REGULATION OF DEFENSE RESPONSES MEDIATED BY BON1 AND BAP2 IN ARABIDOPSIS THALIANA

A Thesis

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Master of Science

by

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ABSTRACT

In modern agriculture, a great deal of time and resources are spent combating widespread insect infestation and microbial infections. Comprehension of the natural defenses against these threats could enable the production of genetically modified plants with enhanced innate immunity without the use of chemicals to which insects and microbes are renown for developing resistances to. Plants have three main defense mechanisms to guard against bacteria: physical tissue barriers, basal defenses, and race-specific resistance. In the latter case, the plant cell perceives foreign proteins and this triggers the hypersensitive response (HR), a programmed cell death (PCD) to prevent further spread of the pathogen. This is mediated by the recognition of specific avirulence (*Avr*) proteins from the pathogen by resistance (*R*) proteins in the plant. A member of the evolutionarily conserved copine family, *BONZAI 1* (*BON 1*) is a key regulator of defense responses in *A. thaliana*. *BON1* has been shown to negatively regulate the *R* gene *suppressor of npr1-1, constitutive 1* (*SNC1*). In the Columbia (Col) accession of *A. thaliana*, the loss-of-function (LOF) *bon1-1* mutant allele exhibits a growth defect phenotype (dwarfed plant). *bon1-1* leads to constitutive activation of *SNC1* which compromises normal plant growth. *BON1* has been shown to interact with two other proteins: *BON1-associated protein 1 and 2* (*BAP1, BAP2*). The molecular link(s) between *BON1* and *BAP*, and their negative regulation of plant defenses are not well understood. We report the identification and characterization of a new component in the BON1 regulatory pathway and our efforts in probing the biochemical activities of BAP2 in regulating defense responses.

BIOGRAPHICAL SKETCH

Stephanie Topp earned her BA from the University of Pennsylvania in May of 2004 and began her graduate work at Cornell University in August of 2004. After completion of first-year courses, she joined the Hua lab in June of 2005 to begin research on the molecular mechanisms of *BON1*-related cell death and defense regulation in *A. thaliana*. During her second year she worked for Dr. Helen Nivison as a teaching assistant for BioBM-440, a biochemistry lab for majors. In August 2006, she participated in a teaching assistant workshop sponsored by the Cornell Teaching and Learning Center. During her third and fourth years at Cornell, Stephanie worked as teaching assistant for Bio109-110 and for her dedication to the students of the course, she received an “Outstanding Teaching Assistant” award. Stephanie plans to pursue a career in college level education after completion of an MS degree.

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LIST OF ABBREVIATIONS

3HA	- 3 tags of human hemagglutinin
Avr	- avirulence
BAP1, 2	- *BON1-associated protein 1, 2*
BON	- BONZAI
CC	- coiled-coil domain
Col-0	- *A. thaliana* accession Columbia-0
CP	- PVX coat protein
DAB	- diaminobenzidine
dCAPS	- derived cleaved amplified polymorphic sites
EBO12	- *enhancer of bon1-2 12*
EDS1	- *enhanced disease susceptibility 1*
flg22	- flagellar protein 22
GFP	- green fluorescent protein
HopAB2	- Hrp-dependent outer protein AB2
HR	- hypersensitive response
HSF3A	- heat-shock factor
IP	- immunoprecipitation
LRR	- leucine-rich repeats
LOF	- loss-of-function
MAPK	- mitogen-activated protein kinase
NBS	- nucleotide-binding site
PAD4	- *phytoalexin deficient 4*
PAMPs	- pathogen-associated molecular patterns
PCD	- programmed cell death
PCR	- polymerase chain reaction
PR1	- *pathogenesis related 1*
Pst. DC3000	- *Pseudomonas syringae* pathovar *tomato DC3000*
Pto	- *P. syringae pv tomato*
RACE-PCR	- rapid amplification of cDNA ends
RIN4	- RPM1-interacting protein 4
ROS	- reactive oxygen species
RPM1	- Resistance to *Pseudomonas syringae pv maculicola 1*
R-proteins/ genes	- resistance proteins/ genes
RPS2	- Resistance to P. syringae 2
RSR-1	- Resistant to Ralstonia Solanacaerum 1
Rx	- potato R protein
SA	- salicylic acid
Ser	- amino acid serine
SFP	- single-feature polymorphism
SNC1	- *suppressor of npr1-1, constitutive1*
SNP	- single nucleotide polymorphism

SRK	- S-receptor kinases
SSLPs	- simple sequence length polymorphisms
TAIL-PCR	- thermoasymmetrical interlaced polymerase chain reaction
Thr	- amino acid threonine
TIR	- *toll-interleukin*-like receptor-like domain
Ws-2	- *A. thaliana* accession Wassilewskija-2
Y2H	- yeast 2 hybrid assay

CHAPTER 1:

General Introduction

As static organisms, competitive adaptability of plants to biotic and abiotic factors is essential to their continued survival. One adaptable feature of particular interest, bearing applications to possibly improving agriculture, is plant innate immunity to microorganisms. Comprehension of this system is valuable to improving agricultural output in a world experiencing a dramatic increase of insects and microbes resistant to chemical control.

Many species of pathogens attempt to colonize the leaf apoplast and circumvent host defenses by secreting proteins, referred to as "effectors", which are highly evolved to facilitate this process, thus conveying virulence **(2)**. In a "compatible" pathogen-plant interaction, the pathogen is able to infect the plant and circumvent defenses without eliciting host defense responses **(36)**. However, in the "incompatible" scenario, the effectors or pathogen-associated molecular patterns (PAMPs) are recognized **(13)**. PAMPs include compounds such as flagellar proteins (flg22), bacterial translation factor (EF-Tu), chitin, and cell wall lipopolysaccharides that are indicative of bacteria and fungi **(16, 21, 30, 48, 47)**. When PAMPs have been detected, some effectors such as HopAB2 and AvrPto can counteract by deactivating basal defenses by targeting specific host proteins in the signaling pathway **(16)**. Plant defenses have coevolved to recognize such virulence factors, henceforth the recognized effectors are termed "avirulence" (*Avr*) proteins. Basal defenses are activated upon recognition of PAMPs, however, when plant resistance (*R*) proteins detect the presence of *Avr* proteins they initiate defense responses in the form of a hypersensitive response (HR) **(13)**. HR enables the plant to survive by

rapidly stemming the spread of the infection. The HR is characterized by a number of events including: transcriptional reprogramming, rapid ion fluxes, extracellular oxidative burst, and programmed cell death (PCD) both within the site of infection and the surrounding tissues **(9, 13, 22, 23, 29, 31, 34)**. Activation of PCD often results in the appearance of necrotic lesions. In the absence of a pathogen, PCD is an essential component in the growth of multicellular organisms, defining their size, shape, and general morphology **(23, 43)**. The *R* gene to *Avr* gene interaction is usually allele-specific thus a "gene-for-gene" model was proposed. Thus, in order for a plant to detect a given *Avr* protein, it must have the corresponding *R* gene to code for the *R* protein that will specifically bind to a given *Avr*. Very often, this recognition leads to the activation of mitogen-activated protein kinase (MAPK) cascades **(31)**, which in turn can signal the activation of defense-related genes. The *R* protein Pto, which conveys "race-specific resistance to *P. syringae*" **(43)**, was the first cloned plant example of gene-for-gene resistance **(27, 33)**. Another well-studied example is RSR-1, which has been proposed to directly recognize

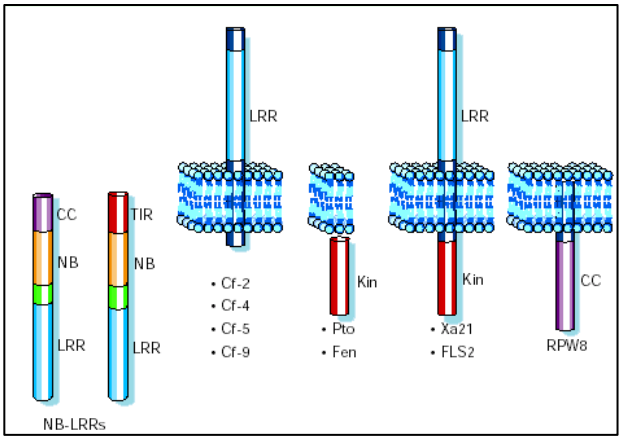

Figure 1.1. *R* protein structures and functional domains.

the *R. solanacearum* effector protein popP2 and then activate plant defense-

related genes **(10)**.

However, it unlikely that all *Avr/R* gene interactions function in this one-on-one manner; there are thousands of different effector proteins in many different species of pathogens whereas there are only a few hundred known *R* genes in plants. In addition, pathogens have generation time far shorter than that of their hosts, therefore can evolve more quickly. To address this anomaly, it has been suggested in the "Guard Hypothesis" that some *R* proteins may monitor or "guard" certain endogenous plant proteins whose function may be modulated by *Avr* proteins **(4, 8, 9, 19, 20, 28, 40).** The *R* proteins may be sensitive to chemical changes, such as phosphorylation, acetylation, or proteolytic cleavage of their guarded proteins and then signal defense pathways without direct detection of the invading *Avr* protein **(8, 20, 28)**. It is logical that *R* proteins could be involved in multiprotein recognition complexes capable of recognizing a wide variety of avirulence factors. The RIN4 host protein provides strong evidence in support of the second model. Two proteins have been found to guard RIN4: RPS2 and RPM1 **(3, 4, 26)**. When RIN4 has been either phosphorylated or cleaved by *Avr* proteins, RPS2 and RPM1 perceive such chemical changes and activate defense responses. Functional RIN4 negatively regulates these two proteins, thus preventing inappropriate activation of defense responses in the absence of a pathogen. This regulation is critical to plant survival as the *rin4* loss-of-function (LOF) mutant is lethal **(4)**. This lethality has been attributed to the constitutive activation of *RPS2*, because the *rin4rps2* double mutant rescues the lethality of *rin4*.*R* proteins have important structural features enabling them to localize within the cell and carry out their biochemical functions. There are five major classes of *R* proteins, which have been grouped by their functional domains

(**9**). Some of these domains include a nucleotide-binding site (NBS) and leucine-rich repeats (LRR). For example, class 2 *R* proteins are collectively known as CC-NBS-LRR due to a C-terminal coiled-coil (CC) domain **(9)** and class 3 *R* proteins possess an N-terminal *toll-interleukin*-like receptor (TIR), (TIR-NBS-LRR) **(1)**. It is believed that some *R* proteins are localized to the plasma membrane, consequently in close proximity to many types of *Avr* proteins, thus facilitating detection in the gene-for-gene hypothesis (**44**). In the guard hypothesis, such localization would permit close monitoring of the guarded host proteins. However, the biochemical means of transmitting a signal indicative of *Avr* protein recognition still remains a mystery for most *R* proteins. Some *R* proteins, such as RSR-1, also contain WRKY domains and are postulated to enter the nucleus upon activation and facilitate defense-

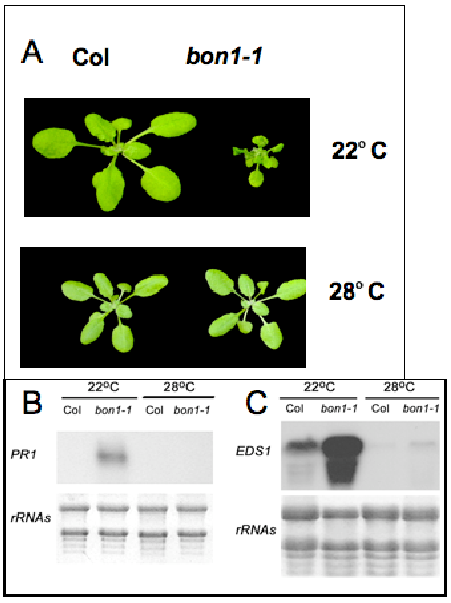

Figure1.2. *bon1-1* has a temperature-dependent growth defect (a). *PR1* (b) and *EDS1* (c) are constitutively activated in *bon1-1* plants grown at 22^{0}C. *Gen. Dev* (2001).

related gene activation **(10)**. Others, such as Pto have a Ser/Thr kinase domain and myristolation motif **(32)**. However, *R* proteins are only the start of the HR-signaling process. Downstream of many TIR-NBS-LRR *R* proteins are several proteins that are critical for the activation of defense responses, some of which are dependent on salicylic acid (SA). Among these are *enhanced disease susceptibility 1* (*EDS1*), and *phytoalexin deficient 4* (*PAD4*), so named for the phenotypes of their respective LOF alleles **(11)**. Both of these genes code for lipase-like proteins and are positive regulators of SA, contributing to SA accumulation, which is important for signaling HR and triggering systemic acquired resistance. Plant survival depends on tight regulation of these pathways as untimely activation or repression can compromise fitness and survival.

