The development of a Niemann-Pick gene-based marker in common bean (*Phaseolus vulgaris* L.) for the selection of common bacterial blight resistance

by

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ABSTRACT

THE DEVELOPMENT OF A NIEMANN-PICK GENE-BASED MARKER IN COMMON BEAN (*PHASEOLUS VULGARIS* L.) FOR THE SELECTION OF COMMON BACTERIAL BLIGHT RESISTANCE

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Common bacterial blight (CBB) is a major disease in common bean (*Phaseolus vulgaris*) caused by *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas fuscans* subsp. *fuscans*. By characterizing alleles in three candidate genes within white bean line OAC Rex, a codominant molecular marker for resistance was created. The Niemann-Pick polymorphism (NPP) marker was developed to screen common beans for CBB resistance, and is anchored in a candidate resistance gene (Niemann-Pick like gene). Individuals in two segregating populations were screened for allelic state and disease susceptibility. It was found that the NPP marker is significantly more efficient (8% decrease in error rate) in comparison to other markers. Within the OAC Rex pedigree, the resistant allele for the NPP marker was found solely within progeny from the introgression of *Phaseolus acutifolius* into *P. vulgaris*. Lastly, the two additional candidate resistance genes investigated (R-genes 231733-8-004 and 231733-8-005), were found to have no exploitable polymorphisms.

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List of Abbreviations

AAFC- Agriculture and Agri-Food Canada

AFLP- amplified fragment length polymorphism

ANOVA- analysis of variance

BP- base pairs

CBB- common bacterial blight

CIAT- International Center for Tropical Agriculture

EST- expressed sequence tag

IDE- insulin degrading enzyme

KB – kilobase pairs

LB- Lysogeny broth

LRR- leucine rich repeats

MAS- marker assisted selection

NBS- nucleotide binding site

OAC- Ontario Agricultural College

PB- potassium phosphate

PI- plant introduction

QTL- quantitative trait loci

RIL- recombinant inbred line

SNP- single nucleotide polymorphism

PCR- polymerase chain reaction

NPP- Niemann-Pick polymorphism

Chapter 1: Literature Review: The incorporation and understanding of common bacterial blight resistance in common bean (*Phaseolus vulgaris* L.) breeding programs

1.1 Introduction

Common bacterial blight (CBB) is a major disease of common bean (*Phaseolus vulgaris* L.) crops worldwide (Munoz and Singh, 1999). CBB resistance within common beans is an active area of research (Singh and Miklas, 2015). In order to be able to more efficiently breed resistant cultivars, the inheritance of resistance within common beans must first be better understood (Urrea *et al.*, 1999). CBB resistance is believed to be quantitatively inherited, and gene for gene interactions between common bean resistance genes and virulence genes within the CBB causing bacteria *Xanthomonas axonopodis* pv. *phaseoli* (Smith) and *Xanthomonas fuscans* subsp. *fuscans* Schadd are not found (Miklas *et al*., 2006; Alavi *et al.,* 2008; Mahuku *et al.*, 2006).

In today's market, there are resistant common bean cultivars that are believed to have gained their resistance to CBB from interspecific hybridizations with *Phaseolus acutifolius* A. Gray followed by back-crossing with *P. vulgaris* varieties (Miklas *et al*., 2006). One of these resistant cultivars, OAC Rex, has been sequenced and assembled allowing the genes in this cultivar to be compared to those in susceptible *P. vulgaris* lines and resistant *P. acutifolius* lines (Perry *et al.,* 2013). In order to investigate the roles that are played by candidate genes this project tested the correlations between CBB disease ratings and marker occurrences for susceptible and resistant lines in two populations segregating for CBB resistance. By testing the correlation between resistance and the marker, more can be discovered about the role that the associated gene, 232701-8-007, may play in CBB resistance. Gene 232701-8-007 is found on contig 232701 at the end of chromosome 8 in OAC Rex (NCBI accession KF429165) (Perry *et al.*, 2013). In addition, this project included an investigation of two R genes for their potential association with

CBB resistance because of their presence in resistant bean line OAC Rex and absence in susceptible G19833 (Perry *et al*., 2013). Sequence data from several bean lines were compared for these two R-genes in order to find genetic polymorphisms that could be exploited for a correlation study, similar to that conducted for gene 232701-8-007. A critical review of current literature is given below of topics and areas of research relevant to the current project.

1.2 *Phaseolus vulgaris*

1.2.1 Plant Biology

P. vulgaris (common bean) is a diploid species (n=11) with diversity in plant architecture and growth (Gepts and Debouck, 1991; Gepts *et al.*, 2008). As the common bean germinates, the seedling emerges with epigeal growth, making young plants susceptible to frost and growth point damage (Micheals, 1991). With the exception of the first pair of leaves, which grow as a unifoliate pair, common bean has pinnately trifoliate leaves based around a main stem (Micheals, 1991). The plant structure can vary between market classes and cultivars, with upright, erect main stems found in small seeded classes and prostrate plants found within large seeded classes (Gepts and Debouck, 1991). There are two main classifications of plant growth: determinate and indeterminate (Gepts and Debouck, 1991). Determinate cultivars have a terminate raceme and are often earlier varieties (Gepts and Debouck, 1991). Indeterminate cultivars continuously produce new flower buds and are limited only by the growth season length. Indeterminate plants can be less upright and more vined in comparison to determinate varieties (Gepts and Debouck, 1991). *P. vulgaris* is a self-pollinating species, with a zygomorphic flower that completely encloses the anthers and stigma, preventing cross pollination (Gepts and Debouck, 1991).

Flowers range from white to purple, with many variations of pink flowers found (Gepts and Debouck, 1991).

Common beans grow in a variety of conditions, ranging from tropical to temperate, though the optimal growth temperatures range from 15-21°C (Micheals, 1991). With ovary number and density highly variable, there are typically 5-8 ovules within each ovary, resulting in 5-8 beans developing within each pod (Gepts and Debouck, 1991). Frequently more ovules develop within an ovary in small seeded market classes, resulting in more beans within in pod in small seeded cultivars in comparison to large seeded cultivars (Micheals, 1991). As a legume species, common bean forms symbiotic relationships with nitrogen fixing *Rhizobium* bacteria (Micheals, 1991; Akter *et al*., 2014). Although common bean symbiosis is typically less efficient than other legume species such as *Glycine max*, the nitrogen fixed from atmospheric nitrogen is still significant for plant growth and development (Akter *et al.*, 2014; Cardoso *et al.*, 2012).

1.2.2 Cultivation

P. vulgaris is grown in diverse environments worldwide with significant cultivation in India, Mexico and Central America (Van Schoonhoven and Voyset, 1991). Common beans are frequently consumed for their high protein content as they are 20-25% protein by weight, and also contain several micronutrients such as iron and vitamins like folate that benefit human health (Akibode and Maredia, 2011). With 249, 400 tonnes produced in Canada in 2016 and 27, 973, 261 tonnes produced worldwide over the same time period, common beans are globally an economically important crop (FAOSTAT, 2018). Common beans can be divided into several classes including: small seeded classes like white beans and black beans, and large seeded

classes such as kidney beans, cranberry beans and pinto beans (Singh *et al*., 1991). Though variation in seed coat colour is found in both large seeded beans and small seeded beans, typically the difference in bean size is linked to the ancestral origin of the bean class (Singh, 2001).

Domesticated over 7 000 years ago in present day Mexico and South America, there are two centres of origin for the genetic diversity of domesticated common beans (Graham and Ranalli, 1997). Based on gene sequence data, it is believed that cultivated common beans originated from the Mesoamerican centre of origin around common day Mexico, but were domesticated a second time near the Andean centre of origin located within the lowlands of the Andes Mountains (Singh *et al*., 1991; Graham and Ranalli, 1997; Bitocchi *et al*., 2012). Large seeded beans descend from the Andean centre of origin, whereas, the majority of small seeded beans are from the Mesoamerican centre of origin (Singh *et al.*, 1991). In addition, chloroplast DNA sequences suggest that the gene pools within each centre of origin are distinct and the result from two independent domestication events (Chaćon *et al.*, 2005).

In comparison to the related wild species, domesticated cultivars are generally photoperiod insensitive, non-dehiscent, and seeds do not have a requirement for an extended period of dormancy (Singh, 2001). The reduction in photoperiod sensitivity has allowed the cultivation of beans to expand from as far north as Canada to Argentina in South America (Singh *et al.*, 1991; Singh, 2001). Additionally, non-dehiscent seed pods have resulted in easier harvests and less seed loss. The loss of seed dormancy results in more uniform establishment of the crop (Singh, 2001). Harvesting methodologies have been adapted to both upright and prostrate plant types; however, more erect plants have been selected in the last four decades (Graham and Ranalli, 1997).

1.3 Common bacterial blight

The vital role that common beans play in the diet of many cultures, and within global markets, supports further research into resistant cultivars for preventing pathogenic diseases such as common bacterial blight (CBB). CBB in beans is caused by an infection by *X. axonopodis* pv. *phaseoli* or *X. fuscans* subsp. *fuscans* (Alavi, 2008). *X. axonopodis* and *X. fuscans* are gram-negative, slightly curved, rod shaped, obligate aerobic bacteria that are found in temperate and tropical climates worldwide (Swings *et al.,* 1993; Vattanaviboon *et al.,* 2005; Saettler, 1991; Mahuku *et al.,* 2006). The bacterial cells range in size from 0.2-0.6 µm wide and 0.8-2.9 µm long (Swings *et al.,* 1993). Both bacterial species can form extracellular polysaccharides coats (of xanthan gums) and have inner and outer membranes that are typical for gram-negative bacteria (Swings *et al.,* 1993). The outer membranes of the bacteria contain protein components responsible for cellular protection, selective permeability, cellular interactions and anchoring the bacteria to plant surfaces (Swings *et al.,* 1993). *Xanthomonas* bacteria can be found as single bacterial cells or in pairs, and do not form bacterial spores (Swings *et al.,* 1993). The cells of both species are motile, with polar monotrichous flagella (Swings *et al.,* 1993).

The *Xanthomonas* species involved in CBB infections can be cultured on simple ammonium-glucose media, however the use of yeast-salt (YS) media allows for faster culture growth (Swings *et al.,* 1993). Both species form convex, round, yellow, mucoid colonies with even colony margins and various colony sizes (Swings *et al.,* 1993). Optimal incubation temperatures vary from 28°C to 37°C, with an optimal pH level of 6.5 (Swings *et al.,* 1993). *X. axonopodis* forms a yellow, water insoluble pigment when grown on yeast-salt growth media (Swings *et al.,* 1993; Andrewes *et al.,* 1973). The yellow pigment produced is classified as a dibromo-aryl polyene or xanthomonadin (Andrewes *et al.,* 1973; Andrewes *et al.,* 1976). In contrast, *X.*

fuscans produces a dark brown to black, soluble pigment when cultured on yeast-salt media (Swings *et al.,* 1993). Cultured bacteria can be utilized to artificially inoculate plants to determine their susceptibility to common bacterial blight (Yu *et al.,* 2000).

CBB is primarily a seed transmitted infection that affects the aerial portions of the plant. The disease symptoms are most evident on leaves and as irregular yellow-brown spots on seeds (Munoz and Singh, 1999; Vidaver, 1993). The primary origin of the bacterial inoculum for CBB is affected seed; however, the disease can be transmitted by infected plant debris and soil inoculum (Saettler, 1991). In addition, exudates can form on the leaves of infected plants and epiphytic bacterial populations can form on the leaves of resistant plants, providing sources for the secondary spread of CBB within a field (Vidaver, 1993; Saettler, 1991). Characteristic CBB symptoms appear four to ten days after infection as water soaked lesions on leaves that progress to zones of necrotic tissue that are surrounded by yellow halos of chlorotic tissue (Vidaver, 1993; Xie *et al.,* 2012). Bacteria accumulate in the zones of active infection, and can cause extensive damage to the leaves, stems, and pods of the plant (Munoz and Singh, 1999). The damage to the leaves of the plant reduces the photosynthetic potential of the plant (Munoz and Singh, 1999). Dark and sunken lesions on the pods can cause seed discolouration, and ultimately reduce seed quality and yield (Vidaver, 1993; Jung *et al.,* 1997). In addition, CBB infections of the pods and seeds can result in the transmission of the bacteria to the next generation (Jung *et al.,* 1997). Furthermore, the production of infected seed from symptomless pods can exacerbate the spread of CBB into the next growing season (Vidaver, 1993).

In favorable growing conditions CBB infection is common in many cultivation regions. Minor CBB infections can result in yield losses and stunted plant growth, with severe infections causing premature plant death (Munoz and Singh, 1999). Yield losses with CBB infections can

be upwards of 40% of the potential yield (Gillard *et al*., 2009), making it a major pathogenic disease of common beans (Saettler, 1991). Globally, the severity of CBB infections can vary depending on the temperature and humidity levels, with the most severe cases occurring in regions with high humidity and heat (Saettler, 1991; Vidaver, 1993). Within common bean germplasm, there are some minor levels of CBB resistance native to cultivars of Mesoamerican ancestry; however, there is little to no resistance found within Andean bean lines (Singh *et al*., 1991). Additional resistance has been incorporated into several navy bean cultivars from introgressions of resistance from *P. acutifolius* (Pratt and Gordon, 1994). Currently, the control of CBB involves an integrative approach focussing on crop rotation, clean seed programs, and the removal and avoidance of secondary plants hosts in and around areas of common bean cultivation (Vidaver, 1993). The utilization of best management practices in order to control CBB allows for the reduction of infection incidences.

1.4 *Phaseolus acutifolius* introgresssion

P. acutifolius, or tepary bean, is mainly a wild accession that is cultivated infrequently in Mexico and Southwestern United States (Blair *et al.,* 2012). Unlike other *Phaseolus* species, tepary beans are rarely adapted to growth outside their centre of domestication and retain photoperiod sensitivity, restricting their potential zones of cultivation (Blair *et al*., 2012). The tepary bean is a small seeded legume plant with indeterminate and viney plant growth (Blair *et al.,* 2012). Tepary beans originated in Northern Mexico and are found native to Mexico and Southwestern United States, overlapping regions of wild growth with the Mesoamerican centre of origin of common beans (Blair *et al.,* 2012; Singh, 2001). Wild landraces of tepary beans are found in the arid deserts of Mexico and Southern United States. It is a species with high

tolerance to drought and salt stress (Blair *et al.*, 2012). Tepary beans are also naturally resistant to common bacterial blight and provide a potential source of resistant germplasm for introgression into common bean cultivars (Munoz and Singh, 1999).

P. acutifolius is the most resistant to common bacterial blight infections out of all the *Phaseolus* species, making it the optimal species to hybridize with in order to create resistant cultivars of common bean (Munoz and Singh, 1999). The F_1s from hybridizations between *P. vulgaris* and *P. acutifolius* are effectively sterile and require embryo rescue and a series of back-crosses in order to form a viable, fertile progeny (Pratt and Gordon, 1994). This difficulty puts *P. acutifolius* in the tertiary gene pool for common bean breeding programs (Singh, 2001). Although difficult, the potential for incorporating CBB resistance into common bean cultivars has created an incentive to overcome the difficulties (Munoz and Singh, 1999). Several breeding lines from interspecific hybridizations have been created (Micheals *et al.*, 2006). One of the successful introgression events resulted in MBE7 (a parent of OAC Rex) and another introgression event resulted in experimental lines HR67 and HR45 (Micheals *et al.*, 2006; Yu *et al.*, 2000).

1.4.1 OAC Rex

OAC Rex is an indeterminate, upright, bushy white bean variety that was released as the first commercial common bean cultivar with CBB resistance (Micheals *et al.*, 2006). OAC Rex is the result of a cross between MBE7, a line with CBB resistance, and line HR20-728 which was selected for its upright architecture (Micheals *et al.*, 2006). The origin of CBB resistance within the OAC Rex pedigree is an interspecific cross between *P. acutifolius* line PI 440 795 and *P. vulgaris* line ICA Pijao (Micheals *et al.*, 2006). After embryo rescue, the F_1s were back-crossed to Ex Rico 23, a white bean variety, in order to form MBE7 (Micheals *et al.*, 2006). OAC Rex

has molecular markers associated with CBB resistance such as microsatellite marker PvCTT001, Bng 21, and Bng 71, as well as marker SU91 on chromosome eight (Perry *et al.*, 2013; Shi *et al.*, 2012).

The OAC Rex genome was sequenced, assembled and compared to another *P. vulgaris* genome, Andean line G19833 (Perry *et al.*, 2013). G19833 is a large seeded bean and is a parent of the principal mapping population at the International Centre for Tropical Agriculture (CIAT), making it an experimental line of interest for sequencing (Altrock *et al*., 2011). Due to the Andean ancestry of G19833, it is assumed that the line is also susceptible to CBB infection (Perry *et al.*, 2013). By comparing the two genomes, differences between structure and sequences of regions adjacent to molecular markers for CBB resistance have been noted (Perry *et al.*, 2013). Of particular interest are sequences that are not found in the genome of susceptible G19833 material, but are found in OAC Rex (Perry *et al.*, 2013). In total, 95 genes of interest were identified to be unique to the OAC Rex genome, ten of which are homologous to resistance genes (Perry *et al.*, 2013).

Of particular interest for the proposed project are three genes with association to marker SU91 and homology to *P. acutifolius* expressed sequence tags (Perry *et al.*, 2013). The first of the three genes is gene 232701-8-007. Found on contig 232701, it has significant homology with a Niemann-Pick like transporter gene (Perry, *et al.*, 2013). As Figure 1.1 shows, a size polymorphism within gene 232701-8-007 was identified between resistant OAC Rex and susceptible G19833. In particular, the Niemann-Pick like gene in G19833 is 21,340 bp long and is comprised of one open reading frame (Schmutz *et al.*, 2014). In comparison, the Niemann-Pick like gene in OAC Rex is composed of 2 open reading frames that are 16,642 and 6,907 bp long (Perry *et al*., 2013). A Niemann-Pick polymorphism (NPP) marker of 956 bp was

developed, and is located in the larger of the two genes in OAC Rex, encompassing four exons and three introns. In G19833 the NPP marker encompasses three exons and two introns. As Figure 1.2 illustrates, the alignment of available genomic sequences for OAC Rex and G19833 allowed for the NPP marker design in order to amplify the 421 bp polymorphism present between the two bean lines.

In addition to gene 232701-8-007, two R genes within close proximity to marker SU91 were investigated. Genes 231733-8-004 and 231733-8-005 are found on contig 231733 of OAC Rex and have significant homology with *Medicago trancatula* resistance genes that contain nucleotide binding sites (NBS) and leucine rich repeats (LRR) (Perry *et al.*, 2013). The two R-genes of interest (231733-8-004 and 231733-8-005) are unique to OAC Rex, and appear to be in an area of insertion within the OAC Rex genome (Perry *et al.*, 2013). These two R-gene analogues are of more conventional resistance gene structure, and also contain high levels of homology with *P. acutifolius* ESTs (Liu *et al*., 2012; Perry *et al.*, 2013). Although high *P. acutifolius* homology is present, R-genes are highly conserved across species and are often found in clusters suggesting that the gene cluster insertion of interest may possibly be independent of the introgression event (Perry *et al.*, 2013; Meyers *et al.*, 1998). The traditional R-gene structure of a NBS and LRR, aid the resulting protein to take part in pathogen sensing. The R-protein is able to bind to the effector molecules released from the pathogen into the plant cell, effectively sensing the pathogen's presence and allowing the plant to respond accordingly (Yu *et al.*, 2015). The known role(s) that R-genes have in pathogen perception and resistance makes them additional candidates for the gene responsible for CBB resistance.

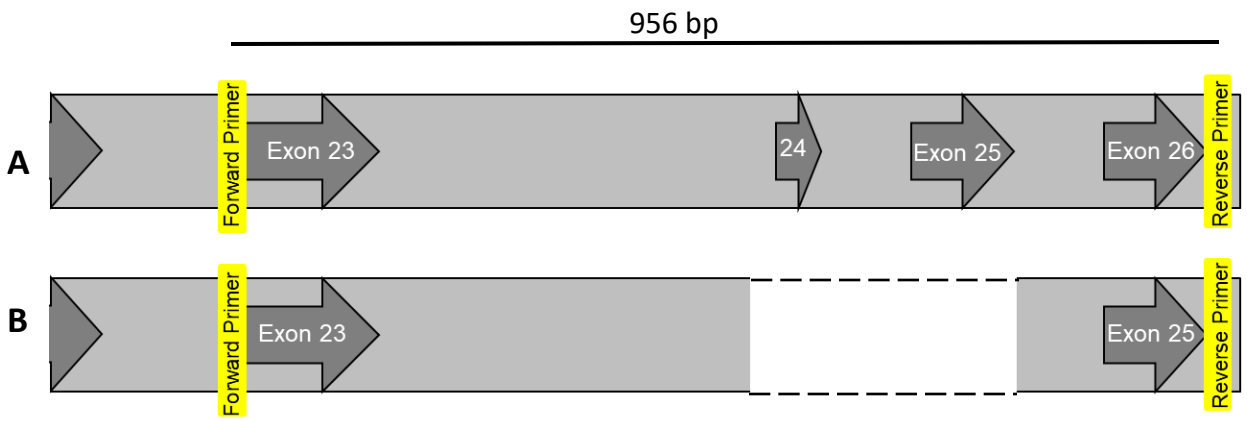


Figure 1.1- The polymorphic region of the Niemann-Pick like gene in OAC Rex and G19833, with primer placement indicated for the NPP marker. A) Illustrating the sequence of OAC Rex, a CBB resistant white bean line. B) Illustrating the sequence of G19833, a CBB susceptible Andean bean line. The dashed lines represent the 421 bp deletion found in susceptible lines.

Figure 1.2- A sequence alignment of available genome sequences for OAC Rex (resistant) and G19833 (susceptible) in the region surrounding a 421 bp polymorphism present in the Niemann-Pick like gene. The primer binding sites designed for the amplification of the polymorphism are highlighted in yellow. The 421 bp polymorphism between the two lines is highlighted in grey.

1.5 Niemann-Pick like proteins

The role of Niemann-Pick like proteins is not well understood in plants. In mammals, Niemann-Pick proteins are responsible for cholesterol transport across membranes (Watari *et al.*, 1999). Specifically, Niemann-Pick proteins control the transport of cholesterol from the endoplasmic reticulum to the exterior of the cell, and from outside of the cell through the plasma membrane (Subramanian and Balch, 2008). Within mammalian systems there are two main Niemann-Pick protein groups, Niemann-Pick C1 proteins are integrated into the plasma membrane, where Niemann-Pick C2 proteins are within the membrane of the endoplasmic reticulum (Subramanian and Balch, 2008). By comparing the mammalian system to plant systems, it has been discovered that within plants Niemann-Pick like proteins play a role in the transport of plant sterols and sphingolipids (Feldman *et al.*, 2015).

Although the roles that Niemann-Pick proteins play within plants are greatly unknown, there are several studies to determine their function(s) in plants. Arabidopsis has two genes homologous to the Niemann-Pick C1 gene found in mammals (Feldman *et al.*, 2015). Null mutations within these genes have been found to effect sphingolipid accumulation, plant growth and reproductive success (Feldman *et al.*, 2015). Plants that were mutated with hemizygous knock out mutations were dwarf and had low reproductive success. In addition, homozygous mutations were lethal within the Arabidopsis model (Feldman *et al.*, 2015). Through tDNA insertions, the role of both Niemann-Pick like proteins were studied. One protein was located in the plasma membrane while the other was located in both the tonoplast and the plasma membranes (Feldman *et al.*, 2015). The structures of the produced proteins were found to have a sterol sensing domain and a total 11 transmembrane domains (Feldman *et al.*, 2015). Within the Arabidopsis model system it was found that hemizygous mutations of the two different

Niemann-Pick like genes resulted in two different phenotypes. While one null mutation resulted in an accumulation of sphingolipids, the other resulted in dwarf plants with poor reproductive success (Feldman *et al*., 2015). The study within Arabidopsis shows an interaction between plant sphingolipids and sterols with the Niemann-pick like proteins.

1.6 Lipid raft theory

Alterations in sterol concentration and location have previously been correlated with resistance in plants to fungal infections (Bhat and Panstruga, 2005; Feldman *et al.*, 2015). The accumulation of saturated lipids within microdomains of the lipid bilayer of cell membranes is referred to as lipid rafts (Bhat and Panstruga, 2005). Lipid rafts can be found in the plasma membranes of eukaryotic cells and contain higher concentrations of sterols and sphingolipids than the remaining membrane (Bhat and Panstruga, 2005). The theory of lipid rafts is based on the non-uniform nature of plasma membranes, and suggests that the outer leaflet of lipid bilayers has regions with a higher percentage of saturated lipids and proteins that anchor within saturated lipids (Bhat and Panstruga, 2005). The alteration of membrane components within lipid rafts has an effect on the membrane characteristics within the region. With the higher percentage of saturated lipids, and the reduction of unsaturated lipids, the lipid rafts are rigid and have low detergent solubility (Bhat and Panstruga, 2005). Within plants, the formation of lipid rafts has shown to alter the rate of endocytosis and the presence of hypersensitive response proteins (Kale *et al.*, 2010; Borner *et al.*, 2005).

There are several pathways for mediating endocytosis in cells, including lipid raft-mediation (Kale *et al.*, 2010). The presence of many lipid-anchored proteins within lipid rafts facilitates their functioning in endocytosis (Lafont *et al.*, 2004). Raft-mediated endocytosis functions in

cell uptake of components from the environment; however, the addition of inhibitors of lipid accumulation, also inhibit raft function in endocytosis (Kale *et al.*, 2010). Pharmaceutical compounds can alter the ability of lipid rafts to form, as well as their ability to come together and interact (Lafont *et al.*, 2004).

By altering the cell's ability the uptake extracellular compounds, the susceptibility of the cell to bacterial and fungal infection can also be altered (Kale *et al*., 2010; Bhat *et al.*, 2005). For some infectious bacteria, the use of effector molecules is required to disable the plants defense mechanisms (Kale *et al.*, 2010). For example, in order for soybean plants to be susceptible to oomycetes a lipid raft anchored signalling protein is needed to induce endocytosis of the effectors (Kale *et al.*, 2010). Without functional lipid rafts and the accumulation of signalling proteins, the plant fails to uptake the effector molecules and becomes resistant to the oomycete infection (Kale *et al.*, 2010).

In comparison, proteins responsible for signalling and initiating the hypersensitive response after fungal infection have also been located to lipid rafts (Bhat *et al.*, 2005). Within the Arabidopsis plant model, the localization of lipid rafts was studied *in situ* in order to determine their role in powdery mildew resistance (Bhat *et al.*, 2005). It was found that when the accumulation of lipid rafts under the point of cellular entry was inhibited, the plant became susceptible to the fungal infection (Bhat *et al.*, 2005). In both Arabidopsis and barley, fungal infections of the cell initiate a redistribution of lipid rafts to the point of cell penetration (Bhat *et al.*, 2005). The inability for a plant cell to relocate the lipid rafts resulted in disease susceptibility (Bhat *et al.*, 2005). In both plant models, a loss of function mutation of mildew resistance locus O resulted in a lack of interaction between lipid rafts and ultimately plant susceptibility (Bhat *et al.*, 2005). The role of lipid rafts is still under investigation; however, it appears they have the

ability to play two distinct roles within disease resistance. Their presence and interactions can both help and hinder a plant's ability to initiate cellular defenses (Kale *et al.,* 2010; Bhat *et al.,* 2005).

