DELINEATION AND CHARACTERIZATION OF RUST RESISTANCE REGIONS IN COMMON BEAN *PHASEOLUS VULGARIS* L.

by

Antonette Todd

A dissertation submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant and Soil Sciences

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ABSTRACT

Common bean (*Phaseolus vulgaris* L.) is an important grain legume in many developing countries throughout the world. It is most useful for its high protein and dietary fiber content. The fungal rust pathogen *Uromyces appendiculatus* (Pers.) Unger can cause crop loss in susceptible cultivars of common bean. Of the 89 races of rust cultivated in the U.S., the *Ur-3* locus provides resistance to 44 of those races along with at least one other gene, *Crg* (*C*omplements *r*esistance *g*ene). *Crg* is required for *Ur-3*-mediated rust resistance. The release of the common bean genome enables the use of a whole transcriptome approach for an improved understanding of regions responsible for rust resistance. In order to better understand this interaction, several common bean genotypes were inoculated with bean rust race 53, leaf RNA isolated at numerous time points, and transcriptomes sequenced. Information generated from Illumina RNA-seq data was then used to analyze five genotypes, 'Sierra' (resistant) and 'Olathe' (susceptible), and three Sierra-derived susceptible mutants; crg, ur3-Δ2 and ur3-Δ3. RNA-seq data were used to identify and characterize the deletion region in crg (which carries a deletion of the *Crg* locus) using comparative gene expression analysis against Sierra. Genomic pcr and rt-pcr with candidate gene primers from this region indicates no amplification in crg, but indicates

amplification in Sierra. Subsequently, Mock Inoculated (MI) and Inoculated (I) samples of Sierra leaf RNA were compared for differential expression among candidate genes in the deletion region, which is approximately 250 kb. A similar approach was used in an attempt to identify deletion regions in ur3-Δ2 and ur3-Δ3. However, differential genomic pcr amplifications of selected molecular markers between Sierra and ur3-Δ2/ur3-Δ3 did not yield an alignment to any gene. Since identity of a mutation in any particular gene(s) was unidentifiable, data were collected and compared expression across the transcriptomes of Sierra MI and I and ur3-Δ2 I. This research demonstrates the identification of a disease resistance cluster located on chromosome 10 in common bean. It also employs methods that use both genomic DNA amplification of deletion mutants, paired with RNA-seq to identify possible genomic locations of interest in regard to pathogen resistance in common bean. Identification of differential expression among resistant and susceptible genotypes in disease resistance clusters in the bean genome may elucidate important genes underlying resistance. Detecting candidate gene regions may help in yield loss of common bean due to virulent races of rust on susceptible genotypes. Besides preserving favorable traits in the crop, but can also help in global sustainability of food stocks necessary for many populations.

INTRODUCTION

Throughout history, human beings have learned to manage crops for consumption to sustain the health and livelihood of themselves as well as livestock. In many cases, crops have evolved into the current varieties that are now consumed by people around the world. Common bean (*Phaseolus vulgaris L*.) has been cultivated for more than 8000 years, and contains two centers of domestication, which include Central and South America. It serves as a food staple for many developing countries throughout the world as well as being a protein replacement for many people with vegetarian diets. As a legume, common bean has a symbiotic relationship with rhizobia, forming nodules which aid in nitrogen uptake.

There are many examples of plant diseases that have had devastating effects on the population. One example in particular is the Irish potato famine in the mid-1800's. *Phytophthora infestans*, the most virulent pathogen on potato, is the oomycete that caused major crop loss during this time (Haas et al., 2009). More than 1 million people died due to lack of food (Woodham-Smith, 1991). Another example is Southern Corn Leaf Blight epidemic in the US (Tatum, 1971), which caused major damage to corn crops.

The fungal pathogen *Uromyces appendiculatus* (Pers.) Unger is virulent on susceptible cultivars of common bean. It is an obligate biotroph and reduces crop yield by colonizing mostly on the leaf surface of common bean. The pathogen may also colonize on other surfaces such as pods and stems.

In this study, methods were employed to challenge common bean with fungal rust in an effort to better understand the reaction of gene expression between the plant and pathogen. Candidate rust resistance gene regions were explored and annotate the delineated region on chromosome 10. Also, a transcriptome comparison study using RNA-seq data generated from rust resistant and susceptible bean plants was used to identify disease resistant clusters.

The goal of this research is to characterize gene regions responsible for fungal rust resistance in common bean. By identifying and characterizing gene regions, there may be a greater knowledge of resistance gene function and discovery of novel domain structure. Employed methods combine genetic mutation and deletion with gene expression data to obtain information of disease resistance in common bean. A greater appreciation of this pathosystem may help reduce global yield loss of bean that is normally susceptible to bean rust while preserving favorable traits. Maintaining higher bean yields may not only help economically, but is greatly important for global food security as it serves as an essential food crop throughout the world.

Chapter 1

LITERATURE REVIEW

1.1 Bean-Bean Rust Interaction

1.1.1 Common Bean

Common bean (*Phaseolus vulgaris L*.) is an economically important grain legume, particularly in many developing countries throughout the world. Diverging from a common ancestor over 100,000 years ago (Mamidi et al., 2013), two groups were first independently domesticated more than 8000 years ago in Central and South America (Schmutz et al., 2014). The most common landraces are generally divided into two groups; Meso (Middle) American and Andean. Meso American consists of wild beans that contain varieties from Mexico to Colombia, while Andean consists of beans from Peru to Argentina (Freyre et al., 1996). Meso American beans, such as pinto, have a tendency to be smaller than Andean beans, such as dark and light red kidney beans. Dry beans can be stored indefinitely under proper environmental conditions, lending to their value as an excellent food stock for developing and underdeveloped populations. Common bean's crop value in the US alone is more than 1 billion dollars (Bailey, 2014). Along with its economic importance, common bean is also

generally high in protein, about 30% of daily value (McClean et al., 2008), accounting for approximately 22% of its weight. The fiber content of bean makes it a great food for stabilizing blood sugar and cholesterol. Because of this, it also aids in the fight against obesity and diabetes (Chandalia et al., 2000).

Common bean is a diploid plant consisting of 22 chromosomes (2n=22) and has a genome size of about 587 MB, of which 473 MB has been assembled (Schmutz et al., 2014). It is a dicotelydon, meaning its seed has two embryonic leaves that emerge from the seed coat after germination. Growth habits are variable, growing anywhere from two to three meters tall. The flowers produce pods that contain usually four to six seeds per pod. Pods are harvested in both its dry stage and as fresh green beans. Beans harvested in their green stage include string beans, while beans harvested in the dry stage include the pinto and kidney market types. The pods and seeds may exhibit a spotted coat or they may be neutral in color. Common bean grows best in day neutral conditions, meaning it requires equal amounts of light and dark hours each day.

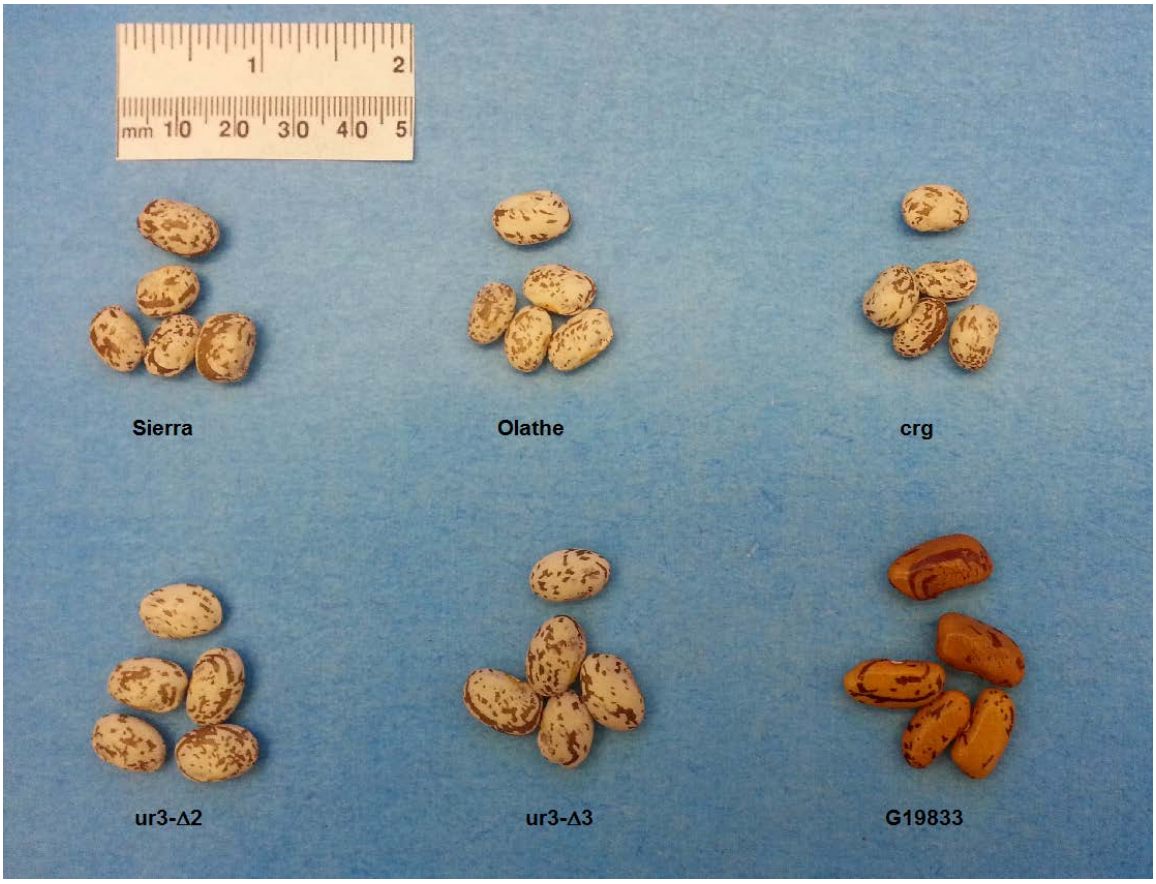


Figure 1.1 Six genotypes of common bean used in this research, including one reference genome (G19833). Sierra (resistant to rust race 53), Olathe (susceptible to rust race 53), crg (Sierra-derived mutant, susceptible to rust race 53), ur3-Δ2 and ur3-Δ3 (both Sierra derived mutants, susceptible to rust race 53).

Common bean is also valuable in its ability to fix nitrogen through root nodules in the presence of rhizobia (Mylona et al., 1995). In this symbiotic relationship, rhizobia take atmospheric nitrogen from the air into the soil where it is transformed into ammonia (NH_3). The ammonia is later converted into ammonium (NH_4) which can be used by the plant. Nitrogen is necessary for overall plant health. Nitrogen deficiencies may cause premature senescence, leaf yellowing and dropping.

Common bean is a member of the order Fabales, and is most closely related to soybean (*Glycine max*). According to the Delaware Department of Natural Resources and Environmental control (DNREC) (http://www.dnrec.delaware.gov/ClimateChange/Pages/ClimateChangeDelawareAgriculture.aspx), in Delaware, soybean yields $28 million a year, second only to corn ($36 million). Michigan State University released the pinto common bean variety Sierra in 1989 (Kelly and Copeland, 1994). It was the first of many released, and was chosen because of its competitive yield, resistance to the fungal rust pathogen *Uromyces appendiculatus* (Pers.) Unger and its type two growth habit, upright. It was bred using nine commercial pinto varieties and 16 navy and black bean breeding lines (Kelly and Copeland, 1994). Although Sierra is resistant to all rust races in Michigan and many in other production areas, it appears to flower later than other pinto varieties, adding approximately 10 days to maturation. The Sierra cultivar is important as it serves as the progenitor for the mutants derived in this research. Comparatively, the Olathe (G18350) cultivar

serves as a race 53 susceptible wild type bean in our research, used as a naturally susceptible genotype to compare to Sierra (Figure 1.1).

The common bean cultivar Sierra exhibits a hypersensitive resistance response when it is challenged with fungal pathogen bean rust race 53. In this response, necrotic legions will form at the point of rust pathogen's entry into resistant plants. The pathogen must penetrate the leaf tissue in order to produce a response. Conversely, the common bean variety Olathe is susceptible to race 53 because it does not contain the *Ur-3* locus. In this response, no necrotic legions will be apparent, rather small white spots followed by rust colored uredia. Similarly, the three mutant genotypes are susceptible to the bean rust pathogen. The mutants, crg (*Ur-3*, *crg crg*), ur3-Δ2 (*ur-3*, *Crg Crg*), and ur3-Δ3 (*ur-3*, *Crg Crg*) were identified during a forward genetics study conducted by Kalavacharla et al., aimed at identifying rust susceptible mutants in an effort to isolate resistance loci (Kalavacharla et al., 2000). In forward genetics, the particular phenotype is known, but the genotype is unknown. In order to match the phenotype with the genotype(s), the plant is mutated to create the desired phenotype. Then the desired phenotype in molecularly screened to identify the genotype.

1.1.2 Fungal Rust

Basidiomycetes are the cause of disruptive pathogenic groups that attack many plants. These include fungal smuts and rusts. Fungal rust has been the cause of

economic ruin in areas as large as an entire country (Helfer, 2014). The *Puccinia* genus is the largest group of rusts, including *P. graminis*, which causes rust on rye, barley, and wheat. Some rust also have special forms that are of the same species but infect different hosts, i.e., f. sp. (formae specialis) *tritici* on wheat and f. sp. *hordei* on barley.

The Basidiomycete fungal pathogen *Uromyces appendiculatus* (Pers.) Unger, which affects common bean, causes the development of rust pustules on susceptible varieties. Although some rusts can be grown in special media in the laboratory, most are obligate biotrophs. As an obligate biotroph, *U. appendiculatus* needs a living plant host in order to propagate and cannot be cultured. *Uromyces appendiculatus* flourishes in temperatures between 17-25 degrees and high humidity (>95%). In optimal cases, the fungal pathogen can cause major crop losses when the temperature and humidity are favorable for fungal growth. Tissue that is penetrable by fungal urediospore germination is also necessary for the fungus to gain entrance. Once the urediospore germinates, it forms a germ tube, which contains cytoplasm and two nuclei (Cooper et al., 2007). When the germ tube recognizes a height difference between the leaf surface and the stomata, an appressorium forms. At this point, the two nuclei are released through the stomata. Hyphae form and increase throughout the leaf area. The haustorial mother cell is responsible for entering the leaf cell and obtaining nutrients (Cooper et al., 2007), resulting in a parasitism relationship. Once this occurs, uredia will begin to form.

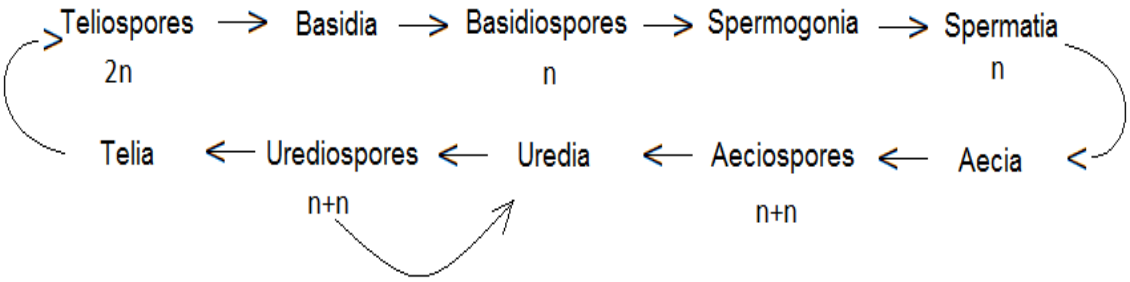


Figure 1.2 Macrocyclic rust cycle showing all spore types. Urediospores can repeatedly infect the same host plant by geminating and creating more uredia.

Uromyces appendiculatus is macrocyclic and has five distinct spore stages: teliospores, basidiospores, spermatia (pycniospores), aeciospores, and urediospores (Figure 1.2). An asexual part of the cycle, teliospore production, is a prerequisite to the development of the spore stages linked with the sexual cycle (basidiospores, urediospores, and aeciospores). Teliospores (Figure 1.3) are the hibernating, overwintering spores that lay dormant when conditions for spore growth are less optimal, such as cold weather in winter months. In optimal conditions, the germinating teliospores will produce basidia that in turn release basidiospores. Basidiospores will germinate on the leaf surface, and produce spermagonia. Spermagonia contain both haploid spermatia (male gamete) and receptive hyphae to produce dikaryotic mycelia, which form aecia. Aecia produce aeciospores and travel by wind or other contact to other beans. Dikaryotic mycelia form and produce uredia that produce urediospores (Figure 1.3). Urediospores will germinate, entering through the stoma, which act as guards on the leaf surface, allowing water in and out when needed. At this point, the urediospore can keep reproducing uredia if the conditions are favorable, causing repeating cycles of infection. If conditions are not favorable, they will produce the overwintering telia.

Unlike heteroecious rusts, such as some cereal stem rusts, common bean rust *U. appendiculatus* is autoecious and do not require an alternate host to complete its life cycle (Cooper et al., 2007). This indicates that *U. appendiculatus* is specific to bean and cannot infect any other plant type. An example of a heteroecious rust is *Puccinia graminis*, which begins its cycle on barberry and completes it on wheat (Figueroa et al., 2013).

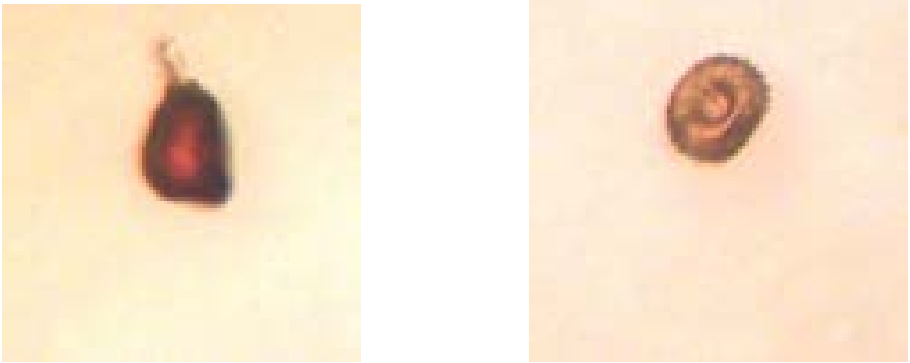


Figure 1.3 Teliospore (left panel) and urediospore (right panel) at 40x magnification from a single collection from inoculated common bean plants. The teliospore is the overwintering spore type. The urediospore is the repeating spore stage.

Rust spores are not visible to the naked eye immediately after infection. After a few days to a week, small white spots will begin to form on the leaf surface. The infected leaf has visible rust colored pustules that are more frequent on the adaxial (upper) surface a week to 10 days after the initial inoculation. On infected, susceptible leaves, there are hundreds of uredia visible. Each uredium is also characterized by a yellow halo (Figure 1.4). Eventually, the spore number inside the uredium becomes so large it bursts, releasing more urediospores (Figure 1.4). Here, the urediospores can move to other susceptible plants and infect them. Uredia are the only spore types that are capable of reinfection of the same host (Bolton et al., 2008).

Figure 1.4 Susceptible bean leaf inoculated with race 53 fungal rust pathogen.

The inset shows a cluster of urediospores that have burst open from one pustule.

1.1.3 Global Impact of Common Bean and Bean Rust

Globally, common bean is nutritionally important, especially in places such as eastern Africa and Latin America. Not only does common bean supply nutrients such as protein, carbohydrates, and other vitamins (Namugwanya et al., 2014), they also store indefinitely in their dry form, lending to cost effectiveness. Throughout the world, more than 12 million metric tons of beans are produced yearly, with most (5.5 million metric tons) being produced in Latin America (Petry et al., 2015). Common bean is also highly produced in India (4 million metric tons), followed by 2.5 million metric tons produced in eastern Africa. The per capita amount consumed is highest in southern and eastern Africa (Buruchara et al., 2011). However, consumption and production data sometimes go undocumented due to information not reported from rural areas.

