyA* *phyB* double mutant on the SA-mediated pathway suggests that light perception has a cumulative effect on SA signaling and plant defense (Griebel et al., 2008). Recent analysis has suggested a major role for *PHYA PHYB* in systemic immunity and a rather minor role in local defense response (Griebel et al., 2008).

Previous work showed that light is required for resistance to Turnip Crinkle Virus (TCV) in Arabidopsis (Chandra-Shekara et al., 2006). Resistance to TCV is dependent on the R protein HRT, which contains a coiled coil, nucleotide binding, and leucine-rich-repeat domain (Cooley et al., 2000). However, *HRT* by itself is insufficient and requires the recessive allele of an as yet unidentified locus, *rrt*, to confer resistance (Chandra-Shekara et al., 2004; Kachroo et al., 2000). Following TCV inoculation, *HRT rrt* plants develop HR, induce defense gene expression, and accumulate SA (Chandra-Shekara et al., 2004; Kachroo et al., 2000). Plants lacking *HRT* fail to develop HR and allow systemic spread of the virus, resulting in a crinkled leaf/drooping bolt appearance, followed by death of the plant (Kachroo et al., 2000). The requirement for *rrt* can be overcome by increasing the levels of *HRT* transcript via exogenous application of SA (Chandra-Shekara et al., 2004; 2006; 2007). Strikingly, unlike resistance, TCV-induced HR and *PR-1* gene expression function independent of the SA pathway and *rrt*.

This study was undertaken to decipher the genetic and biochemical basis of dark-conferred susceptibility using Arabidopsis-TCV as a model system. Results here demonstrate that the blue-light photoreceptors, CRY2 and PHOT2, are specifically required for the stability of HRT, whereas CRY1 and PHOT1 influence HRT-mediated resistance without affecting the stability of the R protein. Furthermore, HRT, as well as CRY2 and PHOT2, interact with the E3 ubiquitin ligase COP1, which is known to target proteins for 26S proteasome-mediated degradation. Conversely, inhibition of the 26S proteasome restores HRT levels and resistance to TCV under blue light. I conclude that CRY2/PHOT2 negatively regulate COP1 activity, thereby maintaining the stability of the HRT protein.
HRT is a plasma membrane-localized protein that is degraded in the dark

Previously it was shown that a critical period of illumination, immediately after TCV inoculation, is essential for HRT-mediated resistance to the virus (Chandra-Shekara et al., 2006). To monitor the effects of light versus dark on the R protein HRT, the epitope (FLAG)-tagged HRT protein was expressed via its native promoter in Dijon (Di)-17 (HRT/rrt, resistant) and Columbia (Col)-0 (hrt/RRT, susceptible) ecotypes (Fig. 4.1A). Inoculation of TCV on the Col-0 HRT-FLAG lines induced HR formation (Fig. 4.1B) and PR-1 gene expression (Fig. 4.1C), similar to that in Di-17 or Di-17 HRT-FLAG plants, indicating that the HRT-FLAG fusion protein was functional. Consistent with the requirement of the recessive locus rrt for TCV resistance, Col-0 HRT-FLAG lines remained susceptible to TCV (Fig. 4.1D). To test whether the dark-triggered susceptibility in Di-17 plants was associated with a change in HRT levels, HRT-FLAG levels in Di-17 HRT-FLAG plants grown under 14 h light and 10 h dark (14h L:10h D) photocycles were compared to those kept continuously in the dark for 24, 48, or 72 h. As expected, dark-treatment caused susceptibility in Di-17 HRT-FLAG plants (Fig. 4.2A & 2B). Western analysis showed that although HRT-FLAG was detectable in the dark treated plants these levels were greatly reduced compared to plants grown under a normal day and night cycle (Fig. 4.3A). Unlike HRT protein, dark treatment did not alter HRT transcript levels (Fig. 4.3B & Fig. 4.2C), suggesting that light was required for the post-transcriptional stability of the HRT protein. Together, these results suggest that lack of light promoted susceptibility in Di-17 HRT-FLAG plants by reducing the levels of HRT protein.

RPM1, a peripheral plasma membrane protein, which confers resistance to Pseudomonas syringae expressing avrRpm1, is specifically degraded in response to HR (Boyes et al., 1998). However, unlike HRT, dark treatment did not result in degradation of the RPM1-MYC protein (Fig. 4.3C). Normal levels of HRT-FLAG during early and late stages of HR development suggests that HRT was not degraded in response to HR (Fig. 4.3A). Similarly, no significant changes in HRT-FLAG levels were observed during HR to P. syringae expressing avrRpt2 (Fig. 4.3D), even though HR to P. syringae covered the
entire leaf. Together, these results suggest that HRT is not degraded during HR formation and that light is specifically required for the stability of HRT.

Next, I evaluated whether, in addition to altering HRT levels, absence of light also affected the localization of HRT. HRT-FLAG protein was detected in the membrane fraction of leaf extracts from healthy plants grown under 14h L:10h D photocycles, with no visible protein in the soluble fraction (Fig. 4.3E). Further sub-fractionation localized HRT-FLAG to the plasma membrane (Fig. 4.3F). This was confirmed using transgenic lines expressing HRT-GFP under the HRT promoter, where HRT-GFP was localized exclusively to the periphery of the cell, compared to GFP alone which was distributed uniformly throughout the cell (Fig. 4.3G). The release of HRT into the soluble fraction by treatment with either Triton X-100 or urea argues that it is a peripheral plasma membrane protein (Fig. 4.3H). These treatments also released PHOT1, a plasma membrane-localized blue-light photoreceptor that relocalizes to the cytosol in the presence of blue light (Sakamoto et al., 2000). These data confirmed the peripheral plasma membrane localization of both HRT and PHOT1. The possible TCV-responsive release of HRT from plasma membranes was analyzed at 24, 48, and 72 h post TCV inoculation. HRT was detected only in the microsomal fractions at all time points (Fig. 4.3I), suggesting that HRT-mediated resistance signaling was likely not associated with the relocalization of this R protein into the soluble fraction. Similarly, HRT-FLAG protein was not found in soluble fractions of leaves subjected to dark treatment (Fig. 4.3J).

**Mutation in blue-light photoreceptors compromises HRT-mediated resistance**

Epistatic mutant analysis was next used to determine whether light-absorbing photoreceptors participated in HRT-mediated resistance. The phyC, phyD, phyE, cry1, cry2, phot1, phot2 or phyA phyB mutations were crossed into the Di-17 background and F2 plants were analyzed for defense phenotypes (Tables 4.1). All hrt/hrt F2 progeny showed susceptibility to TCV. Approximately 75% (homo/heterozygous for HRT) of F2 progeny from an HRT rrt X hrt RRT crosses developed HR upon TCV infection. However, only 25% (homo/heterozygous for HRT, but homozygous for rrt) of these HR-
developing progeny were able to resist TCV infection (Table 4.1). The resistance phenotype in \textit{HRT phyC}, \textit{HRT phyD}, \textit{HRT phyE} and \textit{HRT phyA phyB} plants also showed expected Mendelian segregation (Table 4.1), suggesting that mutations in \textit{PHYC}, \textit{PHYD}, or \textit{PHYE} do not affect HRT-mediated resistance. Furthermore, \textit{phyA} and \textit{phyB} single or \textit{phyA phyB} double mutations did not alter HRT-mediated resistance. In contrast, mutations in \textit{cry1}, \textit{cry2}, \textit{phot1} or \textit{phot2} abrogated HRT-mediated resistance; all plants containing \textit{HRT} and mutant \textit{cry1}, \textit{cry2}, \textit{phot1} or \textit{phot2} loci showed typical phenotypes associated with susceptible plants (Fig. 4.4A, Table 4.1). The appearance of disease symptoms also correlated with the presence of TCV transcript in the systemic uninoculated tissues (Fig. 4.4A). Together, these data suggest that blue-light photoreceptors are required for HRT-mediated resistance.

**CRY2 and PHOT2 are required for post-transcriptional stability of HRT**

To determine whether mutations in the \textit{CRY} or \textit{PHOT} genes caused susceptibility by altering HRT levels, I introduced the \textit{HRT-FLAG} transgene into \textit{cry1}, \textit{cry2}, \textit{phot1} or \textit{phot2} mutant plants. Interestingly, HRT-FLAG levels were significantly reduced in \textit{HRT cry2} and \textit{HRT phot2} plants but not in \textit{HRT cry1} or \textit{HRT phot1} plants (Fig. 4.4B). Reduced levels of the HRT protein in \textit{HRT cry2} or \textit{HRT phot2} plants were not attributable to transcript instability because these plants contained wt levels of the \textit{HRT} transcript (Fig. 4.4C & Fig. 4.5B). Together, these data suggested that CRY2 and PHOT2 were required for stability of the HRT protein. Furthermore, normal levels of HRT in \textit{cry1} and \textit{phot1} plants suggest that CRY1 and PHOT1 were not required for stability of the HRT protein and likely functioned elsewhere in the HRT-mediated resistance signaling pathway.

To determine if \textit{cry1} and \textit{phot1} mutations and/or reduced levels of HRT protein in \textit{HRT cry2} and \textit{HRT phot2} plants impaired HRT-mediated downstream signaling, I analyzed SA levels, \textit{PR-1} expression and HR phenotypes post TCV inoculation. Interestingly, TCV-induced levels of SA did not correlate with the levels of HRT protein in the various mutant backgrounds. The \textit{HRT cry1} (wt levels of HRT protein) and \textit{HRT cry2} (low levels
of HRT protein) plants accumulated marginally lower levels of SA, and similar levels of SAG, as Di-17 plants (Fig. 4.5C & 4.5D). In contrast, HRT phot1 (wt levels of HRT protein) and HRT phot2 (low levels of HRT protein) plants accumulated significantly lower levels of both SA and SAG compared to Di-17 (Fig. 4.5C & 4.5D). Normal increase of SA in TCV inoculated HRT cry2 plants suggest that reduced HRT protein in these plants is sufficient to trigger a signaling response related to SA accumulation but not resistance. This was further evident upon evaluation of the HR response in HRT cry2 and HRT phot2 plants; plants containing mutations in PHY, CRY, or PHOT genes showed normal visible and microscopic HR (Fig. 4.4D&E) and induced normal expression of the PR-1 gene (Fig. 4.4F). However, the HR phenotype in these mutant backgrounds did not correlate with the fact that dark-treated plants showed compromised HR (Chandra-Shekara et al., 2006). One possibility is that multiple photoreceptors might be involved in regulating HR against TCV. However, normal HR and PR-1 expression phenotypes were seen in double and triple mutant plants including, HRT cry1 cry2, HRT phot1 phot2, HRT phyA phyB, and HRT phyC phyD phyE plants (Fig. 4.4D and Fig. 4.5E). These data suggest that the PHOT and CRY photoreceptors might act in a redundant manner, and/or factors other than photoreceptors regulate the effect of light on HR to TCV.

**Overexpression of HRT increases HRT levels in phot2 but not in cry2 background**

To determine if overexpression of HRT-FLAG was able to compensate for reduced stability of HRT-FLAG in the cry2 and phot2 backgrounds, the plants were treated with SA or its active analog benzo(1,2,3)thiadiazole-7-carbothioic acid (BTH), which induced HRT transcription by ~6-fold (Fig. 4.6A & 4.6B). However, BTH application increased HRT protein levels only in HRT cry1, HRT phot1 or HRT phot2 plants, but not in HRT cry2 plants (Fig. 4.7A & Fig. 4.6C). Inability to accumulate HRT protein in cry2 background was not related to SA responsiveness or transcription of HRT since BTH application induced wt-like levels of HRT transcript and PR-1 expression in HRT cry2 and cry2 backgrounds, respectively (Fig. 4.6D). The reduced accumulation of HRT protein in the cry2 background was further confirmed using a bioassay involving HR formation. This bioassay is based on the rationale that plants overexpressing HRT do not produce visible HR upon TCV inoculation (Chandra-Shekara et al., 2004; 2006). Thus,
absence of HR after TCV inoculation of BTH pre-treated plants would indicate the presence of increased levels of HRT in those plants. As expected, TCV inoculation did not produce visible HR in BTH pre-treated HRT cry1 (Fig. 4.6E) or HRT phot1 plants. In contrast, TCV inoculation continued to induce HR lesions on HRT cry2 plants even when they were pre-treated with BTH. Interestingly, HRT phot2 leaves showed an intermediate phenotype; visible HR formation on HRT phot2 leaves was reduced but not abolished (Fig. 4.6E). HR phenotype correlated well with resistance. BTH treatment led to a significant increase in the number of resistant HRT cry1, HRT phot1 and HRT phot2 plants (Fig. 4.6F & 4.6G). In contrast, HRT cry2 plants continued to show high levels of susceptibility; these plants showed typical morphology associated with susceptible plants and accumulated virus in their systemic tissues. The percentage of BTH-treated HRT phot2 plants showing resistance to TCV was lower compared to HRT cry1 or HRT phot1 plants, and was likely related to the reduced levels of HRT accumulating in response to BTH in these plants. Together, these data suggest that increased expression of HRT overcomes a requirement for CRY1, PHOT1 and PHOT2 to varying degrees, but not CRY2.

