UNDERSTANDING PROGRAMMED CELL DEATH PATHWAYS BY CHARACTERIZING BARLEY DISEASE LESION MIMIC MUTANTS

A Dissertation
Submitted to the Graduate Faculty
of the
North Dakota State University
of Agriculture and Applied Science

By

Wail Alsolami

In Partial Fulfillment of the Requirements
for the Degree of
DOCTOR OF PHILOSOPHY

Major Department:
Plant Pathology

November 2020

Fargo, North Dakota

North Dakota State University
Graduate School

Title

Understanding Programmed Cell Death Pathways by Characterizing Barley Disease Lesion Mimic Mutants

By

Wail Alsolami

The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

SUPERVISORY COMMITTEE:

Dr. Jack Rasmussen
Chair

Dr. Robert S. Brueggeman

Dr. Phillip McClean

Dr. Luis Del Rio Mendoza

Dr. Zhaohui Liu

Approved:

12/28/2020 — Date

Dr. Jack Rasmussen — Department Chair

ABSTRACT

Programmed cell death (PCD) in plants refers to rapid and localized cell death that occurs as a result of regular biological processes, or in response to infection or injury. Understanding the molecular mechanisms of PCD will fill key knowledge gaps in regard to plant development or resistance mechanisms. Upon pathogen invasion salicylic acid and reactive oxygen intermediate (ROI) signaling responses are triggered that contribute to the PCD outcome. Substantial research efforts have been conducted to identify genes involved in these PCD pathways including the generation and characterization of disease lesion mimic mutants (DLMMs) that spontaneously express PCD and necrotic lesions that resemble necrotrophic diseases in the absence of infection by a pathogen. The constitutive expression of PCD may be a result of the deletion of gene/s that directly or indirectly suppress PCD when it is not needed. Five fast neutron generated DLMMs in the cultivar (cv) Steptoe background (FN360, FN361, FN365, FN370 and FN396) were crossed with the cv Morex and 400 F_2 individuals of each population were screened for the mutant phenotype. Allele frequency mapping was used to map the mutation in each population to different chromosomal locations showing that each was caused by a different gene. Thus, each phenotype is the result of different mutated gene(s) in the PCD pathway that needs to be functionally validated.

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my major advisor, Dr. Robert Brueggeman, for his excellent guidance, support, patience, and encouragement throughout my research. I thank my committee members Dr. Jack Rasmussen, Dr. Luis Del Rio Mendoza, Dr. Phillip McClean, and Dr. Zhaohui Liu, for their advice and support. To my lab mates and colleagues, especially Dr. Roshan Sharma Poudel, Dr. Shyam Solanki, Dr. Gazala Ameen, Adbullah Alhashel and my lab mates for being there when needed. Also, I would like thank Shaun Clare for his big help.

A very special gratitude goes out to my government Saudi Arabia Culture Mission and Jazan University for helping and providing the funding for my PhD scholarship.

I owe my sincere appreciation to my parents whom I am grateful to them for their inspiration, love, and encouragement.

Finally, I would like to thank my beloved wife and the joy of my heart for her continuous support and endurance.

DEDICATION

This dissertation is dedicated to my beloved parents, and also to my loving and supportive wife.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

PCD ..... Programmed Cell Death

LMM..... Lesion Mimic Mutant

FN ..... Fast Neutron

NBS ..... Nucleotide Binding Site

LRR ..... Leucine Rich Repeats

cv ..... Cultivar

HR..... Hypersensitive Response

NLR ..... Nucleotide Binding Leucine Rich Repeat

DLMM..... Disease lesion mimic mutant

TILLING ..... Targeting Induced Local Lesions in Genome

CHAPTER 1: LITERATURE REVIEW

Introduction

Barley production ranks fourth amongst the major cereal crops grown worldwide behind maize, rice and wheat (Zhou, 2009). The majority of the barley crop (~75%) is used as animal feed yet the explosion of corn acreage in the Midwestern US in the past decade has supplanted barley as a major feed crop due to its consistent availability and low price. This has led to a drastic decrease in barley acreage across the US. Barley grown for malting, ~20% of the barley crop, is primarily used for brewing beer and distilling spirits. Malt barley demands a premium price that is very profitable for producers, however, it must meet strict quality parameters (IBGSC, 2012). Thus, in the face of drought and disease malt barley can be a risky crop to grow due to dockage at the silo. This is due to low quality malt barley being bought at feed grade prices and combined with the lack of adequate crop insurance to protect against quality losses malt barley acreage across the US has also declined in the last couple decades.

The evolution and diversification of barley within the *Poaceae* family resulted in a grain that was domesticated and has been used in brewing and spirit fermentation for over 10,000 years (Ullrich, 2010), a use for which barley will not be replaced by other grains. The social and economic added values of beer and spirits has led to malt quality trait selection for specific uses in the brewing and distilling industries. Also, the resiliency and adaptability of barley to harsh environments and its uses as animal feed, human food, and for brewing and distilling contributed to its dissemination throughout the world.

In modern times barley has become a model cereal crop in research due to its true diploid genome, which makes it easier to utilize in genetic and genomic studies. It was used to develop one of the first cereal genetic maps using molecular markers (Kleinhofs et al., 1993) and

bacterial artificial chromosome (BAC) libraries for gene discovery and genome analysis (Yu et al., 2000). In the last two decades the barley research community has developed powerful molecular tools such as a reference genome sequence (Schreiber et al., 2019), TILLING populations, (Schreiber et al., 2019), mutant populations (Uauy et al., 2009) and exome capture arrays (Mascher et al., 2013) that allowed for the advancement of genetic, genomic and gene function analysis that can also be applied to the more economically and calorically important wheat crop. Although wheat is considered a more important crop as it supplies ~ 25% of worldwide needed calories and protein (Hubbard et al., 2015), barley shares common genome architecture and physiological processes as well as many common diseases. Thus, barley research can easily be translated to wheat.

Recent progress in the research of plant programmed cell death (PCD) using barley mutagenesis induced disease lesion mimic mutants (DLMMs) will be summarized here. It is important to utilize barley to characterize these conserved PCD pathways as mutations of single genes in these conserved pathways cannot be identified in common hexaploid bread wheat or tetraploid Durum wheat as the ploidy of these genomes means redundancy of conserved housekeeping genes within these large complex grass genomes. The research presented in this dissertation is focused on PCD signaling in barley, which can be translated to wheat and other diverse plant systems.

The Evolution of Barley (*Hordeum vulgare* L.)

