MECHANISM OF RESISTANCE TO *BEAN COMMON MOSAIC VIRUS* CONFERRED BY THE *I* LOCUS IN *PHASEOLUS VULGARIS L.*

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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August 2005

MECHANISM OF RESISTANCE TO *BEAN COMMON MOSAIC VIRUS* CONFERRED BY THE *I* LOCUS IN *PHASEOLUS VULGARIS L.*

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Cornell University 2005

Resistance employed by plants to combat infection by pathogens from a broad range of species is frequently mediated by resistance genes (R genes). While R genes are known to be involved in pathogen recognition, how they convey this message to host defense machinery is not completely understood. These studies employ genetics and cell biology to evaluate the interaction of pathogen infection in resistant and susceptible host plants. The system used here is the *I* locus of *Phaseolus vulgaris L.* and the *Potyvirus, Bean common mosaic virus* (BCMV). Near isogenic lines for the *I* locus were challenged with BCMV at 20°C, 26°C and 34°C, and assayed over time using a number of different techniques. A protoplast system was developed for use in transfection experiments for determination of viral replication in the presence of the *I* allele. Confocal laser scanning microscopy was used in combination with fluorescence immunostaining to localize viral coat protein in resistant and susceptible responses. Genes that are differentially expressed in these isolines at 26°C and 34°C following inoculation with BCMV were detected using cDNA-AFLP. Protoplast experiments revealed that BCMV is able to accumulate in genotypes containing zero, one, or two copies of the *I* allele, although at different rates. Results from microscopic observations support the protoplast data and show that BCMV infects *II*, *Ii* and *ii* plants but that movement is restricted in resistant genotypes (*II* and *Ii*). cDNA-AFLP analysis revealed 20 genes that are differentially expressed during the infection process. Sequence analysis demonstrated that several of these genes are *Phaseolus* homologs of those known to be involved in plant defense responses in other well-characterized systems.

BIOGRAPHICAL SKETCH

And so it happened, that events and people coalesced in the late 1960s in central Illinois so that Molly Cadle arrived into the world. From this auspicious beginning she made her way through the St. Joseph, IL school system and, when the time came, started working for the soybean breeder at Northrup King seeds company. Molly's interest in genetics had been sparked that year by her seventh grade science teacher, Mr. Walton, who introduced the class to Mendelian genetics. Molly worked at breeding soybeans—making crosses, taking notes, inoculated cotyledons—for the next five summers. At the end of this time and following a year at the local junior college (a year for which she's always been thankful), she got on a bus and moved to Davis, CA where she earned her Bachelor's degree in Genetics in 1991. From there she moved to Pullman, WA and worked as a technician in a wheat genetics and breeding lab for several years. During this time she also earned her Masters degree studying a fungal pathogen of wheat under Dr. Timothy Murray. Upon completion of this degree in 1997, Molly again moved across the country to Ithaca, NY where she embarked upon the endeavors presented in these pages. Fatefully, she also moved into a house just two doors down from the most wonderful man in the world, Lance Davidson, to whom she was married in May of 2003.

ACKNOWLEDGMENTS

First and foremost, I thank my major advisor, Molly Jahn, and special committee members, Candace Collmer, Sondra Lazarowitz, Phillip Griffiths, Susan McCouch and Tom Zitter. I appreciate the time and effort that all members put into helping me see this through. I am grateful to all members of the Jahn and Lazarowitz labs, each and every one of whom are wonderful friends, excellent labmates, ice cream buddies, and science geeks. I would like to specifically mention Becky Grube, Troy Thorup, Tim Porch, James Frantz, Byoung-Cheorl Kang, Roisin McGarry, Miguel Carvalho, Wen Ling Deng, and Jenn Lewis for their incredible friendship, scientific discourse and support. Without their presence in my life I would certainly be a different person now. I thank George Moriarty and John Jantz for the expert care of my bean plants in the greenhouse. I acknowledge all members of the breakfast club. I have made more and deeper friendships during the course of these studies than at any other time in my life—I think this reliance on other people is part and parcel of graduate school—and I list them here, knowing this is insufficient but hoping they already know how I feel: Lance Cadle-Davidson, Esther van der Knaap, Nicole Donofrio, Clemencia Rojas, Paul Mason, Alice Mason, Susan Switras Meyer, Carl Meyer, Clint Nesbitt, Leslie Patton, Shawn Puller, Jason Palter, Chris Gee, Peter Cousins, Huver Posada-Suarez, Maria Del Pilar Moncada. I am, of course, eternally grateful for the incredible support I've received from my family. They have backed me in every decision I have made, been happy to receive me when I needed a break, humored me when I have been opinionated, tolerant when I have been grumpy, and cooperative when asked to wear suboptimal bridesmaids' dresses. Finally, I would like to thank my husband, Lance. I started this degree without him, but I could never have finished in that state.

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LIST OF ABBREVIATIONS

Bean common mosaic necrosis virus	BCMNV
Bean common mosaic virus	BCMV
cDNA-Amplified fragment length polymorphism	cDNA-AFLP
Coat protein	CP
Confocal laser scanning microscopy	CLSM
Cowpea aphid-borne mosaic virus	CabMV
Cucumber mosaic virus	CMV
cv. Black Turtle Soup	BT
heterozygous *Ii* NIL	BT_{Ii}
homozygous dominant *II* NIL	BT_{II}
homozygous recessive *ii* NIL	BT_{ii}
Cylindrical inclusion	CI
Extreme resistance	ER
Helper component proteinase	HC-Pro
Hypersensitive response	HR
Near isoline	NIL
Nuclear inclusion A proteinase	NIa
Nuclear inclusion B replicase	NIb
Passionfruit woodiness virus strain K	PWV-K
Potyvirus protein 1, proteinase	P1
Potyvirus protein 3	P3
Resistance gene	R gene
RNA-dependent RNA polymerase	RdRp
Soybean mosaic virus	SMV
Thailand Passiflora mosaic virus	ThPV

Tobacco etch virus	TEV
Tomato bushy stunt virus	TBSV
Tomato spotted wilt virus	TSWV
Viral genome-linked protein	VPg
Virus-induced gene silencing	VIGS
Watermelon mosaic virus	WMV
Zucchini yellow mosaic virus	ZYMV

CHAPTER 1

Literature Review

Disease Resistance

In order to resist disease, an organism must first know that it is under attack by a pathogen. Organisms that possess immune systems have cells that specifically recognize foreign objects and communicate this recognition to cells that can release molecules that effect targeted destruction of the invader. While plants do not have immune systems, the cytoplasm is confluent throughout most of the plant and we could, therefore, argue for the presence of a "circulatory" system through which signals may pass. In fact, it is established that small molecules, protein and RNA can move between cells and have effects in locations other than their cell of origination (Haywood *et al.*, 2002); (Vollbrecht *et al.*, 1991). Until the 1990s research into plant disease resistance could identify genes important in the resistance response, but specific biochemical models showing how it functions were lacking. As a fallout of H. H. Flor's "gene-for-gene" model, we expected to find the plant version of an immune system—some sort of receptor ligand interaction (Ellingboe, 1981; Flor, 1955). While this expectation has not proved to be entirely correct, it has been shown that the single dominant resistance genes (R genes) typically used in plant breeding frequently encode receptor type molecules (Table A.1) (Martin *et al.*, 2003).

In 1992 *Pto*, which confers resistance against *Pseudomonas syringae* pv. tomato (bacterial speck) in tomato, was the first classical R gene to be cloned (Martin

et al., 2003). This gene encodes a protein kinase and has been shown to directly interact with *AvrPto*—the avirulence factor or effector molecule produced by the pathogen (kim *et al.*, 2002). However, since 1992 more than 40 additional R genes have been cloned and none of them are simply protein kinases and very few of them interact with their corresponding avirulence genes (Martin *et al.*, 2003). On the other hand, they do resemble each other and are easily categorized into groups: leucine zipper-nucleotide binding site-leucine rich repeat (LZ-NBS-LRR) (including coiled-coil (CC) and Toll-interleukin receptor (TIR) types), membrane-bound LRR, Kinase, membrane bound LRR-Kinase, and membrane-bound CC (Martin *et al.*, 2003).

While we know a great deal about the structure of these cloned genes, very little is known about their mechanism. In most cases, a discrete effector molecule is expressed by the pathogen that is either directly or indirectly recognized by plant R genes. This recognition has been shown to be direct for a small number of cases including *Pto-AvrPto*/*AvrPtoB*, *PiTa-AvrPita*, and *RPS2-AvrRpt2* although many attempts have been made to show this for other pathosystems (Jia *et al.*, 2000; kim *et al.*, 2002; Leister and Katagiri, 2000; Luderer *et al.*, 2001; Martin *et al.*, 2003). The employment of genetic mutant screens has given us insight into the events that occur following the recognition. Downstream of the R gene are signal transduction networks that lead to induction of various combinations of defense response genes (Glazebrook, 2001; Glazebrook *et al.*, 2003; Kachroo *et al.*, 2000). Generally these cascades are considered either salicylic acid dependent or independent and lead to changes in "pathogenesis related" (PR) protein expression patterns. However, how these changes in expression lead to the hypersensitive response (HR) or other resistance phenotypes is, as yet, unknown.

There are currently three main models or strategies for resistance being explored at a molecular level: receptor-ligand interaction of an R gene-encoded protein and pathogen avr protein, the 'guard hypothesis,' and non-host resistance. The first of these—the receptor-ligand model—is the simplest molecular model that can be used to explain Flor's gene-for-gene hypothesis (Ellingboe, 1981; Flor, 1955). This hypothesis states that when a dominant R gene is present in a host under attack by a pathogen with a recognizable *avr* protein, resistance occurs. In all other cases, disease can occur as represented in the following diagram:

	Avr	avr
R	Resistance	Disease
r	Disease	Disease

Using this genetic model as a starting point, a mechanism can be envisioned wherein the host specifically recognizes the pathogen and triggers a signal transduction cascade ultimately leading to resistance. The recognition event is mediated by direct interaction between the R and *Avr* gene products (Ellingboe, 1981). Such a model is attractive because it provides a direct link between pathogen attack and host responses. Further, it can account for the rapid evolution of new virulences in some pathogens because loss of recognition, which could be accomplished through a simple base change in the *Avr* gene, will allow disease to occur. However, as already stated, in only a very small number of cases have R gene products been shown to directly interact with *Avr* gene products.

A second model predicts an indirect rather than direct detection of the avr protein by the R protein and is termed the 'guard hypothesis' (Dangl and Jones, 2001; van der Biezen and Jones, 1998). Much like models for enzyme reaction kinetics, the

guard hypothesis predicts that additional host molecules other than simply R gene products are necessary for the trigger of host defenses. That is, the *avr* protein from the invading pathogen may recognize and bind (perhaps for the purposes of its virulence) a host protein, causing some conformational change or disrupting some normal interaction for this target protein. This proposed interaction is precedented in what we already know of competitive, noncompetitive and uncompetitive enzyme-catalyzed reactions (Voet and Voet, 2003). The function of the R protein is then to recognize either the *avr*-target complex or some other cell perturbation based on the entry of the *avr* protein (Dangl and Jones, 2001; van der Biezen and Jones, 1998). In light of the extreme difficulty in demonstrating direct R gene product-*avr* protein interactions, this hypothesis is very attractive (Luderer *et al.*, 2001). Further, it is testable through the use of some rather basic biochemical approaches for detection of protein complexes as well as more modern proteomic techniques. It will not be long before these first steps of host detection of pathogen invasion are exposed.

Nonhost resistance has been and will continue to be one of the most important types of resistance in agriculture. The fact that most plants are resistant to most diseases is an important point that is not often acknowledged. While not always as dramatic as resistance conferred by an R gene, the level of protection is generally sufficient to ward off pestilence in natural populations. This basal level of disease resistance is apparent in genetic screens in which mutants such as *eds1*, *sid2*, *ndr1* and *pad4*, are detected with increased susceptibility to pathogens and non-pathogens alike (Century *et al.*, 1997; Falk *et al.*, 1999; Glazebrook *et al.*, 1997; Nawrath and Metraux, 1999). The effect of these genes is seen in microarray profiling studies comparing compatible, incompatible and non-host expression when challenged with the relevant pathogen (Glazebrook *et al.*, 2003; Tao *et al.*, 2003). Differences between these

classes of interactions are based primarily on the timing and amplitude of their responses, suggesting that all the right machinery for defense is present, even in susceptible and non-host interactions (Tao *et al.*, 2003). In fact, the only elements that are lacking from these systems are those enabling specific recognition of a pathogen. Studies searching for proteins that interact with known defense signal transduction molecules such as mitogen-activated protein kinases (MAPKs) have revealed molecules that play important rolls in non-host responses in addition to those involved in R gene pathways. In *Nicotiana benthamiana* the MAPK interacter HSP90 has been silenced using virus-induced gene silencing (VIGS) and results in a phenotype of stunting, no HR when challenged with both incompatible and non-host pathogens (that typically give an HR), reduced PR gene expression, and defective non-host resistance to a non-host pathogen (Kanzaki *et al.*, 2003). Similar results were found when HSP70 was silenced in the same system (Kanzaki *et al.*, 2003). These experiments demonstrate that, while perhaps a different set of genes may be involved in non-host resistance, they are deeply linked to R gene associated pathways. Given the mutable nature of R gene specificity (i.e. the leucine rich repeat) this opens the door for rapid evolution of and selection for a higher level of resistance.

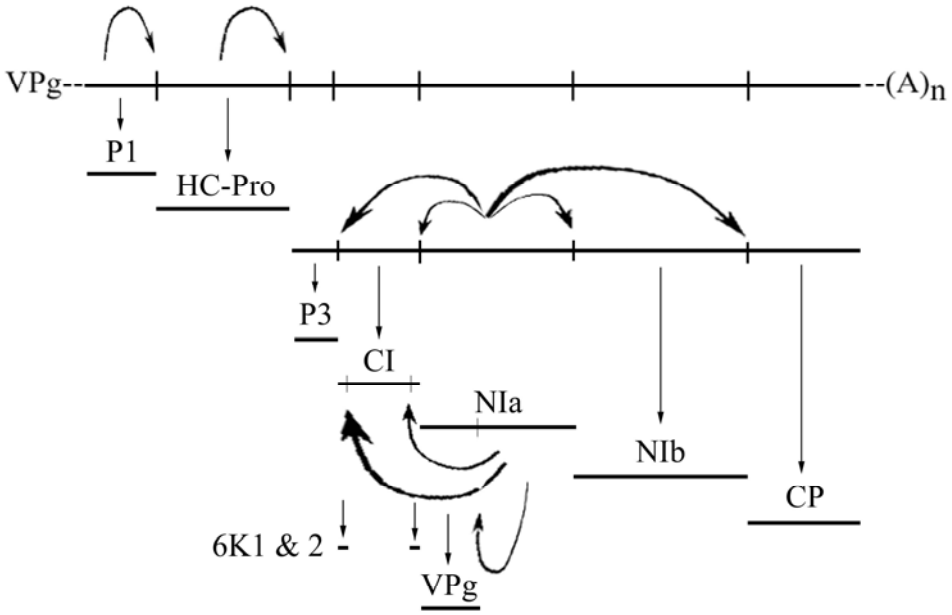
Biology of Potyviruses

Viruses are unique among organisms. They are composed solely of nucleic acid packaged in protein (with slight variation among the virus groups) and, given the right host, are capable of diverting host resources for self-replication, assembly, propagation and dissemination (Hull, 2002). Viruses achieve astounding feats of

adaptation to survive in novel environments by any number of evolutionary "tricks." They employ strategies to block host defense mechanisms such as shutting down host translation, inducing host translation, or suppressing VIGS (Aranda *et al.*, 1996; Brigneti *et al.*, 1998; Ratcliff *et al.*, 1997). They can reassort and recombine to result in virions with novel antigenic and virulence properties (Silbernagel *et al.*, 2001). They exist as swarms of individuals, with only a consensus genotype, allowing rapid selection for the fittest population (Hull, 2002). Given their remarkable life strategies, it is no wonder that viral diseases are the most interesting as well as the most feared.