1.7 Hypothesis and objectives

In order to identify the gene(s) responsible for common bacterial blight (CBB) resistance within common bean this project aimed to develop and determine the usefulness of molecular markers closely linked to candidate genes in a major quantitative trait loci (QTL) for CBB resistance on chromosome 8, identified by the SU91 marker (O'Boyle *et al.,* 2007). The hypothesis of this thesis is that a marker for CBB resistance, better than SU91, can be developed by exploiting polymorphisms within the most closely linked candidate gene for resistance that was previously identified in the QTL.

This thesis has the following three objectives. The first objective is to develop and validate a molecular marker for CBB resistance within the Niemann-Pick gene (232701-8-007), a candidate gene for CBB resistance. The second objective is to determine if the closely linked R-genes, also linked to SU91 at the end of chromosome 8, have polymorphisms between OAC Rex and susceptible bean genotypes; and if they may be used to develop molecular markers for CBB resistance. The final objective is to characterize the patterns of expression of the candidate genes in the SU91 QTL in OAC Rex by real time PCR to determine their involvement in CBB resistance.

It was anticipated that the combination of genomic and expression data obtained in the current study would allow for candidate genes of interest to be evaluated for possible roles they might play in CBB resistance in common bean.

Chapter 2: Development of co-dominant polymorphic molecular marker within a Niemann-Pick like gene on chromosome eight of *P. vulgaris*

2.1 Abstract

In order to validate a molecular marker for common bacterial blight (CBB), two populations segregating for CBB resistance were screened for the marker alleles and phenotypic responses to common bacterial blight. The effectiveness of the candidate codominant molecular marker (called the Niemann-Pick Polymorphism (NPP) marker) was evaluated by comparing correlations between genotypic and phenotypic scores in the populations with those measured for established molecular markers for common bacterial blight (such as SU91). In addition, the NPP marker was tracked through the pedigree of OAC Rex (a CBB resistant line) and in several commercial and experimental lines (with various levels of disease resistance). Although it was found that within the OAC Rex/OAC Seaforth population the NPP marker was not significantly different in distinguishing between CBB resistant and susceptible lines from the SU91 marker, the NPP marker distinguished susceptible individuals from the resistant individuals with 99% accuracy. In comparison, the SU91 analog SU91-CG5 had the next best accuracy with 90.5%. In the Compass//Rexeter/Apex population, the NPP marker discriminated between susceptible and resistant lines with 99% accuracy in comparison to the 76% accuracy for SU91. Although further investigations into the function of the Niemann-Pick like gene are required before it can be deemed to be responsible for CBB resistance in common bean, the NPP marker, which is based on a polymorphism it contains, has been validated as a superior marker to the current industry standards for CBB resistance screening.

2.2 Introduction

Common bacterial blight (CBB) is a major disease that affects common bean crops worldwide (Vidaver, 1993). With the severity of the disease dependent on the local environment, the bacteria responsible for CBB can thrive in climates ranging from tropical to temperate (Vidaver, 1993). Common bacterial blight infection symptoms can vary from minimal lesions on aerial portions of the plant, to stunted growth and plant death (Rudolf, 1993). CBB resistance is a quantitative trait within common bean, and several quantitative trait loci (QTL) on multiple chromosomes have been identified that correlate with CBB resistance (Singh and Miklas, 2015).

With resistant germplasm in the primary, secondary and tertiary gene pools, the development of resistant cultivars is a focus of breeding programs worldwide (Singh and Miklas, 2015). The use of tertiary gene pools has led to several successful introgression events of CBB resistance into the common bean genome (Singh and Miklas, 2015). Such introgression events have resulted in the incorporation of novel DNA encoding CBB resistance, including the region linked to the molecular marker SU91 (Duncan *et al.,* 2011). In order to efficiently incorporate resistance into new cultivars within a breeding program, the genes responsible for CBB resistance must be better understood.

The QTL that explains the largest proportion of phenotypic variation for CBB resistance in a range of bean germplasm is located at the end of chromosome 8 and includes the established molecular marker SU91 (Miklas *et al*., 2003). This QTL region has been studied previously in order to characterize the genes it contains (Perry *et al.,* 2013). The genes within this region were compared between CBB susceptible bean line G19833, and CBB resistant line OAC Rex. Genes novel to OAC Rex were run through BLAST in order to identify likely functions (Perry *et al.,*

2013). In addition, the genes were compared to *P. acutifolius* expressed sequence tags (ESTs), the source of CBB resistance within the pedigree of several CBB resistant common beans (Perry *et al.,* 2013).

By comparing the genes present, ESTs from *P. acutifolius*, and conserved sequences of known functions, three candidate genes were identified in OAC Rex (Perry *et al.,* 2013). The candidate genes identified were denoted as 231733-8-004, 231733-8-005, and 232701-8-007 (Perry *et al.,* 2013). Genes 231733-8-004 and 231733-8-005 are typical R-genes that contain highly conserved regions characteristic of resistance genes in many plant species (Perry *et al.,* 2013). Gene 232701-8-007, however, is homologous to Niemann-Pick like gene sequences found in many plants and animals. Although an atypical source for conveying resistance, its homology to *P. acutifolius* sequences made it a candidate gene for conveying resistance to CBB in select common bean populations.

2.2.1 Molecular markers

There are several molecular markers that are utilized for marker assisted selection of CBB resistant lines. These markers include SAP6 on chromosome 10, BC420 on chromosome 6 and SU91 on chromosome 8 (Miklas *et al.,* 2000; Yu *et al.,* 2000). In the CBB resistance QTL region located on chromosome 8, five markers have been identified (Shi *et al.,* 2012). The molecular marker SU91, and its analogs, are located in close proximity to both the previously determined peak for the QTL for CBB resistance and the Niemann-Pick like gene under investigation (Perry *et al.,* 2013; Shi *et al.,* 2012). The molecular marker SU91 is a dominant marker that has been extensively utilized to screen for CBB resistance within common bean populations (Miklas *et al.,* 2006). Associated with the introgression event between *P. vulgaris*

and *P. acutifolius*, SU91 has been utilized as one of the three main CBB markers to date (Liu *et al.,* 2008; Miklas *et al.,* 2006). Located 33 kb from the Niemann-Pick like gene is SU91-CG5, the closest of the five SU91 analogs that have been developed. As shown in Figure 2.1, SU91 and its analogs are all located within 106 kb of the Niemann-Pick like gene. Although they are effective markers for the selection of CBB resistant plant material, there are some recombinant lines that have broken the linkage between SU91 and the gene responsible for CBB resistance, at the end of chromosome 8. This led to the development of a molecular marker based in the gene of interest in order to study the linkage between the Niemann-Pick like gene and CBB resistance.

2.2.2 Niemann-Pick gene

A previous *in silico* study of the Niemann-Pick like gene sequences in OAC Rex and G19833 resulted in a translated and annotated amino acid sequence for the Niemann-Pick gene. The resulting amino acid alignment can be seen in Figure 2.2. A comparison between OAC Rex and G19833 alluded to the presence of a total of 13 transmembrane domains across two separate genes in OAC Rex, and 15 transmembrane domains in G19833. As seen in Figure 2.3, the differences in genomic sequence between OAC Rex and G19833, specifically the Niemann-Pick polymorphism (NPP), results in the theoretical separation of the Niemann-Pick protein into two separate proteins. The separation of the Niemann-Pick protein in OAC Rex occurs in the cysteine residue region of the protein. This protein division has an unknown effect on the function of the Niemann-Pick protein in OAC Rex.

2.2.3 Populations

In order to validate the linkage between resistance and the NPP marker, the correlation between the presence of the resistant allele of the NPP marker and CBB resistance was studied in

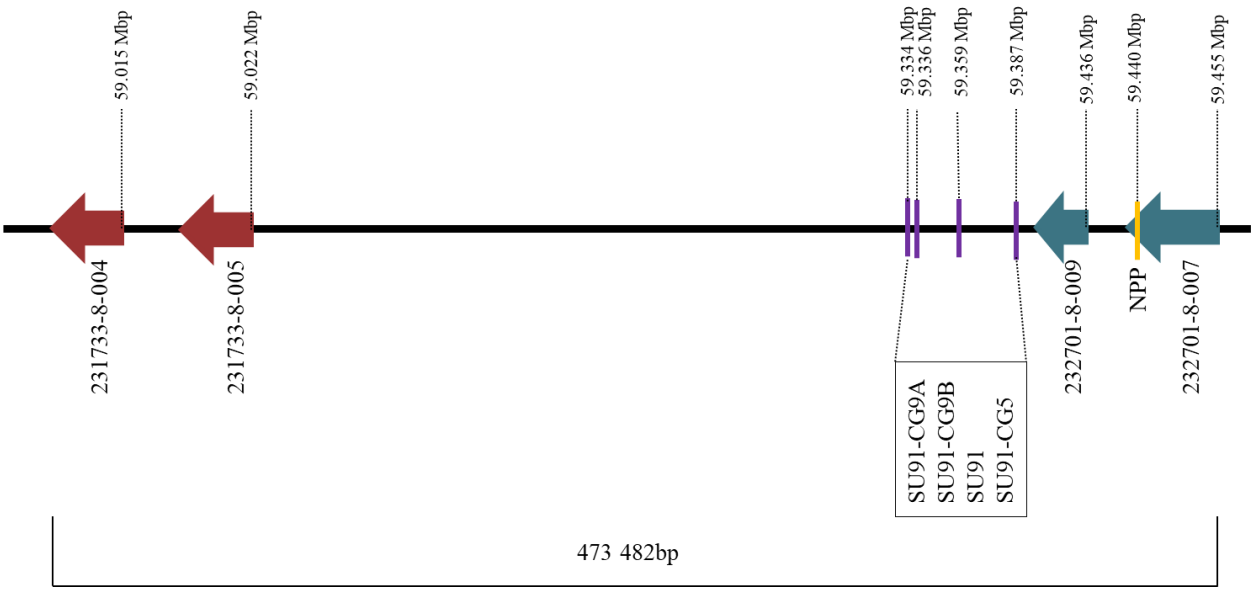


Figure 2.1- A diagrammatic representation of the end of chromosome eight of OAC Rex, illustrating the physical locations of five CBB markers as well as three candidate resistance genes that are novel to OAC Rex (resistant) in comparison to line G19833 (susceptible). Physical locations of each gene and marker are based on OAC Rex genomic sequence data (modified from Perry *et al.*, 2013).

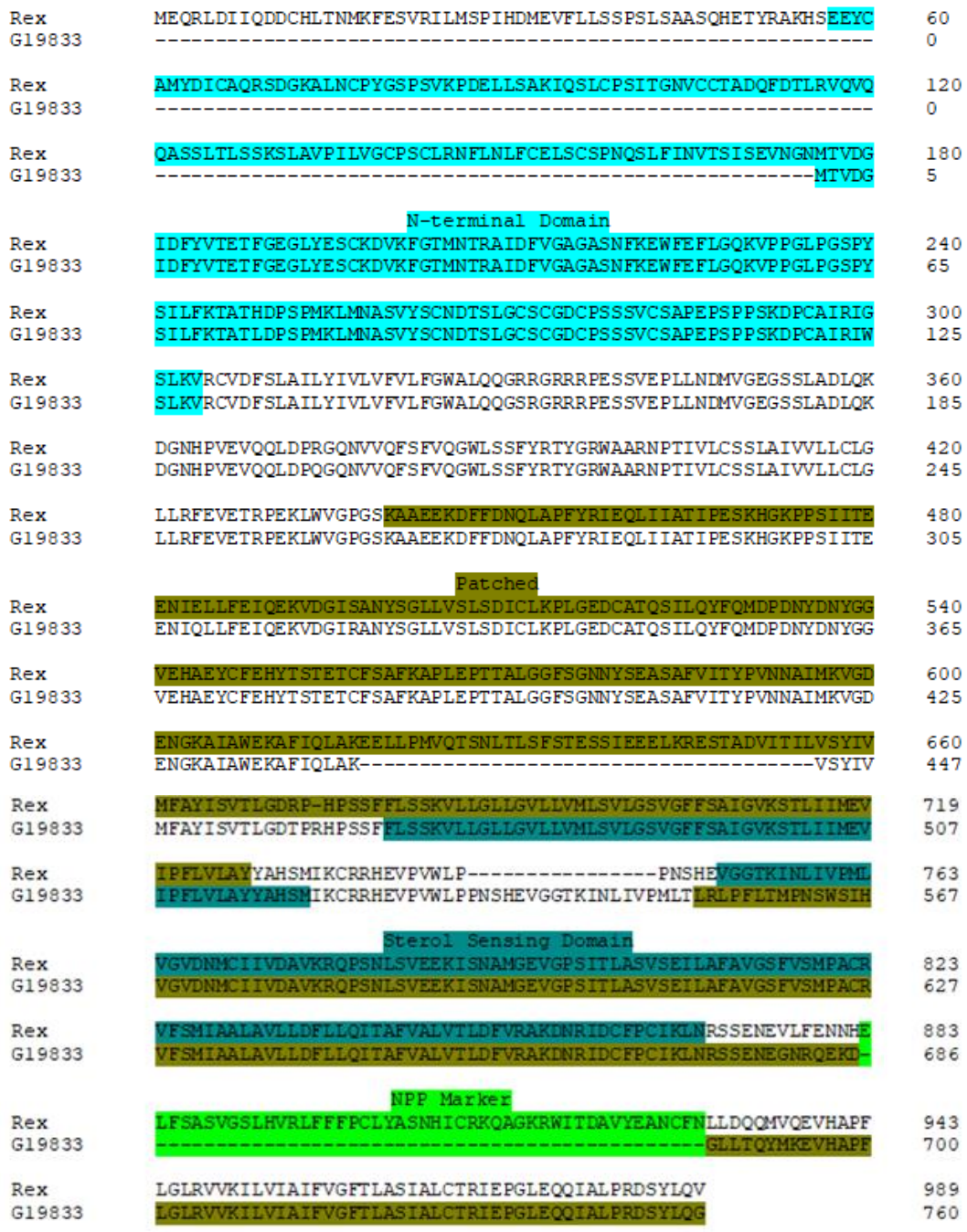


Figure 2.2- The amino acid sequence alignment between OAC Rex (resistant) and G19833 (susceptible) for the resulting protein from gene 232701-8-007, with protein domains and the NPP marker location highlighted. The N-terminal domain responsible for sterol sensing and containing a leucine zipper is highlighted in light blue. The patched protein domain is highlighted in brown. A secondary sterol sensing domain is highlighted in dark blue. The location of the NPP marker is highlighted in light green.

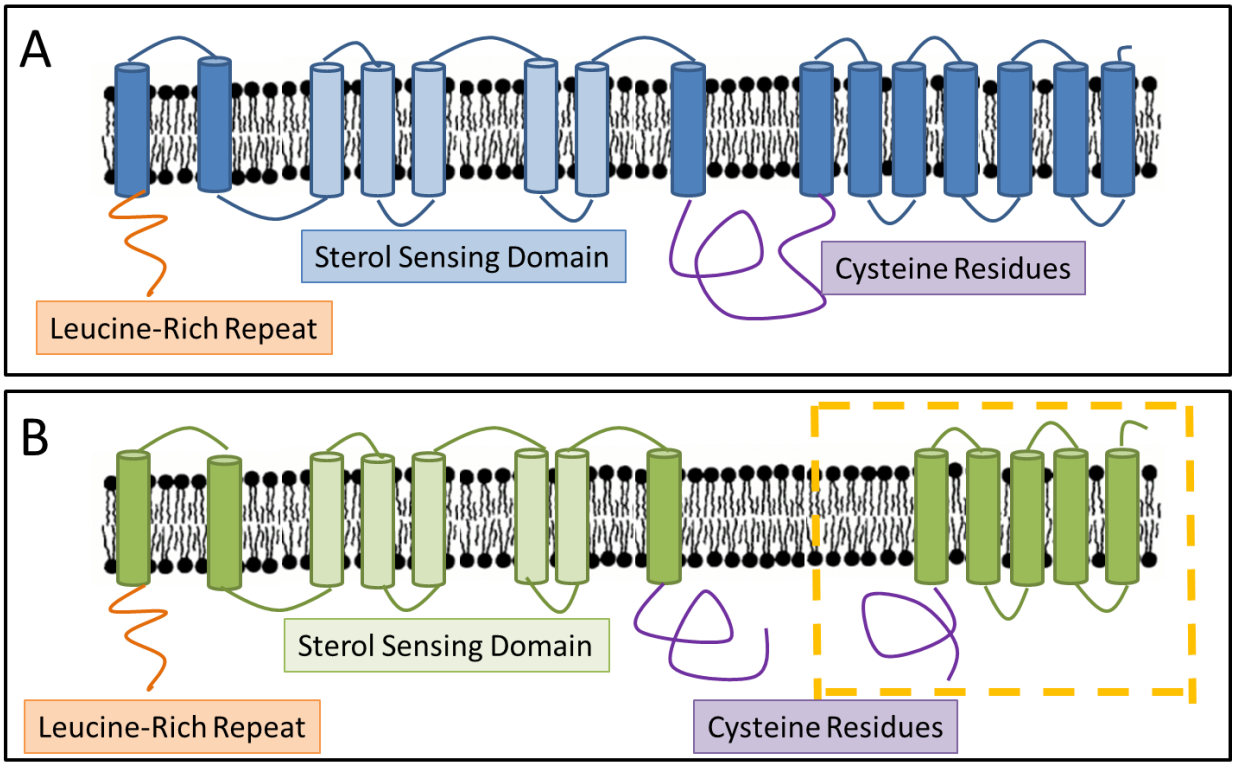


Figure 2.3- An illustration based off of amino acid sequence annotations, comparing the putative Niemann-Pick protein structures in G19833 (A) and OAC Rex (B). The frequency and number of transmembrane domains between the two bean lines differ, with OAC Rex having two fewer transmembrane domains. A) Protein structure based on the single Niemann-Pick like gene in the susceptible bean line G19833; with 15 transmembrane domains and an intact cysteine residue region. B) Protein structure based on the two Niemann-Pick like genes in the resistant bean line OAC Rex; with 13 transmembrane domains and a fragmented cysteine residue region.

two genetically distinct populations. The first population was composed of $F_{6:7}$ recombinant inbred lines (RIL) from the biparental cross of OAC Seaforth and OAC Rex, containing 72 individuals. The susceptible parent, OAC Seaforth, is the result of six backcrosses between Seafarer and PI 326 418 where Seafarer is the recurrent parent and PI 326 418 is the donor parent (Beversdorf and Buzzell, 1984). OAC Rex is the resistant parent of the population with the pedigree HR20-728/MBE7 (Micheals *et al*., 2006). HR20 has the pedigree Ex Rico 23-Are/Midnight, and was utilized for its plant architecture (Micheals *et al*., 2006). MBE7 is the result of a cross between Ex Rico 23 and a F_1 embryo rescue from the crossing of ICA Pijao with PI 440 795 (*P. acutifolius*) which is the source of CBB resistance for OAC Rex (Micheals *et al*., 2006). The subsequent selfing of the progeny of OAC Rex /OAC Seaforth resulted in the first population that was utilized for this study.

The second population that was utilized was created by backcrossing Compass//Rexeter/Apex back to Compass. The recurrent parent Compass has the pedigree W1285c-42603/OAC Laser and is susceptible to CBB (Park and Rupert, 2000). The additional parents are both resistant to CBB, with pedigrees of Centralia/NY5268//HR67/3/AC Cruiser and OAC Rex/AC Kippen for Apex and Rexeter, respectively (Navabi *et al.,* 2013; Smith *et al*., 2012). With 161 individuals, the $F_{4:5}$ population has segregated into homozygous resistant and susceptible lines. With the additional backcrossing to a susceptible parent, the population contains more individuals that are susceptible. However it was also predicted that this could provide further breaks between the molecular markers under investigation and the gene responsible for CBB resistance. The second population was utilized to increase the number of individuals screened, as well as to validate the developed NPP marker in an alternative genetic background.

The following study aimed to investigate the usefulness of a Niemann-Pick gene based marker as a molecular marker for CBB resistance. The objective was to evaluate the marker within multiple genetic backgrounds to determine its reliability. In order to be able to determine the marker's efficiency, comparisons were also made with established markers for CBB resistance. The efficiency of the developed NPP marker was compared to the efficiency of the previously validated SU91 marker and its analogs. In addition, the NPP marker was tested with several commercial and experimental lines to evaluate the marker in a wide range of genetic backgrounds.

2.3 Materials and methods

2.3.1 Sequencing

The primers developed for the Niemann-Pick like gene polymorphism were utilized to amplify the gene in several genetic backgrounds in order to determine the specificity of the primers. Four bean lines were sequenced for the NPP region of the Niemann-Pick like gene. OAC Rex, OAC Seaforth, PI 440 795, and G19833 were all sequenced for the amplicon from the NPP primers. OAC Rex and OAC Seaforth are the parents for one of the two populations used, PI 440 795 is the *P. acutifolius* line within the OAC Rex pedigree and G19833 is a susceptible line with genomic sequence data available. The amplified PCR product was utilized as the insert for TOPO-TA Cloning by Thermo Fisher Scientific (2014). The PCR product was inserted into the pCR™2.1-TOPO® vector as the kit protocol specifies. The vector was then transformed into competent DH5α-T1 *E. coli* cells following the One Shot chemical transformation protocol. In summary, the vector solution is added to cells in solution, heat shocked and then incubated. The

liquid culture is plated and blue/white selection is utilized in order to select successful transformation events.

After 12 hours, 6 successfully transformed colonies were selected off of the plate and placed in individual LB liquid media cultures. Each culture had 3 mL of LB media and was placed on a shaker for overnight incubation. After 24 hours of incubation, the bacterial cultures went through the plasmid miniprep protocol to isolate the plasmids for sequencing. The Purelink Quick Plasmid Miniprep Kit was used to purify the plasmid DNA with centrifugation. The kit protocol was followed, and in summary the bacterial cells were lysed, the DNA was bound to the spin column, and eluted after several washing steps. The purified plasmids were double checked for insert size using PCR with the NPP primers. One successful transformation event for each of the four bean lines was sent to the Genomics Facility at the University of Guelph's Advanced Analysis Centre. The returned sequence data was analyzed using the CLC Genomics Workbench.

2.3.2 Phenotypic screening

In order to screen both populations, they were incorporated into the CBB nursery of the Agriculture and Agri-Food Canada Research and Development Centre in Harrow, Ontario. For population one (OAC Rex/OAC Seaforth), the population was screened for CBB susceptibility for three years and planted on June 12, 21 and 9 in 2011, 2013, and 2016 respectively. For population two (Compass//Rexeter/Apex), the population was screened for two years and planted on June 17 and 21 in 2012 and 2013 respectively. Within both populations, each year's entries were randomized and planted into hill plots following a simple lattice design with two replications. The parents of each population were randomly incorporated as disease checks, as

well as several additional lines with known disease symptoms. Each plot contained seven plants and they were spaced with 70 cm between plots and 60cm between rows. Each year within the experiment, the plots were inoculated twice, first 35 days after planting (DAP) and again at 42 DAP. Two inoculation times were utilized to ensure that late geminating plants were inoculated at the appropriate growth stage for disease infection. The second timing also allowed for maximum exposure to the bacteria, and therefore the development of the most severe disease symptoms possible. The plots were inoculated using bacterial culture suspended in deionized water, between 8 and 8:30 am to ensure a damp canopy. Plots were sprayed using a high pressure sprayer which injures the leaf canopy as it sprays the plant with the inoculum, thus allowing for efficient infection of the leaf tissue (Gilbertson *et al.*, 1988). The sprayer was pulled at a rate of 3 km/h, and the inoculum was sprayed at a pressure of 200 psi. The inoculated fields were irrigated bi-daily to maintain high humidity and to promote bacterial growth.

The bacterial inoculum was prepared following a modified protocol outlined in Yu *et al.*, (2000), utilizing dry leaf inoculum as described in Gilbertson *et al.*, (1988). In summary, two strains of *X. axonopodis* pv. *phaseoli* (strains 18 and 98), and two strains of *X. fuscans* subsp. *fuscans* (strains 12 and 118) were suspended from dried, infected leaves into potassium phosphate buffer (PB). The bacterial suspension was left to soak for two hours and then streak plated separately onto YS medium and incubated at 37 °C for 48h. Single colonies of each strain were individually dissolved into neutral PB buffer, and pour plated back onto new YS salt medium plates. Each pour plate had 600 µL of bacterial solution spread evenly onto the media with a sterilized, bent glass rod. These plates were incubated at 37 °C for an additional 48 h. Lastly, to form the inoculum concentrate each plate was flooded with deionized water, the bacterial lawn was dissolved and poured into 1750 mL of deionized water. The concentration of

the solution was adjusted to 10^7 CFU mL^{-1} using a Klett-Summerson Photoelectric Colorimeter fitted with a red filter (640 -700 nm). The concentrated inoculum for each *Xanthomonas* strain (1750 mL per strain) was added to a 150 L spray tank and diluted with water, to a total volume of 150 L, before inoculating the field.

Rating for disease severity was completed twice, first two weeks after the second inoculation (56 DAP), followed by a second rating ten days later (66 DAP). Each hill plot was rated for overall disease severity across plants, as well as the lesion leaf cover on each individual plant. Total active lesion cover is determined as a percentage and rated accordingly on a scale from 0 -5. Plots with no active lesions were rated a 0; plots with 1-10% of leaf area under active infection were rated a 1; 11-30% infected leaf area was rated a 2; 31-50% infected leaf area was rated a 3; 51-80% infected leaf area was rated a 4; and plots with greater than 80% of leaf area under active infection were rated a 5. In order to normalize the disease severity by the timing of the disease rating, the area under the disease progressive curve (AUDPC) was calculated. The equation for AUDPC is:

$$AUDPC = \sum_{i=1}^{n} \left(\frac{y_i + y_{i+1}}{2}\right)(t_{i+1} - t_i)$$

where *n* represents the number of observations, *i* represents the *i*th observation, *t* represents the time point of the observation in days after inoculation (DAI) and *y* represents the disease severity. The unit of AUDPC %-developmental stage unit or in the case of CBB it is the % disease coverage per day after bacterial inoculation (Campbell and Madden, 1990). Figure 2.4 illustrates the calculation of AUDPC for two example RILs. The AUDPC value standardizes the disease ratings across a population; however, differentiating the timing of disease ratings results in varying ranges of values for populations screened at different timings post inoculation.