The *Poaceae* family of monocotyledonous angiosperms (the cereal grasses, bamboos, and wild grasses) contains all cereal crop species (Kellogg, 1998) including barley (*Hordeum vulgare* L.). The *Poaceae* consists of two major clades, which are the PACMAD and BOP clades, that diverged about 47.9-61.9 MYA (Cotton et al., 2015; Paterson et al., 2003). The Panicoideae

subfamily of the PACMAD clade includes the grass crops maize, sorghum, and sugarcane, along with the independent evolution of C4 grasses (Cotton et al., 2015) while the BOP clade has the remaining cereals with rice (Oryzoideae subfamily), which diverged from temperate grasses (Pooideae subfamily) about 40 – 54 MYA (Opanowicz et al., 2008). There are around 400-500 species, including wheat, barley, and rye, found in the monophyletic *Triticeae* tribe within the Pooideae (Middleton et al., 2014). The divergence of barley from wheat and rye was ~8-9 MYA (Middleton et al., 2014), and therefore the degree of synteny between the genomes is high with both containing a core set of 7 chromosomes. However, polyploid wheat has complete duplicated sets of these 7 homoeologous chromosomes including tetraploid Duram wheat (2n = 4x = 28) and hexaploid Bread Wheat (2n = 6x = 42) (Moore et al. 1995). There are thirty-two species within the *Hordeum* genera, including diploid (2n = 2x = 14), tetraploid (2n = 4x = 28) and hexaploid (2n = 6x = 42) cytotypes, which can be hybridized (Bothmer et al., 1995; Pourkheirandish & Komatsuda, 2007). Modern domesticated barley (*H. vulgare* ssp. *vulgare*) was derived from the wild progenitor barley (*H. vulgare* ssp. *spontaneum*), which was considered as a separate species in the past (Pourkheirandish & Komatsuda, 2007), but can easily be hybridized to domesticated barley. Usually, double haploid (DH) populations are formed from the barley species (*H. bulbosum*) followed by colchicin treatment (Chen & Hayes, 1989).

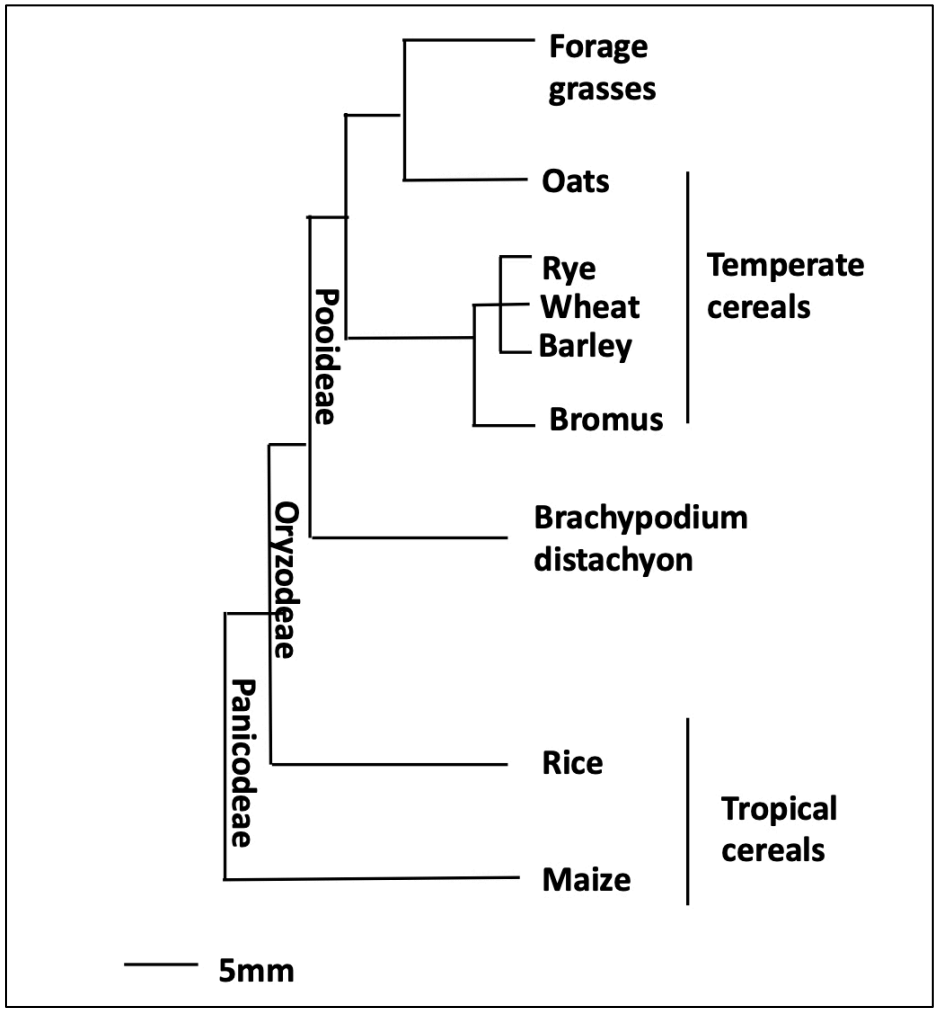

Figure 1.1:The Phylogenetic analysis of the relationships between Brachypodium and the small grain cereals (rye, barley, wheat, and oat) modified from Opanowicz et al. (2008).

The Domestication of the Species

According to Pourkheirandish et al. (2015), the first evidence of barley seed collection dates back to ~23 thousand years ago (KYA) and the domestication of barley from its wild progenitor *H. vulgare* ssp. *spontaneum* began approximately 10-12 KYA (IBGSC 2012, Paterson et al., 2003). The transition from the wild progenitor to a domesticated cereal is determined by the selection of principle morphological characteristics, including seed size, ear rachis stiffness, and seed release (Salamini et al., 2002). Domesticated barley evolved some characteristics: wider leaves, longer stems, awns, reduced seed dormancy, and semi-brittle rachis if harvested before

full maturity (Pourkheirandish & Komatsuda 2007; Salamini et al., 2002). Four important traits diversified modern barley cultivars post-domestication and are not fixed within all barley classes, including winter and spring growth habit, two- and six-row spikelets, hulled and hulless, and semi-dwarf varieties (Pourkheirandish & Komatsuda, 2007; Xu et al., 2017).