Copines are an evolutionarily conserved family of proteins and have been found in plants, worms, mice, and humans. This protein family is characterized by the presence of two C2 domains for calcium-dependent phospholipid binding, and an A domain. The latter has sequence homology to the von Willebrand domain in integrin, possibly conferring protein-protein interactions between this copine and other proteins **(45)**. One member of this family was identified during a screen for mutants with temperature-dependent growth defects in *Arabidopsis* **(18)**. The gene was cloned and named *BONZAI* 1 (*BON1*) for its temperature-dependent dwarfed phenotype (Figure 1.2) caused by the recessive LOF allele (*bon1-1*). This dwarfed phenotype is the result of significant growth defects in the plant during early development that continues even after entering the reproductive phase. (Figure 1.3). This protein has been shown to associate with the plasma membrane and it binds to phospholipids in a calcium-dependent manner **(18)**. There are two

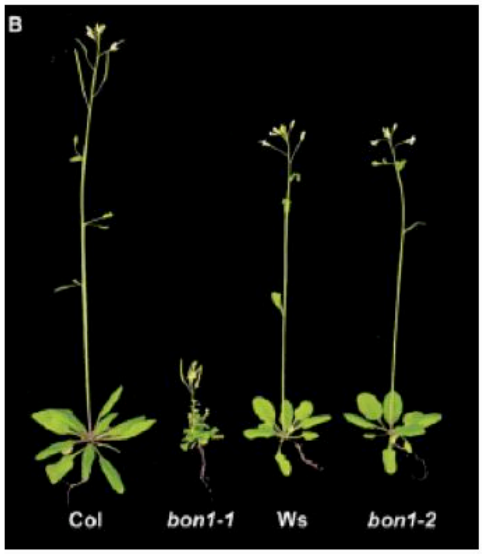

Figure 1.3 Comparison of Col & Ws to their respective LOF *bon1* alleles after bolting. *Gen. Dev* (2001).

additional *BON* genes in *A. thaliana*, however, *BON1* has a dominant role over these other copines. The *bon1-1* mutant exhibits microlesions but other *BON* family mutants do not. There is an apparent functional redundancy in the gene family as the *BON* family knockdown has a lethal phenotype (**45**).

It was later determined that these dwarfed *bon1-1* plants exhibit increased resistance to disease and constitutive expression (Figure 1.2) of several downstream resistance-indicative genes, such as *pathogenesis related 1* (*PR1*), are possible causes of the dwarf phenotype **(19, 45)**. It is possible that the compromised cell growth may be the result of reallocation of cellular resources, energetically costly defensive measures, a combination of both, or other unknown factors. Thus, it is likely that the biochemical processes of cell death and disease resistance may be intimately connected to those of growth homeostasis. The defense pathway is apparently sensitive to perturbation of the norm with regard to cellular metabolism, whether caused by

biotic or abiotic factors, and necrotic lesions may result **(18, 45)**.

As to how the *BON* genes mediate normal growth under standard growth conditions (*see Methods*) remains a mystery. The *bon1-1*-like phenotype has been observed in Col-0 but not in the Wassilewskija (specifically, Ws-2) accession (Figure 1.3). The LOF allele *bon1-2* in Ws-2 grows normally and has no discernable enhancement in disease resistance over wild-type Ws-2. However, about four years ago, our lab reported a natural modifier of *BON1* in the Col-0 accession **(44)**. This modifier was identified as the haplotype-specific *R* gene, *suppressor of npr1-1, constitutive1* (*SNC1*). This gene codes for a TIR-NBS-LRR *R* protein. In *bon1-1*, *SNC1* has a higher activity causing the observed phenotype and enhanced disease resistance of *bon1-1*. The LOF allele *snc1*-11 completely rescues the *bon1-1* (Figure 1.4) and suppresses activation of defense-related genes caused by *bon1-1*. SNC1 functions in a defense-activating pathway involving SA, which positively feeds back to *SNC1* expression. The *SNC1*-mediated resistance is also sensitive to ambient temperature. How this *R* gene is subject to the modulation of *BON1* and how temperature modulates disease resistance are unknown.

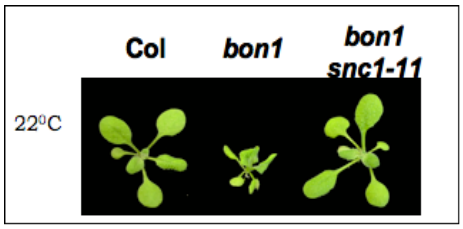

Figure 1.4 LOF *snc1-11* rescues *bon1-1*
Yang and Hua (2004) *The Plant Cell*

The *BON* genes do not function alone in the roles of regulating cell death and normal growth. Through in a Y2H assay, using *BON1* as bait, we have identified a strongly interacting protein: *BON1-associated protein 1* (*BAP1*).

We later identified a homolog of *BAP1* on chromosome 2, *At2g45760*, and named it *BAP2*. The BAP proteins contain a single C2 domain but they have no A domain **(42)** and we have discovered that these proteins have an apparent functional redundancy. The *bap1-1* LOF mutant has a weak phenotype similar to *bon1-1* (Figure1. 5) that can be rescued at 28^0C, whereas *bap2-4* is always wild-type (not shown). Interestingly, the double homozygous *bap1-1bap2-4* plants are seedling lethal and cannot be rescued by higher temperatures.

We have also shown that these two proteins also negatively regulate cell death **(45, 46)**. Double heterozygous plants exhibit increased cell death and ROS accumulation in comparison to Col-0, *bap1-1*, and *bap2-4* plants **(39)**. BON1, BAP1, and BAP2 have also been shown to suppress cell death in yeast stressed by H_2O_2 and delayed HR in *A. thaliana* and *N. benthamiana*

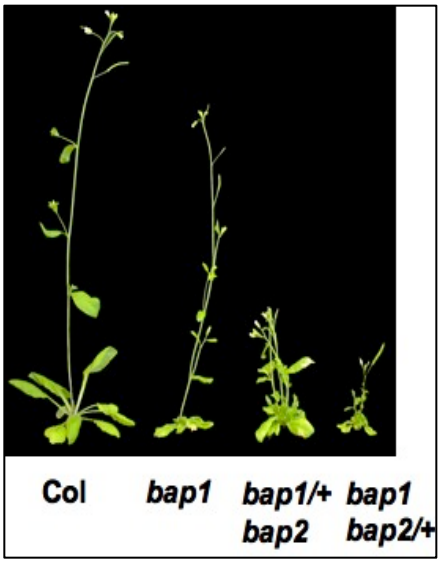

Figure 1.5. Comparison of Col-0, *bap1, bap1/+bap2* and *bap1bap2/+* after bolting. The phenotype of the double heterozygous plants is more severe than the single mutants. *bap2* (not shown) has a wild-type phenotype.

leaves. When *A. thaliana* plants were challenged with avriulent strains of *Pst DC3000* (*avrRpm1*, *avrRpt2*), suppression or strong delay of HR was seen in Col-0 plants that were co-infiltrated with *p35S::BAP1* and *p35S::BON1* as opposed to separate infiltrations. Similar results were observed in *N. benthamiana* plants transiently expressing the potato R protein (Rx) and its elicitor PVX coat protein (CP) **(46)** when co-infiltrated with *p35S::BAP1* and *p35S::BON1*. These results indicate these proteins are functional partners and that their overexpression can suppress cell death elicited by a number of biotic and abiotic factors.

As with *BON1*, we have discovered a connection to *R* genes as the *bap1-1* phenotype is mediated through *SNC1* and can be rescued with the LOF allele *snc1-11* **(24, 45, 46)**. *bap1-1bap2-4* plants (hereon referred to as *bap1bap2*), however, cannot be rescued by *snc1-11* but can be rescued by the LOF alleles of *EDS1* and *PAD4* (Figure. 1.6). This finding suggests that other proteins, or perhaps other *R* genes, are targets of the BAP proteins in addition to *SNC1.* It is unknown if the relationship between the *BON* and *BAP*

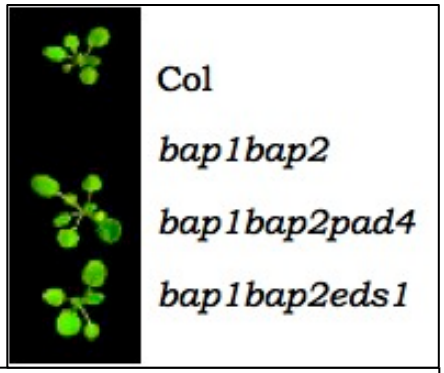

Figure 1.6. Double homozygous *bap*1bap2, is seedling lethal. Crossing double heterozygous plants to *pad4* or *eds1* completely rescues *bap1bap2*, suggesting that activation of *R* genes occurs in the *bap* mutants.

gene families to *R* gene regulation is direct or not. If the relationship is direct, it is possible that the *BAP* genes may function in a larger protein complex regulating the activity of SNC1 and other *R* proteins, or they may function in separate, yet related metabolic pathways. However, if the regulation of *R* genes is indirect, unknown proteins could function in monitoring the biochemical status of the BAP proteins, and this may prove to be another supporting example of the guard hypothesis **(8, 20, 28)**. Currently, the specific mechanism of regulation by *BON1* on plant defense responses is unknown. It is necessary to understand the specific role of this gene, biochemical activity of the protein, and what other genes function in this metabolic pathway to explain how and why the loss of *BON1* and *BAP* function effect plant morphology and resistance so dramatically.