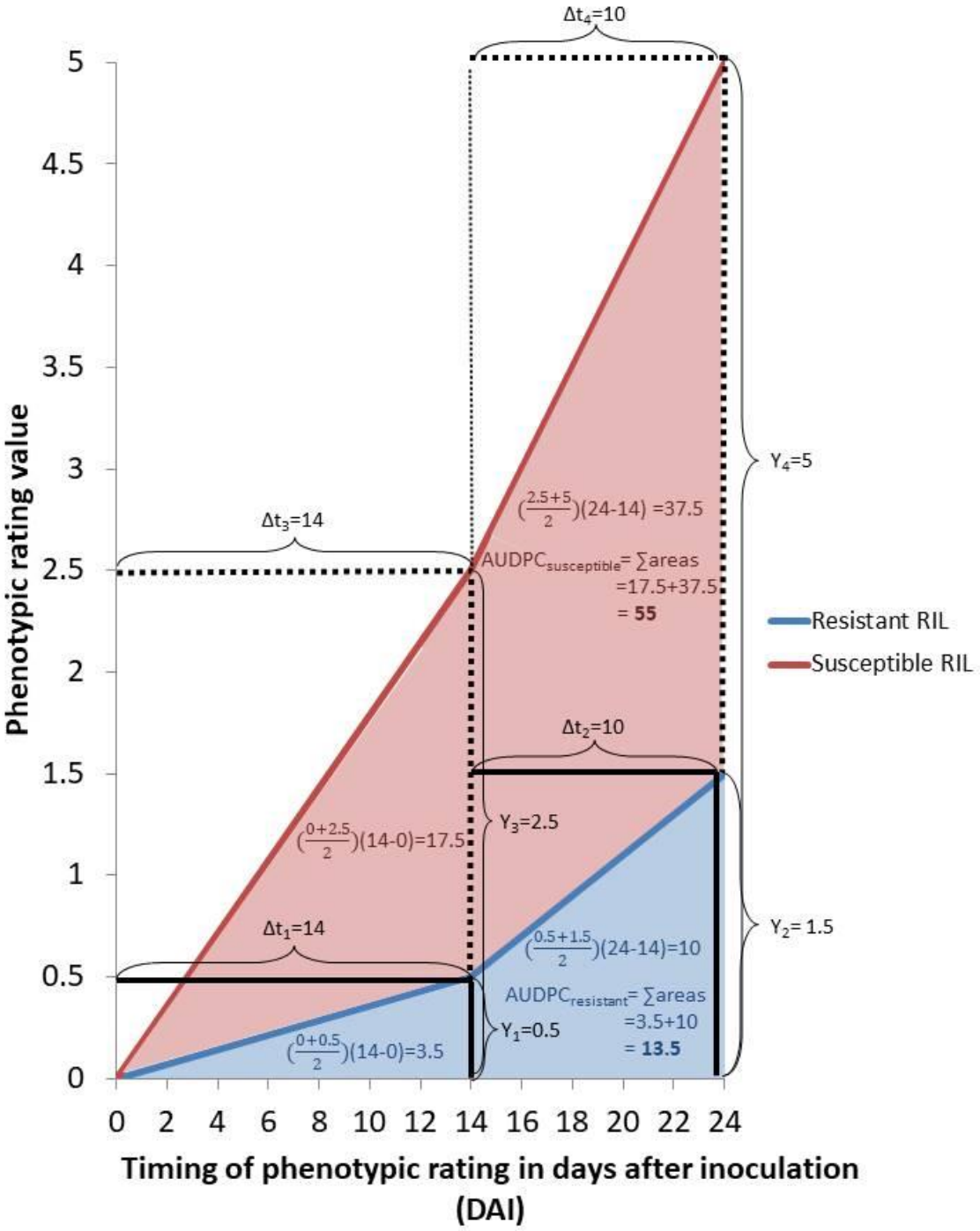


Figure 2.4- A graphical representation of the AUDPC calculation for the CBB scores of two example RILs. The calculation of the area under the disease progression curve standardizes the disease scores for each individual, taking into account the duration post inoculation, and the progression of disease over time.

2.3.3 Genotypic screening

Both populations were grown out in a growth room at 16 hour/25 °C days and 8 hour/20 °C nights. After 20 days, once the first trifoliate started to open, tissue samples were taken by placing leaf tissue in liquid nitrogen and storing at -80°C. In order to extract the DNA from each RIL in both populations, a Sigma-Aldrich GenElute Plant Genomic DNA Miniprep Kit was utilized. The protocol for the kit was followed, and in summary the frozen tissue was ground so that the cells could be lysed and homogenized. The impurities were removed from the samples and the DNA was bound to a silica column. The column was then washed several times and the DNA was eluted. The extracted DNA was stored at -20˚C until polymerase chain reactions were utilized to screen the population for the NPP marker.

The DNA extracted from each RIL in both populations was utilized as DNA template in a series of PCR reactions. For each reaction 0.5 µL of template (50-100 ng), 0.5 µL of dNTP's (2 mM), 0.2 µL of Taq polymerase (1 unit), 0.5 µL of the NPP primers (3 µg/µL), 17.8 µL of PCR grade water, 2.5 µL of PCR buffer and 3 µL of $MgCl_2$ (25 mM) were combined and dispersed into PCR tubes. The primer sequences for the NPP marker were 5'-GCTTCTGTTGGTAGTTTGCAT-3' (forward) and 5'-TAGGAATCTCGTGGA AGAGC-3' (reverse). The PCR reaction ran with an annealing temperature of 56 °C. After the PCR products were formed, they were run on a 1% agarose gel and visualized with ethidium bromide in order to determine the size of the amplicon produced. Each RIL was classified as having band size of "A", or that of the susceptible parent (535 bp) or a band size of "B" (956 bp) found in the resistant parent. Each RIL of the population was compared to the parental NPP amplicons to label the allele present in each individual. The data was inputted into Excel for later statistical analysis. The data for the NPP marker in the OAC Rex/OAC Seaforth population was added to

previously compiled data for the SU91 marker and its analogs. This additional marker data was previously obtained from samples sent for genome wide genotyping by a SNP chip. For the Compass//Rexeter/Apex population, the extracted DNA was also utilized to screen for the SU91 marker in a similar fashion as the NPP marker. Both marker alleles were compiled into excel for further analysis.

2.3.4 Pedigree Screening

In order to be able to determine the origin of the resistant allele for the NPP marker, the lines within the pedigree for OAC Rex were screened for the NPP marker. Similar to the screening of the populations, the lines were grown in a growth room at 16 hour/25˚Cdays and 8 hour/20˚C nights until several trifoliate leaves formed. At that point the leaves were frozen in liquid nitrogen, and then ground into a powder for DNA extraction. The same extraction protocol from Sigma-Aldrich was followed. The lines that were screened are the following: ICA Pijao, PI 440 795 (*P. acutifolius*), Midnight, Ex Rico 23, HR 20, and MBE7. All lines within the pedigree, except for PI 440 795, are *P. vulgaris* lines utilized in the breeding program. In addition to the lines within the OAC Rex pedigree, select lines were chosen for addition to the screening panel for various reasons. Lines HR 45, HR 67, Vax 4, Rexeter, X159, ACUG 11-7, Mist, and Apex were all screened because they have *P. acutifolius* and/or OAC Rex in their pedigrees. PI 319443 is a *P. acutifolius* plant introduction that was screened because of its occurrence in the pedigree of HR45 and HR67. BAT 93 was screened since it is a resistant white bean line. Also screened were several lines that are known to be susceptible. These lines included Nautica, Compass, and OAC Thunder. Lastly, the *P. acutifolius* line Mex was screened as it is a susceptible *P. acutifolius* line. Just as in the population, extracted DNA was used a

template for PCR reactions and the resulting products were run on a 1% agarose gel to visualize the allele present in each line.

2.3.5 Statistical Analysis

In order to determine the effectiveness of the NPP marker in CBB screening, the rate of error was investigated and compared to the effectiveness of current molecular markers that are utilized to screen populations for CBB resistance within the same QTL. The population data was segregated into two classes based on the identification of a small marker size (A) or a larger marker band (B). The two classes were utilized to separate AUDPC values into two groups. A t-test and tests for model assumptions were conducted using Glimmix and Univariate procedures within SAS® software version 9.4 (SAS Institute Inc., Cary, NC). A t-test compared the least square means of the two classes to determine if the marker had significantly divided the population according to CBB susceptibility. The model assumed that the data had a binomial distribution of individual scores. The magnitude of the error associated with each mean and the distribution of scores was considered when determining the efficiency of the marker in segregating the population based on CBB resistance. By plotting out a scatter plot of the mean AUDPC scores for each individual based on the marker variant, outliers were observed and considered when determining the efficiency of the NPP marker to identify CBB resistant lines within the population. Studentized residuals were calculated in order to formally test for the presence of outliers. Individuals with a studentized residual greater than 3.4 or less than -3.4 were considered to be outliers. A type I error rate of $\alpha=0.05$ was specified for all tests conducted.

In order to analyze the co-segregation of the NPP marker and CBB resistance, a mixed model was specified within SAS. The recombinant inbred line was considered to be a fixed effect, with the marker variant for each line being a random effect. The AUDPC score resulting from phenotypic observation was the dependent variable being tested. The logit link option [$\eta=\log(\mu l-\mu)$] was added to fit residuals to a normal distribution. The fit of the model was tested using a likelihood ratio test. An analysis of model assumptions was conducted to determine if the residuals followed a normal distribution, were homogeneous, random and had a mean of zero. A Shapiro-Wilk test for normality was conducted to determine if the distribution of residuals was normal. The studentized residuals were plotted in order to determine homogeneity and to detect possible non-random patterns. Before pooling scores a test of heterogeneity was conducted to ensure data was homogeneous and could be pooled. Basic statistics of the residuals were calculated in order to determine the mean. Data was divided by allele*marker subcategories creating 10 classes that were compared by a pairwise test for the OAC Rex/OAC Seaforth population. For the Compass//Rexeter/Apex population, 4 classes were compared. In the end, the utilization of a t-test to compare the means of two classes, created by the segregation of the data based on marker variant, allowed for the efficiency of the NPP marker to be tested.

2.4 Results

2.4.1 Sequencing Niemann-Pick Alleles

A Niemann-Pick polymorphism (NPP) marker was developed based on polymorphisms (Figure 1.1) in the larger fragment of the Niemann-Pick like gene (Figure 2.1) between OAC Rex and G19833, identified previously *in silico* (Perry et al, 2013). Previous work (Morneau *et al.*,, unpublished data) had validated the polymorphism between the two bean genotypes by

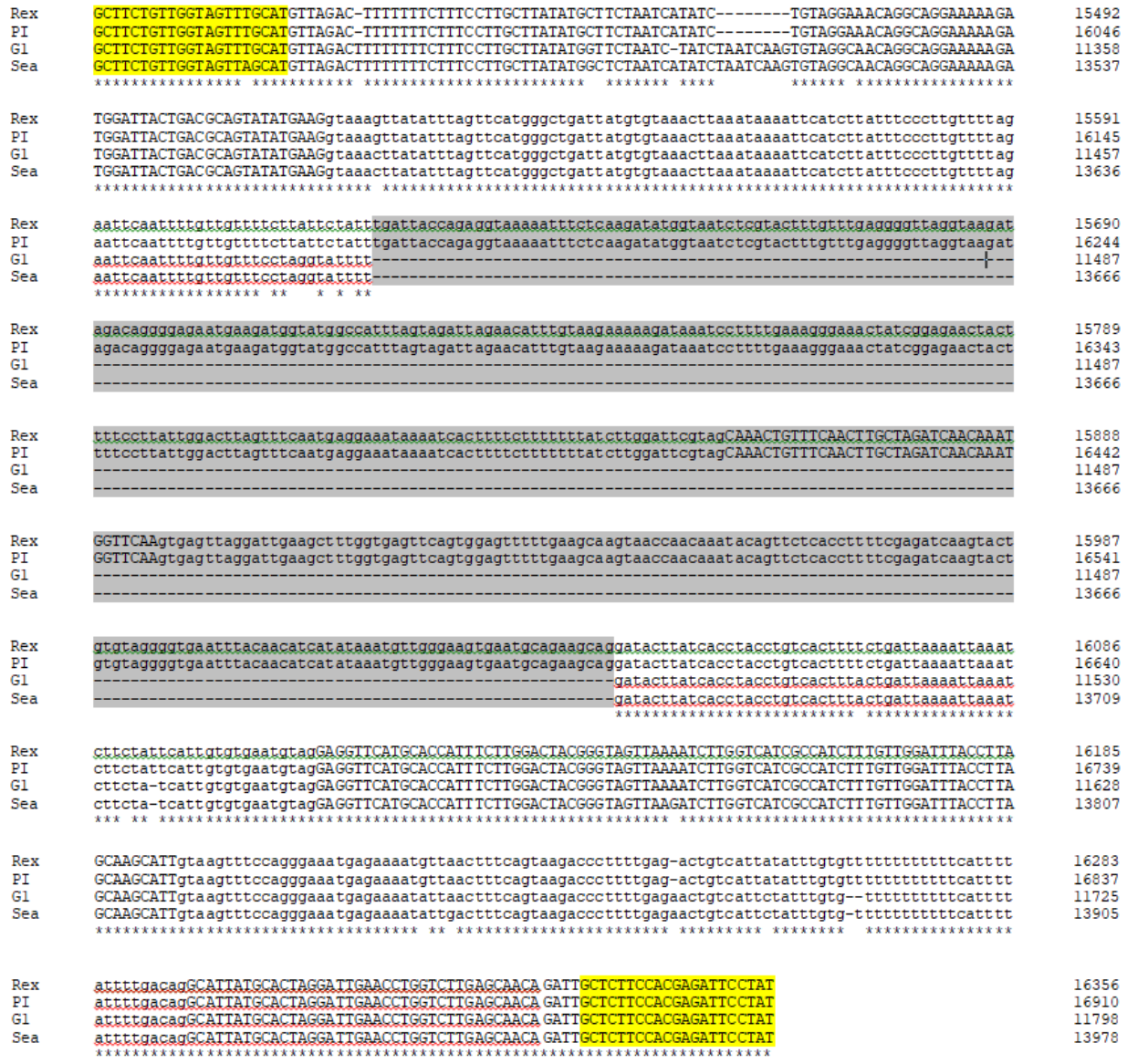


Figure 2.5- The alignment of sequenced amplicons resulting from the NPP marker, including sequences from OAC Rex (Rex), PI 440 795 (PI), G19833 (G1), and OAC Seaforth (Sea). Primer binding sites are indicated by the yellow highlighted regions, and the 421 bp insertion in resistant plant material is indicated by the grey highlighted region.

sequencing amplicons from primers [5'-GCTTCTGTTGGTAGTTTGCAT-3' (forward) and 5'-ATAGGAATCTCGTGGAAGAGC-3' (reverse)] spanning the putative polymorphism, but it had not been validated as an effective marker. Figure 2.5 shows the alignment of the resulting sequences for the NPP amplicons from genomic DNA from four bean lines, including the source of CBB resistance, PI 440 795 (*P. acutifolius).* The sequencing results confirmed the polymorphism in the sizes of the amplicons from OAC Rex and G19833 and the previous *in silico* comparisons between and the genomic regions for these genotypes. In particular, the sequence from OAC Rex was 421 bp larger than the one obtained from G19833 and the insertion was annotated as two additional exons. The sequence for OAC Seaforth, a CBB susceptible plant line, was 100% identical to the amplicon sequence obtained for G19833. The amplicon sequence for the *P. acutifolius* line PI 440795, the source of the CBB resistance in OAC Rex, was 100% identical to the sequence for OAC Rex.

A comparison of the gene structures for the NPP marker region of the Niemann-Pick like gene, in these four bean lines, is shown in Figure 2.6. Common bean lines that are resistant to CBB, which come from crosses with *P. acutifolius*, have two open reading frame gene models. The larger of the two genes in resistant OAC Rex (232701-8-007) is 16 642 bp in length and has 26 exons and 28 introns, including 4 exons that are covered by the NPP marker. The smaller of the two genes in OAC Rex (resistant) is 6 907 bp in length and contains 15 exons and 17 introns. In G19833 (susceptible), the Niemann-Pick like gene is 18 724 bp in length, and encompasses 37 exons and 38 introns. The difference in exon structure and quantity is the result of additional exons flanking the smaller gene in OAC Rex, as well as the additional two exons present in the 421 bp insertion that the NPP marker encompasses within OAC Rex.

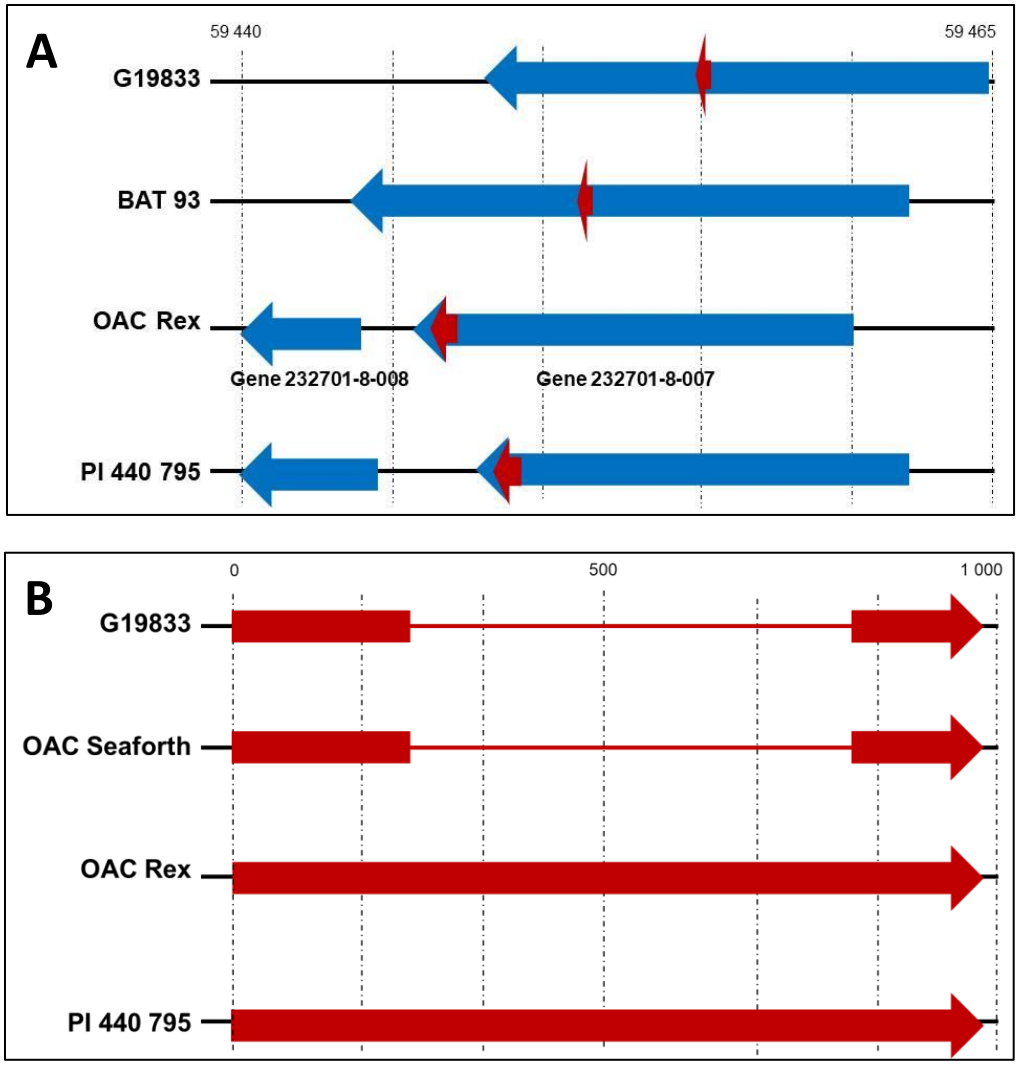


Figure 2.6- An illustration based on amplicon sequence data, showing the differences between bean lines for the Niemann-Pick like gene structure and the Niemann-Pick polymorphism. A) A comparison of gene structure, in reference to the physical location in the OAC Rex genome (in kb), shows the presence of two open reading frames for two of the bean lines. The region highlighted by red represents the location of the NPP marker amplicon. B) The alignment of the Niemann-Pick polymorphism amplicon, showing the insertion present in two of the bean lines: OAC Rex and PI 440 795.

2.4.2 NPP Marker Screening

In order to screen the population for the NPP marker, a series of DNA extractions were conducted. These extractions yielded DNA concentrations ranging from 41.5-132 ng/µL. The quality of the DNA was also screened to ensure that contaminants in the sample were not going to affect the resulting PCR. The 280 nm/260 nm ratio was measured and all samples fell below the ratio value of 2. The resulting ratios were acceptable and therefore no extractions were redone or excluded. After each series of PCR reactions, the products were run on a 1% agarose gel, and stained with ethidium bromide in order to image the gel and determine the amplicon size. As seen in Figure 2.7, the resulting images were compiled and each RIL was denoted as having either the "A" or "B" allele for the NPP marker. Individuals with the smaller, 535bp allele, the same as OAC Seaforth, are labeled as having the "A" allele. The "B" allele denoted individuals with the larger (956 bp) allele similar to OAC Rex. The allele distribution for each population is illustrated in Figure 2.8. In summary, for the OAC Rex/OAC Seaforth population there were 25 RILs that carried the "A" allele and 47 RILs that had the "B" allele for the NPP marker. For the Compass//Rexeter/Apex population there were 109 individuals that had the "A" allele and 52 that had the "B" allele for the NPP marker.

2.4.3 Pedigree Screening

The lines that are in the pedigree of OAC Rex were phenotypically and genotypically screened in order to investigate the source of the resistant NPP allele. As shown in Figure 2.9 the resistant NPP allele (B) can be traced back to the *P. acutifolius* Plant *I*ntroduction 440 795. The interspecific cross between this line and ICA Pijao resulted in a F_1 line, after embryo rescue (Micheals *et al*., 2006). This material is no longer available, but MBE7, which was created by

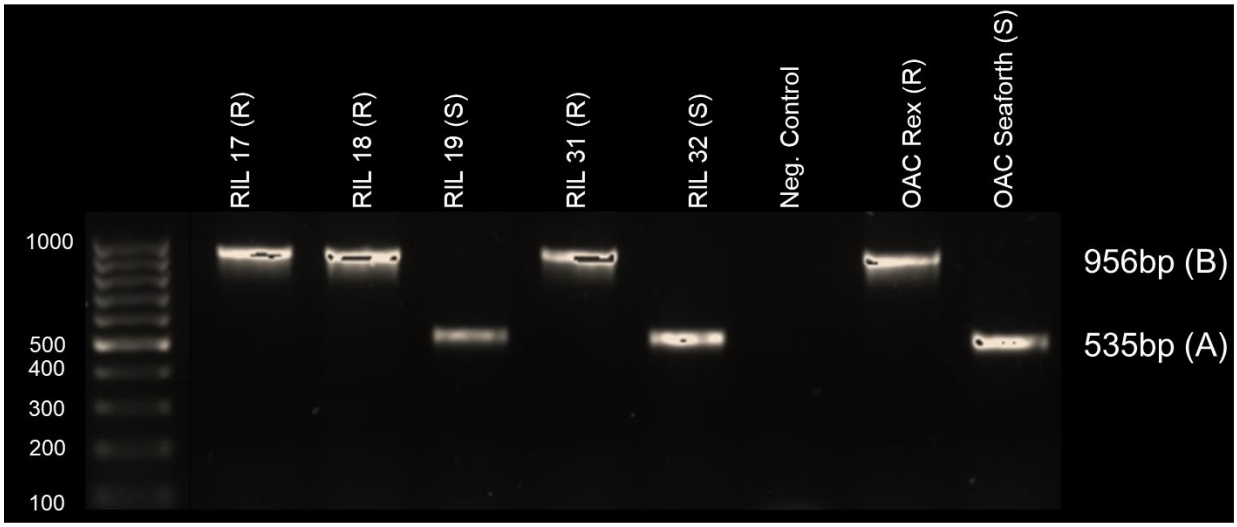


Figure 2.7- A sample 1% agarose gel showing several RILs, and the parental lines, screened in order to determine the allele present for each individual. Shown above are 5 RILs from the OAC Rex/OAC Seaforth population in the first 5 lanes, a negative control, OAC Rex and OAC Seaforth. The band sizes for the two NPP alleles are indicated alongside the allele labels utilized (A and B). The disease susceptibility for each RIL and parent are indicated in brackets with (R) representing resistance and (S) representing susceptibility.

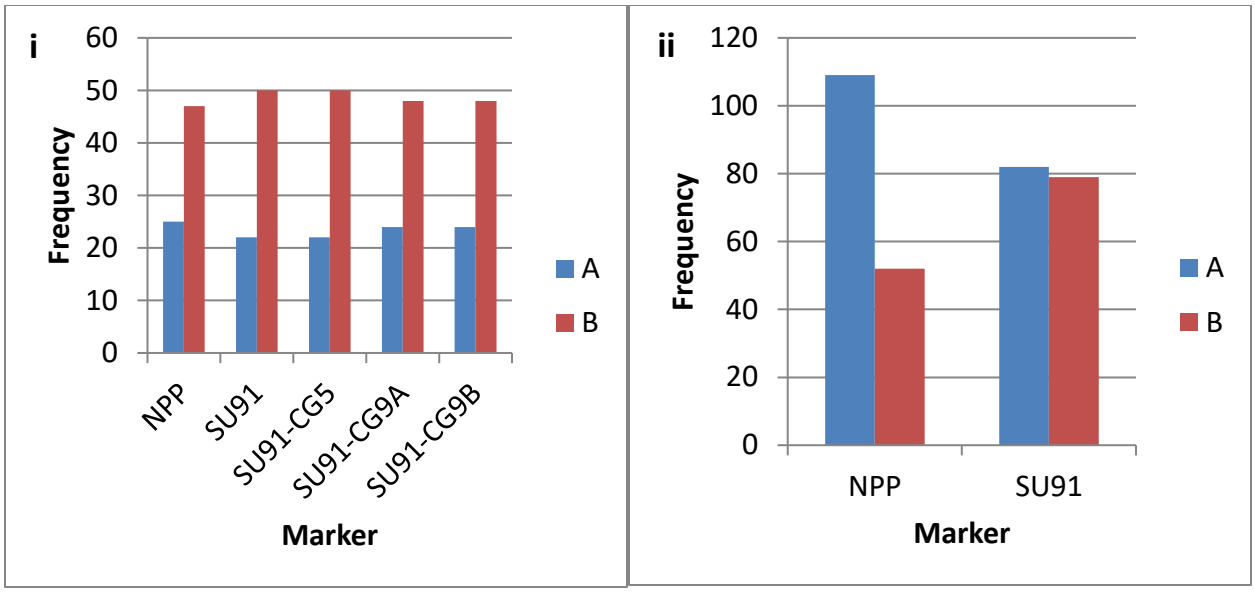


Figure 2.8- A comparison of the population distribution for each marker, for both of the two populations. i) The frequency of each CBB marker screened for the OAC Rex/OAC Seaforth population with the susceptible alleles, or absence of an allele, indicated by "A" and resistant alleles, or presence of an allele, indicated by "B". ii) The frequency of each CBB marker screened for the Compass//Rexeter/Apex population with the susceptible allele, or absence of an allele, indicated by "A" and the resistant allele, or presence of an allele, indicated by "B".