Greater than 10% of all food crops cultivated for consumption is lost to plant pathogens (Strange and Scott, 2005). Fourteen crops make up the bulk of food produced for the entire world (Strange and Scott, 2005), and common bean is one of those crops. Fungal rust plays an important role in common bean crop loss, particularly in places with tropical and subtropical climates. This is due to the high humidity and optimal temperature and environment that causes disease in places such as Brazil (Souza et al., 2013) and Uganda (Odogwu et al.). Aside from abiotic stresses, such as drought conditions in some parts of Africa, rising problems with rust also leads to lowering crop yields in places where bean is a

necessary part of the local diet. Understanding growth conditions and plant-pathogen interactions in areas with an increased disease environment is paramount in dealing with major crop yield loss. This is especially necessary with a crop as important as common bean, upon which global population is heavily dependent.

1.1.4 Plant Disease

Plant disease occurs when a plant is overcome by an outside bacterial, viral, or fungal pathogen. In most cases, plants are able to use physical barriers to keep out pathogens and to resist succumbing to them. Sometimes, plants are unable to protect themselves from ravaging effects of these virulent pests. When this occurs, host and pathogen interaction is said to be compatible, meaning the pathogen is able to use the host for a source of survival. In some cases, the pathogen feeds off of dead tissue and needs to kill the plant first. In other cases, pathogens need a live source of nutrients in order to propagate. This is the case in common bean and fungal rust.

1.1.5 Types of Plant Disease Resistance

1.1.5.1 Non-host Resistance

Some plants will be attacked by plant pathogens that will have no effect on them at all. This is the case in non-host resistance. The pathogen can do no harm because the environment and conditions are not suitable enough to sustain itself

(Hammond-Kosack and Jones, 1996). In other cases, plants will respond with various levels of basal or adaptive defenses such as basal, adaptive, or lack sufficient defense, which results in susceptibility.

There are two types of non-host resistance; Type I and Type II. In type I, which is also the most common type, there is no hypersensitive response (HR) and usually no symptoms are visible (Uma et al., 2011). In this case, fungal pathogens are not even afforded the opportunity to penetrate the plant surface. In type II, HR induces necrosis quickly as the pathogens are overcome by preformed and induced defenses. Plants release enzymes that are detoxifying, allowing the plant to use HR as a defense mechanism (Uma et al., 2011). The plant is not susceptible to the pathogen; therefore the pathogen will not have the opportunity to propagate.

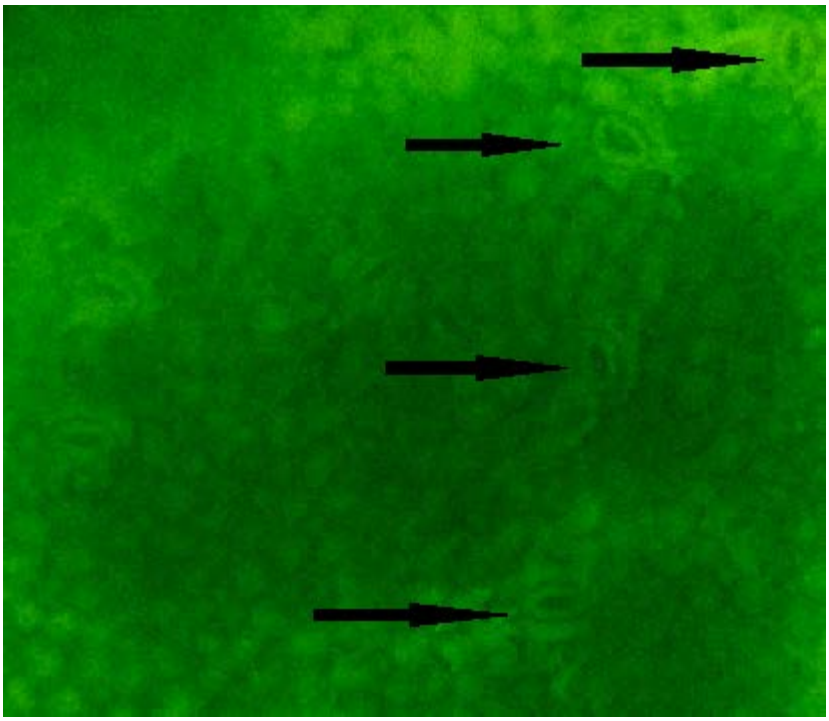


Figure 1.5 Common bean leaf surface at 40X magnification on a standard light microscope. Several stomata are labeled with arrows pointing towards their openings. Here, urediospores will germinate and use the stomata to penetrate the surface.

1.1.5.2 Basal Defense

Unlike animals, plants are not able to move away from outside pathogenic and physical attack. However, plants have an innate ability to protect themselves from outside attack through many different avenues. Some plants have thick, wax-covered leaves or needles to ward off being eaten by insects and other animals. A plant may use its physical barrier to keep viral, bacterial, and fungal pathogens from gaining access to their vascular system. When this does not work, through a network of signals, plants can use its basal defenses. The plant may use pattern recognition receptors that react to pathogen associated molecular patterns (PAMPs), sometimes termed microbe associated molecular patterns (MAMPs) (Jones and Dangl, 2006). P/MAMPs are crucial components of pathogens that allow the host to distinguish itself from the pathogen and stimulate signs connected with innate immunity (Tang et al., 2012). These terms are often associated with PAMP triggered immunity (PTI).

There are several different categories of molecular patterns and effectors that induce plant defenses. P/MAMPs can induce PTI through the recognition of specific patterns by a plant. Aside from P/MAMPs, there are also microbe induced molecular patterns (MIMPs) (da Cunha et al., 2006) and wound herbivory induced molecular patterns (WHIMPs) that induce resistance in plants (collectively called "DAMPs" for damage associated molecular patterns). As a plant is wounded by insect chewing, basal defenses help protect it from being

completely destroyed. In some cases, the plant will use pattern recognition and close its stomata to prevent the pathogen from entering (Zeng, 2010). As mentioned earlier, the stomata are guard cells on the hosts' surface that allow moisture in and out (Figure 1.5). It can also serve as an entrance for pathogens to gain access to the hosts' system. In the case of common bean, stomata pressure relaxes in low light, causing them to open wider. This may allow the pathogen to penetrate the leaf surface in lower light (Lawson, 2009).

1.1.5.3 Adaptive Defense

Unlike basal defense, in adaptive defense, resistance (R) genes are present in the host plant. *R* genes in the host interact with avirulence genes in the pathogen to induce adapted defenses. Jones and Dangl (2006) describe the network of pattern recognition and plant immunity through the zig zag model. Molecular patterns caused by the pathogen triggers a plant immunity response. Sometimes pathogens are able to successfully bypass the PTI response and release effectors into the plant. Effector triggered susceptibility (ETS) occurs when the pathogen releases effectors that infect the host, and go unrecognized by the plant. Effector triggered immunity (ETI) happens when specific proteins in the plant recognize pathogen effectors. This recognition of pathogen effectors by the host receptors is the avirulence gene-resistance gene interaction that provides gene-for-gene resistance in the host.

1.1.6 The Guard Hypothesis

The two types of plant immunity, PAMP triggered immunity (PTI) and Effector triggered immunity (ETI), are used by the host to combat pathogen attack. PTI is the initial step in plant immunity. PTI triggers innate immunity, and is associated with low resistance. The pathogen will deploy effector proteins into the host, resulting in ETI. ETI is more durable and is recognized by R proteins. Suppression occurs in several ways including vesicle trafficking, organelle alteration, interference of immune receptor signaling. Through a needle-like

structure, the bacterial pathogen will use a secretion system to inject effectors into the host.

In PTI, there are proteins such as flagellin, a prokaryotic elongation factor called EF-Tu, and chitin that are recognized by receptor-like kinases (RLK) (Block and Alfano, 2011). The proteins are recognized by immune receptor complexes that include EF-Tu Receptor (EFR), recognized by EF-Tu, Chitin Elicitor Receptor Kinase 1 (CERK1), recognized by chitin, and Flagellin-sensitive 2 (FLS2), which is recognized by flagellin (Block and Alfano, 2011). Once PTI is overcome, ETI is activated by proteins in the host that monitor for modifications caused by the pathogen. Nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) are responsible for guarding host proteins that attract effectors (Nishimura and Dangl, 2014). The R protein will wait for T3Es to make modifications in plant proteins and release effectors. The host will produce the “bait” and the R protein will act as a “switch” that is turned on or off depending upon it’s recognition of the bait. This is the case in which the “guard hypothesis” works.

A large family of WRKY transcription factors is essential in disease resistance and response to stress. The WRKY domain serves as a target for plant pathogen effectors. There are WRKY-like “decoys” used to attract pathogen effectors that deflect attention from the true target in order to suppress ETI. In other cases, the NLRs work in pairs to trigger effector responses (Dangl et al., 2013). They work

as “sensor” and “helper” NLRs where one will attract the effector and become activated by the effector, while the other is the functioning NLR. Because there are typically more pathogens than R proteins, it is postulated that the R protein typically guards the host protective proteins (Birch et al., 2006).

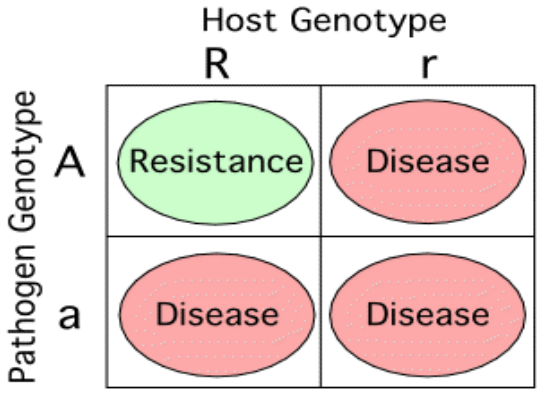
1.1.7 Local and Systemic Acquired Resistance

Local acquired resistance (LAR) and systemic acquired resistance (SAR) are obtained when a biotic or abiotic stress is introduced to the plant. These interactions in turn cause the hosts to achieve resistance, not just at the initial point of contact as is the case with LAR, but throughout the entire plant in SAR. Both terms were first described by Ross in 1961 while studying tobacco plant infected with tobacco mosaic virus (TMV) (Ross, 1961a; Ross, 1961b). He first noticed with LAR that a 1-2 mm area around the site of pathogenicity would gain resistance to TMV. In SAR, he challenged half of the leaves on a specific variety of tobacco and noticed that the unchallenged half also gained resistance when challenged with the virus several days later.

1.1.8 Gene-for-Gene Mediated Disease Resistance

In 1942, H.H. Flor first published his work on genetic pathogenicity in the flax (*Linum usitatissimum*) rust pathogen *Melampsora lini* L. The genetic foundation of the gene-for-gene theory was first demonstrated by Flor (Flor, 1956) when he conducted research on the interaction between flax and the flax rust pathogen. He was the first to study pathosystems, the pathogen and host simultaneously.

He discovered that plants inherited resistance and pathogens inherited the ability to cause virulence on hosts in gene pairs. In gene-for-gene interactions, a host plant carries the resistance genes that interacts with corresponding avirulence (avr) genes in the pathogen. In Flor's gene-for-gene theory, every *R* gene in the host has a corresponding, complementary avirulence gene in the pathogen (Figure 1.6). The corresponding avirulence gene interacts with the *R* gene in the host. If either the *R* gene in the host or the avirulence gene in the pathogen is missing, there will be no gene-for-gene resistance. Even if a host carries an *R* gene, but is attacked by a pathogen that does not hold a corresponding avirulence gene, disease is inevitable.



http://www.uvm.edu/~tpdelane/lab/images/Quadratic_GforG.gif

Figure 1.6 Punnett square of pathogen Avirulence (*A*) genes interacting with host Resistance (*R*) genes. Resistance can only occur when the resistance gene is present in the host and the avirulence gene is present in the pathogen.

1.1.9 Cloned *R* Genes

Cloning has opened many opportunities for analysis of function and structure in proteins encoding *R* genes (Bent, 1996). The first *R* gene cloned was *Helminthosporium* 1 (Hm1) in in maize (Johal and Briggs, 1992). Although this gene does not follow the gene-for-gene theory, study of the structure and function revealed that it specifically controls resistance against *Cochliobolus carbonum* race 1. It does this by directly disabling the toxin through encoding a NAPDH-dependent reductase that inactivates the *Helminthosporium carbonum* (HC) toxin (Sindhu et al., 2008).

The first *R* gene cloned to follow Flor's gene-for-gene theory was *Pto* in tomato (Martin et al., 1993). It confers resistance to *Pseudomonas syringae* strains that contain the avrPto protein kinase. *Pseudomonas syringae* is a bacterial pathogen that causes speck disease on tomato. In the gene-for-gene interaction, tomato plants will become necrotic at the site of infection when challenged with the pathogen.

Hypersensitive response (HR) causes the plant cells to commit apoptosis in order to save the plant. This typically occurs within the first few hours of pathogenesis (Petre et al., 2012), but can happen as late as 10 days as in the case of common bean challenged with fungal rust. The HR between obligate biotrophs such as fungal rust and bean is clearer than those between hemi-biotrophs and necrotrophs and their hosts. Obligate biotrophs need live tissue in

order to propagate whereas necrotrophs need dead tissue. One the other hand, hemi-biotrophs start with live tissue to gain access to the host. Once it colonizes the host, it will cause necrosis and live off of the dead tissue (Glazebrook, 2005). Therefore, when plant cells become necrotic at the site of pathogen infection, the obligate biotroph will no longer be able to survive due to lack of nourishment. There are several classes of *R* genes that are marked by specific motifs that are synonymous with disease resistance.

1.1.10 Functional Domains in *R* genes

1.1.10.1 Leucine Rich repeats

Leucine Rich Repeats (LRR) are a set of about 24 amino acid first described by Takahashi (Takahashi et al., 1985), and play an important role in pathogen recognition (Bell et al., 2003). The leucines or other hydrophobic residues, such as prolines and asparagines, are spread regularly throughout the chain (Bent, 1996). One LRR crystal structure contains a porcine RNase inhibitor, which produces a tertiary structure. This tertiary structure resembles a fist, with the "fingers" being a LRR domain. In porcine RNase inhibitor structures, repeats are longer than usual, as opposed to Beta helical structured, which are shorter (Bent, 1996).

LRR proteins from yeast, humans, and other species seem to control protein-protein interactions (Bent, 1996). Several examples include enzymatic interactions with its enzyme inhibitor, transmembrane receptors binding of peptide hormones, and a signal transduction's cascade from the interaction of intracellular components. Leucine-rich repeats may also be a facilitator in the interaction of defense signal transduction with *R* gene products (Bent, 1996). *R* genes in this class include the *Cf-2* (Dixon et al., 1996), *Cf-5* (Dixon et al., 1998), and *Cf-9* (Jones et al., 1994) in tomato. LRRs are the most common structural domain among *R* genes.

1.1.10.2 Nucleotide Binding Site

The nucleotide binding site (NBS) (also referenced as P-loop) domain is a highly conserved region in the *R* gene and is essential for protein function (Bent, 1996). They are necessary to bind TGTP and ATP. Their conserved presence among *R* genes indicates that they are essential for protein function, making them an important component of disease resistance. Most *R* genes contain the domain, in many cases being flanked by two other conserved domains on its ends, including Leucine Rich repeats and TIR (Toll/Interleukin-1 Receptor).

1.1.10.3 TIR (Toll/Interleukin-1 Receptor)

NBS-LRR domains can be classified into subdivisions of families that include TIR and Non TIR (Toll/Interleukin-1 Receptor) domains (Garzón et al., 2013). Toll receptors play a key role in innate immune response. Originally, they were described through comparisons between *Drosophila* and mammals. We now know that Toll receptors are also abundant in *Arabidopsis* (Jebanathirajah et al., 2002). The tobacco *N* gene was the first to be characterized, which encoded a TIR domain (Whitman et al., 1996).

1.1.11 Current Perspective of Disease Resistance

Plant-pathogen interactions have been a necessary study to understand the relationships and mechanisms that drive disease resistance (Gururani et al., 2012). For more than a hundred years, people have used methods such as

breeding to try to control immune receptors in plants (Nishimura et al., 2015). The number of cloned *R* genes has greatly increased over the last 20 years. As more plant *R* genes are isolated and cloned, there is new promise that scientists may obtain a better understanding pathogenicity and gene function, which can ultimately be used to create more resistant plant varieties. The identification and isolation of *R* genes is advantageous, as developing disease resistant varieties in plants may be a more suitable approach than the use of pesticides to manage virulent pathogens on crops. Because the long term effects of pesticide use is not completely known, many consumers now opt for organically raised crops. While humans have been purposely studying *R* genes for a hundred years, pathogens have been evolving for much longer (Nishimura et al., 2015).

Disease resistance genes usually contain signature motifs that are recognizable as such. It is understood that NBS, LRR, and TIR domains are normally present in *R* genes, making them easy to identify in a group of amino acid sequences. However, recent work by two groups, (Le Roux et al., 2015) and (Sarris et al., 2015), reveal unusual domains anchoring themselves to nucleotide binding oligomerization domain(NOD)-like receptors (NLRs) (Nishimura and Dangl, 2014). One of these unusual domains is the aforementioned WRKY-like or WRKY transcription factor-like domain that is fused to the RRS1 gene in Arabidopsis. The RRS1 and RPS4 complex are paired with the WRKY domain to provide recognition of two bacterial effectors. However, when the WRKY-like domain anchors itself to this complex instead, the bacterial effectors never

interact with the true target. Therefore, instead of pathogen effectors being intercepted by true WRKY domains, they are intercepted by an imposter. Hence the WRKY-like domain attracts the pathogen effectors and suppresses the immune response (Le Roux et al., 2015; Sarris et al., 2015). The current plant disease perspectives seek to use the pathogen effectors as a means to exploit *R* gene mediated resistance (Vleeshouwers and Oliver, 2014). Initially, pathogen effectors were believed to directly interact with host *R* genes. It is now understood that the primary role of pathogen effectors is to alter the host's cellular function and create a better environment for itself (Link et al., 2014). The hemibiotrophic oomycete pathogen *Phytophthora infestans* secretes the effector protein AVR3a into the host, potato. There are two forms of AVR3a, which are $AVR3a^{KI}$ and $AVR3a^{EM}$. $AVR3a^{KI}$ strongly suppresses infestin 1 (INF1) induced cell death (ICD) in potato interacting with the R3a protein. $AVR3a^{EM}$ weakly suppresses INF1 ICD when interacting with R3a (Bos et al., 2010). This interaction suppresses HR by recognition of the R3a protein in the host by AVR3a (Engelhardt et al., 2012).

In obligate biotrophs, such as *Puccinia graminis* and *Melampsora larici*, two advances in genome sequencing have allowed the identification of predicted gene sequences, which also include conserved domains present in powdery mildew (Rafiqi et al., 2012). This conserved domain consists of an 8-cysteine or 10-cysteine pattern. Observing conserved domains among pathogens may be the key to identifying effector function.

Recently, transcriptome information for *Uromyces appendiculatus* and *Phakopsora pachyrhizi* was published, which also categorized candidate effector families (Link et al., 2014). Rust pathogen effectors are introduced into the host through haustoria. Link et al., 2014 isolated the transcripts of haustoria from two rust fungi in an attempt to identify pathogen effectors. Currently, there is no whole genome sequence or EST (expressed sequence tags) database available for *U. appendiculatus*, which allowed novelty in their approach. There is no publicly available information for which to compare the resulting transcript data. However, through comparison to *P. pachyrhizi* data that is available, they were able to identify candidate effector proteins that were associated with haustoria as opposed to resting or water-grown urediospores (Link et al., 2014).