CHAPTER 2:

Cloning and Characterization of EBO12

Introduction

We are hypothesizing that there exist other molecules functioning together with *BON1* and the *BAP*s in the negative regulation of plant cell death. One research focus has been to investigate the role of new components in this regulation through a *bon1* enhancer screen. We used the phenotypically wild-type *bon1-2* line (Figure. 2.1) in the Ws-2 background to perform a sensitive enhancer screen. We have seen that double mutants of the *BON* family cannot be rescued by *snc1-11*, but can be rescued by LOF alleles of *EDS1* and *PAD4* (**45**). We believe that the lack of *SNC1* in Ws-2 could lead to the discovery of other *R* genes or other proteins that are in the *BON1* pathway or in a parallel pathway regulating defense responses.

Figure 2.1 *ebo12bon1-2* exhibits a strong growth defect phenotype and necrotic lesions at 22 ^{0}C. By Dr. S. Yang.

Results

Isolation of a bon1-2 enhancer

To identify additional components in the *BON1* regulatory pathway, Dr. Shuhua Yang performed an enhancer screen by activation tagging in *bon1-2* plants (**38**). One such enhancer was identified and named *enhancer of bon1-2 12*, (*ebo12) (*Figure 2.1). To establish that the mutant was a true enhancer of *bon1*, it was crossed to Ws to determine if the *ebo12* phenotype was linked to *bon1-2*. This was found to be the case as the strong phenotype is only present in *bon1-2* plants. *ebo12bon1-2* plants exhibit a *bon1-like* phenotype when grown under standard conditions (*see Methods)* and complete rescue at 28^0C, indicative of temperature sensitivity. *ebo12bon1-2* plants have leaves that are often small and convex, and have random necrotic lesions that are indicative of PCD. Dr. S. Yang determined that *ebo12bon1-2* is dominant over *bon1-2* (Table 2.1). Roughly $^3/_{16}$ of the F2 population from an *ebo12bon1-2 x*

Table 2.1 Phenotypic and BASTA™ resistance data for *ebo12bon1-2* x *bon1-2*. No wild-type plants survived in F2, confirming that the *ebo12* gene alone was segregating and the mutation is dominant.

Generation	Wild-type	*ebo12*-like
F1	None	All
F2	14 (0 BASTA™+)	36 (36 BASTA™+)

Ws-2 cross (Table 2.2) had an *ebo12-like* phenotype indicating the

Table 2.2 Phenotypic and BASTA™ resistance data for *ebo12bon1-2* x Ws

Generation	Wild-type	*ebo12*-like
F1	All	All
F2	93 (70 BASTA™ +)	8 (8 BASTA™ +)

segregation of a dominant (*ebo12*) and a recessive gene (*bon1-2*).

The activation tagging vector, pSKI015, conveys resistance to the herbicide BASTA™. To assess if the phenotype was T-DNA linked, the F2 progeny from crosses to *bon1-2* and Ws were sprayed to select for plants carrying the transgene. All plants with *ebo12-like* phenotype were resistant to BASTA™, and some of the wild-type looking plants died (Tables 2.1 & 2.2). Wild-type looking plants resistant to BASTA™ were not *bon1-2* as determined

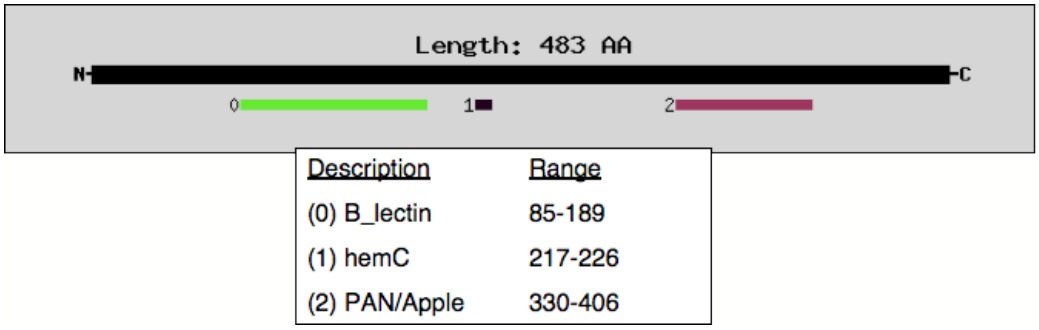

Figure 2.2 Functional domains of the protein for *At5g03700*. The protein contains 3 putative domains. The PAN/Apple domain is a component found in S-locus glycoproteins and S-receptor kinases. Figure courtesy of SubCellular Proteomic Database (SUBA)

by genotyping. In conclusion, the *ebo12* mutation is dominant, and the phenotype is *bon1-2* dependent and T-DNA-linked.

Overexpression of proximal genes

As the enhancer phenotype type is associated with the T-DNA insertion, Dr. Yang determined the insertion locus of the specific T-DNA linked to *ebo12* phenotype. This was accomplished with thermoasymmetrical interlaced polymerase chain reaction (TAIL-PCR) to identify the flanking regions of the T-DNA inserts, followed by genotyping of *ebo12* plants with primers for candidate loci to establish which T-DNA was phenotypically linked. To ascertain if the activation tagging was linked to *ebo12* by altering gene expression, genes in close proximity to the T-DNA insert were tested via Northern blotting for significant changes in expression level. One particular T-DNA insert was found to be linked to the *ebo12bon1-2* phenotype and is inserted 923 bp into the 3' end of *At5g03700*. This gene codes for a 428 amino acid protein with three putative functional domains (Figure 2.5). One of these domains is a PAN/Apple domain found in the S-locus glycoproteins and S-receptor kinases of plants. Thus, we have temporarily named this allele *srk1-1*. To determine whether or not the presence of this T-DNA influenced expression of nearby genes Dr. Yang performed a Northern blot on this mutant and compared it to Ws-2 and *bon1-2* plants. She determined that the 3' end of the gene (about 600bp) is overexpressed, the 5' end cannot be detected, and an aberrant transcript runs at approximately 5kb (Figure 2.3). By employing *rapid amplification of cDNA ends* (RACE)-PCR Dr. Hua identified the RNA transcripts from *ebo12* to be a chimera of the T-DNA insert and the 3' end of

the *SRK-like* gene, however, the full length of the larger transcripts has yet to be accounted for. The nearby gene, *At5g03690*, which codes for a putative fructose-bisphosphate aldolase, was also overexpressed in *ebo12* plants, in comparison to Ws and *bon1-2* controls (Figure 2.3). A downstream heat-shock factor (HSF3A), annotated *At5g03720,* did not show any change in expression (data not shown).

To determine if overexpression of the 3' end of the *SRK* gene could recapitulate the enhancer phenotype, Dr. Rena Shimizu engineered a *35S:srk1-1* chimera construct based on the RACE-PCR sequence data into the pGREEN vector. The construct was transformed into *bon1-2* plants. We phenotyped more than 30 BASTA® resistant lines and no *ebo12*-like phenotype was observed. Overexpression of *At5g03690* in *bon1-2* plants did not confer an *ebo12*-like phenotype either.

Enhanced cell death in ebo12bon1-2

We have seen that *bon1-1* causes increased cell death in plant tissues and accumulates ROS. We suspected an increase cell death in *ebo12* plants because the leaves consistently show necrotic lesions, possibly due to PCD. To determine if the *bon1-2* enhancer was functioning in a similar manner to *bon1-1*, Dr. Shimizu stained *ebo12bon1-2* to look for changes in cell death rate and increased H_2O_2 accumulation. Trypan Blue staining revealed an increase in the number of dead cells in *ebo12bon1-2* compared to Ws and *bon1-2* (Figure 2.4). This stain is impermeable to living cell membranes, thus it will permeate any damaged or dead cells, regardless of the cause. Diaminobenzidine (DAB) reacts with H_2O_2 and produces a reddish-brown

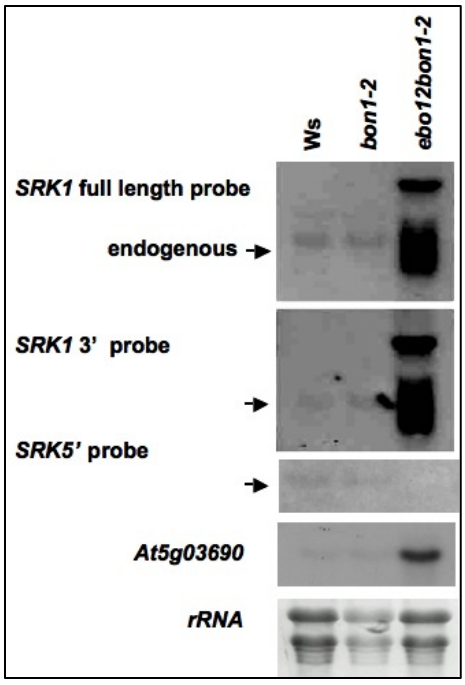

Figure 2.3 Northern blot of Ws, *bon1-2,* and *ebo12bon1-2*. By Dr. S. Yang

precipitate. This staining revealed strong accumulation of brown precipitates, indicating that these tissues were also accumulating H_2O_2, similar to *bon1-1* **(45)**. (Fig 2.5) These stains also accumulated strongly around what appeared to be necrotic lesions on the *ebo12bon1-2* leaves. Taken together, these results indicate that the necrotic lesions and increased cell death in *ebo12bon1-2* leaf tissues are likely due to H_2O_2 accumulation.

Map-based cloning

In order to identify the gene responsible for the enhancer phenotype in *ebo12bon1-2* we employed a number of approaches, including map-based cloning to narrow down the genetic region of interest and identify the *EBO12* gene. *ebo12-bon1-2* (Ws-2) plants were crossed to *bon1-1snc1-11* (Col-0)

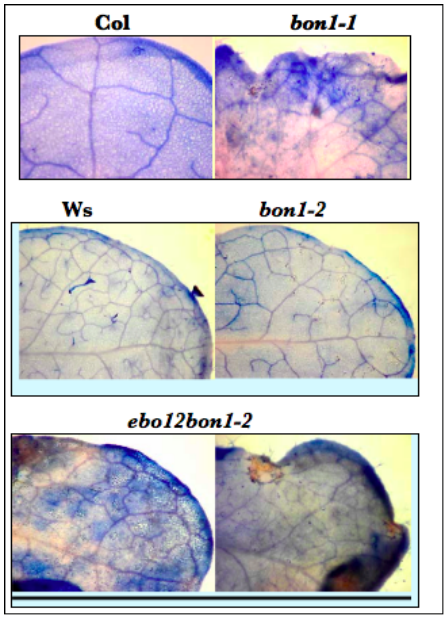

Figure 2.4 Trypan Blue staining of *ebo12bon1-2* tissues indicates increased number of dead cells in comparison to Ws and *bon-2.* By Dr. Shimizu.

and the F2 population was generated for mapping. A hundred plants were genotyped for the presence of the T-DNA (*srk1-1* allele) within the *SRK* gene and all mutant plants contained this T-DNA. Therefore, the gene must be close to the T-DNA, within 1cM (250,000bp) of the T-DNA insertion site. The same population was checked for recombination events using the markersCTR1 (in *At5g03720*) and nga249. These markers confirmed that the

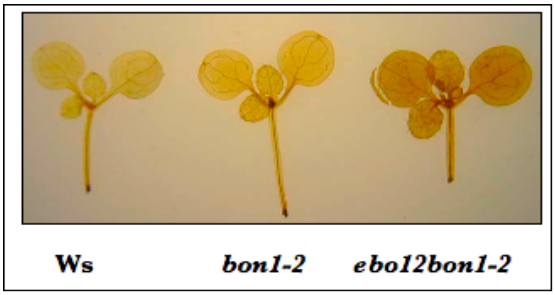

Figure 2.5 Comparison of DAB staining. Dr. Shimizu.

linkage to the *ebo12* phenotype was located upstream and within about 1cM of CTR1. According to current online databases, there are very few simple sequence length polymorphisms (SSLPs), derived cleaved amplified polymorphic sites (dCAPS) markers within this region of chromosome 5.