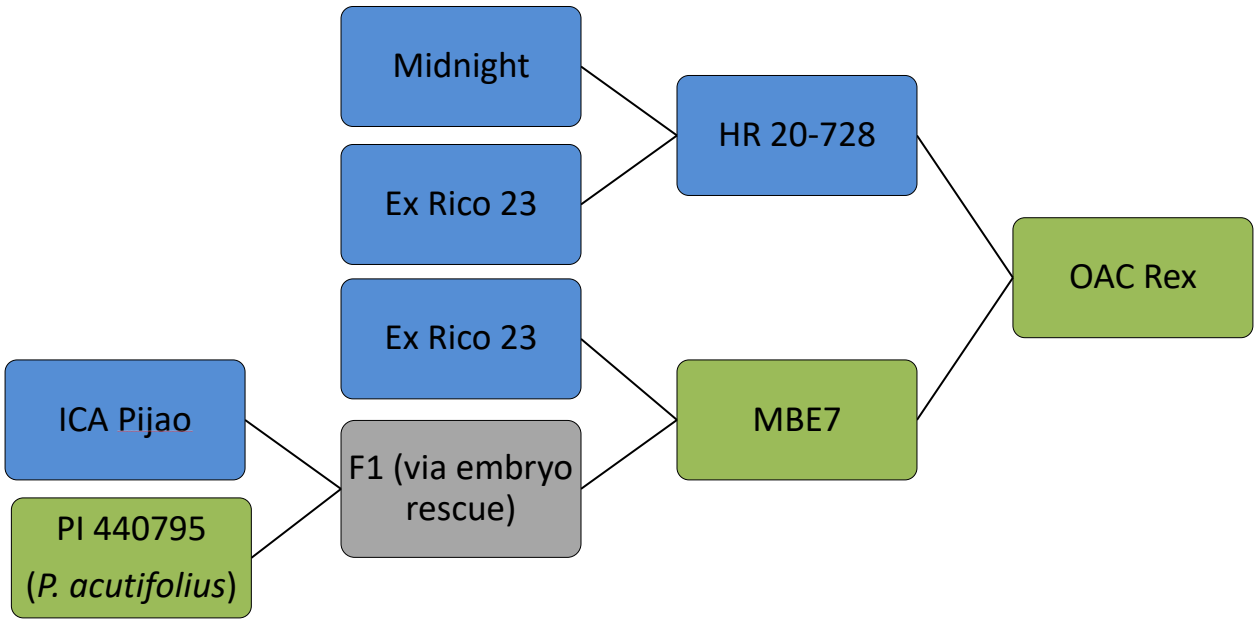


Figure 2.9- The pedigree of the CBB resistant line OAC Rex with each line's CBB reaction and NPP marker variant indicated by colour. Lines shaded blue are CBB susceptible and screened positive for the "A" allele of the NPP marker. Lines shaded green are CBB resistant and screened positive for the "B" allele for the NPP marker. The F_1 line was not screened for disease reaction and was unavailable for marker screening (shaded grey).

crossing the F_1 with Ex Rico, is also CBB resistant. MBE7 inherited the NPP allele B from that side of the lineage since Ex Rico has the susceptible allele A. OAC Rex was selected from a cross between MBE7 and HR20-728. The latter contributed good architecture and yield performance characteristics to OAC Rex but was susceptible to CBB and carries the NPP susceptible allele A. Similarly earlier parents in HR20-728's lineage all showed the smaller band size and therefore had the susceptible allele. In addition to lines in OAC Rex's pedigree, 11 other experimental and commercial lines were screened with the NPP marker. The *P. acutifolius* line Mex, which was found to be susceptible to CBB, showed the susceptible NPP allele. The PI 319 443 is a *P. acutifolius* line that is resistant to CBB and was introgressed into the lines that became experimental lines HR45 and HR 67 (Park and Dhanvantari, 1994; Park *et al.*, 2007). All three lines (PI 319 443, HR45 and HR67) were screened and they were all found to have the resistant NPP allele. Vax 4 and X159 are also experimental lines that have an introgression event in their pedigrees, and were also found to have the resistant NPP allele. Rexeter and Apex were included as parents to the Compass//Rexeter/Apex population utilized, to ensure the presence of the NPP marker. It was found that both Apex and Rexeter have the larger NPP allele (B). The remaining resistant lines (Mist and ACUG 11-7) contain OAC Rex in their pedigrees, and were found to have the resistant NPP allele (B). A summary of the lines screened is found in Table 2.1.

2.4.4 Phenotypic Screening

Figure 2.10 shows the range of CBB lesion development on unifoliate leaves 24 days after inoculation in a growth room, on RILs with different susceptibilities to the disease. For a susceptible individual with a rating of 4 (Figure 2.10A), inoculation resulted in the development

Table 2.1- The results of line screening of various commercial and experimental lines, in addition to the disease reactions of the same lines. NPP marker allele "A" denotes the 535 bp amplicon similar to the CBB susceptible line OAC Seaforth. NPP marker allele "B" denotes the 956 bp amplicon similar to the CBB resistant line OAC Rex.

Line	NPP Marker Allele	CBB Disease Reaction
HR 45	B	Resistant (Park and Dhanvantari, 1994)
HR 67	B	Resistant (Park *et al.*, 2007)
Vax 4	B	Resistant (Singh *et al.*, 2001)
Rexeter	B	Resistant (Smith *et al.*, 2012)
X159	B	Resistant (Park *et al.*, 2007)
ACUG 11-7	B	Resistant
Mist	B	Resistant (Khanal *et al.*, 2017)
Apex	B	Resistant (Navabi *et al*., 2013)
BAT 93	A	Resistant (Singh and Munoz, 1999)
PI 319 443	B	Resistant (Drijfhout and Blok, 1987)
Mex	A	Susceptible
Nautica	A	Susceptible
Compass	A	Susceptible
OAC Thunder	A	Susceptible

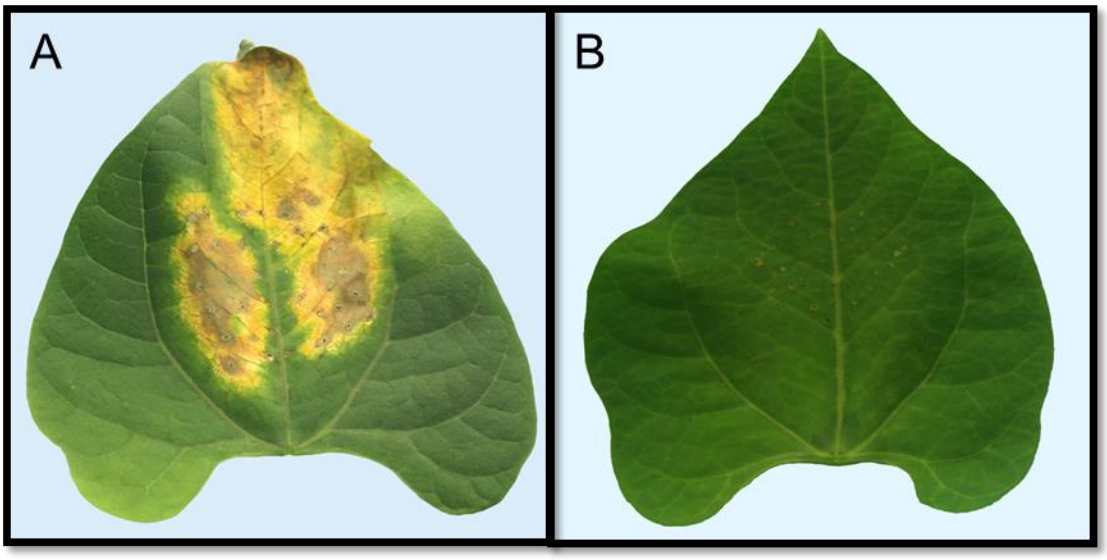


Figure 2.10- The phenotype of a susceptible individual (A) and resistant individual (B) illustrating phenotypic ratings of 4 (A) and 0 (B). The inoculation of these leaves was completed in a climate-controlled growth room with 25˚C 16 hour days and 20˚C 8 hour nights. The plants were exposed to 100% humidity for 48 hours prior to inoculation and 48 hours after inoculation. After an initial exposure to 100% humidity, plants were kept at 90% humidity. Images were taken 24 days after inoculation.

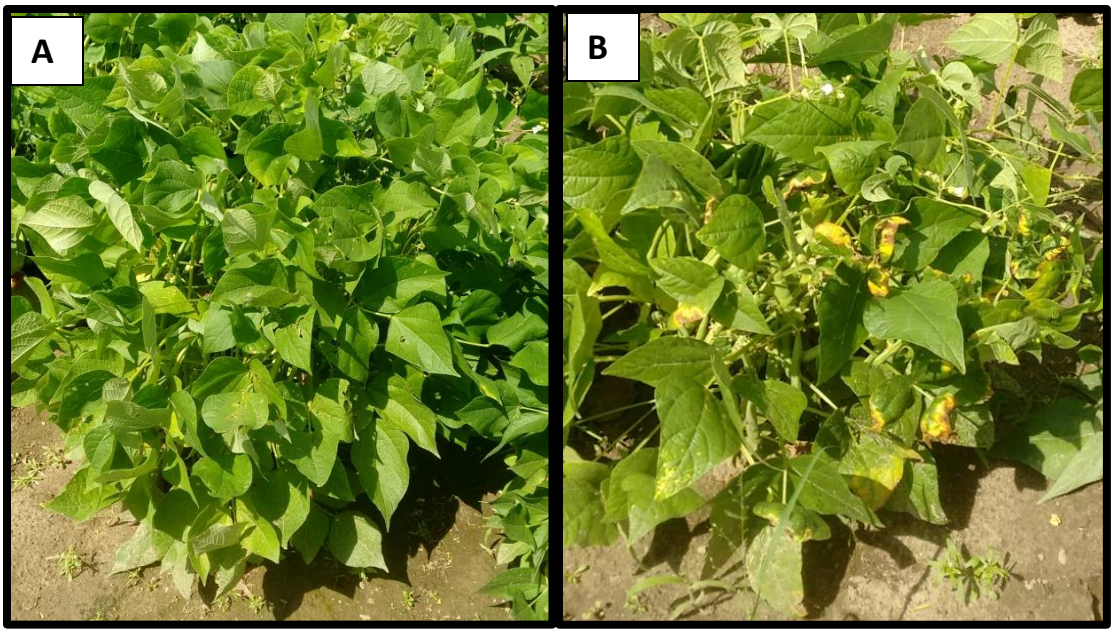


Figure 2.11- Images of hill plots in the CBB nursery located at the Agriculture and Agri-Food Canada station in Harrow, ON 14 days after artificial inoculation. Plots were planted 1m apart and contained 7 plants per hill. A) A hill plot of resistant parent OAC Rex 14 days after inoculation. B) A hill plot of susceptible parent OAC Seaforth 14 days after inoculation.

of typical CBB lesions that had a necrotic centre and a bright yellow halo. In contrast, a leaf showing a resistant reaction, with a rating of 0, is shown in Figure 2.10B. The leaf showed a small amount of necrosis around the inoculation wounds but no lesion development. In addition, Figure 2.11 shows two hill plots 14 days post inoculation in the field nursery in Harrow, ON. Figure 2.11A shows a resistant plot (OAC Rex), with little to no disease symptoms in comparison to the susceptible plot (OAC Seaforth) shown in Figure 2.11B.

The OAC Rex/OAC Seaforth population was screened for three years, two replications each year, within a field CBB nursery. The phenotypic rating for disease susceptibility was averaged within the year and across years. All resulting statistics were run within years as well as across years. Table 2.2 shows a summary of the combined data. Based on an approximate susceptibility threshold of a rating of 3 (24 DAI), the population contains 49 resistant and 25 susceptible RILs. As indicated, averages within years and across years were compared to determine outliers. There were two lines that were removed based on inconsistent scores within years, alluding to poor plot inoculation. After the removal of the two lines, the remaining 72 lines could be separated as 48 resistant lines and 24 susceptible lines based on an approximate threshold of a CBB rating of 3. The distribution of disease severity, based on AUDPC (Figure 2.6), is illustrated in Figure 2.12. The resulting AUDPC range for the OAC Rex/OAC Seaforth population was 17.50-83.67. The resistant parent, OAC Rex, scored an average of 20.75 and the susceptible parent OAC Seaforth scored an average of 83.67. All RILs scored lower than the susceptible parent.

The disease ratings for the Compass//Rexeter/Apex population were completed for two years, with two repetitions each year. The disease severity summary can be seen in Table 2.3. Based on the same approximate susceptibility threshold of a CBB score of 3, the

Table 2.2- Population averages for the CBB disease scoring of the OAC Rex/OAC Seaforth population over three years (2011, 2013, 2016); shown in area under the disease progress curve (AUDPC). The population was divided on the basis of NPP alleles (A and B) carried by the individuals, and the means of the groups were tested with a t-test against the population mean.

	Number of Individuals	**Mean AUDPC[y]**	**p-value***	**Minimum AUDPC[y]**	**Maximum AUDPC[y]**
NPP A	25	62.6	<0.0001	41.3	83.7
NPP B	47	33.8	<0.0001	17.5	69.6
Total Population	72	43.8		17.5	83.7

[y] Measured in % disease coverage day^{-1} after bacterial inoculation

*α =0.05

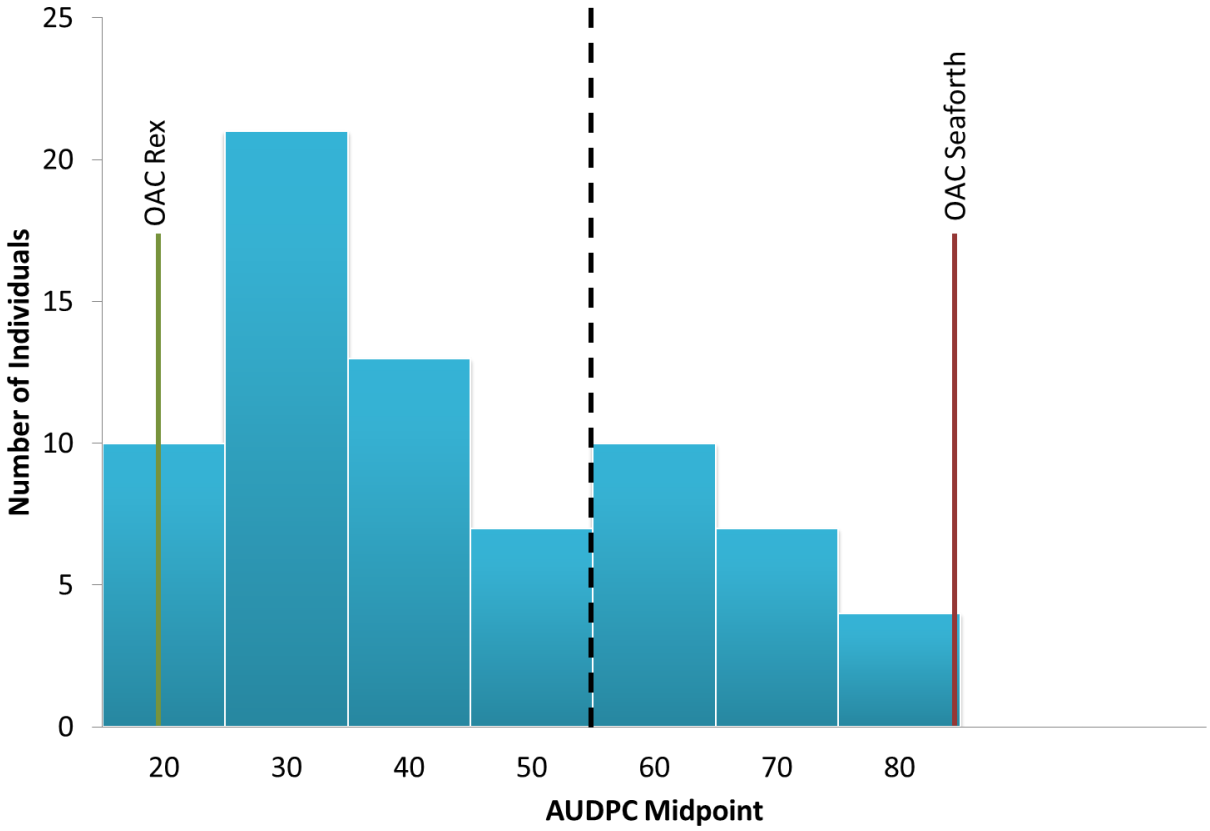


Figure 2.12- The distribution of CBB disease scores, shown in AUDPC, for the OAC Rex/OAC Seaforth population averaged across three years (2011, 2013, and 2016). The threshold of resistance, determined by a rating of 2.5, is indicated by a dashed line, and the scores of the population parents are labeled.

Table 2.3- Population averages for the CBB disease scoring of the Compass//Rexeter/Apex population over two years (2012 and 2013); shown in area under the disease progressive curve (AUDPC). The population was segregated by the NPP allele, and utilizing a t-test the allelic means were tested against the population mean to determine if the marker was separating the population based on disease susceptibility.

	Number of Individuals	**Mean AUDPC*y***	**p-value***	**Minimum AUDPC*y***	**Maximum AUDPC*y***
NPP A	109	122.92	<0.0001	89.69	161.50
NPP B	52	70.48	<0.0001	38.88	105.88
Total Population	161	105.99		38.88	161.50

y Measured in % disease coverage day^{-1} after bacterial inoculation

*α =0.05

Compass//Rexeter/Apex population contained 108 susceptible RILs and 53 resistant RILs. Similar to the OAC Rex/OAC Seaforth population, the CBB averages within years and across years were evaluated in order to detect outliers and remove any plot data that may have resulted from ineffective plant inoculation. In the Compass//Rexeter/Apex population, no lines were identified as outliers, nor removed from the data based on inconsistent CBB scores. The distribution of AUDPC values is illustrated in Figure 2.13. The resulting AUDPC range for the Compass//Rexeter/Apex population was 38.88-161.50. The susceptible parent, Compass, scored an average of 132.88, while Rexeter scored an average of 38.88 and Apex scored an average of 45.50. This places Rexeter at the very resistant end of the range of values; however, there are several RILs that were more susceptible than the susceptible parent Compass.

The phenotypic data supported the differences in population structure. The OAC Rex/OAC Seaforth population was selfed for several generations to form homologous recombinant inbred lines. This resulted in a population with various levels of resistance, and a distribution of disease scores skewed slightly to the resistant. In comparison, the Compass//Rexeter/Apex population was backcrossed to the susceptible parent, Compass. This skewed the distribution of disease scores to the susceptible, so that more than half of the population exceeded the susceptibility threshold. The ranges of AUDPC values also varied between the populations. This is due to differences in rating timings between the populations. The Compass//Rexeter/Apex population was scored with more time elapsed between ratings in comparison to the OAC Rex/OAC Seaforth population. The difference in timing prevented direct comparison between populations, but does not inhibit comparisons within populations.

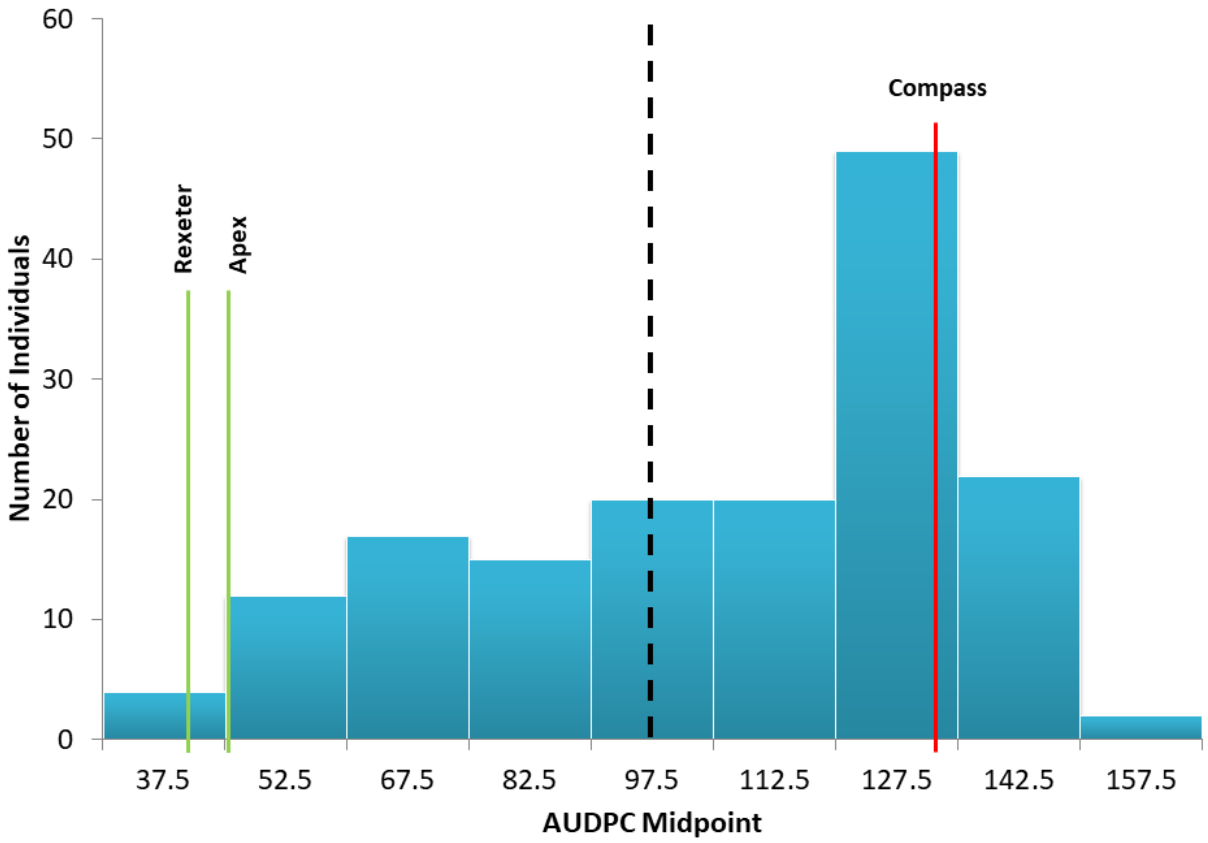


Figure 2.13- The distribution of CBB disease scores, shown in AUDPC, for the Compass//Rexeter/Apex population averaged across two years (2012 and 2013). The threshold level of resistance, determined by a rating of 2.5, is indicated by a dashed line, and the scores of the population parents are labeled.

Table 2.4- Summary of marker efficiency within two populations to compare the NPP marker against established markers utilized for CBB resistance screening in current breeding programs, given in percent of the population (%). False positives and negatives are based on discrepancies between phenotypic data and marker allele.

	NPP		**SU91**		**SU91-CG5**		**SU91-CG9A**		**SU91-CG9B**	
Population	False (+)	False (-)	False (+)	False (-)	False (+)	False (-)	False (+)	False (-)	False (+)	False (-)
OAC Rex/ OAC Seaforth	0%	0%	4%	2%	3%	5%	8%	4%	8%	4%
Compass// Rexeter/ Apex	1%	2%	24%	33%						

2.4.5 Genotypic Screening

Table 2.4 combines the previously obtained data for the SU91 marker and its analogs and the current NPP marker data with the disease scoring data. In summary, the NPP marker resulted in fewer false positives and false negatives than SU91, SU91-CG5, SU91-CG9A, and SU91-CG9B in both populations. In particular, the NPP marker decreased false positives by 23% and false negatives by 31% in comparison to the SU91 marker within the Compass//Rexeter/Apex population. Within the OAC Rex/OAC Seaforth population the NPP marker decreased false positives by an average of 6% and false negatives by an average of 4% in comparison to SU91 and the SU91 analogs.

2.4.6 Statistical Analysis

In order to determine if the variation of data within years and across years is homogeneous, the data was tested for variance heterogeneity. Ultimately, the data was determined to have homogeneous variance and therefore the data was pooled and the AUDPC values for all years were included. When each population was separated based on the NPP marker allele, the least square means for the AUDPC scores for each group created by the allelic division were significantly different from each other based on the resulting *p-value* (Appendix 1, Table 1). Each allele of the NPP marker successfully separated the populations into the resistant and the susceptible individuals. As seen in Figure 2.14, the NPP marker was effective in both populations, but was found to be significantly more effective in comparison to SU91 in the Compass//Rexeter/Apex population (Figure 2.15). After separating the population by the single NPP marker, the same separation was conducted with SU91 (for both populations) and the SU91 analogs (for the OAC Rex/OAC Seaforth population only) (Appendix 1, Tables 2-7). This

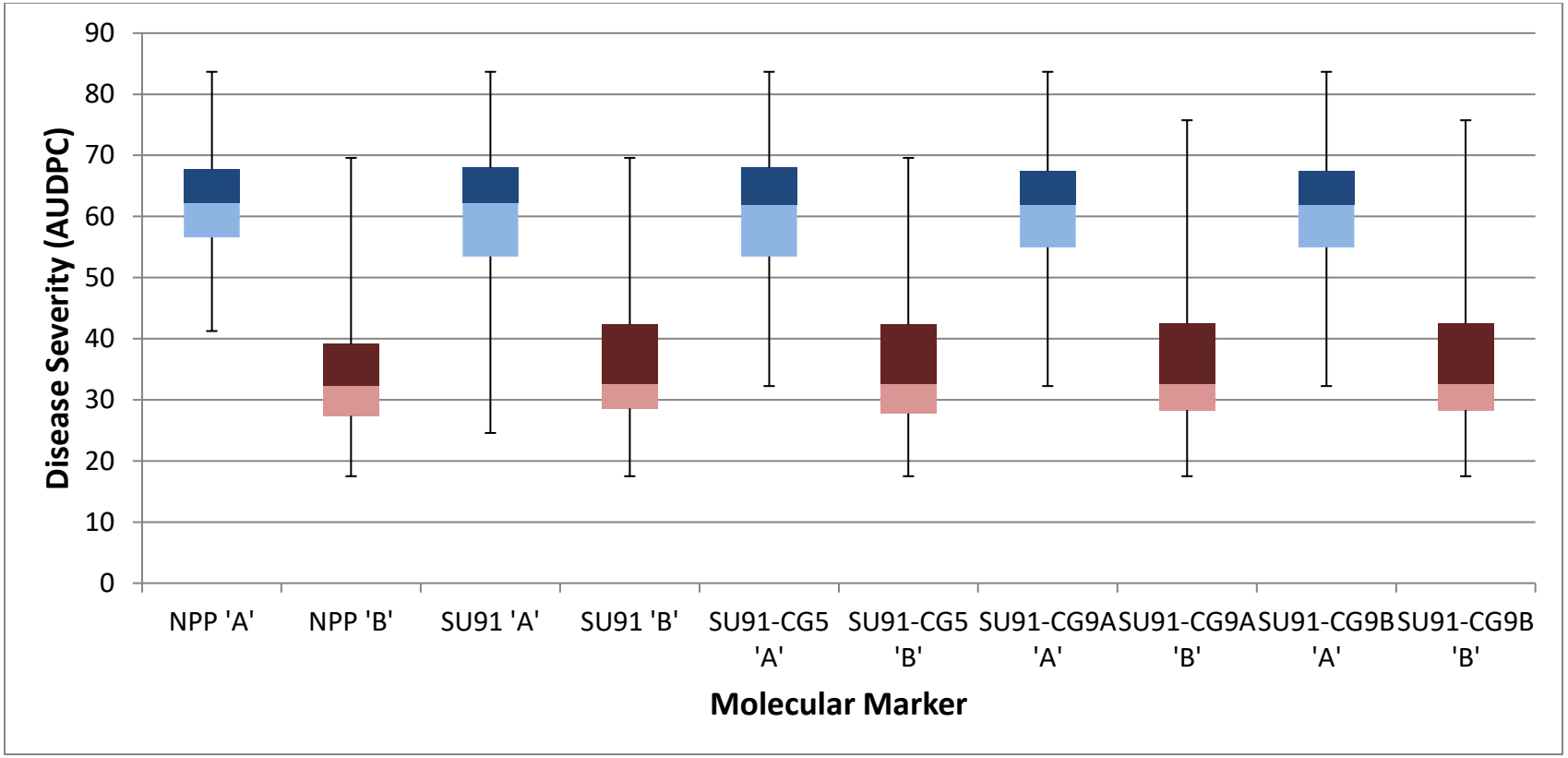


Figure 2.14- Boxplots showing how each molecular marker separated individuals of the OAC Rex/OAC Seaforth population by allele. Allele A represents the allelic form of the susceptible parent (blue), and the absence of an allele for the dominant markers (SU91 and analogs). Allele B represents the allelic form of the resistant parent (red) and the presence of an amplified allele for the dominant markers. The median AUDPC score for each marker allele is indicated at the point of colour shade change in each boxplot.