Arguably one of the most important traits needed for selection of a cereal grain for domestication is seed size and is a trait that is selected upon for further yield enhancement. For barley an increase in seed size can be detected ~500-1000 years after cultivation began (Purugganan & Fuller, 2009). Another very important domestication trait is non-brittle rachis. The wild barley trait, brittle rachis, allows the ear to shatter and the rough awns promoted seed dispersal as the seed would stick to the fur or hair of passing animals (Pourkheirandish & Komatsuda, 2007). Thus, a key trait of domestication, which aids in efficient seed harvest after senescence is non-brittle rachi, which is controlled by two independent genes (*btr1* and *btr2*), that are tightly linked ~100kb apart on the short arm of chromosome 3HS (Civáň & Brown, 2017). The recessive nature of the non-brittle rachis loci suggests loss of function mutations that include a 1 bp deletion or single point mutation in *Btr1* or an 11 bp deletion in *Btr2* (Civáň & Brown, 2017; Pourkheirandish et al., 2015). Epistatic interactions also exist with *Btr1.a* and *Btr2.k* that produce the brittle rachis phenotype in the presence of D gene, whereas *Btr1.h* and *Btr2.h* do not (Komatsuda et al., 2004).

Barley is the only modern cereal to retain a hulled caryopsis and is therefore not free threshing like wheat (Sang, 2009). Naked, or hulless barley allows for easier release from the husk, occurred post selection of non-brittle rachi and is in higher proportions in Asian barley suggesting the trait arose in the region (Pourkheirandish & Komatsuda, 2007). The trait is caused by a single recessive gene, *nud* on the long arm of chromosome 7HL, from a 17 kb deletion of

the region (Sang, 2009). Hulled or covered barley is important in feed and malting varieties, whereas hulless barley is typically only used for human consumption (Sang, 2009). Hooded barley (extra flower of inverse polarity) is used in feed varieties, controlled by ectopic expression of the *Knox3 gene* (Müller et al., 1995; Schulte et al., 2009). Reduced seed dormancy was required for wild varieties to survive adverse conditions and disperse over a wider region (Pourkheirandish & Komatsuda, 2007). In modern cultivation, a fine balance between dormant seed and pre-harvest sprouting is desired, especially for malting. Seed dormancy is a quantitative trait with many environmental influences and so far, the two loci seed dormancy 1 and 2 (*SD1* and *SD2)* have been identified on chromosome 5H (Gong et al., 2014).

Barley can be classified into two-row and six-row and were previously considered separate species based on spikelet inflorescence (Zohary & Hopf, 2000). However, two- and six-row varieties hybridize (Iriondo et al., 2018), therefore are not a separate species (Muñoz-Amatriaín et al., 2014) and the phenotype is predominately controlled by the single gene, *Vrs1* on chromosoms 2H (Komatsuda et al., 2007). The two-row phenotype is the dominant original ecotype controlled by the functional *Vrs1* gene and the mutant recessive *vrs1* allele gives rise to the six-row phenotype. There are three additional minor effect loci that contribute to the six-row phenotype, *vrs2*, *vrs3*, and *vrs4* on chromosomes 5HL, 1HL and 3HL, respectively (Pourkheirandish & Komatsuda, 2007). Additionally, allelic variation at the locus (*vrs1.a, Vrs1.b, vrs1.c, Vrs1.p, Vrs1.t*) have been shown to control awn length (Komatsuda et al., 2007), which is a component of the highly quantitative and complex yield trait (Liller et al., 2017).

The transition from winter to spring growth habit arose from reduced vernalization requirements and increased photoperiod insensitivity (Pourkheirandish & Komatsuda, 2007). There are four major barley genes *Vrn-H1* (*Sgh2*), *Vrn-H2* (*Sgh1*), *Vrn-H3* (*Sgh3*) and *Vrn4*

located on chromosomes 5H, 4H, 7H and 5H, respectively, that contribute to vernalization (Sasani et al., 2009; Trevaskis et al., 2003; Distelfeld & Dubcovsky, 2009; Laurie et al., 1995; Karsai et al., 2005; Kippes et al., 2015; Kato et al., 2003). These four genes are also conserved in wheat. In both barley and wheat these four genes epistatically interact and function in vernalization sensitivity (Cuesta-Marcos et al., 2010). Mutations arrose in *Sgh2*, many times, and multiple alleles of *Sgh2I* and *Sgh2II* exhibit a gradation of vernalization requirements (Takahashi & Yasuda, 1956). Also, the lines possessing *Sgh1* revert back to winter growth habit. Spring barley also requires reduced photoperiod sensitivity to flower earlier under longer photoperiod (13 – 16 h), which occur at higher and lower latitudes (Laurie et al., 1995). Reduced photoperiod sensitivity is primarily controlled by the *Ppd-H1* gene on chromosome 2HS (Laurie et al., 1995). More than 14 other loci have been detected that influence photoperiod insensitivity, such as *Ppd-H2* gene that controls flowering time under short-day (10 h) conditions (Laurie et al., 1995).

The semi-dwarf trait was used in multiple crops to increase yield during the Green Revolution, with *uzu1* barley varieties having an average 4.7-fold increase over older cultivars (Xu et al., 2017). There are three principal types of semi-dwarf varieties in barley including *semi-brachytic 1* (*uzu1*; 3HL), *breviaristatum-e* (*ari-e; 5HL*), and *semi-dwarf 1*(*sdw1/denso; 3HL*), used in Asian, early European, and modern European, American and Australian varieties (Xu et al., 2017). The *sdw1* locus contains an allelic series including *sdw1.a*, *sdw1.c* (*denso*), *sdw1.d*, and *sdw1.e* (Xu et al 2017).

Dissemination of the Domesticated Lines

Wild barley (*H. vulgare* ssp. *spontaneum*) evolved ~8-9 million years ago in the Fertile Crescent in the Near East, the center of barley (Middleton et al., 2014). There are two *H. vulgare* ssp. *spontaneum* subpopulations centered in the Fertile Crescent and Tibetan Plateau that were