Blue-light treatment causes degradation of HRT

Next, a time-course study was carried out to determine if resistance was associated with changes in CRY or PHOT proteins and if HRT levels in cry or phot backgrounds changed in response to TCV inoculation. TCV inoculation did not alter HRT levels significantly in cry1, cry2, phot1 or phot2 backgrounds (Fig. 4.7B). TCV inoculation also did not alter levels of CRY or PHOT proteins in HRT plants (Fig. 4.7C). However, lack of light, which lowered HRT levels (Fig. 4.1A & 4.1J), caused a gradual decline in CRY2, but not CRY1 or either PHOT proteins (Fig. 4.7D). A role for CRY2 in anion channel-mediated currents across the plasma membrane (Yu et al., 2007), and the fact that HRT is a plasma membrane protein, prompted me to determine if the dark-triggered decrease in CRY2 was associated with relocalization of CRY2 into the membranous fraction. Absence of CRY2 in dark-treated plants was not associated with the relocalization of this protein to the membrane (Fig. 4.7E). The dark-induced changes in the levels of CRY2 were consistent with diurnal rhythms and maximal transcript seen during the light phase (Toth
et al., 2001) as well as a previous report showing reduction in CRY2 levels in dark-treated seedlings (Lin et al., 1998). On the other hand, the CRY2 protein has also been shown to exhibit a short-day specific diurnal rhythm, with increased accumulation of CRY2 at the end of the dark period (Mockler et al., 2003). The fact that this study was carried out using continuous short-day photocycles versus a shift from 14h L:10h D photocycles to dark, could account for this discrepancy. Nonetheless, in my study, reduction in HRT levels did correlate with a decrease in CRY2 levels. However, at this point it is unclear whether photoactivated form of CRY2 is required for HRT stability since most, if not all of CRY2, is thought to be inactive in the dark.

Since CRY2 protein is degraded in the presence of blue light (Ahmad et al., 1998; Lin et al., 1998), we next exposed the Di-17 HRT-FLAG plants to blue-light. The blue light-mediated disappearance of CRY2 correlated with degradation of HRT-FLAG; the levels of both CRY2 and HRT-FLAG were reduced significantly 6h after blue-light treatment (Fig. 4.7F). Notably, reduction in HRT levels was quicker (within 6h) than that in CRY2, which reduced more gradually. This suggested that a certain threshold level of CRY2 might be required for the stability of HRT. Consistent with the degradation of HRT, the Di-17 HRT-FLAG plants exposed to blue light showed susceptibility to TCV (Fig. 4.7G) and supported the systemic movement of the virus (Fig. 4.7H). Interestingly, treatment with blue light also compromised HR to TCV (Fig. 4.7I, upper panel) and these plants showed marked reduction in PR-1 expression (Fig. 4.7I, lower panel).

HRT interacts with COP1 and is degraded in a 26S proteasome-dependent manner

To determine if CRY2 and/or PHOT2 contributed to the stability of HRT by direct association with the R protein, interactions between HRT and CRY2/PHOT2 using Bimolecular fluorescence complementation assays (BiFC) were tested (Fig. 4.8A & Fig. 4.9B). Interaction between CRY2 and CIB1 (Liu et al., 2008) and HRT and CRT1 (Kang et al., 2010) were used as positive controls. No interaction was detected between CRY2 and HRT or PHOT2 and HRT, suggesting that CRY2 or PHOT2 do not affect stability of HRT via direct interactions with the R protein (Fig. 4.8A). As expected, CRY2 interacted
with CIB1 in the nucleus (Fig. 4.8A). Similarly, HRT interacted with CRT1 and this interaction occurred in the endosomes, the site of CRT1 localization (Fig. 4.8A). To determine if the indirect association between HRT and its cognate avirulence factor CP is mediated via CRY2 or PHOT2, we evaluated interaction between CP and CRY2/PHOT2 proteins. The fact that CP was present in both soluble and membranous fractions of TCV-infected cells (Fig. 4.9A) further supported a possibility for its interaction with the soluble CRY2 and the membranous PHOT2 proteins (Shalitin et al., 2003; Kong et al., 2006). In addition to these, we also tested interaction between HRT and CP. However, CP did not interact with HRT, CRY2 or PHOT2 in the BiFC assays (Fig. 4.8A & Fig. 4.9B). All proteins were expressed at detectable levels indicating that the lack of interactions in BiFC assays was not due to insufficient protein expression (data not shown).

Interactions of HRT with CIB1 and COP1 proteins were tested next, since both CIB1 and COP1 are known to interact with CRY2 (Liu et al., 2008; Wang et al., 2001). Consistent with earlier results (Kang et al., 2010), CRY2 interacted with COP1 in the nucleus (Fig. 4.8A). Interestingly, HRT interacted with COP1 but not with CIB1 (Fig. 4.8A). Consistent with the plasma membrane localization of HRT, fluorescence was seen in the periphery of the cell. This is in contrast to the HRT-CRT1 interaction, which occurs primarily in the endosomes. Thus, although HRT normally localizes to the plasma membrane, it associates with proteins present in different cellular compartments. The interaction between HRT and COP1 was further confirmed by co-immunoprecipitation (Fig. 4.8B). The COP1 protein also interacted with PHOT2 (Fig. 4.8A & 4.8C), which is consistent with fact that COP1 serves as a negative regulator of PHOT2-mediated signaling (Mao et al., 2005). The interaction between PHOT2 and COP1 was further confirmed by co-immunoprecipitation (Fig. 4.8C). Interestingly, the COP1-PHOT2 interaction was detected both inside and outside the nucleus, suggesting that, besides the golgi apparatus (Kong et al., 2006), PHOT2 also relocalizes to the nucleus. However, at this point I am unable to discount the possibility that the apparent nuclear localization of PHOT2 may be due to its increased expression.
COP1 is an E3 ubiquitin ligase that tags proteins with ubiquitin thereby subjecting them to degradation via the 26S proteasome pathway. I therefore examined the effects of the 26S proteasome-specific inhibitor MG132 on blue light-dependent HRT degradation. The Di-17 HRT-FLAG leaves pretreated with MG132 accumulated ~85% of HRT-FLAG protein after 24 h of blue light treatment, compared to <15% HRT-FLAG in leaves treated with a protease inhibitor cocktail and <10% in control plants (Fig. 4.8D). Consistent with this result, plants exposed to blue light showed resistance to TCV when they were pretreated with MG132 (Fig. 4.8E). It is possible that HRT levels are maintained because of interactions of CRY2 or PHOT2 with COP1, both of which are thought to repress COP1 activity (Wang et al., 2006; Mao et al., 2005). Under dark or blue-light conditions, CRY2 degradation and possible conformational changes in PHOT2 might relieve their repression of COP1 activity, enabling COP1 to interact with HRT, thereby targeting HRT for degradation. The slow kinetics of nuclear and cytoplasmic relocalization of COP1 in dark and light, respectively (Von et al., 1997), suggests that a sufficient amount of COP1 might be present in the cytoplasm in the dark to interact with HRT. While the above data clearly show that blue light induces the degradation of HRT in a 26S proteasome-specific manner, a direct role for COP1 in this degradation remains unclear. Likewise, the exact role of COP1 in CRY1 and CRY2 stability remains unresolved. Notably, although both CRY1 and CRY2 interact with COP1, only CRY2 undergoes blue light-dependent degradation (Shalitin et al., 2002; 2003; Ahmad et al., 1998; Lin et al., 1998; Wang et al., 2001; Yang et al., 2001).

**Mutations in blue-light photoreceptors do not alter resistance to bacterial pathogen**

To determine if blue-light photoreceptors play a specific or generalized role in R protein-mediated resistance, I evaluated RPS2- and RPS4-mediated resistance to the bacterial pathogen *Pseudomonas syringae* expressing avrRpt2 (Fig. 4.10A) or avrRps4 (Fig. 4.10B), respectively. Evaluating RPS2-mediated resistance was particularly relevant because PHOT2 was recently shown to co-immunoprecipitate with RPS2 (Qi et al., 2008). Notably, *cry1*, *cry2*, *phot1* and *phot2* mutations did not alter *RPS2*- or *RPS4*-
mediated resistance to *P. syringae* expressing *avrRpt2* or *avrRps4*, respectively (Fig. 4.10A & 4.10B) or PR-1 expression induced in response to *P. syringae* expressing *avrRpt2* (Fig. 4.10C). Furthermore, *cry1 cry2* and *phot1 phot2* double mutants also showed normal resistance to *P. syringae* expressing *avrRpt2*. These data suggest that RPS2- and RPS4-mediated resistance likely do not require the CRY or PHOT blue-light photoreceptors. These results, however, disagree with a recent report where CRY1 was shown to positively regulate RPS2-mediated resistance (Wu et al., 2010). One possible explanation is that Wu and Yang (Wu et al., 2010) studied resistance under continuous light whereas I used 10h L:14h D photocycles for bacterial infections. It is quite likely that photoreceptors function differently under different light conditions and/or that they play redundant roles in mediating bacterial resistance.