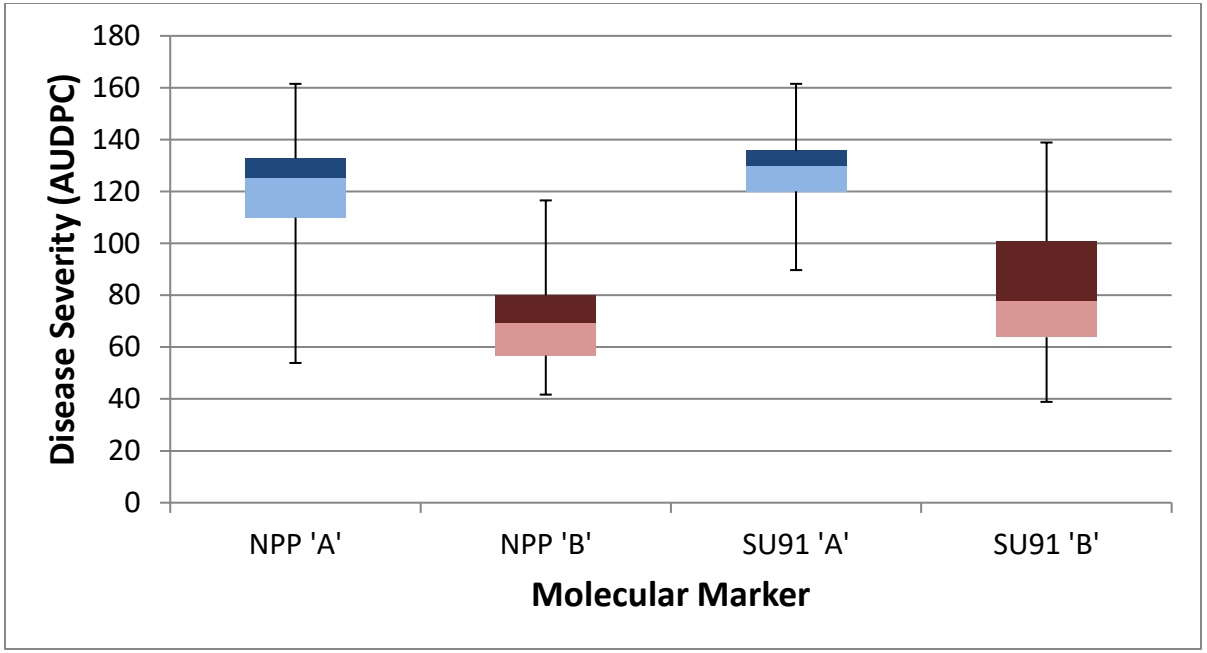


Figure 2.15- Boxplots showing how each molecular marker separated individuals of the Compass//Rexeter/Apex population by allele. Allele A (blue) represents the allelic form of the susceptible parent (NPP) and the absence of an amplified allele for the dominant marker SU91. Allele B (red) represents the allelic form of the resistant parent (NPP) and the presence of an amplified allele for the SU91 marker. The median AUDPC score for each marker allele is indicated at the point of colour shade change in each boxplot.

Table 2.5- A summary of the pairwise comparison of mean AUDPC scores for each molecular marker screened in the OAC Rex/OAC Seaforth population. AUDPC means were compared for each marker*allele combination (α=0.05).

Marker	Allele	Mean AUDPC[y]	Estimate	SE	
NPP	A	62.6	4.14	0.039	*b*
NPP	B	33.8	3.52	0.054	*a*
SU91	A	60.1	4.10	0.042	*b*
SU91	B	35.7	3.57	0.050	*a*
SU91-CG5	A	60.4	4.10	0.042	*b*
SU91-CG5	B	35.5	3.57	0.051	*a*
SU91-CG9A	A	61.0	4.11	0.043	*b*
SU91-CG9A	B	36.2	3.59	0.048	*a*
SU91-CG9B	A	61.0	4.11	0.043	*b*
SU91-CG9B	B	36.2	3.59	0.048	*a*

a-b Means followed by the same letter are not significantly different (α=0.05), N=6
y Measured in % disease coverage day^{-1} after bacterial inoculation
SE- standard error (α=0.05)

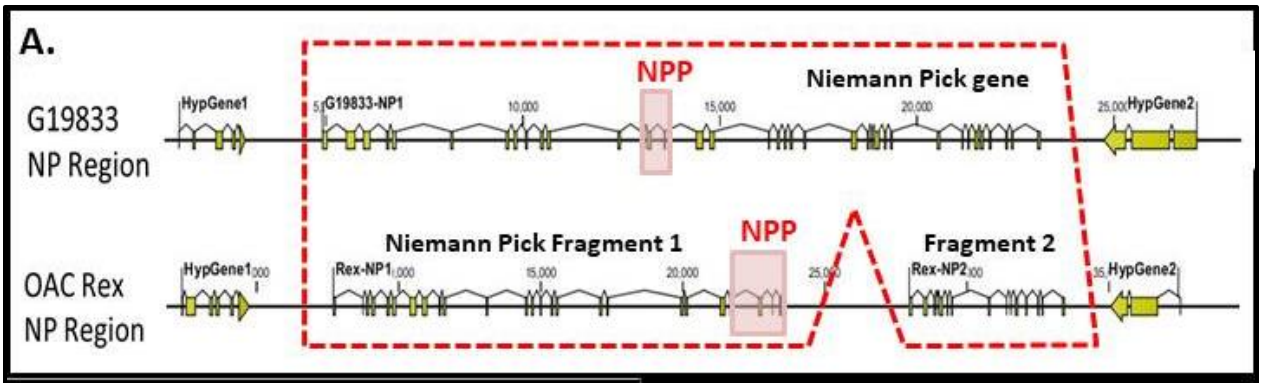
resulted in 10 classes for the OAC Rex/OAC Seaforth population (five markers with two alleles each) and 4 classes for the Compass//Rexeter/Apex population (two markers with two alleles each). The means for these classes were compared pairwise in order to determine if the division of the population by the NPP marker was more efficient than the pre-existing markers.

The summary of group means and marker comparisons for the OAC Rex/OAC Seaforth population can be found in Table 2.5. Similarly, for the Compass//Rexeter/Apex population the AUDPC scores were separated based on the NPP allele and the least square means were calculated. The same was done for the SU91 marker. The four marker*allele subgroups were compared pairwise. For the OAC Rex/OAC Seaforth population, there was no significant difference between the segregation based on the NPP marker and any other marker (SU91 and the three analogs used) but the NPP marker was significantly more efficient in separating the resistant individuals. The means for the resistant alleles for the NPP marker and the SU91 marker were significantly different, with the mean for the NPP subgroup being significantly lower than the SU91 subgroup. This suggests that the NPP marker incorporates fewer false positives when screening a population for CBB resistance based on the molecular marker. A summary of the least square means and marker comparisons for the Compass//Rexeter/Apex population can be found in Table 2.6. Ultimately, the NPP marker was a significantly more efficient marker to utilize within the Compass//Rexeter/Apex population in comparison to the SU91 marker. Within susceptible individuals, there was no significant difference between the NPP marker and the SU91 marker. In resistant individuals, there was found to be a significant difference between NPP and SU91, with the NPP marker being significantly better than SU91.

Table 2.6- A summary of the pairwise comparison of mean AUDPC scores for each molecular marker screened in the Compass//Rexeter/Apex population. AUDPC means were compared for each marker*allele combination (α=0.05).

Marker	Allele	Mean AUDPC[y]	SE	
NPP	A	122.92	1.63	*a*
NPP	B	70.48	2.36	*c*
SU91	A	126.93	2.31	*a*
SU91	B	84.24	2.35	*b*

a-c Means followed by the same letter are not significantly different (α=0.05), N=4
y Measured in % disease coverage day^{-1} after bacterial inoculation
SE- standard error (α=0.05)



B. Niemann-Pick Polymorphism Marker

Forward Primer (5'-3')	GCTTCTGTTGGTAGTTTGCAT
Reverse Primer (5'-3')	ATAGGAATCTCGTGGAAGAGC
Susceptible Amplicon Size	535 bp
Resistant Amplicon Size	956 bp
Annealing Temperature	56°C

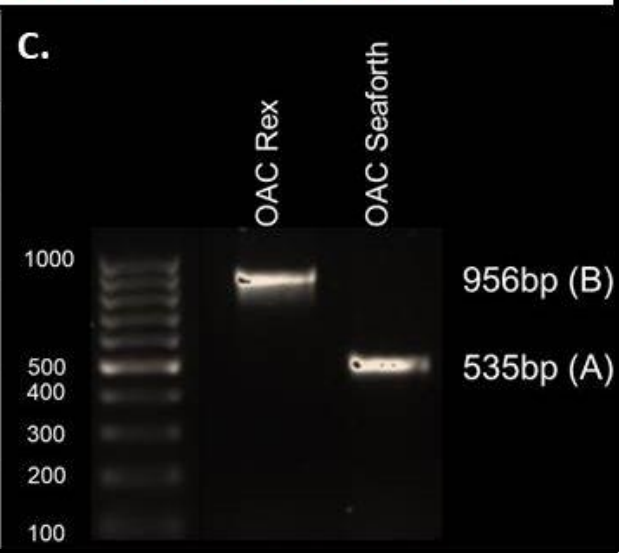


Figure 2.16- A summary of the NPP marker, showing the amplicon placement, primer details and protocol, and the use of the NPP marker as a codominant marker for CBB resistance. A) The NPP marker amplifies a 535 bp region in susceptible bean material such as line G19833, and a 956 bp region in resistant plant material such as OAC Rex. B) The primers for the NPP marker and the PCR protocol for the use of the marker. C) A resulting agarose gel showing the amplified band for a resistant line (OAC Rex) and a susceptible line (OAC Seaforth).

2.5 Discussion and Implications

By validating the NPP marker, it has been shown that a polymorphism in the Niemann-Pick like gene of *P. vulgaris* is collocated with a CBB resistance QTL. Although not conclusive, the close linkage between the marker and CBB resistance, established in the current work, resulted in the development of a molecular marker for CBB resistance that is superior to the currently available molecular markers. The summary of the NPP marker including its protocol, location and amplicon size can be found in Figure 2.16. When utilized to screen the two populations for CBB resistance, the NPP marker was equivalent to the current markers for the OAC Rex/OAC Seaforth population, and was significantly more efficient than the SU91 marker within the Compass//Rexeter/Apex population. Within the Compass//Rexeter/Apex population, the NPP marker divided the individuals into 109 susceptible individuals and 52 resistant individuals, whereas the SU91 marker divided the same population into 81 susceptible individuals and 79 resistant individuals. The AUDPC means of these groupings were significantly different, but the ability for the NPP marker to divide the population appropriately based on disease susceptibility scores is of greater significance. Based on disease ratings with a threshold rating of 3, the population has 108 susceptible individuals and 53 resistant individuals. Therefore NPP is considerably more efficient in comparison to the SU91 marker. The SU91 marker had a 33% error rate for the Compass//Rexeter/Apex population in comparison to the NPP marker's error rate of 1.2%.

In addition, the NPP marker is a codominant marker, allowing for the detection of both the resistant and susceptible allele. The use of a codominant marker allows for the certain discernment between resistant and susceptible material within marker assisted selection. In comparison, the use of a dominant marker within marker assisted selection can add uncertainty.

The lack of a susceptible allele creates a potential for additional false negatives, therefore increasing the error rate of the marker. The codominant nature of the NPP allele is a benefit for its use within marker assisted selection.

The screening of the OAC Rex pedigree allowed for the tracking of the allele through the introgression of *P. acutifolius* into the *P. vulgaris* genome. The presence of the resistant allele in PI 440 795 and resulting progeny, but not in any other *P. vulgaris* line supports the theory that the candidate gene originated from the introgression event in OAC Rex's pedigree. In addition, the susceptible *P. acutifolius* line, Mex, was screened with a resulting 'A' allele which is consistent with susceptible *P. vulgaris* lines. Although the complete structure of the Niemann-Pick gene in Mex was not determined, the current results support the hypothesis that the polymorphism present in the Niemann-Pick like gene in PI 440 795 is also related to the CBB resistance in *P. acutifolius.* Further *P. acutifolius* lines would have to be screened in order to confirm the interaction with disease susceptibility within *P. acutifolius.* In order to further validate the NPP marker, additional screening would have to be conducted, but within the two populations utilized the NPP marker was validated as an effective molecular marker.

Due to the anchoring of the NPP marker in the Niemann-Pick like gene at the end of chromosome 8, further investigation into the role that the Niemann-Pick like gene may play in CBB resistance is required. The role that the Niemann-Pick like protein plays in disease resistance is unknown but may be related to the proposed role that the Niemann-Pick like protein plays in plants in the localization of sterols and sphingolipids within the plant cell (Huckelhoven, 2007; Kale *et al.*, 2010; Borner *et al.*, 2005). There are several theories that can explain how the Niemann-Pick like protein, and sterol transport, can be involved in CBB resistance, including that modification of the lipid composition of membrane micro-domains (or rafts) may cause: re-

localization of proteins involved in the hyper sensitive response; reductions in the penetration of plant cells by bacterial effector molecule injection mechanisms; and reductions in the endocytosis of extracellular effector molecules (Huckelhoven, 2007; Kale *et al.*, 2010; Borner *et al.*, 2005).

The proposal that the Niemann-Pick like gene may play in plant disease defense by altering the incorporation of hyper sensitive response proteins into the plasma membrane is based on observations that the signal proteins are anchored in sphingolipid and sterol rich membrane micro-domains (Borner *et al.*, 2005). The formation of these micro-domains, also known as lipid rafts, requires the localization of sterols and sphingolipids to the plasma membrane (Borner *et al.*, 2005). If the Niemann-Pick like protein is responsible for sterol transport, alterations to these lipid rafts could hypothetically affect the signaling system for the hyper sensitive response. Potentially, if the alteration to the gene structure of the Niemann-Pick like gene in resistant plant material leads to additional sterols being localized to the plasma membrane, there may be additional hyper sensitive response proteins being localized to the plasma membrane as well. This potential alteration of protein localization is one area of research that could be studied further in order to understand how Niemann-Pick proteins might function in plant disease defense mechanisms.

The connection of the Niemann-Pick protein in animals to cell sterol transport is well supported and is the basis for speculation that increased activity may result in increases in the quantity of saturated sterols present in lipid rafts, making them more rigid and thus preventing the penetration of plant cells by effector molecule injection systems (Kale *et al.*, 2010) present in bacterial pathogens, like *Xanthomonas*. Pathogens that utilize type III effector molecule injection systems penetrate the plant cell with a syringe like protein complex, allowing for

effector molecules to be directly inserted into the plant cell (Troisfontaines and Cornelis, 2005). *X. axonopodius* and *X. fuscans* both utilize type III effector molecule injection systems (Perry and Pauls, 2012). By incorporating more saturated lipids into lipid rafts, the rafts become more rigid resulting in reduced penetration by pathogens (Kale *et al.*, 2010). This theory is supported by work on the penetration of the fungi responsible for rust (*Uromyces appendiculatus* Pers.) in common bean and oats (Harder and Mendgen, 1982). By staining the sterol complexes in the plasma membrane with filipin stain, it was found that resistant plants were able to relocate sterol rich domains to the site of penetration in order to prevent infection (Harder and Mendgen, 1982). Possibly, if sterol domain relocation can prevent fungal infection, it could similarly prevent the penetration of the cell by injection systems involved in bacterial infection.

The alteration of lipid rafts may also reduce the ability for plant cells to envelope extracellular material through endocytosis (Huckelhoven, 2007). Endocytosis is one pathway in which pathogen effector molecules enter the plant cell. Therefore, by reducing the rate of endocytosis the plant may also be able to reduce the rate of effector molecule uptake (Huckelhoven, 2007). Lipid raft mediated endocytosis represents a significant rate of endocytosis in plants, therefore if the lipid raft accumulation is altered, the rate of endocytosis may be decreased (Huckelhoven, 2007).

The endocytosis explanation would function in an opposite direction to the previous two theories, in that it would suggest that the Niemann-Pick polymorphism is responsible for a reduction of function as opposed to an increase in function. As illustrated in protein structure comparison in Figure 2.3, in resistant plant material, such as OAC Rex, the Niemann-Pick protein is proposed to exist in two fragments in comparison to the whole protein in susceptible plant material such as G19833. This division of the protein makes the theory of a reduction in

function theoretically plausible. The reduced function could lead to the prevention of saturated sterols being located to the plasma membrane, therefore reducing lipid raft mediated endocytosis. The lack of knowledge of what the Niemann-Pick protein does in plants means that there is no certainty about the role that Niemann-Pick like gene may play in CBB resistance.

This work has validated the NPP marker as an efficient molecular marker for use in CBB resistance screening. By testing the NPP marker in two genetically distinct populations the superiority of this marker over the current industry standard markers was demonstrated. However, the use of the NPP marker would be limited to breeding populations that have used a resistant *P. acutifolius* line as the source of resistance. Its location in the Niemann-Pick like gene and strong linkage to CBB resistance makes the Niemann-Pick like gene a strong candidate for the gene responsible for CBB resistance but the mechanism for resistance is only speculative.

Chapter 3: Investigation into novel R-genes within the OAC Rex genome as potential sources of common bacterial blight resistance

3.1 Abstract

In order to identify the gene responsible for common bacterial blight resistance within a major resistance QTL at the end of chromosome 8, the genomic sequences for OAC Rex and G19833 were compared and three candidate genes were identified. Two of the three genes contained a nucleotide binding site and leucine rich repeats; motifs consistent with those found in classical plant resistance genes (R-genes). Genes 231733-8-004 and 231733-8-005 were studied in both resistant and susceptible bean lines of interest to determine if they might have a role in CBB resistance. Both genes were found in available sequence data for resistant lines OAC Rex and BAT 93, but not in G19833. R-gene 231733-8-004 was present in OAC Seaforth, a susceptible common bean line, thus, excluding it from being considered as a resistance gene that was transferred into the *P. vulgaris* genome from an introgression event with *P. acutifolius*. Sequencing of gene 231733-8-004, and sequence alignment across the three bean lines led to the discovery of two single nucleotide polymorphisms (SNPs) in gene 231733-8-004, 6002 bp and 6087 bp from the beginning of the gene, which may play some role in CBB resistance. It was concluded that gene 231733-8-005 has no role in CBB resistance, as it was found to have 100% homology between OAC Rex and BAT 93.

3.2 Introduction

Breeding common bean lines for common bacterial blight (CBB) resistance is a reliable form of disease prevention (Singh and Miklas, 2015). The use of molecular markers in marker assisted selection is an efficient method to select lines for CBB resistance. Several markers have

been created and utilized to screen breeding populations for CBB resistance (Singh and Miklas, 2015). These markers are located on several chromosomes and are linked to QTLs for CBB resistance. Of these markers, marker SU91 is of interest as it is located in close proximity to the main CBB resistance QTL that is located at the end of chromosome 8 (Miklas *et al.,* 2006). Although the SU91 marker is efficient, when used in some genetic backgrounds there is a significant rate of error, and therefore improvements in the marker placement have been made, thus creating several SU91 analogs based in candidate genes (Shi *et al.,* 2012).

In an attempt to find the gene responsible for CBB resistance, candidate resistance genes were identified within the QTL region at the end of chromosome 8 that were novel to the resistant OAC Rex genome in comparison to the susceptible G19833 genome (Perry *et al.,* 2013). Each novel gene within the region was BLAST'd to determine its most likely function, and this analysis identified three candidate resistance genes in proximity to the SU91 marker. The first of the three genes, labeled 232701-8-007, was found to be homologous to a Niemann-Pick like gene. As previously described (Chapter 2), this candidate gene was utilized as the starting point for the development of an improved marker for CBB resistance. The other two candidate genes were classified as R-genes, each with leucine rich repeats and a nucleotide binding site. The two R-genes of interest have sequences that are very conserved amongst other known R-genes in plants (Ellis *et al.,* 2000; Meyers *et al.,* 1999). This typical R-gene structure, including several conserved motifs, makes genes 231733-8-004 and 231733-8-005, found near SU91 (Figure 3.1) logical candidates for CBB resistance genes in OAC Rex (Rivkin *et al.,* 1999). The first R-gene, gene 231733-8-004 is 340 kb from the SU91 marker. The second R-gene, gene 231733-8-005, is located 336 kb from the SU91 marker.

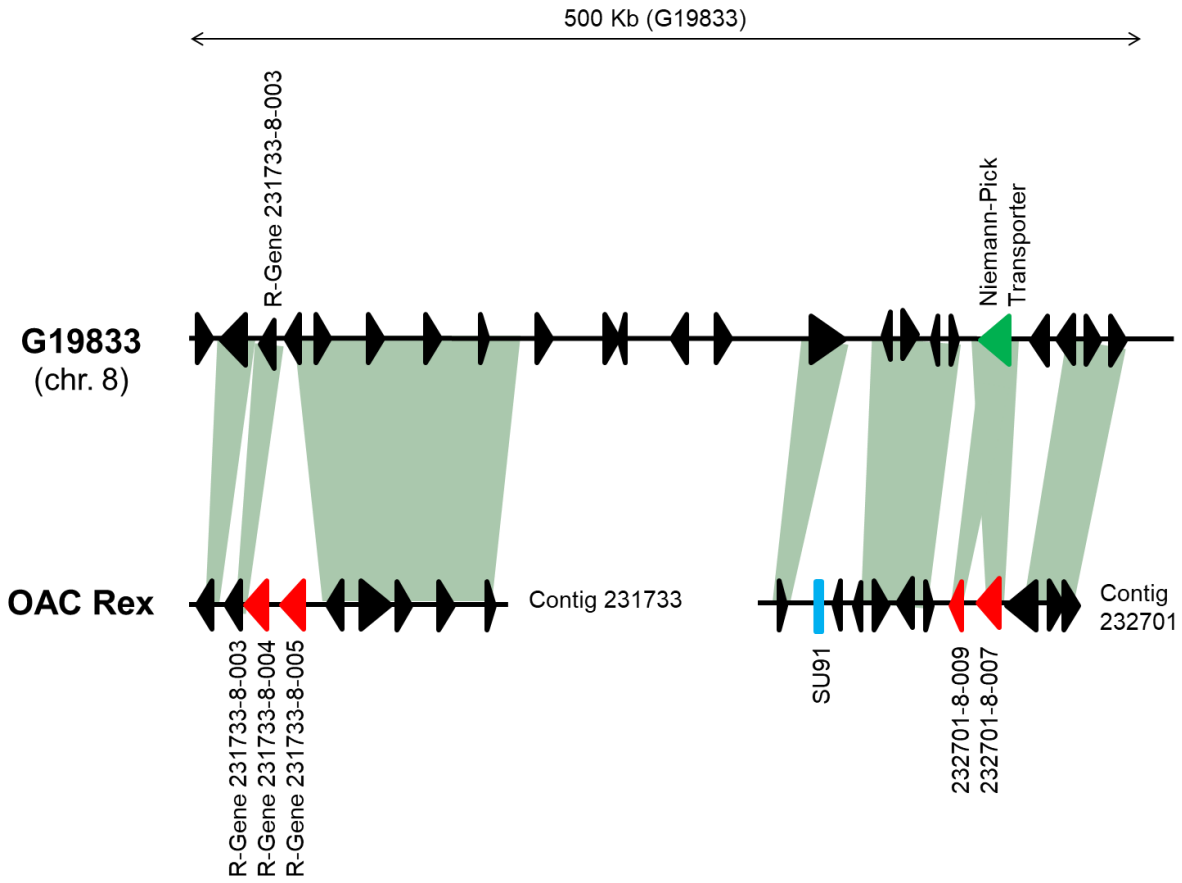


Figure 3.1- A diagram illustrating the location of novel genes present in CBB resistant OAC Rex (red), in comparison to CBB susceptible G19833. Regions compared are located at the end of chromosome eight in both cultivars. The major molecular marker SU91 is also highlighted to show proximity to the candidate genes identified (modified from Perry *et al.*, 2013).

Resistance (R) genes are a highly effective mechanism in a plant's defence against pathogenic attack from various bacteria, viruses and fungi (Liu *et al.,* 2007). Each R-gene specifically recognizes a single pathogen and initiates a cascade of events that leads to a localized plant defence against the pathogen (Liu *et al.,* 2007). This immediate, site specific response allows for the limitation of the disease progression (Ellis *et al.,* 2000). In order to maintain a defence against the multitude of pathogens that can affect plants, plant species maintain many pathogen specific R-gene copies, each with individualized compatibility to a single pathogen (Ellis *et al.,* 2000). This leads to a large portion of the genome of some plant species being composed of R-gene copies (Ellis *et al.,* 2000). These copies are often clustered within the genome (Ellis *et al.,* 2000). This organization facilitates the evolution of pathogen-specific copies of R-genes as the close proximity supports gene duplication and recombination that ultimately leads to polymorphic changes in the gene copies (Ellis *et al.,* 2000).

R-genes have a number of characteristics that identify them, and that allow for the recognition of pathogens and the initiation of a defence response (Martin, 1999). Nucleotide binding sites are a crucial characteristic of R-genes that allow for the initiation of resistance responses (Meyers *et al.*, 1999; Martin, 1999). Other characteristics that are typical of a R-gene include leucine-rich repeats, leucine zippers, transmembrane motifs and recognition sites for corresponding pathogen derived avirulence proteins (Martin, 1999). Leucine-rich repeats enable protein to protein interactions that allow for the recognition of avirulence proteins (Martin, 1999). Leucine zippers are thought to play a role in inducing transcription of downstream genes involved in plant resistance (Takken and Joosten, 2000). Transmembrane motifs result in resistance proteins that are located within cellular membranes and can allow for the detection of

secreted effector molecules in the extracellular space as well as within the cytoplasmic space (Martin, 1999; Takken and Joosten, 2000).

The presence or absence of these various features in proteins encoded by R-genes forms the basis for the definition of five classes of R-genes (Martin, 1999). The R-genes detected in close proximity to the SU91 marker both contain a nucleotide binding site (NBS) and leucine-rich repeats (LRR) (Perry *et al.,* 2013) making them a member of the LLR-NBS class of R genes (Martin, 1999; Ellis *et al.,* 2000). Gene 231733-8-004 also contains a coiled coil motif in addition to the NBS and LRR which is found in several subclasses of LLR-NBS R genes. These motifs are the basis of the homologies between the candidate genes 231733-8-004 and 231733-8-005, and resistance proteins found in *Medicago truncatula* (Perry *et al.,* 2013).

Based on this evidence it was hypothesized that the R genes located in chromosome 8, close to marker SU91, are involved in the CBB resistance in OAC Rex. The approach was to compare their sequences with R-gene sequences found in susceptible common bean lines. The hypothesis that was tested in this experiment was that the R-genes (231733-8-004 and 231733-8-005) located at the end of chromosome 8 in OAC Rex, contain polymorphic regions when compared to susceptible common bean lines G19833 and OAC Seaforth.

3.3 Materials and Methods

3.3.1 In silico comparison of R-genes

The sequences found within the OAC Rex genomic sequence, for both R-gene 231733-8-004 and R-gene 231733-8-005, were compared to the available genome sequences for common bean lines G19833 and BAT 93 (GenBank 864298 and 2828258 respectively). Lines G19833 and BAT 93 were used since they are the two available and published genome sequences for

common bean (Schmutz *et al.*, 2014; Vlasova *et al.*, 2016). The sequences used were all obtained from the National Center for Biotechnology Information website, and compared within the CLC Genomics Workbench. The gene sequences from OAC Rex for R-genes 231733-8-004 and 231733-8-005 were compared against the entire genome for both BAT 93 and G19833. All sequences with 90% homology or higher were further investigated.

In addition to sequence alignments, the pre-existing SNP data available for OAC Rex (Diaz Castro, 2015) was entered into Graphical Genotypes software (GGT2) in order to determine if any of the SNP markers lay close enough to the R-genes to suggest the origin of the R-genes within the OAC Rex pedigree. The SNP data for 72 RILs from an OAC Rex/OAC Seaforth population was entered, alongside the SNP data for the parents OAC Rex and OAC Seaforth. The region of the two R-genes in question was evaluated for recombination events near the physical location of the two genes within the population. The physical locations of the genes were determined in reference to their physical locations within the OAC Rex genomic sequence.