Members of the genus *Potyvirus* are single-stranded, positive-sense RNA viruses, with a 5' genome-linked protein (VPg) and a 3' poly adenosine tail (Hull, 2002). Most members are transmitted by aphids although a few are carried by whiteflies (Colinet *et al.*, 1996; Hull, 2002). Once a potyvirus enters the host cell, it first uncoats and is translated into its polyprotein by host translational machinery. This polyprotein is cotranslationally cleaved by its own encoded proteinases into six to eight proteins: P1 proteinase, Helper component-proteinase (HC-Pro), P3, Cylindrical inclusion helicase (CI), Viral genomic protein (VPg), Nuclear inclusion A proteinase (NIa), Nuclear inclusion B RNA-dependent RNA polymerase (NIb/RdRp), and Coat protein (CP) (Hull, 2002). The P1 proteinase and HC-Pro are responsible for cleavage of the N-terminal third of the polyprotein and the NIa proteinase the C-terminal two-thirds (Revers *et al.*, 1999) (Figure 1.1). HC-Pro has been implicated in suppression of host gene silencing and is thought to enable genome amplification, aphid transmission, and play a roll in synergistic viral infection (Revers *et al.*, 1999; Urcuqui-Inchima *et al.*, 2001). It is likely that CI is involved in cell-to-cell movement as it has been shown to align its central pore with the plasmodesmata (Revers *et al.*, 1999). In several cases of recessive resistance genes, the VPg has been shown to play

an avirulence role (Jenner *et al.*, 2000; Keller *et al.*, 1998; Masuta *et al.*, 1999). Its more general function is to act as a 5' cap and to direct initiation of translation (Revers *et al.*, 1999). Finally, the NIb protein is the replicase protein for a potyvirus and following the initial translation and cleavage of the polyprotein, this molecule initiates



P1 = protein 1, proteinase
HC-Pro = Helper component proteinase
P3 = protein 3
CI = Cylindrical inclusion
6K1 & 2 = 6 kDa proteins
NIa = Nuclear inclusion a, proteinase
VPg = Viral genome-linked protein
NIb = Nuclear inclusion b, RNA-dependent RNA polymerase
CP = Coat protein

Figure 1.1. Potyviral genome strategy showing processing sites and presumed protein function. (Derived from Urcuqui-Inchima *et al.*, 2001)

synthesis of a negative-sense genome copy (Revers *et al.*, 1999). From this antisense RNA many more genomic-sense RNAs are made and new viruses can be assembled.

Bean common mosaic virus

Bean common mosaic virus is a typical *Potyvirus* at 750 nm x 15 nm and a genome size of 9.6 Kb (Bos, 1971; Urcuqui-Inchima *et al.*, 2001). Its virion is a flexuous rod that is made up of approximately 2000 CP monomers encapsidating the RNA genome (Urcuqui-Inchima *et al.*, 2001). BCMV is an important pathogen of *Phaseolus vulgaris* genotypes worldwide and can infect a wide range of crop legume species (Bos, 1971). Seed transmission is an important source of initial infections with up to 83% of the seed from an infected plant carrying the virus (Bos, 1971). Several strains of BCMV exist with different virulences and have been categorized into pathogenicity groups I (NL 1, US 1, PR 1), II (NL 7), III (NL 8), IVa (US 5), IVb (US 4;, US 3, NL 6), Va (US 2), Vb (NL 2), VIa (NL 3), VIb (NL 5), and VII (US 6, NL 4) based on their virulence on 11 differential cultivars established by Drijfhout (Drijfhout, 1978). These strains fall into two different serogroups, type A including NL 8, NL 3 and NL 5, while type B encompasses the remainder (Vetten *et al.*, 1992). BCMV serotype A has been renamed as *Bean common mosaic necrotic virus* (BCMNV) based on serological and symptomatic differences between the two groups (McKern *et al.*, 1992a; Vetten *et al.*, 1992).

In *Phaseolus* spp. BCMV produces several distinct symptoms. In susceptible genotypes at typical growing temperatures (26-28˚C), a severe mosaic, curling of the

leaves, vein banding and mottled and malformed pods can appear (Figure 1.2) (Bos, 1971). At elevated temperatures (above 30˚C)

	26°C	34°C
BT_{ii}	Necrotic lesions, Mosaic	Systemic necrosis Rapid death
BT_{II}	No symptoms	Vascular necrosis

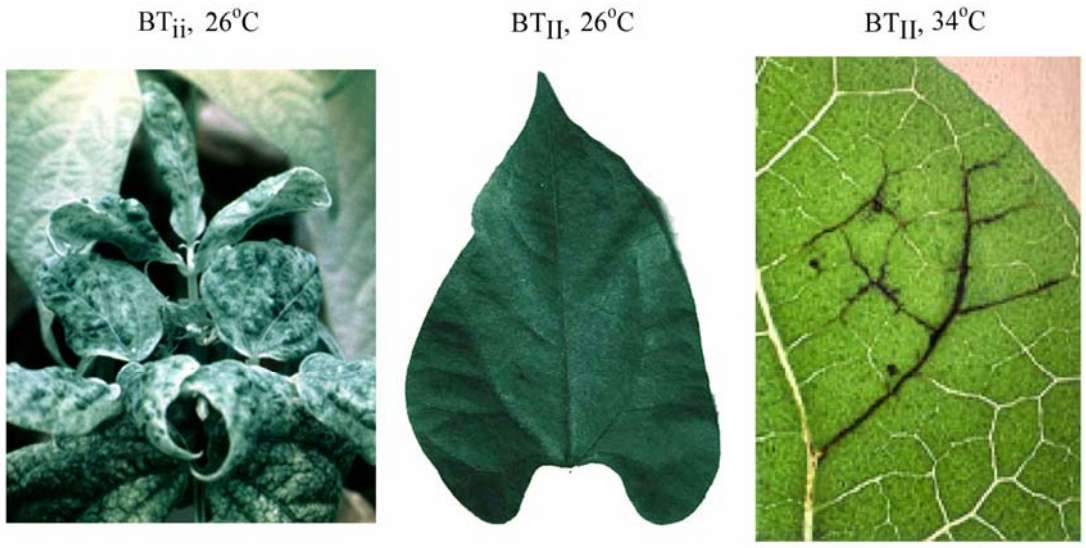


Figure 1.2. Symptoms typical of *I* gene-containing germplasm when infected with *Bean common mosaic virus* NY15. Images are of 'Black Turtle Soup' near isogenic lines. Subscripts denote genotype at the *I* locus. 34ºC image by M.M. Jahn.

these plants show stunting and "black root" or systemic necrosis symptoms when infected with the type strain (US 1) (Bos, 1971). Tolerant varieties can become systemically infected but show only a mild deformation or narrowing of the leaves (Bos, 1971). Some genotypes show an extreme resistance (ER) against the type strain at typical growing temperatures that manifests no visible symptoms (Figure 1.2) (Bos, 1971; Fisher and Kyle, 1994). At higher temperatures (above 30˚C) the spreading

vascular necrosis appears and often death, typical of "black root" (Figure 1.2) (Bos, 1971).

Virus Resistance

Just as viruses have many unique features among the plant pathogens, so are there also unique ways in which plants can resist viral diseases. When a virus infects a plant it must accomplish three things in order to systemically invade its host: uncoat and begin replicating, move cell-to-cell, and move into and through the vasculature to traverse long distances (Hull, 2002). Virologists have typically broken down the viral life cycle in this fairly simplistic way due to the fact that these are three checkpoints at which it is relatively easy to assay viral infection. Does a virus replicate? Can it move between cells? Can it move long distance? This way of thinking ignores the complexity of virus-host cellular interactions and has led us to speculate that these are the only points at which a host can resist viral attack. It is a narrow view.

What we know through the tools of molecular and cell biology is that when a virus enters a cell, it interacts with a multitude of host proteins in order to initiate its own replication. We suspect that host proteins exist to monitor the normal or abnormal interactions of its own proteome (Dangl and Jones, 2001; van der Biezen and Jones, 1998). When progeny viral genomes or virions attempt to leave the initial cell, they must move along the host cell framework, using host motor proteins, and, in some cases, effect a change in the size of their exit route (i.e. the plasmodesmata) (Cruz *et al.*, 1998; Hull, 2002; Reichel *et al.*, 1999; Roberts and Oparka, 2003). Having made its way into the phloem, viral movement is mainly passive and may be

unchecked. However, we are learning that some proteins are specifically expressed in the vascular tissue, including those involved in viral trafficking (Carrington *et al.*, 1996; Chisholm *et al.*, 2001; Chisholm *et al.*, 2000; Haywood *et al.*, 2002). There is no free ride for a virus—we are learning that they interact with and manipulate a myriad of host processes that are continuous along their life cycles. Their invasion of a host cell should not be pigeon-holed into three technique-driven areas. Things are getting "Curiouser and curiouser," as Alice might say, the more we learn (Carroll, 1946).

Table A.1 is a list of cloned and uncloned virus resistance genes from a number of species and are effective against different viruses. As with the vast majority of cloned resistance genes to date, it is notable that they are mostly of the NB-LRR class. Only the *RTM* genes differ; however, these were discovered using a novel gain of function technique rather than traditional positional cloning of a known gene (Chisholm *et al.*, 2000; Whitham *et al.*, 2000). Based on structural similarity with other systems, it is assumed that these NB-LRR R genes will be receptors. Depending on which of the current schools of thought apply to these systems (see above), this could mean that R genes against viral pathogens recognize either a component of the viral proteome or some interaction between a viral protein and a host protein. What this means, actually, is that resistance to viral disease at this level is no different from any other type of resistance—the same type of molecules are used to initiate the defense signal, only the specificities change.

However, there is a known, very powerful resistance mechanism against viruses that does not seem to be connected with R genes. This type was first discovered as an unfortunate consequence of plant transformation and termed co-

suppression or gene silencing (Flavell, 1994; Kumagai *et al.*, 1995; Napoli *et al.*, 1990; Vanderkrol *et al.*, 1990). As the details of this phenomenon are being established it has become suggestive of a mechanism by which plants halt viral infection at a very basic level (Covey *et al.*, 1997; Ratcliff *et al.*, 1997). Symptomatically, its hallmark is a recovery from viral infection. At a molecular level, the host plant recognizes "over expression" of some RNA perhaps by presence of a double-stranded RNA intermediate or some other structure in the problematic RNA (Agrawal *et al.*, 2003). Recognition is followed by cleavage of the invading RNA, producing 21-23 nt RNAs that are then incorporated into a nuclease complex that degrades the invading ssRNA in a sequence-specific manner (Voinnet, 2001). The role of the RNase responsible for cleavage of dsRNAs may be filled by a homolog of the *Drosophila melanogaster*, Dicer, an RNase known to be functional in RNAi (RNA interference) in that organism (Bernstein *et al.*, 2001). The genome of *Arabidopsis thaliana* contains a four member gene family of similar RNases, including the floral development RNase, CAF (Jacobsen *et al.*, 1999; Papp *et al.*, 2003). This entire phenomenon has been termed Virus-Induced Gene Silencing, or VIGS.

Interestingly, viruses have evolved a means of circumventing this type of defense system. Several viruses have been demonstrated to suppress gene silencing and this ability has been associated with specific viral proteins: HC-Pro of TEV, 2b of CMV, P19 of TBSV and NSs of TSWV, to name a few (Brigneti *et al.*, 1998; Kasschau and Carrington, 1998; Takeda *et al.*, 2002; Voinnet *et al.*, 1999). While this ability is key to a particular virus' ability to infect, it also may explain what has historically been termed a synegistic infection. Potyviruses are often found in mixed infections and their ability to suppress host defenses (silencing) has been shown to allow other viruses to replicate and move (Vance *et al.*, 1995; Yang and

Ravelonandro, 2002). In such cases, the levels of potyviral expression has remained at typical levels while the complementing virus (often a potexvirus) is able to replicate to very high levels and symptom expression is more severe than a single infection (Yang and Ravelonandro, 2002). The ability of a potyvirus to enhance potexvirus infection has been assigned to the N-terminus of the HC-Pro protein—the same region that confers suppression of gene silencing (Kasschau and Carrington, 1998; Vance *et al.*, 1995). These facts are highly suggestive of an evolutionarily successful potyviral infection strategy onto which potexviruses have piggybacked.

The Common Bean: *Phaseolus vulgaris* L.

Plants classified under the taxonomic distinction of *Phaseolus vulgaris* L. are of worldwide distribution and are used by humans for a myriad of functions including food, animal feed, medicine, ornamentation and poison (GRIN; http://www.ars-grin.gov/npgs/). In the United States, they are harvested fresh as green or snap beans as well as dry, such as with kidney or pinto beans (Gepts, 1998). Approximately 1.6 million acres were planted to dry beans in 2005 in the U.S., making it a relatively minor crop in this country when compared with corn (81.6 million acres) or soybean (73.3 million acres) (USDA - National Agricultural Statistics Service). According to UN food production statistics, 19 million metric tons were produced worldwide in 2002 with Brazil being the top producer at over 3 million tons (http://faostat.fao.org/).

Bean is a new world crop and can be divided into two gene pools: Andean and Mesoamerican (Gepts, 1998). While still the same species, the gene pools are nearly reproductively isolated and may be on an evolutionary path to speciation (Gepts, 1998). Interestingly, two complementary genes, termed *Dosage dependent lethal* (DL_1

and *DL*$_2$), have been identified as contributing to the lack of vigor in F_1s between the Andean and Mesoamerican gene pools (Shii *et al.*, 1980). Hannah and coworkers (Hannah *et al.*, 2000) demonstrated that the lethal combination of alleles at these loci can be reproduced in grafted *Phaseolus* material and results in significantly decreased root development. However, not all inter-gene pool crosses are lethal and, following initial reduced vigor generations, segregation of the *DL* genes can lead to a return to type. In fact, most Chilean landraces (Andean) are derived from crosses between the gene pools (Gepts, 1998). Also, snap beans generally tend to be intermediate between Andean and Mesoamerican and are likely the results of breeding between the two pools (Gepts, 1998).

One implication of the existence of *Phaseolus* gene pools for plant breeding is that coevolution with interacting organisms has also occurred. Specifically, pathogens that have coevolved with the Mesoamerican gene pool more readily affect those genotypes than Andean genotypes and vice versa (Gepts, 1998). For plant breeders this means that the contrasting gene pool can be a good source of resistance genes for future varieties, assuming that one can bridge the loss of vigor associated with intercrossing. Such variation is critical when considering bean—as a genus it is susceptible to all pathogen types, yet resistance genes have been identified for all of the major disease problems (Ali, 1950; Alzate-Marin *et al.*, 2004; Chen and Roberts, 2003; Jung *et al.*, 1996; Kalavacharla *et al.*, 2000; Kelly and Vallejo, 2004; Mahuku *et al.*, 2004; Velez *et al.*, 1998).

I Gene-Mediated Resistance

Resistance conferred by the *I* gene was discovered in the early 1930s by Ralph Corbett while working for the Sioux City Seed Company in Sioux City, IA (Pierce, 1934). The variety 'Corbett Refugee' was selected as a surviving plant out of a field of the susceptible 'Refugee Green' (Pierce, 1934) and the resistance it imbues has been incorporated into bean cultivars worldwide ever since. In the late 1930s an apparently new disease termed "Black root" arose, infecting materials bred from 'Corbett Refugee' (Jenkins, 1940). This was a severe wilt followed by chlorosis of the lower leaves and necrotic streaks running along the stem both above and below the cotyledonary node (Jenkins, 1940). Today we recognize these symptoms as being associated with the *I* locus when challenged with different isolates of BCMV or even other legume-infecting potyviruses (Bos, 1971; Fisher and Kyle, 1994). When *I* gene-containing genotypes (without any additional resistance genes) are inoculated with BCMV a so-called extreme resistance (ER) is the result. No notable symptoms occur at 25˚C and no virus has been recoverable from inoculated leaves (Bos, 1971; Fisher and Kyle, 1994). However, at temperatures over 30˚C this resistance fails and a systemic veinal necrosis results (Bos, 1971; Fisher and Kyle, 1994). Further, when inoculated with strains of BCMV that have since been re-classified as *Bean common mosaic necrotic virus* (BCMNV) or other necrosis-inducing viruses, the spreading veinal necrosis occurs regardless of temperature (McKern *et al.*, 1992c).

The genetics of this virus-bean pathosystem was first explored by Ali in the 1950s (Ali, 1950). He determined that two factors were involved in resistance to BCMV: dominant *I* and recessive *a* (Ali, 1950). Further work was performed in the 1970s by Drijfhout who made great advances in definition of differential cultivars and

virus strains. He showed that the "immunity" against some strains of BCMV was conferred by the *I* locus alone whereas the strain specific and nonspecific resistance was conferred by a set of recessive *bc* genes (*bc-1*, *bc-1*^{2}, *bc-2*, *bc-2*^{2}, *bc-3*, *bc-u*) (Drijfhout, 1978). It has recently been suggested that these recessive resistance genes follow the trend of several other more fully characterized recessive virus resistance genes that have been shown to be putative translation initiation factors, eIF4E and eIF(iso)4E (Kang *et al.*, In Press).

The broad-spectrum nature of the *I* locus was defined by research performed at Cornell University. In these studies, workers were able to show the cosegregation of resistance against eight potyviruses with the *I* locus (Fisher and Kyle, 1994; Fisher and Kyle, 1996; Kyle and Dickson, 1988; Kyle and Provvidenti, 1993). Table 1.1 lists the viruses that interact with *I* and the classes of phenotypes that they exhibit (Fisher and Kyle, 1994).

Table 1.1. Disease phenotypes displayed by *I* gene-containing material when mechanically inoculated with one of 8 potyviruses.

Temperature-independent necrosis	**Temperature-dependent necrosis**	**Non-necrotic**
SMV	BCMV**	ZYMV
ThPV	CabMV	PWV-K
BCMNV*	WMV	

* Formerly BCMV serotype A

** *Azuki bean mosaic virus* and *Black-eye cowpea mosaic virus* were previously considered independent viruses that interacted with the *I* locus with temperature-dependent symptoms (Fisher and Kyle, 1994; Fisher and Kyle, 1996; Provvidenti *et al.*, 1983). However sequence data and phylogenetic analysis show that these are instead, strains of BCMV and is reflected in the table (Berger *et al.*, 1997; Collmer *et al.*, 1996; McKern *et al.*, 1992b).