1.2 Common Bean and Race 53

There are over 250 known races of fungal rust, with 89 races being curated at the USDA in Beltsville Maryland. Of the 89 races curated in the US, the *Ur-3* gene controls hypersensitive resistance response to more than 40 races of fungal rust. The rust resistance gene *Ur-3* was discovered by B.J. Ballantyne (Ballantyne, 1978). The gene is located on linkage group 11 (chromosome 11) in bean (Freyre et al., 1998; Kelly et al., 2003) and is linked to the *Co-2* gene which is responsible for anthracnose resistance (Mastenbroek, 1960). To date, the Ur-3 gene has not been isolated. Therefore, we are unsure of the conserved domains within the genes.

The common bean cultivar Sierra was derived from a number of crossings between nine pinto bean varieties along with 16 navy and black bean varieties over a period of about 4 years (Kelly and Copeland, 1994). It is characterized by cordate (heart-shaped) to ovate (oval-shaped) shaped leaves and bears small seeds which are usually found in Meso American varieties of bean. Depending on temperature and daylight conditions, Sierra can go from seed to seed in approximately 99 days (Kelly and Copeland, 1994). Sierra contains the rust resistance gene, *Ur-3*, which confers resistance to 44 of 89 races of rust curated in Beltsville, Maryland USDA.

In order to identify rust resistance genes in Sierra, Kalavacharla et al. (2000) used a forward genetics approach. They used fast neutron bombardment at a dosage level of 6.0 Gy (International Atomic Energy Agency) to irradiate large, random portions of the genome. Irradiated seeds (M_1) were planted to propagate more seeds. The collected M_2 seeds were placed in bulks and later screened with several races of rust, including race 53. Of 10,000 seeds initially screened, one was susceptible to race 53. After screening several thousand more, two additional race 53-susceptible plants were identified. The plants were named ur3-Δ1 (later named crg), ur3-Δ2, and ur3-Δ3 (Table 1.1) (Kalavacharla et al., 2000). The resulting mutants became the basis of research which helped to identify differential amplification among several molecular markers matched with rust-susceptible phenotypes.

Table 1.1 (Adapted from Kalavacharla et al., 2000) Phenotypic distribution of pathogenic response of F1 and F2 plants of populations of crosses involving mutants crg and ur3-Δ3 to *Uromyces appendiculatus* race 53.
[a] HR = hypersensitive response; grade = 2 (chlorotic or necrotic hypersensitive lesions less than 0.3-mm diameter in size with no sporulation).
[b] Uredinia = presence of uredinia. Rust uredinia grades: 3 = sporulating uredinia less than 0.3 mm in size; 4 = sporulating uredinia 0.3 to 0.5 mm in size; 5 = sporulating uredinia 0.5 to 0.8 mm in size; and 6 = sporulating uredinia greater than 0.8 mm in size.

	F_1 pathogenic response		F_2 pathogenic response		
Cross	**HR[a]**	**Uredinia[b]**	**HR**	**Uredinia**	**Ratio**
crg × cv. Sierra	5	…	74	24	3:1
ur3-Δ3 × Sierra	2	…	44	9	3:1
crg × ur3-Δ3	4	…	121	91	9:7
	…	…	…	…	1:1
crg × cv. Olathe	…	6	144	91	9:7
	…	…	…	…	3:1
ur3-Δ3 × Olathe	…	2	0	71	0:1

The resistance gene analog (RGA) SB1 primer (Rivkin et al., 1999) was used to screen Sierra, Olathe, and the three Sierra-derived mutants through genomic pcr. The SB1 molecular marker amplifies in all genotypes except for crg. Of all five genotypes analyzed, Sierra is the only one resistant to rust race 53. Previous information provided from the Bean Improvement Cooperative published linkage map suggests that the *Ur-3* locus is on chromosome 11 in bean. We initially focused on chromosome 11 to detect differential expression between the two wild type genotypes. Two more molecular markers SK14 and SAE19 co-segregate with a rust resistant phenotype in Sierra and are believed to co-segregate with the *Ur-3* gene.

Chapter 2

DELINEATION AND CHARACTERIZATION OF A RESISTANCE GENE REGION IN COMMON BEAN MUTANT "crg"

Common bean (*Phaseolus vulgaris* L.) is an economically and nutritionally important crop throughout the world. It is a high protein, low cost food. It is also rich in dietary fiber, which may help lower blood sugar in type-2 diabetes (Chandalia et al., 2000). With the advent of the fully sequenced bean genome, scientists are now able to characterize regions that were previously a mystery.

Pathogens can impede growth when a plant does not have the means to protect itself from virulent diseases, resulting in a susceptible plant. In other cases, plants defend themselves from disease-causing pathogens through several types of resistance, including non-host, basal, and adaptive defense. The fungal rust pathogen *Uromyces appendiculatus* affects common bean by infecting leaf, stem, and pod tissues, causing lower crop yield and disease propagation. In the optimal conditions, yield loss may approach 100 % (Pastor-Corrales and Lieberman, 2010). Resistance (*R*) genes are adaptive defenses and provide protection from devastating effects of pathogen virulence on host plants. Pathogens will induce a signal cascade in which an *R* gene will receive the

signal. The *R* gene will induce protein expression at various levels to provide *R* gene-mediated resistance. Reported in this dissertation is the delineation of a cluster of genes associated with disease resistance in mutated common bean on chromosome 10. Isolated was the location of a molecular marker associated with the resistant genotype ‘Sierra’ and correlated the absence of this marker in a rust susceptible mutant, crg. There are several genes in this region were identified that exhibit differential expression between mock inoculated (MI) and rust inoculated (I) samples of resistant Sierra, including a gene with previous unknown function.

An early version of a common bean global transcriptome analysis was generated using the Sierra cultivar (Kalavacharla et al., 2013). More recently, a common bean transcriptome, which is publicly available, was generated using an Andean landrace (Schmutz et al., 2014). Chaucha Chuga, or more commonly known as G19833, originates from the South American country of Peru. It serves as the Meso-American control in this study. G19833 is a common bean plant with an intermediate growth type. Comparatively, the leaves of G19833 are larger than those of genotype Sierra (Figure 2.1). G19833 also produces seeds during times of the year when days are shorter. Unlike cultivar Sierra, G19833 develops more during the winter months than in spring and summer.

Although the genotypes appear to have several physical and morphological differences, the ability to use the recently published genome as a source for gene

comparison will propel the study of disease resistance within this pathosystem. The goal of this research is to identify disease resistance gene loci, characterize, and conceive approaches for future use.

In gene-for-gene interactions, for every *R* gene present in the host, there is a corresponding avirulence gene in the pathogen (Flor, 1942). Common bean variety 'Sierra' confers resistance to 44 of 89 races of fungal rust races curated at the USDA in Beltsville, Maryland. A particular interest lies in the *Ur-3* resistance gene and its resistance to rust race 53. *Ur-3* genotypes are usually resistant to rust race 53 because there are no signs of disease when common bean plants containing this gene are challenged with this particular rust race. However, Sierra may display a hypersensitive resistance (HR) response when challenged with fungal rust spores. Mutated seeds from the common bean cultivar Sierra were generated using fast neutron bombarded with radiation, which randomly deletes large segments of DNA from the genome. This forward genetics technique can be used to screen for mutants for a specified trait of interest in the attempt to identify genes associated with the trait. The first group of mutated seeds planted was called the M_1 population. This generation of seeds is planted and then self-pollinated to produce the M_2 population. Therefore, to screen for mutations in disease resistance genes, a large number of M_2 progeny resulting from the seed exposed to the fast-neutron bombardment have to be screened properly. Conversely, a Sierra-derived mutant 'crg', resulting from fast neutron bombardment, is susceptible to rust race 53, allowing the growth of rust pustules

on leaves, stems, and pods. Along with crg, two more Sierra-derived mutants (ur3-Δ2 and ur3-Δ3) have been identified through classical breeding (Kalavacharla et al., 2000). In order to verify if the mutations were in a single locus or multiple loci, Mendelian crosses were made among all mutant pairs. A cross between ur3-Δ2 and ur3-Δ3 yielded all race 53-susceptible progeny in the F_1 and F_2 generations. However, crosses made between crg and ur3-Δ2/Δ3 yielded all race 53 resistant progeny in the F_1 generation and a 9:7 resistant to susceptible ratio for the F_2 generation. This meant that two different genes were responsible for collectively providing resistance to race 53. Aside from differing phenotypic ratios in crg and ur3-Δ2/Δ3, they also appeared to give varying levels of susceptibility when challenged with rust race 53. Mutant crg has smaller pustules than ur3-Δ2/Δ3. Molecular analysis through polymerase chain reaction (pcr) also identified amplifications of the resistance gene analog (RGA) SB1 in genotypes including Sierra, Olathe (a naturally-occurring race 53-susceptible wild type), and susceptible mutants ur3-Δ2/Δ3. The SB1marker does not amplify in crg (Table 2.1). This information further confirmed that the mutation in crg is in a different locus than that of ur3-Δ2/Δ3.

In this study, we sought to identify deletion regions in mutated Sierra genotypes. By using this information, we sought to analyze differential expression of genes among Sierra MI and I within the deletion region identified in the mutated genotypes.

Figure 2.1 Six genotypes at approximately three weeks old. 1. Sierra, 2. Olathe, 3. crg, 4. ur3-Δ2, 5. ur3-Δ3, 6. G19833. Sierra and crg are markedly shorter than the rest, lacking the apical shoot apparent in the others.

Table 2.1 All genotypes used in this study with genotype, molecular marker and phenotype information. SAE19 with the noted asterisk (*) amplifies in mutants ur3-Δ2 and ur3-Δ3 at a size of ~2100bp as opposed to ~890bp in Sierra and crg.

	Genotype	Markers	Phenotype
Sierra	(*Ur-3,Ur-3/Crg,Crg*)	SK14/SB1/SAE19	Resistant to race 53
Olathe	(*ur-3,ur-3/Crg,Crg*)	SB1	Susceptible to race 53
crg	(*Ur-3,Ur-3/crg,crg*)	SK14/SAE19	Susceptible to race 53
ur3-Δ2	(*ur-3,ur-3/Crg,Crg*)	SB1/SAE19*	Susceptible to race 53
ur3-Δ3	(*ur-3,ur-3/Crg,Crg*)	SB1/SAE19*	Susceptible to race 53

2.1 Materials and Methods

In this study, six common bean genotypes were used (Figure 2.1), which includes race 53 rust resistant 'Sierra', race 53 rust susceptible 'Olathe', and three race 53 rust susceptible mutants crg, ur3-Δ2 and ur3-Δ3, which were derived from Sierra. Additionally, for verification of IDT designed primers (Integrated DNA Technology, Iowa 52241), common bean genotype G19833 was used for *In-Silico* pcr (Hinrichs et al., 2006). This allowed the identification of primer pairs that would amplify in the published common bean genomic DNA without ordering faulty primers. Plants were grown in the greenhouse as per standard conditions to collect leaves for isolating DNA. Plants were grown long enough to collect at least one leaf. After collection, plants were maintained for seed growth and propagation.

2.1.1 DNA Isolation

DNA isolation was done according to the CTAB method (Doyle, 1991). Plant leaves were collected by genotype and flash frozen in liquid nitrogen and stored at -80˚C until use. Occasionally, leaves were processed immediately after collection, being first flash frozen in liquid nitrogen. Approximately two grams of each leaf tissue were ground and added into a 50 milliliters (ml) screw cap tube with 10 ml of preheated 60˚C CTAB buffer (CTAB (Hexadecyl Trimethyl-Ammonium Bromide), Sodium Chloride, 2 M Tris-HCL (pH 8.0), 0.5 M EDTA (pH 8.0), 2-mercaptoethanol, and distilled water). The ground leaf tissue in tubes was

incubated at 60˚C for 30 minutes in a hot water bath. A mixture of 24:1 Chloroform: Isoamyl alcohol (10 ml) was added to each tube and shaken vigorously. Pressure was released by uncapping the tubes periodically after shaking. The tubes were centrifuged at 3500 reps per minute (rpm) for 15 minutes using a refrigerated ultracentrifuge. The supernatant was removed from each of the tubes and placed in clean 50 ml centrifuge tubes. Cheese cloth was used sometimes to avoid getting large pieces of leaf tissue in the clean supernatant. Next, 5 ml of isopropyl alcohol was added to precipitate the DNA strands. Here, the samples usually stayed in the -20°C freezer for a half hour to overnight to precipitate the DNA as much as possible. Next, the samples were centrifuged at 3,000 rpm for 15 minutes. The supernatant was discarded and the pellet was washed with 10 ml of DNA wash solution (7.5 M Ammonium Acetate (pH 7.7), 95% Ethanol, brought up to one liter in distilled water) and kept in the cooler for at least 20 minutes. This step removes traces of chloroform. A second wash step was done. The supernatant was removed from each sample and the pellet DNA was placed upside down and allowed to dry. The pellets were then re-suspended in 200 microliters (µl) of TE buffer with RNase A (10 µl of 10 mg/ml RNase A per ml of TE).

2.1.2 DNA Clean-Up

All samples were then cleaned up using a 50:50 combination of phenol: chloroform (Sambrook and Russell, 2001). An equal amount of combination was

added to each of the tubes and shaken until the solutions were one. Samples were then centrifuged for one minute to separate the layer containing the DNA from the other. The top layer of liquid containing the DNA was then transferred to a clean micro centrifuge tube and kept for further clean-up. An equal amount of chloroform was added to the sample and shaken until an emulsion forms. The tubes were centrifuged for one minute at 3,000 rpm. The top layer was then transferred into a fresh micro centrifuge tube. Ethanol precipitation was then performed on the samples. Two times the amount of ice-cold ethanol was added to each tube and mixed well. The samples were precipitated for at least half an hour on ice. DNA recovery occurred by centrifugation at 0˚C for one minute at 3,000 rpm. The supernatant was then discarded. Seventy percent ethanol was added to each 1.5ml tube hallway and centrifuged at 4˚C at top speed for 2 minutes. This step was repeated a second time and supernatant was removed. The liquid was then allowed to evaporate from the pelleted DNA in an open area. After the liquid evaporated, TE buffer was added to each tube. The amount of TE buffer added was depended upon the size of the DNA pellet at the end of the isolation process. The amount usually ranged from 200-500 µl.

2.1.3 DNA Quantification

After extraction, all DNAs were quantified by NanoDrop 2000 spectrophotometer (ThermoScientific, US), and diluted to a standard concentration of 100 ng/µl. Simultaneously, the DNA was quantified by gel electrophoresis to ensure the

quantity and quality of each genotype can be visualized in agarose. We performed polymerase chain reaction (pcr) with the resistance gene analog (RGA) primer set SB1 and sequence characterized amplified region (SCAR) marker primer set SK14 in order to differentiate amplification patterns between the five genotypes used. Molecular markers SK14 and SB1 were used to confirm the integrity of each isolated DNA. Each 25 µl pcr reaction contained 16.25 µl of distilled autoclaved water, 5 µl of 5x taq buffer with $MgCl_2$, 1 µl each of forward and reverse primers, 0.50 µl of dNTPs, 0.25 µl of taq polymerase, and 1 µl of DNA. The optimized pcr protocol for SK14 amplification was 34 cycles of 10 seconds at 94°C, 40 seconds at 63°C, 2 minutes at 72°C, 1 cycle of 5 minutes at 72°C, and a final holding cycle at 4°C. The pcr protocol for molecular marker SB1 was 1 cycle of 94° for 3 minutes, 30 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72° C for 2 minutes, 1 cycle of 72°C for 5 minutes, and an infinite cycle of 4°C. Pcr amplifications were visualized on a 1% agarose gel containing 0.008% ethidium bromide staining solution.

Once all genotypes were confirmed via pcr, seed collected from each of the original plants were planted and labeled accordingly. Plants were grown in the greenhouse during early spring under seasonal conditions.

2.1.4 Maintenance of Pathogen and Inoculum Preparation

Original urediospores received from USDA-ARS were stored at -80°C. A 0.1% Tween 20 solution was prepared to serve as a surfactant and spreader for the

fungal spores. The amount of Tween 20 solution was divided to accommodate both inoculating and mock inoculating solutions. Urediospores were added to one aliquot of Tween 20 solution and spun for at least 3 hours before inoculations. The final volume of urediospores in the inoculating solution was quantified to 20,000 spores per milliliters. This amount was quantified by using a hemocytometer and light microscope. A total of 50 ml of urediospore solution was used for 15 plants.

2.1.5 Plant Inoculation

The seeds were germinated in Petri dishes prior to being planted in soil pots. Germination usually took two to three days, with mutants ur3-Δ2 and ur3-Δ3 germinating more quickly. Once plants were transferred to pots, they were first grown in the greenhouse. Prior to being spray inoculated, plants were placed in a Conviron Growth room under 12 hrs photoperiod (daylight) conditions. The temperature was set to 25˚C during the day and 18˚C at night. Plant growth times vary with temperature and watering. To ensure that all plants were treated equally, watering was done by measured amounts. Also, plants were rotated within flats to guarantee that each plant received an equal distribution of light and temperature fluctuation.

Once plants were ready for inoculation, approximately 10 days after germination began, the growth room humidity was adjusted to 95-100% and the lights were turned off. Plants were sprayed with inoculum on both leaf surfaces evenly.

Inoculated plants were placed in the growth room's humidity chamber for 16 hrs overnight in the dark.

A total of two sets of plants, first set then a biological replicate, were inoculated on two separate days in this study. Garden five plants were inoculated with rust race 53 approximately 10 days after germination, consisting of the inoculation of the first two leaves that emerged after the cotyledon. Plants were rested for five minutes to allow inoculum to dry a bit. Zero hours post inoculated (hpi) samples were collected from both inoculated (I) and mock inoculated (MI) plants and were flash frozen in liquid nitrogen before being stored in the -80˚C freezer. Plants were placed in the Percival growth room dark box with >95% humidity at 19˚C. Samples were collected again at 12hpi and 84hpi, flash frozen, and kept at -80˚C. Before further processing, plants were evaluated for uredia growth (Figure 2.2). Uredia growth was monitored until urediospore content caused them to burst. Urediospores were collected by placing a clean sheet of aluminum foil beneath the infected leaves. Infected leaves were lightly tapped to release the urediospores. Collected urediospores were validated using a light microscope and stored in a 1.5ml micro centrifuge tube in the -80°C freezer.

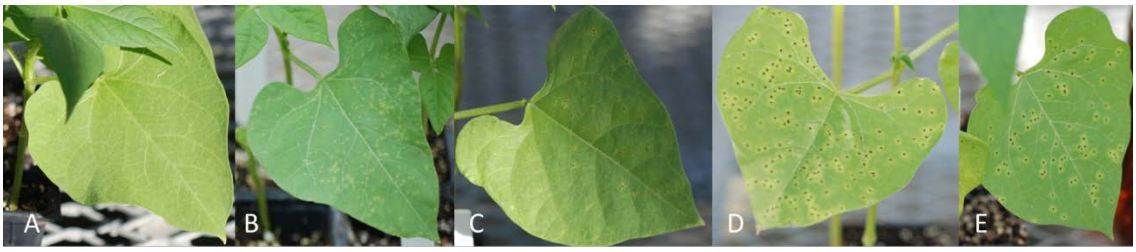


Figure 2.2 Five genotypes of common bean inoculated with fungal rust pathogen *Uromyces appendiculatus* race 53. A. Sierra B. Olathe C. crg D. ur3-Δ2 E. ur3-Δ3. Sierra shows no sign of pathogen growth. All susceptible genotypes vary in degree of uredia formation, with mutant crg exhibiting the mildest reaction.