3.3.2 Sequencing R-gene 231733-8-004

The discovery of a 20 bp deletion in the sequence for BAT 93 (Figure 3.2) led to the re-sequencing of the region surrounding the deletion. The deletion found in the sequence alignment was present in the fourth of six exons; therefore, primers were designed to amplify the entire fourth exon. Primers 5'-AACCCAAGTTGAGCTTCCAGA-3' and 5'-AATTTTGTCCTCGGATCTCCTG-3' were designed using the primer design tool in CLC Genomics Workbench and anchored within the exon. They were utilized to amplify a predicted amplicon size of 163 bp with genomic DNA from OAC Rex, OAC Seaforth, and BAT 93.

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Bat93       TGATATACAGATGATGAGACCACAAGTGAATTTAAAGCTAGAATATTTGGATTTGAAAAA   5313
Rex         TGATATACAGATGATGAGACCACAAGTGAATTTAAAGCTAGAATATTTGGATTTGAAAAA   5699

Bat93       TCTACCTCAAATGACTAATATTTGGGAGGCTACCAAGAACACATTTACCCTCCAACATCT   5373
Rex         TCTACCTCAAATGACTAATATTTGGGAGGCTACCAAGAACACATTTACCCTCCAACATCT   5759

Bat93       CAAGTCAATACAAATAATGGGATGTGAAAAATTGGAAGTAATATTTTCTCAGTCTGTTTT   5433
Rex         CAAGTCAATACAAATAATGGGATGTGAAAAATTGGAAGTAATATTTTCTCAGTCTGTTTT   5819

Bat93       GAGATGCCTACCAGAATTAAATAGCCTAAAGGtaagagaatgcaaggaattaagacaaat   5493
Rex         GAGATGCCTACCAGAATTAAATAGCCTAAAGGTAAGAGAATGCAAGGAATTAAGACAAAT   5879

Bat93       tattgaagaggatttggaggataaaaaattgtccaatctccttctccacaaccatgctt   5553
Rex         TATTGAAGAGGATTTGGAGGATAAAAAATTGTCCAATCTCCTTTCTCCACAACCATGCTT   5939

Bat93       ccccaaacttgtgatattgattgttgaacaatgccacaagttgaaatatttcacctctct   5613
Rex         CCCCAAACTTGTGATATTGATTGTTGAACAATGCCACAAGTTGAAATATTTCACCTCTCT   5999

Bat93       atctgcatctaatgattttcccaacttggagattctagccattaatggagccactgaact   5673
Rex         ATTTGCATCTAATGATTTTCCCAACTTGGAGATTCTAGCCATTAATGGAGCCACTGAACT   6059

Bat93       actacagtttaatgagacaggaaaaactcaagttgagcttccagaactaaaatttttaat   5733
Rex         ACTACAGTTTAATGAGACAGGAAAAACTCAAGTTGAGCTTCCAGAACTAAAATTTTTAAT   6119

Bat93       atttatacatctttcaaagttttgccaagagactcaatttttaaatgtgaagcttcgcat   5793
Rex         ATTTATACATCTTTCAAAGTTTTGCCAAGAGACTCAATTTTTAAATGTGAAGCTTCGCAT   6179

Bat93       tgttcta-------------------------aacaactactcctcaggagatccgagg   5827
Rex         TGTTCTCAATTGTCCAAAACTCTCTTTGACTTCAACAACTACTCCTCAGGAGATCCGAGG   6239

Bat93       acaaaatttttgataatctagGTAAATAAAATATTATGCATGGATATTTTGCTGTAAATAT   5887
Rex         ACAAAATTTTGATAATCTAGgtaaataaaatattatgcatggatattttgctgtaaatat   6299
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Figure 3.2- A genomic alignment based on available sequence data for R-gene 231733-8-004 in OAC Rex and BAT 93. The primer binding locations used for the screening of several lines are highlighted in yellow, and the 20 bp deletion in BAT 93 is shown.

The plants used for DNA extraction were grown in a growth room with 16 hour days (25 ºC) and 8 hour nights (20 ºC). Leaves were flash frozen in liquid nitrogen after the expansion of the first trifoliate and stored at -80 ºC. The frozen leaves were ground with a pestle and mortar, and DNA was extracted utilizing the Sigma-Aldrich GenElute Genomic DNA Miniprep Kit. The protocol for the kit was followed and, in summary: plant tissue was lysed, impurities were removed and the DNA was bound to a silica column. The column was washed with the included wash buffer and the DNA was eluted from the column with nuclease free water.

The eluted DNA was used as the template for PCR to amplify the region surrounding the predicted deletion in exon 4 of 231733-8-004. For each reaction 0.5 µL of template (50-100 ng), 0.5 µL of dNTP's (2 mM), 0.2 µL of Taq polymerase (1 unit), 0.5 µL of the primers (3 µg/µL), 17.8 µL of PCR grade water, 2.5 µL of PCR buffer and 3 µL of $MgCl_2$ (25 mM) were combined and dispersed into PCR tubes. An annealing temperature of 56 ℃ was utilized for each PCR reaction.

In order to sequence the amplicons, primers 5'-ACAGATGATGAGACCACAAGTGA-3'and 5'-AATTTTGTCCTCGGATCTCCTG-3' were utilized to amplify a region of 602 bp that encompassed the previously studied 163 bp amplicon. The PCR products were inserted into TOPO-TA plasmids and transformed into competent *E. coli* cells. The protocol included in the TOPO-TA Cloning kit by Thermo Fisher Scientific was used to clone the amplicons and the One Shot Chemical Transformation Protocol was followed in order to insert the desired plasmid into the bacterial cells. The transformed bacterial cells were grown in liquid S.O.C. media overnight at 37 °C, then transferred to solid LB media with kanamycin (50 µg/mL) incorporated to select transformed bacterial cells only. After 24 hours at 37°C, 6 colonies were selected for each bean line, and grown in individual liquid culture (LB media) for plasmid extraction. The Purelink

Quick Plasmid Miniprep Kit (2011) was used to purify the plasmid DNA with centrifugation according to the manufacturer's instructions. In summary, the bacterial cells were isolated, lysed to release the plasmids, the plasmid DNA was bound to a silica column and the column was washed with the included wash buffer, and eluted with nuclease free water.

The extracted plasmid DNA was used as a template for PCR with primers 5'-ACAGATGATGAGACCACAAGTGA-3' and 5'-AATTTTGTCCTCGGATCTCCTG-3' to confirm the insert size and one successful transformation was selected for each of the three bean lines for sequencing. The plasmid DNA was sent to the Genomics Facility at the University of Guelph's Advanced Analysis Centre for Sanger sequencing of the insert. The resulting sequence data was aligned using the CLC Genomics workbench to determine if the deletion in BAT 93 was present.

3.4 Results

3.4.1 In silico comparison of R-genes

When the sequences for R-genes 231733-8-004 and 231733-8-005 were compared within the genomes of G19833 and BAT 93, there were two distinctly different results for each gene. The alignment of the OAC Rex sequence with the CBB susceptible, G19833 genome, confirmed previous results, which indicated that neither R-gene 231733-8-004 nor R-gene 231733-8-005 were present in G19833 (Perry *et al*, 2013). When the OAC Rex genome was compared to BAT 93, a 20 bp deletion was present in gene 231733-8-004 in the sequence for BAT 93 (Figure 3.2) and the sequences for 231733-8-005 were identical. The presence of the deletion in 231733-8-004 led to the re-sequencing of a portion of R-gene 231733-8-004 within OAC Rex, BAT 93 and OAC Seaforth. The lack of exploitable differences between the OAC Rex sequence and that of

BAT 93 led to the conclusion that R-gene 231733-8-005 was not a suitable candidate for further screening.

For R-gene 231733-8-004, the deletion within BAT 93 led to further analysis of the gene through re-sequencing of R-gene 231733-8-004 within several common bean lines. Reanalysis of the SNP data available for OAC Rex and a population of 72 RILs from the OAC Rex/OAC Seaforth cross (Diaz Castro, 2015) was also completed to determine if pre-existing SNP markers are in close enough in proximity to lend evidence to the origin of R-gene 231733-8-004 within the OAC Rex pedigree. Figure 3.3 shows that the two R-genes are bordered by two SNP markers SS715646763 and SS715647398, which are 470 kb apart. As seen in Figure 3.3, there are recombination events present between these two SNP markers for some of the population, however the distance between these two markers is too great to be able to know if it lies in the region near R-gene231733-8-004.

Although the location of the recombination events between SNPs SS715646763 and SS715647398 cannot be known based on the information gained from the Graphical Genotypes Software (GGT2) study, the parental source of the genetic material in the region can be studied. By studying adjacent SNPs, the parental source of the genetic material can be determined based on the allele present for each SNP. For 18% of the population, the genetic material in the region of R-genes 231733-8-004 and 23733-8-005 is from a parental line that is inconsistent with the CBB susceptibility of the RIL. For five individuals that are susceptible to CBB, the 470 kb surrounding R genes 231733-8-004 and 231733-8-005 are from parent OAC Rex, a resistant bean cultivar. For eight resistant individuals the region surrounding R genes 231733-8-004 and 231733-8-005 has been inherited from susceptible parent OAC Seaforth. These inconsistencies in genetic material origins can be seen in Figure 3.3 as blue bars with no recombination events in

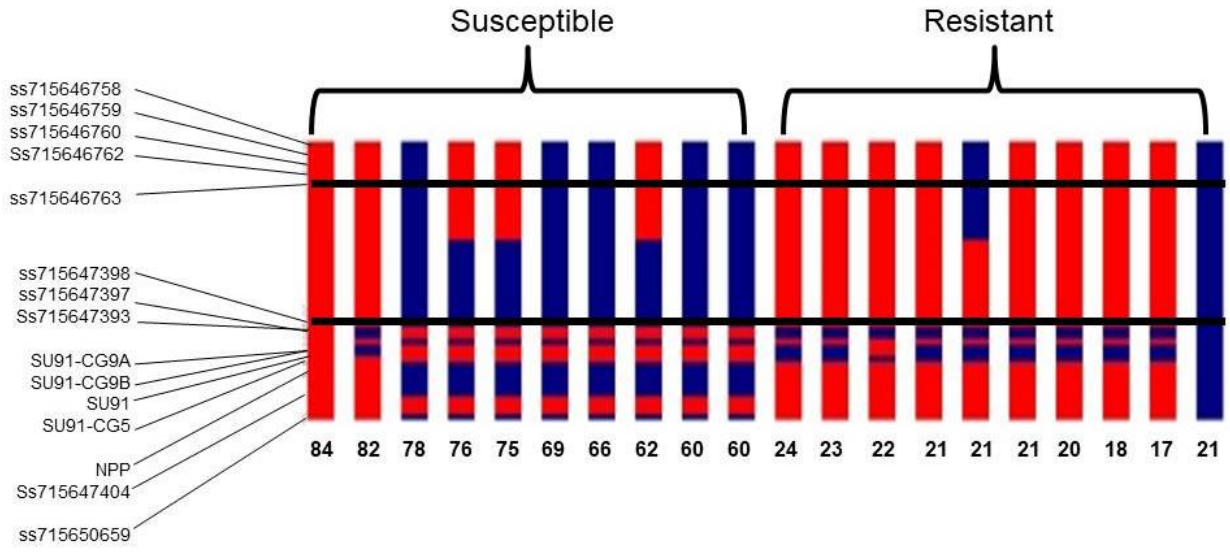

Figure 3.3- A diagram illustrating points of recombination within RILs obtained from the OAC Rex/OAC Seaforth cross, where red represents alleles from OAC Seaforth and blue represents genetic material from OAC Rex. The R-genes identified as candidate CBB resistance genes are both located between the black bars, and within the 470 kb region between SNPs ss715647398 and ss715646763. The individuals shown represent the most susceptible and most resistant RILs of the OAC Rex/OAC Seaforth population.

individuals that are susceptible, and red bars with no recombination events in individuals that are resistant. These inconsistencies are based on a 470 kb region between SNPs SS715646763 and SS715647398 that contains no observed recombination events. What cannot be concluded is what percentage of the remaining 82% of the population also has inconsistent genotypes for their given phenotype (CBB score) in this region. Without additional SNPs in this region, the rate of recombination surrounding the R-genes cannot be concluded.

3.4.2 Sequencing R-gene 231733-8-004

The initial analysis of exon 4 of R gene 231733-8-004 in BAT 93, OAC Rex, and OAC Seaforth, showed that the BAT 93 sequence contained a 20 bp deletion. However, PCR amplification of fragments encompassing this region from genomic DNA of these three lines resulted in PCR products that were all 163 bp in size (Figure 3.4). This initial screen suggested that the deletion within the published BAT 93 sequence (Vlasova *et al.*, 2016) could potentially be due to sequencing error instead of a true polymorphism within R-gene 231733-8-004. Therefore, the amplicons of the fourth exon of R gene 231733-8-004 in OAC Rex, BAT 93 and Seaforth were re-sequenced and compared. The alignment of the sequences obtained for the three lines showed that all of the sequences were very similar and do not include a 20 bp deletion in BAT 93 (Figure 3.5).

A further comparison of the sequences for OAC Rex and OAC Seaforth identified two single nucleotide polymorphisms (SNPs) between OAC Rex and OAC Seaforth, spaced 84 bp apart within the fourth exon of R-gene 231733-8-004 (Figure 3.5). SNP 1, located 6002 bp into R-gene 231733-8-004, would change an adenine in OAC Rex to a guanine in OAC Seaforth and BAT 93. SNP 2, located 6087 bp into R-gene 231733-8-004, would change a guanine in OAC

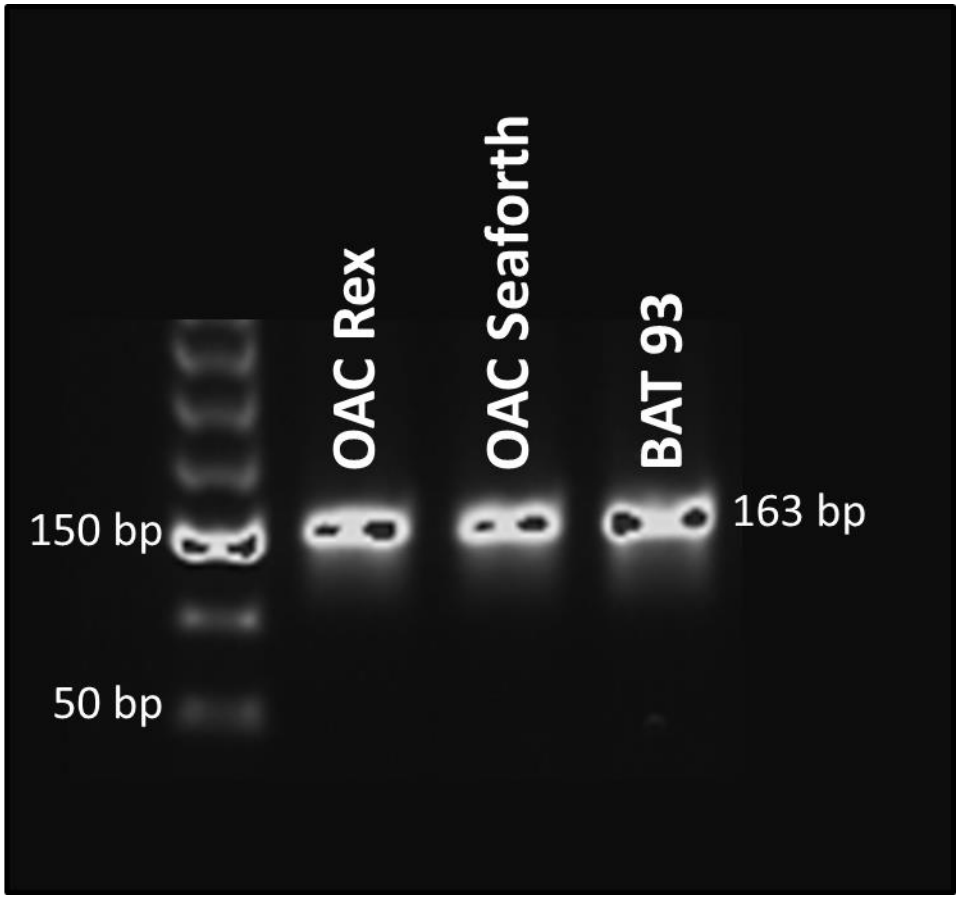

Figure 3.4- A 1% agarose gel image of exon 4 amplicons from R-gene 231733-8-004 in OAC Rex (resistant), OAC Seaforth (susceptible), and BAT 93 (resistant). All of the bands were 163 bp in size.

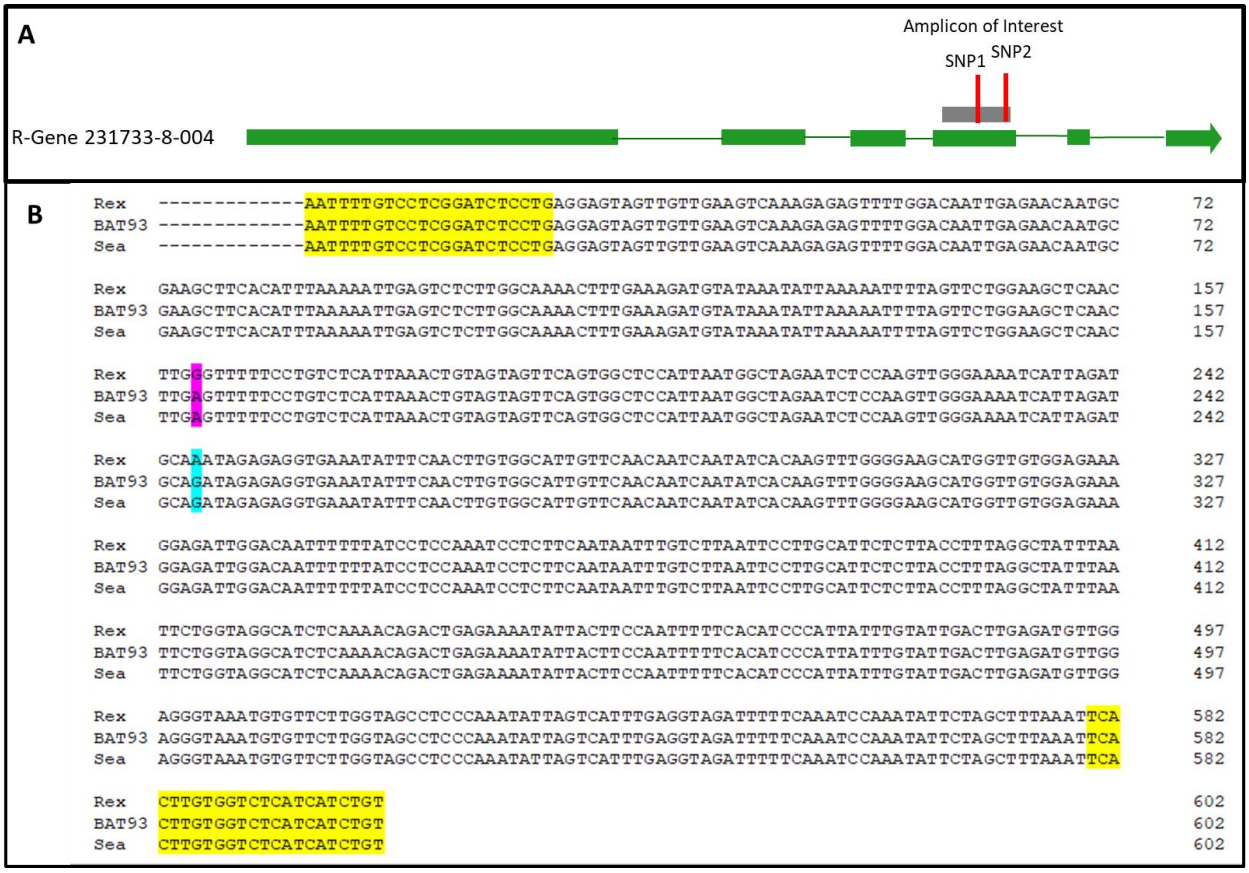

Figure 3.5- R-gene 231733-8-004 alignment in OAC Rex (R), BAT 93 (R) and OAC Seaforth (S). A) R-gene 231733-8-004 showing the DNA sequence amplified from exon 4 (highlighted in grey) with the locations of the two SNPs labelled. The comparison illustrated is based on the sequence for OAC Rex. B) An alignment of sequences obtained by amplifying exon 4 of R-gene 231733-8-004. The primer binding sites are highlighted in yellow, SNP 1 is highlighted in purple and SNP 2 is highlighted in blue.

A)

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Rex   MDTIFDFVADILKDLVRGAMKELHYSFCFNDFVKELEKEENKFIETRKSVEDRVTHARKQ      60
Rex   TLKTAEVMDKWVENVKIDAEEVNRLLKEAKTKKSCCLGYCPNWIWRYRLGKNLENKKMDL     120
Rex   QNIIQEGKQYIQLERTASIPSSTLDILTEKCMNFKSRKFASDKLMEALKDDGVAMIGLYG     180
Rex   MGGCGKTTLAMEIKKMAKDEHLFDKIIFVPVSSIVEVPRIQEKIASSLEYKFPENEEMER     240
Rex   AQRLCMRLTQEKNILMIIDDVWEKLDFGRIGIPSFEHHKGCKILITTRSEEVCTLMDCQR     300
Rex   KIYLPILNDEEAWALFQNKAFISEDTHETIKHLAKSISNECKGLPVAIAAVASSLKGKVE     360
Rex   VVWSVALNRLKSSKPINFGKGLSDPFKCLQLSYDNLDTEEAKSLFLLCSVFPEDYEIPVE     420
Rex   CLIRCAIGLGMAGEVNSYEEARSEVMTTKIKLVSSCLLLDTNYKSVKMHDLVRDVAQWIA     480
Rex   KNENNIIKCEMENDVTSEQSSIRYLWCVKFPDDMDCSNLEFLCIQAKFEVSDGIFETMEK     540
Rex   LRVLIIIGPKKYDRSSLSTRSFKTLTNLRCIIFQYWKLSDISFVRYMKKLQSFSLHGCSW     600
Rex   PPFIDLQTDIAFTQLKNLKLLEFNGCDIEVKNLEEIKSIPLLEELYIIQTESKYNDRKLI     660
Rex   ECFNLFSFVQTLQRYGIVLGQKFSPLYIPSEFFSCERTLLVNYFNISNEVIKGLAKEANE     720
Rex   LFVANIEGGVKNMMPDIFEIEGGMNELNGLGIRNCEEIEYLVDTGNDLSKVRNLFSKLHF     780
Rex   LRIYNMKHLRALWHGCVPGNGSFEKLEKLYLRDCPKLKSLFTYVIARGDYAVGHSLKSKI     840
Rex   FQNLQILMIDNCGELNHVFSSSTIGDLSQLKKLSIYNCDMLEQIIGDDVHEKKERDEIIE     900
Rex   EDKHQHFESNHFKTTSIPSLTGVNRNTGSITLFNLVVLQIWSCPMLGSLFEISVANTLTS     960
Rex   LRNLTIQHFHGLKDIITQAKVKRNKKENMVEDGNDFQSDFSMFLNLEALYIEECDLLEYI    1020
Rex   FLESFVEDMVKLNDTRNKETSNSKDNTCHQHKKNTQIELPTLQELELDCIPNYIIPYSYY    1080
Rex   VRCPSLETLSLGVGRYVEFFTVNCSSNTSEAKHSDYIKIKISNSDSLHLFESSQYLAEQP    1140
Rex   QGLNFLIMCNIRVIHLKGFDNAKYLFDLSIASLLMLQNLCIENCPRLQHIIDIGDEYESK    1200
Rex   NWDVIFPKLKYLLVYNCDQLEYMIGQYPIDDKNDKKIHLHFPTLEELYLRNLPNFISICA    1260
Rex   TNSLSMAWPSLKKFECSGCSQLVNISTSHKDDIQMMRPQVNLKLEYLDLKNLPQMTNIWE    1320
Rex   ATKNTFTLQHLKSIQIMGCEKLEVIFSQSVLRCLPELNSLKVRECKELRQIIEEDLEDKK    1380
Rex   LSNLLSPQPCFPKLVILIVEQCHKLKYFTSLFASNDFPNLEILAINGATELLQFNETGKT    1440
Rex   QVELPELKFLIFIHLSKFCQETQFLNVKLRIVLNCPKLSLTSTTTPQEIRGQNFDNLGLK    1500
Rex   NSRTHWWEFRSMIDKIKESDKVSTSNNELPSSKLEEDYMSENTSPLQMNQEDPPISKNKP    1560
Rex   CSSQVSDNNQSTEEIGRQKIEDGPPSEGVVTKTLSVGIDNISLDRGVTIHKSSGASILPQ    1620
Rex   DFQWMYYMDGVK                                                    1632
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B)

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Rex         MDLQNIIQEGRQYIQLERTASIPSNTLDILTEKCMNFDSRKIAYDQLVEALKDNGIAMIG     60
Rex         LYGMGGCGKTTLAMEIKKMAEDEHLFEKICFVSVSSIVEVPRIQEKIGSSLEYKFLENEE    120
Rex         MERAQRLCIRLIQEKNILMILDDVWEKLDF-GRIGIPXQYIQLERTASIPSNTLDILTEK    179
Rex         CMNFDSRKIAYDQLVEALKDNGIAMIGLYGMGGCGKTTLAMEIKKMAEDEHLFEKICFVS    239
Rex         VSSIVEVPRIQEKIGSSLEYKFLENEEMERAQRLCIRLIQEKNILMILDDVWEKLDFGRI    299
Rex         GIPSSKHHKGCKILTTTRSEEVCTSMDCQRNIYLPILTDEEAWILFQKKAVIYENTPETI    359
Rex         KHLAKSISNECKGLPVAIATVASSLKGKKDVIWSVALNRLRSSKPINIGKGLQDPYKCLQ    419
Rex         LSYDNLDSEEAKSLFLLCSVFPEDYEIPVECLIRYAIGLGVVGEVNSYENARSEVMAAKI    479
Rex         KLVSCCLILDADYESVKMHDLVRDVAHWIAKNDNNIITCETEKDVTLEQSPIRYLWCVKF    539
Rex         PDDMDCSNLEFLCIKAKLEVSDEIFERMGKLRVLIINNPTTHVRLLLSTRSFKTLTNLRC    599
Rex         LVLQYCKLSDISFVRDMKKLQSLSLHDCSWPPLTDMQTDVAFTQLKNLKVLEFNRCDIEI    659
Rex         KNLEEIKSIPLLEELYIIQTGSKYNDRKFIECFNLFSFVQTLQRCGIVLGQDFSPIDIPS    719
Rex         KNFSCQRTIRINCFNISNEGIKGLTKEANELFVGNIEGGVKNMMPGIFEVEGGMNELNEL    779
Rex         RIMSSEEIEYLVETSNDPRKVGNFFSKLQYLKIEKMKHLRALWHGCVLGNESFEKLEKLY    839
Rex         LTNCPELTSLFTYVITRGDYAAGHFLQSKIFQNLKEVEIEDCGKLSHVFSASIIGDLSQL    899
Rex         KKLSIKRCDMLEEIIGDVVDEKEERDEIIEESNHFKTTSIPSPTTLNRNTGSLTLVNLVI    959
Rex         LEIFACLVLDSLFEISVAKTLTSLRYLTIEDCDGLKHIVTQARLKRNEKENVVEDGHDFQ   1019
Rex         THLSMFPTLEKLRIERCELLQHIFLESFVGELPALKELRLDYLPNYIIPYSYNVRCPSLE   1079
Rex         TLSLSVGRYVEFSTVNSSSNASEMRHSDYIKIKISSLDSLHLFESSQYLAEQPQGLNFLI   1139
Rex         KHNISEINLNGFDNAKYLFDLSVASLLKLQILHIKNCSGLQHIIDIGEEYESKNWDAIFP   1199
Rex         NLKSLSVFCCKKLKYMIGQYPLDNKNYKEIHLHFPTLEKLYLDYLPNFISICATDSPSMA   1259
Rex         WPSLKKLDYSGCSQLVNINTSGKDAIWVQNHFLTLQTLRMVNAKVEAIFFLNGHQTMGQQ   1319
Rex         VNLKLEKMHLQNLPKMDNIWEATKNTFTLKHLQSLEIIGCEKLEVIFPQSVLRCLPELNT   1379
Rex         LEVRECKELRQIIEEDLEDKKLSNPLSPQPCFPKLTTLIVEQCHKLKYLTSVSASNDFPN   1439
Rex         LEFLVINGATELLQFNETGKTQVELPELKLLIFIHLSKFRQETQFLNVEHRIVRNCPKLS   1499
Rex         FTSTTTLDEIKQKFGFLGFTKNFIIDRWEFGWLINRIKRSDGVSTNDNELPSSQNIEEIG   1559
Rex         RQKIEDGPPSEGVVTKTLSTSVDNISLDRGVTIHKSSGASILPQDSQIIKQDDKMNEDEE   1619
Rex         GIILFL                                                         1625
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Figure 3.6- Putative amino acid sequences for R-genes 231733-8-004 and 231733-8-005 in OAC Rex. A) Amino acid sequence for R-gene 231733-8-004 in OAC Rex, with the nucleotide binding ARC domain highlighted in pink, leucine rich repeats highlighted in green and the amino acid altered by a single nucleotide polymorphism highlighted in red. B) Amino acid sequence for R-gene 231733-8-005 in OAC Rex with the nucleotide binding ARC domain (pink), and leucine rich repeats (green) highlighted.