reported to have diverged ~2.76 MYA (Dai et al., 2012). The primary domestication event occurred in the Fertile Crescent during the Neolithic revolution ~10-12 KYA alongside wheat, rye, and oats (Salamini et al., 2002). This was determined using genetic, archaeological and phytogeographical methods. The progenitor species of wheat, barley and rye intersect in the Fertile Crescent, suggesting that modern cereal species radiated out from the region (Doebley et al., 2006; Salamini et al., 2002). Seed of the wild species have been found in early archaeological sites of the area and were radiocarbon dated and shown to be the oldest known samples deposited from human collection and storage activity (Salamini et al., 2002). Studies assessing 400 polymorphic amplified fragment length polymorphism (AFLP) markers amplified from 317 wild and 57 domestic barley lines (Badr et al., 2000) and chloroplast DNA (Neale et al., 1988) suggested domestication occurred in the region (Salamini et al., 2002). Future studies narrowed the first domestication event down to the Jerusalem - Jordan area (Badr et al., 2000), with 6,000-year-old samples from the Yorum Cave in the West Bank around the Dead Sea area showing similar genetic composition to modern cultivated lines (Mascher et al., 2016). However, molecular evidence of *btr1* (Fertile Crescent) and *btr2* (Tibetan Plateau), the *NAM-1* locus and *RPB2* (largest subunit of RNA Polymerase 2) genes suggested a second domestication event occurred in the Tibetain Plateau, ~1,500-3000 km east of the Fertile Crescent (Russell et al., 2016; Wang et al., 2015; Wang et al.,2016). Badr et al (2000) previously noted this region as a region of diversification, not domestication. A third domestication event may have occurred in Africa (Orabi et al., 2007). There are four to five subpopulations of barley including two- and six-row Central European, Coastal Mediterranean, East African and Asian subpopulations (Muñoz-Amatriaín et al., 2014; Poets et al., 2015). Subpopulation 1 consists of Mediterranean six-row barley, also present in Australia and Central and South America. Subpopulation 2

consists of Asian six-row barley. Population 3 is European two-row barley, also present in New Zealand, Canada, Brazil and Chile. Subpopulation 4 consists of six-row barley breeding lines from Europe, US, Canada, and landraces from Macedonia and Asia. Subpopulation 5 consists of two- and six-row landraces from east Africa (Muñoz-Amatriaín et al., 2014). The subpopulations can be explained by the migration pathways of barley.

Barley migrated east to India with migrants accounting for the Asian landraces in subpopulation 4, and subsequently diversified throughout the Himalayas ~9-10 KYA (Salamini et al., 2010; Wang et al., 2015). Barley also spread with early Neolithic farmers from the Fertile Crescent southeast through Saudi Arabia, southwest through to Northern Africa and northeast through Europe along two principle routes; the Mediterranean coast and valleys of central Europe (Jones et al., 2012). Barley arrived in the Mediterranean (Turkey, Greece, and Spain) along the Mediterranean coastal route ~7–8 KYA (Jones et al., 2011), the United Kingdom ~5 KYA, and Scandinavia ~4 KYA through the valleys of central Europe (Newman & Newman, 2006). This movement from its center of origin closer to the equator radiating towards the higher latitudes facilitated the selection of barley land races resulting in the transition from winter to spring growth habit in northwestern Europe (Russell et al., 2016; Wang et al., 2016). Barley spread into the rest of Asia ~4-5 KYA from both sources of domestication (Newman & Newman, 2006; Wang et al., 2015), also resulting in spring barley types (Russell et al., 2016). Barley arrived in Central and South America with Spanish explorers ~500 years ago, and in North America and Australia with European settlers ~500 years ago (Muñoz-Amatriaín et al., 2014). Today, barley can be cultivated from the Arctic Circle to equatorial highlands and southern latitudes due to its adaptability (Russell et al., 2016). The Fertile Crescent and Tibetan Plateau domestication events are proposed to contribute most of the genetic diversity to

occidental (Western) and oriental (east Asian) cultivars, respectively (Morrell & Clegg, 2007). Two- and six-row European barley remain as distinct subpopulations (Muñoz-Amatriaín et al., 2014).

US Commercialization of the Crop

Barley first reached North America in 1494 via Columbus' second trip to the new world (Newman & Newman, 2006), but production of the English two-row barley landraces, such as 'Chevalier' first began in 1602 in New England as European settlement increased. However, these English two-row landraces were poorly adapted to the region (Schwarz et al., 2011). North American barley production increased and expanded westward into New York during the 1700s after Scottish six-row landraces were found to be more suitable to the region (Schwarz et al., 2011). In addition, in the late 1700s, Spanish six-row barley originating from Northern Africa (subpopulation 1) was grown in California due to the dry climate that was more suited to these genotypes (Muñoz-Amatriaín et al., 2014; Schwarz et al., 2011). Barley would continue to migrate westward from the eastern US to the Midwest and West with North Dakota becoming a major producer of barley in the early 1900s and Montana and Idaho joining in the mid-1950s (Schwarz et al., 2011).

Diversity studies using SNP data show the majority of American barley is descended from European six-row barley (subpopulation 4) (Muñoz-Amatriaín et al., 2014), with all upper Midwest six-row barley derived from 10 major accessions (Horsley et al., 1995). Major developments in US barley came from the development of Manchurian spring six-row types (Muñoz-Amatriaín et al., 2014) in the upper Midwest.

Currently six-row spring barley dominate the major barley growing regions of the northern Great Plains (Minnesota, North Dakota, and South Dakota) and two-row spring barley

dominate the major Western barley growing regions (Idaho, Colorado, Montana, Oregon, Washington, and Wyoming) (Mikel & Kolb., 2008). Diversity in feed barley exceeds that of malt barley in North American germplasm (Mikel & Kolb, 2008). The dwarfing *sdw1.a* allele is widely used to develop feed varieties, whereas the *sdw.d* allele has been used extensively in malt varieties (Li et al., 2015; Xu et al., 2017). Additionally, there is sub-population structure of US barley corresponding to the originating breeding program (Hamblin et al., 2010). The cultivar Morex was a popular malting variety in the northern Great Plains during the 80s, remaining a malting quality check (Smith et al., 2010). Morex along with Steptoe would become vital in molecular studies of barley due to their agronomic trait diversity and production of one of the earliest DH genetic mapping population of barley (Kleinhofs et al., 1993).

Barley is now the fourth most produced cereal crop worldwide (IBGSC, 2012) with 148 million metric tons produced in the 2016/17 season. The US produced 8.5 million metric tons in 1961, peaking at 13.2 million metric tons in 1986. Currently, the US produces approximately 4 million metric tons (ranking 10^{th}) from 1 million ha (ranking 10^{th}) (http://faostat.fao.org). The US yields 3.91 t / ha^{-1}, (ranking 15^{th}), approximately half of the leading country Belgium, which yields ~8.47 t / ha^{-1}. For comparison, the leading barley producer, Russia produces 20.4 metric tons from 9 million ha, equaling 2.27 t / ha^{-1} (http://faostat.fao.org). The economic value of barley is calculated at over $270 billion annually for the US; with over 90% generated from beverages (AMBA, 2017).