**Discussion**

The above results provide the first definitive evidence for a mechanistic role for blue-light photoreceptors in R protein-mediated resistance to TCV. These blue-light photoreceptors appear to affect multiple steps in the resistance signaling pathway, including their indirect roles in maintaining R protein stability by repressing COP1 activity as well as by regulating pathogen-responsive accumulation of SA and downstream resistance signaling.
Table 4.1. Epistatic analysis of F2 population derived from crosses between Di-17 and various wild-type or photoreceptor mutants.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Plants analyzed</th>
<th>Genotype*</th>
<th>Plants obtained</th>
<th>HR b</th>
<th>R c</th>
<th>S d</th>
<th>χ² e</th>
<th>P f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Di-17 x Col-0</td>
<td>135</td>
<td>HRT/-</td>
<td>97</td>
<td>+</td>
<td>22</td>
<td>75</td>
<td>0.27</td>
<td>0.59</td>
</tr>
<tr>
<td>Di-17 x Ler</td>
<td>128</td>
<td>HRT/-</td>
<td>89</td>
<td>+</td>
<td>21</td>
<td>68</td>
<td>0.09</td>
<td>0.75</td>
</tr>
<tr>
<td>Di-17 x phyC</td>
<td>265</td>
<td>HRT/- phyC</td>
<td>38</td>
<td>+</td>
<td>8</td>
<td>43</td>
<td>30</td>
<td>0.31</td>
</tr>
<tr>
<td>Di-17 x phyD</td>
<td>272</td>
<td>HRT/- phyD</td>
<td>43</td>
<td>+</td>
<td>13</td>
<td>35</td>
<td>30</td>
<td>0.62</td>
</tr>
<tr>
<td>Di-17 x phyE</td>
<td>388</td>
<td>HRT/- phyE</td>
<td>60</td>
<td>+</td>
<td>13</td>
<td>51</td>
<td>47</td>
<td>0.35</td>
</tr>
<tr>
<td>Di-17 x cry1</td>
<td>326</td>
<td>HRT/- cry1</td>
<td>56</td>
<td>+</td>
<td>0</td>
<td>39</td>
<td>56</td>
<td>18.6</td>
</tr>
<tr>
<td>Di-17 x cry2</td>
<td>340</td>
<td>HRT/- cry2</td>
<td>57</td>
<td>+</td>
<td>0</td>
<td>43</td>
<td>26</td>
<td>19.0</td>
</tr>
<tr>
<td>Di-17 x phot1</td>
<td>248</td>
<td>HRT/- phot1</td>
<td>42</td>
<td>+</td>
<td>0</td>
<td>35</td>
<td>42</td>
<td>14.0</td>
</tr>
<tr>
<td>Di-17 x phot2</td>
<td>256</td>
<td>HRT/- phot2</td>
<td>53</td>
<td>+</td>
<td>0</td>
<td>33</td>
<td>53</td>
<td>17.6</td>
</tr>
<tr>
<td>Di-17 x phyA phyB</td>
<td></td>
<td>HRT/- phyA phyB</td>
<td>12</td>
<td>+</td>
<td>3</td>
<td>42</td>
<td>9</td>
<td>0.00</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The genotype at HRT and various mutant loci was determined by CAPS analysis
\textsuperscript{b} HR, hypersensitive response
\textsuperscript{c} Resistant
\textsuperscript{d} Susceptible
\textsuperscript{e} One degree of freedom; based on segregation of 3 susceptible:1 resistant plants
\textsuperscript{f} Statistically significant
Figure 4. 1. Characterization of transgenic plants Di-17 and Col-0 lines expressing *HRT-FLAG* transgene. (A) Western blot showing HRT-FLAG levels in Di-17 and Col-0 plants expressing the *HRT-FLAG* transgene. Wild-type Di-17 and Col-0 plants were used as controls. Ponceau-S staining of the western blots was used as the loading control. (B) HR formation in Di-17 and Col-0 leaves expressing the *HRT-FLAG* transgene, 3 days post inoculation (dpi) with TCV. (C) *PR-1* gene expression in mock and TCV-inoculated Di-17, Col-0 and *HRT-FLAG* transgenic plants. The samples were collected at 3 dpi. Ethidium bromide staining of rRNA was used as a loading control. (D) Typical morphological phenotypes of TCV-inoculated genotypes. The susceptible plants showed a stunted, crinkling phenotype and accumulated virus in their systemic tissues.
Figure 4.2. Morphological phenotype, systemic spread and HRT expression levels in dark-treated plants. (A) The typical susceptible phenotype shown by TCV-inoculated Di-17 HRT-FLAG subjected to 48 h of dark treatment. (B) Systemic spread of TCV to uninoculated tissue in TCV-inoculated Di-17 HRT-FLAG plants exposed to dark or normal day and night cycles. Ethidium bromide staining of rRNA was used as a loading control. (C) Quantitative RT-PCR analysis showing relative levels of HRT transcript in plants kept in dark for 0-72 hr.
Figure. 4. 3. HRT-FLAG protein is degraded in dark. (A) Western blot showing HRT-FLAG levels in plants kept under 14h L: 10h D photocycles or in dark after TCV inoculation for indicated hours. Total proteins were extracted and analyzed by immunoblotting. (B) RT-PCR analysis showing HRT transcript levels after 0-72h of dark treatment. The level of β-tubulin was used as an internal control to normalize the amount of cDNA template. (C) RPM1-MYC levels in soluble (S) and microsomal (M) fractions extracted from plants kept in dark for 0-72 h. (D) HRT-FLAG levels in S and M fractions extracted from plants inoculated with P. syringae expressing avrRpt2 at indicated hours post-inoculation (hpi). (E) HRT-FLAG levels in total (T), S and M fractions. (F) Subcellular localization of HRT-FLAG and PHOT1 proteins to the plasma membrane (P) fractions. E indicates endomembranes. (G) Confocal image showing the subcellular localization of GFP and HRT-GFP expressed under 35S or HRT promoters, respectively. (H) HRT-FLAG and PHOT1 levels in M or S fractions before (M-I; input) and after treatment with 2M urea or 1% Triton X-100. (I) HRT-FLAG levels in S and M fractions extracted from TCV-inoculated plants at indicated hpi. (J) HRT-FLAG levels in S and M fractions extracted from plants kept under 14h L: 10h D photocycles or in dark for 48h. Ponceau-S staining of the western blots shown in A, C, D, E, H, I and J were used as the loading control.
Figure 4. Mutations in blue-light photoreceptors compromise HRT-mediated resistance. (A) Typical morphological phenotypes of TCV-inoculated genotypes. The susceptible plants showed stunted, crinkling phenotypes and accumulated virus in their systemic tissues (Fig. S3A). (B) Western blot showing HRT-FLAG levels in indicated genotypes. Wild-type Di-17 was used as a negative control (last lane). Total proteins were extracted and analyzed by immunoblotting. Ponceau-S staining of the western blots was used as the loading control. (C) RT-PCR analysis showing HRT transcript levels in indicated genotypes. The level of β-tubulin was used as an internal control to normalize the amount of cDNA template. (D) Typical HR in TCV-inoculated leaves at 3 days post-inoculation (dpi). The indicated genotypes were homozygous for the mutant loci and contain at least one copy of the HRT gene. (E) Microscopy of trypan blue-stained leaves from TCV-inoculated plants shown in B (scale bars, 270 microns). (F) PR-1 gene expression in mock- or TCV-inoculated plants. Leaves were sampled at 3 dpi. Ethidium bromide staining of rRNA was used as a loading control.
Figure 4. 5. Systemic spread HRT expression, HR formation, and levels of SA and SAG. (A) Levels of TCV-CP transcript in systemic tissues of mock- and TCV-inoculated plants. RNA was extracted from the uninoculated tissues at 18 days postinoculation (dpi) and analyzed for the presence of the CP transcript. Ethidium bromide staining of rRNA was used as a loading control. (B) Quantitative RT-PCR analysis showing relative levels of HRT transcript in indicated genotypes. (C) Endogenous SA and SAG levels in mock (gray bars) leaves of indicated genotypes. Samples were harvested at 3 dpi with TCV. The values are presented as a mean of three replicates. The error bars represent SD. Asterisks denote samples showing significant differences from wild-type (t test, P < 0.05). (E) Typical HR phenotype of TCV-inoculated HRT phyA phyB and HRT phyC phyD phyE plants kept in 14-h L:10-h D photoperiod. Leaves were photographed at 3 dpi.
Figure F: Bar graph showing the percentage of resistant plants under different treatments. The graph compares the effects of Water and BTH (Bromegrass Herbicide Treatment) on plant resistance across six conditions: HRT, hrt, HRT cry1, HRT cry2, HRT phot1, and HRT phot2.

Figure G: Images of plant growth under different treatments, illustrating the visual differences in plant health and growth under Water and BTH conditions.
Figure 4. 6. HRT-FLAG protein and transcript levels, PR-1 expression, HR and resistance to TCV in plants pretreated with BTH. (A) RT-PCR analysis showing HRT transcript levels in indicated genotypes treated with water or BTH for 48 h before sampling. Like other genotypes, BTH-treated HRT phot1 plants also showed induction in HRT expression. The level of β-tubulin was used as an internal control to normalize the amount of cDNA template. (B) Quantitative RT-PCR analysis showing relative levels of HRT transcript in plants treated with water (gray bars) or BTH (black bars) for 48 h. (C) HRT-FLAG levels in water-and BTH-treated HRT phot1 plants. Ponceau-S staining of the western blots was used as the loading control. (D) PR-1 gene expression in water- and BTH-treated plants. The samples were collected 48 h posttreatments. Ethidium bromide staining of rRNA was used as a loading control. (E) HR formation in indicated genotypes that were treated with water or BTH for 48 h before TCV inoculation. (F). Percentage of resistant plants obtained in indicated genotypes that were treated with water (gray bars) or BTH (black bars) for 48 h before TCV inoculation. Approximately 40-50 plants were inoculated in four separate experiments and analyzed for HR and resistance phenotype. Asterisks denote 100% susceptibility. (G) Typical morphological phenotype of TCV-inoculated genotypes that were treated with water or BTH for 48 h before TCV inoculation. Approximately 40-50 plants were inoculated in four separate experiments and analyzed for HR and resistance phenotypes. Both susceptible (Center) and resistant plants (Right) are shown for BTH-treated HRT phot2 genotype.
Figure 4. 7. Blue light causes degradation of HRT-FLAG and overexpression of HRT is unable to increase HRT-FLAG levels in cry2 plants. (A) Western blot showing HRT-FLAG levels in indicated genotypes treated with water or BTH for 48 h prior to sampling. Total proteins were extracted and analyzed by immunoblotting. HRT-FLAG levels in BTH-treated HRT phot1 plants were similar to those observed in BTH-treated Di-17 plants (Fig. S4C). (B) HRT-FLAG levels in HRT cry1, HRT cry2, HRT phot1 and HRT phot2 plants before (0h) and after TCV inoculation. (C) CRY1, CRY2, PHOT1 and PHOT2 levels in total proteins extracted from Di-17 plants before (0h) and after TCV inoculation. Arrows indicate respective proteins. (D) Levels of CRY1, CRY2, PHOT1 and PHOT2 at various time points after dark treatment. Arrows indicate respective proteins. (E) CRY2 levels in proteins extracted from soluble (S) and membrane (M) fractions before (0h) and after dark treatments. (F) Immunoblot showing HRT-FLAG and CRY2 levels in total proteins extracted from Di-17 HRT-FLAG plants exposed to blue light (7 \(\mu\text{mol m}^{-1} \text{s}^{-1}\)) for 3-24 h. (G) Typical morphological phenotypes of TCV inoculated Di-17 HRT-FLAG plants kept in 14h L:10h D photocycles or under blue light for 48h post-inoculation. (H) Immunoblot showing levels of TCV coat protein (CP) in total proteins extracted from systemic tissues of plants shown in G. (I) Typical HR (upper panel) and PR-1 expression (lower panel) phenotypes of TCV inoculated Di-17 HRT-FLAG plants kept under 14h L:10h D photocycles or under blue light for 72h. Ponceau-S staining of the western blots shown in A, B, C, D, E, F and H were used as the loading control.
Figure 4. 8. HRT interacts with COP1 and is degraded via the 26S proteasome pathway. (A) Confocal micrographs showing Bimolecular fluorescence complementation for indicated proteins. Agroinfiltration was used to express protein in transgenic *N. benthamiana* plants expressing the nuclear marker CFP-H2B or wild-type plants (see Fig. S5B). The micrographs shown are CFP and YFP overlay images. The arrow indicates nucleus. Scale bars: 10 µM. (B) Co-immunoprecipitation of HRT-MYC with α-FLAG. *N. benthamiana* plants were Agroinfiltrated with COP1-FLAG or HRT-MYC or both. Total extract (input) and immunoprecipitated proteins were analyzed using immunoblotting with α-MYC and reprobed with α-FLAG. (C) Co-immunoprecipitation of PHOT2-MYC with α-FLAG. *Nicotiana benthamiana* plants were Agroinfiltrated with COP1-FLAG or PHOT2-MYC or both. Total extract (input) and immunoprecipitated proteins were analyzed using immunoblotting with α-MYC and reprobed with α-FLAG. (D) Immunoblot showing HRT-FLAG levels in total proteins extracted from Di-17 HRT-FLAG plants infiltrated with mock solutions (0.1% DMSO, control), plant protease inhibitor cocktail (protease) or the 26S proteasome specific inhibitor (MG132), prior to normal or blue-light treatments (7 µmol m⁻¹ s⁻¹) for 24 h. (E) Typical morphological phenotypes of TCV-inoculated Di-17 plants kept under blue light for 48h after treatment with DMSO or MG132.
Figure 4.9. Localization of TCV-CP and bimolecular fluorescence complementation assays showing interaction between COP1-HRT and PHOT2-COP1. (A) TCV-CP levels in soluble and microsomal fractions extracted from plants infected with TCV at 72 hr postinoculations. Ponceau-S staining of the Western blots was used as the control loading control. (B) Confocal micrographs showing bimolecular fluorescence complementation
for indicated proteins. Agroinfiltration was used to express protein in wild-type or transgenic (Bottom showing PHOT2-GST and COP1-GST) *N. benthamiana* plants expressing the nuclear marker CFP-H2B. The arrow indicates nucleus. The micrographs shown are CFP and YFP overlay images (Scale bars : 10 µM).
Figure 4. 10. RPS2- and RPS4-mediated resistance are not dependent on blue-light photoreceptors. (A and B) Growth of virulent or avirulent P. syringae expressing avrRpt2 (A) or avrRps4 (B) on indicated genotypes. The error bars indicate SD (n=3). (C) PR-1 gene expression in mock- or P. syringae (avrRpt2) - inoculated plants. Leaves were sampled at 3 dpi. Ethidium bromide staining of rRNA was used as a loading control.
CHAPTER 5

Multiprotein complex containing RNA silencing components mediate resistance signaling against turnip crinkle virus

The various modes of defense induced upon the recognition of pathogen-derived molecules provide species level resistance to non-host pathogens, local and systemic resistance to race-specific pathogens, and basal resistance to virulent pathogens. Resistance (R) gene-mediated or species-specific immunity is induced when a strain-specific avirulent (avr) protein from the pathogen associates directly/indirectly with a cognate plant R protein (reviewed in Martin et al., 2003; Chisholm et al., 2006; Jones and Dangl, 2006; Kachroo et al., 2006). R proteins mediating defense against bacteria, fungi, viruses, oomycetes, nematodes and insects have been identified from a variety of plants. A majority of the known R proteins belong either to the coiled coil (CC)-nucleotide binding site (NBS)-leucine rich repeat (LRR) or toll-interleukin 1 receptor (TIR)-NBS-LRR class. Several components of R-mediated signaling have been identified and many of these participate in basal defenses as well (Kachroo and Kachroo, 2007).