2.1.6 RNA Isolation

Samples that were collected at 12 hpi were processed further for RNA isolation. Isolations were performed using the Trizol method (Invitrogen, CA). 1 mL of TRIzol Reagent was added per 50–100 mg of tissue sample. The tissue was ground with either a glass mortar & pestle, or in a micro centrifuge tube with micro pestles. The samples were then kept at room temperature for five minutes in a micro centrifuge tube. Two hundred microliters of chloroform was added to each tube and mixed well. They were incubated again for another two to three minutes. The samples were then centrifuged at 12,000 rpm in a 5810R ultra centrifuge (Eppendorf) for 10 minutes at 4°C. After centrifuging, the top layer was pipetted into a separate tube for further cleaning. Five hundred microliters of 100% isopropanol was added to each tube and incubated at room temperature for 10 minutes. Next, each tube was centrifuged for 10 minutes at 4°C. After removing the supernatant from each tube, each pellet was washed with 1 ml of 75% ethanol. The samples were briefly vortexed, then centrifuged again to remove any excess traces of TRIzol or chloroform. The supernatant was carefully removed by pipetting and the pellet was dried almost completely. Over drying the pellet may make it more difficult to go into solution when hydrating with nuclease free water. The RNA was quantified by gel electrophoresis, NanoDrop 2000 (ThermoScientific, US), and Qubit Fluorometer (Invitrogen, Ca).

2.1.7 cDNA Synthesis

For quality control purposes, RNA was then converted to cDNA using ProtoScript II (NEB, MA). Approximately 1 µg of RNA was mixed with d (T) 23 VN (50 µM) nucleotides and nuclease free water and incubated at 65 degrees Celsius for five minutes. The tube was immediately placed on ice to keep the RNA denatured. 10 µl of ProtoScript II Reaction Mix (2X) and 2 µl of ProtoScript II Enzyme Mix (10X) was added to the denatured RNA for a total of 20 µl and incubated at 42 degrees Celsius for one hour. Samples were incubated at 80 degrees Celsius to inactivate the enzyme. Synthesized cDNA was then quantified by NanoDrop (ThermoScientific, US) and analyzed through pcr. The pcr analysis with molecular marker SK14 primers and SB1 primers was done to confirm that the cDNA was free of genomic DNA contamination. We also used a set of primers derived from cDNA pcr with soybean (*Glycine max*). Constitutive gene 7, or cons 7, is one of several genes in soybean that always express. The cons 7 gene was used to check the integrity of RNA isolation. Because common bean and soy bean are so closely related, we used it as a control. We expected all cDNA samples to amplify cons 7 at equal intensities.

2.1.8 Illumina Library Prep

Total RNA was used to build sequencing libraries with TruSeq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA). The Illumina Library prep included the purification and fragmentation of mRNA from total RNA, first and second

strand cDNA synthesis, end repair, adapter ligation, pcr amplification, library validation, normalization and pooling. Of the 20 libraries built, there were four libraries to represent each genotype. Two of each genotype was mock inoculated and two were inoculated. First, Sierra and Olathe samples were made into libraries and run in a single lane. A total of eight libraries were made to accommodate two replicates of Sierra and Olathe mock inoculated and inoculated samples. Each sample was tagged with a specific nucleotide sequence to distinguish each separately in the pooled sample. Mutant crg was processed in a single lane, while mutants ur3-Δ2 and ur3-Δ3 shared a sequencing lane. Libraries were sequenced at the University of Delaware Sequencing & Genotyping Center at Delaware Biotechnology Institute (DBI, Newark, DE).

2.1.9 Data Analysis

Sequencing reads were analyzed using CLC Genomics workbench (Qiagen, Denmark). Each set of reads were processed individually using the RNA-seq mapping tool. Reads were mapped to the publicly available common bean transcript as well as the publicly available common bean genome. Read samples were compared to each other in groups and one against another. Sierra MI and crg MI were first compared to identify differential expression among genes specifically on chromosome 10, the location of molecular marker SB1. Heat maps were generated to compare regions of interest on chromosome 10 in

Sierra and crg. Empirical analysis of digital gene expression (EDGE) test tag wise dispersions were completed on all possible sample combinations (including Sierra, Olathe, crg, ur3-Δ2, and ur3-Δ3) MI and I, generating P-value and fold change data. Additional primer sets were designed using Integrated DNA Technology (IDTDNA.com) Primer Quest tool (IDT, IA) to identify presence/absence particularly in crg genomic pcr when compared to Sierra genomic pcr. Primers were designed upstream and downstream of gene Phvul.010G025000, which contains molecular marker SB1. The designed primers correlated with genes in the region of interest on chromosome 10.

2.1.10 Quantitative Real Time pcr (rt-pcr)

For q-pcr/rt-pcr, the Applied Biosystems 7500 Real Time platform and Power SYBR green Master mixes were used. cDNA was quantified to 200 ng/µl in each sample before analysis. Twenty five µl reactions prepared for each processed sample including 12.5 µl of Power SYBR Master mix, 1 µl each of forward and reverse primer, 1 µl of cDNA and 9.5ul of nuclease free water.

2.2 Results

Ur-3 was previously genetically mapped to linkage group 11, chromosome 11, (Freyre et al., 1998); (Kelly et al., 2003), while *Crg* was mapped to linkage group 8, chromosome 8 (Freyre et al., 1998). The recent release of the common bean genome has allowed us to locate molecular markers that genetically differentiate mutant crg from mutant(s) ur3-Δ2/Δ3. The RGA SB1 sequence, which is missing in mutant crg (Figure 2.3), was BLASTed (Basic Local Alignment & Search Tool) to the common bean genome (http://phytozome.jgi.doe.gov/) to identify a specific location on chromosome 8 in the attempt to create primers upstream and downstream of this region. Interestingly, SB1 actually mapped to a single location (Phvul.010G025000) on chromosome 10, with no alignments to chromosome 8. Since the alignment occurred on chromosome 10 at about position 3,750,000 bp, primer design began within the first 100,000 bases to determine the range of the mutation. The designed primers amplified in all genotypes, including crg. Next, primers were designed closer to the deletion region, but still came to the same result. It was then realized that although the mutations were random, the size would be much smaller than millions of base pairs. Primers were designed outwardly, designing specifically from genes adjacent to Phvul.010G025000 as opposed to intronic regions farther away. First genomic DNA was amplified using primers designed from a gene directly beside Phvul.010G025000, Phvul.010G024900. A successfully designed a primer set with the same amplification/deletion pattern as SB1 was obtained. The next primer was

designed from the gene to the right of Phvul.010G025000, Phvul.010G025100. In order to identify genes in the deletion region, a presence-absence-presence (+-+) pattern needed to be shown in the cluster of genes on chromosome 10 (Figure 2.4) using genomic pcr. Pcr was performed to identify where the deletion began and ended in crg.

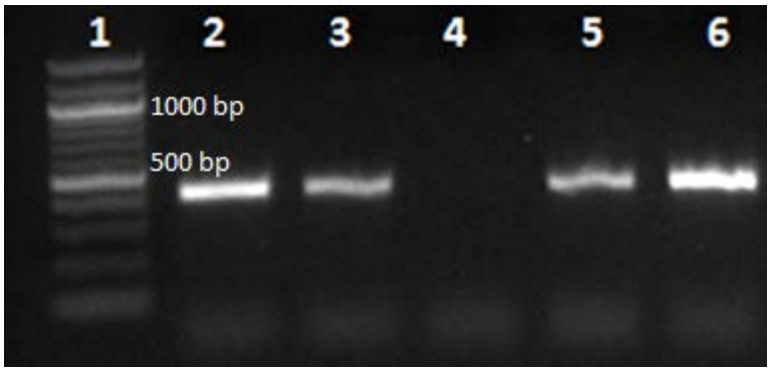


Figure 2.3 Pcr completed for SB1. 1.100 bp ladder 2.Sierra 3.Olathe 4.crg 5.ur3-Δ2 6.ur3-Δ3. SB1 is missing in crg but amplifies in all other genotypes, indicating that the mutation causing susceptibility to race 53 is in a different region.

Simultaneously, RNA-seq data were generated from a race 53 inoculation time course study to compliment the transcriptomic data from the deletion mutants. Marrying the two approaches allowed the deduction of the deletion region in crg, which permitted a focal point on a candidate gene area for *Crg*. Comparatively, Sierra and crg differ only on chromosome 10 in a 250 kb stretch of the genome. Within the RNA-seq data generated from Illumina sequencing and CLC analysis, expression data were analyzed in the region of the deletion on chromosome 10 (Figure 2.5). The cluster of genes used to design primers to amplify in genomic Sierra and crg differed in expression value. The cluster of 17 genes, including Phvul.010G024900 through Phvul.010G026500, showed no expression in crg as opposed to little to moderate expression in Sierra MI and I.

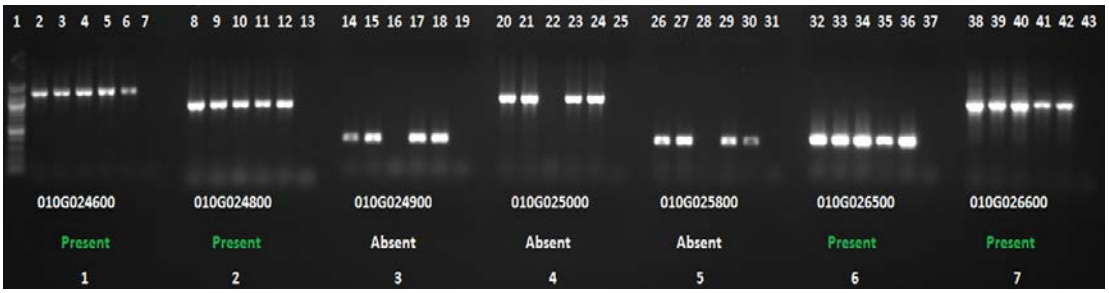


Figure 2.4 Two hundred and fifty kb delineated deletion region on chromosome 10. Pcr completed for seven different primer sets. 1. 100 bp ladder 2. Sierra . Olathe 4. **crg** 5. ur3-Δ2 6. ur3-Δ3 7. H2O control 8.Sierra 9.Olathe 10.**crg** 11.ur3-Δ2 12. ur3-Δ3 13.H2O control 14.Sierra 15.Olathe 16.**crg** 17.ur3-Δ2 18.ur3-Δ3 19.H2O control 20.Sierra 21. Olathe 22.crg 23.ur3-Δ2 24.ur3-Δ3 25.H2O control 26.Sierra 27.Olathe 28.**crg** 29.ur3-Δ2 30.ur3-Δ3 31.H2O control 32.Sierra 33.Olathe 34.**crg** 35.ur3-Δ2 36.ur3-Δ3 37.H2O control 38.Sierra 39.Olathe 40.**crg** 41.ur3-Δ2 42.ur3-Δ3 43.H2O control

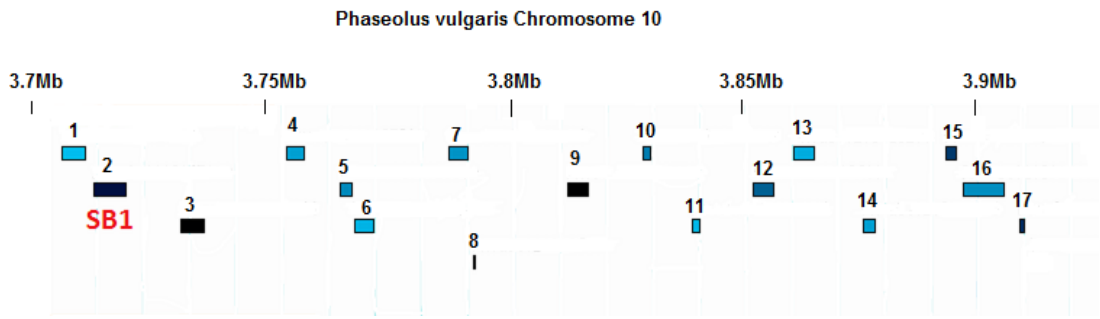


Figure 2.5 A portion of chromosome 10 from the published common bean genome from the Phytozome website (http://phytozome.jgi.doe.gov/). The darker color indicates lesser amounts of tissues with expression for that particular gene. The lighter colored genes express in more tissues. According to the published data, the gene underwritten with SB1 expresses in two tissues only, roots and nodules. 1. Phvul.010G024900 2.Phvul.010G025000 3.Phvul.010G025100 4.Phvul.010G025200 5.Phvul.010G025300 6.Phvul.010G025400 7.Phvul.010G025500 8.Phvul.010G025600 9.Phvul.010G025700 10.Phvul.010G025800 11.Phvul.010G025900 12. Phvul.010G026000 13.Phvul.010G026100 14.Phvul.010G026200 15.Phvul.010G026300 16.Phvul.010G026400 17.Phvul.010G026500 Molecular marker SB1 aligns with gene Phvul.010G025000, and is missing in crg only. Reverse transcriptase pcr has been carried out with several genes in this region, including Phvul.010G025800.

Sequence alignment was previously used to identify several BACs (Bacterial artificial chromosomes) in the region of *Crg*, which co-segregates with molecular marker SB1 (Kalavacharla et al., 2000). However, because the whole genome was not available, the area was never delineated until now. It is now known that this region is dense in disease resistance-related genes and contains the molecular marker SB1. It was previously believed that SB1 was part of the intronic region in the genome, located on chromosome 8. In fact, the findings and resources show that it is part of a gene, but may not express in the processed leaf tissue or at the selected time point. BLAST analysis for SB1 to the common bean genome indicated that SB1 maps to gene Phvul.10G025000, located on chromosome 10. The RPKM (Reads per Kilo base per Million Mapped Reads) data for the SB1 mapped gene Phvul.010G025000 expression level is zero in all analyzed samples. This confirms that SB1 does not express in our Sierra leaf tissue. Information from the Phytozome website also shows that the SB1 gene expression is present in flowers and nodules only, and does not express in leaf tissue of the G19833 genotype. Several genes in the delineated region have been verified by genomic pcr as well as reverse transcriptase pcr, using cDNA made from isolated messenger RNAs. One gene in particular had higher expression in cDNA pcr among inoculated Sierra samples than in mock inoculated samples. The gene Phvul.010G025800 is the most differentially expressed gene between Sierra MI and I 12 hpi samples in the deletion region.

There were no amplifications in any crg samples, mock inoculated or inoculated (Figure 2.6), which further confirms the mutation in this region in crg. Phvul.010G025800 has the highest gene expression, represented in the heat map (Figure 2.7).

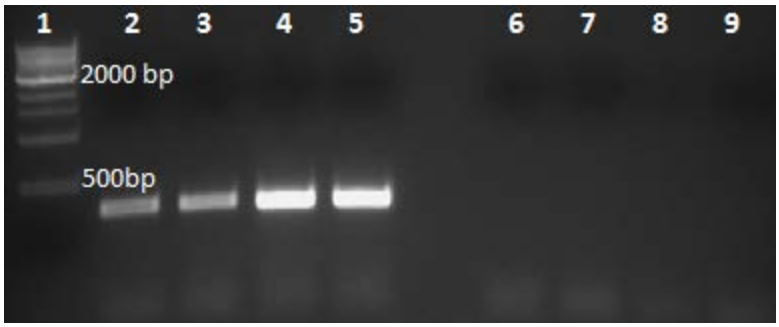


Figure 2.6 cDNA pcr with select primer set designed from Phvul010G025800 reveals increased expression in Sierra I versus Sierra MI 1. 1 kb ladder 2. Sierra MI 3. Sierra MI 4. Sierra I 5. Sierra I 6. crg MI 7. crg MI 8. crg I 9. crg I

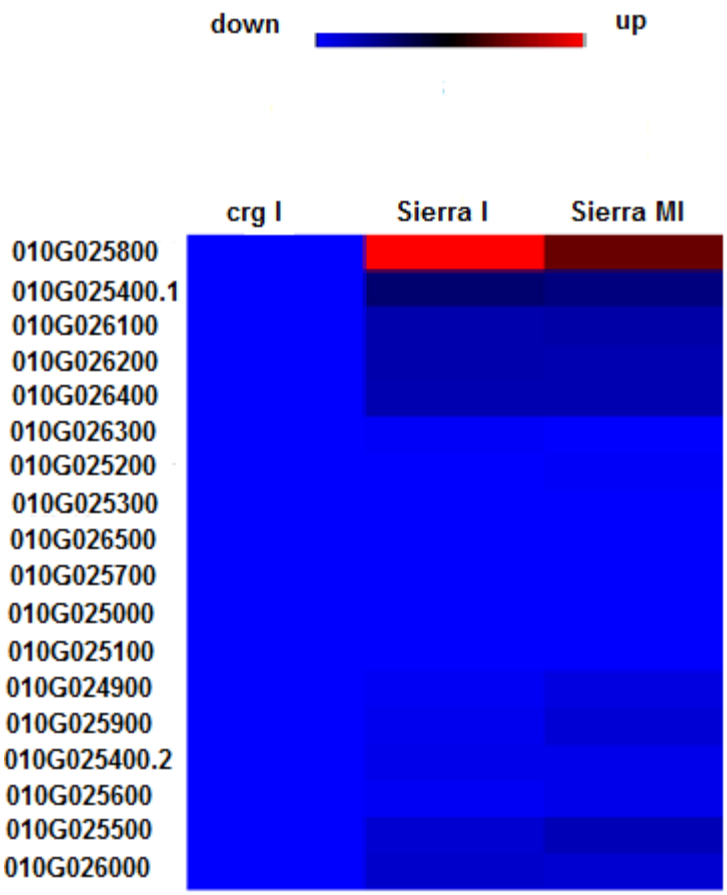


Figure 2.7 Heat map comparison of crg I vs. Sierra I vs. Sierra MI. Genes listed in delineated region in order of differential expression from highest to lowest (top to bottom). Phvul.010G025800 is most highly expressed and most differentially expressed across samples.

The group of genes present in the delineated region of chromosome 10 mostly belongs to the disease resistance protein (TIR-NBS-LRR class) family. They include a mitochondrial processing peptidase beta subunit insulinase protein (MPPBETA) belonging to the Peptidase family M16, an NB-ARC domain-containing disease resistance protein, target of AvrB operation1 (TAO1), and three proteins of unknown function (Table 3.1).

TAO1 is a disease resistance protein induced by the AvrB effector in *Pseudomonas syringae* (Eitas et al., 2008). The TAO1 protein works in tandem with the RPM1 plants, a gene conferring resistance to *Pseudomonas syringae* in Arabidopsis and soybean (Eitas et al., 2008). It also works with *Pto* in RPM1 plants, which also confers resistance to *P. syringae* in tomato and was the first *R* gene cloned that followed Flor's gene-for-gene theory (Martin et al., 1993). It is required for full resistance against the DC3000(avrB) strain of *P. syringae (*Eitas et al., 2008).

The NB-ARC protein regulates *R* gene activity (Van Ooijen et al., 2008). It serves as a signaling motif in plant cell regulatory systems (van der Biezen and Jones, 1998). It is instrumental in programmed cell death, which may be one of the main factors in HR in the resistant plant, Sierra. The NB-ARC domain is also present in at least five other plant *R* genes, including *RPM1* (Grant et al., 1995), *RPS2* (Bent et al., 1994; Mindrinos et al., 1994), *RPP5* (Parker et al., 1997), *N* (Whitman et al., 1996), and L^6 (Lawrence et al., 1995). All of the mentioned

genes are also encoded with a C-terminal end of leucine rich repeats (LRR). They are then divided into two groups, differing only at the N-terminal, which are composed of either Leucine zippers (*RPS2* and *RPM1*) or Toll/Interleukin-1 (*N*, L^6, and *RPP5*). The gene Phvul.010G025100 also contains a C-terminal LRR domain as well. However, the N-terminal end is not identifiable.

Table 3.1 Seventeen genes within the delineated region of mutant crg. P-values and fold change values represent Sierra MI (Rep 1and 2) vs. Sierra I (Rep 1and 2).