The work of Fisher and Kyle presents convincing evidence that resistance against all eight potyviruses segregates as a single locus, however it does not preclude the existence of a tightly linked gene cluster at this locus (Fisher and Kyle, 1994). Interestingly, recent attempts to positionally clone the *I* gene have so far been fruitless, however the genomic region in which it lies has several NB-LRR type sequences (see Table A.1) (Astua-Monge *et al.*, 2000). Should *I* be revealed to be a typical NB-LRR resistance gene it will join the many cloned R genes with such a predicted structure.

One of the most exciting features of the *I* locus is its genetic action. Collmer and colleagues demonstrated its incompletely dominant nature by measuring lesion size and number in resistant and susceptible lines of 'Black Turtle Soup' (BT)(Collmer *et al.*, 2000). Of the cloned resistance genes, $Tm2^2$, conferring resistance to *Tomato mosaic virus* (ToMV) has also been shown to be incompletely dominant and encodes a putative CC-NB-LRR type protein (Hall, 1980; Lanfermeijer *et al.*, 2003). Similar to *I* gene-mediated resistance, its phenotype is that of extreme resistance or spreading necrosis at elevated temperatures or when in the heterozygous state (Hall, 1980). Very few studies on the gene action of R genes in uniform backgrounds have been conducted. It is possible that incomplete dominance occurs more frequently, however we must wait for this work to be reported.

The present study

In the work presented here I took approaches to study some very basic properties of BCMV survival in resistant and susceptible near isogenic lines (NILs) of BT (BT_{II}, BT_{Ii} and BT_{ii}). These genotypes were generated by selecting both resistant

(*I*-) and susceptible (*ii*) individuals from the same seed lot of BT (BT-1 and BT-2)(Provvidenti, 1983), followed by a backcrossing program between the two lines to obtain a genetic background as nearly uniform as possible. At the time these studies began, the NILs had been backcrossed five times which, in theory, would maintain approximately 1.5% of the donor genome intact. Considering that the original lines were selections from the same variety, this estimate of residual donor genome is probably higher than is actually the case. Throughout this volume I refer to these lines as being nearly-isogenic lines (NILs) and assume that there is genetic variation at the *I* locus and very little elsewhere in the genome.

Questions regarding whether or not BCMV can replicate in gentoypes containing one or more copies of the *I* allele were addressed using a protoplast transfection system. Reliable methods for isolation and transfection of bean protoplasts were developed in these studies. Semiquantitative reverse transcriptase PCR (sqRT-PCR) was used to measure the amount of viral RNA present in protoplasts isogenic for the *I* gene over time. This assay measures steady state levels of RNA in a cell rather than rate of accumulation, so replication, in the strict sense was not measured. However, the ability of the virus to accumulate and exist in the presence of varying states of host genetic resistance was addressed and is the first step in determining the mechanism of *I* gene-mediated resistance.

A second approach was to look at differential gene expression in the resistant and susceptible isolines. The two homozygous isolines were compared using cDNA-AFLP on samples that were inoculated or mock-inoculated and grown at either 26˚C or 34˚C (8 conditions total). The purpose of this assay was to discover genes that were differentially expressed during the resistant, susceptible and hypersensitive responses

and to attempt to align events in the BCMV-*I* gene pathosystem with other pathosystems that are more fully characterized molecularly.

Finally, the activity of BCMV in resistant, intermediate and susceptible responses was followed using Confocal Laser Scanning Microscopy (CLSM). These experiments were designed to answer questions about the ability of BCMV to replicate and move cell-to-cell in the face of resistance and in plants growing at both 26˚C and 34˚C. Time course studies were designed to show patterns or trends in viral accumulation and movement with the ultimate goal being the discovery of a checkpoint at which resistance is affected.

CHAPTER 2*

Resistance Conferred against *Bean common mosaic virus* by the Incompletely Dominant *I* Locus of *Phaseolus vulgaris* L.is Active at the Single Cell Level

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Running title: *Phaseolus vulgaris I* locus confers single cell level BCMV resistance

*A version of this chapter is published as:

Cadle-Davidson, M M and Jahn, M. In Press. Resistance conferred against Bean common mosaic virus by the incompletely dominant *I* locus of *Phaseolus vulgaris* is active at the single cell level. *Archives of Virology*.

Summary

A protoplast transfection system was used in *Phaseolus vulgaris* to study the incompletely dominant resistance locus *I*. The genetic materials in the study were cultivar 'Black Turtle Soup' (BT) lines nearly isogenic for *I* and their F_1. Accumulation of *Bean common mosaic virus* (BCMV; genus *Potyvirus*) RNA and virions was assayed following BCMV RNA electrotransfection of protoplasts from each genotype. BCMV RNA and virions accumulated in all genotypes tested but the relative rates of RNA accumulation differed. This suggests that the *I* allele is active at the single cell level and in a dosage-dependent fashion and supports previous work in this area.

The research reported here addresses the mechanism of the resistance conferred by the *I* locus in *P. vulgaris* (Drijfhout *et al.*, 1978). This locus controls resistance against *Bean common mosaic virus* (BCMV; genus *Potyvirus*), a virus that is frequently seed-transmitted and that can cause devastating crop losses worldwide (Drijfhout, 1991). The *I* locus has been incorporated into bean varieties worldwide, thereby conferring resistance to BCMV and preventing seed transmission. The existence of temperature-independent necrosis-inducing BCMV strains has necessitated attempts to protect the *I* locus by pyramiding it with additional resistance genes (Drijfhout, 1991; Kelly *et al.*, 1995). This locus has been characterized as conferring extreme resistance (Collmer *et al.*, 2000) however, plants carrying the *I* allele can be inoculated with BCMV at high temperature (34^{o}C) or, following

inoculation at 26°C, transferred to high temperature for at least four days and elicit a systemic, vascular necrosis that results in plant death (Fisher, 1995) and Cadle-Davidson, unpublished data). Collmer and colleagues (Collmer *et al.*, 2000) have demonstrated that the heterozygote displays an intermediate resistance phenotype and therefore, that the gene is incompletely dominant.

We tested the null hypothesis that BCMV accumulates in genotypes carrying one and two copies of the *I* allele at the same rate and to the same extent as in the absence of this allele. *P. vulgaris* protoplasts were isolated and transfected, and rates of BCMV RNA accumulation were compared in homozygous resistant and susceptible near-isogenic lines (NILs) of BT (BT_{II} and BT_{ii}, respectively). The heterozygous genotype (BT_{Ii}) was generated by crossing the two homozygous NILs using BT_{ii} as the female parent. The susceptibility phenotypes of BT_{II}, BT_{ii} and BT_{Ii} under the conditions tested in the present study matched those previously described (Collmer *et al.*, 2000). Both healthy and inoculated plant materials were maintained in a growth chamber at 25°C day/22°C night with 16 hours photoperiod.

Protoplasts of BT_{II}, BT_{Ii} and BT_{ii} were isolated using a modified version of the procedure published by Bajet and Goodman (Bajet and Goodman, 1981). The predominate alterations of this procedure were: an increase in pH from 6.5 to 6.9 for all solutions, extension of incubation times to 18-20 hours, the use of 8-day-old, growth chamber-grown, bean primary leaves, and incubation of isolated protoplasts in constant darkness at 25ºC. Following isolation, protoplast samples were incubated on ice for approximately 1 hour before further manipulation. Cell viability was determined by double staining protoplasts with fluoroscein diacetate (FDA) and propidium iodide (PI) using the method of Fowke and Cutler (Fowke and Cutler, 1994). Viability was assayed (number of fluorescent cells/total number of cells) for each batch of protoplasts isolated and averaged 78.4% for transfected protoplasts through five days post transfection for all three NILs over three experiments (Table

2.1). Isolation of protoplasts following this method repeatedly yielded on the order of 1 x 10^6 *P. vulgaris* protoplasts per gram of fresh leaf tissue.

To assess the competence of protoplasts from each genotype to be transfected equally, a preliminary experiment was carried out. Cells (1 x 10^6 in 1 ml) of each genotype were electroporated in 0.6 M mannitol, pH 6.9 with 15 μg plasmid containing GFP under the transcriptional control of the *Cauliflower mosaic virus* 35S promoter (p35S-GFP). Sheared salmon sperm DNA (25 μg) was added as a carrier. Each sample was electroporated (300V) twice with gentle mixing between the applications of voltage. The samples were placed on ice for 15 minutes, after which the mannitol was replaced with incubation medium (0.6 M mannitol pH 6.9, 100 μg/ml Ampicillin, 50 μg/ml Rimocidin), and protoplasts were incubated at 25°C in the dark. Negative controls for these experiments were protoplasts transfected only with carrier DNA and no GFP expression was detected in these samples. Transfection efficiencies for the plasmid DNA are given in Table 2.1.

To assay the effect of the *I* allele on viral RNA accumulation, NIL protoplasts were transfected with the genomic RNA of BCMV strain NY15 68/95 (pathogroup V), a severe strain of NY15 obtained from R. Provvidenti (Geneva, NY) (Kyle and Provvidenti, 1987b). This strain was maintained on cv. 'California Light Red Kidney,' a mosaic-producing (*ii*) host. All viral transfers were accomplished using the method of Provvidenti (Provvidenti, 2001). Virions were isolated according to Hill and Benner (Hill and Benner, 1980) except that virions were precipitated in the presence of 6% PEG. Viral RNA was isolated from virions using the method of Warren and Murphy (Warren and Murphy, 2003), of which 5 μg was used for each transfection. RNA was extracted from protoplasts immediately following their collection using the same procedure (Warren and Murphy, 2003), and the concentrations determined by absorbance at 260 nm.

Table 2.1. Protoplast viability over time and DNA and RNA transfection efficiencies for each of the three *I*-gene genotypes.

	Percent viable cells			Transfection Efficiency	
Genotype	1 dpt *	3 dpt	5 dpt	DNA (p35S-GFP)	RNA (BCMV)
BT_{II}	80.5% ** (116/144)	81.10% (103/127)	70.00% (84/120)	12.3%† ± 2.2	65.5%‡ ± 1.3
BT_{Ii} (F_1)	96.80% (154/159)	83.00% (54/65)	85.20% (52/61)	11.4% ± 2.7	47.2% ± 1.8
BT_{ii}	88.60% (172/194)	83.30% (75/90)	88.30% (53/60)	16.7% ± 7.8	61.6% ± 1.1

* Days post transfection

** Percent viability was determined by staining with fluoroscein diacetate and is expressed as a percentage of fluorescent cells out of total intact cells. Cells used in viability counts were transfected with carrier DNA (Salmon testes DNA) only.

† Transfection efficiencies for protoplasts transfected with p35S-GFP plasmid DNA (GFP expressing cells/total number of cells) are averaged from at least three protoplast batches, with standard error shown.

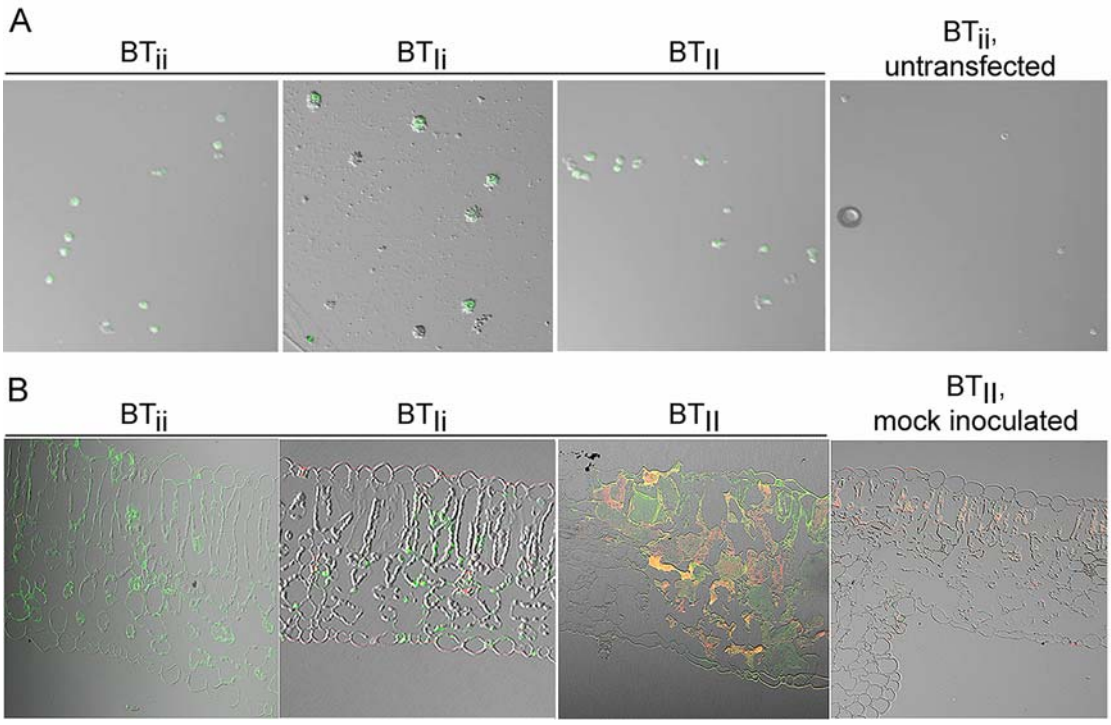
‡ Transfection efficiencies for protoplasts transfected with purified *Bean common mosaic virus* genomic RNA (number of positively α–CP immunostained cells/total number of cells) are averaged from three replicates of a single experiment, with standard error shown.

RNA transfection efficiency was evaluated by transfecting protoplasts of each genotype with BCMV RNA, followed by fixation and immunostaining using anti-BCMV antiserum (Uyemoto *et al.*, 1972) (Figure 2.1A) according to Mas and Beachy (Mas and Beachy, 1999). Alexafluor488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) was used as the secondary antibody. Transfection efficiencies were calculated by dividing the number of intact, stained protoplasts by the total number of intact protoplasts and are listed in Table 2.1. BCMV was detected in protoplasts of all three NILs (Figure 2.1A), indicating that the protoplasts were competent to translate the viral RNA regardless of genotype tested. The negative controls for RNA

transfection were either untransfected or transfected with only carrier DNA and were processed in the same fashion as experimental samples. No staining was observed in these controls.

Time course experiments were conducted to quantify the accumulation of viral RNA over time in the three NILs. Transfected protoplast samples were collected each day, one to five days post transfection (dpt). The amount of viral RNA present in the protoplast total RNA samples was assayed by RNA gel blot (northern) analysis or semi-quantitative reverse transcriptase PCR (sqRT-PCR, described below) (Figure 2.2). Bands on RNA gel blots and sqRT-PCR agarose gels were quantified using the pixel volume function of Adobe Photoshop 6.0 software (Adobe Systems Inc., San Jose, CA). Northern analysis using protoplast RNA from two independent experiments (batches of protoplasts) demonstrated an increase in BCMV RNA (normalized to ethidium bromide-stained rRNA) through five dpt in all three genotypes in both experiments. Figure 2.2 (A and B) shows a representative example of the results from these experiments and a plot of the raw data normalized to the amount of rRNA over time. Further, viral RNA appeared to increase at a faster rate in BT_{ii} than in either BT_{II} or BT_{Ii}.

To quantify viral RNA accumulation more precisely in the three genotypes, sqRT-PCR was conducted with three biological replicates (transfected protoplast batches) and between two and four technical replicates (sqRT-PCR replicates) for each biological replicate. Primers used for 18S rRNA were (5'-3'): reverse transcription, AGTCTGTCAATCCTTACTAT; forward, CTGGCGACGCATCATTC; and reverse, GAATTACCGCGGCTGCT. Primers for the BCMV coat protein gene were those developed by Xu and Hampton for specific BCMV detection (Xu and Hampton, 1996). Omniscript reverse transcriptase (Qiagen, CA) was used to generate first strand cDNA from protoplast total RNA following the manufacturer's recommended protocol. The subsequent PCR was carried out in 1X Promega Mg-free buffer; 1mM



$MgCl_2$; 0.1mM each dNTP; 0.67 μM each PCR primer; and 2.5 U *Taq* DNA polymerase (Promega, Madison, WI). A standard curve was constructed using

Figure 2.1. Immunostained transfected protoplasts and inoculated, embedded *Phaseolus* tissue. A. At 1 day post transfection, cells were fixed and then immunostained with anti-BCMV primary antibody and Alexafluor488 secondary antibody (green). Untransfected samples were immunostained in the same fashion as transfected samples. B. Confocal images of inoculated and mock-inoculated immunostained sections. Images representative of four experiments are shown. Red signal is chloroplast autofluorescence or propidium iodide staining

samples containing 200, 400, 600, 800, and 1000 ng total RNA starting material in order to determine the number of PCR cycles that give a linear response for PCR amplification. The cycle number at which the standard curve was linear was the point at which the remainder of the experiment was evaluated (23 cyles for BCMV; 17 for 18S rRNA). The data (ethidium bromide-stained band intensities normalized using 18S rRNA band intensities) were analyzed using regression analysis to determine if

viral RNA increased over time and if there were any differences in the rates of viral RNA accumulation among BT_{ii}, BT_{Ii}, and BT_{II}. Minitab statistical software release 14 (Minitab Inc, State College, PA) was used to run backward stepwise regression using α=0.10 to remove predictors from the model (Table 2.2). The predictor "hours post transfection" reflected a statistically significant increase in viral RNA over time, but had to be square root-transformed because the normal probability plot was skewed. Regression analysis of these data suggested that viral RNA increased over time for all genotypes tested (i.e., rate of viral RNA increase > 0; P=0.003). Furthermore, this

Table 2.2 Statistical significance of parameters used in the regression analysis testing the null hypothesis that there is not difference between BCMV RNA accumulation in *P. vulgaris* BT_{II}, BT_{Ii} and BT_{ii} protoplasts. Analysis was carried out using Minitab statistical software release 14.