Rex to an adenine in OAC Seaforth and BAT 93 (Figure 3.5). SNP 1 results in a predicted amino acid change from a phenylalanine in OAC Rex to a serine in OAC Seaforth and BAT 93 (Figure 3.6). SNP 2 does not result in a predicted amino acid change between OAC Rex and OAC Seaforth and BAT93.

3.5 Discussion and Implications

The use of sequencing data allowed susceptible and resistant bean lines to be compared to examine possible relationships between CBB susceptibility/resistance and the presence of R-genes 231733-8-004 and 231733-8-005. Originally, a comparison between OAC Rex, a resistant white bean line, and G19833, a susceptible Andean bean line, revealed that R-genes 231733-8-004 and 231733-8-005 are absent from G19833 but present in OAC Rex (Perry *et al.,* 2013). This led to a second comparison against the CBB resistant line BAT 93, which was also found to contain R-genes 231733-8-004 and 231733-8-005. This result was consistent with the proposal that R-genes 231733-8-004 and 231733-8-005 are potentially responsible for CBB resistance in white beans.

However, the examination in the current study of the susceptible OAC Seaforth variety that showed that it also contained R-gene 231733-8-004, was inconsistent with the original proposal for the role of this gene in CBB resistance in OAC Rex and in particular with the possibility that it was derived from *P. acutifolius*. After comparing sequence data for the region, it was found that the sequences for R-gene 231733-8-005 are identical in BAT 93 and OAC Rex. This led to the conclusion that R-gene 231733-8-005 is unlikely to be a CBB resistance gene introgressed into common bean variety OAC Rex from a *P. acutifolius* source since the BAT93

line is of Mesoamerican origin (*P. vulgaris*). The R-gene 231733-8-005 was not investigated further in this project.

The single nucleotide polymorphisms found between OAC Rex and OAC Seaforth within R-gene 231733-8-004 are interesting for roles they may play in CBB resistance. SNP 1 in R-gene 231733-8-004 results in a predicted amino acid change from a phenylalanine in OAC Rex to a serine in OAC Seaforth. This amino acid change could potentially alter the protein structure of the resulting resistance protein, however further investigation would be required in order to verify the alteration. The second SNP at 6087 bp does not result in an amino acid change; therefore there is no predicted functional change due to this polymorphism. The amino acid sequence for R-gene 231733-8-004 in OAC Rex can be found in Figure 3.6, with the altered amino acid highlighted in red. Ultimately, the first of the two SNPs shows the potential for R-gene 231733-8-004 to remain a candidate resistance gene in OAC Rex.

The identical sequence between OAC Rex and BAT 93 does not support the theory that R-gene 231733-8-004 could be the result of introgression of CBB resistance from *P. acutifolius* to *P. vulgaris*. In addition, OAC Seaforth does not have an introgression event in its pedigree yet has R-gene 231733-8-004 present with nearly identical homology to OAC Rex. Thus, R-gene 231733-8-004 must originate from the *P. vulgaris* genome and may be a common feature of Mesoamerican germplasm. This does not exclude it as a candidate resistance gene; however it does exclude it as a candidate gene from an introgression event with *P. acutifolius*.

The study conducted with the GGT2 software confirms that the region surrounding R-genes 231733-8-004 and 231733-8-005 in OAC Rx x Seaforth RILS suggests that it is not inherited in a manner consistent with what would be expected for a resistance locus inherited from OAC Rex and indicates that these R-genes (231733-8-004 and 231733-8-005) are not likely

responsible for CBB resistance. However, it must also be considered that the region spanning the two flanking SNPs is 470 kb, which is large and could be mapped more precisely to identify with additional SNPs.

Although the current results appear to rule out the possibility that R-genes 231733-8-004 and 231733-8-005 are the sources of CBB resistance, additional experiments could be conducted to further investigate their potential roles in CBB resistance, including knock-down expression studies. However this type of experiment is very difficult in common bean. In addition, their roles in disease resistance could be further investigated by screening many more susceptible and resistant individuals for their presence and absence. It would also be interesting to examine possible interactions between these R-genes and other pathogens.

Chapter 4: Expression analysis of Niemann-Pick like gene in a *P. vulgaris* population under active infection with *Xanthomonas axonopodis* pv. *phaseoli* (Smith) and *Xanthomonas fuscans* subsp. *fuscans* Schadd

4.1 Abstract

In order to test the possibility that the Niemann-Pick gene is involved in CBB resistance in common bean, RNA was extracted at several time points after inoculation with *X. axonopodis* and *X. fuscans* in order to measure expression levels in leaf tissue. The leaves were collected 0, 1, 2, 4, 8, 24, and 48 hours after infection, flash frozen and the RNA was extracted. Primers were tested in order to find optimal housekeeping genes to be used in the creation of a standard curve. Ultimately the endogenous genes, Actin-11 and Insulin Degrading Enzyme (IDE), were found to be the best for use in creating a standard curve of expression in order to determine relative expression levels of the Niemann-Pick gene. However, after several unsuccessful attempts to troubleshoot the quantitative PCR assays, it was determined that the protocol prior to tissue sampling must be altered in order for a successful expression study.

4.2 Introduction

Expression analysis can be used to validate the associations between genetic polymorphisms and phenotypic variation. The stages of candidate gene validation include: identification of candidate genes by functional analysis or positional association, characterization of polymorphic regions among alleles that can be exploited for marker development, and statistical correlation between the candidate gene and the trait of interest (linkage) (Pflieger *et al.,* 2001). In order to validate a candidate gene the role of the gene must be studied by genetic transformation, complementation, or a physiological analysis (Pflieger *et al.,* 2001). In the case of common bean, transformation is not a well-developed option as many market classes do not

have transformation protocols with acceptable rates of success. Therefore, to validate candidate genes for common bacterial blight (CBB) resistance in common beans, the best option is to conduct a physiological analysis that can provide evidence to support an argument for or against a candidate gene (Pflieger *et al.*, 2001).

Studies of the expression levels of candidate genes during disease challenge of resistant and susceptible bean germplasm can provide strong evidence, for or against, their involvement in resistance. Real-time polymerase chain reaction (RT-PCR) analyses provide measures of relative expression for genes of interest in comparison to constitutively expressed genes, referred to as housekeeping genes (Borges *et al.*, 2012). To ensure that the measurements of expression level are conducted with the lowest rates of error, reference housekeeping genes must be best suited for the circumstances of the experiment (Borges *et al.*, 2012). Borges *et al.* (2012) has compared the efficiency of reference genes under both biotic and abiotic stresses, and found that the Actin-11 (*Act11*) and the Insulin degrading enzyme (*IDE*) genes are best suited for use in common bean studies with biotic stressors.

In the current study, the expression of a Niemann-Pick like gene was investigated in infected tissue to support the use a molecular marker based in the Niemann-Pick gene for selecting CBB resistant genotypes. The NPP marker has been shown to be consistently associated with CBB resistance in two populations, as well as in a variety of bean cultivars. Although the NPP marker appears to be valid, the lack of knowledge about the function of Niemann-Pick genes in plants, in general, and in disease resistance, indicates a need for more information on Niemann-Pick expression in beans. The Niemann-Pick like gene was identified as a candidate gene based on its positional association to the major resistance QTL on chromosome 8 and its homology to *P. acutifolius* ESTs. Within the Niemann-Pick like gene, a

421 bp insertion was identified in resistant lines, like OAC Rex, that was absent from susceptible lines like G19833 and OAC Seaforth. This polymorphism was consistently associated with CBB resistance in a RIL population from a biparental cross between OAC Rex and OAC Seaforth. The previous validation makes the Niemann-Pick like gene a candidate for physiological analysis in order to complete the marker validation process.

The hypothesis for this study was that, since the NPP marker is anchored in the Niemann-Pick gene and has been found to be closely linked to CBB resistance, the expression of the Niemann-Pick gene will be upregulated in resistant bean tissue shortly after inoculation with CBB causing bacteria (*X. axonopodis* pv. *phaseoli* and *X. fuscans* subsp. *fuscans*). The objective was to gain further evidence, for or against, the proposal that the altered Niemann-Pick gene in OAC Rex confers CBB resistance to the variety.

4.3 Materials and methods

4.3.1 Plant inoculation and tissue collection

The most resistant (RILs 31, 57, 81, 110 and 121) and the most susceptible (RILS 21, 33, 37, 120, and 138) individuals (5% either way) of the OAC Rex/OAC Seaforth population and the population parents were utilized to test whether the expression of the Niemann-Pick like gene is correlated with the levels of resistance within a plant. For each line that was tested, 24 plants were grown, in a randomized block design, to allow for seven time points of tissue collection, a mock inoculated control, and three biological replicates for each. The plants that were mock-inoculated were kept separate from the inoculated plants to prevent contamination. The plants were planted in one litre pots and grown under 16 hour/25 ℃ days and 8 hour/20 ℃ nights. The

seedlings were grown for 16 days before inoculation to allow the first unifoliate leaves to fully expand and first trifoliate leaves to partially expand.

Inoculum was created by combining both *X. axonopodis* pv. *phaseoli* and *X. fuscans* subsp. *fuscans* isolates. Specifically *X. fuscans* isolates 18-2 and 98-12 were combined with *X. axonopodis* isolate 118-5 (Xie *et al.,* unpublished). The three isolates were taken from frozen culture and used to inoculate liquid YS media, which was incubated, while shaking, at 37 ℃ for 24 hours. The liquid culture was used to streak solid YS plates in order to obtain single colonies. The plates were incubated at 37 ℃ for 48 hours. After 48 hours the single colonies were selected for typical colony appearance, and scraped off of the YS plates with a wire loop, then suspended in 5 mL of liquid YS media. Typical colony appearance is small, convex, shiny, yellow colonies with entire, smooth margins. The bacterial suspensions were used to create pour plates on solid YS media. For each pour plate 600 µL of bacterial suspension was aliquoted onto the plate and spread using a sterile, bent glass rod. Bacterial lawns were incubated at 37 ℃ for 48 hours. For each of the three isolates, 20 pour plates were created to ensure enough bacterial growth for inoculation.

To create the bacterial inoculum the bacterial lawns for each isolate were suspended in double distilled water using a bent glass rod to separate the bacteria from the growth medium. Each bacterial preparation was agitated to create single cell suspensions and adjusted to $1x10^8$ CFU/mL. Equal volumes of each bacterial suspension were combined to a total volume of 4 L. Approximately 12 plates of each isolate were used, although uneven bacterial growth meant that each isolate required a slightly different number of bacterial plates. The mixed inoculum was utilized to inoculate each plant using the multi-needle protocol (Haygood and Strider, 1982). A multi-needle device containing 36 needles, evenly placed into a 6x6 square and secured into a

foam base was used to wound the first two leaves of the plants. For the inoculated plants the needles pierced the leaves and were forced into a sponge saturated with the inoculum. A second sponge saturated with inoculum was utilized to apply pressure to the opposing side of the leaf after the wounds were made. Three plants of each line were inoculated with sterile double distilled water to act as a negative control. This mock inoculation occurred before the bacterial inoculum was created, and was done with the multi-needle protocol just as the bacterial inoculations were conducted.

The plants were placed in 100% humidity 48 hours prior to inoculation, and remained at 100% humidity in a mist chamber for 48 hours after inoculation to promote bacterial growth. Plants were maintained at 25 ℃ days and 20 ℃ nights throughout the process, including the 48 hours after inoculation. The chamber in which the plants were kept misted the plants from above every 15 minutes for duration of 30 seconds. The chamber was enclosed with plastic sheeting in order to ensure a consistent 100% humidity around the plants. Extra plants for each RIL planted, as well as the parental lines, were inoculated in order to ensure enough plant material for tissue collection. This alleviated the concern of premature leaf senescence of the first leaves before tissue samples were taken. Tissue collection was done at seven time points post inoculation: 0 hours, 1 hour, 2 hours, 4 hours, 8 hours, 24 hours, and 48 hours. The timing of tissue collection was chosen on the basis of previous studies that found that expression peaks of pathogenesis-related candidate genes occurred between 8 and 24 hours post inoculation (Shi *et al.*, 2011). For each of the seven time points, as well as the water inoculated group, three plants were sampled to act as three biological replicates. For each plant sampled, the two leaves that were inoculated were cut from the plant and immediately frozen in liquid nitrogen. Any leaf that was not completely frozen within five seconds from being cut from the plant was discarded. The

frozen leaves were stored in tin foil packets, and placed in a -80 ℃ freezer until RNA extractions could be performed.

4.3.2 RNA extraction

Leaf samples were taken from the -80 ℃ freezer and placed in liquid nitrogen to prevent any thawing of tissue. The samples were then processed following the Macherey-Nagel NucleoSpin® RNA Plant Kit protocol (2015). In summary, approximately 70 mg of frozen tissue was placed in a pre-frozen mortar along with a small amount of liquid nitrogen. As soon as the liquid nitrogen evaporated the leaf tissue was ground with the mortar and pestle until a fine powder was achieved. The powder was scooped into a frozen micro centrifuge tube using a frozen spatula, and the tube of powder was placed back into liquid nitrogen. The tubes of frozen leaf powder were brought into a flow hood, with all surfaces being treated with RNAseZap Solution (Ambion), before proceeding with the rest of the protocol. Frozen leaf tissue was only allowed to thaw once in contact with the lysis buffer containing β-mercaptoethanol to prevent RNA degradation. The cells were lysed, the samples were homogenized using the filter columns, and the RNA was bound to a silica column. The column was washed by guanidinium thiocyanate to desalt the column. The DNA on the column was degraded using an on column DNase treatment. The column was washed once with guanidine hydrochloride and twice with the wash buffer. Finally, the RNA was eluted into treated, RNase free, storage micro centrifuge tubes and placed back in the -80 ℃ freezer for storage.

The RNA samples were run through the QIAxpert System to determine the quality and concentration of each sample. RNA extractions with a low concentration or poor quality were redone. The concentrations of the samples were recorded for cDNA synthesis. cDNA was

synthesized using the iScript Reverse Transcription Supermix for RT-qPCR and associated protocol (2018). In summary, a master mix of reagents including reverse transcriptase was aliquoted into PCR reaction tubes, and the RNA for each sample was added to the respective tube. Each reaction was topped off with nuclease free water to a total volume of 20 µL. The reactions were placed in a thermo-cycler, and underwent three stages: priming for 5 minutes at 25 ℃, reverse transcription for 20 minutes at 46 ℃, and reverse transcription inactivation for 1 minute at 95 ℃. All synthesized cDNA was quantified with the QIAxpert System and the concentrations of cDNA were recorded for further conversions for expression analysis. The cDNA for each sample was stored at -20 ℃ until needed.

4.3.3 Expression analysis

In order to compare expression levels for the Niemann-Pick gene, real time PCR (RT-qPCR) was conducted using a 48 well plate. Each biological replicate by time point sample was given a numerical label and cDNA for each sample was loaded into the plate. Each well contained template cDNA (100 ng), forward and reverse primers (3 µg/µL), 10 µL iQ SYBR Green PCR Supermix, and nuclease free water to bring the total volume to 20 µL. Included on each plate were a negative control (no template), a mock-inoculated control (cDNA isolated from mock-inoculated leaf tissue), a positive control (genomic DNA), repeats of the samples containing the two housekeeping genes (Actin-11 and Insulin degrading enzyme), and a series of dilutions of the NPP marker amplicon for comparison. All samples had three biological replicates and three technical replicates. One bean line was completed on each 48 well plate, and in total 10 plates were run through. Plates were placed in the thermocycler and underwent the reaction outlined in Table 4.1.

Table 4.1- The reaction protocol designed for the RT-PCR reactions intended for the expression study planned. The temperatures indicated are based on the annealing temperature of the primers utilized.

Step	Temperature (°C)	Time (minutes)	Number of Cycles
Initial denaturation	95	15:00	1
Denaturing	95	0:10-0:15	
Annealing	56	0:15-0:30	40
Extension	72	1:00	
Melting Curve	55-95 (in 0.5 increments)	0:10-0:30	1

4.4 Results

4.4.1 Plant inoculation and tissue collection

The leaves were slightly yellow due to the saturation of the potting mix after 48 hours at 100% humidity. The unifoliate leaves were flash frozen instead of trifoliate tissue to ensure that leaves collected were exposed with inoculum.

4.4.2 RNA extraction

Initially RNA extractions were unsuccessful. Both the concentration and quality of the RNA were affected. Most samples had a very low amount of RNA, to no detectable RNA. In the few samples that contained RNA it was of poor quality. The extractions were repeated several times in attempt to improve recoveries without success. A different extraction kit was utilized, but extractions with the second kit were also unsuccessful in two attempts. A third kit was utilized with tissue samples taken prior to 8 hours post inoculation which resulted in the recovery of RNA with a low concentration (9-15 µg/µL), and quality scores within an acceptable range. Samples taken after the 8 hour mark still had very low RNA concentrations to no RNA recovered. After modifying the protocol to add an additional wash step during the extraction, and to incubate the silica column at 28 ℃ for 10 minutes prior to elution, the tissue samples taken at 8 hours post inoculation were successfully extracted, however RNA was not recovered from tissue samples taken at 24 hours and longer. Therefore, the 24 hour and 48 hour time points were removed from the experiment.

4.4.3 Expression analysis

cDNA was synthesized from each of the RNA samples and quantified cDNA samples from extracts from leaf tissue were used in PCR reactions to test for the expression of the Niemann-Pick gene. The primers utilized were anchored in exons and therefore the same primers that were used for genomic DNA screening were utilized for the expression study. There were no bands amplified by the PCR. The quantity of cDNA was increased twice to determine if it was the quantity of template that was effecting the PCR reaction. When template quantities were at 1 µg there was still no amplification of the Niemann-Pick gene. The lack of amplification resulted in several steps of troubleshooting.

4.4.4 Troubleshooting

Several attempts were made to troubleshoot the PCR reaction with the cDNA samples from infected tissue and primers for the Niemann-Pick gene. First the RNA was checked for the amplicon of the Niemann-Pick polymorphism. The NPP marker was not amplified in the RNA extracted from infected leaf tissues. Second the housekeeping genes were amplified from the RNA. Very faint bands were amplified from large amounts of template (12 ng). Lastly, the cDNA was screened through PCR for the NPP marker and housekeeping genes. Neither the NPP marker nor the housekeeping genes were amplified from the cDNA.

In addition, the primers were tested with cDNA preparations from tissue that was not inoculated. Leaf tissue from OAC Rex, frozen for DNA extraction in a previous experiment, was utilized to extract RNA, and ultimately synthesize cDNA. This cDNA was run through a PCR with the NPP primers, and a faint band was amplified (Figure 4.1). Lastly, cDNA samples from the inoculated tissue were added to a series of PCRs at various concentrations to determine

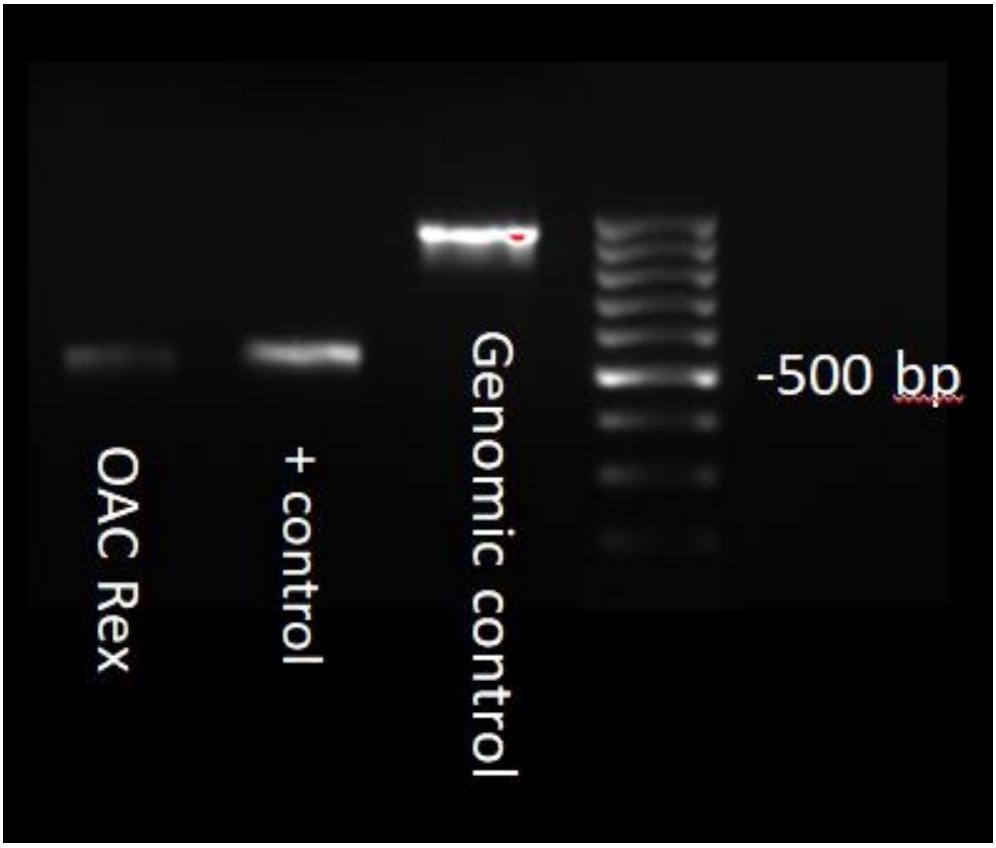

Figure 4.1- A 1% agarose gel illustrating the faint band amplified from RNA extracted from OAC Rex leaf tissue that was sampled in normal growing conditions, without inoculation. The samples shown are a genomic control extracted from OAC Rex leaf tissue; cDNA amplicon with increased concentration used as a positive control from leaf tissue of OAC Rex; and cDNA extracted from OAC Rex leaf tissue with unaltered concentration.

if the amount of gene expression was limiting the amplification. The series contained cDNA samples synthesized from various amounts of RNA from 10 pg to 8 µg. This range surpassed the recommended range (of 10 pg to 5 µg). However, no amplicons of the Niemann-Pick gene were detected. Ultimately, no amplicon from a Niemann-Pick transcript could be detected in cDNA samples prepared from bean leaf tissue inoculated with *X. axonopodis* pv. *phaseoli* and *X. fuscans* subsp. *fuscans* isolates.

4.5 Discussion and implications

The assay for expression of the Niemann-Pick genes in inoculated and mock inoculated tissues of resistant and susceptible genotypes was conducted to obtain correlative evidence for the involvement of the Niemann-Pick like gene in resistance to common bacterial blight. It was hypothesized that if the Niemann-Pick gene plays a role in disease resistance, a difference in transcription rate would be observed between resistant and susceptible genotypes in the hours following exposure to the pathogen (Lawton and Lamb, 1987). However, several issues were encountered with the gene expression assay that were related to the quality and quantity of the RNA that could be extracted and the usefulness of the cDNA template that was synthesized that ultimately, led to the conclusion the tissue samples were not healthy enough to be utilized for an expression study.

There are several reasons why leaf tissue would be negatively affected by the inoculation process. The first stress would have been the mechanical damage the leaves sustained by the inoculation process. Although the physically disrupted tissue represents a small percentage of total leaf surface area, the necrotic tissue likely introduces stressors, like free radicals into the undamaged tissue that would compromise cell function (Beleid El-moshaty *et al.*, 1993). In

addition, the inoculated plants were subjected to several days of 100% humidity before and after inoculation, which saturated the soil in the pots and caused the plants to turn pale green. By the end of tissue collection, the older leaves of the plants began to turn yellow, also indicative of stress, perhaps due to waterlogging. The humidity incorporated into the protocol was required to provide an optimal environment for the bacteria to flourish, and cause aggressive CBB infection, but common beans are susceptible to waterlogging stress.

From the results of the RNA extractions, it was apparent that the longer the leaf tissue was in the misting chamber the less RNA could be recovered to the extreme that no RNA could be recovered from the samples taken after 8 hours. In addition, it appeared that the transcript from the Niemann-Pick gene was not represented in the cDNA samples produced from the early time point, which would not support the hypothesis that it is involved in early resistance mechanisms, but the results are inconclusive because of the technical challenges already discussed.

Based on what was discovered in this experiment, it is suggested that the protocol for inoculation be adjusted in order to collect tissue that is viable for RNA extractions. One suggestion would be to allow for the disease to establish before taking samples for RNA extraction. Another option would be to take samples under field conditions, where favorable environmental conditions exist without the extreme water stress on the plants.

First steps for augmenting the indoor inoculation protocol in order for it to be conducive to RNA extraction includes increasing the volume of soil that the plants are potted in, altering the potting material to include additional drainage, reducing the relative humidity to a more manageable level (85%) and reducing the acclimatization step prior to inoculation. By increasing the volume of soil the plants are potted in, the degree of water stress is reduced by

allowing for additional absorption to occur. In addition, if a large particulate material was added, such as vermiculite, drainage of excess water could occur. This could allow for the root system to remain dry enough to prevent plant stress. By reducing the relative humidity, and the total amount of time spent in the misting chamber, the amount of water sitting on the leaf surface will be reduced. This surface water also plays a large role in causing leaf stress and ultimately damage.

Overall the expression study conducted was unsuccessful; however several lessons were learned with respect to the protocol utilized for expression studies under bacterial inoculation. The protocol utilized for this experiment was unchanged from the inoculation protocol used for disease severity ratings. After the completion of this experiment it can be concluded that a second, less harsh, protocol needs to be developed in order to successfully extract high quality RNA in enough quantity to conduct an expression study.