Sierra MI vs. Sierra I			
Feature ID	P-value	Fold change	Function
Phvul.010G024900.1	1	-1.64	MPPBETA Insulinase (Peptidase family M16) protein
Phvul.010G025000.1	1	-1.05	Disease resistance protein (TIR-NBS-LRR class) family
Phvul.010G025100.1	1	1.01	NB-ARC domain-containing disease resistance protein
Phvul.010G025200.1	1	-1.26	Disease resistance protein (TIR-NBS-LRR class) family
Phvul.010G025300.1	1	1.1	Disease resistance protein (TIR-NBS-LRR class) family
Phvul.010G025400.2	1	1.17	Disease resistance protein (TIR-NBS-LRR class) family
Phvul.010G025400.1	0.73	1.76	Disease resistance protein (TIR-NBS-LRR class) family
Phvul.010G025500.1	1	-1.04	Disease resistance protein (TIR-NBS-LRR class) family
Phvul.010G025600.1	1	1.05	Protein of unknown function
Phvul.010G025700.1	1	1	Disease resistance protein (TIR-NBS-LRR class) family
Phvul.010G025800.1	0.86	-1.12	Protein of unknown function (DUF506)
Phvul.010G025900.1	1	-1.53	Mitochondrial transcription termination factor protein
Phvul.010G026000.1	1	-1.01	Disease resistance protein (TIR-NBS-LRR class) family

Table 3.1 continued

Phvul.010G026100.1	1	-1.06	Disease resistance protein (TIR-NBS-LRR class) family
Phvul.010G026200.1	1	-1.01	Disease resistance protein (TIR-NBS-LRR class) family
Phvul.010G026300.1	1	1.31	TAO1 Target of AVRB operation1
Phvul.010G026400.1	1	-1.04	Disease resistance protein (TIR-NBS-LRR class) family
Phvul.010G026500.1	1	1.31	Protein of unknown function

Table 3.2 RT-pcr with Sierra MI and I samples and primer sets designed from delineated region. Lower CT (cycle threshold) values indicate stronger positive reactions, i.e. higher expression.

Sample Name	Target Name	Reporter	Cycle Threshold	Cycle Threshold Mean
SMI	Cons 7	SYBR	28.87731	28.64317
SMI	Cons 7	SYBR	28.26263	28.64317
SMI	Cons 7	SYBR	28.78955	28.64317
SI	Cons 7	SYBR	28.02788	28.22898
SI	Cons 7	SYBR	28.22381	28.22898
SI	Cons 7	SYBR	28.43523	28.22898
SMI	24900	SYBR	30.75357	31.21148
SMI	24900	SYBR	31.66939	31.21148
SMI	24900	SYBR	Undetermined	31.21148
SI	24900	SYBR	30.48375	33.34883
SI	24900	SYBR	37.1442	33.34883
SI	24900	SYBR	32.41853	33.34883
SMI	25000	SYBR	34.58884	34.89832
SMI	25000	SYBR	34.19945	34.89832
SMI	25000	SYBR	35.90666	34.89832
SI	25000	SYBR	35.85514	36.5375
SI	25000	SYBR	36.9123	36.5375
SI	25000	SYBR	36.84506	36.5375
SMI	25100	SYBR	34.28683	34.58403
SMI	25100	SYBR	35.15882	34.58403
SMI	25100	SYBR	34.30645	34.58403
SI	25100	SYBR	36.18275	36.35098
SI	25100	SYBR	35.89535	36.35098
SI	25100	SYBR	36.97483	36.35098
SMI	25200	SYBR	34.5163	34.56065
SMI	25200	SYBR	33.87501	34.56065
SMI	25200	SYBR	35.29064	34.56065
SI	25200	SYBR	32.40336	32.53666
SI	25200	SYBR	32.39663	32.53666

Table 3.2 continued

SI	25200	SYBR	32.81	32.53666
SMI	25300	SYBR	Undetermined	
SMI	25300	SYBR	Undetermined	
SMI	25300	SYBR	Undetermined	
SI	25300	SYBR	37.03968	37.03968
SI	25300	SYBR	Undetermined	37.03968
SI	25300	SYBR	Undetermined	37.03968
SMI	25400	SYBR	28.87551	28.89649
SMI	25400	SYBR	28.69859	28.89649
SMI	25400	SYBR	29.11537	28.89649
SI	25400	SYBR	27.36451	27.48367
SI	25400	SYBR	27.47783	27.48367
SI	25400	SYBR	27.60867	27.48367
SMI	25500	SYBR	34.79234	34.97721
SMI	25500	SYBR	34.83144	34.97721
SMI	25500	SYBR	35.30784	34.97721
SI	25500	SYBR	30.49621	30.77309
SI	25500	SYBR	30.91353	30.77309
SI	25500	SYBR	30.90952	30.77309
SMI	25600	SYBR	31.89131	32.48995
SMI	25600	SYBR	32.92747	32.48995
SMI	25600	SYBR	32.65107	32.48995
SI	25600	SYBR	32.73882	32.74718
SI	25600	SYBR	32.55291	32.74718
SI	25600	SYBR	32.94981	32.74718
SMI	25700	SYBR	35.31003	34.70147
SMI	25700	SYBR	35.20224	34.70147
SMI	25700	SYBR	33.59216	34.70147
SI	25700	SYBR	35.68888	35.05976
SI	25700	SYBR	33.60799	35.05976
SI	25700	SYBR	35.8824	35.05976

Table 3.3 RT-pcr with Sierra MI and I samples and primer sets designed from delineated region. Lower CT (cycle threshold) values indicate stronger positive reactions, i.e. higher expression. The lowest CT values in the table are associated with gene Phvul.010G025800, particularly in Sierra I samples.

Sample Name	**Target Name**	**Reporter**	**Cycle Threshold**	**Cycle Threshold Mean**
SMI	Cons 7	SYBR	29.88477	30.19886
SMI	Cons 7	SYBR	30.33912	30.19886
SMI	Cons 7	SYBR	30.37269	30.19886
SI	Cons 7	SYBR	28.92787	29.31236
SI	Cons 7	SYBR	29.3696	29.31236
SI	Cons 7	SYBR	29.63963	29.31236
SMI	25800	SYBR	27.765	27.19686
SMI	25800	SYBR	26.9359	27.19686
SMI	25800	SYBR	26.88967	27.19686
SI	25800	SYBR	23.62484	23.73442
SI	25800	SYBR	23.68967	23.73442
SI	25800	SYBR	23.88874	23.73442
SMI	25900	SYBR	34.50357	34.36478
SMI	25900	SYBR	34.89905	34.36478
SMI	25900	SYBR	33.69172	34.36478
SI	25900	SYBR	32.30329	32.21144
SI	25900	SYBR	31.97454	32.21144
SI	25900	SYBR	32.35649	32.21144
SMI	26000	SYBR	35.52095	36.00336
SMI	26000	SYBR	35.37188	36.00336
SMI	26000	SYBR	37.11726	36.00336
SI	26000	SYBR	33.67605	34.55983
SI	26000	SYBR	34.3568	34.55983
SI	26000	SYBR	35.64665	34.55983
SMI	26100	SYBR	32.71664	32.82502
SMI	26100	SYBR	32.89111	32.82502
SMI	26100	SYBR	32.86732	32.82502

Table 3.3 continued

SI	26100	SYBR	30.44485	30.58018
SI	26100	SYBR	30.47906	30.58018
SI	26100	SYBR	30.81661	30.58018
SMI	26200	SYBR	33.30546	32.6739
SMI	26200	SYBR	32.62796	32.6739
SMI	26200	SYBR	32.0883	32.6739
SI	26200	SYBR	29.21747	29.63936
SI	26200	SYBR	29.89228	29.63936
SI	26200	SYBR	29.80833	29.63936
SMI	26300	SYBR	Undetermined	38.00851
SMI	26300	SYBR	Undetermined	38.00851
SMI	26300	SYBR	38.00851	38.00851
SI	26300	SYBR	36.68328	36.82367
SI	26300	SYBR	37.07371	36.82367
SI	26300	SYBR	36.71402	36.82367
SMI	26400	SYBR	33.80589	33.4706
SMI	26400	SYBR	33.15413	33.4706
SMI	26400	SYBR	33.45179	33.4706
SI	26400	SYBR	31.58919	31.67615
SI	26400	SYBR	31.55182	31.67615
SI	26400	SYBR	31.88745	31.67615
SMI	26500	SYBR	35.30411	35.40184
SMI	26500	SYBR	35.87034	35.40184
SMI	26500	SYBR	35.03107	35.40184
SI	26500	SYBR	32.87538	32.70929
SI	26500	SYBR	32.91201	32.70929
SI	26500	SYBR	32.34049	32.70929

In performing RT-pcr, Phvul.010G025800 and Phvul.010G025400 had the lowest cycle threshold number (Table 3.2) and (Table 3.3), which correlates with a higher abundance of target nucleic acid. Genes listed in the two tables represent three technical replicate values for all genes within the deletion region. The endogenous gene cons 7 was including in each plate as a control. Two separate RT-pcr plates were run to accommodate the number of samples. One of three unknown proteins (DUF506), which is associated with Phvul.010G025800, has the highest level of differential expression among genes in region between Sierra MI and Sierra I. The most closely related plant species that has been partially characterized and has a similar sequence to this gene is in cacao (*Theobroma cacao*). The sequence encodes a sulfate/thiosulfate import ATP-binding protein (Motamayor et al., 2013), which may aid in solute transfer across membranes.

Although the deletion region was delineated in crg by using genomic pcr, cDNA pcr, and RNA-seq, our main focus in this study was to identify differential expression between Sierra MI and Sierra I samples when challenged with rust. Since the aim was not to perform a global study on Sierra MI and I, mutated plants were used to identify breakpoints in the genome of susceptible plants. In this way, the focus is on a small group of genes, giving a greater chance at identifying the gene(s) of interest. Three key points were concluded through this research. One, SB1 is located on chromosome 10, not chromosome eight as previously believed.

Two, SB1 is part of a gene, but does not express in leaf tissue at our specific time point (12hpi). Three, the deletion in crg is less than 300 kb, making the list of identified genes missing less than twenty.

Chapter 3

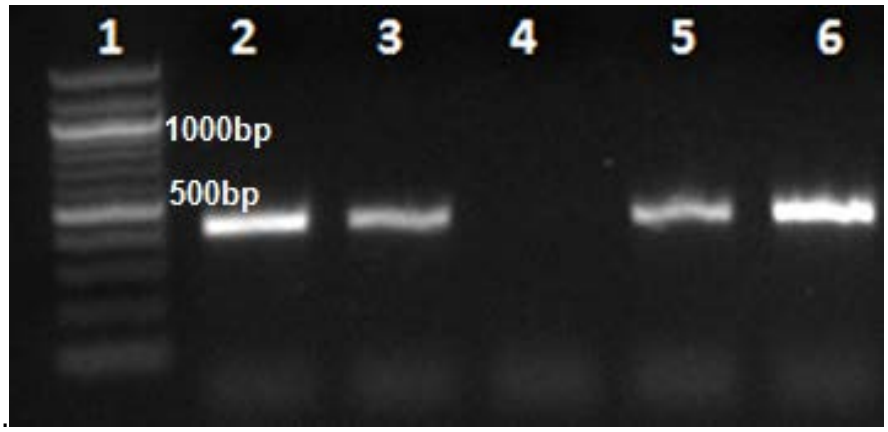
A TRANSCRIPTOME-WIDE APPROACH TO IDENTIFY DELETION REGIONS IN COMMON BEAN MUTANTS ur3-Δ2 and ur3-Δ3

Common bean (*Phaseolus vulgaris* L.) is an important crop throughout the world, as it serves as a staple food for many in developing countries and as a substitute for meat due to its high protein value. Fungal rust can have devastating effects on susceptible bean crops throughout the world. In some cases, where conditions are favorable for the pathogen, rust can overcome the entire crop keep repeating its infection over again. In order to combat this vicious cycle, scientists work with farmers to identify and release crop cultivars to resist fungal disease. In many cases, crops are resistant to specific races or strains of a pathogen, and are susceptible to others.

In this study, the aim is to use a comparative transcriptomics approach to identify differential expression of genes between the Sierra cultivar and Sierra-derived, rust susceptible mutants ur3-Δ2 and ur3-Δ3. The interest lies in disease resistance genes, particularly the *Ur-3* gene, in which both mutants are recessive. The *Ur-3* gene has been genetically linked to molecular marker SK14(Nemchinova and Stavely, 1998), and is also tightly linked to the *Co2*

(Miklas, 2002) gene located on chromosome 11 in common bean. Techniques were employed combining genomic pcr and RNA-seq to identify differential amplification and expression, respectively, among Sierra and the Sierra-derived mutants.

Although both crg and mutants ur3-Δ2/ur3-Δ3 are susceptible to fungal rust race 53, they are distinguished by using molecular marker amplification, or absence, along with the appearance of rust. Since Sierra is not susceptible to race 53, and it amplifies both molecular markers (Figure 3.1), SB1 and SK14, we conclude that there is no mutation present. Of the three mutants, crg has an overall milder susceptibility reaction to race 53 (Figure 2.2), as well as to all races used in work by Kalavacharla et al (2001). In Figure 2.2, crg had less uredia than all other susceptible genotypes. The crg uredia were also smaller in size than the other genotypes. Mutants ur3-Δ2 and ur3-Δ3 amplify the SB1 molecular marker, while crg does not. Also, the SK14 marker does not amplify in ur3-Δ2 and ur3-Δ3, but amplifies in crg. In addition, SCAR marker SAE19 has a different amplification pattern in ur3-Δ2 and ur3-Δ3 than in that of crg. In ur3-Δ2 and ur3-Δ3, there are amplifications, but they appear to be about 2 kb in size. This is more than twice the size of the amplification in Sierra and mutant crg.

1. 

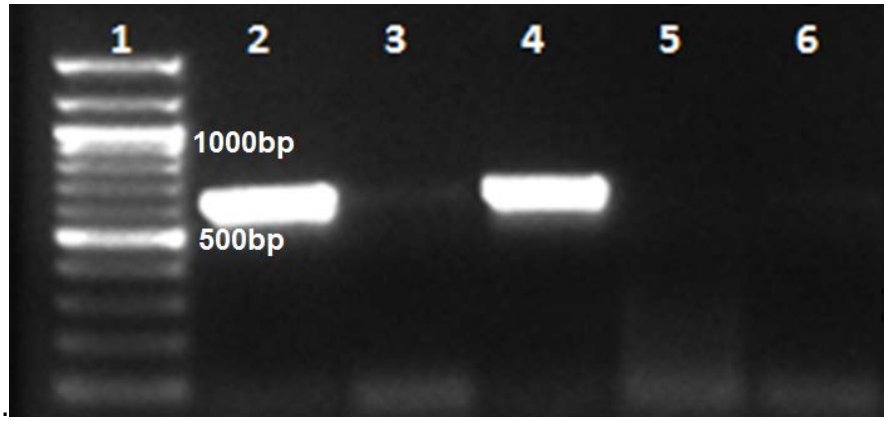
2. 

Figure 3.1 Pcr completed for SB1 (1) and SK14 (2) primer sets. 1. 100bp Ladder 2.Sierra 3.Olathe 4.crg 5.ur3-Δ2 6.ur3-Δ3. SB1 is missing in crg but amplifies in all other genotypes. SK14 amplifies in Sierra and crg only, indicating that the deletion in crg is different than that of ur3-Δ2 and ur3-Δ3.

Unlike SB1, the molecular marker SK14 does not completely align with any particular chromosome. The ~600 bp sequence only aligns partially (approximately 150 bp) to several chromosomes, including chromosomes 8, 3, and 11. For this reason, a transcriptome study approach was used to compare transcript data from Sierra against ur3-Δ2 and ur3-Δ3. We treat ur3-Δ2 and ur3-Δ3 as siblings because they were recovered from the same seed bulk collected after the initial fast neutron bombardment and self-pollinating (Kalavacharla et al., 2000). Mutants ur3-Δ2 and ur3-Δ3 also have identical growth habits, with seeds and plant height growing larger than their progenitor, Sierra. They also flower and set seed up to two weeks earlier than Sierra. In Sierra and crg, the shoot apical meristem is less pronounced than in ur3-Δ2, ur3-Δ3, and G19833.

3.1 Materials and Methods

For DNA Isolation, DNA Clean Up, DNA Quantification, Plant Inoculation, and RNA Isolation & Library Prep, please refer to the Material and Methods section in chapter 2.

3.1.1 Sample Processing

Sequence reads for both Sierra and ur3-Δ2 (MI and I) were analyzed using CLC Genomics Workbench Desktop Software (Qiagen, Denmark). All Illumina reads samples were trimmed to ensure that adapter sequences were removed prior to analysis. Trimmed reads were mapped to the publicly available common bean genome and transcript (http://phytozome.jgi.doe.gov/). Duplicate samples were combined and processed as one. Trimmed, combined samples were assembled into contigs (contiguous sequences) and BLASTed against the Ref-Seq Viridian plant protein database either locally or against NCBI (ncbi.nlm.nih.gov). After contigs were BLASTed, outputs were mapped and annotated using BLAST2GO, a plugin from CLC genomics workbench. Data were generated for biological processes, cellular components, and molecular functions for all mapped contigs in all samples.

3.2 Results

3.2.1 Molecular Marker SK14

The SCAR marker SK14 is one used to molecularly differentiate the rust resistant progenitor Sierra from ur3-Δ2, ur3-Δ3, and Olathe in pcr. SK14 primers were used to amplify a specific region which we believe co-segregates with the *Ur-3* gene. The SK14 sequence was obtained using Sanger sequencing. The original primers are available at the Bean Improvement Cooperative website (http://bic.css.msu.edu/), along with several other SCAR markers. The primers amplify in the Sierra and crg genotypes only, lending to the evidence that there is more than one gene required for resistance to race 53, as in the *Crg* gene.

In order to identify the location of the SK14 marker, we BLASTed the published bean genome as well as transcript data. Unlike SB1, which aligns to a single region on chromosome 10, the SK14 primer does not fully align with any particular gene, nor does it completely align to any part of the genome. Previous literature from a comprehensive linkage map (Miklas et al., 2002) places the *Ur-3* gene on chromosome 11, to which SK14 only partially aligns. However, BLAST alignment of SK14 holds the greatest similarity to a region on chromosome eight shotgun sequence, at approximately 25 MB. Additionally, a similar sequence alignment occurs on chromosome three shotgun sequence, at approximately 52 MB. We designed primers from genes adjacent to partial alignments for pcr.

We were unsuccessful in seeing any differential amplification patterns in genomic pcr using Sierra and ur3-Δ3 (Figure 3.2). Primers designed to the left and right of partial alignments amplified in both genotypes.

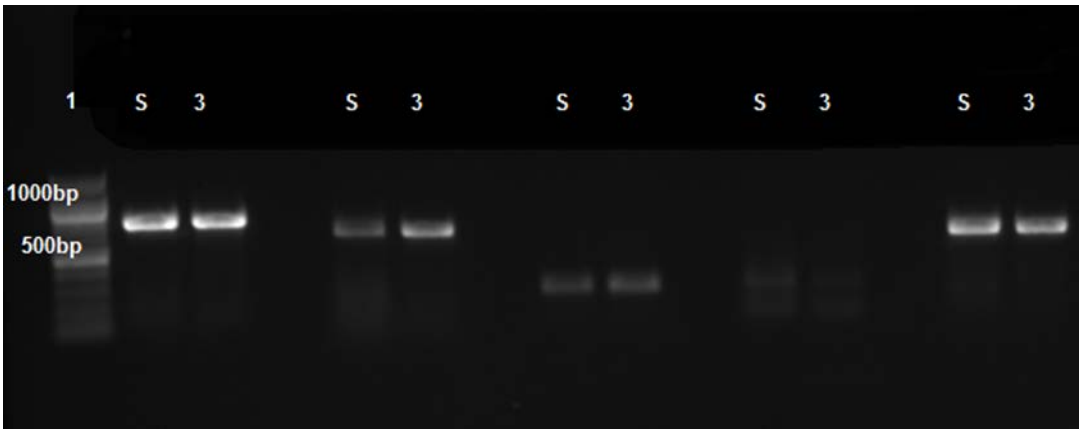


Figure 3.2 Primers designed from partial alignment of molecular marker SK14 to chromosome 8 in common bean. The ‘S’ represents Sierra and ‘3’ represents ur3-Δ3. Since there was no differential amplification pattern between the two, no further pcr was performed on other genotypes.