Regression equation:

Normalized intensity=0.619+0.0887sqT+0.476I_1-0.0882 I_1*sqT-0.0258 I_2*sqT+0.0319 sqT*Exp2

R-Sq=47.2% P=0.000

Predictor†	**Coefficient**	**SE Coefficient**	**T**	**P**
Constant	.6193	.2037	3.04	.004
SqrtTime	.08872	.02800	3.17	.003
I_1	.4757	.2553	1.86	.069
I_1*sqT	-.08219	.03445	-2.39	.021
I_2*sqT	-.02584	.01115	-2.32	.025
sqT*Exp2	.031918	.009834	3.25	.002

Analysis of Variance

Source	**DF**	**SS**	**MS**	**F**	**P**
Regression	5	2.16552	0.43310	8.04	0.000
Residual Error	45	2.42398	0.05387		
Total	50	4.58950			

† sqT = square root-transformed hours post transfection; I_1=presence of a single *I* allele; I_2=presence of a second *I* allele; Exp2=experiment 2; * denotes the interaction between parameters.

analysis revealed that the presence of a single *I* allele decreased the rate of viral RNA accumulation (P=0.021), and that the presence of a second *I* allele decreased the rate even further (P=0.025). Variation was present between experiments (protoplast batches) and between PCR assays; however, statistical interactions between experiments and either genotype, gel, or days after transfection were not significant (removed from the model at $\alpha > 0.10$). In other words, while there were differences between the sqRT-PCR data between independent protoplast experiments, the relative rates of viral RNA accumulation between genotypes were consistent for all experiments.

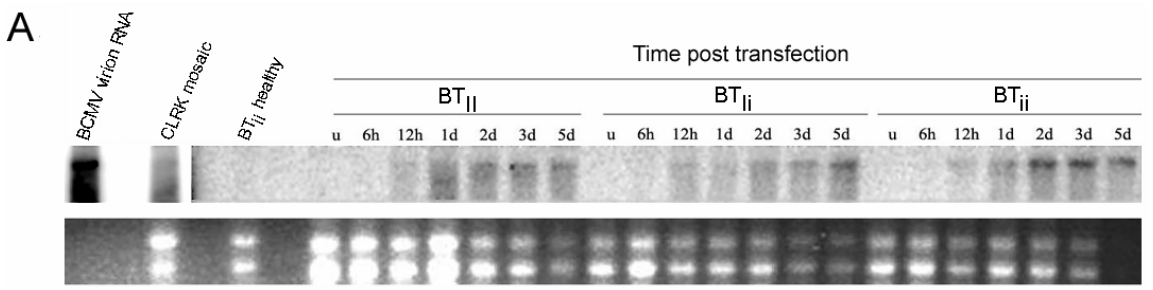
Figure 2.2 (C and D) shows a graph of all experimental data used in the regression analysis and a representative gel from the sqRT-PCR analysis. The data presented in the graph were analyzed using an unpaired t-test and columns with the same letter were not significantly different ($\alpha=0.05$). The graph confirms the results of the northern and regression analyses that BCMV RNA increases in all three genotypes. The data suggest that viral RNA accumulation in BT_{Ii} is intermediate to BT_{ii} and BT_{II} between two and four days post transfection.

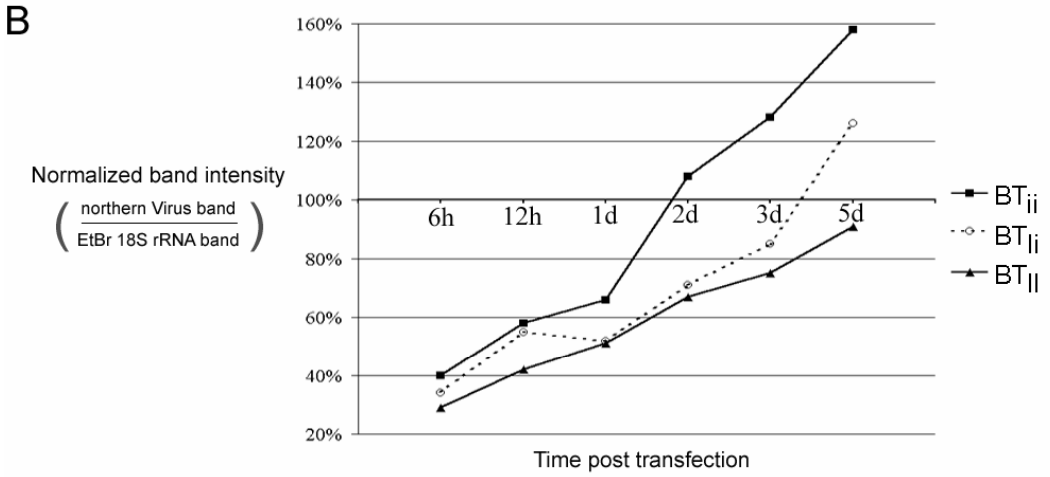
To determine if BCMV can accumulate in the presence of the *I* allele *in planta*, BT_{II}, BT_{Ii} and BT_{ii} whole plants were inoculated with BCMV NY15 68-95 at eight days post germination. Inoculated plants were harvested at 4 days post inoculation (dpi) and leaf, stem and trifoliate leaf samples were collected and fixed. Mock inoculated controls (leaf and stem) were also collected and treated in the same fashion. Samples were fixed, embedded, and immunostained using the anti-BCMV primary antibody and Alexafluor488 goat α-rabbit secondary antibody (Molecular Probes, OR) according to Baskin and colleagues (Baskin *et al.*, 1992). These experiments were designed to assay BCMV accumulation early in the infection cycle and, this being the case, a few chlorotic local lesions were present on BT_{ii} and BT_{Ii} leaves whereas no symptoms were visible by eye in BT_{II} at the time of tissue collection. At least two

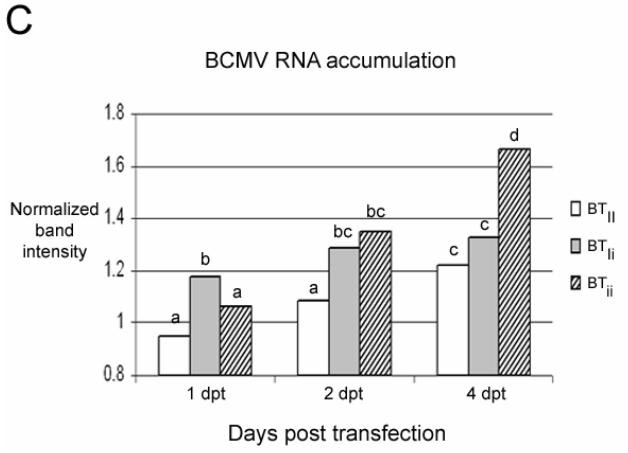
plants per genotype and six embedded tissue blocks per plant were sampled in each of four inoculation experiments. Microscopy was performed with a Leica TCS SP2 laser scanning confocal microscope at the Cornell Biotechnology Resource Center. BCMV accumulation was detected in all three genotypes at 4 dpi in inoculated leaves (Figure 2.1B). Widespread staining was evident in BT_{ii} tissue sections, whereas in BT_{Ii} BCMV was found in discrete areas in a smaller number of cells and in BT_{II} single cell infections or small lesions were detected (Fig. 3B). Figure 1.1B shows a lesion in the BT_{II} sample, which may be a micro-hypersensitive response (HR) based on the presence of viral staining (green) and dead cells (red autofluoresence). These results support the conclusion that BCMV can replicate in the presence of *I* allele and suggest the possibility that short distance movement can also occur.

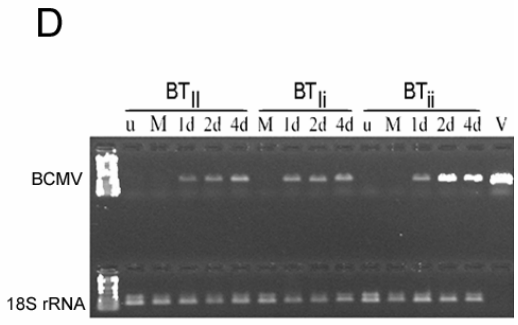
The aim of the current study was to address the basic question of whether or not BCMV can replicate in the presence of the *I* allele and, if so, whether it does so in a dosage-dependent way, thus extending Collmer and colleagues' conclusions to the single cell level (Collmer *et al.*, 2000). The data presented here show that BCMV RNA and protein accumulate in both BT_{II} and BT_{Ii}, suggesting that this virus can replicate in the presence of the *I* allele. Further, our quantitative analysis of accumulation suggests that the *I* allele may affect either the replication or the persistence of BCMV, as each additional allele had a significant effect on viral accumulation ($P \leq 0.025$). Collmer and colleagues (Collmer *et al.*, 2000) have shown that the broad-spectrum resistance locus, *I*, confers incompletely dominant resistance against BCMV at the whole plant level. Further, their work demonstrated that this resistance may function on what can be considered more of a continuum rather than "on" versus "off," depending on the number of *I* alleles present and the environmental conditions tested (Collmer *et al.*, 2000). The concept of resistance as a continuum depending on both genetic and environmental factors is also supported by the present finding that each additional *I* allele incrementally reduces BCMV RNA accumulation

Figure 2.2. Demonstration of *Bean common mosaic virus* (BCMV) replication in protoplasts by measurement of viral RNA over time. A. Northern analysis of BCMV replication (top panel) and ethidium bromide-stained RNA gel (lower panel). The BT_{ii} 5 dpt sample contained very low amounts of total RNA and thus is not visible in the ethidium bromide-stained gel. A riboprobe for the viral coat protein was used as a probe. B**.** Plot of northern band intensities from one of two experiments as a percent of ethidium bromide-stained rRNA. C. Semi-quantitative RT-PCR from protoplast RNA. 18S sequences were used as internal normalization controls . Graph shows the averages of three experiments (protoplast batches), each with internal replication of RT-PCR and agarose gel electrophoresis. Columns with the same letter were not significantly different at α=0.05. D**.** Representative results of the sqRT-PCR experiments. M=mock transfected (carrier DNA only); V=purified BCMV RNA; u=untransfected


A
BCMV virion RNA
CLRK mosaic
BTii healthy
Time post transfection
BTII
BTIi
BTii
u 6h 12h 1d 2d 3d 5d
u 6h 12h 1d 2d 3d 5d
u 6h 12h 1d 2d 3d 5d


B
Normalized band intensity
(northern Virus band / EtBr 18S rRNA band)
160%
140%
120%
100%
80%
60%
40%
20%
6h 12h 1d 2d 3d 5d
Time post transfection
BTii
BTIi
BTII


C
BCMV RNA accumulation
Normalized band intensity
1.8
1.6
1.4
1.2
1
0.8
a b a a bc bc c c d
1 dpt
2 dpt
4 dpt
Days post transfection
BTII
BTIi
BTii


D
BTII
BTIi
BTii
u M 1d 2d 4d M 1d 2d 4d u M 1d 2d 4d V
BCMV
18S rRNA

in protoplasts. The data presented here further suggest that BCMV accumulation also occurs in whole plants with the *I* allele, although the current experiments did not quantify the differences between the NILs at this level. These data, taken together with previous temperature shift data (Fisher, 1995), suggest that *in planta*, BCMV is able to replicate in *I* containing genotypes.

Acknowledgements

We thank Dr. R. Provvidenti for the BCMV isolate used in these experiments. We acknowledge Dr. S. Lazarowitz and members of her lab for technical assistance and the use of the p35S-GFP clone, and Dr. L. Cadle-Davidson for statistical analysis.

CHAPTER 3[†]

Patterns of accumulation of *Bean common mosaic virus* in *Phaseolus vulgaris* L. genotypes nearly isogenic for the *I* locus

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Keywords: Confocal microscopy; immunostaining; Potyvirus; resistance mechanism

Running Title: Patterns of BCMV accumulation in *I* genotypes

[†] Chapter 3 is in preparation for submission to Annals of Applied Biology.

Summary

Resistance conferred by the *I* locus of *Phaseolus vulgaris* L. is notable in that it is effective against at least eight different potyviruses, including *Bean common mosaic virus* (BCMV), has been bred into nearly all common bean germplasm since its discovery and, most importantly, that it continues to be durable when in combination with other recessive resistance genes. However, as with nearly all resistance genes, we take advantage of the effectiveness of the *I* locus without knowledge of its specific mechanism. In the present study we examine near isolines (NILs) of *P. vulgaris* cultivar 'Black Turtle Soup' (BT) that vary at *I*. Homozygous resistant (BT_{II}), susceptible (BT_{ii}) and F_1 (BT_{Ii}) plants were inoculated with BCMV, maintained at both permissive and restrictive temperatures, and then harvested and prepared for immunostaining. Antibodies against the BCMV virion were used to detect the presence of the virus and observations were made by confocal microscopy. The patterns of virion accumulation were distinct for each genotype tested and support previous findings in this pathosystem that BCMV is able to accumulate in the presence of the *I* allele.

Introduction

The *I* locus of *P. vulgaris* was originally identified in the 1930s as conferring asymptomatic resistance against the *Potyvirus Bean common mosaic virus* (BCMV) (Pierce, 1934) and has since been incorporated into most common bean germplasm worldwide (Drijfhout, 1991). Unfortunately, subsequent experience and research

showed that when inoculated at temperatures above 28ºC resistant plants develop a systemic necrosis that ultimately kills the host (Bos, 1971). Further, several potyviruses have been shown to interact with this resistance locus in three general symptom classes: temperature-independent necrosis, temperature-dependent necrosis, and non-necrotic (Fisher and Kyle, 1994; Fisher and Kyle, 1996; Kyle, 1988; Kyle and Provvidenti, 1987a; Kyle and Provvidenti, 1993). The inheritance of the *I* locus has been extensively studied (Ali, 1950; Bos, 1971; Collmer *et al.*, 2000; Drijfhout, 1978; Fisher and Kyle, 1994; Fisher and Kyle, 1996; Kyle and Dickson, 1988; Kyle and Provvidenti, 1993), resulting in a cumulative knowledge that this locus is incompletely dominant, confers broad-spectrum resistance against nine potyviruses, and, when infected by temperature-dependent necrosis-causing viruses above 28ºC or by temperature-independent necrosis-causing viruses, is associated with a vascular necrosis that may kill the host.

While the resistance mechanism of the *I* locus is, as yet, undetermined, several studies exist that can provide insight into what actually occurs during the associated resistance response. In previous work, protoplasts of resistant, susceptible and intermediate (heterozygous) isolines of BT transfected with BCMV RNA showed viral RNA accumulation in all three genotypes at 26ºC, albeit at different rates (Cadle-Davidson and Jahn, In Press). Other experiments demonstrated that *I*/- plants graft-inoculated with infected susceptible plants (*i*/*i*) developed systemic necrosis regardless of temperature (Grogan and Walker, 1948), indicating that I/- plants are competent to support BCMV movement and symptom expression even at low temperatures. Finally, temperature shift experiments in which *I/I* resistant plants are inoculated and shifted from 25ºC to 34ºC or from 34ºC to 25ºC over time have shown that BCMV can persist at low temperatures for at least four days post inoculation and will produce symptoms if the inoculated plant is transferred to high temperatures ((Fisher, 1995), Cadle-Davidson, unpublished data). Together, these experiments show that the BCMV isolate

used in these studies, a temperature-dependent necrosis-inducing isolate, can replicate in the presence of *I* alleles at low temperature and that once the virus reaches some as yet undefined threshold or criteria, the necrotic phenotype can be expressed regardless of temperature.

Still, while a great deal of genetic information is available along with a collection of whole plant observational data, there is a paucity of cell biological data in this pathosystem. The key host-virus interactions dictating resistance and susceptibility occur, by definition, at the cell-level; however, exactly what occurs in an individual cell during the resistance response to BCMV is not known. At the whole plant level it can be seen that plants with and without the *I* allele have vastly different responses to BCMV, but this does not tell us anything about what happens within infected cells. In the present study, the cellular differences in virus accumulation and patterning during BCMV infection and colonization of resistant (*I*/*I*), susceptible (*i*/*i*) and heterozygous (*I*/*i*) genotypes were assayed. Further, high temperature conditions were utilized to induce the temperature-dependent necrosis phenotype associated with the BCMV isolate used here. To address these issues, NILs of BT (BT_{II} and BT_{ii}) and their F_1 (BT_{Ii}) were inoculated and maintained under low (26ºC) and high (34ºC) temperature regimes. Immunostaining and confocal microscopy were used to visualize virion accumulation.