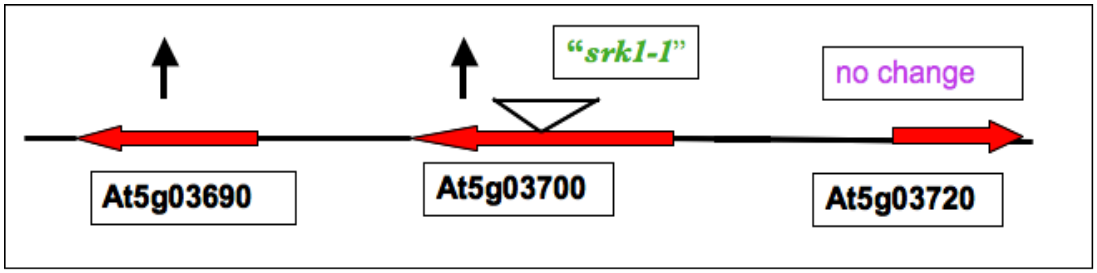

Figure 2.6 Site of *ebo12* T-DNA insert. Arrows indicate expression change in *ebo12* plants.

Thus, single nucleotide polymorphism (SNP) and the single-feature polymorphism (SFP) databases would be useful to search for polymorphisms if we wish continue fine-mapping.

Reverse complementation

We have hypothesized that the *ebo12* phenotype could be caused by the overexpression of genes close to the insertion locus of the T-DNA. To test this hypothesis, we chose to overexpress wild-type genomic fragments of genes *At5g03690* and chimera clone of *srk1-1*. The chimera was designed to contain part of the T-DNA and the 3' end of *At5g03700*, as seen from RACE-PCR data. The expression of these clones was driven with a *35S* promoter but they did not confer an enhancer phenotype in *bon1-2* plants. We also attempted long fragment PCR amplification of this locus, but were unsuccessful. Thus, we constructed a genomic library of *ebo12bon1-2* with a complexity of 10^5, which approximately covers the entire genome. The

purpose of this approach was to duplicate the correct mutant locus as closely as possible in order to best recapitulate the *ebo12-bon1-2* phenotype. It is

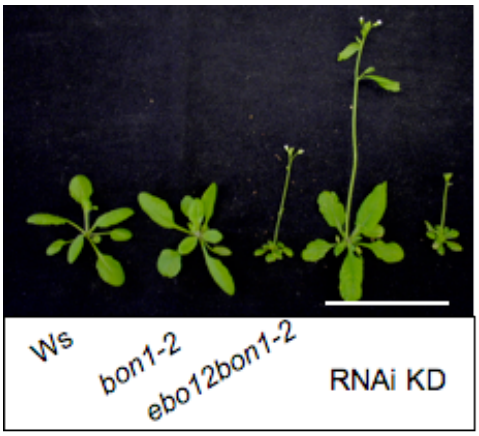

Figure 2.7 Ws, *bon1-2* and *ebo12bon1-2* plants (left) compared against plants carrying the *srk1* chimera RNAi construct (right) after bolting. L7 is second from the right.

possible that other mutations exist in addition to the T-DNA of which we are currently unaware. Clones that covered the *srk1-1* locus were isolated from the *ebo12* library by genotyping individual plaques. One positive clone was identified after screening the library and it covers approximately 20kb, which encompasses the two candidate genes. This fragment covers the entire *srk1-1* allele and up to 500bp downstream of the intergenic (putative promoter) region, but does not extend to *At5g03720*. This fragment was cloned into the pGREEN-0230 binary vector and then transformed into *bon1-2* plants. Transgenic plants in the T1 generation were selected for with a BASTA™ spray. Of the 46 surviving plants, 7 have exhibited a weak *ebo12*-like phenotype at the 4-6 leaf stage. Some of these plants have shown a convex leaf shape and sporadic lesions but not to the same extent as *ebo12bon1-2* plants (data not shown). It is important to ascertain if the phenotype is true and stable thus, we must phenotype the T2 generation and perform a Northern

Figure 2.8 Ws, *bon1-2* and *ebo12bon1-2* plants (top row) compared against plants carrying the *srk1* chimera RNAi construct (bottom). L7 on the bottom left, has some lesions but shows the most rescue.

blot to determine if the desired genes are expressing in a manner reminiscent of *ebo12bon1-2*.

RNAi knockdown of candidate genes

Since activation tagging was used to generate this enhancer line, we hypothesized that the overexpression of *EBO12* gene is the cause the *bon1-2* enhancer phenotype. To test the candidate genes, we used RNAi silencing to knockdown the expression of these genes to determine if suppression of specific transcripts could suppress the enhancer phenotype. We made RNAi constructs in the binary vector pFGC1008 to knockdown the transcripts of *srk1-1*, *At5g03690*, and an LTR-Gypsy retrotransposon sequence 100bp downstream of *At5g03700.* The choice of these candidates gene was based

on the Northern blot data. Although no signal was detected on the Northern blot for the retrotransposon we chose to make an RNAi construct for it as well. 25 lines were obtained for the *srk1-1*, 12 for the retrotransposon. None of the RNAi lines for the retrotransposon rescued the phenotype (data not shown). Some of the lines showed partial rescue in the T1 generation but only one line, L7, showed consistent rescue in the T2 generation (Figure 2.7, 2.8). We performed a Northern blot on Ws, *bon1-2*, *ebo12bon1-2*, and several RNAi lines (with and without rescue) to check the expression levels of our candidate genes. Interestingly, the RNAi not only suppressed the expression of the 3' end of the *srk1-1* allele, but also that of *At5g03690*. This result was unexpected, but could be attributed to the fact that the RNAi construct contains part of the *35S* sequence of the T-DNA. Therefore, there might be additional effects of this particular RNAi construct on the expression of nearby genes. Thus, we designed 2 more RNAi constructs to test this hypothesis, by targeting the 3' end of *At5g03700* and the *35S* promoter, specifically. To expedite the cloning process, we switched to the pSUPER1300+ vector. A total of 5 and 9 lines were obtained for the new constructs, respectively. 3 lines showed partial rescue with the 3' end specific RNAi in the T1 generation. In the T2 generation, no rescue was seen from these lines (Figure 2.9). To more conclusively determine if these KD constructs suppress the phenotype or not, more lines would be necessary in addition to RNA-blotting and probing of this locus to determine if the suppression has indeed occurred.

ebo12 single mutant analysis

The further understand the relationship of *EBO12* to *BON1* we isolated the single mutant by crossing *ebo12bon1-2* plants to Ws-2. Homozygous *ebo12* individuals were isolated and identified in the F2 generation by genotyping and BASTA® spray. To determine the expression pattern of the candidate genes, we performed a Northern blot and probed for the 3' end of *srk1-1* and *At5g03690* in Ws-2, *bon1-2, ebo12bon1-2*, and *ebo12* plants. The results indicated that the overexpression of the 3' end of *srk1-1* and *At5g03690* was present in *ebo12* plants as seen in *ebo12bon1-2* but not Ws-2 or *bon1-2*. This supports the hypothesis that the T-DNA could be responsible for the overexpression of these transcripts and that the enhancer phenotype is dependent on the *bon1-2* mutation.

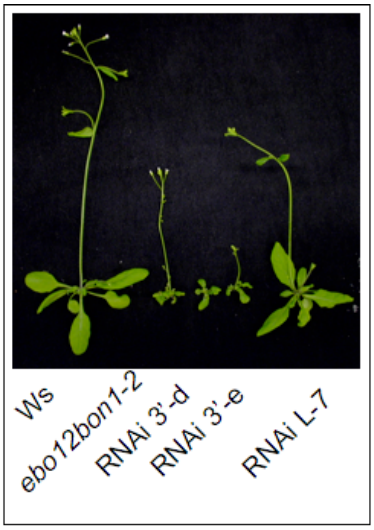

Figure 2.9 *srk1-1* 3'end RNAi lines did not show rescue of *ebo12bon1-2* phenotype.

ebo12 has an enhanced basal disease resistance

To establish the connection of *EBO12* to *BON1* with respect to disease resistance, we investigated whether or not this enhancer influenced resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato DC3000*. We determined that *ebo12bon1-2* has enhanced disease resistance denoted by a substantial growth reduction in comparison to *ebo12*, *bon1-2*, and Ws-2 (Fig. 2.12, 2.13). The results of the resistance assay indicate a synergistic effect on pathogen resistance between *bon1-2* and *ebo12*, suggesting that these genes are in the same regulatory pathway. Thus, the enhancer could be a true component in the *BON1* signaling pathway.

Figure 2.10 *ebo12* does not exhibit a *bon1*-like phenotype.

Additionally, we tested whether the resistance phenotype is dependent on *EDS1,* which is an important signaling component downstream of many *R* genes. These plants were crossed to *eds1-1bon1-2* to determine if LOF *EDS1* allele could rescue the enhancer phenotype. (Figure 2.14). The *ebo12bon1-2eds1-1* mutant plants were obtained and they exhibited a wild-type phenotype and normal plant size comparable to Ws and *bon1-2* under

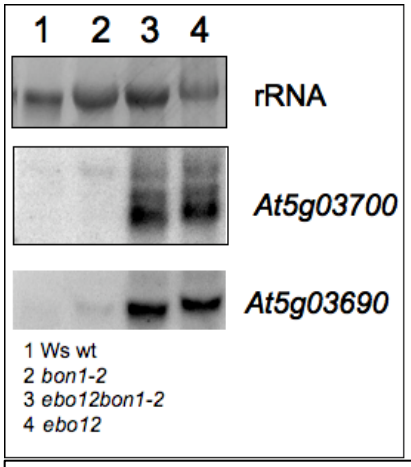

Figure 2.11 Northern blot analysis of *ebo12*. Overexpression of the two candidate genes is not affected by the *bon1-2* mutation.

standard conditions. The necrotic lesions that were apparent on the double mutant leaves were absent in the triple, indicating a reduction in PCD. Thus, the enhancer gene could be in the same pathway as *BON1* and *EDS1* and lies upstream of the latter.

Enhanced basal disease resistance of ebo12bon1-2

As an enhancer of *bon1-2* we expected the necrotic lesions and dwarfed stature of *ebo12bon1-2* to be correlated a heighten state of disease resistance. Preliminary data from Dr. Shimizu suggested a 10^2cfu mg^{-1} fresh weight decrease of *DC3000* growth in *ebo12bon1-2* in comparison to Ws-2 and *bon1-2* plants at 4 days-post inoculation. To determine whether or not the interaction of *bon1-2* and *ebo12* is synergistic with respect to basal disease resistance, *ebo12* plants were grown together with Ws-2, *bon1-2*, and *ebo12bon1-2*. At 4 days post-inoculation (dpi), *ebo12-bon1-2* showed about $2.1x10^4$cfu mg^{-1} fresh weight growth, approximately 100-fold less bacterial

growth than Ws-2 and *bon1-2* (6.4x10^6 and 9.9 x10^{5}cfu mg^{-1} fresh weight, respectively), consistent with previous findings (Figure 2.13). *ebo12* plants showed susceptibility to *DC3000* (2.1x10^{6}cfu mg^{-1} fresh weight), comparable to *bon1-2*, indicating a synergistic effect of the two mutations on the basal disease resistance in the double mutants. This suggests that the two genes could be functioning in parallel pathways governing the regulation of defense responses in *A. thaliana*.