3.2.2 Molecular Marker SAE19

The SAE19 marker co-segregates with the *Co-2* gene and is closely located to the *Ur-3* gene, located on chromosome 11. This molecular marker was used along with SK14 to help identify deleted regions in ur3-Δ2 and ur3-Δ3. The idea was to use markers closely linked to *Ur-3* along with the SK14 marker in an attempt to identify the gene location. SAE19 amplifies in Sierra and crg at a size of approximately 850bp. It does not amplify in Olathe at all. It does, however, amplify in ur3-Δ2 and ur3-Δ3, but at more than twice the size of that in Sierra (Figure 3.3). It also amplifies in G19833, the published common bean genome, at the same size. When BLASTed against the common bean genome, the sequence aligns in an intronic region on chromosome 11. Primers were designed from genes adjacent to the area to which SAE19 aligns. However, the primers amplified in all six genotypes (Figure 3.4). This shows that there are no deletions in this area, unlike the area on chromosome 10 to which SB1 aligns.

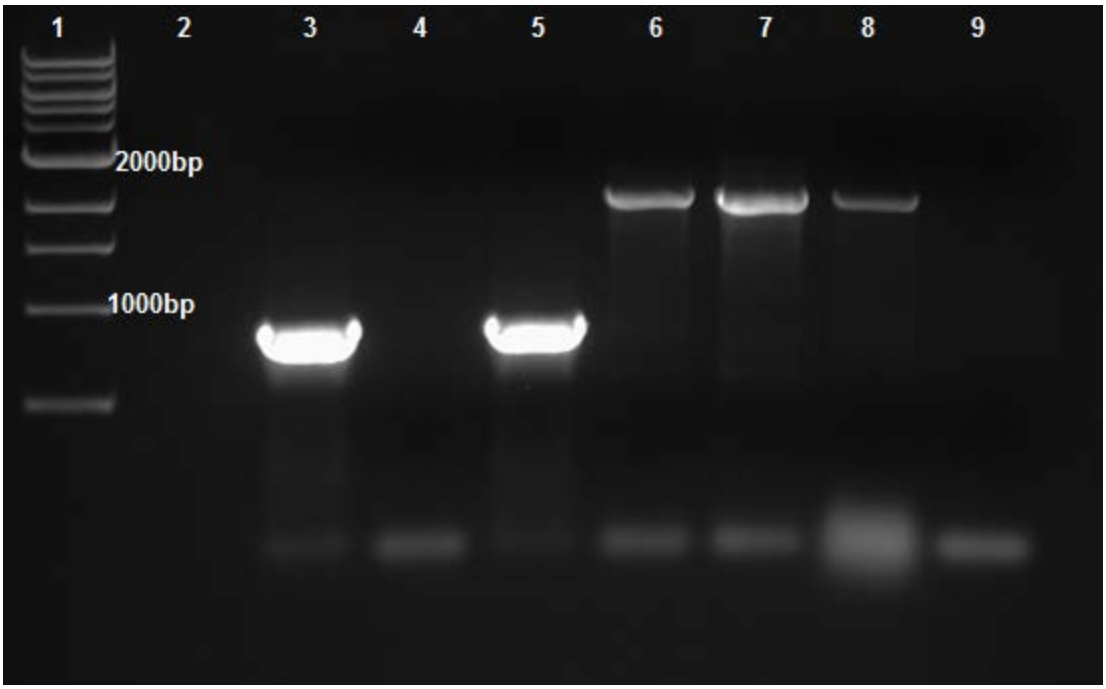


Figure 3.3 Genomic pcr with molecular marker primers SAE19. 1. 1kb ladder, 2. Intentionally left blank, 3. Sierra, 4. Olathe, 5. crg, 6. ur3-Δ2, 7. ur3-Δ3, 8. G19833, 9. Water

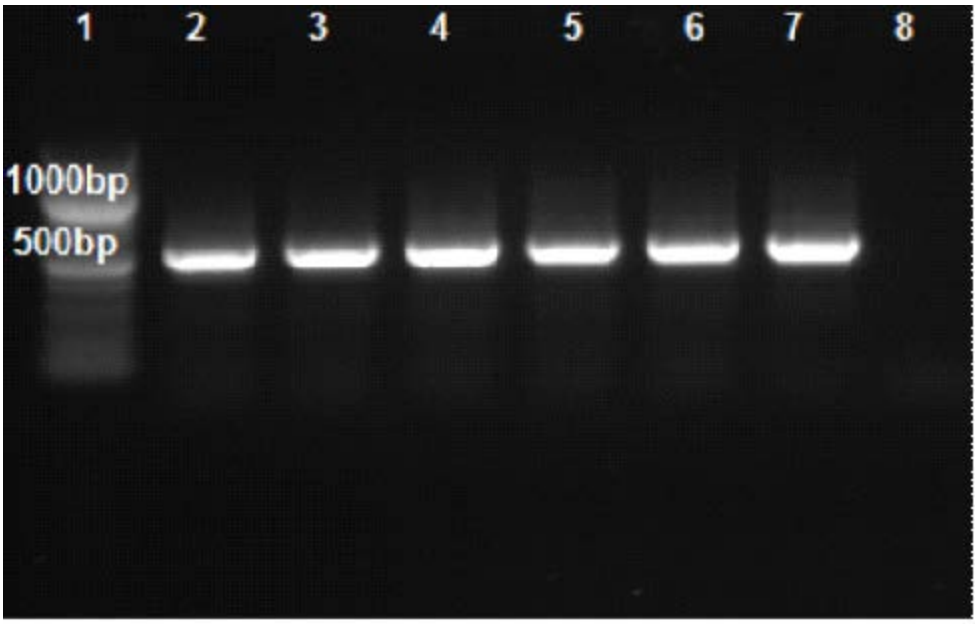


Figure 3.4 Primer set designed from region adjacent to SAE19 alignment on chromosome 11 in common bean (Phvul011G198600). 1. 100 bp ladder 2. Sierra 3. Olathe 4. crg 5. ur3-Δ2 6. ur3-Δ3 7. G19833 8. H_2O

3.2.3 Whole Transcriptome Comparison of Sierra and Mutant ur3-Δ2

Throughout the current research, attempts were made to identify deletion regions by using molecular markers and pcr. However, I was unsuccessful in finding any region where either the SK14 or SAE19 markers completely mapped to the common bean genome or transcriptome. In order to identify differences between Sierra MI, Sierra I, and ur3-Δ2 I, a whole transcriptome comparison approach was used. In this, contigs were built by using De Novo mapping in the CLC software. After building the contigs, BLAST2Go software mapped them to the Plante protein database at NCBI. After mapping the contigs for each particular group of samples, pie graphs were generated identifying cellular, biological, and molecular processes for all mapped sequences. Samples analyzed include Sierra MI (Figure 3.5) (Figure 3.8), Sierra I (Figure 3.6) (Figure 3.9), and ur3-Δ2 I (Figure 3.7) (Figure 3.10). In analyzing the samples, I was able to identify several key differences in SMI and SI versus ur3-Δ2 I. Because SMI and SI are the same genotype, with varying gene expression levels, all of the same cellular, biological, and molecular processes were present in both samples. Conversely, in ur3-Δ2 I, there were several processes that were not present in SMI or SI.

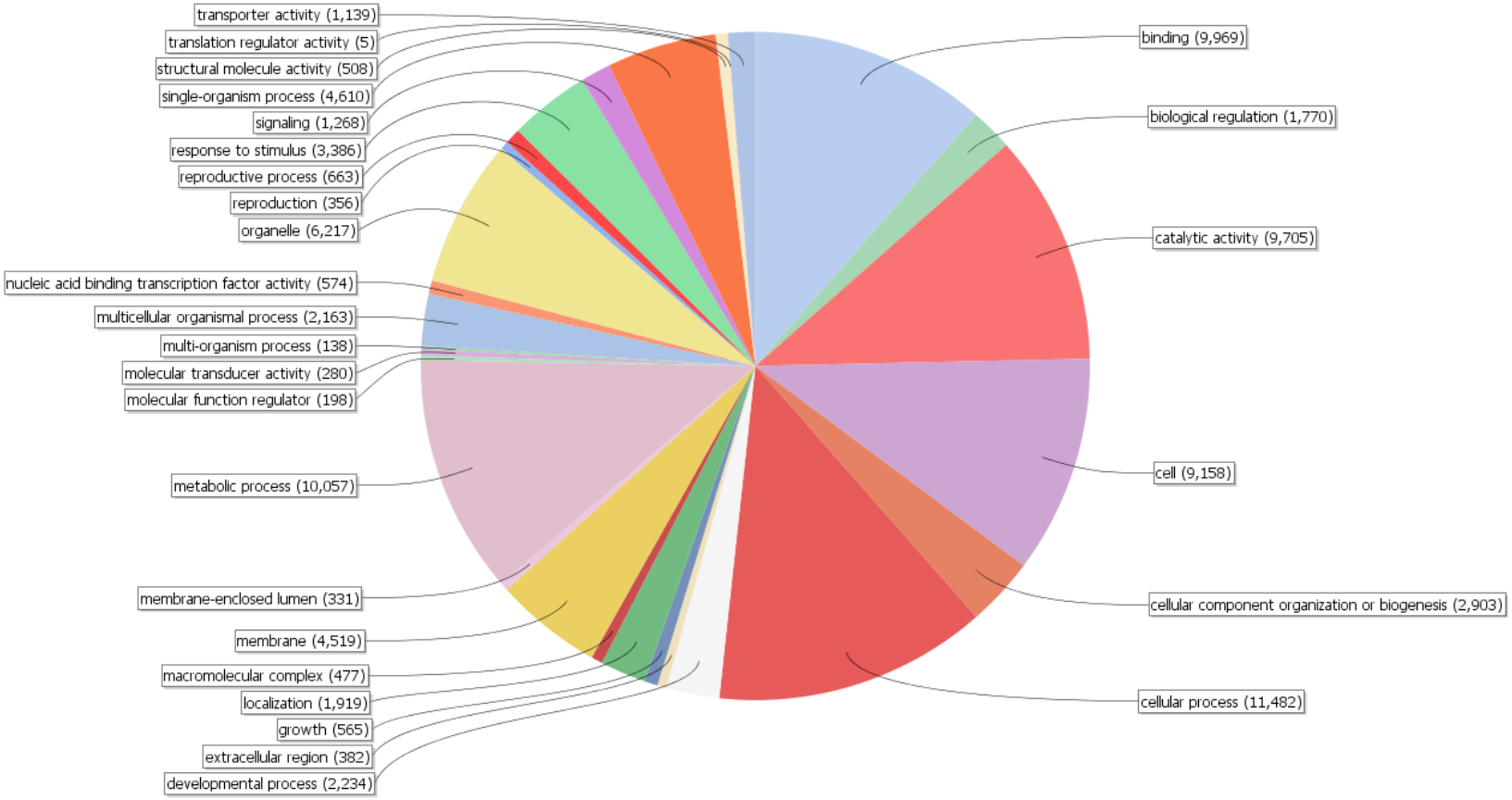


Figure 3.5 Sierra MI contig alignment to all molecular, cellular, and biological processes in Plante samples in GenBank from NCBI

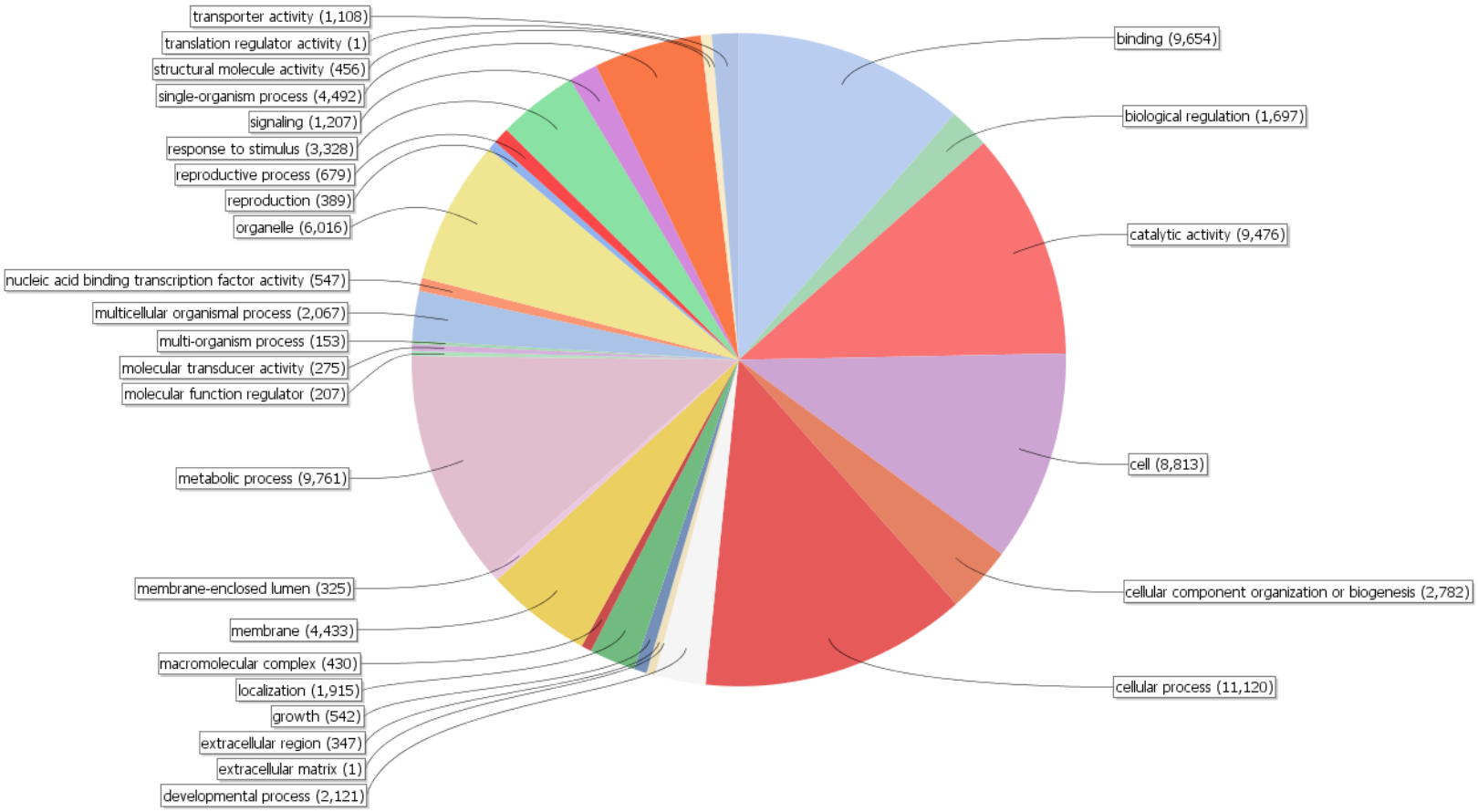


Figure 3.6 Sierra I contig alignment to all molecular, cellular, and biological processes in Plante samples in GenBank from NCBI

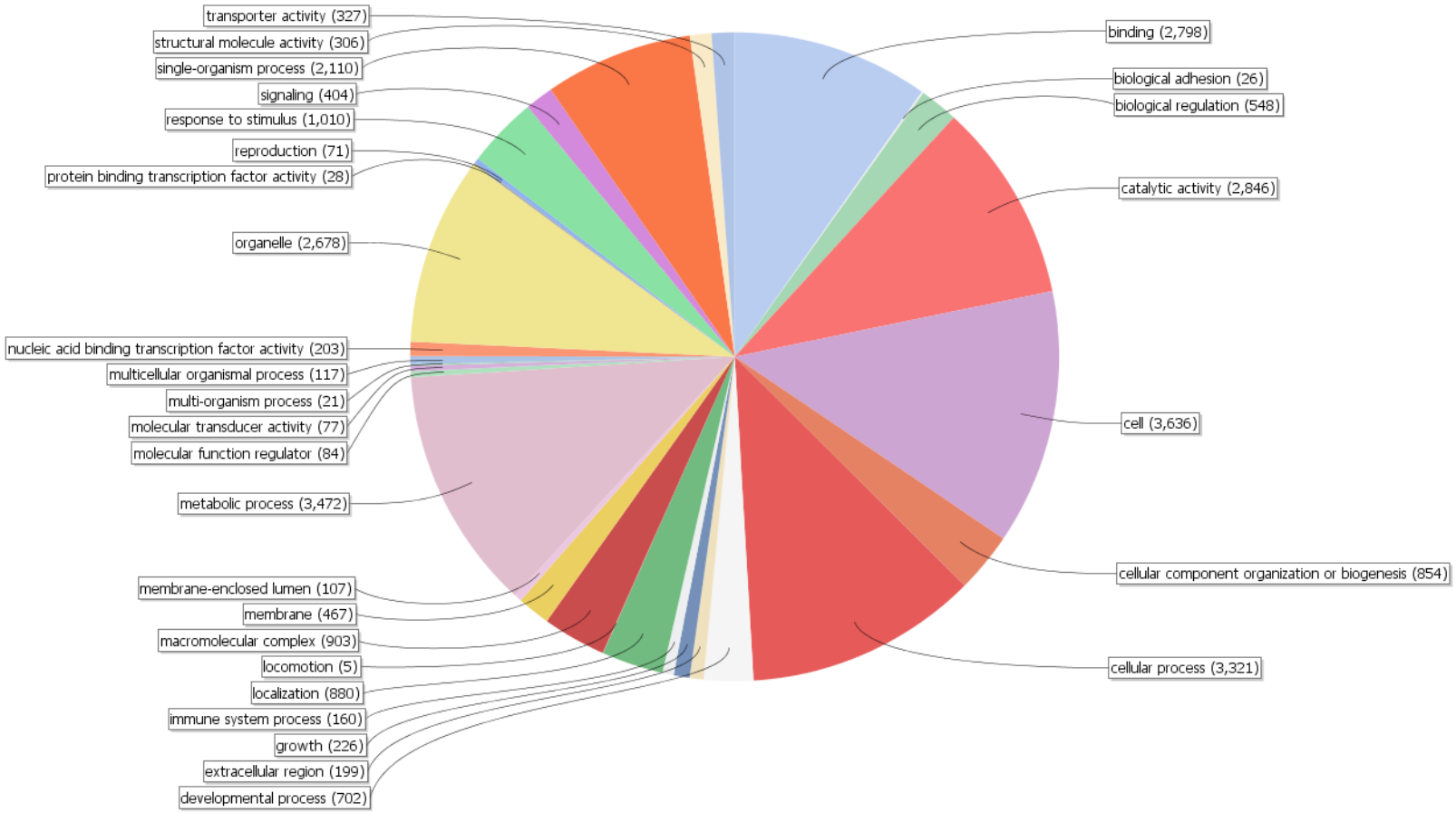


Figure 3.7 All of the processes are identified for each mapped contig in ur3-Δ2. Unlike Sierra MI and Sierra I, ur3-Δ2 has an immune system process that is completely missing in both of the progenitor samples.