Materials and Methods

Germplasm

NILs of *P. vulgaris* variety BT homozygous for either the dominant (BT_{II}) or recessive allele (BT_{ii}) were used in these experiments. F_1 seed of the cross between the two NILs using the susceptible genotype as the female was used as the heterozygote

(BT_{Ii}). Plant material was maintained in a growth chamber at 26°C day/22°C night for low temperature treatments or 34°C day/31°C night for high temperature treatments, both with 16 hours photoperiod. Two plants of each genotype were inoculated for each temperature tested, and two to four samples of each tissue type (primary leaf, stem or trifoliate leaf) were observed. Mock inoculated controls (leaf and stem) were collected and treated in the same fashion as the experimental samples. Complete experiments were carried out twice at both 26ºC and 34ºC.

Viral isolate maintenance and inoculation

Bean Common Mosaic Virus NY15 68/95, a severe, temperature-dependent necrosis-inducing isolate, was obtained from R. Provvidenti (Geneva, NY) (Kyle and Provvidenti, 1987b) and maintained on cv. California Light Red Kidney (CLRK), a mosaic-producing host. Rub inoculations were performed using sap ground from fresh, highly symptomatic CLRK leaves in a chilled mortar with carborundum (400 mesh) and 0.05 M KH_2PO_4 (pH 8.5). For all microscopy analyses, BT_{II}, BT_{ii} and BT_{Ii} were germinated at 26°C, inoculated at 8 days post planting and maintained at either 26°C or 34°C in the growth chamber for the duration of the experiment. All plant material was grown in Cornell potting mix.

Fixation and embedding

Inoculated leaf (primary), stem and trifoliate leaf samples were collected and fixed at 4 days post inoculation. Samples were fixed in 4% paraformaldehyde, 50 mM PIPES, 0.05 mM $CaCl_2$ for two hours followed by three brief washes in 50 mM PIPES, 0.05 mM $CaCl_2$. The material was dehydrated with a series of 10, 25, 50, 75, 95 and 100% ethanol for 30 minutes each step. Methacrylate prepolymer (4:1 n-butyl methacrylate: methyl methacrylate) was gradually introduced into the tissue in 30 minute steps of a methacrylate:ethanol series consisting of 25%:75%; 50%:50%;

75%:25%; and ending in 100% methacrylate at –20°C overnight. Tissue samples were embedded in fresh methacrylate prepolymer in beem capsules (EMS, Hatfield, PA) overnight at 65°C.

Immunostaining and microscopy

Samples for microscopy were sectioned to a thickness of 1 or 2 μm and placed on fresh poly-L-lysine-coated slides. Prior to immunostaining, the samples (slides) were treated with acetone to remove the supporting methacrylate medium. Cells were permeablized using Tween-20, washed three times with 1XPBS, 1% BSA and then incubated with antibody raised against BCMV virions (Provvidenti, 2001; Uyemoto *et al.*, 1972) in 1XPBS, 1% BSA at 37°C for 2 hours. The slides were washed three times 10 minutes in 1XPBS, 1% BSA and goat anti-rabbit Alexafluor488 (Molecular Probes, OR) applied in 1XPBS; 1%BSA at 37°C for 1 hour. Propidium iodide counter stain was used to visualize the nucleus. Microscopy was performed on a Leica TCS SP2 laser scanning confocal microscope or an Olympus Fluoview confocal microscope. Autofluorescence from plant tissues was limited by turning down the photomultiplier (PMT) based on mock controls and using these settings to collect the experimental images.

Results

Whole plant symptom development on BT_{II}, BT_{ii} and BT_{Ii} was observed over four days (Table 3.1). No symptoms appeared on BT_{II} plants at 26ºC whereas both epinasty and necrotic lesions ultimately developed on the heterozygote. Ultimately, only BT_{ii} plants developed mosaic symptoms characteristic of BCMV infection at 26ºC.

At 34°C all genotypes developed necrotic phenotypes within four days of BCMV inoculation (Table 3.1).

A preliminary study using immunodetection of BCMV in tissue prints of BT_{ii} and BT_{II} inoculated leaves demonstrated that BCMV is able to persist and move at least short distances at 26ºC in BT_{II} (data not shown). Cellular observations of BCMV virion accumulation using Confocal Laser Scanning Microscopy (CLSM) at four days post inoculation for 26ºC and 34ºC are summarized in Table 3.1. In total, 113 samples were viewed for 26ºC material and 79 samples for 34ºC material. At least 100 cells per sample were observed depending on the magnification. Mock-inoculated controls were tested for all genotypes and both temperature regimes, although only BT_{II} at 34ºC is presented here (Figure 3.2). No fluorescence due to cross-reaction of the BCMV antibody or unintended presence of virus was observed.

Table 3.1. Symptoms[a] present in BCMV-infected tissue varying for I allele dosage over time.

		Genotype		
Temperature	Time post inoculation	BT_{ii}	BT_{Ii}	BT_{II}
26ºC	12 hpi[b]	none	none	none
	1 dpi[c]	none	none	none
	2 dpi	epinasty	epinasty	none
	4 dpi	epinasty; cll	nl; lsvn	none
34ºC	12 hpi	none	none	none
	1 dpi	none	epinasty	none
	2 dpi	epinasty; cll	nl; lsvn	nl; lsvn
	4 dpi	near death	systemic nl; ad	ad; near death

[a]nl: necrotic lesions; lsvn: local and systemic veinal necrosis; cll: chlorotic local lesions; ad: apical death
[b]hpi = hours post inoculation
[c]dpi = days post inoculation

All three genotypes supported BCMV persistence in the inoculated leaves at 26ºC, but systemic BCMV movement was only detected in BT_{ii} (Table 3.2, Figure 3.1). In 34ºC four dpi samples, all three genotypes tested showed viral staining in primary/inoculated leaves, stem and trifoliate leaves (Table 3.2, Figure 3.2). Stained sections from 34ºC heterozygous (BT_{Ii}) inoculated leaf showed severe cell damage and callose deposition that coincided with visible necrotic lesions on the inoculated leaf. Notably, this coincidence with necrosis was not present in BT_{II} sections and when necrosis was apparent in BT_{II} samples at the whole plant level, virus was not necessarily immunolocalized to these same lesions at the cell level. At this temperature and time point, both infected and non-infected BT_{II} cells appeared to be healthy and undamaged.

Discussion

The observational data presented here help to demonstrate that BCMV is capable of persistence, replication and local movement at 26ºC in the presence of one or more *I* alleles. While susceptible plants allow widespread replication and movement, resistant and heterozygous plants limit virus to a small number of cells that may or may not become necrotic. Interestingly, at 26ºC BCMV was detected in all cell types in BT_{Ii} sections, whereas the virus was only infrequently detected in BT_{II} (Figure 3.1).

At 34ºC necrotic cells were observed in both BT_{ii} and BT_{Ii} sections; however the patterns of necrosis were completely different: BT_{Ii} developed distinct necrotic lesions while BT_{ii} showed sporadic or random necrosis. When necrosis did not occur in BT_{Ii} sections, virus was able to move extensively in the inoculated leaf and a low level of accumulation was detected. The widespread, low-level presence of virus in these

sections was similar in pattern to the generalized BT_{ii} virus accumulation pattern. This is in contrast to viral patterns in BT_{II} at high temperature, in which BCMV is able to accumulate to very high levels and move by a more direct route (i.e. virus is not widespread in any certain tissue) to systemic tissues.

The patterns at both 26ºC and 34ºC support the previous genetic studies that describe the *I* allele as incompletely dominant (Cadle-Davidson and Jahn, In Press; Collmer *et al.*, 2000) in that the heterozygote resembles neither homozygous parent. The data described above may suggest a resistance mechanism that employs programmed cell death to stop viral infection, although the hypersensitive response (HR) has not typically been associated with *I* gene-mediated resistance. First, in BT_{ii}, the presence of BCMV seems to be widespread but lower-level than in either BT_{Ii} or BT_{II}, indicating that the virus is free to move in this genotype and rates of replication are high enough for visualization despite the virus' dilution through many cells. Further, at the higher temperature the heterozygote showed the cell death phenotype displayed by BT_{II} at low temperature and apparently limited virus to that region of necrotized cells, as little virus is detectable in systemic tissues when necrosis was present. However, examples were found in which no necrosis occurred and the virus was distributed in the tissue much as in BT_{ii}. These patterns could translate to a necrotic response that may be a timing, location or quantity-based mechanism, and that should the virus bypass the critical checkpoint, no barrier exists to halt further invasion.

Tissue and cell-level studies have been carried out for several other virus-host interactions (Hinrichs *et al.*, 1998; Kobori *et al.*, 2003; Reichel and Beachy, 1998; Schaad and Carrington, 1996; Shi *et al.*, 2003; Valkonen and Somersalo, 1996). Few of these, however, make direct comparisons between resistant and susceptible genotypes and fewer still can give insight into a temperature-inducible system such as the one

Table 3.2 Typical patterns of BCMV accumulation in cv. Black Turtle Soup (BT) genotypes isogenic for the *I* locus at 4 days post inoculation.

		Genotype					
		BT_{ii}		BT_{Ii}		BT_{II}	
Temperature	Tissue	# samples observed[a]	Localization	# samples observed	Localization	# samples observed	Localization
26°C	Inoculated leaf	21	widespread	23	all cell types; limited distribution; necrosis	21	rare, single or small cell clusters; callose deposition and cell collapse
	Systemic: *stem*	17	variable	4	none	7	none
	trifoliate leaf	9	limited to vascular tissue and adjacent cells	8	none	3	none
34°C	Inoculated leaf	13	widespread, low-level; occasional damage	17	callose deposition, visible necrotic lesions; virus limited to necrotic regions OR no necrosis; low-level, widespread virus	6	high-level; little to no cell damage
	Systemic: *stem*	14	widespread, low-level	4	limited	5	high-level; little to no cell damage
	trifoliate leaf	10	widespread, low-level	3	rare	7	high-level; little to no cell damage

[a]Approximately 100 cells observed per sample

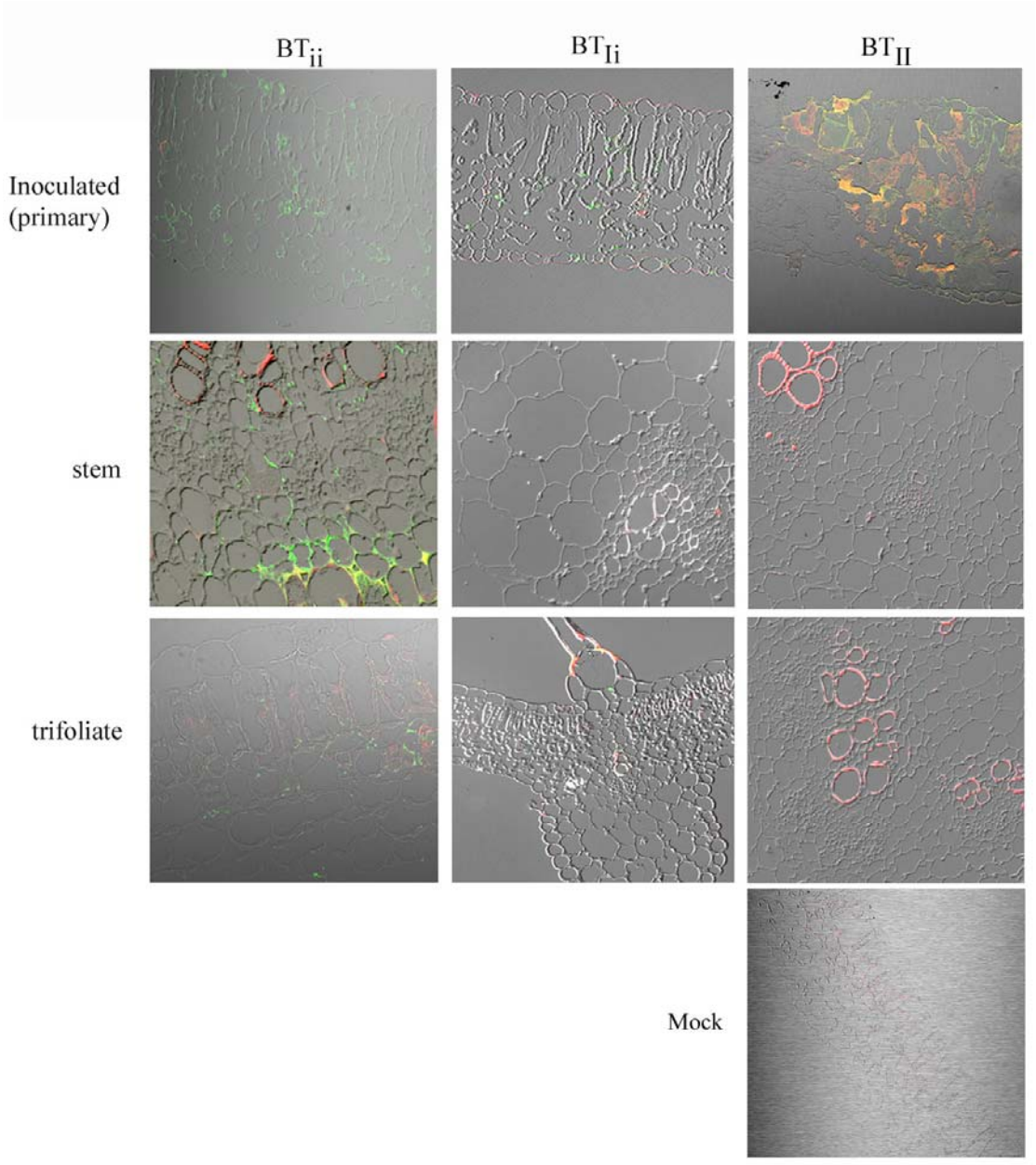

Figure 3.1. Confocal images of fixed tissue sections of resistant (BT_{II}), intermediate (F_1) and susceptible (BT_{ii}) *Phaseolus* grown at 26ºC and harvested at 4 dpi. Images from primary leaf samples also appear on page 34. Green fluorescence is Alexafluor488 secondary antibody recognizing αBCMV. Red fluorescence is chloroplast autofluorescence and PI stained nuclei. Scale bars=50μm.

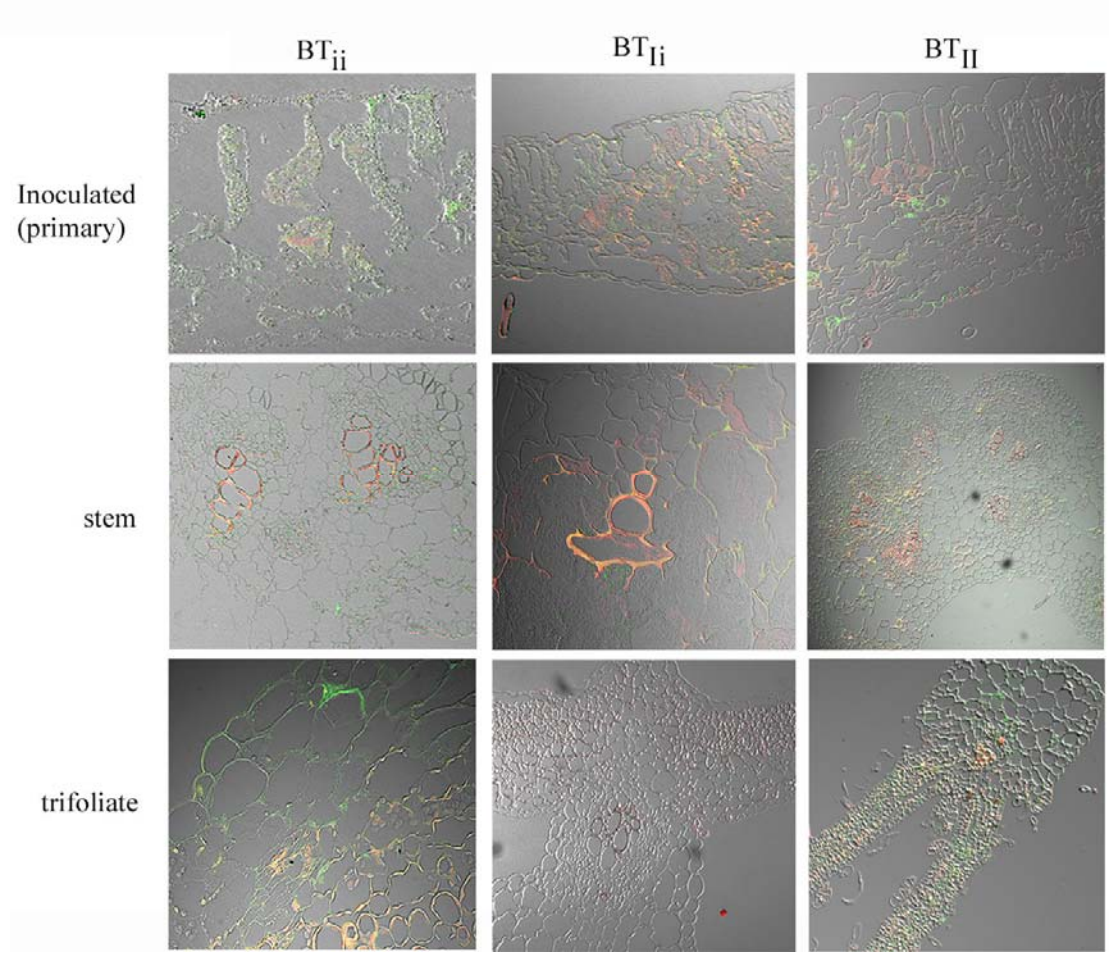

Figure 3.2. Confocal images of fixed tissue sections of resistant (BT_{II}), intermediate (F_1) and susceptible (BT_{ii}) *Phaseolus* grown at 34ºC and harvested at 4 dpi. Green fluorescence is Alexafluor488 secondary antibody recognizing αBCMV. Red fluorescence is chloroplast autofluorescence and PI stained nuclei. Scale bars=50μm.

presented here. On the other hand, a number of studies involving the potyviruses *Potato virus Y* (PVY) and *Tobacco etch virus* (TEV) have been conducted with the aim of resolving the distinction between extreme, hypersensitive and necrotic resistances (Hinrichs *et al.*, 1995; Hinrichs *et al.*, 1998; Hinrichs-Berger *et al.*, 1999; Schaad and Carrington, 1996; Valkonen and Somersalo, 1996). Defined macroscopically, extreme resistance (ER) is that which produces no symptoms whatsoever when the resistance gene is present, while hypersensitive and necrotic resistances display varying degrees

of necrotized tissue rather than developing systemic mosaic, mottle, or other symptoms (Hull, 2002). Resistance conferred by the *I* locus against BCMV has been thought of as being extreme due to the lack of symptoms at 26ºC (Drijfhout, 1991). Yet, in the data presented here, replication and necrosis were apparent in a small number of cells in BT_{II} and BT_{Ii} even at 26ºC. Similarly, Hinrichs and colleagues (1998) showed that both TEV and PVY can replicate in initially infected cells and a few neighboring cells in potato varieties containing the Ry_{sto} gene for ER. In their ER genotype ('Bettina'), infected cells were found in the centers and borders of otherwise necrotic lesions (Hinrichs *et al.*, 1998), which is similar to the pattern of virus infection displayed in the present study (Figure 3.1).