In the absence of the R protein, plants can induce a basal defense response against pathogens, although this mode of defense fails to contain the pathogen in contrast to R gene-mediated resistance. Basal defense to viruses often involves RNA silencing (reviewed in Mlotshwa et al., 2008; Ding and Voinnet, 2007; Ding, 2010; Voinnet, 2010). RNA silencing is induced upon the formation of a double stranded (ds) RNA, which is processed to small (s) 20-30 nucleotide (nt) dsRNA with staggered ends. One strand of the dsRNA is then incorporated into a large ribonucleoprotein complex called the RNA-induced silencing complex (RISC), which then cleaves the target viral RNA. The ribonuclease III enzymes called Dicers, mediate the processing of dsRNA into sRNA. Arabidopsis plants encode four Dicer-like proteins (DCL1 to DCL4) and of these DCL2, DCL3, and DCL4 process long dsRNA molecules of various cellular origins into sRNA that are 22, 24 or 21 nt in length, respectively. RISC complexes are formed of sRNA strand and a member of the Argonaute (AGO) protein family, which are also
called slicer proteins since they cleave target single stranded RNA at the duplex formed with the guide-strand sRNA. There are ten AGO homologues in Arabidopsis, but the functions of most of these remain unclear. Although emerging results implicate small RNA in R protein-mediated resistance (Navarro et al., 2006; Katiyar-Agarwal et al., 2006, 2007; Bhattacharjee et al., 2009), the relationship between R protein-mediated signaling and RNA silencing pathway remains unclear.

Viruses in turn have evolved to express suppressors that target host RNA silencing components and thereby ensure replication in the host (reviewed in Mlotshwa et al., 2008; Ding and Voinnet, 2007; Ding, 2010; Voinnet, 2010). Interestingly, in many cases these suppressors of silencing also act as avr factors and their direct/indirect interaction with the host R proteins leads to activation of defense responses. Most R-avr protein interactions are thought to occur indirectly and involve other host proteins that are targeted by the pathogen-encoded effector proteins. The interaction between R-avr proteins is thought to follow the “guard model” or modified version thereof, where R protein guards other host protein(s) referred to as “guardee” (Van der Biezen and Jones 1998; Collier et al., 2009; Van der Hoorn and Kamoun, 2008). Any alteration in the “guardee” protein, brought about by a pathogen-encoded avr factor, is thought to activate the R protein resulting in initiation of defense responses against the pathogen.

HRT, confers resistance to TCV, a single-stranded, positive-sense RNA virus. Most Arabidopsis ecotypes are susceptible to TCV; however, a resistant line, designated Di-17, was isolated from the Dijon (Di) ecotype (Dempsey et al., 1997). Following TCV infection, Di-17 plants develop a hypersensitive response (HR), express several defense genes, including PR-1 and accumulate SA (Dempsey et al., 1997; Kachroo et al., 2000). In contrast, plants lacking the dominant gene HRT fail to develop HR after TCV infection, do not induce PR-1 expression nor accumulate increased levels of SA. Plants lacking HRT also allow systemic spread of the virus, which is associated with a crinkled leaf and drooping bolt appearance, followed by death of the plant (Chandra-Shekara et al., 2004; Dempsey et al., 1997; Kachroo et al., 2000). However, HRT alone is not sufficient to confer TCV resistance, since all F₁ plants and ~75% of HR-developing F₂
plants derived from a cross between resistant (Di-17) and susceptible (Col-0) ecotypes succumb to disease. Furthermore, ~90% of transgenic Col-0 plants expressing the HRT transgene are susceptible to TCV even though these plants develop HR upon TCV inoculation (Cooley et al., 2000). Subsequent studies showed that the recessive allele of a second, as yet unidentified, locus designated rrt (regulates resistance to TCV), is also required for resistance (Chandra-Shekara et al., 2004; Kachroo et al., 2000). The requirement of rrt for resistance can be overcome by increasing the levels of HRT transcript via exogenous application of SA (Chandra-Shekara et al., 2004).

HRT-mediated signaling is activated in the presence of the coat protein (CP) of the virus (Cooley et al., 2000; Zhao et al., 2000; Jeong et al., 2008), although a direct interaction between HRT and TCV-CP has not been detectable. Resistance to TCV is dependent upon SA, and its signaling components EDS1, PAD4 and EDS5 (Kachroo et al., 2000). Of these, EDS1 and SA function redundantly in HRT-mediated signaling (Venugopal et al., 2009). Resistance is also dependent on blue-light photoreceptors, and of these cryptochrome 2 and phototropin 2 are required for stability of HRT (Chandra-Shekara et al., 2006; Jeong et al., 2010a; Jeong et al., 2010b). HRT interacts with CRT1, an ATPase; and a mutation in CRT1 partially compromises resistance to TCV (Kang, 2008, 2010; Jeong et al., 2010a).

Here, I show that components of RNA silencing and photomorphogenesis pathways are intricately involved in regulation of R-mediated signaling. Genetic analysis showed that RDR6, DCL4 and DRB4 were required for HRT-mediated resistance. Biochemical analysis showed that HRT was present in a complex containing DCL4, AGO1, RDR6 and CRT1. DRB4 and CRT1 were required for stability of HRT and negatively regulate the E3 ubiquitin ligase, COP1 (Chen et al., 2004). The avr factor, CP, specifically resulted in the dissociation of the HRT-DRB4 complex. Furthermore, CP relocalized to the nucleus in the presence of DRB4, which correlated with its avirulent function.
RDR6 and DCL4 are required for HRT-mediated resistance but not HR

To assess the role of RNA silencing components in HRT-mediated signaling, the rdr1, rdr2, rdr6, dcl1, dcl2, dcl3 and dcl4 alleles were crossed into the Di-17 background. F2 progeny homozygous for the mutant allele and containing at least one copy of HRT were tested for defense phenotypes. All of the HRT-containing F2 progeny from these crosses developed visible and microscopic HR following TCV infection (Figure 5.1A&B), and induced PR-1 gene expression (Figure 5.1C). This suggested that these RNA silencing components are not required for HR development or the associated PR-1 expression. All hrt/hrt F2 progeny showed susceptibility to TCV. Approximately 75% (homo/heterozygous for HRT) of F2 progeny from an HRT rrt X hrt RRT cross, developed HR upon TCV infection. However, only 25% (homo/heterozygous for HRT, but homozygous for rrt) of these HR-developing progeny were able to resist TCV infection (Table 5.1). The resistance phenotype in HRT rdr1, HRT rdr2, HRT dcl1, HRT dcl2 and HRT dcl3 also showed expected Mendelian segregation (Table 5.1), suggesting that mutations in RDR1, RDR2, DCL1, DCL2 or DCL3 do not affect HRT-mediated resistance (Figure 5.2A). In contrast, mutations in RDR6 or DCL4 abrogated HRT-mediated resistance; all plants containing HRT and mutant rdr6 or dcl4 loci showed typical phenotypes associated with susceptible plants (Figure 5.1D, Table 5.1). The appearance of disease symptoms also correlated with the presence of TCV transcript in the systemic non-inoculated tissues (Figure 5.2A&B). Together, these data suggest that RDR6 and DCL4 are required for HRT-mediated resistance.

SA accumulation is a critical signaling event required for resistance to TCV. Both free and conjugated (SAG) forms of SA increase by ~10- and ~15-fold, respectively, in TCV-inoculated Di-17 plants (Kachroo, et al., 2000). SA/SAG levels in TCV-inoculated HRT rdr6 and HRT dcl4 plants were tested to determine if the loss of resistance in these mutant backgrounds is due to suppressed induction of SA accumulation. No defects in SA or SAG accumulation were evident (Figure 5.1E). These data correlate well with the fact that the TCV-induced increase in SA is associated with HR, and HRT rdr6 and HRT dcl4 show normal HR to TCV (Figure 5.1A).
The effect of mutations in the RDR6 or DCL4 genes on HRT levels was analyzed using Di-17 HRT-FLAG plants containing rdr6 or dcl4 mutations. Neither mutation significantly altered HRT-FLAG levels (Figure 5.1F). In addition, as in Di-17 plants, HRT-FLAG was only detected in the membraneous fraction (Figure 5.2C) and the HRT levels did not change after TCV inoculation (Figure 5.2D). Exogenous SA (or its biologically functional analog, BTH) induces the accumulation of HRT transcripts (Chandra-Shekara et al., 2004, 2006, 2007) and HRT-FLAG protein (Jeong et al., 2010a). I therefore tested whether exogenous SA or its biologically functional analog, BTH, induces HRT and PR-1 expression in HRT rdr6 and HRT dcl4 plants. Interestingly, BTH application increased the HRT transcript in HRT rdr6 and HRT dcl4 plants (Figure 5.2E). However, HRT protein levels were only induced in HRT rdr6 but not in HRT dcl4 plants (Figure 5.1G). Consistent with these data, exogenous BTH conferred TCV resistance and reduced HR to TCV and PR-1 induction in HRT rdr6, but not in HRT dcl4 plants (Figures 5.1H, 1I, 5.2F). These data suggest that DCL4 and RDR6 likely affect different steps in the HRT-mediated signaling pathway.

**RDR6 associates with the HRT-interacting CRT1**

Since RDR6 and DCL4 are required for TCV resistance, I next used bi-molecular fluorescence complementation (BiFC) and co-immunoprecipitation (IP) assays to determine whether they interact with HRT or its interacting partner CRT1 (Kang et al., 2008). Neither DCL4 nor RDR6 interacted with HRT in BiFC assays; however, RDR6, but not DCL4, interacted with CRT1 (Figure 5.3A). The RDR6-CRT1 interaction was further confirmed by co-IP assay (Figure 5.3B). Unlike the HRT-CRT1, which localizes to the cell periphery and the endosomes, the CRT1-RDR6 complex was primarily detected along the cell periphery (Figure 5.3A). This suggested that CRT1 might form spatially distinct complexes with HRT and RDR6. However, co-expression of RDR6 and CRT1 in N. benthamiana showed that at least some RDR6 protein co-localized with CRT1 (Figure 5.3C), suggesting that HRT, CRT1 and RDR6 might form a complex. This was further supported by the observation that expression of RDR6 and HRT did not alter the HRT-CRT1 or the RDR6-CRT1 complexes, respectively (data not shown). In addition, these results suggested that HRT and RDR6 do not compete for binding with...
CRT1. To determine if these three proteins were present in a single complex, we performed co-IP assays on total protein extracts from plants co-expressing HRT-FLAG, CRT1-MYC and RDR6-MYC. Surprisingly, and unlike BiFC, co-IP with anti-FLAG pulled down CRT1 and also RDR6 even in the absence of CRT1 (Figures 5.3D, 5.4A). Taken together, the BiFC and co-IP results suggested that the interaction between HRT and RDR6 was indirect, and that *N. benthamiana* contained the protein(s) essential for this association. Co-IP did not detect any interaction between HRT and DCL4 (Figure 5.4B).