Chapter 5: Implications and Future Research

5.1 Implications

The primary objective of this thesis was to develop and validate a new marker for screening for resistance to common bacterial blight in common beans. The Niemann-Pick Polymorphism (NPP) marker was shown to be as accurate or significantly better in identifying genotypes with resistance than existing molecular markers in screens of two populations segregating for resistance to CBB. In addition, the NPP marker is a codominant marker, allowing for the detection of heterozygous individuals. By incorporating the NPP marker into current breeding programs for CBB resistant common beans, populations with the introgression of *P. acutifolius* material can be more efficiently screened for CBB resistance. The increased efficiency is based on the significant reduction of false positives (molecular screening labeling a susceptible plant resistant), and the reduction of false negatives (molecular screening labeling a resistant plant as susceptible). Although it has yet to be conclusively shown that the Niemann-Pick gene is the gene responsible for CBB resistance and the QTL for resistance located at the end of chromosome 8, it has been shown to be tightly linked to resistance through the experiments conducted in this thesis.

The ability to improve the efficiency and reliability of disease resistance screening by 23-31% in a breeding program is a considerable improvement to the overall speed and efficiency of breeding for disease resistance because it means that there are fewer lines being advanced to be screened in a field disease nursery, saving time and space. The use of a more reliable molecular marker means that a breeding program can base selection decisions more readily on marker assisted selection (MAS) and allocate the labour, time and cost of disease nurseries for more advanced lines. In addition to making already established breeding programs more efficient, the

development of a highly reliable molecular marker allows for the incorporation of disease resistance into the germplasm of breeding programs that previously did not focus on CBB resistance as a part of their breeding objectives. This could potentially include programs with a focus on Andean germplasm, as a tightly linked marker allows newly incorporated genetic material to be tracked.

In addition to developing the NPP marker for CBB resistance, this project investigated the importance of two candidate R-genes in CBB resistance. The two R-genes that were studied were thought to be associated with resistance based on their position in relation to the SU91 marker and in the QTL located at the end of chromosome eight. The current research provided evidence against their involvement in CBB resistance. The lack of exploitable polymorphisms in these genes, and their presence in susceptible bean lines resulted in the conclusion that these two R-genes are not likely genes conveying CBB resistance at the end of chromosome eight. Although not conclusive, this evidence would suggest that future efforts would be best focused on genes closer to the SU91 marker, and ultimately closer to the peak of the QTL at the end of chromosome eight.

Overall, this research established the utility of an efficient, codominant, molecular marker for selecting for CBB resistance in common beans and validated it in multiple genetic backgrounds for use in common bean breeding programs; it provided additional phenotypic data for a population frequently used in CBB resistance studies; it corrected a section of sequence in the genomic sequence data for *P. vulgaris* line BAT 93.

5.2 Future Research

One aspect of this project that could not be brought to a definitive conclusion is the potential for a single nucleotide polymorphism (SNP) between OAC Rex and OAC Seaforth, within R-gene 231733-8-004, to be a candidate cause of resistance within OAC Rex. The SNP has been predicted to alter the amino acid sequence, changing a serine in OAC Seaforth to a phenylalanine in OAC Rex. This predicted amino acid change could potentially alter the structure and function of the resulting resistance protein. Therefore, in order to rule out this SNP as the source of CBB resistance in OAC Rex, the resulting proteins would have to be compared in structure to determine if this SNP has a downstream effect. This comparison was not in the scope of this project, however further investigation could be helpful, not only to determine if R-gene 231733-8-004 has a role in CBB resistance, but also to determine if an alteration of this type has an effect on R-gene function as a whole. The conservation of R-gene structure allows a finding of this type to be applied to an entire category of genes.

This thesis focused on providing evidence for or against the three candidate genes previously outlined, however other methods of disease resistance should be investigated before the mode of CBB resistance in common bean can be definitively stated. In addition, there are several expansions on the research conducted in this thesis that could increase what is known about the NPP marker and associated genomic region. To investigate the limitations of the NPP marker, its use to screen a genetically diverse and large panel of common bean lines is suggested. The two populations studied in this thesis were distinct from each other; but had a common parent in their pedigrees. The parent of the first population, OAC Rex, is a parent of Rexeter (a parent in the second population, Compass//Rexeter/Apex). This means that the source of CBB resistance stems from the same introgression event in both populations' pedigrees. In addition to

Rexeter, the second population also has Apex as a source of resistance. The use of other populations derived from different introgression events is suggested. In addition, screening a panel of common bean lines with the NPP marker and other CBB resistance markers could easily establish its efficiency to already established markers. By including various common bean cultivars in a screening panel, the limits of the NPP marker and its ability to detect CBB resistance arising from a variety of sources can be tested. In addition to testing the NPP marker in additional genetic backgrounds, investigating the expression levels of the Niemann-Pick gene is suggested. The last experiment of this thesis was unsuccessful in providing expression data, due to what is believed to be errors in inoculation protocol prior to tissue sampling. For future research, an adjusted protocol should be utilized.

Chapter 6: Literature Cited

Akibode, S., and M. Maredia. Consultative Group on International Agricultural Research. 2011. Global and regional trends in production, trade and consumption of food legume crops. Michigan State University, Lansing, MI.

Akter, Z., B.B. Pageni, N.Z. Lupwayi and P.M. Balasubramanian. 2014. Biological nitrogen fixation and *nif*H gene expression in dry beans (*Phaseolus vulgaris* L.). Can. J. Plant Sci. 94:203-212.

Alavi, S.M., S. Sanjari, F. Durand, C. Brin, C. Manceau, and S. Poussier. 2008.Assessment of the genetic diversity of *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas fuscans* subsp. *fuscans* as a basis to identify putative pathogenicity genes and a type III secretion system of the SPI-1 family by multiple suppression subtractive hybridizations. Appl. Environ. Microbiol. 74:3295-3301.

Altrock, S., A. Fonseca, and A. Pedrosa-Harand. 2011. Chromosome identification in the Andean common bean accession G19833 (*Phaseolus vulgaris* L. *Fabeceae*). Genet. Molec. Biol. 34:459-463.

Andrewes, A.G., S. Hertzberg, S. Liaaen-Jensen, and M.P. Starr. 1973, Xanthomonas pigments: The Xanthomonas "Carotenoids"- Non-carotenoid brominated aryl-polyene esters. Acta. Chem. Scand. 27:2383-2395.

Andrewes, A.G., C.L. Jenkins, M.P. Starr, J. Shepard, and H. Hope. 1976. Structure of Xanthomonadin I, a novel dibrominated aryl-polyene pigment produced by the bacterium *Xanthomonas juglandis*. Tetrahedron Letters 45:4023-4024.

Beleid El-moshaty, F.I., S.M. Pike, A.J. Novacky, and O.P. Sehgal. 1993. Lipid peroxidation and superoxide production in cowpea (*Vigna unguiculata*) leaves infected with tobacco ringspot virus or southern bean mosaic virus. Physiol. Molec. Plant Path. 43:109- 119.

Beversdorf, W.D. and R.I. Buzzell. 1984. OAC Seaforth Field Bean. Can. J. Plant Sci. 64:757 -758.

Bhat, R.A. and R. Panstruga. 2005. Lipid rafts in plants. Planta 223:5-19

Bhat, R.A, M. Miklis, E. Schmelzer, P. Schulze-Lefert and R. Panstruga. 2005. Recruitment and interaction dynamics of plant penetration resistance components in a plasma membrane microdomain. PNAS 102:3135-3140.

Bitocchi, E., L. Nanni, E. Bellucci, M. Rossi, A. Giardini, P.S. Zeuli, G. Logozzo, J. Stougaard, P. McClean, G. Attene, and R. Papa. 2012. Mesoamerican origin of the common bean (*Phaseolus vulgaris* L.) is revealed by sequence data. PNAS 109:788-796.

Blair, M.W., and W. Pantoja. 2012. First use of microsatellite markers in a large collection of cultivated and wild accessions of tepary bean (*Phaseolus acutifolius* A. Gray). Theor. Appl. Genet. 125:1137-1147.

Borges, A., S.M. Tsai, and D.G.G. Caldas. 2012. Validation of reference genes for RT-qPCR normalization in common bean during biotic and abiotic stresses. Plant Cell Rep. 31:827-838.

Borner, G.H.H., D.J. Sherrier, T. Weimar, L.V. Michaelson, N.D. Hawkins, A. MacAskill, J.A. Napier, M.H. Beale, K.S. Lilley and P. Dupree. 2005. Analysis of detergent-resistant membranes in Arabidopsis. Evidence for plasma membrane lipid rafts. Plant Physiol. 137:104-116.

Campbell, C.L., and L.V. Madden. 1990. Introduction to Plant Disease Epidemiology. Pg. 532. John Wiley and Sons, New York.

Cardoso, J.D., M. Hungria and D.S. Andrade. 2012. Polyphasic approach for the characterization of rhizobial symbionts effective in fixing N_2 with common bean (*Phaseolus vulgaris* L.). Appl. Microbiol. Biotechnol. 93:2035-2049.

Chaćon, M.I., S.B. Pickersgill, and D.G. Debouck. 2005. Domestication patterns in common bean (*Phaseolus vulgaris* L.) and the origin of the Mesoamerican and Andean cultivated races. Theor. Appl. Genet. 110:432-444.

Diaz Castro, E. 2015. Quantitative trait loci (QTL) for common bacterial blight resistance and agronomic and developmental traits in common bean (*Phaseolus vulgaris* L.). University of Guelph.

Drijfhout, E., and W.J. Blok. 1987. Inheritance of resistance to *Xanthomonas campestris* Pv. *Phaseoli* in tepary bean (*Phaseolus acutifolius).* Euphytica 36:803-808.

Duncan, R.W., S.P. Singh, and R.L. Gilbertson. 2011. Interaction of common bacterial blight bacteria with disease resistance quantitative trait loci in common bean. Phytopathology 101:425-435.

Ellis, J., P. Dodds, and T. Pryor. 2000. Structure, function and evolutions of plant disease resistance genes. Plant Biol. 3:278-284.

Feldman, M.J., B.C. Poirier and B.M. Lange. 2015. Misexpression of the Niemann-Pick disease type C1 (NPC1)-like protein in *Arabidopsis* causes sphingolipid accumulation and reproductive defects. Planta 242:921-933.

Food and Agriculture Organization of the United Nations Statistics Division (FAOSTAT), 2018, http://www.fao.org/faostat/en/#data/QC

Gepts, P., F.J.L. Aragao, E. de Barros, M.W. Blair, R. Brondani, W. Broughton, I. Galasso, G. Hernandez, J. Kami, P. Lariguet, P. McClean, M. Melotto, P. Miklas, P. Pauls, A. Pedrosa-Harand, T. Porch, F. Sanchez, F. Sparvoli, and K. Yu. 2008. Genomics of Phaseolus beans, a major source of dietary protein and micronutrients in the tropics. Genomics of Tropical Crop Plant, pg. 113-143. Springer Publishing.

Gepts, P., and D.G. Debouck. 1991. Origin, domestication, and evolution of the common bean (Phaseolus vulgaris L.). Pages 7-53 in: Common beans: research for crop improvement.

Van Schoonhoven, A., and O. Voyset. ed. Commonwealth Agricultural Bureaux International, Wallingford, United Kingdom.

Gilbertson, R.L., R.E. Rand, E. Carlson, and D.J. Hagedorn. 1988. The use of dry-leaf inoculum for establishment of common bacterial blight of beans. Plant Disease 72:385-389.

Gillard, C.L., R.L. Conner, R.J. Howard, K.P. Pauls, L. Shaw, and B. Taran. 2009. The performance of dry bean cultivars with and without common bacterial blight resistance in field studies across Canada. Can. J. Plant Sci. 89:405-410.

Graham, P.H., and P. Ranalli. 1997. Common bean (*Phaseolus vulgaris* L.). Field Crops Research 53:131-146.

Harder, D.E., and K. Mendgen. 1982. Filipin-sterol complexes in bean rust- and oat crown rust-fungal/plant intractions: Freeze-etch electron microscopy. Protoplasma 112:46-54.

Haygood, R.A and D.L. Strider. 1982. A comparison of inoculation methods of *Erwinia chrysanthemi* in greenhouse ornamentals. Plant Disease 66:461-463.

Huckelhoven, R. 2007. Cell wall-associated mechanisms of disease resistance and susceptibility. Annu. Rev. Phytopathol. 45:101-127.

Jung, G., P.W. Skroch, D.P. Coyne, J. Nienhuis, E. Arnaud-Santana, H.M. Ariyarathne, S.M. Kaeppler, and M.J. Bassett. 1997. Molecular-marker-based genetic analysis of tepary bean-derived common bacterial blight resistance in different developmental stages of common bean. J. Amer. Soc. Hort. Sci. 122:329-337.

Kale, S.D., B. Gu, D.G.S. Capelluto, D. Dou, E. Feldman, A. Rumore, F.D. Arredondo, R. Hanlon, I. Fudal, T. Rouxel, C.B. Lawrence, W. Shan and B.M. Tyler. 2010. External lipid PI3P mediates entry of eukaryotic pathogen effectors into plant and animal host cells. Cell 142:284-295.

Khanal, R., T. Rupert, A. Navabi, T.H. Smith, T.E. Michaels, A.J. Burt, and K.P. Pauls. 2017. Mist common bean. Can. J. Plant Sci. 97:192-195.

Lafont, F., L. Abrami and F. Gisou van der Goot. 2004. Bacterial subversion of lipid rafts. Curr. Opin. Microbiol. 7:4-10.

Lawton, M.A., and C.J. Lamb. 1987. Transcriptional activation of plant defense genes by fungal elicitor, wounding, and infection. Mol. Cell Biol. 7:335-341.

Liu, Z., M. Crampton, A. Todd, and V. Kalavacharla. 2012. Identification of expressed resistance gene-like sequences by data mining in 454-derived transcriptomic sequences of common bean (*Phaseolus vulgaris* L.). BMC Plant Biol. 12:42-60.

Liu, J., X. Liu, L. Dai, and G. Wang. 2007. Recent progress in elucidating the structure, function and evolution of disease resistance genes in plants. J. Genet. Genomics 34:765-776.

Liu, S., K. Yu, and S.J. Park. 2008. Development of STS markers and QTL validation for common bacterial blight resistance in common bean. Plant Breeding 127:62-68.

Mahuku, G.S., C. Jara, M.A. Henriquez, G. Castellanos, and J. Cuasquer. 2006. Genotypic characterization of the common bean bacterial blight pathogens, *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* by rep-PCR and PCR-RFLP of the ribosomal genes. J. Phytopathology 154:35-44.

Martin, G.B. 1999. Functional analysis of plant disease resistance genes and their downstream effectors. Plant Biol. 2:273-279.

Meyers, B.C., A.W. Dickerman, R.W. Michelmore, S. Sivaramakrishnan, B.W. Sobral and N.D. Young. 1999. Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. Plant J. 20:317-332.

Meyers, B.C., D.B. Chin, K.A. Shen, S. Sivaramakrishanan, D.O. Lavelle, and Z. Zhang. 1998. The major resistance gene cluster in lettuce is highly duplicated and spans several megabases. Plant Cell 10:1817-1832.

Micheals, T.E. 1991. The bean plant. Pages 1-4 in: Compendium of Bean Diseases. R. Hall, ed. American Phytopathological Society Press, St. Paul, MN.

Michaels, T.E., T.H. Smith, J. Larsen, A.D. Beattie, and K.P. Pauls. 2006. OAC Rex common bean. Can. J. Plant Sci. 86: 733-736.

Miklas, P.N, D.P. Coyne, K.F. Grafton, N. Mutlu, J. Reiser, D.T. Lindgren and S.P. Singh. 2003. A major QTL for common bacterial blight resistance derives from the common bean great northern landrace cultivar Montana No. 5. Euphytica 131:137-146.

Miklas, P.N., J.D. Kelly, S.E. Beebe, and M.W. Blair. 2006. Common bean breeding for resistance against biotic and abiotic stresses: from classical to MAS breeding. Euphytica 147:105-131.

Miklas, P.N., J.R. Smith, R. Riley, K.F. Grafton, S.P. Singh, G. Jung, and D.P. Coyne. 2000. Marker-assisted breeding for pyramided resistance to common bacterial blight in common bean. Annu. Rep. Bean Inprov. Coop. 43:39-40.

Morneau, E. 2015. An expression analysis of candidate common bacterial blight resistance genes in common bean (*Phaseolus vulgaris*). Unpublished.

Munoz, C.G., and S.P. Singh. 1999. Resistance to common bacterial blight among *Phaseolus* species and common bean improvement. Crop Science 39:80.

Navabi, A., T. Rupert, S.J. Park, K. Yu, T.H. Smith and K.P. Pauls. 2013. Apex common bean. Can. J. Plant Sci. 93:131-135.

O'Boyle, P.D., J.D. Kelly, and W.W. Kirk. 2007. Use of marker-assisted selection to breed for resistance to common bacterial blight in common bean. J. Amer. Soc. Hort. Sci. 132:381-386.

Park, S.J. and B.N. Dhanvantari. 1994. Registration of common bean blight-resistant germplasm, HR45. Crop Science 34:548.

Park, S.J. and T. Rupert. 2000. AC Compass navy bean. Can. J. Plant Sci. 80: 341–342.

Park, S.J., K. Yu, S. Liu, and T. Rupert. 2007. Release of common bean germplasm line HR67. BIC Annual Report 50:221-222.

Perry, G., C. DiNatale, W. Xie, A. Navabi, Y. Reinprecht, W. Crosby, K. Yu, C. Shi, and K.P. Pauls. 2013. A comparison of the molecular organization of genomic regions associated with resistance to common bacterial blight in two *Phaseolus vulgaris* genotypes. Front. Plant Science 4:318-334.

Perry, G., and K.P. Pauls. 2012. Common bacterial blight in *Phaseolus vulgaris*. Pages 239-264 in: Agricultural Research Updates Vol. 2. B. Hendricks, ed. Nova Science Publishers, Hauppage, NY.

Pflieger, S., V. Lefebvre, and M. Causse. 2001. The candidate gene approach in plant genetics: a review. Molecular Breeding 7:275-291.

Pratt, R.C., and S.G. Gordon. 1994. Introgression of *Phaseolus acutifolius* A. Gray genes into the *Phaseolus vulgaris* L. genome. Plant Breeding 113:137-149.

Rivkin, M.I., C.E. Vallejos, and P.E. McClean. 1999. Disease-resistance related sequences in common bean. Genome 42:41-47.

Rudolf, K. 1993. Infection of the plant by Xanthomonas. Pages 193-245 in: Xanthomonas. J.G Swings and E.L. Giverolo, ed. Chapman & Hall, London, England.

Saettler, A.W. 1991. Common bacterial blight. Pages 29-30 in: Compendium of Bean Diseases. R. Hall, ed. American Phytopathological Society Press, St. Paul, MN.

SAS Institute Inc. 2017. Base SAS® 9.4 Procedures Guide. Cary, NC. USA: SAS Institute.

Schmutz, J., P.E. McClean, S. Mamidi, G.A. Wu, S.B. Cannon, J. Grimwood, J. Jenkins, S. Shu, Q. Song, C. Chavarro, M. Torres-Torres, V. Geffroy, S.M. Moghaddam, D. Gao, B. Abernathy, K. Barry, M. Blair, M.A. Brick, M. Chovatia, P. Gepts, D.M. Goodstein, M. Gonzales, U. Hellsten, D.L. Hyten, and G. Jia. A reference genome for common bean and genome-wide analysis of dual domestications. Nature Genetics, published online 2014 June 08.

Shi, C., S. Chaudhary, K. Yu, S.J. Park, A. Navabi, and P.E. McClean. 2011. Identification of candidate genes associated with CBB resistance in common bean HR45 (*Phaseolus vulgaris* L.) using cDNA-AFLP. Mol. Biol. Rep. 38:75-81.

Shi, C., K. Yu, W. Xie, G. Perry, A. Navabi, K.P. Pauls, P.N. Miklas, and D. Fourie. 2012. Development of candidate gene markers associated to common bacterial blight resistance in common bean. Theor. Appl. Genet. 10.1007:1-13.

Singh, S.P. 2001. Broadening the genetic base of common bean cultivars: A review. Crop Sci. 41:1659-1675.

Singh, S.P., and C.G. Munoz. 1999. Resistance to common bacterial blight among *Phaseolus* species and common bean improvement. Crop Sci. 39:80-89.

Singh, S.P., C.G. Munoz, and H. Teran. 2001. Registration of common bacterial blight resistant dry bean germplasm VAX 1, VAX 3 and VAX 4. Crop Sci. 41:275-276.

Singh, S.P. and P.N. Miklas. 2015. Breeding common bean for resistance to common blight: A review. Crop Sci. 55:971-984.

Singh, S.P., P. Gepts, and D.G. Debouck. 1991. Races of common bean (*Phaseolus vulgaris, Fabaceae*). Economic Botany 45: 379-396.

Swings, J., L. Vauterin, and K. Kersters. 1993. The bacterium Xanthomonas. Pages 121-146 in: Xanthomonas. J.G Swings and E.L. Giverolo, ed. Chapman & Hall, London, England.

Smith, T.H., T.E. Michaels, A. Navabi, and K.P. Pauls. 2012. Rexeter common bean. Can. J. Plant Sci. 92:351-353.

Subramanian, K., and W.E. Balch. 2008. NPC1/NPC2 function as a tag team duo to mobilize cholesterol. PNAS 105:15223-15224.

Takken, F.L.W., and M.H.A.J. Joosten. 2000. Plant resistance genes: their structure, function and evolution. Eur. J. Plant Pathol. 106:699-713.

Troisfontaines, P., and G.R. Cornelis. 2005. Type III secretion: More systems than you think. Physiology 20:326-339.

Urrea, C.A., P.N. Miklas, and J.S. Beaver. 1999. Inheritance of resistance to common bacterial blight in four tepary bean lines. J. Amer. Soc. Hort. Sci. 124:24-27.

Van Schoonhoven, A., and O. Voysest. 1991. Common Beans: Research for crop improvement. 1st ed. CAB International, Wallingford, UK.

Vattanaviboon, P., C. Seeanukun, W. Whangsuk, S. Utamapongchai, and S. Mongkolsuk. 2005. Important role for methionine sulfoxide reductase in the oxidative stress response of *Xanthomonas campestris* pv. p*haseoli*. Jour. Bacteriol. 187:5831-5836.

Vidaver, A.K. 1993. *Xanthomonas campestris* pv. *phaseoli:* Cause of common bacterial blight of bean. Pages 40-44 in: Xanthomonas. J.G Swings and E.L. Giverolo, ed. Chapman & Hall, London, England.

Vlasova, A., S. Capella-Gutierrez, M. Rendon-Anaya, M. Hernandez-Onate, A.E. Minoche, I. Erb, F. Camara, P. Prieto-Barja, A. Corvelo, W. Sanseverino, G. Westergaard, J.C. Dohm, G.J. Pappas, S. Saburido-Alvarez, D. Kedra, I. Gonzalez, L. Cozzuto, J. Gomez-Garrido, M.A. Aguilar-Moron, N. Andreu, O.M. Aguilar, J. Garcia-Mas, M. Zehnsdorf, M.P. Vasquez, A. Delgado-Salinas, L. Delaye, E. Lowy, A. Mentaberry, R.P. Vianello-Brondani, J.L. Garcia, T. Alioto, F. Sanchez, H. Himmelbauer, M.

Santalla, C. Notredame, T. Gabaldon, A. Herrera-Estrella, and R. Guigo. 2016. Genome and transcriptome analysis of the Mesoamerican common bean and the role of gene duplications in establishing tissue and temporal specialization of genes. Genome Biol. 17:32.

Watari, H., E.J. Blanchette-Mackie, N.K. Dwyer, J.M. Glick, S. Patel, E.B. Neufeld, R.O. Brady, P.G. Pentchev, and J.F. Strauss III. 1999. Niemann-Pick C1 protein: Obligatory roles for N-terminal domains and lysosomal targeting in cholesterol mobilization. Proc. Natl. Acad. Sci. 96:805-810.

Xie, W., G. Perry, B. Zhang, K. Yu, A. Navabi, and K.P. Pauls. Genome sequencing reveals polymorphism of transcription activator-like effectors in bacterial blight pathogen isolates with differential aggressiveness on bean. Unpublished.

Xie, W., K. Yu, K.P. Pauls, and A. Navabi. 2012. Application of image analysis in studies of quantitative disease resistance, exemplified using common bacterial blight-common bean pathosystem. Phytopathology 102:434-442.

Yu, K., S.J. Park, and V. Poysa. 2000. Marker-assisted selection of common beans for resistance to common bacterial blight: efficacy and economics. Plant Breeding 119:411-415.

Yu, N., M. Kim, Z.R. King, D.K. Harris, J.W. Buck, Z. Li and B.W.Diers. 2015. Fine mapping of the Asian soybean rust resistance gene *Rpp*2 from soybean PI 230970. Theor. Appl. Genet. 128:387-396.

Appendix I: ANOVA Tables

Table 1: ANOVA table resulting from the separation of AUDPC scores for the OAC Rex/OAC Seaforth population based on the NPP allele (A or B allele). AUDPC scores for three years (2011, 2013 and 2016) are included for the 72 individuals in the population.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F*
Model	1	13593.58912	13593.58912	125.23	<.0001
Error	70	7598.60126	108.55145		
Corrected Total	71	21192.19039			

*$\alpha = 0.05$

Table 2: ANOVA table resulting from the separation of AUDPC scores of the OAC Rex/OAC Seaforth population based on the SU91 allele (A or B allele). AUDPC scores for three years (2011, 2013 and 2016) are included for the 72 individuals in the population.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F*
Model	1	9553.10760	9553.10760	57.45	<.0001
Error	70	11639.08279	166.27261		
Corrected Total	71	21192.19039			

*$\alpha = 0.05$

Table 3: ANOVA table resulting from the separation of AUDPC scores for the OAC Rex/OAC Seaforth population based on the SU91-CG9A allele (A or B allele). AUDPC scores for three years (2011, 2013 and 2016) are included for the 72 individuals in the population.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F*
Model	1	9363.91576	9363.91576	55.42	<.0001
Error	70	11828.27463	168.97535		
Corrected Total	71	21192.19039			

*$\alpha = 0.05$

Table 4: ANOVA table resulting from the separation of AUDPC scores for the OAC Rex/OAC Seaforth population based on the SU91-CG9B allele (A or B allele). AUDPC scores for three years (2011, 2013 and 2016) are included for the 72 individuals in the population.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F*
Model	1	9363.91576	9363.91576	55.42	<.0001
Error	70	11828.27463	168.97535		
Corrected Total	71	21192.19039			

*$\alpha = 0.05$

Table 5: ANOVA table resulting from the separation of AUDPC scores for the OAC Rex/OAC Seaforth population based on the SU91-CG5 allele (A or B allele). AUDPC scores for three years (2011, 2013 and 2016) are included for the 72 individuals in the population.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F*
Model	1	9940.09000	9940.09000	61.84	<.0001
Error	70	11252.10039	160.74429		
Corrected Total	71	21192.19039			

*$\alpha = 0.05$

Table 6: ANOVA table resulting from the separation of AUDPC scores for the Compass//Rexeter/Apex population based on the NPP allele (A or B allele). AUDPC scores for two years (2012 and 2013) are included for the 161 individuals in the population.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F*
Model	1	96826.5650	96826.5650	334.55	<.0001
Error	159	46018.0031	289.4214		
Corrected Total	160	142844.5681			

*$\alpha = 0.05$

Table 7: ANOVA table resulting from the separation of AUDPC scores for the Compass//Rexeter/Apex population based on the SU91 allele (A or B allele). AUDPC scores for two years (2012 and 2013) are included for the 161 individuals in the population.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F*
Model	1	73322.0797	73322.0797	167.69	<.0001
Error	159	69522.4885	437.2484		
Corrected Total	160	142844.5681			

*$\alpha = 0.05$