The *I* gene is likely an NBS-LRR type resistance protein with potential receptor function, which, according to current thinking, recognizes some viral factor in resistant cultivars and not in susceptible cultivars (Astua-Monge *et al.*, 2000; Martin *et al.*, 2003). Currently, the viral effecter molecule for the I protein is unknown. Even at low temperature (26ºC), BCMV is capable of replication in the presence of the *I* allele (Cadle-Davidson and Jahn, In Press) with the appearance of occasional necrosis in small numbers of cells (present study). This data suggests that in *I*/- genotypes, when BCMV moves out of an initially infected cell, recognition and necrosis occur. This argues for the existence of a host protein that is perhaps involved in plasmodesmal transport or gating that interacts (either directly or indirectly) with the BCMV movement complex in order to trigger the necrotic response. Such a protein could be the I protein itself or another with which the I protein interacts. The fact that only a single locus has been identified as being responsible for the *I*-BCMV defense response among 1000 F_3 families screened (Fisher and Kyle, 1994), further supports the idea that either the I protein is directly involved in BCMV recognition or that its interacting

protein is recessive lethal (although no evidence of lethality has been observed in this system).

CHAPTER 4[‡]

Differential gene expression in nearly isogenic lines for the *I* locus of *Phaseolus vulgaris* L. challenged with *Bean common mosaic virus*

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Keywords: cDNA-AFLP; Potyvirus; Defense response; Signal transduction; Expression analysis

[‡] Chapter 4 is in preparation for submission to Theoretical and Applied Genetics.

Summary

The *Phaseolus vulgaris I* locus-*Bean common mosaic virus* (BCMV; Potyviridae) pathosystem is of critical importance to bean geneticists, breeders and pathologists because of the worldwide distribution of both the virus as well as germplasm containing this resistance gene. In order to learn more about the molecular responses characteristic of this resistance gene, we have conducted a cDNA-AFLP screen on homozygous nearly isogenic lines of *P. vulgaris* variety 'Black Turtle Soup' (BT), containing either the *I* locus allele for resistance (BT_{II}) or susceptibility (BT_{ii}) to BCMV. Eight conditions were compared in a factorial analysis: BT_{II} versus BT_{ii}; mock inoculated versus BCMV inoculated; 26˚C versus 34˚C. Transcripts induced in response to viral infection and that were further responsive to temperature, genotype or both were isolated and cloned. Sequence analysis of the resultant clones revealed several classes of putative genes, including transcription-related and signal transduction-related genes. Review of disease resistance literature suggests further avenues of research involving the candidates isolated in this screen.

Introduction

Disease resistance research is currently at the forefront in the field of plant biology and much effort has gone into the elucidation of what occurs between the recognition of a pathogen and the onset of defense. The endpoint of known pathogen recognition signal transduction pathways is labeled, very generally, as "resistance." The reason for this lack of precision is the fact that, while genetic evidence confirms roles for several genes in the defense response, the actual chemical, biochemical and

physical reactions that produce the resistance phenotype are unknown. Pathogenesis related (PR) genes are often expressed as a result of signal transduction (Maleck *et al.*, 2000; Shah *et al.*, 2001) and specific transcription factors have been identified whose interaction with PR gene promoters is required for their salicylic acid-dependent induction (Zhang *et al.*, 2003; Zhang *et al.*, 1999; Zhou *et al.*, 2000). Yet most PR genes have neither anti-microbial activity nor any direct effect on pathogen attack at all. What are the molecules that literally and immediately confer resistance? What stops the pathogen attack? Possible candidate molecules for the resistance endpoint are those, such as ubiquitin and protease inhibitors, that in some way modify or inhibit invading pathogen molecules (Heath *et al.*, 1997; Jones and Takemoto, 2004). However, these defense genes are hard to discover due to the likely scenario that the ultimate inhibition or death of the pathogen may be multifaceted.

Efforts to dissect the resistance response employ both classical genetics and genomics—ranging from mutant screens to microarray studies. The use of transformation technology to confirm gene function in species such as *Arabidopsis* and *Nicotiana* has resulted in large databases of phenotypic data. However, mutant screens can be confounded by epistasis and dominance, and microarrays can be too costly to examine all the relevant genotypes, environments, and pathogens and the microarrays themselves may not even include all the relevant genes. A great deal of data has been generated using these methods, but the complete picture still eludes us. An alternative approach, differential display, can be sensitive enough to detect flux through a pathway while being cost-effective enough to screen multiple genotypes under multiple conditions. cDNA-AFLP can be performed under conditions of much higher stringency than other differential display methods, thus eliminating some potential for false positives (Bachem *et al.*, 1996). Further, small variations at multiple points along a pathway as well as differences in timing or tissue-specificity of expression can be

revealed (Bachem *et al.*, 1996). As a result, differential screens often identify housekeeping or seemingly nonsensical genes; however, this is not entirely unreasonable especially in light of the "Guard hypothesis" (Dangl and Jones, 2001; van der Biezen and Jones, 1998). That is, if a cell is under attack by a pathogen it is feasible that normal metabolism or functioning may be perturbed and, in fact, this may well be what an R gene monitors (Dangl and Jones, 2001; van der Biezen and Jones, 1998).

In order to discover some of the transcriptional variation that occurs as either resistance or susceptibility develops, we evaluated a genetically simplified, yet agronomically relevant pathosystem: *Bean common mosaic virus* (BCMV) infection in *I* gene-containing *P. vulgaris*. BCMV is a member of the *Potyviridae* and, as such, encodes only eight proteins, whose functions have been characterized in BCMV and closely related Potyviruses (Bos, 1971; Urcuqui-Inchima *et al.*, 2001). No single virulence factor or movement protein for this virus is known at this time, although several genes have been implicated as being involved with the latter (Urcuqui-Inchima *et al.*, 2001). The *P. vulgaris* host genotypes used here are inbred, near-isogenic lines (NILs) varying at the *I* locus, so variation detected in a differential expression study should be attributable to this locus (See Chapter 1 for a description of the NILs). Resistance to BCMV conferred by the incompletely dominant *I* allele has been considered to be extreme at 26°C (Cadle-Davidson and Jahn, In Press; Collmer *et al.*, 2000; Drijfhout, 1991). The "extreme" descriptor refers to the lack of any visible symptoms and the inability to recover virus from inoculated plants. However, upon inoculation at higher temperatures (above 30°C), resistant plants develop necrotic lesions, systemic veinal necrosis and, ultimately, apical death (Drijfhout, 1991). Further, we have shown that BCMV is able to accumulate, persist and move short distances in the presence of the *I* allele using protoplasts and fixed tissue sections

(Cadle-Davidson and Jahn, In Press; Collmer *et al.*, 2000; Drijfhout, 1991)). Susceptible plants show chlorotic (sometimes necrotic) lesions and systemic mosaic at either temperature, although at high temperature symptoms develop faster and often lead to premature death (Drijfhout, 1991).

In the present study we conducted a factorial cDNA-AFLP screen to compare expression between resistant and susceptible isolines of BT, BT_{II} and BT_{ii}, at 26˚C and 34˚C. Polymorphic bands were cloned and sequenced and their sequences submitted for BLAST searches against the nonredundant and EST databases in Genbank. The results of these sequence analyses were compared with known genes involved in plant disease resistance responses.

Materials and Methods

Germplasm.

NILs of BT homozygous for either the dominant (BT_{II}) or recessive allele (BT_{ii}) were used in these experiments. Plant material was planted in Cornell potting mix and maintained in a growth chamber at 26°C days/22° nights or 34˚C days/31˚C nights with 16 hour photoperiod.

Viral isolate maintenance and inoculation.

Bean common mosaic virus NY15 68/95 (BCMV) was obtained from R. Provvidenti (Geneva, NY) and maintained on the *P. vulgaris* variety 'California Light Red Kidney' (CLRK), a mosaic-producing host. Rub inoculations were performed using sap extracted from fresh, highly symptomatic CLRK leaves by grinding in a chilled mortar with carborundum (400 mesh) and 0.05M KH_2PO_4.

RNA isolation and cDNA-AFLP

Samples for RNA extraction and cDNA-AFLP analysis were collected at four days post inoculation and immediately frozen in liquid nitrogen and was stored at -80˚C until the RNA extraction. This material consisted of: BT_{II} mock-inoculated (buffer only), 26˚C; BT_{II} BCMV-inoculated, 26˚C; BT_{II} mock-inoculated, 34˚C; BT_{II} BCMV-inoculated, 34˚C; BT_{ii} mock-inoculated (buffer only), 26˚C; BT_{ii} BCMV-inoculated, 26˚C; BT_{ii} mock-inoculated, 34˚C; BT_{ii} BCMV-inoculated, 34˚C. A single primary (inoculated) leaf from each sample was used as starting material for the RNA preparation and extraction volumes were scaled down to one forth of the original protocol to account for the small quantity of starting material. RNA isolation and cDNA-AFLP procedures and primer sequences were as in Bachem et al. (1996). In brief, mRNA was isolated from total RNA using the PolyAtract paramagnetic beads (Promega, Madison, WI USA) and converted to double-stranded cDNA using M-MLV reverse transcriptase for first strand synthesis and Klenow DNA polymerase and RNase H for second strand synthesis. The cDNA was digested with *Ase*I and *Taq*I and then ligated with following adaptors:

Taq I adaptor: 5'-GACGATGAGTCCTGAC
TACTCAGGACTGGC- 5'

*Ase*l adaptor: 5'-CTCGTAGACTGCGTACC
CTGACGCATGGAT- 5'

To increase the amount of primary template a preamplification was carried out using primers specific to the adaptors:

*Taq*l pre-amplification primer: 5'-GACGATGAGTCCTGACCGA

*Ase*l pre-amplification primer: 5'-CTCGTAGACTGCGTACCTAAT

The final amplification was performed using additional adaptor-specific primers that contained two variable nucleotides at the 3’ ends, thus reducing the number of bands

expected per lane and increasing potential stringency. A total of 256 primer combinations was tested. The selective primer sequences were:

*Taq*I amplification primer: 5'-GATGAGTCCTGACCGANN

*Ase*I amplification primer: 5'-GACTGCGTACCTAATNN

All PCR steps were carried out using standard conditions to incorporate P^{33}-dATP and all enzymes were obtained from Promega (Madison, WI USA). Following the final amplification, samples were electrophoresed through a 6% acrylamide Tris-borate-EDTA/Urea sequencing gel. These gels were dried on filter paper and exposed to Kodak XOmat film.

Clone isolation and validation.

Polymorphic bands that were relevant to the pathosystem being studied were isolated and cloned. Selection criteria consisted of: 1) Responsiveness to viral infection; 2) Responsiveness to temperature 3) Responsiveness to genotype, specifically the presence of the *I* allele; and 4) Combinations of 1 – 3. Gel slices corresponding to the bands of interest were boiled and used as template for PCR using the primer sets from the original amplification. Following this PCR and confirmation of product by agarose gel electrophoresis, the PCR product was cloned into *Eco*RV-digested pBlueScript KS- to which a T overhang had been added by incubation at 70˚C with 1 U Taq DNA polymerase and 50 mM dTTP. Cloning was confirmed by β-galactosidase blue/white screening and all clones were sequenced using the M13 forward primers by the Bioresource Center, Cornell University, Ithaca, NY. Validation of clones was achieved by northern analysis of a panel of RNAs extracted using the same genotypes and environmental conditions as were used for the original screen.

Sequence analysis

All sequence analysis was performed using the Lasergene suite of sequence analysis programs (DNAstar) and Sequencher (Gene Codes Corporation). Redundant clones were eliminated at this point. Following annotation of sequence data, all clones were submitted to NCBI BLAST comparisons to attempt to determine their identities. EXPECT (E) values of 10^{-5} or less were considered to be significant. The procedure used for identifying each clone was first to submit each sequence to BLAST in the nonredundant (nr) database. If no hits were found in this database that met the E criterion, the sequence was then submitted to BLAST in the EST database. The original sequence was also used to perform a translated BLAST on the protein database. If the original sequence returned a significant match only in the EST database, the resultant EST was submitted to BLAST in the nr and protein databases.

Results

Several banding patterns were present in the materials tested here including uniform expression for the majority of bands detected. This was expected due to the fact that the starting plant materials are NILs that differ at the *I* locus. Seven different banding patterns (Figure 4.1) were considered interesting in the context of these experiments and only bands that fit into these categories (labeled a-g) were extracted and cloned. With the exception of (e), all bands cloned were virus-dependent. The relevant banding patterns were: a, temperature-dependent size polymorphism (both bands were isolated); b, BT_{II} specific, temperature-independent; c, genotype-dependent differential abundance; d, heat responsive, virus-dependent; e, heat responsive, virus-independent; f, high temperature induction; and g, BT_{II}-specific, high temperature only.

In total, 59 bands were identified as being meaningfully polymorphic and extracted from the gels.

Sequence analysis revealed that most of the clones were redundant and the original number of cloned bands was reduced to 21 unique clones (Table 4.1) with 19 of these being confirmed by northern analysis. Table 4.1 shows the results of the sequence analyses conducted on the cDNA-AFLP clones. The sequences identified by the cDNA-AFLP experiments presented here are comprised of genes that can be categorized as transcription-related, signal transduction-related, defense-related, "housekeeping" genes and others that do not give insight into their relevance to this study.

Discussion

In the present study we screened *P. vulgaris* NILs that contrasted at the virus resistance locus, *I*, using cDNA-AFLP. The rigor of the experimental design aided the discovery of candidate genes induced by the interaction of BCMV and the *I* locus due to the fact that genotype, environment and infection state were controlled. Specifically, resistant and susceptible NILs were tested at both normal and elevated temperatures and in both the inoculated and mock-inoculated condition. The factorial design eliminates selection of many false-positive banding patterns and allows for the identification of bands that are present only in the presence of viral infection as well as in association with resistance. This combination of genetics and differential expression screen is very powerful; because there are so few genetic differences between the BT NILs, any differences in gene expression could very well be a result of the influence of the *I* locus. Further, the conditions we tested included virus-infected samples at

"permissive" (34ºC) and "restrictive" (26ºC) temperatures. It is well known that viral infection leads to alteration of gene expression and that this, in part, is due to viral recruitment of the cellular transcription machinery for its own use (Hull, 2002). Therefore, the differential expression seen in this study may be viewed as having two sources: 1. Defensive regulation controlled by host cells in order to combat infection and heat shock and 2. Viral manipulation of cellular transcription for the purpose of replication and movement.

In a similar study to the one presented here, Vallejos and colleagues (2000) compared bulked samples homozygous for either *I* or *i* from a population of recombinant inbred lines (76 lines in the complete population). This study did much to account for the genetic background and identified RNA-level polymorphism between resistance and susceptibility. A further result of their research was the discovery of sequences genetically linked to the *I* locus that may prove beneficial in the positional cloning of this resistance locus (Vallejos *et al.*, 2000). However, they did not address the complete interaction of the *I* locus with pathogen or environment either by inoculation with BCMV or by exposure to the permissive and restrictive temperatures (Vallejos *et al.*, 2000). Further, the clones isolated in the Vallejos et al. (2000) study were not sequenced and as a result, no additional comparisons between their study and the present one may be made.