**The DCL4-interacting DRB4 associates with HRT and regulates its stability**

The dsRNA-binding protein 4 (DRB4) is known to interact with DCL4 (Hiraguri et al., 2004). I tested if like RDR6, DCL4 associated indirectly with HRT via the interacting protein DRB4. Both BiFC (Figure 5.5A) and co-IP assays (Figure 5.5B) revealed that DRB4 did interact with HRT. Furthermore, co-IP assays showed that the HRT, DRB4, and DCL4 proteins were present in a single complex (Figure 5.5C). To verify the role of DRB4 in TCV resistance, HR and resistance phenotypes were evaluated in *HRT drb4* F2 plants obtained from a Di-17 x *drb4* cross. DRB4 was indeed found to be required for HRT-mediated resistance (Figure 5.5D, Table 5.1), but not HR formation, or *PR-I* gene expression (Figures 5.5E, 5.5F). SA/SAG levels in TCV-inoculated *HRT drb4* plants were tested to determine if the loss of resistance in these mutant backgrounds was due to a lack/reduction of SA accumulation. No significant defects in SA or SAG accumulation were evident (Figures 5.6A, 5.6B) and these data correlated well with the normal HR to TCV in *HRT drb4* plants (Figure 5.5E).

The effect of the *drb4* mutation on HRT levels was analyzed in Di-17 *HRT-FLAG drb4* plants. Notably, unlike *HRT dcl4*, the *HRT drb4* plants showed a significant reduction in HRT protein (Figure 5.5G), but not transcript levels (Figure 5.6C). However, similar to *HRT dcl4* plants, BTH pretreatment increased *HRT* transcript, but not protein levels, in *HRT drb4* plants (Figures 5.5H, S3C). Together, these results suggested that while both DRB4 and DCL4 were required for HRT-mediated resistance to TCV and SA-mediated induction of HRT expression, only DRB4 was required for stability of HRT.
CRT1 interacts with DRB4 and is required for the stability of HRT
Like DRB4, CRT1 too interacts with HRT (Kang et al., 2008), leading to the possibility that CRT1 might also be required for the stability of HRT. Indeed, similar to HRT drb4, the HRT crt1 plants showed a significant reduction in HRT levels (Figure 5.7A). As in drb4, HRT transcript levels were normal in HRT crt1 plants (Figure 5.8A), suggesting a post-transcriptional role for CRT1 in HRT stability. This result prompted us to check the interaction between CRT1 and DRB4. Interestingly, both BiFC and co-IP analysis showed interaction between CRT1 and DRB4 (Figures 5.7B, 5.7C). In addition, the HRT-DRB4 or the HRT-CRT1 interactions were not altered by the presence of CRT1 or DRB4, respectively (Figure 5.7D). Together, these data suggested that CRT1 and DRB4 likely do not compete with each other for interaction with HRT and that HRT, CRT1 and DRB4 likely existed as a complex.

CRT1 was earlier shown to bind the NBS domain of HRT (Kang et al., 2008). In comparison, DRB4 bound to the CC, NBS and LRR domains (Figures 5.7E, 5.8B). This suggested that the N-terminal CC and C-terminal LRR domains of the HRT protein may fold over the NBS domain, thereby facilitating the interaction of DRB4 with multiple domains of HRT (Figure 5.8C). Furthermore, both CC and NBS domains self-interacted (Figure 5.7E), suggesting intra-molecular interactions within HRT (Figure 5.8C). To assess this further I studied self-interaction of the full-length HRT protein. HRT did interact with itself (Figure 5.7F), suggesting that it was present as a dimer or multimer. Interestingly, DRB4 also self-interacted (Figures 5.7E, 5.8D), suggesting that the HRT complex likely contains multiple units of these proteins.

In chapter 4, I showed that HRT interacted with the E3 ubiquitin ligase, COP1, and was degraded in a proteasome-dependent manner in the absence of blue-light photoreceptors that negatively regulate COP1 (Jeong et al., 2010a). This and the reduced stability of HRT in drb4 and crt1 mutant plants raised the possibility that these proteins might also negatively regulate COP1. To test this, I first assessed if COP1 ubiquitinated HRT. HRT was expressed alone or together with COP1 in N. benthamiana and the immunoprecipitated HRT was analyzed using anti-ubiquitin antibodies. Indeed, co-
expression of HRT and COP1, but not HRT alone, resulted in ubiquitination of HRT (Figure 5.7G). Next, I assessed the interaction between COP1 and DRB4 and/or CRT1 proteins, since all three showed direct interaction with HRT. DRB4 and CRT1, like HRT, interacted with COP1 in BiFC and co-IP assays (Figures 5.7H, 5.9A, 5.9B). Notably, the COP1-DRB4 complex was also present in the nucleus and formed nuclear speckles (Figure 5.7H), a common feature exhibited by several COP1-interacting proteins (Holm et al., 2002). Together, these data suggest that DRB4 and CRT1 likely prevent COP1-mediated degradation of HRT by negatively regulating COP1 activity.

**Viral avirulence effector, coat protein, dissociates the HRT-DRB4 complex**

I next assayed the effect of TCV-CP on the HRT-CRT1 and HRT-DRB4 interactions. Interestingly, while CP did not alter interaction of HRT with CRT1, it abolished the HRT-DRB4 interaction (Figures 5.10A, 5.10B). CP also did not affect interaction of DRB4 with DCL4 (Figure 5.11A), suggesting a specific effect of CP on the HRT-DRB4 complex. To determine if dissociation of the HRT-DRB4 complex involved binding of CP to one or both proteins, interaction of CP with HRT and DRB4 were analyzed. Co-IP assays between CP and HRT or DRB4 gave inconsistent results, so the CP-DRB4/HRT interactions were assayed using yeast-two hybrid assays, which detected a weak, but specific interaction between CP and DRB4 (Figures 5.11B, 5.11C). To determine if the CP-mediated dissociation of the HRT-DRB4 complex was required for HRT-mediated resistance, the HRT-DRB4 interaction was evaluated in the presence of mutant CP, R8A. As shown previously (Choi et al., 2004), TCV containing the R8A mutation in CP was virulent on Di-17 (Figure 5.11D). Surprisingly, R8A also resulted in the dissociation of the HRT-DRB4 complex (Figure 5.11E). This prompted me to assess if R8A was capable of initiating HRT-mediated signaling. Interestingly, inoculation with R8A did induce HR, although the lesion size was smaller compared to HR triggered in response to wt TCV (Figure 5.11F). This was further reconfirmed using *N. benthamiana*; coexpression of HRT with either CP or R8A induced HR and, as in Arabidopsis, HRT+CP induced a stronger HR compared to HRT+R8A (Figure 5.10C). Together, these results suggest that R8A was able to induce HRT-mediated signaling and that the dissociation of the HRT-DRB4 complex alone was not responsible for the activation of HRT.
I next studied if CP affects the localization of HRT or DRB4 and vice-versa. HRT and DRB4 were tagged with GFP and CP was tagged with RFP. As expected, when expressed alone HRT and DRB4 localized to the periphery (plasma membrane; Jeong et al., 2010a) or nucleus (Hiraguri et al., 2005), respectively (Figure 5.10D). In comparison, CP or its mutant derivative R8A, were primarily seen in inclusion bodies (Figure 5.10D). Interestingly, coexpression of CP-RFP or R8A-RFP with HRT-GFP resulted in localization of CP/R8A to the periphery of the cell (Figure 5.10E). No change was observed in the localization of HRT-GFP. Moreover, coexpression of CP-RFP with DRB4-GFP resulted in the localization of CP to the nucleus and DRB4 to the nucleus as well as periphery (Figure 5.10E). The nuclear localization of CP was consistent with the presence of both mono and bipartite nuclear localization signals (NLS) at the N-terminus of CP (Figure 5.11D). Furthermore, DRB4 was unable to alter localization of CP derivatives, which carry mutations in the monopartite (R6A) or the bipartite (R8A) NLS (Figures 5.10E, 5.11G). Like R8A, TCV containing the R6A mutation also confers virulence on Di-17 plants (Figure 5.11D; Choi et al., 2004). Notably, DRB4 co-localized with CP/R6A/R8A when these proteins were co-expressed (Figures 5.10E, 5.11G).

These results prompted me to assess whether CRT1 co-localized with CP. CRT1-GFP localized to endosome-like vesicles and the periphery of the cell (Figure 5.10D). However, when co-expressed with CP, both CRT1 and CP were located in the nucleus (Figure 5.10E). Unlike wt CP, mutant CP derivatives (R8A or R6A) were unable to change the location of CRT1 from vesicles to the nucleus. Conversely, the presence of CRT1 did not direct the R8A or R6A mutants to the nucleus (Figures 5.10E, 5.11H). These results suggest that a dynamic flux in the sub-cellular localization of TCV-CP and some defense-related host proteins might contribute to the activation of HRT.

**HEN1 is not required for HRT-mediated signaling**

The requirements for DCL4/RDR6/DRB4 suggest that the RNA-silencing pathway may participate in HRT-mediated resistance. This was tested by analyzing the involvement of HEN1, an essential, common regulator of RNA silencing that functions downstream of
DCL4, RDR6, and DRB4. The HEN1 methylase methylates the 2’ hydroxyl group at the 3’-terminus of cellular and viral sRNAs to protect them from degradation, and is required for all RNA-silencing pathways (Yu et al., 2005; Ding and Voinnet, 2007; Ding, 2010; Voinnet, 2010). Notably, a KO mutation in HEN1 did not impair HRT-mediated resistance to TCV (Table 5.1) or TCV-induced HR and PR-1 expression (Figures 5.12A, 5.12B). Thus, the requirement for DCL4, RDR6, and DRB4 in HRT-mediated resistance appears to be unrelated to their roles in the generation/amplification of sRNAs during RNA silencing. This unexpected finding suggests that HRT-mediated signaling does not enlist the RNA-silencing pathway itself. This conclusion is further supported by the fact that the avirulence function of CP is distinct from its function as a suppressor of RNA silencing (Figure S6D; Choi et al., 2004).

**AGO1 interacts with DRB4 and is part of the HRT complex**

Recent studies have shown that the TCV avirulence effector CP interacts with AGO1 (Azevedo et al., 2010), suggesting the possibility that AGO1 might be present in a complex containing HRT and/or contribute to its activation. To test this, interaction between AGO1 and HRT was assayed first (Figures 5.13A, 5.13B). Both BiFC and co-IP assays failed to detect interaction between AGO1 and HRT. To test if AGO1 was part of a complex containing HRT, we assayed interaction between AGO1 and the HRT-interacting proteins, DRB4 and CRT1. No interaction was detected between AGO1 and CRT1, but AGO1 did interact with DRB4 (Figures 5.13A, 5.13B, 5.14A). To determine if AGO1 was a part of the larger complex, HRT, DRB4, and AGO1 were co-expressed together. AGO1 immunoprecipitated using antibodies specific to the tag on HRT (Figure 5.13B). This data suggests that AGO1 was present in the HRT complex. I next tested if CP dissociated DRB4-AGO1 or the HRT-DRB4-AGO1 complex. Notably, CP did not affect interaction of DRB4 with AGO1 (Figure 5.14A), but it did dissociate DRB4 from the HRT-DRB4-AGO1 complex (Figure 5.13B). Surprisingly, dissociation of DRB4 from the HRT-DRB4-AGO1 complex did not reduce AGO1 levels (Figure 5.13B), suggesting that perhaps CP was replacing DRB4 in this complex. To test this I assessed interaction between HRT-AGO1 in the presence of CP. Indeed, HRT interacted with AGO1, but only when CP was present (Figures 5.13C, 5.14B). Together, these results
suggest that CP replaces DRB4 in the HRT-DRB4-AGO1 complex and serves as a bridge protein between HRT and AGO1. The similar size of the CP and DRB4 proteins (~38 kDa), may further facilitate substitution of DRB4 by CP in the HRT-DRB4-AGO1 complex.