Molecular Characterization of the Species

Modern cultivated barley is predominantly a self-fertilizing diploid cereal, with a 5.1 Gb genome containing seven chromosomes (designated H) and represent the primary number of chromosomes of the triticeae cereal crops (IBGSC, 2012). The barley genome is comparably

smaller than bread wheat's 17 Gb (A, B and D) genomes (IWGSC, 2014) and rye's 8.1 Gb (R) genome (Martis et al., 2013). The first draft genome of barley was generated from the North American cultivar Morex, using a BAC physical map anchored to a genetic map and enhanced using whole genome shotgun sequencing (IBGSC, 2012). The Morex BAC library was developed by Yu et al. (2000) and has been used for positional gene cloning, comparative sequence analysis and physical mapping (Muñoz-Amatriaín et al., 2015). Other barley genomes sequenced include 'Bowman', 'Barke', 'Igri' (IBGSC, 2012), a Tibeten hulless variety (Zeng et al., 2015), Tibetan hulless barley (Xingquan et al., 2020), Golden Promise (Schreiber et al., 2020) and 'Haruna Nijo' (IBGSC, 2012; Sato et al., 2016). The annotation contains a total of 26,159 high confidence gene models with ~ 84% of the genome made up of repetitive DNA, of which 99.7% is accounted for by long terminal repeat retrotransposons (IBGSC, 2012). Recently, a high-quality reference genome was completed of Morex using a chromosome conformation capture mapping and whole shotgun sequencing approach (Beier et al., 2017; Mascher et al., 2017).

The first partial molecular map of barley was generated (parents not stated) utilizing restriction fragment length polymorphisms (RFLPs), isozyme and morphological trait loci as markers (Shin et al., 1990). The first full molecular map of barley was developed utilizing 114 RFLP markers on a Igri x 'Franka' DH population which spanned 870 cM (Graner et al., 1991). Subsequent maps were constructed utilizing RFLPs, RAPDs, isozymes, and. morphological trait loci or AFLPs and were generated from a Steptoe x Morex DH population (Kleinhofs et al., 1993) and 'L94' x 'Vada' recombinant inbred line (RIL) population (Qi et al., 1998), respectively. The lines were chosen due to their differing agronomic traits or leaf rust reaction and spanned 1,250 cM for the Steptoe x Morex DH map (Kleinhofs et al., 1993) and 1062 cM

for the ‘L94’ x ‘Vada’ RIL population (Qi et al., 1998), respectively. As part of the North American Barley Genome Mapping Project 45 microsatellite markers were anchored to the existing RFLP maps using four DH populations to futher saturate the existing barley genetic maps (Liu et al., 1996; Ramsey et al., 2000). From the mid-2000s there was a drive towards consensus maps that utilized multiple mapping populations using a variety of marker types (Close et al., 2009). The first single nucleotide polymorphism (SNP) based consensus map of barley was constructed by merging the four DH mapping populations Steptoe x Morex, Morex x Barke, ‘Oregon Wolfe Barley’, and Haruna Nijo x ‘OHU602’ (Close et al., 2009). The map consisted of 2,943 SNPs in 975 unique bins spanning 1,099 cM, produced from two barley oligonucleotide pool assays (BOPAs) (Close et al., 2009). Continued advancement in SNP genotyping resulted in an improved consensus map from 11 mapping populations (Muñoz-Amatriaín et al., 2011). The most recent advancement in barley genotyping arrived with the 50k iSelect SNP array (Bayer et al., 2017), an upgrade from the 9k iSelect SNP Array (Comadran et al., 2012), which was evaluated on the ‘Golden Promise’ x Morex RIL population produced by Liu et al. (2014), as they were elite two-row European and six-row American cultivars, respectively. (Liu et al., 2014). The 50k iSelect SNP Array resulted in a 14,626 SNP genetic map spanning 914 cM (Bayer et al., 2017).

Mascher et al. (2013) integrated sequencing data from the Morex x Barke RIL population (POPSEQ) using multiple genetic maps to the draft genome of Morex by the international barley genome sequencing consortium (IBGSC, 2012), which lead to the publication of a barley genomic resource that spans 98% of the genome anchored with two million SNPs (Ariyadassa et al., 2014). The recent trend in barley genetic mapping has seen a move away from bi-parental mapping which is time and resource intensive towards utilizing natural populations for

association mapping (Herzig et al., 2018; Muñoz-Amatriaín et al., 2014; Richards et al., 2017; Tamang et al., 2015; Visioni, 2020). Association mapping carried out on 2,417 barley accessions, known as the barley core collection from the USDA National Small Grains Collection of 33,176 accessions, representing over 100 countries for maximum diversity has. Provided a powerful tool to identify marker trait associations for traits of interest including resistance to *Pyrenophora teres* f. *teres* and *Pyrenophora teres* f. *maculata* the causal agents of. the diseases net form net blotch and spot form net blotch, respectively (Muñoz-Amatriaín et al., 2014; Richards et al., 2017; Tamang et al., 2015).

Genetic Improvement of Barley in Africa

Barley plays a key role as a source of feed, food, and malt around the world (IBGSC, 2012). Approximately 75% of global barley production is used for feed, 20% for beverages and 5% for food. Although barley as a food crop is considered a minor use globally, today it still remains an important food crop in Northern Africa (Amezrou et al., 2018). Barley exhibits substantial genetic diversity within its germplasm related to many agronomically important traits including disease resistance, seasonal habitat, head type, growth habit, abiotic stress tolerance (drought, cold, salt), yield and earliness (Molnar-Lang et al., 2014). Many of these traits could potentially mitigate the effects of climate change on barley production in the future (Ellis et al., 2000; IBGSC, 2012), with the International Center for Agricultural Research in the Dry Areas (ICARDA) having a global mandate to improve barley (Amezrou et al., 2018). Barley is one of the most important cereal crops in Morocco, accounting for 38% of cereal production in the country (Taibi et al., 2016). However, average Moroccan yield (1.38 t ha^{-1}) is considerably lower than the world (2.90 t ha^{-1}) and European (3.49 t ha^{-1}) averages (Taibi et al., 2016). Yield gaps are due to the lack of improved germplasm with resistance to biotic and abiotic stresses (Taibi et

al., 2016). The two forms of the net blotch disease, net form net blotch and spot form net blotch, are the most important disease factor limiting barley production in Morocco, with average yield losses of 29% and as high as 39% (Taibi et al., 2016). As Moroccan farmers do not use fungicide applications, genetic resistance is the only effective method of disease management (Taibi et al., 2016). The population of *Pyrenophora teres* f. *teres* the causal agent of net form net blotch in the North African region is highly variably and lines with resistance to the majority of isolates are not available (Bouajila et al., 2012; Taibi et al., 2016). Association mapping of ICARDA barley breeding germplasm has shown considerable genetic diversity among agronomic traits and net blotch resistance that could be used to introgress suitable genes into adapted germplasm (Amezrou et al., 2018). Barley is a model cereal crop used to study physiological processes in plants including plant innate immunity responses and programmed cell death (PCD).