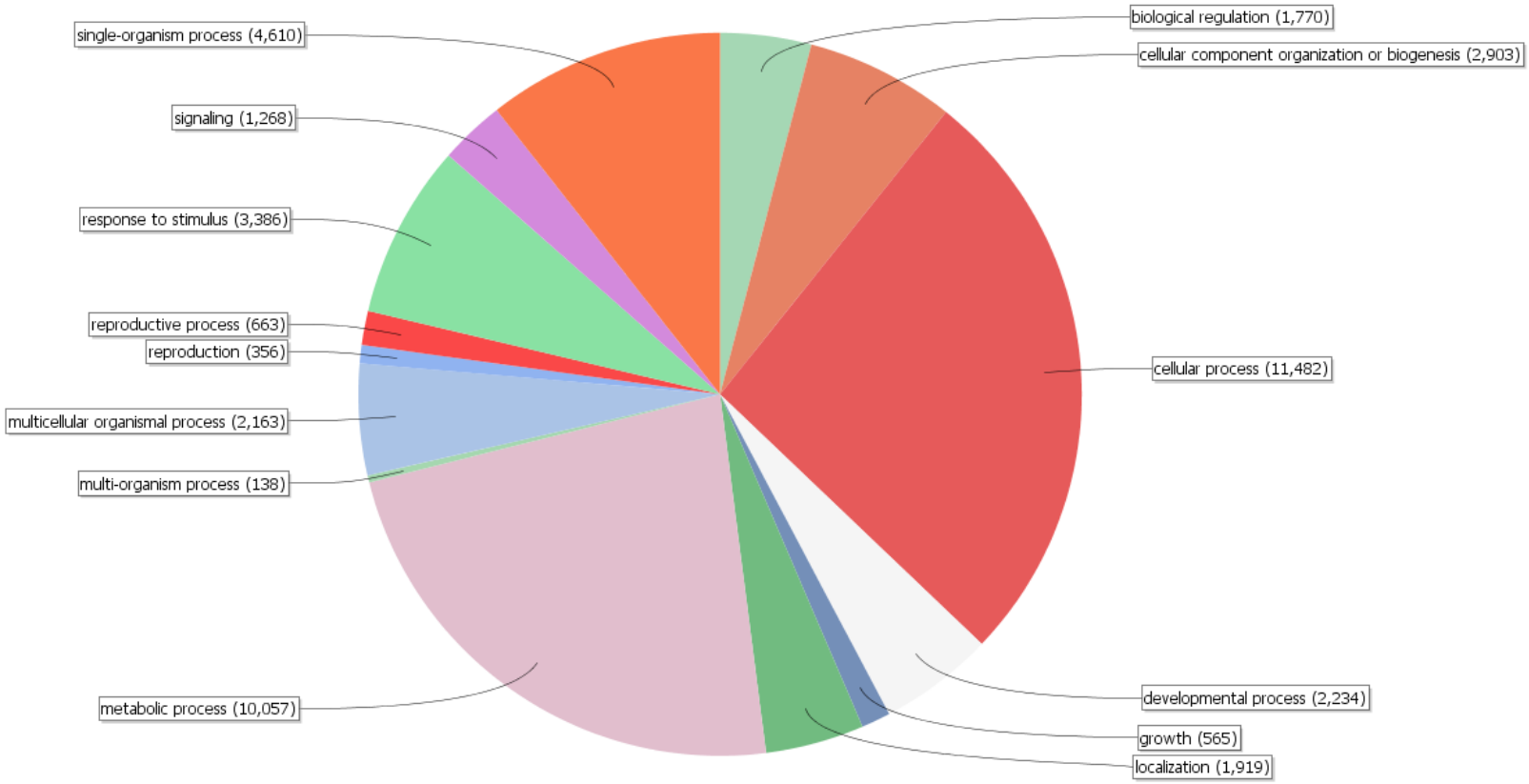


Figure 3.8 Sierra MI biological processes in Plante samples in GenBank from NCBI

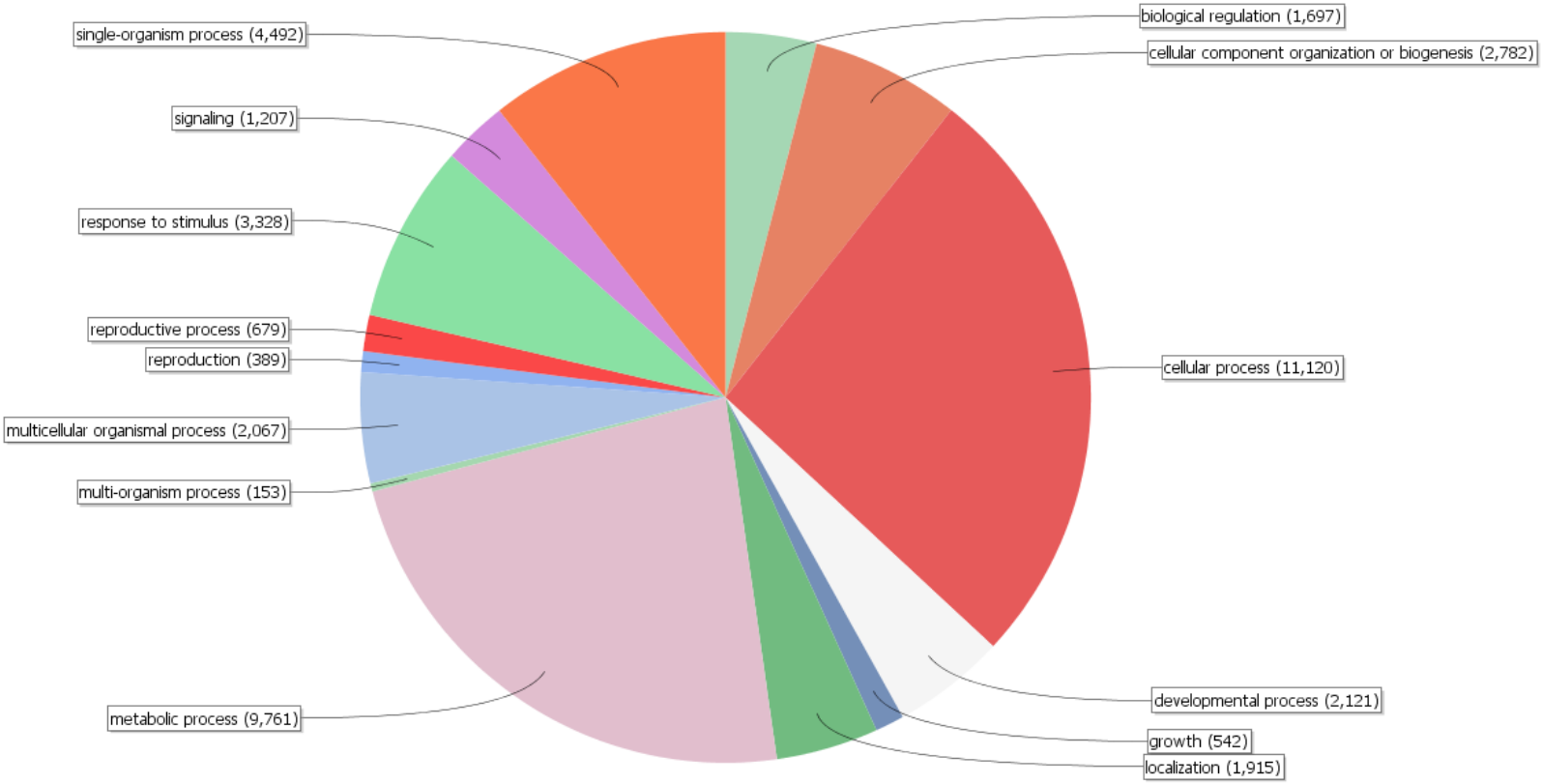


Figure 3.9 Sierra I biological processes in Plante samples in GenBank from NCBI

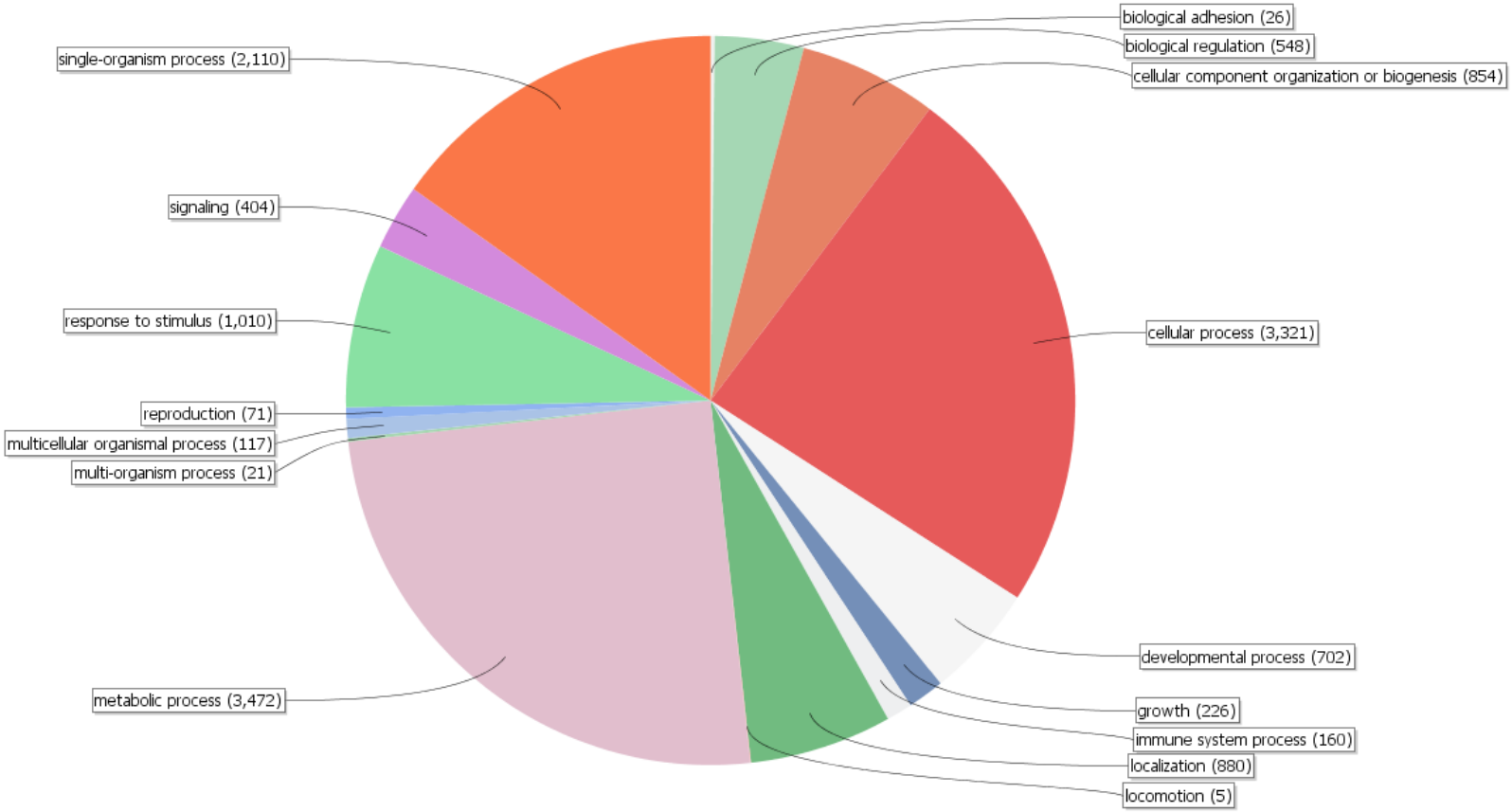


Figure 3.10 Within ur3-Δ2 I, there are immune system processes that are not present in Sierra MI and I.

The identification of an entire group of biological processes that is totally absent in either Sierra sample (MI and I) shows that there is differential expression of genes among between the resistant and susceptible genotypes. The Gene Ontology term refers to “any process involved in the development or functioning of the immune system, an organismal system for calibrated responses to potential internal or invasive threats.”

In addition to the identification of the immune system process in ur3-Δ2 I, also identified was a cluster of differentially expressed genes on chromosome 11, which were more highly expressed in ur3-Δ2 and ur3-Δ3 compared to Sierra. This cluster was, in fact, located on the distal end of chromosome 11. It was located downstream of the SAE19 amplification region. However, the group of genes did not contain any conserved domain information as in the case of the chromosome 10 deletion region genes. Each gene sequence was searched in the NCBI Conserved Domain database to try and identify if they contained any NBS, LRR, or TIR domains. I was unsuccessful in identifying any of the probable disease resistance domains.

Chapter 4

DISCUSSION AND CONCLUSIONS

4.1 Identification of SB1 in Gene Phvul.010G025000

The release of the version 1.0 common bean genome (phytozome.jgi.doe.gov) has allowed information to be obtained that was not before available for public use. Hence, we were able to BLAST several of our molecular marker sequences to identify their location in the genome. Molecular marker SB1, which is located on chromosome 10 in genic sequence Phvul.010G025000, amplifies in all genomic DNA samples except for crg. For this reason, the focal point included the regions to the left and right of Phvul.010G025000 to deduce the group of genes involved in crg's reduced resistance to race 53 rust. When using cDNA from Sierra and crg (MI and I) we found that Phvul.010G025000 does not amplify in either group of samples. This is further confirmed by information provided by Phytozome, showing that this gene only amplifies in the roots and nodules of common bean genotype G19833. Therefore, a conclusion was made that Phvul.010G025000 is most likely not the *Crg* gene, although it is probably located in the vicinity. In order to positively confirm this, additional time course studies will need to be performed. This includes collecting samples at closer time points after initial inoculation.

A brief study in the summer of 2014 allowed work to begin on this next phase. However, due to time constraints and resources, we decided to focus on time point collections that followed literature that was available. In this brief study, we inoculated common bean leaves from Sierra, Olathe, and the three Sierra derived mutants. Tissue collections were made every two hours after the initial inoculation, including a collection at zero hour. RNA was isolated and converted to cDNA for standard pcr. Next, primers derived from Phvul.010G025800 were used to perform pcr on Sierra and crg (MI and I) at zero, two, four, six, and 24 hours post inoculated. Two biological replicates appeared to have the greatest expression in Sierra samples isolated four hours post inoculation. At hours zero and two, there was no expression at all in Sierra MI samples, as opposed to that of inoculated Sierra samples. It was believed that the expression that was observed was due to possible cDNA amplification of pathogen cDNA that was inadvertently converted when we processed the inoculated leaf tissue.

Since there is no way to physically separate fungal rust from the tissue once the inoculum is applied, there will always be residual amounts of pathogen that is processed along with the tissue. One way to confirm or disprove the expression seen in the cDNA pcr at so many alternate time points would be to sequence RNA libraries derived from all time points and map to common bean and available pathogen genomes. Expression data can be collected from reads mapped to target genes and their values can be calculated. Based on expression values between MI and I sample, true differential expression can be determined.

Advances in sequencing and the lowering of cost can make doing this possible. Furthermore, it is unlikely that residual fungal RNA caused expression observed in inoculated Sierra samples because the fungal RNA on crg would have caused expression there as well.

4.2 Delineation of crg Deletion Region

Throughout this research, I was able to identify clusters of differentially expressed genes among genotypes of mock inoculated versus inoculated species. The most promising find was in the disease resistance gene cluster on chromosome 10, which is missing in race 53 rust susceptible crg. Through genomic and cDNA pcr, I was not only able to delineate the 250KB region on chromosome 10, but also able to show expression in Sierra and no expression in crg. Differential expression of a cluster of disease resistance genes was shown between Sierra MI and Sierra I.

The unknown gene, Phvul.010G025800, within the delineated region was most differentially expressed between Sierra MI and I. However, because it does not contain the disease resistance-related conserved domain, such as NBS, TIR, LRR, its role is not yet completely understood. However, one end represents a domain found near the C-terminus of many plant proteins that are uncharacterized. According to the conserved domain search on NCBI website (http://www.ncbi.nlm.nih.gov/), part of the sequence is a probable serine/threonine-protein kinase. Serine/threonine kinases are known to be active

in signal transduction (Martin et al., 1993). If Phvul.010G025800 truly contains a serine/threonine kinase domain, this may explain the gene's role in signal transduction between fungal pathogen effectors and host genes. Also within this region is the *TAO1* gene, which serves as a target of operation of the avirulence gene B in *P. syringae*. Amplification of cDNA paired with Rt-pcr confirm that *TAO1* does not express in either Sierra sample presented in this study. Similarly, the unknown gene Phvul.010G025800 may be a target for effectors from avirulence gene in *U. appendiculatus* in order to contribute to rust resistance. Furthermore, since this study primarily included data from 12hpi only, the expression levels for *TAO1* or Phvul.010G025800 are not completely known throughout pathogenesis.

4.3 Confirmation of SK14 as Non-Genic

In order to identify whether or not SK14 is part of a gene, the zero-24 hour cDNA was analyzed through pcr using the primer set. The results concluded that SK14 does not express at any level in any leaf genotype in this study, leading us to conclude that SK14 is not part of a genic region. Further, there is no evidence that SK14 expresses in residual fungal rust RNA isolated during the leaf tissue isolation as in the case of SB1.

Although there is now a published common bean genome, specific molecular markers, such as SK14, may not be present in certain genomes. Genomic pcr

with SK14 primers on G19833 result in no amplification. Therefore, the likelihood of SK14 aligning with any part of this genome is low to nonexistent.

To date, there is no published information for race 53 rust on common bean genotype G19833. Several attempts have been made to inoculate G19833 with race 53 along with the other five genotypes in this study. The findings have shown no response, as opposed to HR which is seen with Sierra.

4.4 Next Steps

Now that candidate genes have been identified, future research can be done to clone and transfer genes into susceptible genotype crg in an attempt to recover resistance. There is little plausible research available for gene transformation in common bean. Successful transformations have mostly been achieved in cereal crops. However, there has been at least one study identified that describes transformation of the common bean, including the Olathe genotype (Kwapata et al., 2012). In this study, three transgenes were bombarded into selected apical meristems of common bean. The *Gus* reporter gene marker (β-glucuronidase) (Jefferson et al., 1987), the bar herbicide marker (Thompson et al., 1987; White et al., 1990), and the HVA1protein for barley late embryogenesis (Straub et al., 1994; Xu et al., 1996) were particle bombarded into common bean. Since we have not yet done this type of procedure, it would be necessary to collaborate with a lab group that has expertise in this area.

Another approach to exploring the importance of the selected group of genes is to use viral induced RNA silencing (Weitzman, 2002). The process is sometimes termed viral induced gene silencing or 'VIGS.' In this technique, a silencing suppressor is used to degrade a specific sequence of RNA. By targeting and silencing each specific gene separately, gene importance can be identified.

Performing a global transcriptome analysis across all genotypes used in this study may help better understand rust-induced gene expression. Already available is Sierra 454 transcriptome sequencing data (Kalavacharla et al., 2013), which can serve as a guide for future analysis. By doing comparative analysis of all five genotypes, plus the sequenced genome, genes or gene clusters with increased or decreased expression values may be identified.

There are several ways to approach the global transcriptome analysis across genotypes. Available for each genotype are MI and I samples. Comparisons can be done within one genotype, MI and I, as well as among several genotypes. Comparisons among samples include comparing all genotypes that have been inoculated against one another to identify differential expression. Within the crg deletion region, the *TAO1* gene was identified, but was not highly expressed in either Sierra (MI and I) sample. The *TAO1* gene serves as an operational target of *AvrB.* Future work seeks to identify the role of *TAO1* in Olathe and mutants ur3-Δ2/3, if any. Does *TAO1* serve as an effector target in other genotypes, creating an increase or decrease in expression? There may be other genes,

globally, that are involved in ETI. Performing a global analysis may allow the identification of genes that are possibly triggered by pathogen effectors.

Also of importance is time of disease resistance gene expression in common bean. Currently, the tissues available in this study include those collected at zero, 12, and 84 hpi. In order to have a better understanding of gene expression, shorter time intervals need to be collected, i.e. every one hour. By collecting tissue at every hour between one and 12, there may be a clearer understanding of when gene expression begins to occur.

The research presented in this study explains the necessity of continually identifying disease resistance genes in food crops as a means of global sustainability. As pathogens eventually overcome known disease resistance gene, it is important to isolate new genes that confer resistance for the sake of the global population's nutrition and economy. As the population continues to grow, it is necessary to make sure we can continue to produce food, especially in places where particular crops are relied on heavily. In many of the places across the globe, application of pesticides are not always available due to costs, nor are they popular due to unknown long term effects of use.