To determine if the HRT-CP-AGO1 complex was formed when CP was replaced with R8A, I assessed interaction between AGO1 and the R8A mutant of TCV-CP. Notably, AGO1 interacted with R8A as efficiently as with the wt CP (Figure 5.13D) and this was further consistent with R8A-mediated dissociation of the HRT-DRB4 complex (Figure 5.13E). Since the R8A mutant of TCV-CP retains its function as a suppressor of RNA silencing (Figure 5.13D, Choi et al., 2004), the RNA-silencing activity of TCV-CP is likely not associated with its avirulence function. I next monitored the effect of CP/R8A proteins on localization of AGO1 and vice-versa. When expressed alone, AGO1-GFP and CP/R8A-RFP localized to the periphery and inclusion bodies, respectively (Figure 5.13E). However, when co-expressed with CP, both AGO1 and CP were located in the nucleus (Figure 5.13E). Unlike wt CP, the R8A mutant was unable to change the location of AGO1 from the periphery to the nucleus. Conversely, the presence of AGO1 did not direct the R8A mutant to the nucleus (Figure 5.13E).

To determine a role for AGO1 in HRT-mediated signaling, HR and resistance in HRT ago1-11 plants were analyzed. Notably, the HRT ago1-11 plants did not form a distinct HR (Figure 5.13F), and this correlated with a significant reduction in PR-1 expression (Figure 5.13G). These data suggest that AGO1 was required for normal induction of these defense responses. The role of AGO1 in HRT-mediated resistance could not be properly assessed since the ago1-11 mutant exhibits severe developmental defects. Nonetheless, the four HRT ago1-11 F2 plants that survived through the duration of our experiments showed susceptible phenotypes (Figure 5.13H).

Besides AGO1, the Arabidopsis genome encodes nine other AGO proteins and all participate in RNA silencing. The roles of AGO 2, 3, 4, 5, 7 and 9 in HRT-mediated resistance were evaluated by analyzing F2 plants derived from a cross between Di-17 and
the respective ago mutants. All of the HRT-containing F2 progeny from these crosses developed visible and microscopic HR (Figures 5.13I, 5.13J) and induction of PR-1 expression (Figure 5.13K) following TCV infection, suggesting that these RNA-silencing components are not required for these defense responses. Furthermore, the resistant phenotypes of HRT ago2, HRT ago3, HRT ago4, HRT ago5, HRT ago7 and HRT ago9 segregated in the expected Mendelian fashion (Table 5.1, Figure 5.15), arguing that AGO2, AGO3, AGO4, AGO5, AGO7 or AGO9 are not required for HRT-mediated resistance.

**DRB4 is required for RPS2- and RPM1-mediated resistance to bacterial pathogens**

The roles of RDR6, DCL4, and DRB4 in bacterial resistance were evaluated next, to determine whether these proteins played specific or generalized roles in R protein-mediated plant defenses. Mutations in RDR6 or DCL4 did not compromise HR or resistance mediated by the R protein RPS2 against *P. syringae* expressing *avrRpt2* (data not shown). In contrast, a mutation in DRB4 compromised RPS2- as well as RPM1-mediated HR as well as resistance against *P. syringae* expressing *avrRpt2* and *avrRpm1*, respectively; the *drb4* plants showed increased necrosis (Figure 5.16A) and supported ~5-7-fold higher growth of bacteria (Figures 5.16B, 5.16C). A requirement for DRB4 further correlated with its interaction with RPS2 and RPM1 (Figures 5.16D, 5.16E). Unlike DRB4, RDR6 and DCL4 did not interact with RPS2 or RPM1 (data not shown).

Together, these results suggest that DRB4 plays a general role in R protein-mediated defense against both viral and bacterial pathogens while RDR6 and DCL4 might be specific to HRT-mediated resistance.

**Discussion**

RNA silencing is one of the conserved pathways in plants and animals, which has been mostly studied with regards to basal resistance to viral pathogens. In plants the RNA-silencing pathway targets viral RNA for degradation. Viruses, in turn, encode suppressors that target one or more components of the host RNA-silencing machinery. Compromised RNA silencing in the host leads to increased accumulation of viral RNA, thereby enhancing susceptibility to the pathogen. Intriguingly, even though a number of viral
encoded suppressors of RNA-silencing serve as avirulence effectors that trigger species-specific defense in hosts containing the cognate R gene, a relationship between RNA silencing and R protein-mediated defense has not been established. In this study, I show that components of the Arabidopsis RNA-silencing machinery, including RDR6, DCL4, DRB4 and AGO1, are present as a multiprotein complex with the R protein, HRT. This complex also contains a previously characterized ATPase, CRT1, which together with DRB4 directly associates with HRT and protects HRT from COP1-mediated ubiquitination and degradation. Thus, DRB4 and CRT1 likely repress COP1 activity to stabilize the R protein. Similarly, the blue-light photoreceptors CRY2 and PHOT2 also stabilize HRT by negatively regulating COP1 (Jeong et al., 2010a). A role for light signaling components in the RNA-silencing pathway is also supported by a recent report showing the importance of light intensity in RNA silencing of transgenes (Kotakis et al., 2010). Together, these results present a complex picture wherein R protein-mediated defense intricately overlaps with that of photomorphogenesis and RNA silencing.

Results indicate that, under non-induced conditions (ie. in the absence of a pathogen-derived effector), HRT forms a multiprotein complex, involving inter- and intramolecular interactions with itself as well as with several other host proteins. The pathogen encoded avr effector, coat protein, specifically disrupts the HRT-DRB4 interaction and replaces DRB4 in this multiprotein complex. The CP-mediated release of DRB4 is also associated with the dissociation of DCL4 from the multiprotein complex (see model in Figure S8C). Thus, CP acts as a bridge facilitating interaction between HRT and AGO1. It is likely that this interaction in turn activates HRT-mediated signaling. This is further supported by the observation that AGO1 is required for HRT-mediated signaling.

Intriguingly, the TCV CP mutant R8A, which is able to induce HRT-mediated signaling, dissociates the HRT-DRB4 interaction, interacts with AGO1, and remains virulent on Di-17 plants. This suggests that the HRT-CP-AGO1 complex formation alone may not be sufficient for the activation of HRT. The fact that AGO1, DRB4 and CRT1 direct CP, but not the R8A mutants to the nucleus, correlates with loss of avirulence in the R8A mutant.
on Di-17 plants. This result suggests that the nuclear localization of CP might also be important for the resistance response. The ability of CP, but not the R8A mutant, to redirect AGO1/CRT1 and DRB4 to the nucleus and periphery, respectively, further suggests that the resistance response might involve the redistribution of cellular proteins, a combined effect of which is required to prevent the spread of the virus.

Our results also demonstrate a unique function for components of the RNA silencing pathway, which does not appear to overlap with their role in basal resistance to viral pathogens. For instance, DCL2 serves as a substitute for DCL4 and mutations in both DCL2 and DCL4 are required to compromise basal resistance to TCV (Bouché et al., 2006; Qu et al., 2008). Clearly, this is not the case for HRT-derived resistance since the dcl4 mutation alone is sufficient to compromise HRT-mediated resistance. Conversely, while basal resistance to TCV requires HEN1 (data not shown), it is not essential for HRT-mediated resistance. In this regard, DCL4, RDR6, DRB4 differ from other host components like EDS1 and SID2, which play roles in both basal and R protein-mediated resistance against TCV. It is possible that components of the RNA-silencing pathway interact with other downstream signaling components to modulate defense against pathogens. This is particularly evident in the case of pad4, cry2, and dcl4 plants, all of which are impaired in SA-mediated induction of HRT protein levels (Chandra-Shekara et al., 2004; Jeong et al., 2010a, 2010b). It will therefore be useful to study the relationship among components of the RNA-silencing machinery with other downstream signaling components of the HRT-mediated pathway.
Table 5.1. Epistatic analysis of F2 populations derived from crosses between Di-17 and various wild-type or mutant lines.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Plants analyzed</th>
<th>Genotype</th>
<th>Plants obtained</th>
<th>HR</th>
<th>R</th>
<th>S</th>
<th>χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Di-17 x Col-0</td>
<td>165</td>
<td>HRT/-</td>
<td>97</td>
<td>+</td>
<td>22</td>
<td>75</td>
<td>0.58</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRT/- rdr1</td>
<td>74</td>
<td>+</td>
<td>14</td>
<td>60</td>
<td>1.45</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRT/- RDR1/-</td>
<td>238</td>
<td>+</td>
<td>52</td>
<td>186</td>
<td>1.26</td>
<td>0.26</td>
</tr>
<tr>
<td>Di-17 x rdr2-1</td>
<td>498</td>
<td>HRT/- rdr2</td>
<td>78</td>
<td>+</td>
<td>21</td>
<td>57</td>
<td>0.15</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRT/- RDR2/-</td>
<td>263</td>
<td>+</td>
<td>59</td>
<td>204</td>
<td>0.92</td>
<td>0.33</td>
</tr>
<tr>
<td>Di-17 x rdr6-15</td>
<td>373</td>
<td>HRT/- rdr6</td>
<td>53</td>
<td>+</td>
<td>0</td>
<td>53</td>
<td>17.6</td>
<td>&lt;0.0001 ^</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRT/- RDR6/-</td>
<td>201</td>
<td>+</td>
<td>53</td>
<td>148</td>
<td>0.20</td>
<td>0.65</td>
</tr>
<tr>
<td>Di-17 x dcl1-7</td>
<td>176</td>
<td>HRT/- dcl1</td>
<td>29</td>
<td>+</td>
<td>9</td>
<td>20</td>
<td>0.56</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRT/- DCL1/-</td>
<td>94</td>
<td>+</td>
<td>21</td>
<td>73</td>
<td>0.35</td>
<td>0.55</td>
</tr>
<tr>
<td>Di-17 x dcl2-1</td>
<td>476</td>
<td>HRT/- dcl2</td>
<td>77</td>
<td>+</td>
<td>14</td>
<td>63</td>
<td>1.9</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRT/- DCL1/-</td>
<td>263</td>
<td>+</td>
<td>58</td>
<td>205</td>
<td>1.2</td>
<td>0.26</td>
</tr>
<tr>
<td>Di-17 x dcl3-1</td>
<td>549</td>
<td>HRT/- dcl3</td>
<td>99</td>
<td>+</td>
<td>25</td>
<td>74</td>
<td>0.03</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRT/- DCL1/-</td>
<td>306</td>
<td>+</td>
<td>72</td>
<td>234</td>
<td>0.35</td>
<td>0.55</td>
</tr>
<tr>
<td>Di-17 x dcl4-2</td>
<td>534</td>
<td>HRT/- dcl4</td>
<td>84</td>
<td>+</td>
<td>0</td>
<td>84</td>
<td>28.0</td>
<td>&lt;0.0001 ^</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRT/- DCL4/-</td>
<td>301</td>
<td>+</td>
<td>74</td>
<td>227</td>
<td>0.02</td>
<td>0.86</td>
</tr>
<tr>
<td>Di-17 x ago2</td>
<td>129</td>
<td>HRT/- ago2</td>
<td>20</td>
<td>+</td>
<td>4</td>
<td>16</td>
<td>0.26</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRT/- AGO2/-</td>
<td>74</td>
<td>+</td>
<td>23</td>
<td>51</td>
<td>1.45</td>
<td>0.22</td>
</tr>
<tr>
<td>Di-17 x ago3</td>
<td>134</td>
<td>HRT/- ago3</td>
<td>23</td>
<td>+</td>
<td>3</td>
<td>23</td>
<td>1.75</td>
<td>0.18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRT/- AGO3/-</td>
<td>72</td>
<td>+</td>
<td>20</td>
<td>52</td>
<td>0.29</td>
<td>0.58</td>
</tr>
<tr>
<td>Di-17 x ago4</td>
<td>133</td>
<td>HRT/- ago4</td>
<td>17</td>
<td>+</td>
<td>4</td>
<td>13</td>
<td>0.02</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRT/- AGO4/-</td>
<td>90</td>
<td>+</td>
<td>27</td>
<td>63</td>
<td>1.20</td>
<td>0.27</td>
</tr>
<tr>
<td>Di-17 x ago5</td>
<td>133</td>
<td>HRT/- ago5</td>
<td>22</td>
<td>+</td>
<td>3</td>
<td>19</td>
<td>1.09</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRT/- AGO5/-</td>
<td>74</td>
<td>+</td>
<td>24</td>
<td>50</td>
<td>1.51</td>
<td>0.21</td>
</tr>
<tr>
<td>Di-17 x ago7</td>
<td>240</td>
<td>HRT/- ago7</td>
<td>42</td>
<td>+</td>
<td>5</td>
<td>37</td>
<td>3.84</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRT/- AGO7/-</td>
<td>113</td>
<td>+</td>
<td>38</td>
<td>75</td>
<td>1.07</td>
<td>0.29</td>
</tr>
<tr>
<td>Di-17 x ago9</td>
<td>129</td>
<td>HRT/- ago9</td>
<td>18</td>
<td>+</td>
<td>2</td>
<td>16</td>
<td>1.85</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRT/- AGO9/-</td>
<td>79</td>
<td>+</td>
<td>27</td>
<td>52</td>
<td>3.54</td>
<td>0.05</td>
</tr>
<tr>
<td>Di-17 x drb4-1</td>
<td>256</td>
<td>HRT/- drb4</td>
<td>33</td>
<td>+</td>
<td>0</td>
<td>33</td>
<td>11.0</td>
<td>0.0009 ^</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRT/- DRB4/-</td>
<td>106</td>
<td>+</td>
<td>24</td>
<td>82</td>
<td>0.31</td>
<td>0.57</td>
</tr>
<tr>
<td>Di-17 x hen1-7</td>
<td>133</td>
<td>HRT/- hen1</td>
<td>20</td>
<td>+</td>
<td>7</td>
<td>13</td>
<td>1.06</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HRT/- HEN1/-</td>
<td>70</td>
<td>+</td>
<td>20</td>
<td>50</td>
<td>0.47</td>
<td>0.49</td>
</tr>
</tbody>
</table>
The genotype at $HRT$ and various mutant loci was determined by CAPS analysis