The Plant Innate Immune System

Animals possess circulating immune cells to restrict pathogen infection, in which antibodies are produced that identify pathogen antigens in order to elicit defense responses (Duxbury et al., 2016). The plant innate immune system is cell autonomous implying that each cell possesses its own preformed innate immune system to intercept pathogen infection at the site by using two types of immunity receptors; the cell surface pattern recognition receptors (PRRs) and cytoplasmically localized nucleotide binding-leucine rich repeat (NLR) receptors (Jacob et al., 2013; Monaghan & Zipfel, 2012). PRRs are membrane bound receptors that recognize microbe/pathogen associated molecular patterns (MAMP/PAMP), which are conserved microbial structures such as chitin a major component of fungal cell walls (Shinya et al., 2015) or flg22 a 22-amino-acid peptide subunit of bacterial flagella required for motility (Felix et al., 1999). Plants also recognize damage associated molecular patterns (DAMPs), which are self-

molecules that are disrupted due to pathogen infection such as oligogalacturonides (OGs) that are released as a result of plant cell wall degradation induced by pathogen cell wall degradation enzymes (Ferrari et al., 2013). In the process of pathogen recognition, plant immunity receptors recognize MAMPs/PAMPs or DAMPs to initiate defense responses that include reactive oxygen species (ROS) bursts, ion fluxes, and activation of mitogen activated protein kinase (MAPK) signaling pathways. The elicitation of these early defense responses can lead to PCD, but typically activate lower amplitude immunity responses that do not result in PCD. Although, immunity receptors have shared immunity signaling mechanisms they can trigger responses of differing amplitude based on differential spatial and temporal activation (Pieterse et al., 2012).

Pattern Recognition Receptors

Plant PRRs are transmembrane receptors that are known as the first line of defense in plants (Coll et al., 2011). Receptor like kinases (RLKs) are a class of PRRs that contain an extracellular receptor domain commonly in the class of the leucine-rich repeats (LRRs) that evolved to recognize MAMPs, which then relays the signal to the intracellular serine threonine protein kinase (S/TPK) cytosolic signaling domain (Jones & Dangl, 2006). PRRs result in MAMP/PAMP triggered immunity M/PTI, and the best characterized mechanism for the function of these receptors is the recognition of bacterial flagella by the flagellin sensing 2 (FLS2) PRR receptor. In plants FLS2 is an LRR-STPK class of RLK receptor, that functions in the detection of the N-terminal epitope of flagellin, which is a 22 amino acid peptide designated flg22 that was originally identified from *Pseudomonas aeruginosa* (Gomez-Gomez and Boller, 2000; Macho & Zipfel, 2014).

Typically, RLKs contain a variable ectodomain or extracellular receptor domain that is responsible for ligand binding, a single-pass transmembrane domain, an intracellular

juxtamembrane domain, and a cytoplasmic S/TPK signaling domain. There are other cell surface proteins that have been shown to associate with FLS2 forming cell surface receptor complexes that function to detect flg22. One important FLS2 coreceptor protein is BAK1 (BRI1-associated receptor kinase 1) (Heese et al., 2007). FLS2 and BAK1 form the complex that detects flg22, which then translocate the signal across the plasma membrane to the intracellular signal transduction pathway (Lu et al., 2010) that leads to protein phosphorylation. There is another protein called Botrytis-Induced Kinase 1 (BIK1) within the receptor complex that interacts with the FLS2 – BAK1 complex at the cytoplasm interface. After flg22 elicitation, BIK1 phosphorylates the FLS2 – BAK1 complex, then disassociated from the complex (Lu et al, 2010). BIK1 mediates PTI signal transduction resulting in resistance response to pathogens (Lu et al., 2010) by playing roles in the activation of both RbohD-dependent ROS production and AtMPK3/6 (Liu & He, 2017).

Plant Intracellular Immune Receptors

Pathogens evolve effectors and deliver them into plant cells to suppress PAMP/MAMP triggered immunity (PTI/MTI) in order to colonize the host, which is referred to as effector triggered susceptibility (ETS). However, plants counter evolved to deal with ETS through the selection of intracellular receptor that function to detect these effectors. This second level of the plant innate immune system is controlled through nucleotide binding site- leucine-rich repeat (NLR) cytoplasm localized receptors. The NLR receptors can recognize pathogen effectors through direct binding but more commonly detect modifications induced by effectors on the host susceptibility targets in the cell (Chiang & Coaker, 2015). The typical structure of plant NLR immunity receptors are an N-terminal coiled-coil domain (CC) or Toll/interleukin-1 receptor domain (TIR) (Meyers et al., 2003) followed by a nucleotide binding site and a C-terminal

Leucine Rich Repeats (LRRs). The LRR domains are under diversifying selection, which reflects their role in the recognition of diverse effectors (Qi & Innes, 2013). In the absence of pathogen detection, the LRR domain keeps the NLR in the "off " state, in which the nucleotide binding site is bound with ADP while the activation of the NLR by effector binding or recognition of the effector action on the guardee protein activates the NLR by ATP binding, which switches the NLR from the closed structure "Off" state to the open structure "On" state (Qi and Innes, 2013). The N-terminal domain either CC or TIR have been shown for some NLR-mediated immunity responses to control the downstream signaling that activates defense responses (Qi and Innes, 2013). For some characterized NLR-mediated defense response it has also been shown that after effector detection the activated NLR protein is trafficked from the cytoplasm to the nucleus activating transcriptional reprogrammed and elicitation of defense responses (Chiang & Coaker, 2015; Qi & Innes, 2013). The second line of active defense responses in plants that is typically mediated by NLR immunity receptors is called effector triggered immunity (ETI), which is typically characterized be the hypersensitive response (HR), a form of programmed cell death further discussed below (Adachi & Tsuda, 2019).

Both PTI and ETI pathways have crosstalk that interconnect with hormone signaling mechanisms including salicylic acid, Jasmonic acid, and ethylene signaling mechanisms that are involved in pathways and signaling that lead to PCD responses (Staal & Dixelius, 2009). Also, the MAPK cascades are early responses in PTI or ETI mechanisms that regulate NO in cell that may result in PCD responses (Liu & He, 2017; Meng & Zhang, 2013). Thus, these two layers of innate immunity in plants share similar signaling pathways that trigger defense responses against pathogens that result in PCD and HR.