REFERENCES

Bailey P. (2014) Nothing common abouth these beans, Food Blog: Food news from the UC Division of Agriculture and Natural Resources.

Ballantyne B. (1978) The genetic basis of resistance to rust, caused by *Uromyces appendiculatus* in bean (*Phaseolus vulgaris*). Agric. Dept, Univ. Sydney,Australia Sydney. pp. 262

Bell J.K., Mullen G.E., Leifer C.A., Mazzoni A., Davies D.R., Segal D.M. (2003) Leucine-rich repeats and pathogen recognition in Toll-like receptors. Trends in immunology 24:528-533.

Bent A. (1996) Plant disease resistance genes: Function meets structure. Plant Cell 8:1757–1771.

Bent A., Kunkel B., Dahlbeck D., Brown K., Schmidt R., Giraudat J., Leung J., Staskawicz B. (1994) RPS2 of *Arabidopsis thaliana*: a leucine-rich repeat class of plant disease resistance genes. Science 265:1856-1860.

B.I.C. (2015) Bean Improvement Cooperative (http://bic.css.msu.edu/) November 24, 2015.

Birch P.R.J., Rehmany A.P., Pritchard L., Kamoun S., Beynon J.L. (2006) Trafficking arms: oomycete effectors enter host plant cells. Trends in Microbiology 14:8-11.

Block A., Alfano J.R. (2011) Plant targets for *Pseudomonas syringae* type III effectors: virulence targets or guarded decoys? Current Opinion in Microbiology 14:39-46.

Bolton M., Kolmer J., Garvin D. (2008) Wheat leaf rust caused by *Puccinia triticina*. Molecular Plant Pathology 9:563-575.

Bos J.I.B., Armstrong M.R., Gilroy E.M., Boevink P.C., Hein I., Taylor R.M., Zhendong T., Engelhardt S., Vetukuri R.R., Harrower B., Dixelius C., Bryan G., Sadanandom A., Whisson S.C., Kamoun S., Birch P.R.J. (2010) *Phytophthora infestans* effector AVR3a is essential for virulence and manipulates plant immunity by stabilizing host E3 ligase CMPG1. Proceedings of the National Academy of Sciences 107:9909-9914.

Buruchara R., Chirwa R., Sperling L., Mukankusi C., Rubyogo J.C., Mutonhi R., Abang M. (2011) Development and delivery of bean varieties in Africa: the Pan-Africa Bean Research Alliance (PABRA) model. African crop science journal 19:227-245.

Chandalia M., Garg A., Lutjohann D., von Bergmann K., Grundy S.M., Brinkley L.J. (2000) Beneficial effects of high dietary fiber intake in patients with type 2 diabetes mellitus. New England Journal of Medicine 342:1392-1398.

Cooper B., Neelam A., Campbell K.B., Lee J., Liu G., Garrett W.M., Scheffler B., Tucker M.L. (2007) Protein Accumulation in the Germinating Uromyces appendiculatus Uredospore. Molecular plant-microbe interactions 20:857-866.

da Cunha L., McFall A.J., Mackey D. (2006) Innate immunity in plants: a continuum of layered defenses. Microbes and Infection 8:1372-1381.

Dangl J.L., Horvath D.M., Staskawicz B.J. (2013) Pivoting the Plant Immune System from Dissection to Deployment. Science 341:746-751.

Dixon M., Hatzixanthis K., Jones D., Harrison K., Jones J. (1998) The tomato Cf-5 disease resistance gene and six homologs show pronounced allelic variation in leucine-rich repeat copy number. Plant Cell 10:1915-1925.

Dixon M., Jones D., Keddie J., Thomas C., Harrison K., Jones J. (1996) The tomato Cf-2 disease resistance locus comprises two functional genes encoding leucine rich repeat proteins. Cell 84:451-459.

DNREC Delaware Department of Natural Resources and Environmental Control (http://www.dnrec.delaware.gov/ClimateChange/Pages/ClimateChangeDelawareAgriculture.aspx) November 24, 2015

Doyle J. (1991) DNA protocols for plants, Molecular techniques in taxonomy, Springer. pp. 283-293.

Eitas T., Nimchuk Z., Dangl J. (2008) Arabidopsis TAO1 is a TIR-NB-LRR protein that contributes to disease resistance induced by the *Pseudomonas syringae* effector AvrB. Proceedings of the National Academy of Sciences 105:6475-6480.

Engelhardt S., Boevink P.C., Armstrong M.R., Ramos M.B., Hein I., Birch P.R. (2012) Relocalization of late blight resistance protein R3a to endosomal compartments is associated with effector recognition and required for the immune response. The Plant Cell 24:5142-5158.

Figueroa, M., Alderman, S., Garvin, D. F., & Pfender, W. F. (2013). Infection of Brachypodium distachyon by formae speciales of *Puccinia graminis*: early infection events and host-pathogen incompatibility. PLoS One, 8(2), e56857.

Flor H. (1956) The complementary genic systems in flax and flax rust. Advanced Genetics 8:29-54.

Freyre R., Ríos R., Guzmán L., Debouck D., Gepts P. (1996) Ecogeographic distribution of *Phaseolus* spp. (Fabaceae) in Bolivia. Economic Botany 50:195-215.

Freyre R., Skroch P.W., Geffroy V., Adam-Blondon A.F., Shirmohamadali A., Johnson W.C., Llaca V., Nodari R.O., Pereira P.A., Tsai S.M., Tohme J., Dron M., Nienhuis J., Vallejos C.E., Gepts P. (1998) Towards an integrated linkage map of common bean. 4. Development of a core linkage map and alignment of RFLP maps. TAG Theoretical and Applied Genetics 97:847-856.

Garzón L.N., Oliveros O.A., Rosen B., Ligarreto G.A., Cook D.R., Blair M.W. (2013) Isolation and characterization of nucleotide-binding site resistance gene homologues in common bean (*Phaseolus vulgaris*). Phytopathology 103:156-168.

Grant M., Godiard L., Straube E., Ashfield T., Lewald J., Sattler A., Innes R., Dangl J. (1995) Structure of the Arabidopsis *RPM1* gene enabling dual specificity disease resistance. Science 269:843-846.

Gururani M.A., Venkatesh J., Upadhyaya C.P., Nookaraju A., Pandey S.K., Park S.W. (2012) Plant disease resistance genes: Current status and future directions. Physiological and Molecular Plant Pathology 78:51-65.

Haas B.J., Kamoun S., Zody M.C., Jiang R.H.Y., Handsaker R.E., Cano L.M., Grabherr M., Kodira C.D., Raffaele S., Torto-Alalibo T., Bozkurt T.O., Ah-Fong A.M.V., Alvarado L., Anderson V.L., Armstrong M.R., Avrova A., Baxter L., Beynon J., Boevink P.C., Bollmann S.R., Bos J.I.B., Bulone V., Cai G., Cakir C., Carrington J.C., Chawner M., Conti L., Costanzo S., Ewan R., Fahlgren N., Fischbach M.A., Fugelstad J., Gilroy E.M., Gnerre S., Green P.J., Grenville-Briggs L.J., Griffith J., Grunwald N.J., Horn K., Horner N.R., Hu C.-H., Huitema E., Jeong D.-H., Jones A.M.E., Jones J.D.G., Jones R.W., Karlsson E.K., Kunjeti S.G., Lamour K., Liu Z., Ma L., MacLean D., Chibucos M.C., McDonald H., McWalters J., Meijer H.J.G., Morgan W., Morris P.F., Munro C.A., O'Neill K., Ospina-Giraldo M., Pinzon A., Pritchard L., Ramsahoye B., Ren Q., Restrepo S., Roy S., Sadanandom A., Savidor A., Schornack S., Schwartz D.C., Schumann U.D., Schwessinger B., Seyer L., Sharpe T., Silvar C., Song J., Studholme D.J., Sykes S., Thines M., van de Vondervoort P.J.I., Phuntumart V., Wawra S., Weide R., Win J., Young C., Zhou S., Fry W., Meyers B.C., van West P., Ristaino J., Govers F., Birch P.R.J., Whisson S.C., Judelson H.S., Nusbaum C. (2009) Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. Nature 461:393-398.

Hammond-Kosack K.E., Jones J.D. (1996) Resistance gene-dependent plant defense responses. The Plant Cell 8:1773-1791.

Helfer, S. (2014). Rust fungi and global change. New Phytologist, 201(3), 770-780.

Hinrichs A.S., Karolchik D., Baertsch R., Barber G.P., Bejerano G., Clawson H., Diekhans M., Furey T.S., Harte R.A., Hsu F., Hillman-Jackson J., Kuhn R.M., Pedersen J.S., Pohl A., Raney B.J., Rosenbloom K.R., Siepel A., Smith K.E., Sugnet C.W., Sultan-Qurraie A., Thomas D.J., Trumbower H., Weber R.J., Weirauch M., Zweig A.S., Haussler D., Kent W.J. (2006) The UCSC Genome Browser Database: update 2006. Nucleic Acids Research 34:D590-D598.

Jebanathirajah J.A., Peri S., Pandey A. (2002) Toll and interleukin-1 receptor (TIR) domain-containing proteins in plants: a genomic perspective. Trends in Plant Science 7:388-391.

Johal G., Briggs S. (1992) Reductase activity encoded by the HM1 disease resistance gene in maize. Science 258:985-987.

Jones D.A., Thomas C.M., Hammond-Kosack K.E., Balint-Kurti P.J., Jones J.D. (1994) Isolation of the tomato Cf-9 gene for resistance to *Cladosporium fulvum* by transposon tagging. Science 266:789-793.

Jones J., Dangl J. (2006) The plant immune system. Nature 444:1-7.

Kalavacharla V., Liu Z., Meyers B.C., Thimmapuram J., Melmaiee K. (2011) Identification and analysis of common bean (*Phaseolus vulgaris* L.) transcriptomes by massively parallel pyrosequencing. BMC Plant Biology.

Kalavacharla V., Stavely J., Myers J., McClean P. (2000) *Crg*, a Gene required for *Ur-3*-mediated rust resistance in common bean, maps to a resistance gene analog cluster. Molecular Plant-Microbe Interactions 13:1237-1242.

Kelly J.D., Copeland J.O. (1994) Sierra. a new Pinto bean for Michigan. Ext. Bull.

Kelly J.D., Gepts P., Miklas P.N., Coyne D.P. (2003) Tagging and mapping of genes and QTL and molecular marker-assisted selection for traits of economic importance in bean and cowpea. Field Crops Research 82:135-154.

Lawrence G.J., Finnegan E.J., Ayliffe M.A., Ellis J.G. (1995) The *L6* gene for flax rust resistance is related to the Arabidopsis bacterial resistance gene *RPS2* and the tobacco viral resistance gene *N*. Plant Cell 7:1195-1206.

Le Roux C., Huet G., Jauneau A., Camborde L., Trémousaygue D., Kraut A., Zhou B., Levaillant M., Adachi H., Yoshioka H., Raffaele S., Berthomé R., Couté Y., Parker Jane E., Deslandes L. (2015) A Receptor Pair with an Integrated Decoy Converts Pathogen Disabling of Transcription Factors to Immunity. Cell 161:1074-1088.

Link T.I., Lang P., Scheffler B.E., Duke M.V., Graham M.A., Cooper B., Tucker M.L., van de Mortel M., Voegele R.T., Mendgen K., Baum T.J., Whitham S.A. (2014) The haustorial transcriptomes of *Uromyces appendiculatus*

and *Phakopsora pachyrhizi* and their candidate effector families. Molecular Plant Pathology 15:379-393.

Mamidi S., Rossi M., Moghaddam S.M., Annam D., Lee R., Papa R., McClean P.E. (2013) Demographic factors shaped diversity in the two gene pools of wild common bean *Phaseolus vulgaris* L. Heredity 110:267-276.

Martin G.B., Brommonschenkel S., Chunwongse J., Frary A., Ganal M.W., Spivey R., Wu T., Earle E.D., Tanksley S.D. (1993) Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science 262:1432-1436.

Mastenbroek C. (1960) A breeding programme for resistance to anthracnose in dry shell haricot beans, based on a new gene. Euphytica 9:177-184.

McClean P., Cannon S., Gepts P., Hudson M., Jackson S., Rokhsar D., Schmutz J., Vance C. (2008) Towards a whole genome sequence of common bean, (*Phaseolus vulgaris*): background, approaches, applications.

Miklas P., Pastor-Corrales M., Jung G., Coyne D., Kelly J., McClean P., Gepts P. (2002) Comprehensive linkage map of bean rust resistance genes. ANNUAL REPORT-BEAN IMPROVEMENT COOPERATIVE 45:125-129.

Miklas P.N. (2002) Marker-assisted selection for disease resistance in common bean. ANNUAL REPORT-BEAN IMPROVEMENT COOPERATIVE 45:1-3.

Mindrinos M., Katagiri F., Yu G., Ausubel F. (1994) The A. thaliana disease resistance gene RPS2 encodes a protein containing a nucleotide-binding site and leucine-rich repeats. Cell 78:1089-1099.

Motamayor J.C., Mockaitis K., Schmutz J., Haiminen N., Iii D.L., Cornejo O., Findley S.D., Zheng P., Utro F., Royaert S., Saski C., Jenkins J., Podicheti R., Zhao M., Scheffler B.E., Stack J.C., Feltus F.A., Mustiga G.M., Amores F., Phillips W., Marelli J.P., May G.D., Shapiro H., Ma J., Bustamante C.D., Schnell R.J., Main D., Gilbert D., Parida L., Kuhn D.N. (2013) The genome sequence of the most widely cultivated cacao type and its use to identify candidate genes regulating pod color. Genome Biology 14:r53-r53.

Mylona P., Pawlowski K., Bisseling T. (1995) Symbiotic nitrogen fixation. The Plant Cell 7:869-885.

Namugwanya M., Tenywa J.S., Otabbong E., Mubiru D.N., Masamba T.A. (2014) Development of Common Bean (*Phaseolus Vulgaris* L.) Production Under Low Soil Phosphorus and Drought in Sub-Saharan Africa: A Review. Journal of Sustainable Development 7:p128.

Nemchinova Y., Stavely J. (1998) Development of SCAR primers for the *Ur-3* rust resistance gene in common bean. Phytopathology 88:S67.

Nishimura M.T., Dangl J.L. (2014) Paired Plant Immune Receptors. Science 344:267-268.

Nishimura Marc T., Monteiro F., Dangl Jeffery L. (2015) Treasure Your Exceptions: Unusual Domains in Immune Receptors Reveal Host Virulence Targets. Cell 161:957-960.

Odogwu B., Nkalubo S., Rubaihayo P. Breeding for common bean rust in Uganda.

Parker J.E., Coleman M.J., Szabò V., Frost L.N., Schmidt R., van der Biezen E.A., Moores T., Dean C., Daniels M.J., Jones J. (1997) The Arabidopsis downy mildew resistance gene RPP5 shares similarity to the toll and interleukin-1 receptors with N and L6. The Plant Cell 9:879-894.

Petre, Benjamin, et al. "RNA-Seq of early-infected poplar leaves by the rust pathogen *Melampsora larici-populina* uncovers PtSultr3; 5, a fungal-induced host sulfate transporter." (2012): e44408.

Petry N., Boy E., Wirth J.P., Hurrell R.F. (2015) Review: The Potential of the Common Bean (*Phaseolus vulgaris*) as a Vehicle for Iron Biofortification. Nutrients 7:1144-1173.

Rafiqi M., Ellis J.G., Ludowici V.A., Hardham A.R., Dodds P.N. (2012) Challenges and progress towards understanding the role of effectors in plant–fungal interactions. Current Opinion in Plant Biology 15:477-482.

Rivkin M.I., Vallejos C.E., McClean P.E. (1999) Disease-resistance related sequences in common bean. Genome 42:1-7.

Ross A.F. (1961a) Localized acquired resistance to plant virus infection in hypersensitive hosts. Virology 14:329-339.

Ross A.F. (1961b) Systemic acquired resistance induced by localized virus infections in plants. Virology 14:340-358.

Sambrook J., Russell D.W. (2001) Molecular Cloning: A Laboratory Manual . 2001, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sarris Panagiotis F., Duxbury Z., Huh Sung U., Ma Y., Segonzac C., Sklenar J., Derbyshire P., Cevik V., Rallapalli G., Saucet Simon B., Wirthmueller L., Menke Frank L.H., Sohn Kee H., Jones Jonathan D.G. (2015) A Plant Immune Receptor Detects Pathogen Effectors that Target WRKY Transcription Factors. Cell 161:1089-1100.

Schmutz J., McClean P.E., Mamidi S., Wu G.A., Cannon S.B., Grimwood J., Jenkins J., Shu S., Song Q., Chavarro C., Torres-Torres M., Geffroy V., Moghaddam S.M., Gao D., Abernathy B., Barry K., Blair M., Brick M.A., Chovatia M., Gepts P., Goodstein D.M., Gonzales M., Hellsten U., Hyten D.L., Jia G., Kelly J.D., Kudrna D., Lee R., Richard M.M.S., Miklas P.N., Osorno J.M., Rodrigues J., Thareau V., Urrea C.A., Wang M., Yu Y., Zhang M., Wing R.A., Cregan P.B., Rokhsar D.S., Jackson S.A. (2014) A reference genome for common bean and genome-wide analysis of dual domestications. Nat Genet 46:707-713.

Sindhu A., Chintamanani S., Brandt A.S., Zanis M., Scofield S.R., Johal G.S. (2008) A guardian of grasses: Specific origin and conservation of a unique disease-resistance gene in the grass lineage. Proceedings of the National Academy of Sciences of the United States of America 105:1762-1767.

Souza T.L.P.O., Faleiro F.G., Dessaune S.N., Paula-Junior T.J.d., Moreira M.A., Barros E.G.d. (2013) Breeding for common bean (*Phaseolus vulgaris* L.) rust resistance in Brazil. Tropical Plant Pathology 38:361-374.

Strange R.N., Scott P.R. (2005) Plant Disease: A Threat to Global Food Security. Annual Review of Phytopathology 43:83-116. DOI: 10.1146/annurev.phyto.43.113004.133839.

Takahashi N., Takahashi Y., Putnam F.W. (1985) Periodicity of leucine and tandem repetition of a 24-amino acid segment in the primary structure of leucine-rich alpha 2-glycoprotein of human serum. Proceedings of the National Academy of Sciences 82:1906-1910.

Tang D., Kang R., Coyne C.B., Zeh H.J., Lotze M.T. (2012) PAMPs and DAMPs: Signal 0s that Spur Autophagy and Immunity. Immunological reviews 249:158-175..

Tatum L.A. 1971, The southern corn leaf blight epidemic, Science, 171, 1113–6.

Uma B., Rani T.S., Podile A.R. (2011) Warriors at the gate that never sleep: non-host resistance in plants. Journal of plant physiology 168:2141-2152.

van der Biezen E.A., Jones J.D.G. (1998) The NB-ARC domain: a novel signalling motif shared by plant resistance gene products and regulators of cell death in animals. Current Biology 8:R226-R228.

Van Ooijen G., Mayr G., Kasiem M.M., Albrecht M., Cornelissen B.J., Takken F.L. (2008) Structure–function analysis of the NB-ARC domain of plant disease resistance proteins. Journal of Experimental Botany 59:1383-1397.

Vleeshouwers V.G.A.A., Oliver R.P. (2014) Effectors as Tools in Disease Resistance Breeding Against Biotrophic, Hemibiotrophic, and Necrotrophic Plant Pathogens. Molecular plant-microbe interactions 27:196-206.

Whitman S., McCormick S., Baker B. (1996) The *N* gene of tobacco confers resistance to tobacco mosaic virus in transgenic tomato. Proc. Natl. Acad. Sci. USA 93:8776-8781.

Woodham-Smith C. (1991) The great hunger: Ireland 1845-1849 Penguin books.