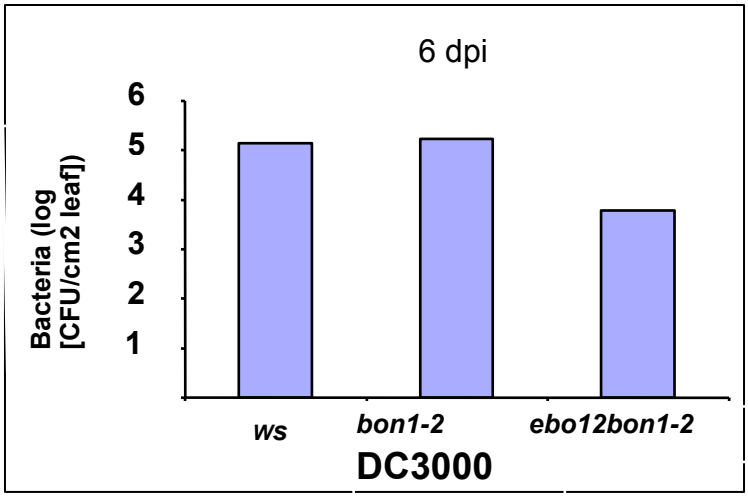

Figure 2.12 Disease resistance assay of *ebo12bon1-2* compared to *bon1-2* and Ws. By Dr. Shimizu.

ebo12 function is mediated by eds1-1

Because the defense-related genes *PAD4* and *EDS1* mediate the function of many R--genes, we wanted to determine if this was also true for *EBO12*. If the function of *ebo12* is mediated through *EDS1*, then we would expect that the *eds1-1* mutation would abolish the enhanced disease resistance of *ebo12-bon1-2.* Thus, *ebo12bon1-2* plants were crossed to *eds1-1bon1-2*, and homozygous *ebo12bon1-2eds1-1* plants (triple mutants) were isolated in the

F2 generation (Figure 2.14). To determine the relative resistance/susceptibility of these triple mutants, they were grown alongside Ws-2, *bon1-2*, *eds1-1*, *eds1-1bon1-2*, and *ebo12bon1-2* plants. As expected, *eds1-1* and *eds1-1bon1-2* (5.4 x10^7 and 8.3 x10^7 cfu mg^{-1} fresh weight, respectively) had greater susceptibility to the pathogen at 4 days-post inoculation compared to Ws-2 (6.4 x10^6 cfu mg^{-1} fresh weight) (Figure 2.13). Interestingly, the *ebo12bon1-2eds1-1* plants were susceptible to the pathogen (5.2 x10^6 cfu mg^{-1} fresh weight), relative to Ws-2, *bon1-2*, and *ebo12* plants. Since these plants are more resistant than *eds1-1* or *eds1-1bon1-2*, it is possible that the function of *ebo12* is not completely mediated by *EDS1*. Crosses have been made between *ebo12bon1-2* plants and *pad4-5* to ascertain the importance of *PAD4* in *EBO12* function. In *bon1-1 and bap1bap2* plants the *eds1* and *pad4* mutations also abolished the observed ROS production, noted by a reduction in DAB staining **(45, 46)**. It remains to be seen if this is also the case with *ebo12bon1-2*.

Discussion

We report that *ebo12* is an enhancer of *bon1-2* and that its morphological phenotype and enhanced disease resistance are dependent on the T-DNA inserted into *At5g03700* and the *bon1-2* mutation. From Northern blot analysis of the single mutant we have seen that *srk1-1* is sufficient to cause the overexpression of two neighboring genes, likely by the T-DNA. It still remains to be seen if there are additional mutations linked to the phenotype. *At5g03700* is more strongly affected by this T-DNA because only the 3' end of the gene is expressed in addition to several aberrant transcripts.

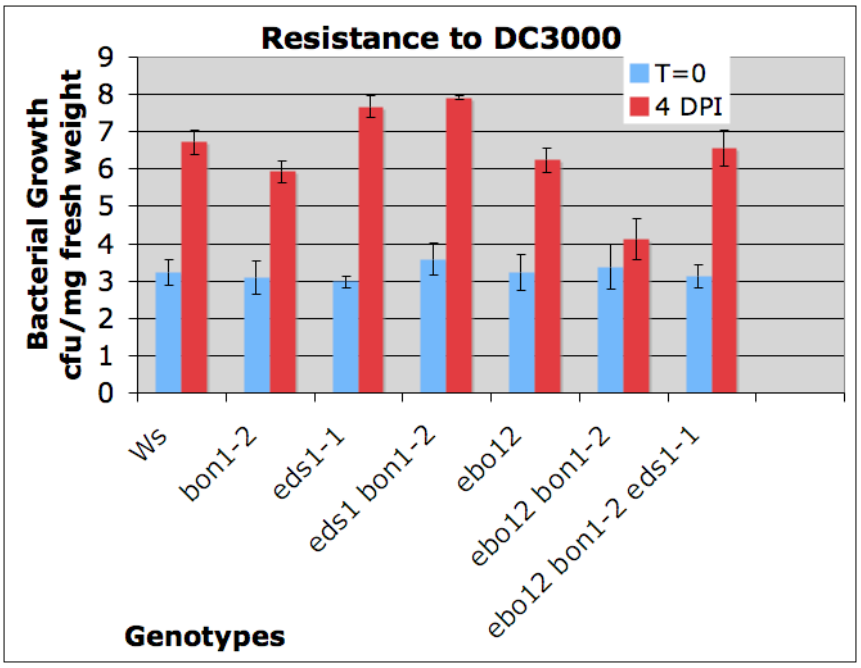

Figure 2.13 Resistance to *DC3000*. *ebo12* double= *ebo12bon1-2*, *ebo12* triple= *edbo12bon1-2eds1-1*.

The phenotype and disease resistance of *ebo12* are dependent on *bon1-2* as the single mutant, which lacks a *bon1*-like phenotype, is as susceptible to *DC3000* as Ws-2. These results indicate that there is a synergistic effect of *ebo12* and *bon1-2* with respect to phenotype, cell death, and disease resistance.

In an effort to conclusively clone the *EBO12*, we have attempted to recapitulate the phenotype by cloning and overexpressing the gene candidates, the *srk1-1* allele. We have not seen *ebo12*-like phenotype with overexpressed *srk1-1* chimera or *At5g03690* which may suggest that neither of these genes is the true *EBO12*. It is possible that there are additional mutations present that we are currently unaware of that are necessary to cause an *ebo12* phenotype. We are certain that a single T-DNA is linked to the phenotype due to numbers of BASTA™ resistant offspring of *ebo12bon1-2* x *bon1-2* cross and TAIL-PCR, which identified only one linked T-DNA. It is

Figure 2.14 Rescue of *ebo12bon1-2* by *eds1-1*. By. Dr. Shimizu.

important to note that this T-DNA was identified only by the right border. We have not been successful with PCR-amplification of the left border. The insertion of the T-DNA insert could have caused small insertion/deletion mutations. Our *srk1-1* chimera is an approximation of what we believe to be a prominent transcript in *ebo12* plants. We have seen an unexplained larger transcript by Northern blot analysis in *ebo12* and *ebo12bon1-2* plants. If this transcript is indeed necessary for the enhancer phenotype in *bon1-2*, perhaps we have not expressed all of the critical regions in *srk1-1,* which could account for a lack of recapitulation of the *ebo12* phenotype.

To test this hypothesis, it was necessary to employ either large fragment PCR of the genomic DNA or isolation of a clone from a library of *ebo12* plants such that the genetic conditions could be duplicated as closely as possible. Preliminary data has suggested that the transformed genomic DNA clone encompassing this region may yield an *ebo12*-like phenotype. However, it is important to note that recapitulation of the phenotype with *ebo12* genomic DNA would only indicate whether or not we have the correct region

isolated. This would not be conclusive evidence to identify a specific gene, however, it could support cloning of a larger chimera construct.

In another effort to identify the gene, we attempted to knockdown the overexpression of the candidates with RNAi. We engineered an RNAi construct for the *srk1-1* chimera and saw only one line capable of rescuing the *ebo12* phenotype. In this line, both the aberrant transcripts of *At5g03700* and overexpression of *At5g03690* were suppressed. A reduction in expression levels was noted in other lines showing a weaker degree of rescue. This could be a result of differential expression of the RNAi construct or efficiency of the knockdown between independent transgenic lines. Suppression of *At5g03690* was unexpected as the *srk1-1* chimera was not designed to target the expression of this gene. It is possible that knocking down the T-DNA has a pleiotropic effect on gene expression, or perhaps this locus is under a more interconnected manner of gene regulation. This data suggests that perhaps targeting the T-DNA, the 3'end of *At5g03700*, or both is sufficient to suppress the *ebo12*-like phenotype.

We have shown that the *ebo12* mutant not only enhances a *bon1* morphological phenotype in Ws-2, but that these genes together, synergistically enhancing the basal disease resistance of *A. thaliana* (Figure 2.13). *bon1-2* and *ebo12* alone do not confer resistance, but in concert these mutations cause the plants to exhibit 10^2 cfu mg^{-1} fresh weight less bacterial growth after 4 days. The *eds1-1* mutation abolishes the enhancer phenotype of *ebo12bon1-2,* but the disease resistance is only compromised to wild-type levels, not to those of *eds1-1* mutants (Figure 2.12). This suggests a partial mediation of *ebo12* function through *EDS1*, which may suggest alternate

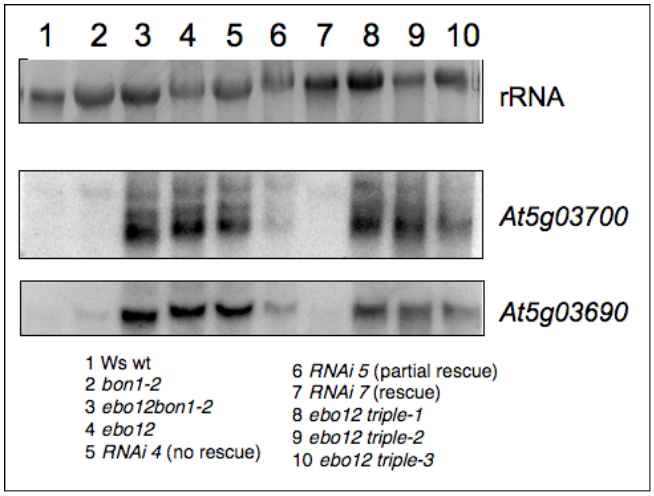

Figure 2.15 Overexpression of the two candidate genes is dependent on the T-DNA in *At5g03700*. Expression is altered in L7 RNAi plants. *eds1-1* does not alter gene expression but rescues phenotype.

means of regulating defense responses and additional downstream components in the signaling pathway.