ETI responses that are manifested by PCD mediated HR responses and result in resistance to biotrophic pathogens in a gene-for-gene interaction (Glazebrook, 2005; Zhang et al., 2013). However, these same immunity mechanisms can result in susceptibility to necrotrophic pathogens in an inverse gene-for-gene interaction where the necrotrophic pathogen hijacks the plants defense mechanisms to elicit PCD and disease susceptibility. Research on necrotrophic host-parasite interactions has shown that necrotrophic pathogens that colonize and extract nutrient from dead and dying tissues produce small secreted necrotrophic effector (NE) proteins, to induce PCD to colonize and complete their life cycle on the host (Faris et al., 2010; Oliver & Solomon, 2010). The cloning of host NE-targets showed that some are classical NLRs that elicit ETI-mediated HR responses (Faris et al., 2010; Lorang et al., 2007). This was well exemplified by the wheat susceptibility target *Tsn1* and the cognate *Parastagonospora nodorum* NE SnToxA showing that the NLR once triggered by SnToxA activates ETI-mediated PCD that the pathogen colonizes to cause further disease (Faris et al., 2010; Pandelova et al., 2009). Thus, necrotrophic specialists elicit immunity responses that rely on PCD, which is effective against biotrophic pathogens yet facilitate disease development by the necrotophs via necrotrophic effector triggered susceptibility (NETS) (Liu et al., 2015; Vleeshouwers & Oliver, 2014).

This type of NETS also occurs when necrotrophic pathogens hijack other mechanisms other than R-protein-mediated HR responses. A recent study showed that the hemibiotrophic pathogen *Phythophthora infestans* hijack autophagy process to uptake nutrients by redirecting autophagy in potato towards the pathogen feeding structures during the necrotrophic phase of its lifecycle on the host (Pandy et al., 2020). *Phythophthora infestans* delivered the effector PexRD54 to mimic carbon starvation to neutralize defense-related autophagy allowing other

autophagy pathways that are induced under carbon starvation for its own benefit (Pandy et al, 2020).

Programmed Cell Death

Programmed Cell death is a process in multicellular organisms that plays a critical role in the production of new cells to fulfill the function of specific tissues at different developmental stages or to stop the function of old cells that are no longer needed (Daneva et al., 2016). The original term, apoptosis (Greek: apoptosis, apo- from and ptosis- falling) is often used in literature as a form of PCD that occurs in both animals and plants (Smetana, 2010). Although PCD originally evolved as a developmental process it also evolved as a major component of plant defenses against pathogens (Daneva et al., 2016; Pennell and Lamb, 1997). Plant PCD has features including changes in cell morphology that are based on the type of PCD. The PCD can be either developmental, which is linked with a vacuolar-type of cell death or PCD that occurs during plant-pathogen interactions result in DNA shredding into oligonucleosome sized fragments giving rise to the hallmark DNA laddering that is used to characterize HR responses in plants (Huysmans et al., 2017; Pennell & Lamb, 1997; Van Doorn et al., 2011).

Plant PCD is involved in development processes in plants such as leaf and petal senescence, seed development and germination, which requires the removal of embryonic suspensors and aleurone layers via PCD (Pennell & Lamb, 1997). PCD also occurs in the ontogenesis of the xylem of vascular bundles such as vessels and tracheids, and it occurs in the depletion of tissues of reproductive organs, including stomium, tapetum, and ovaries (Pennell and Lamb, 1997). Programmed cell death also occurs in parenchyma cells when they differentiate into xylem, which surrounds wounding sites (Greenberg, 1996). There are also proteins involve in PCD that loosen the cell wall to modify the cells such as arabinogalactan

proteins in maize coleoptiles (Schindler et al., 1995). Furthermore, vessels and tracheids, which are vascular bundles in plants, are generated from aligned dead tracheary elements (TEs), and the transformation of parenchyma cells into TEs requires three stages (Fukuda, 1997) where the last stage involves PCD and secondary wall formation (Fukuda, 1997).

Environmental Cues Elicit Plant PCD Responses

Environmental stresses and conditions induce PCD responses that evolved to help the plant adapt and survive abiotic stressors including waterlogging and hypoxia (Pennell & Lamb, 1997). In the cortex of roots and stems Aerenchyma form that serve as an aerating tissue to provide more efficient transfer of O_2 to waterlogged stem bases and roots (Armstrong, 1979). as In maize it was shown that hypoxic conditions due to waterlogging induced programmed cell death that leads to the rapid formation of aerenchyma formation; a major structure required for the plant's survival (Pennell & Lamb, 1997).

Plants are under constant attack by diverse pathogens and because of their sessile nature they require an effective defense system to react and protect themselves from diverse biotic threats. Thus, plant-pathogen interactions that lead to incompatible (resistance) interactions illicit plant innate immunity responses that depend on PCD at the site of infection to deprive biotrophic pathogens from acquiring nutrient from the living cells (Pennell and Lamb, 1997). These biotrophic pathogens require living host cells to acquire nutrient from the host in order to complete its life cycle. The tightly regulated PCD response that sequesters the pathogens in a foci of dead cells is called the hypersensitive response (HR), which is the major mechanism of plant immunity. These resistance responses involve the recognition of the pathogen via immunity receptors that activate protein kinase signaling molecules leading to the accumulation of reactive

oxygen species (ROS) like O_2 and H_2O_2 that contribute to the activation of PCD (Levine et al., 1996; Mehdy, 1994; Pennell & Lamb, 1997).

Control of PCD in the Plant

The regulation of signals controlling PCD in plants is relatively unknown. However, many studies reported the accumulation of ROS in plants prior to PCD suggesting that these ROS molecules are involved in the elicitation and/or signaling that leads to PCD (Pennell & Lamb, 1997). When plants recognize pathogens via immunity receptors the defense signaling pathways lead to ROS accumulation at the sites of infection as a result of O_2 reduction, which occurs in organelles such as mitochondria, chloroplasts, or peroxisomes. The accumulation of ROS also occurs as a result of DNA damage as a protection mechanism (Smetana, 2010).