We isolated several transcripts that are up-regulated in response to host genotype interaction with both viral infection and heat stress. Putative genes for malate

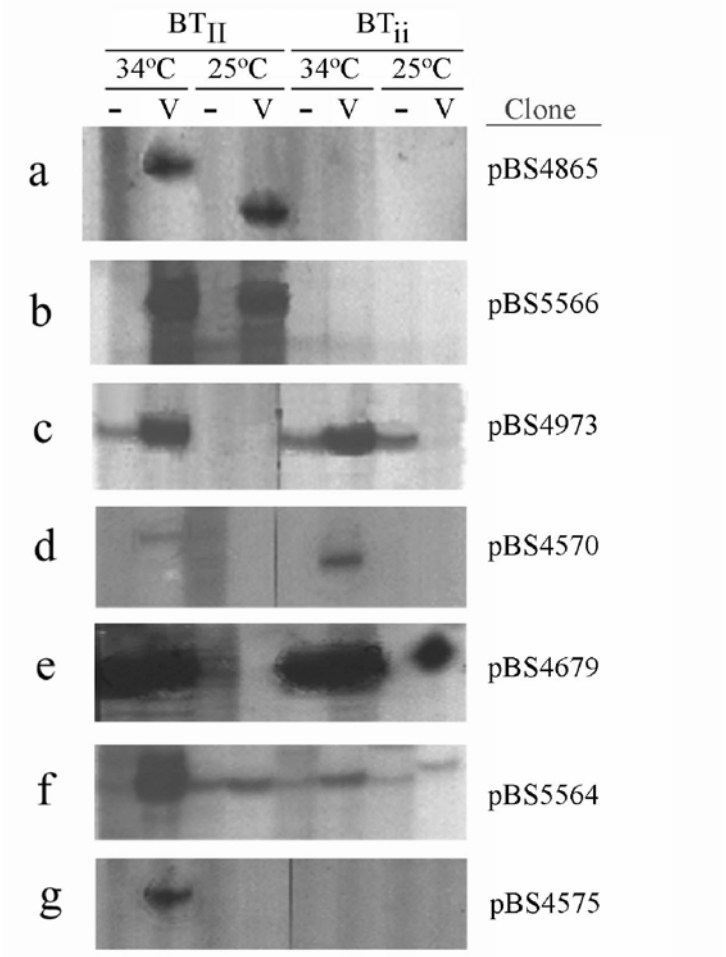

Figure 4.1. Examples of expression patterns from cDNA-AFLP gels when BT_{II} and BT_{ii} were compared at 25˚C and 34˚C. RNA for this analysis was collected four days post inoculation or mock inoculation. Letters a-g represent classes of banding patterns and are represented by images of single clones each. V = BCMV-inoculated sample - = mock inoculated sample. a=temperature-dependent size polymorphism; b=BT_{II} specific, temperature-independent; c=genotype-dependent differential abundance; d=heat responsive, virus-dependent; e=heat responsive, virus-independent; f=high temperature induction; g=BT_{II}-specific, high temperature only.

Table 4.1 cDNA-AFLP clones showing differential expression between *P. vulgaris* BT_{II} and BT_{ii} NILs and at 25˚C vs 34˚C. Banding patterns were grouped into seven different classes as indicated by letters a through g

		BLAST results		
Clone	**Banding pattern***	GenBank accession	E value	Description
Transcription				
pBS4979	g	NP_197184	6.00E-20	*Arabidopsis* heat shock transcription factor 3 (HSF3)
Signal Transduction				
pBS4773	g	AY463016.1	2.00E-11	*Musa acuminata* putative beta family G-protein
pBS4866	g	AF096249	1.00E-100	*S. lycopersicon* ethylene-responsive small GTP-binding
pBS5077	g	BAC05575.1	4.00E-74	*Oryza sativa* protein phosphatase 2C-like protein
Defense				
pBS4575	g	AY248742	4.00E-28	*S. lycopersicon* omega-3 fatty acid desaturase gene
pBS4771	b	AJ506739.1	2.00E-05	*Oryza sativa* mRNA for beta 1,3-glycosyltransferase-like protein II/Avr9 elicitor response protein-like (3e-75, BAB09796.1)
Housekeeping				
pBS4571	c	AJ320268	3.00E-26	*P. paniceum* malate deshydrogenase
pBS4665	a	X75082	6.00E-38	*S. tuberosum* mRNA for mitochondrial citrate-synthase
pBS4679	e	X14060	8.00E-41	*S. lycopersicon* nia gene for nitrate reductase (EC 1.6.6.1)
pBS4764	e	X05984.1	9.00E-08	*S. lycopersicon* rbcS3A gene for ribulose 1,5-bisphosphate carboxylae/oxygenase
pBS4973	c	K00507	6.00E-50	Tobacco chloroplast atpase gene (b and e subunits)
Unclassified				
pBS4777	g	AAM63708.1	1.00E-71	*Oryza sativa* putative zinc-finger, heat shock protein
pBS4865	a	AF233745	1.00E-06	*S. lycopersicon* chaperonin 21 precursor
pBS4872		BI931463	1.00E-95	Tomato EST; predicted transmembrane domain
pBS4968-1	a	AC123524.	7.90E-01	Unknown protein; translated sequence contains putative transmembrane domain
pBS4968-2	a	BQ514455	8.00E-73	Ripening tomato est; translated sequence contains putative transmembrane domain and conserved DUF609 domain of unknown function

Table 4.1 (Continued)

pBS5265	g	CB483497	2.60E-02	*Oryza sativa* aluminium-induced EST
pBS5564	f			No hits
pBS5566	b	AAC98059.1	3.00E-26	*Arabidopsis* chloroplast lumen common protein
pBS5667	g	BG128668	2.00E-26	*S. lycopersicon* shoot/meristem EST

* a=temperature-dependent size polymorphism
b=BT_{II} specific, temperature-independent
c=genotype-dependent differential abundance
d=heat responsive, virus-dependent
e=heat responsive, virus-independent
f=high temperature induction
g=BT_{II}-specific, high temperature only.

dehydrogenase and a chloroplast ATPase were expressed in a genotype-dependent fashion whereas expression of putative citrate synthase and nitrate reductase was heat-responsive. Should the involvement of these genes in *I* gene-mediated resistance be further verified, it could point to differential partitioning of cell resources in cells undergoing basal metabolism as compared to cells undergoing heat shock or viral invasion or defense response. One of the big holes in plant disease resistance research is information regarding how recognition by an R gene (or any type of resistance gene) leads to a phenotype. Knowledge about the effect of resistance or infection on basal cell metabolism may be key in elucidating the final steps in these pathways.

Clones pBS4773, 4866 and 5077 show significant sequence similarity to components of an ethylene-responsive G-protein signaling system (4773, 4866) and an ABA sensitive protein phosphatase 2C (5077) (Table 4.1) (Roehl *et al.*, 1995; Zegzouti *et al.*, 1999). At this time, stress responsive ABA signaling is the only pathway in plants known to function via G-protein signaling (Leung *et al.*, 1997); (Jones, 2002). It is reassuring that we identified three potential components of the same signal

transduction pathway in our screen and that they share the same banding pattern. The possibility that a stress-related signaling pathway is induced in a resistant-genotype-dependent manner in response to viral infection is inviting in that it suggests that general, not specialized, pathways are used by a plant host in order to combat infection and is consistent with the growing evidence for crosstalk between signaling pathways (Kachroo *et al.*, 2003).

Evidence for crosstalk between the jasmonic acid (JA), salicylic acid (SA), and ethylene signaling pathways, and specifically that JA is antagonistic to the SA pathway, is derived from both classical mutant studies and microarray experiments (Kachroo *et al.*, 2003); (Creelman and Mullet, 1997; Seo *et al.*, 1997). Interestingly, we isolated clone pBS4575, a putative omega 3-fatty acid desaturase, from a sample in which resistance was breaking down—i.e. at high temperatures the apparent extreme resistance of BT_{II} gives way to systemic necrosis. The tomato homolog of the sequence identified here (Table 4.1) was originally identified as a mutant with interrupted jasmonic acid signaling (Li *et al.*, 2003; Li *et al.*, 2002). The discovery of this candidate gene in our study suggests the testable hypothesis that *I* locus-mediated resistance may be based on SA signaling that is suppressed by JA at high temperatures, leading to break down of resistance.

Our study has additionally revealed a putative defense-related candidate pBS4771, which shows similarity to an *Avr9*-responsive protein as well as a rice β 1,3-glycosyltransferase-like protein. These two BLAST hits are of interest due to the fact that *Avr9* contains potential glycosylation sites and is found in both glycosylated and nonglycosylated forms in tomato and tobacco (Kooman-Gersmann *et al.*, 1996; Kooman-Gersmann *et al.*, 1998). This type of modification is generally associated with protein targeting to a membrane and, in the case of tomato, the ability of Avr9 to induce

necrosis is associated with its host plasma membrane binding site (Kooman-Gersmann *et al.*, 1996; Kooman-Gersmann *et al.*, 1998). In potyviral pathosystems, glycosylation has been shown to occur in the coat proteins (CP) of Plum pox virus (PPV) and Potato virus A (PVA), leading to an altered ability of the CP to bind nucleic acid (Fernandez-Fernandez *et al.*, 2002; Ivanov *et al.*, 2001; Ivanov *et al.*, 2003). Considering the broad-ranging effects of glycosylation both on symptom expression (necrosis) and on viral replication and/or movement (CP binding of nucleic acid binding), further analysis of this candidate gene may uncover important details about the high temperature necrotic response associated with the *I* allele.

By reviewing the molecular plant pathology literature, we can guess at some of these genes' functions; however, this is only useful in hypothesis generation. In this regard, the clones generated in these experiments are candidates for further hypothesis-driven assays into the molecular interactions of the *I* locus-BCMV pathosystem and, perhaps, virus resistance, in general. Additional progress may be made by the placement of these candidates on linkage maps for future comparative studies and, further, by their use as probes for *in situ* localization in parallel with BCMV immunodetection in infected tissues.

CHAPTER 5

Summary, Conclusions and Future Directions

In the preceding chapters, experiments designed to elucidate some of the mechanisms involved in *P. vulgaris I* locus-mediated resistance against *Bean common mosaic potyvirus* NY15 68/95 (BCMV) were described. Chapter two is published in Archives of Virology and provides data to suggest strongly that resistance is active at the single cell level. In these studies, protoplasts of NILs of *P. vulgaris* cv. 'Black Turtle Soup,' BT_{II}, BT_{Ii} and BT_{ii}, were transfected with BCMV RNA. Total RNA was isolated from these protoplasts over time and the amount of viral RNA amplified and quantitated in relation to host 18S rRNA using semiquantitative RT-PCR. By performing a regression analysis on the resultant data, the slopes of the lines for each genotype were shown to be different from each other, with RNA accumulation occurring fastest in the susceptible isoline (BT_{ii}). RNA did accumulate in both BT_{II} and BT_{Ii}, indicating that BCMV may very well be replicating in the presence of the *I* allele. Further, the regression analysis suggested that each copy of the *I* allele contributed to the reduction of RNA accumulation.

Chapter three attempts to define the infection route taken by BCMV and host cellular responses during the susceptible, intermediate and resistant responses. Studies at both 26ºC and 34ºC were conducted in order to compare both the restrictive and permissive *I* locus states. Distinct differences between homozygous resistant and susceptible isolines were identified: only single cells or small clusters of cells were infected in BT_{II} at low temperatures, but viral accumulation was high at 26ºC. At high temperatures, more cells were infected but strictly along a path towards the vascular

tissue. In contrast, infection was widespread and accumulation per cell was lower in BT_{ii} at both temperatures, and the BT_{Ii} response was intermediate at both temperatures.

Chapter four describes a differential expression analysis between BT_{II} and BT_{ii} when challenged and mock-challenged with BCMV at both 26ºC and 34ºC. The number of candidate genes for the resistance response was small in this study due to the robustness of the experimental design, and several genes potentially along a single signaling pathway were identified and confirmed.

The data presented here are all in keeping with the hypothesis that the differences between BT_{II}, BT_{Ii}, and BT_{ii} lie in BCMV's ability to replicate in each, but that the root of this difference may be in the signaling capabilities of the respective genotypes. Chapter four identified several genes in the G-protein signaling pathway that are upregulated in infected BT_{II} at high temperature. This condition is also the one in which BCMV was observed moving between cells in a direct line to the vasculature (Chapter 3) and in which run-away vascular necrosis is characteristic. It was hypothesized in Chapter 3 that a host factor associated with the plasmodesmata or in the vicinity could interact at some level with the virus and subsequently transmit a signal to initiate an HR or not. Perhaps a component of a G-protein signaling pathway fits this niche.

A current hindrance to studying BCMV biology is the lack of an infectious clone. It was decided at the outset of this project not to pursue cloning the virus due to the complexity of the process and the news that another group had nearly accomplished the feat. By the completion of these experiments, an infectious clone still has not been produced and it is recommended that anyone continuing this project do so as a high priority. Such a tool would allow for the production of unlimited inoculum for both plants and protoplasts but also allow for the directed manipulation of the viral genome. A GFP-expressing clone would be extremely useful in following up on the confocal

microscopic studies presented here. The ability to make chimeric genomes with non-infectious or temperature-independent potyviruses would allow for the functional dissection of critical genes as well as assist in identifying a viral effector molecule.

Also in progress yet still incomplete during the course of these studies is the cloning of the *I* gene. This work is being undertaken by Vallejos and colleagues and a 320 kb BAC containing the *I* locus has been identified. Once this has been achieved, one of the next logical steps is to test for protein-protein interactions between this gene and others in the *Phaseolus* and BCMV genomes. While we have near isolines in hand, it may also be of interest to then knock out the *I* gene in order to identify the effect of tightly linked genes not apparent using the isolines. Should the knockout phenotype be identical to the BT_{ii} phenotype this would be proof that this locus is, in fact, a single gene that imparts broad-spectrum virus resistance. Alternatively, if the knockout phenotype is novel, further analysis of the *i* allele as well as ORFs on the same BAC should be carried out in order to elucidate the more complicated genetic interactions. Cloning and sequence analysis of the *I* locus will also allow for the construction of I promoter-reporter gene fusions. As with potential BCMV-GFP fusions, such constructs will enable the direct assay of the *I*-encoded protein for its expression timing and location.

As noted in the Appendix, several interesting observations were made concerning BCMV-inoculated BT_{II} at 20ºC. It appears, from this preliminary data, that BCMV is able to persist, move and cause mild symptoms on BT_{II} when inoculated at 26ºC and held for extended periods of time at 20ºC. The ability of the virus to replicate and move at this temperature suggests that the *I* locus-BCMV interaction is most active (i.e. the resistance is most effective) at the environmental optimum of approximately 26ºC. There are many examples in the literature of resistances that are overcome at high temperatures (Moury *et al.*, 1998; Ohashi and Shimomura, 1971; Pilowsky *et al.*, 1981) but few to none that discuss altered resistance phenotypes at lower temperatures.

The *I* locus-BCMV pathosystem is an established system in which the resistance response can be fine-tuned by adjusting either environment or allele number or both (Collmer *et al.*, 2000). The observations made here suggest that this interaction is sensitive to a wider environmental range than previously thought. The study of a pathosystem that is adjustable in this way could open the door to understanding plant-virus interactions in that small shifts in experimental conditions could be the difference, for example, between binding and not binding or movement and no movement. Were *P. vulgaris* a model system or BCMV a cloned virus, studies at this level might come to pass. In the absence of model system status, it is more conceivable that the type of environmentally-dependent resistance optimum detected here could guide research questions in more easily manipulated systems, such as *Arabidopsis*-TEV or *N. tobaccum*-TMV.

A final question to broach in this research area is: Can we breed resistance similar to *I* but without a necrotic phenotype? Through the studies presented here it is apparent that BCMV does replicate in resistant genotypes and, further, is able to move. At some point, a signal is created that triggers the necrotic response. What is this signal? Where does the signal have its effect? These are the key questions in developing non-necrotic resistance. Part of the beauty of the *I* locus is its broad-spectrum nature—it confers resistance to nine potyviruses whose phenotypic responses fall into temperature-independent necrosis, temperature-dependent necrosis, and non-necrotic classes. What are the differences between the viruses in these phenotypic classes? Having the answer to this question may very well point to the residues or structure of the *I* gene product that invokes the necrotic trigger. And knowing this may allow us to modify the structure of this gene such that all potyviruses interact with it in a non-necrotic fashion.