HR, hypersensitive response

Resistant

Susceptible

One degree of freedom; based on segregation of 3 susceptible:1 resistant plants

Statistically significant
Figure 5.1. HRT-mediated resistance is dependent on RDR6 and DCL4. (A) HR formation in indicated genotypes at 72 h post inoculation with TCV. (B) Trypan blue-stained leaf showing microscopic cell death phenotype on TCV-inoculated leaves. Scale bars, 270 microns. (C) Expression of PR-1 gene in indicated genotypes after mock- or TCV-inoculation. Total RNA was extracted from inoculated leaves at 3 dpi. Ethidium bromide staining of rRNA was used as the loading control. The PR-1 levels in TCV inoculated HRT dcl1 plants were similar to those in Di-17 (data not shown) (D) Typical morphological phenotypes of TCV-inoculated Di-17, HRT rdr6, HRT dcl4 and Col-0 plants. Plants were photographed at 14 dpi. (E) SA and SAG levels in indicated genotypes at 72 h post inoculation with buffer (Mock) or TCV. Asterisks indicate data statistically significant from wt Di-17 (P<0.05, n=6). The error bars indicate SD. (F) Western blot showing HRT-FLAG levels in indicated genotypes. Ponceau-S staining of the Western blot was used as the loading control. (G) Western blot showing HRT-FLAG levels in indicated genotypes 48h after water (-) or BTH (+) treatments. Ponceau-S staining of the Western blot was used as the loading control. (H) Percentage resistant plants obtained in indicated genotypes that were treated with water (gray bars) or BTH (black bars) for 48 h before TCV inoculation. Approximately 40–50 plants were inoculated in four separate experiments and analyzed for resistance phenotype. Asterisks denote 100% susceptibility. (I) HR formation in indicated genotypes at 72 h post inoculation with TCV. Plants were treated with water (-) or BTH (+) for 48 h prior to TCV inoculation.
Figure 5.2. Morphological and biochemical phenotypes of *HRT rdr* and *HRT dcl* plants. (A) Typical morphological phenotypes of TCV-inoculated Di-17, *HRT rdr* and *HRT dcl* plants. Plants were photographed at 18 dpi. (B) Systemic spread of TCV to uninoculated tissue in TCV-inoculated plants. The plants were sampled at 18 dpi. Ethidium bromide staining of rRNA was used as a loading control. (C) HRT-FLAG levels in total (T), soluble (S) and membrane (M) fractions extracted from indicated genotypes. Ponceau-S staining of the Western blot was used as the loading control. (D) HRT-FLAG levels in TCV-inoculated plants sampled at indicated hours post-inoculation (hpi). Ponceau-S staining of the Western blot was used as the loading control. (E) Quantitative RT-PCR analysis showing relative levels of *HRT* transcript in water- and BTH-treated plants sampled 48 h post treatments. (F) Expression of *PR-1* gene in indicated genotypes after water or BTH treatments and/or mock- or TCV-inoculation. Total RNA was extracted from inoculated leaves at 3 dpi. Ethidium bromide staining of rRNA was used as the loading control.
Figure 5.3. CRT1 interacts with RDR6. (A) Confocal micrographs showing BiFC for indicated proteins. Agroinfiltration was used to express protein in transgenic *Nicotiana benthamiana* plants expressing the nuclear marker CFP-H2B (Scale bar, 10 µM). (B) Coimmunoprecipitation of RDR6-MYC with CRT1-FLAG. *N. benthamiana* plants were agroinfiltrated and total extracts (input) and immunoprecipitated proteins were analyzed with α-MYC and α-FLAG. (C) Confocal micrographs showing localization of RDR6-GFP and CRT1-RFP when they were co-expressed. (D) Coimmunoprecipitation of RDR6-MYC with HRT-FLAG. *N. benthamiana* plants were agroinfiltrated and total extracts (input) and immunoprecipitated proteins were analyzed with α-MYC and α-FLAG.
Figure 5.4. HRT does not interact with DCL4. (A) Coimmunoprecipitation of CRT1-MYC and RDR6-MYC with HRT-FLAG. *N. benthamiana* plants were agroinfiltrated and total extracts (input) and immunoprecipitated proteins were analyzed with α-MYC and α-FLAG. (B) Coimmunoprecipitation assay showing no interaction between DCL4-MYC and HRT-FLAG proteins. *N. benthamiana* plants were agroinfiltrated and total extracts (input) and immunoprecipitated proteins were analyzed with α-MYC and α-FLAG.
Figure 5.5. HRT-mediated resistance is dependent on DRB4. (A) Confocal micrographs showing BiFC for indicated proteins. Agroinfiltration was used to express protein in transgenic *Nicotiana benthamiana* plants expressing the nuclear marker CFP-H2B (Scale bar, 10 µM). (B) Coimmunoprecipitation of DRB4-MYC with HRT-FLAG. *N. benthamiana* plants were agroinfiltrated and total extracts (input) and immunoprecipitated proteins were analyzed with α-MYC and α-FLAG. (C) Coimmunoprecipitation of DRB4-MYC and HRT-MYC with DCL4-FLAG. *N. benthamiana* plants were agroinfiltrated and total extracts (input) and immunoprecipitated proteins were analyzed with α-MYC and α-FLAG. (D) Typical morphological phenotypes of TCV-inoculated Di-17, *HRT drb4* and Col-0 plants. Plants were photographed at 14 dpi. (E) Visible (upper panel) and microscopic (bottom panel) HR formation in indicated genotypes at 72 h post inoculation with TCV. Scale bars, 270 microns. (F) Expression of *PR-1* gene in indicated genotypes after mock- or TCV-inoculation. Total RNA was extracted from inoculated leaves at 3 dpi. Ethidium bromide staining of rRNA was used as the loading control. (G) Western blot showing HRT-FLAG levels in indicated genotypes. Ponceau-S staining of the Western blot was used as the loading control. (H) Western blot showing HRT-FLAG levels in indicated genotypes 48h after water (-) or BTH (+) treatments. Ponceau-S staining of the Western blot was used as the loading control.
Figure 5.6. *HRT* *drb4* plants accumulate normal levels of SA and *HRT* transcript. SA (A) and SAG (B) levels in indicated genotypes at 72 h post inoculation with buffer (Mock) or TCV. Asterisks indicate data statistically significant from wt Di-17 (P<0.05, n=3). The error bars indicate SD. (C) Quantitative RT-PCR analysis showing relative levels of *HRT* transcript in water- and BTH-treated plants sampled 48 h post treatments.
Figure 5.7. CRT1 and DRB4 form a complex with HRT and negatively regulate COP1. (A) Western blot showing HRT-FLAG levels in indicated genotypes. Ponceau-S staining of the Western blot was used as the loading control. (B) Confocal micrographs showing BiFC for indicated proteins. Agroinfiltration was used to express protein in transgenic Nicotiana benthamiana plants expressing the nuclear marker CFP-H2B (Scale bar, 10 µM). (C) Coimmunoprecipitation of DRB4-MYC with CRT1-FLAG. N. benthamiana plants were agroinfiltrated and total extracts (input) and immunoprecipitated proteins were analyzed with α-MYC and α-FLAG. (D) Confocal micrographs showing BiFC for HRT-DRB4 and HRT-CRT1 in the presence or absence of CRT1 and DRB4, respectively. CRT1 and DRB4 were expressed as MYC-tagged proteins. (E) Confocal micrographs showing BiFC for indicated proteins. Agroinfiltration was used to express protein in transgenic Nicotiana benthamiana plants expressing the nuclear marker CFP-H2B (Scale bar, 10 µM). (F) Coimmunoprecipitation of HRT-MYC with HRT-FLAG. N. benthamiana plants were agroinfiltrated and total extracts (input) and immunoprecipitated proteins were analyzed with α-MYC and α-FLAG. (G) Immunoblot showing ubiquitination of HRT in presence of COP1. HRT-FLAG was expressed alone or with COP1, immunoprecipitated with α-FLAG and probed with α-Ub, α-MYC and α-FLAG. (H) Confocal micrographs showing BiFC for indicated proteins. Agroinfiltration was used to express protein in transgenic Nicotiana benthamiana plants expressing the nuclear marker CFP-H2B (Scale bar, 10 µM).
Figure 5.8. DRB4 and CRT1 self interact with each other. (A) Quantitative RT-PCR analysis showing relative levels of *HRT* transcript in water- and BTH-treated plants sampled 48 h post treatments. (B) Coimmunoprecipitation of DRB4-MYC with CC-FLAG, NBS-FLAG and LRR-FLAG. *N. benthamiana* plants were agroinfiltrated and total extracts (input) and immunoprecipitated proteins were analyzed with α-MYC and α-FLAG. (C) Hypothetical model showing possible arrangement of HRT and DRB4 proteins. Yellow, orange and blue indicate CC, NBS, and LRR domains, respectively. (D) Coimmunoprecipitation of DRB4-MYC with DRB4-FLAG. *N. benthamiana* plants were agroinfiltrated and total extracts (input) and immunoprecipitated proteins were analyzed with α-MYC and α-FLAG. Similar to CRT1, two DRB4 forms were observed occasionally.
Figure 5.9. DRB4 and CRT1 interact with COP1. (A) Coimmunoprecipitation of COP1-MYC with DRB4-FLAG. *N. benthamiana* plants were agroinfiltrated and total extracts (input) and immunoprecipitated proteins were analyzed with *α*-MYC and *α*-FLAG. (B) Coimmunoprecipitation of COP1-MYC with CRT1-FLAG. *N. benthamiana* plants were agroinfiltrated and total extracts (input) and immunoprecipitated proteins were analyzed with *α*-MYC and *α*-FLAG.
Figure 5.10. CP dissociates HRT-DRB4 complex and relocalizes to nucleus in presence of CRT1 and DRB4. (A) Coimmunoprecipitation of DRB4-MYC with HRT-FLAG in presence and absence of CP. *N. benthamiana* plants were agroinfiltrated and total extracts (input) and immunoprecipitated proteins were analyzed with α-MYC, α-CP and α-FLAG. (B) Coimmunoprecipitation of CRT1-MYC with HRT-FLAG in presence and absence of CP. *N. benthamiana* plants were agroinfiltrated and total extracts (input) and immunoprecipitated proteins were analyzed with α-MYC, α-CP and α-FLAG. (C) Typical phenotypes seen on *N. benthamiana* leaves infiltrated with indicated constructs. Agroinfiltration was used to express HRT, CP and R8A proteins and the leaf was photographed at 5 days post treatment. Small and big circles indicate point of infiltration and zone of visible lesion, respectively. (D) Confocal micrographs showing localization of HRT, CRT1, DRB4, CP and its mutant derivative R8A. (E) Confocal micrographs showing localization of indicated proteins co-expressed in pairs (Scale bar, 10 μM).
A