To further understand the interaction of *EBO12* and *EDS1* in disease resistance, we could also investigate the ROS accumulation in these plants as well as the expression of other genes whose function is dependent on *EDS1*. We have seen that ROS accumulation occurs in *ebo12bon1-2* leaves, a possible cause of necrotic lesions and heighten levels of cell death, and this may be affected by *eds1-1*. Further RNA expression analysis of Ws, *bon1-2, eds1-1, ebo12bon1-2*, and *ebo12bon1-2eds1-1* for other defense-related genes could enable us to determine what genes are still activated in *ebo12bon1-2eds1-1* plants and may contribute to their relative resistance.

If the *srk1-1* is the real cause of the *ebo12bon1-2* phenotype, this may be a gain-of-function mutation for *At5g03700*. The implication of this work is that a new method of regulating defense responses could be identified in a

gene that would not normally have been suspected of such, based on its putative functional domains. By using 5' and 3' end specific probes for *At5g03700*, the RNA-analysis has revealed that only the 3'end of this gene is overexpressed in *ebo12* mutants (Figure 2.3). The T-DNA is inserted at 923bp (amino 307), therefore only the PAN/Apple domain is expressed. It is unknown what the normal function is for this protein and we have yet to determine if the overexpression of this portion of the gene is required for the enhancer phenotype. If it is, we can speculate that perhaps this aberrant protein could have a greater activity than the wild-type, or it may altogether have a novel function which has a significant impact on cell death regulation.

CHAPTER 3:

Structure & Function Analysis of BAP2

Introduction

We have shown that the *BAP* gene family function is essential in regulating cell death in plants and it can function as such in yeast **(46)**. However, the molecular mechanism of this regulation and interacting proteins in addition to BON1 are currently unknown. To answer questions regarding the molecular mechanism it is necessary to first understand the normal function, localization, and biochemical activities of these proteins. It has been previously established that BON1 and BAP1 both bind to the phospholipids in a Ca^{2+}-dependent manner **(46)**. However, we have yet to determine if BAP2 binds in a similar manner or what the functional mechanism is of this protein. The research focus with this protein has been to determine the normal mechanism of function by mutagenesis of known functional domains. We wished to answer the following questions: Does BAP2 also function in a Ca^{2+}-dependent manner? Which amino acids are essential for this function and alter the membrane binding affinity? Is Ca^{2+}-dependent phospholipid binding essential for normal cellular localization of this protein? With human cytosolic phospholipase A_2, Bittova et al' used specific mutations to identify which aspartates were important in coordinating Ca^{2+} ions **(5)**. Thus, we decided to mutate specific residues chosen by protein domain alignment in the C2 domain, and then test the functionality of the mutant protein by a complementation assay. Once a non-functional mutant is identified, we will test if the protein is properly expressed and localized.

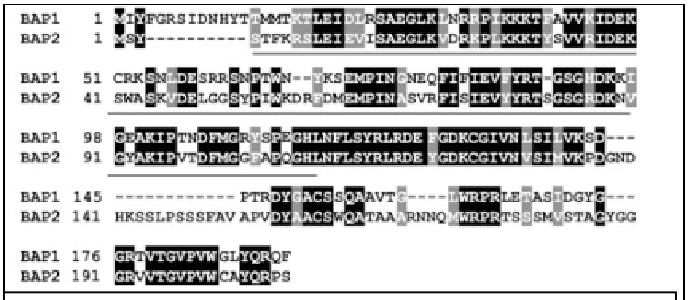

Figure 3.1 Alignment of BAP1 and BAP2.

Results

Mutation of the C2 domain

In order to ascertain if the C2 domain is important for the localization and function for BAP2, we chose to mutate highly conserved residues in this domain. We aligned BON1, the BAP proteins, and human phospholipase 2 (**5, 46**) and identified 2 conserved aspartate residues potentially required for coordinating Ca^{2+} ligands. The native promoter of *BAP2,* though not specifically determined, was used to preserve the natural pattern of expression. The utilized promoter region spans -1 to -1500 bp in front of the start of the reading frame. A 3HA tag was added to the N-terminus of the BAP2 protein with the start codon of *BAP2* removed. The following two constructs were made: *pBAP2:3HA-BAP2* (wild-type) and *pBAP2:3HA-BAP2 D38A, D88A* (mutant).

To determine if the mutated residues were important for the biological function of BAP2, we transformed these constructs into *bap1/+bap2* plants and looked for phenotypic complementation. It was also important to determine if BAP2 tagged with 3HA could function *in vivo*. We chose this mutant because the *bap2* single mutant does not exhibit an obvious

phenotype while *bap1/+bap2* has a strong phenotype and *bap1bap2* is lethal. If *D38* and *D88* were critical to the function of BAP2, then the mutant construct would not be able to complement the strong phenotype of *bap1/+bap2* or *bap1bap2.* We are assuming that the mutations made to BAP2 will not destabilize the protein nor cause major conformational changes. The *pBAP2::3HA-BAP2* construct also served as a control of whether or not the expression level of the transgene expression is sufficient and that the fusion tag does not compromise BAP2 function. A total of 52 total transgenic lines were obtained from floral dipping 4-week old *bap1/+bap2* plants and selecting the transgenic seedlings on ½ MS hygromycin plates. Due to the segregation of *bap1* in this population, it was necessary to genotype the transgenic lines to determine whether the lines were *bap1bap2*, bap1/+*bap2,* or and *BAP1bap2* (Table 3.1). A total of 28 plants with a *bap1/+bap2* genotype were identified. All lines were checked in the T1-T3 generations for complementation. All plants with a *BAP1bap2* genotype were wild-type regardless of the transgene they carried and therefore gave no information about the transgene functionality. The transgenic lines with the BAP2 transgene did not completely complement the *bap1/+bap2* phenotype as expected (Fig. 3.2). No double homozygotes were ever observed in T1; and 1/4 of the progeny of *bap1/+bap2* T1 plants did not germinate or survive the early seedling stage, regardless of the transgene. 17 lines with a *bap1/+bap2* background carried the transgenic *BAP2* and 10 of these exhibited variations in the degree of rescue (Figure 3.2). None of the 11*bap1/+bap2* lines bearing the mutant *BAP2* transgene showed any rescue at all. Of the T1 plants with a *bap1/+bap2* genotype, ¼ of their T2 offspring also did not germinate. Thus, we concluded that the wild-type *3HA-*

BAP2 transgene is functional and it is possible that the two mutated amino acids are essential to normal protein function.

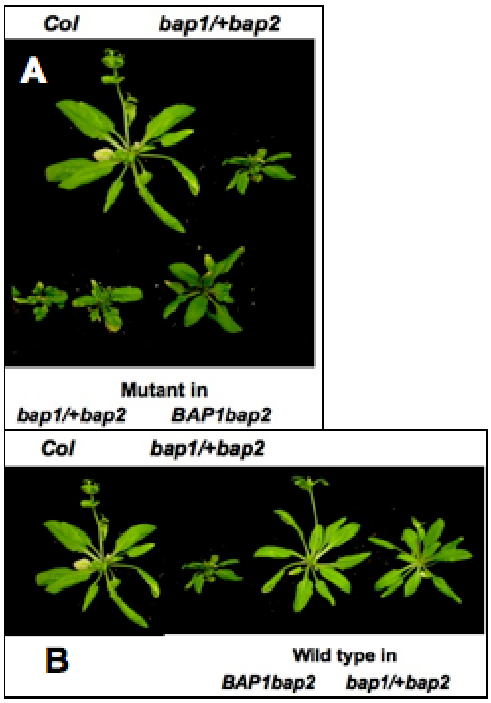

Figure 3.2 (a) T3 plants carrying the *D38A D88A* mutant transgene do not exhibit any phenotypic rescue. (b) T3 plants transformed with *BAP2* transgene exhibit varying degrees of rescue from none to complete. Some plants have normal leaf morphology but display multiple inflorescent shoots at time of bolting.

Detection of 3HA-BAP2 by Western Blotting

To ascertain that the phenotypic rescue with the *BAP2* transgene and lack thereof with the mutant was not due to a difference in expression level, we performed a Western blot on total protein extracts from multiple transgenic lines to detect the fusion protein. Since the variability of the wild-type

transgene rescue was prevalent, we decided that the lines showing the strongest rescue should be used. We selected the lines carrying the mutant transgene at random. The total length of the coding region of the transgene is 724 bp so we expected to see protein signal at approximately 26 kDa. However, we could not detect any signal from the desired protein on a Western blot (data not shown). The positive control for the Western blot primary antibody, BON1-3HA, was easily detected. Since the expression of *BAP2* is normally less than either *BON1* or *BAP1*, we hypothesized that the protein is either not highly expressed, or it could be under a rapid turn-over. Thus, we attempted immunoprecipitation (IP) enrichment to enhance the signal from any present protein. Unfortunately, the IGg bands of the monoclonal antibody run at ~50 and ~25kDa on the gel, thus their strong signal on the Western blot obscured any signal from the 3HA tag. A longer runtime on the protein gel did not improve the resolution nor enable us to discern signal from the primary antibody or fusion protein.

Table 3.1 T1 data of *pBAP2::3HA-BAP2* constructs transformed into *bap1/+bap2* plants. Rescue seen in 1/2 of plants bearing wild-type BAP2. No double homozygotes were found .

Transgene	*BAP1bap2* (phenotype)	*bap1/+bap2* (phenotype)	*bap1bap2*
pBAP2::3HA-BAP2 (wt)	5 (all wt)	17 (10 wt)	0
pBAP2::3HA-BAP2 (mut)	19 (all wt)	11 (all mut)	0

When expression of BAP2 protein could not be detected by Western blot of total protein or an IP-enriched extract, we decided that a stronger

promoter should be used to drive the expression. DIfficult detection with the BAP1 protein under its native promoter was also noted Dr. H. Yang and Yongqing Li. They were able to detect the protein when they expressed *35::MYC-BAP1* in *N. benthamiana*. We therefore cloned the *3HA-BAP2* transgene into the pSUPER 1300+ binary vector with a strong promoter and transformed the construct into GV3101 and C58C1 *A. tumafaciens* lines for generating stable transgenic lines in *A. thaliana* and transient expression in *N. benthamiana*, respectively. Again, stable transgenic seedlings were identified

Table 3.2 T1 data of *35S::3HA-BAP2* constructs transformed into *bap1/+bap2* plants. Rescue seen in 1/3 of plants bearing wild-type BAP2.

Transgene	*BAP1bap2* (phenotype)	*bap1/+bap2* (phenotype)	*bap1bap2*
35S::3HA-BAP2 (wt)	4 (all wt)	6 (2 wt)	0
35S::3HA-BAP2 (mut)	19 (all wt)	15 (all mut)	0

on ½ MS hygromycin plates. 10 lines with *BAP2* were identified and 34 with mutant transgene (Table 3.2). Among these, 6 and 15 lines had a *bap1/+bap2* background, respectively (Table 3.2.). No *bap1bap2* plants were identified and none of the lines bearing the mutant transgene showed any phenotypic rescue. We noted some partial rescue with the *BAP2* transgene, similar to what was seen in the other transgenic lines with the native *BAP2* promoter.