APPENDIX

A.1 Temperature shifts reveal environmental optima for expression of resistance against *Bean common mosaic virus* in *I* locus near isolines of *Phaseolus vulgaris*

During the course of the preceding experiments, *P. vulgaris* Black Turtle Soup isolines were routinely grown and inoculated in the growth chamber. Following an experiment at 26ºC during which BT_{II}, BT_{Ii} and BT_{ii} plants had been inoculated with BCMV NY15 68/95 the temperature of the chamber was lowered to 20ºC for a different experiment. Nine days following this temperature shift, BT_{II} developed an apparent mosaic in uninoculated, systemic leaves. This phenomenon was investigated further by inoculating five plants each of BT_{II} and BT_{ii} at 26ºC and transferring them to 20ºC at different intervals. The result of this experiment was the appearance of a mild mosaic on BT_{II} and yellowing often followed by death in BT_{ii} by around 20 days post inoculation (Figure A.1 and 9). Tissue prints were made of inoculated leaves when transferred to 20ºC after zero, two and four days post inoculation and were subsequently probed with anti-BCMV virion antibody. Figure A.3 shows the results of these tissue prints. This experiment adds to the results from Chapters 2 and 3 of this volume that BCMV does persist in the presence of the *I* allele and may even move somewhat. Further, it appears that at 20ºC, is able to accumulate to much higher levels than at 26ºC and is able to move out of the inoculated
leaf (as is evidenced by signal in the leaf petiole). These experiments were not repeated or pursued any further than this point.

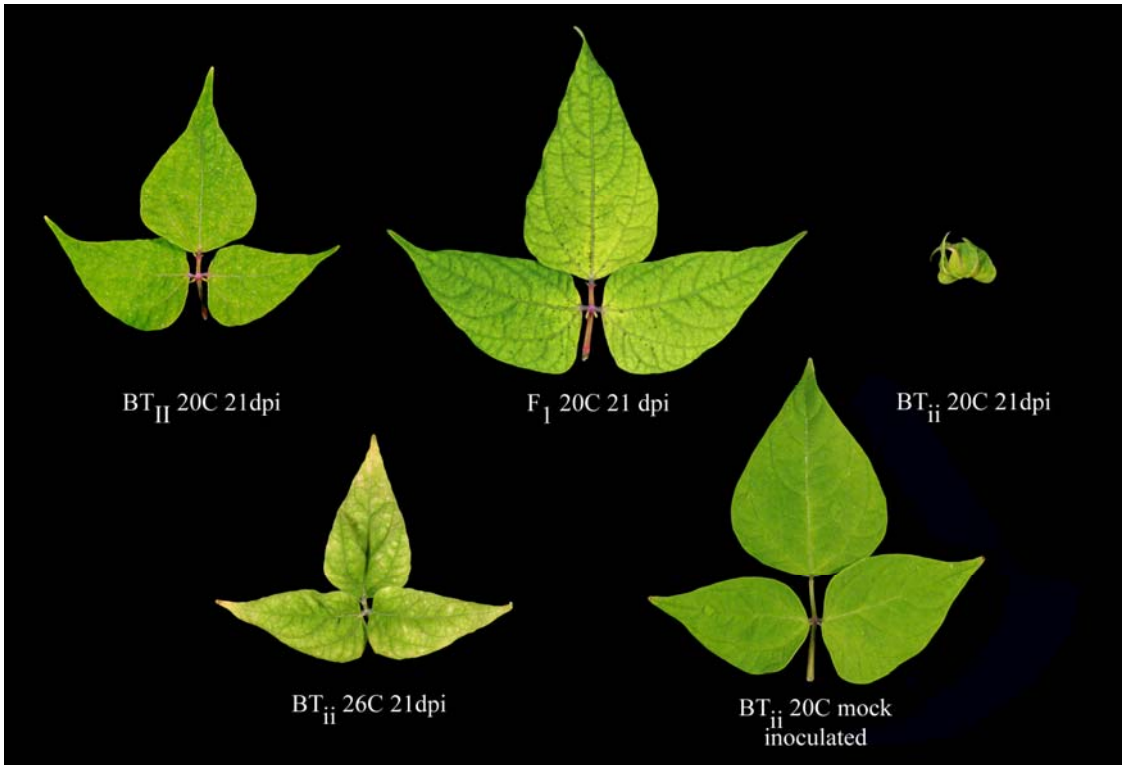

Figure A.1. Images of 'Black Turtle' near-isolines 21 days post inoculation. Experimental samples were inoculated at 26ºC and immediately transferred to 20ºC for the duration of the experiment.

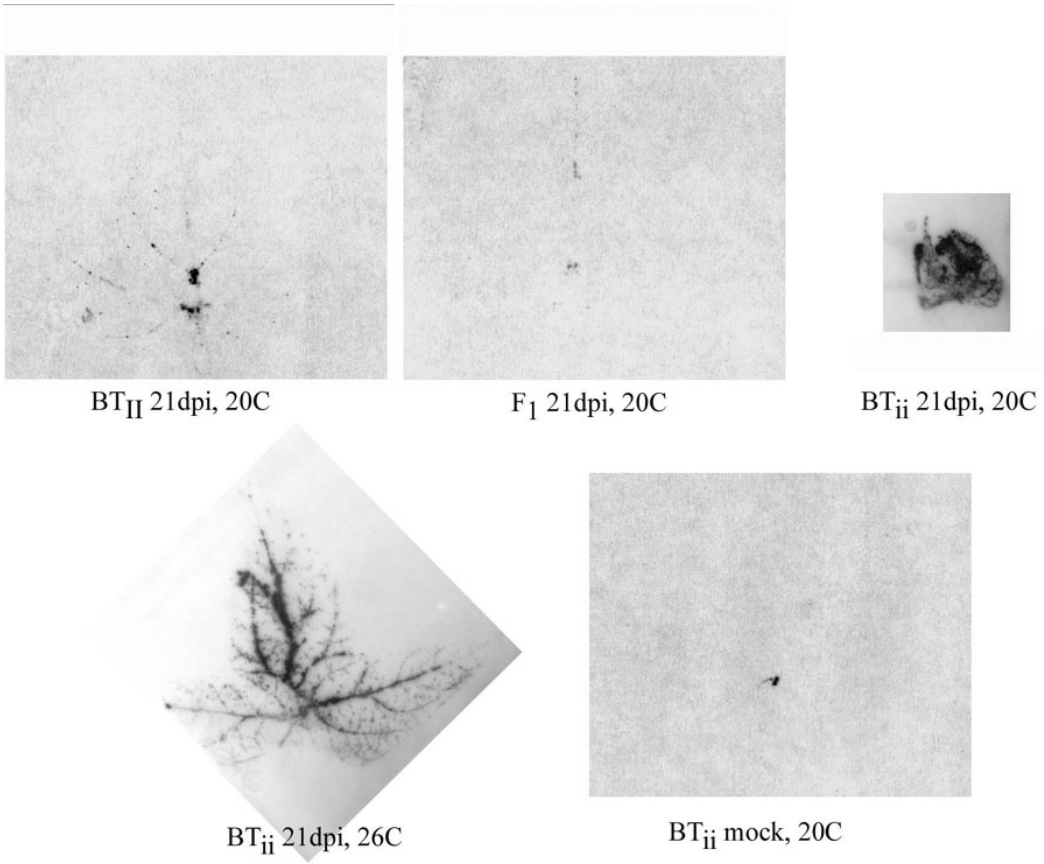

Figure A.2. Tissue prints of samples from Figure A.1 after immunodetection using anti-BCMV antiserum.

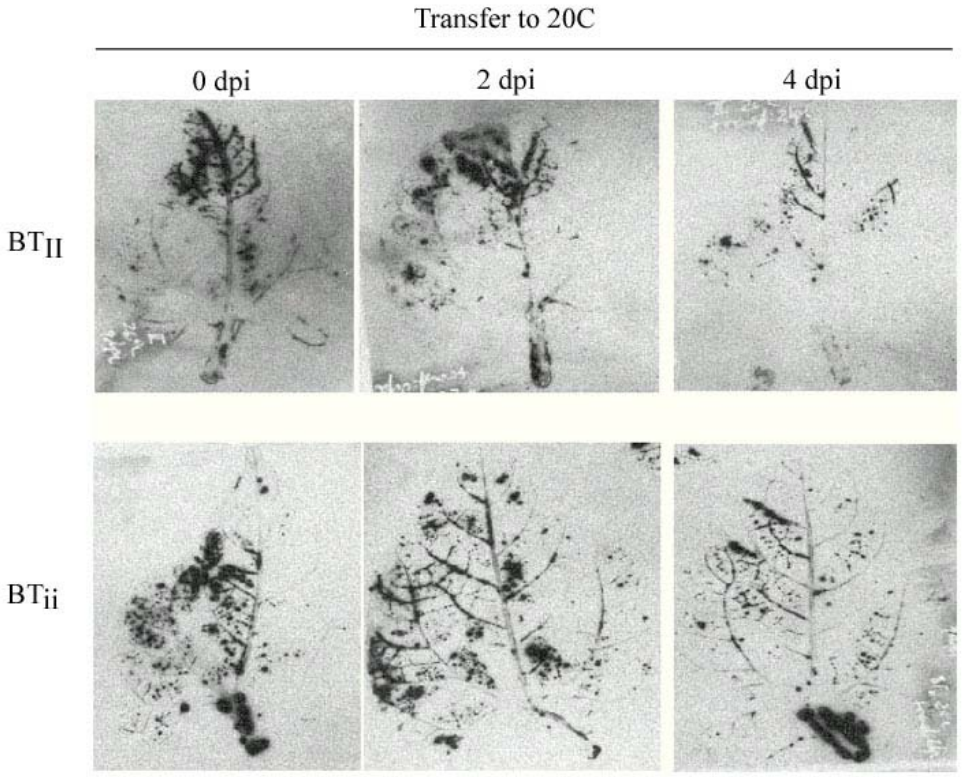

Figure A.3. Tissue prints of BT_{II} and BT_{ii} leaves following inoculation at 26ºC with BCMV and subsequent transfer to 20ºC. All samples were harvested and printed at 4 dpi.

A.2 BCMV NY15 68/95 is able to persist in the presence of the *I* allele at 26ºC without symptom induction for at least 30 days post inoculation.

Several experiments were carried out in which BT_{II} was inoculated at 26ºC and transferred at various times to 34ºC. Previous work using this BCMV isolate indicated that the virus can persist in *I*/- genotypes for up to four days post inoculation (Fisher, 1995). In those studies, inoculated plants were shifted between 25ºC and 34ºC and necrosis development observed. It was determined that if an inoculated *I*/- plant is maintained at 25ºC it can be shifted to 34ºC up to four dpi and necrotic symptoms will still develop. Conversely, an inoculated *I*/- plant must be maintained at 34ºC for at least three dpi prior to shifting down to 25ºC in order for the necrotic response to be induced (Fisher, 1995). In the experiments described in the present volume, inoculated *I*/- plants were occasionally maintained for extended periods at 26ºC. It was observed that after nine days at 26ºC, BT_{II} plants could be shifted to 34ºC and necrotic lesions would develop on systemic leaves. Shifting these same plants back to 26ºC halted symptom spread, however subsequently increasing the temperature could, again, induce necrosis in systemic tissue. It was observed that this temperature-dependent induction and release of necrosis development could be manipulated until at least 30 dpi.

A.3 *Bean common mosaic virus NY15 68/95* coat protein is required for full symptom development on inoculated leaves of BT_{ii}.

Inoculation experiments were carried out using sap from highly symptomatic leaves in 0.05M phosphate buffer (pH 8.5), purified BCMV virions and RNA extracted from purified BCMV virions. It was observed that chlorotic and occasional necrotic lesions developed within four days post inoculation on BT_{ii} following inoculation with either infected plant sap or purified virions at 26°C. Inoculations using purified BCMV RNA produced noticeably fewer chlorotic and necrotic lesions within the same timeframe. Systemic mosaic symptoms appeared at similar rates and intensities regardless of the inoculum used.

A.4 Genes conferring resistance against plant viruses.

At the time of this writing there are over 200 virus resistance genes known in plants (Kang *et al.*, In Press). The studies described in the present volume address the mechanism concerning one of these, *I*. Table A.1 lists many other resistance genes that interact with viruses as well as their protein structure (presumed or actual), mechanism of resistance, genetic action and notable symptoms. Those listed are either unique in some way, are representative of a general class of resistance or are directly related to topics discussed in the papers presented here. A comprehensive list may be found in Kang and collegues (Kang *et al.*, 2005 In Press; http://arjournals.annualreviews.org/doi/abs/10.1146/annurev.phyto.43.011205.141140) in which all virus resistance loci published as of December 2004 are compiled. Their supplementary table is organized based on host species and includes summarized data concerning dominance, mechanism as well as known alleles and alternate (historical) nomenclature) for these resistances.

Table A.1 Plant virus resistance genes.

Cloned virus resistance genes

Gene	Host	Gene action[a]	Predicted protein structure	Virus(es) influenced	Viral Avr genes	Resistance mechanism	Temp?[b]	Symptoms	Citation
HRT	Arabidopsis	D	LZ-NB-ARC-LRR	TCV	CP	?	No	HR	(Cooley *et al.*, 2000;Oh *et al.*, 1995)
RTM1	Arabidopsis	D	Jacalin-like repeats	TEV	?	Restricts systemic movement	No	ER/no PR-1a	(Chisholm *et al.*, 2000)
RTM2	Arabidopsis	D	Small heat shock-like	TEV	?	Restricts systemic movement	No	ER/no PR-1a	(Whitham *et al.*, 2000)
TM-2^2	Tomato	I	CC-NBS-ARC-LRR	ToMV	?	?	Yes	ER	(Hall, 1980;Lanfermeijer *et al.*, 2003;Pilowsky *et al.*, 1981)
Sw-5	Tomato	D	CC-NB-ARC-LRR	TSWV	M RNA	?	No	HR	(Hoffman *et al.*, 2001;Spassova *et al.*, 2001)
N	Tobacco	D	TIR-NB-ARC-LRR	TMV	RdRp	Movement	Yes	HR	(Otsuki *et al.*, 1972;Padgett *et al.*, 1997)

Table A.1 (Continued).

Gene	Host	Gene action[a]	Predicted protein structure	Virus(es) influenced	Viral Avr genes	Resistance mechanism	Temp?[b]	Symptoms	Citation
Rx1	Potato	D	LZ-NB-ARC-LRR	PVX	CP	Eliminates replication	No	ER	(Adams *et al.*, 1986;Kohm *et al.*, 1993)
Rx2	Potato	D	LZ-NB-ARC-LRR	PVX	CP		No	ER	(Bendahmane *et al.*, 2000)
Ry	Potato	D	CC-NBS-ARC-LRR	PVY, TEV	NIa protease	Restricts local movement	No	HR	(Barker, 1997;Cockerham, 1970;Hinrichs *et al.*, 1995;Hinrichs-Berger *et al.*, 1999;Mestre *et al.*, 2000)
Uncloned virus resistance genes									
I	*Phaseolus vulgaris*	D?	LZ-NB-ARC-LRR?	SMV, BCNMV, ThPV	?	?	No	SN	(Astua-Monge *et al.*, 2000;Fisher and Kyle, 1994)
		I	LZ-NB-ARC-LRR?	BCMV	?	Reduces replication	Yes	ER/SN	(Cadle-Davidson and Jahn, In Press;Collmer *et al.*, 2000;Fisher and Kyle, 1994)

Table A.1 (Continued).

Gene	Host	Gene action[a]	Predicted protein structure	Virus(es) influenced	Viral Avr genes	Resistance mechanism	Temp?[b]	Symptoms	Citation
		D	LZ-NB-ARC-LRR?	WMV, CAbMV	?	?	Yes	ER/SN	(Fisher and Kyle, 1994)
		D	LZ-NB-ARC-LRR?	ZYMV, PWV	?	?	No	ER	(Fisher and Kyle, 1994)
ICGR 95-5383	*Glycine max*	I	?	SMV	?	?	?	ER	(Zheng *et al.*, 2003)
Ry_{sto}	Potato	D?	?	PVY, TEV	?	?	?	ER/SN	(Hinrichs *et al.*, 1998;Hinrichs-Berger *et al.*, 1999)
Ry_{adg}	Potato	D	?	PVY/PVA		Restricts systemic movement	?	ER/HR	(Hamalainen *et al.*, 2000)
ra	Potato	R	?	PVA	VPg/HC-Pro/CP	Restricts systemic movement	?	HR	(Hamalainen *et al.*, 2000)

Table A.1 (Continued).

Gene	Host	Gene action[a]	Predicted protein structure	Virus(es) influenced	Viral Avr genes	Resistance mechanism	Temp?[b]	Symptoms	Citation
Rsv1	*Glycine max*	D	?	SMV-G2/N	?	post virion disassembly	?	ER	(Hajimorad and Hill, 2001)
sbm1	*Pisum sativum*	R		PSbMV	VPg	Elimination of replication	no	ER	(Keller *et al.*, 1998)
pvr1	*Capsicum chinense*	R	?	TEV, PepMoV		Reduction in replication	no	ER	(Murphy *et al.*, 1998)
pvr2	*Capsicum annuum*	R	4E (eIF4e)	PVY	VPg	?			(Ruffel *et al.*, 2002)
desc	*Phaseolus vulgaris*	R	?	ClYVV	VPg	(Elimination of replication)	no	ER	(Sato *et al.*, 2003)
Tsw	*Capsicum annuum*	I	?	TSWV	S RNA	?	yes	HR	(Jahn *et al.*, 2000;Moury *et al.*, 1998)

[a]D=dominant; I=incompletely dominant; R=recessive

[b]Temperature dependence?

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