<table>
<thead>
<tr>
<th>DCL4-DRB4</th>
<th>DCL4-DRB4-CP</th>
<th>DCL4-DRB4</th>
<th>DCL4-DRB4-CP</th>
<th>DRB4-MYC</th>
<th>DCL4-FLAG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>α-MYC</td>
<td>α-FLAG</td>
</tr>
</tbody>
</table>

Input    IP with α-FLAG

B

- Leucin
- Tryptophane

- Leucin
- Tryptophane
- Histidine

pGADT7-CP + pGBK7-DRB4
pGADT7-CP + pGBK7
pGADT7-CP + pGBK7-HRT

C

β-galactosidase activity (units/min/cell)

pGADT7-CP + pGBK7
pGADT7-CP + pGBK7-DRB4
D

WT: MENDPRVRKFSAGDQAQWAIKWQKKG
R6A: MENDPRVRKFSAGDQAQWAIKWQKKG
R8A: MENDPRVAKFSAGDQAQWAIKWQKKG

<table>
<thead>
<tr>
<th>Phenotype on Col-0</th>
<th>Phenotype on Di-17</th>
<th>RNA silencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>R</td>
<td>+</td>
</tr>
<tr>
<td>S</td>
<td>S</td>
<td>+</td>
</tr>
<tr>
<td>S</td>
<td>S</td>
<td>+</td>
</tr>
</tbody>
</table>

E

HRT-DRB4
HRT-DRB4-CP
HRT-DRB4-R8A
DRB4-MYC
HRT-FLAG

α-MYC
α-FLAG

IP with α-FLAG

F

TCV
R8A

G

DRB4-GFP
R6A-RFP
Merged

H

CRT1-GFP
R6A-RFP
Merged
CP does not dissociate DCL4-DRB4 and AGO1-DRB4 complexes. (A) Coimmunoprecipitation of DRB4-MYC with DCL4-FLAG in presence and absence of CP. *N. benthamiana* plants were agroinfiltrated and total extracts (input) and immunoprecipitated proteins were analyzed with α-MYC and α-FLAG. (B & C) Yeast-two hybrid assay showing growth on selection medium (B) and β-glactosidase assay (C). Yeast colonies co-expressing bait (pGADT7) and prey (pGBK7T7) plasmids were streaked on plates without (-) leucine and tryptophane or without leucine, tryptophane, and histidine. (D) Partial sequences of CP indicating mono- (asterisks) and bi-partite (underlined) nuclear localization sequences. Amino acids mutated in R6A and R8A are indicated by red and blue, respectively. Corresponding table shows disease phenotypes of TCV containing CP, R6A and R8A and their RNA-silencing activity (Choi et al., 2004). (E) Coimmunoprecipitation of DRB4-MYC with HRT-FLAG in the presence and the absence of CP and R8A. *N. benthamiana* plants were agroinfiltrated and immunoprecipitated proteins were analyzed with α-MYC and α-FLAG. (F) Trypan blue-stained leaves showing microscopic cell death phenotypes of TCV and R8A inoculated plants. Arrows indicate zones corresponding to HR lesions. (G) Confocal micrographs showing localization of DRB4-GFP and R6A-RFP when they were co-expressed (Scale bar, 10 µM). (H) Confocal micrographs showing localization of CRT1-GFP and R6A-RFP when they were co-expressed (Scale bar, 10 µM).
Figure 5.12. HEN1 is not required for TCV-induced HR or PR-1 expression. (A) HR formation in indicated genotypes at 72 hpi with TCV. (B) Expression of PR-1 gene in indicated genotypes after mock- or TCV-inoculation. Total RNA was extracted from inoculated leaves at 3 dpi. Ethidium bromide staining of rRNA was used as the loading control. (C) Typical morphological phenotypes of TCV-inoculated Di-17, HRT hen1 and Col-0 plants. Plants were photographed at 14 dpi.
Figure 5.13. AGO1 interacts with HRT via DRB4. (A) Confocal micrographs showing BiFC for indicated proteins. Agroinfiltration was used to express protein in transgenic *Nicotiana benthamiana* plants expressing the nuclear marker CFP-H2B (Scale bar, 10 μM). Interaction between AGO1 and 2b protein from cucumber mosaic virus (CMV) was used as a positive control. (B) Coimmunoprecipitation of DRB4-MYC and AGO1-MYC with HRT-FLAG in the presence or absence of CP. *N. benthamiana* plants were agroinfiltrated and total extracts (input) and immunoprecipitated proteins were analyzed with α-MYC and α-FLAG. (C) Coimmunoprecipitation of AGO1-MYC with HRT-FLAG in presence of CP. *N. benthamiana* plants were agroinfiltrated and total extracts (input) and immunoprecipitated proteins were analyzed with α-MYC, α-CP and α-FLAG. (D) Coimmunoprecipitation of CP and R8A mutant of CP with AGO1-FLAG. *N. benthamiana* plants were agroinfiltrated and total extracts (input) and immunoprecipitated proteins were analyzed with α-CP and α-FLAG. (E) Confocal micrographs showing localization of AGO1, CP and its mutant derivative R8A expressed individually or in pairs (Scale bar, 10 μM). (F) Visible HR formation in *HRT ago1* plants at 72 h post inoculation with TCV. Enlarged view of *HRT ago1* plants is shown. (G) Expression of PR-1 gene in indicated genotypes after mock- or TCV-inoculation. Total RNA was extracted from inoculated leaves at 3 dpi. Ethidium bromide staining of rRNA was used as the loading control. (H) Typical morphological phenotypes of TCV-inoculated Di-17, *HRT ago1* plants. Enlarged view of *HRT ago1* plants is shown, since they show severe developmental phenotype. Plants were photographed at 18 dpi. (I) HR formation in indicated genotypes at 72 h post inoculation with TCV. (J) Trypan blue stained leaf showing microscopic cell death phenotype on TCV inoculated leaves. Scale bars, 270 microns. (K) Expression of PR-1 gene in indicated genotypes after mock- or TCV-inoculation. The *PR-1* expression levels in *HRT ago7* were similar to those in Di-17 and other *HRT ago* plants shown here (data not shown). Total RNA was extracted from inoculated leaves at 3 dpi. Ethidium bromide staining of rRNA was used as the loading control.
Coimmunoprecipitation of DRB4-MYC with AGO1-FLAG in the presence and absence of CP. *N. benthamiana* plants were agroinfiltrated and total extracts (input) and immunoprecipitated proteins were analyzed with α-MYC and α-FLAG. (B) Confocal micrographs showing BiFC for HRT and AGO1 in the presence of CP. Agroinfiltration was used to express protein in transgenic *N. benthamiana* plants expressing the nuclear marker CFP-H2B (Scale bar, 10 µM). (C) Model showing possible arrangement of proteins in multiprotein complex containing HRT, in absence and presence of CP. AGO1 does not interact with DCL4 and in the presence of CP both DRB4 and DCL4 are released from this complex (data not shown).
Figure 5.15. Typical morphological phenotypes of TCV-inoculated Di-17, HRT ago and Col-0 plants. Plants were photographed at 14 dpi.
Figure 5.16. DRB4 is required for RPS2- and RPM1-mediated resistance. (A) Photograph showing phenotypes produced upon infiltration of $10^5$ CFU/ml *avrRpt2* or *avrRpm1* bacteria. All genotypes are in the Col-0 background. The leaves were photographed at 4 days post inoculation (dpi). (B) Growth of *avrRpt2* bacteria on indicated genotypes. The error bars indicate SD. Asterisks indicate data statistically significant from wt (Col-0, P<0.05 n=4). (C) Growth of *avrRpm1* bacteria on indicated genotypes. The error bars indicate SD. Asterisks indicate data statistically significant from wt (Col-0, P<0.05 n=4). (D) Confocal micrographs showing BiFC for indicated proteins. Agroinfiltration was used to express protein in transgenic *Nicotiana benthamiana* plants expressing the nuclear marker CFP-H2B (Scale bar, 10 µM). (E) Coimmunoprecipitation of DRB4-MYC with RPS2-FLAG and RPM1-FLAG. *N. benthamiana* plants were agroinfiltrated and immunoprecipitated proteins were analyzed with α-MYC and α-FLAG.
## APPENDIX-A

### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Acronym/abbreviation</th>
<th>Expansion</th>
</tr>
</thead>
<tbody>
<tr>
<td>L/mL/µL</td>
<td>Liter/ milliliter/ microliter</td>
</tr>
<tr>
<td>M/mM/µM</td>
<td>Molar/millimolar/ micromolar</td>
</tr>
<tr>
<td>g/mg/µg/ng</td>
<td>Gram/ milligram/ microgram/ nanogram</td>
</tr>
<tr>
<td>h/min/sec</td>
<td>Hours/minutes/seconds</td>
</tr>
<tr>
<td>Rh</td>
<td>Relative humidity</td>
</tr>
<tr>
<td>°C</td>
<td>Degree centigrade</td>
</tr>
<tr>
<td>BiFC</td>
<td>Bi-molecular fluorescence complementation</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BTH</td>
<td>Benzo[1,2,3]thiadiazole-7-carbothioic Acid S-Methyl Ester</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>Calcium chloride</td>
</tr>
<tr>
<td>CAPS</td>
<td>Cleaved Amplified Polymorphic Sequences</td>
</tr>
<tr>
<td>Co-IP</td>
<td>Co-immunoprecipitation</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxyribo adenosine triphosphate</td>
</tr>
<tr>
<td>dCAPS</td>
<td>Derived Cleaved Amplified Polymorphic Sequences</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxyribo cytosine triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrrocarbonate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxyribo nucleic triphosphate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DPI</td>
<td>Days post inoculation</td>
</tr>
<tr>
<td>DPT</td>
<td>Days post treatment</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>Potassium phosphate, dibasic</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>Potassium phosphate, monobasic</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)proanesulfonic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and skoog</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NaOAc</td>
<td>Sodium acetate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>Sodium hydrogen phosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>NaN</td>
<td>Sodium azide</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFD</td>
<td>Photon flux density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>R</td>
<td>Resistant or resistance</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SA</td>
<td>Salicylic acid</td>
</tr>
<tr>
<td>SAG</td>
<td>Salicylic acid glucoside</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SSC</td>
<td>Sodium chloride, sodium citrate</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris- borate/ EDTA electrophoresis buffer</td>
</tr>
<tr>
<td>TE</td>
<td>TRIS-EDTA</td>
</tr>
<tr>
<td>Tfb</td>
<td>Transformation buffer</td>
</tr>
<tr>
<td>TRIS</td>
<td>Hydroxymethyl Aminomethane</td>
</tr>
<tr>
<td>Wt</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
REFERENCES


phosphorylation in its COP1 binding domain. EMBO J. 19: 4997–5006.


Vita

Birth place - Taebaek, South Korea

Birth date - Jan 25th 1978

Education

1. Master of Science (Agriculture) Plant Pathology
   Seoul National University (Seoul), South Korea
   February 2005

2. Bachelor of Science (Agriculture) Agricultural Biology
   Kangwon National University (Chuncheon), South Korea
   February 2003

Professional positions held

1. Graduate Research Assistant (August 2006-May 2011), University of Kentucky, USA
2. Inspector (August 2005-July 2006), National Plant Quarantine Service, Suwon, South Korea

Scholastic and professional honors

1. Honored with the Myrle E. and Verle D. Nietzel Visiting Distinguished Faculty Award in conjunction with Ph.D. defense, University of Kentucky.
2. Travel award: American Society of Plant Biologists (2010)
3. March 2003-February 2005: Graduate fellowship from the Ministry of Education, South Korea
4. March 2000-February 2003: Yun-Kok Fellowship, Kangwon National University, Chuncheon, South Korea

Professional publications (* indicates first co-authors)


Rae-Dong Jeong
May 12th 2011