Transient expression in N. benthamiana

Since the best detection of BAP1 was accomplished with transiently expressed *35S::MYC-BAP1* in *N. benthamiana*, we chose to employ this

strategy for *BAP2* expression. Two-month-old plants were infiltrated with sterile infiltration media (mock treatment), C58C1 cells carrying an empty vector, or the wild-type or mutant *3HA-BAP2* transgene. Because the time of strongest expression of the transgene had yet to be determined, we collected tissue samples from the leaves at 2, 3, and 4 days post-infiltration. Total protein was extracted and run on 12% polyacrylamide gels. Western blotting revealed strong binding of the HA monoclonal antibody to a protein running at

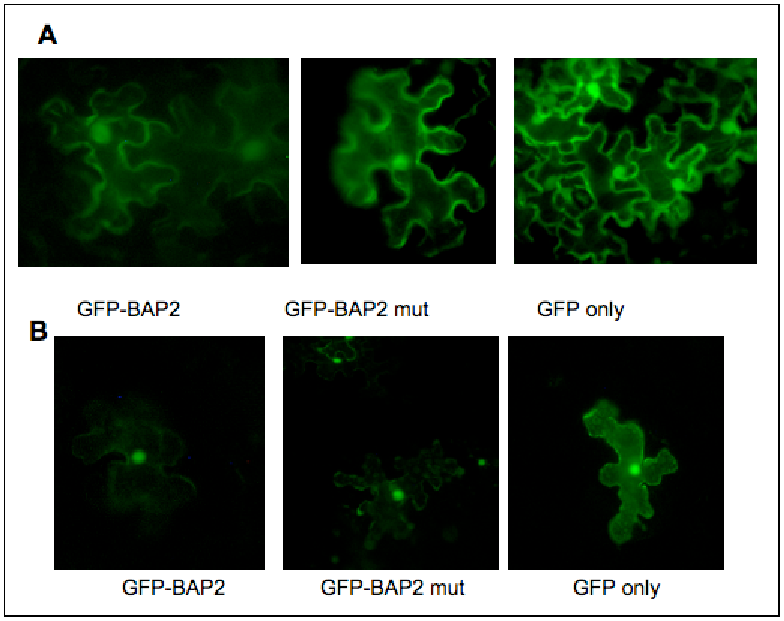

Figure 3.3 GFP fluorescence 2 dpi, multiple layers (a), thin layer peel (b). Exposure time 0.5 sec.

approximately 26 kDa. This band was seen uniformly in all samples, regardless of the treatment. Thus, this protein is likely an endogenous protein from *N. benthamiana* that the antibody binds to with sufficient affinity to be detected on the blot. Concurrently, this band size is precisely the expected size of the expressed 3HA-BAP2 fusion protein and therefore could mask any

signal from 3HA-BAP2 present in the samples. Thus, further work with 3HA tags in *N. benthamiana* was discontinued.

BAP2 localization

We subsequently fused a larger tag, GFP to BAP2 and expressed the fusion under the strong *35S* promoter. The *35S::GFP-BAP2* construct in GV3101 was transformed into *bap1/+bap2* to determine the functionality of the fusion protein. The constructs in C58C1 *A. tumafaciens* were infiltrated into ~2 month-old *N. benthamiana* plants. To control for individual differences in the leaves an infiltrated spot of each of the following samples was made on the same leaf with a total of 4 leaves per trial: *GFP* alone, untransformed C58C1, wild-type and mutant *GFP-BAP2*. Small samples of the spots were cut and examined under a compound fluorescence microscope at 2, 3, and 4 days post-infiltration. Days 2-4 post infiltration showed strong expression of GFP-alone and GFP-BAP2 wild-type and mutant. Nothing beyond background fluorescence was observed at any time point for C58C1-infiltrated and untreated tissues (data not shown). Continuous patches of cells exhibited GFP fluorescence 2 and 3 days post-infiltration in *GFP-BAP2* infiltrated tissues (Figure 3.3). GFP-only tissues showed ubiquitous cellular expression (Figure 3.3). GFP-BAP2 wild-type and mutant tissues did not fluoresce as strongly as GFP-only but there appeared to be strong signal originating from the nucleus and plasma membrane of the cells. Strongly fluorescing tissues were collected for *in vitro* detection of GFP-BAP2 by Western blotting. GFP runs at approximately 26 kDa, BAP2 is expected to run at 23 kDa, thus we expected to find the fusion protein close to the 50 kDa standard band. The GFP band

appears, as expected, between the 25 and 35 kDa standard markers (Figure 3.4). A band running at 35 kDa is present in all samples and is therefore likely to be the result of non-specific binding of the monoclonal GFP antibody to an endogenous *N. benthamiana* protein (Figure 3.4). Bands seen between 25-35 kDa are also visible in lanes 6-9 and could be free GFP. If these are true, a portion of the signal we've seen in the microscope could be partially attributed to GFP and not GFP-BAP2. A series of four bands between 50-40 kDa can be clearly seen in lanes for GFP-BAP2 wild-type and mutant (2 and 3 dpi). The smallest of these four appears to be shared in the GFP-only lane, thus might be background. The others, however, (see arrow) appear to be unique to the GFP-BAP2-infilitrated tissues (Figure 3.4, lanes 6-9). It is possible, although they run shorter than expected, that these bands could in fact be the GFP-BAP2 fusion. The difference in band intensity between lanes 4 & 5, 6 & 8 could be attributed to the higher expression of this fusion seen at 2 dpi, for this particular experimental run. From these results, we know that the GFP-BAP2 protein can be expressed and detected it by Western blotting.

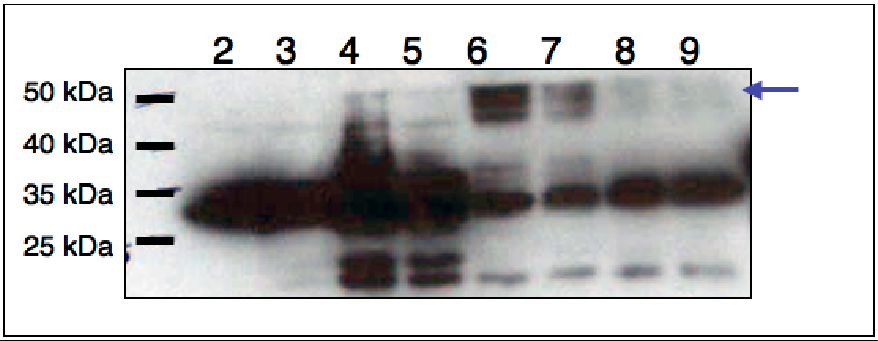

Figure 3.4 Western blot of GFP-BAP2 infiltrated in to *N. benthamiana.* Lanes 2-3: C58C1 only, lanes 4-5: GFP only, lanes 6 & 8: GFP -BAP2 wt, lanes 7 & 9: GFP-BAP2 mut. Lanes 2, 4, 6, & 7, are 2dpi. Lanes 3, 5, 8, & 9 are 3dpi.

Because multiple layers of tissue can blur the specific protein localization in plant leaves, single layer peeling or protoplast expression can be better alternatives. Single layer peeling revealed *GFP-BAP2* mutant expressing tissues showing stronger signal in the nucleus, as opposed to ubiquitous expression in *GFP*-only expressing cells (Figure 3.3). In tissues expressing wild-type *GFP-BAP2*, the nucleus was well-defined in the cells and the plasma membrane also had a strong fluorescent signal. There appeared to be less fluorescence in the cytoplasm of these tissues. However, these results may be due to either the true localization of BAP2 in the cells or the pattern could be due to overexpression and consequential mislocalization of the protein.

Depending on the strength and flexibility of individual leaves, single layer peeling is not always successful, thus, expression in *A. thaliana* protoplast cells provided another means of visualizing the protein localization. *35S::GFP* and *35S::GFP-BAP2* (wild-type and mutant) in the pSATC1 vector were transformed into *A. thaliana* (Col-0) protoplasts by Dr. Ying Zhu. Strong fluorescent signal was detected in the *GFP* control, and the *GFP-BAP2* wild-type and mutant-transformed cells (Figure 3.5). Expression of GFP under the *35S* promoter yielded strong fluorescence seen in both the nucleus and cytoplasm. Some distinctive “network” fluorescence was also seen, which may be cytoskeletal (Figure 3.5). In *GFP-BAP2* transformed cells, the same fluorescent pattern was observed with addition of small dots scattered about the cytoplasm (Figure 3.5). This was seen more often with the mutant transgene than the wild-type but it needs to be quantified. These dots could be an artefact of the protein overexpression in these cells, and thus would be an inaccurate representation of native protein localization. To determine if the

protein has been compartmentalized to specific organelles or vesicles, co-expression with organelle or vesicle-specific markers labelled with other fluorescent tags would enable us to determine if there is any co-localization of the proteins.

Discussion

Expressing and detecting BAP2

We expected that introducing functional BAP2 protein would rescue *bap1/+bap2* and *bap1bap2* phenotypes in *A. thaliana* if the expression was appropriate and the 3HA tag did not impair normal function and localization. The rescue was only partial with *BAP2* wild-type and varied between independent lines, however, no rescue was seen at all with the mutant *BAP2*. The variation with the wild-type transgene may be due to different levels of expression in each line. Partial rescue may indicate that expression level and/ or pattern did not replicate those of the endogenous gene. Another explanation could be that the tag itself impairs normal function or destabilizes the protein; and it is not uncommon that tagged proteins can mislocalize, become unstable, or form aggregates *in vivo* **(37)**. Nevertheless, the wild-type tagged protein can partially rescue the *bap1/+bap2* phenotype while the mutant tagged protein cannot. This evidence indicates that *D23* and *D88* are indeed important amino acids for the biological function of this protein.

To determine whether these mutations affect the biochemical activities or the stability of the BAP2 protein, we set out to compare the expression level and localization of the wild-type and the mutant BAP2 tagged proteins. BAP1

was proven to be difficult to detect until it was expressed in *N. benthamiana*, driven with a *35S* promoter. However, due to the non-specific binding of the HA antibody to an endogenous protein in *N. benthamiana* of similar molecular

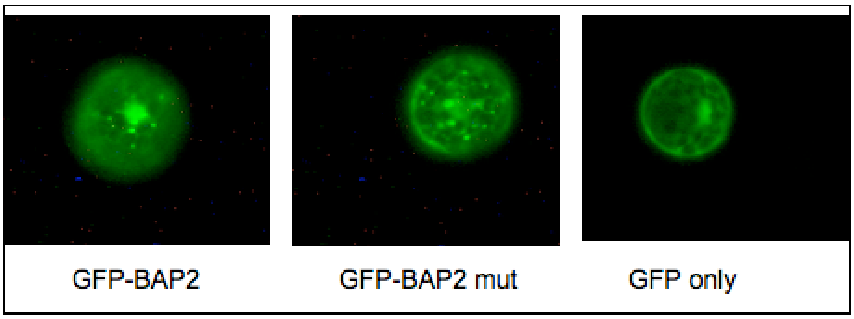

Figure 3.5 GFP-BAP2 fluorescence in Col-0

weight to 3HA-BAP2, we were unable to reliably distinguish these proteins. Although the wild-type BAP2 is capable of rescuing the *bap1/+bap2* phenotype to some extent, our attempts to detect the 3HA-BAP2 fusion protein were not very successful. One possibility is that the fusion protein is not very stable and thus it degrades before we can probe for it on the Western blot. The GFP-BAP2 fusion appears to be more stable, therefore, stable transgenic lines bearing this construct may provide a more robust means of expressing and detecting the protein *in vivo*. Additionally, the wild-type *GFP-BAP2* may better rescue *bap1/+bap2* plants and may perhaps even rescue *bap1bap2.* This would provide additional support for our conclusion that *D23* and *D88* are essential to BAP2 function and may indirectly indicate that the 3HA was interfering in the previous protein fusion.

From expression and detection of the GFP-BAP2 fusion protein we found more than one band specific to GFP-BAP2. By performing Western blot analysis on *A. thaliana* plants transformed with the GFP-BAP2 construct, we could confirm whether or not that these bands are real or background from *N.*

benthamiana. If these are real, this may suggest that some post-translational modification could be at work. The bands correspond to a mass less than we expected, but it is possible that different chemical modification, such as peptide cleavage and/or phosphorylation could have occurred. To ascertain if such changes have occurred to the protein, we could attempt to purify the protein from the gel and do peptide finger printing mass-spectrometry on the bands to specifically identify them. In addition, treating the total protein extracts with phosphotases would remove phosphorlyation, if it has indeed occurred. Mutagenesis would provide another means of identifying the necessity of specific post-translational modifications to protein function.

The preliminary data of *GFP-BAP2* expression has shown that this protein fusion can be expressed and is stable enough for *in vitro* detection. However, it is too soon to conclude whether or not the *D23AD88A* mutations we engineered in *BAP2* have significantly influenced the *in vivo* protein localization. It is possible that any differences between wild-type and mutant BAP2 localization may have been obscured, due to overexpression by the *35S* promoter. It is equally possible that there is no alteration in protein localization although the biochemical activity of the mutant protein has apparently been compromised. One means of circumventing potential complications of overexpression would be to use the native promoter of *BAP2*. Using a weaker promoter may also determine if the GFP tag is causing any mislocalizing of the fusion protein due to its intrinsic chemical nature **(37)**. By using Ca^{2+} binding assays we should be able to better answer the question of whether or not this specific biochemical activity of the protein has been abolished. It also remains to be seen if the putative C2 domain does indeed function normally in Ca^{2+}

binding and if these residues are components in the coordination of such cations.

These findings could better enable us to understand the essential function of the *BAP* genes in the negative regulation of cell death in *A. thaliana*. We have shown that the LOF alleles of these genes have a synergistic effect on cell death **(46)** and we wish to determine how and why. We have seen with *BON1* and *BAP1* that certain functional domains are important to the biochemical activity and protein-protein interactions. Much remains to be uncovered with BAP2 with respect to its individual function. Evidently, *BAP1* and *BAP2* have overlapping functions, and it possible that they may function in a larger protein complex or in related molecular pathways in cell death regulation **(46)**. It also remains to be seen if the function of these genes with respect to cell death is direct or indirect. If the latter is true, the strong cell death response in *bap1/+bap2, bap1bap2/+*, and *bap1bap2* could be the result of "guarding" by specific *R* proteins, perhaps operating in a manner reminiscent of *RPS2* and *RPM1* on *RIN4* **(3, 4, 26)**. By developing a deeper understanding of the *BAP* gene family function, we can better our understanding of the complex molecular mechanisms governing the intricacies of normal cell growth and plant defense responses.

CHAPTER 4:

Perspectives

We have shown that the *BON* and *BAP* gene families are essential to normal growth and development in *A. thaliana*. Specific LOF mutants in these genes leads to increased ROS production, cell death, dwarfism, and constitutive activation of defense-related genes. It is possible that the role of these proteins is a direct regulation of cell death by suppressing protein activity and/or gene expression during plant development, a function perhaps independent of plant immunity. However, we have seen that these proteins have an ancestral role in cell death regulation in other organisms **(46)**. Therefore, it possible that these gene families are more closely linked to innate immunity and therefore could be targets of effector proteins during pathogen infection. If in accordance with the guard hypothesis, then *R* proteins may respond to the LOF mutations by constitutively activating basal defenses **(8, 20)**. We have seen that combining LOF *BON* or *BAP* alleles results in more severe cell death phenotypes and that *snc1-11* cannot fully rescue these plants**(45, 46)**. Thus, it is likely that other *R* genes are involved and have yet to be identified. Our enhancer screen in Ws-2 could lead to the identification of accession-specific defense signaling components because Ws-2 lacks *SNC1*. Thus, by uncovering additional components in the signaling pathway, and by understanding the molecular mechanism of the protein activity, we aim to answer the greater question of how these gene families function and their contribution to the delicate balance of defense activation, fitness, and survival.

METHODS

Growth Conditions

All plants are vernalized at 4^{0}C for three days before germination in the growth facility. Standard growth conditions include: constant light, 22 ^{0}C, 30-70% relative humidity. Metro Mix™ 220 and Redi Earth® topsoil were used as growth media. 1tsp Marathon® was applied to the flat trays during planting to repel aphids in all plants except those used for disease resistance assays.

Genotyping

For amplifying the *ebo12* T-DNA the following primers were used*:* *srk-2*, *35S-5*

For amplifying of the wild-type sequence the following primers were used: s*rk-1*, *srk-2*

SRK-2: agcaatcacagcaagcaaagaca

35S-5: cgtcttcaaagcaagtggattgatgtg

500-up primers are design to PCR-amplify the region downstream on *At5g03700*, encompassing the gypsy LTR retrotransposon.

500 up (rev): CCTTCCATttttggaaacttgaatc

500 up (for): cagtacatcaatgtatgtatacac

VRN2a GGGAGATTAAAGAAGCCTTTGC

VRN2b GTGCGGTTAACTGTTCGGTTACC

Mapping Population

This population is the F2 generation of crossing *ebo12bon1-2* (Ws-2) to *bon1-1snc1-11* (Col-0). F1 plants were genotyped with VRN2 primers to ensure that crossing but not self-fertilization or contamination had occurred. TAIR, SNP, and SFP databases provide information for SSLP and DCAPS marker design.

Genomic Library Construction

This process was derived from several protocols: Promega (phage vector source) *Molecular Cloning Handbook* (plaque lifts, phage amplification, screening, isolation), and Gan and Nasrallah labs (phage DNA prep). LambdaPhage vector: Lambda Dash II[(a)]. Host strain: XL1Blue (MRA) P2

RNAi Constructs

The cloning adaptors were designed by Yongqing Li, based on information from the ChromDB website (www.chromdb.org). The binary vector pFGC1008 (from Chromdb) with chloramphenicol and hygromycin selection was used. Later constructs were cloned in pSUPER1300+ with Amp and hygromycin selection.

Design of **BAP2** *transgenes*

The choice of residues to mutate was based on protein alignment in Vector NTI. A fusion tag was added to determine the location of the protein. Other members of our lab have already done similar experiments with BON1 and BAP1. Native promoter of BAP2 was to be used for determining the natural localization pattern. PCR was used to amplify a 1.5kbp region in front of the 3' UTR to encompass the promoter region. A 3HA tag was added to the N-terminus of the protein. The EcoR1 restriction site was employed to join *pBAP2* and *3HA-BAP2* to complete the construct. Two constructs were made: *pBAP2:3HA-BAP2* and *pBAP2:3HA-BAP2 D41A, D87A.*

3HAsequence:

TACCCATACGACGTTCCTGACTATGCGGGCTATCCCTATGACGTCCCGG

ACTAT GCAGGATCCTATCCATATGACGTTCCAGATTACGCTGCTCAG

construct primers: BAL TAABam: ccggatccagacggccgttgatacgc (adds BamH1 site, removes TAA from *BAP2*)

BamH1-3HA for: GGGATCCCGTACCCATACGACGTTCCAGAC (adds BamH1 site, removes ATG on the 5' end of 3HA, specific to the 1st HA)

SpeI-TAA-3HA rev: GACTAGTCTTACTGAGCAGCGTAGTCTGGG (adds TAA onto the 3' end of 3HA, specific to the 3rd HA)

NotI-ATG-BAP2 for: TTGCGGCCGCAAATGTCGTATTCAACATTC (adds NotI site on the front of BAP2)

Not1-3HA-BAP2 for: ttgcggccgcatgtacccatacgacgttccagac (installs Not1 site on 3' end, for cloning 3HA-BAP2 into pBSK for mobilization into pFGC1008 so the gene fusion is under a 35S promoter). The following vectors were used for constructing the transgene prior to cloning the completed version into

pCAMBIA-NOS (modified from pCAMBIA 1301 by Dr. H. Yang): pBSK SK+/-, Promega pGEM®-T EASY Vector System I. Plasmid DNA was prepared with Qiagen™ and Fermentas™ Miniprep kits. DNA extraction from gels was done with the Wizard® SV Gel & PCR Clean-up System.

Southern, Northern, & Western blotting

Samples were prepared with 0.1-0.15g of tissue with 2.5ml/1.0g protein grinding buffer. Experiments done according to *Molecular Cloning Handbook*, 2nd Edition, Vol1-2. The monoclonal antibodies for the HA tag (HA.11 clone 16B12) were synthesized and shipped to us from Convance™. GFP primary antibodies suspended in 3% BSA in TTBS buffer instead of 2% milk.

Immunoblotting

3HA and GFP probing was done with monoclonal mouse antibodies from Covance™. The secondary antibody was an antimouse goat, also from Covance™. Blots were washed 3 times for 20 min with TTBS (50mM Tris-HCl pH 7.5, 150mM NaCl, 0.1% Tween 20) in between blocking and antibody incubation. ECL detection was accomplish by mixing 1:1 lab-made solutions 1 & 2 which were stored @ 4^0C in the dark.

Solution 1: H_2O_2, Tris-HCl pH 8.8

Solution 2: Tris-HCl pH 8.8, *p*-coumaric acid, luminol

3HA-BAP2

To mutate the chosen amino acids (*D23, D88*) to alanines, we used the Strategene® Quik Change® II XL kit to introduce site-specific mutations.

GFP-BAP2

First, Dr. H. Yang cloned *BAP2* into the pSATC1 vector. We then mobilized the GFP containing cassette from pSATC1 by restriction enzyme digest with PI-psp1. The insert was cloned into pHTP vector, also cut with the same enzyme and treated with CIP to prevent self-ligation. Colonies were identified by colony-PCR and then the *35S::GFP-BAP2* containing the pHTP binary vector were transformed into C58C1 or GV3101 *A. tumafaciens* lines.

Protein expression in *E. coli*

Cloned *3HA-BAP2* wild-type and mutant from pGEM vector to pGEX5.1 by cutting with EcoR1 and **(?)**. Constructs were sequenced using pGEX5.1 primers as described in Strategene® manual. Used BL21-DE3 cells from Strategene®, standard protocol. Cells were grown overnight in LB+ Ampicillin media. Then added to 50ml culture, 100-fold dilution. Cells were grown to an OD_{600nm} of 0.5 and then induced with 0.5 mM IPTG. Measurements of cell growth were taken hourly and samples of pre and post-induction were saved for total protein gel. All gels were 12% acrylamide.

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