There are other regulators that play a crucial role in PCD in plants such as the Bcl-2 family, p53, caspases, or Apaf1 proteins (Smetana, 2010). Bcl-2 was transferred from animal systems to plants in order to study the function of the gene, and the results indicated a conservation of PCD signaling in both animals and plants which includes immunity signaling-mediated HR in plants (Smetana, 2010). The plant hormone ethylene also contributes to the induction of PCD due to the stimulation of Ca2+ influx and induced modification of protein phosphorylation patterns (Guzman & Ecker, 1990; Pennell & Lamb, 1997; Raz & Fluhr, 1992; Raz & Fluhr,1993). Another plant hormone that plays a role in PCD immunity responses against biotrophic pathogens is salicylic acid (SA), however, these pathways that lead to PCD responses that would typically provide resistance to a biotrophic pathogen can result in susceptibility to necrotrophic pathogens. This antagonistic response of the plant innate immunity system is due to the nature of the pathogen's lifestyle. The necrotroph's strategy of colonizing dead and dying tissue allows them to hijack the plant's immunity responses because they purposefully elicit

immunity responses that trigger PCD through necrotrophic triggered susceptibility (NETS) (Birkenbihl & Somssich, 2011; Pieterse et al., 2009). However, pathogens evolve effectors to overcome SA impact by increasing auxin level in plants, which leads to the suppression and loss of the effectiveness of SA-mediated defense responses (Kazan & Lyons, 2014).

In Arabidopsis, resistance to powdery mildew resistance involves the nonrace-specific disease resistance gene 1 (NDRl) located on chromosome 3 encoding a 660-base pair open reading frame (Century et al., 1997). Based on the predicted amino acid sequence of NDR1, it may be associated with cellular membranes (Century et al., 1997). Another gene involves in powdery mildew resistance in barley, RAR1, encodes a cytoplasmic Zn^{2+} binding protein that functions upstream of a cellular H_2O_2 burst (Zhou et al., 2001). RAR1 is required for hypersensitive reaction (HR) to occur in barley (Halterman et al., 2001; Zhou et al., 2001). The *Arabidopsis* R gene RPM1 confers resistance to *Pseudomonas syringae* strains expressing either avrRpm1 or avrB (Tornero et al., 2002). In *Arabidopsis*, both RAR1 and NDR1 function in parallel signaling pathways, and double mutants of these genes results in cell death (Tornero et al., 2002). AtRAR1 contributes to RPM1-mediated resistance and functions in the same pathways as NDR1 which result in the elicitation of the HR (Tornero et al., 2002). Thus, HR responses are controlled by multiple proteins in the pathways to condition resistance in plants (Balint-Kurti, 2019; Dodds et al., 2006; Tornero et al., 2002).

Lesion Mimic Mutants

Disease lesion mimic mutants (DLMMs) are plant with mutations in a gene or genes that results in the expression of spontaneous lesions that are similar to the hypersensitive responses on leaves as a result of immunity responses or pathogen infection. DLMMs are a valuable tool to understand PCD pathways, and many efforts have been made to unravel PCD in plants utilizing

DLMMs to identify genes that are involved in the regulation or execution of this process (Moeder & Yoshioka, 2008). DLMMs have been studied in many crops such as rice, maize, and barley (Lorrain et al., 2003).

Many of the genes characterized to date that are involved in PCD responses are conserved genes that span diverse species (Olvera-Carrillo et al., 2015). Thus, mutagenesis methods are utilized to understand the gap between the lesion mimic mutants and the biochemical disorder caused by the miscues in the PCD signalling pathways. DLMMs have two types: initiation and propagation mutations. The initiation mutations show constitutive development of lesion of restricted sizes. However, in contrast, the propagation mutants cannot control lesion expansion once initiated. A phenomenon known as run-away programmed cell death (Lorrain et al., 2003, Moeder & Yoshioka, 2008).

Disease lesion mimic mutants can be utilized as a tool to dissect PCD function and signaling pathways that are either initiated or suppressed in response to developmental or environmental stresses (Moeder & Yoshioka, 2008). What makes these mechanisms complex and difficult to elucidate is partially due to the fact that many of the signals molecules and pathways identified and elicited during PCD overlap with other physiological pathways in plants (Moeder and Yoshioka, 2008).

Disease Lesion Mimic Mutant Genes Involved in Cellular Processes

The limited number of genes identified to date underlying DLMM phenotypes have shown that they also function in other cellular processes aside from PCD. For example, plant Sphingolipid molecules are also involved in many essential plant developmental and physiological processes including pollen development and signal transduction. Two genes, ACD5 and ACD11, identified utilizing Arabidopsis DLMMs were involved in sphingolipid

metabolisms. ACD5 and ACD11 encode a ceramide kinase and a sphingosine transfer protein, respectively (Brodersen et al. 2002; Liang et al., 2003). The acd5 mutant shows extremely reduced levels of ceramide kinase activity, causing a high level of precursor molecules, which may be either ceramide or sphinganine (Liang et al., 2004). In addition, Greenberg et al. (2000) determined that the acd5 mutant of Arabidopsis challenged with *Pseudomonas syringae* resulted in the accumulation of SA which enhanced PCD. Moeder and Yoshioka (2008) suggested both ceramides /sphingolipids and their phosphorylated derivatives modify cell death in plants.

The connection between Ca_2^+ ion influx and PCD has been known for a long time (Atkinson et al., 1996: Levine et al., 1996). Cytosolic Ca_2^+ concentrations rise in plant cells in response to infected by bacteria (Grant et al., 2000). Characterization of DLMMs in plants has led to the identification of three cyclic nucleotide-gated ion channel genes (CNGC) (Moeder & Yoshioka, 2008). DND1 in *Arabidopsis* encodes AtCNGC2 (Clough et al., 2000) while the Arabidopsis DND2 and HLM1 encode AtCNGC4 (Balagué et al., 2003; Jurkowski et al., 2004). In barley, the *nec1* gene which causes a DLMM phenotype is a homolog of DND2/HLM1 (Rostoks et al., 2006). Two other mutant genes identified via DLMM characterization were copine1 and bonzai1, which are also involved in Ca_2^+ signaling. Their functions are not known, but they are connected to membrane trafficking and Ca2+ signal transduction (Moeder & Yoshioka, 2008), which supports the connection between Ca_2^+signalling and PCD (Hua et al., 2001; Jambunathan et al., 2001).

Disease Lesion Mimic Mutants in Breeding

Genes underlying DLMMS have been identified from Arabidopsis (Zhang et al., 2008) barley (Ameen et al., 2020 unpublished; Jorgensen, 1992; Rostoks et al., 2006), maize (Johal et al., 1995), wheat, (Li & Bai, 2009) and rice (Wang et al., 2015). Some of these genes also play

roles in resistance responses against pathogens, suggesting their involvement in PCD responses elicited by the plants innate immunity system (Zeng, 2005). The goal of plant breeding is to improve both crop yield and quality to maximize economic benefits for producers, end users and consumers as well as enhance qualities such as nutritional value, flavor and aesthetic appeal. Thus, efforts have been made to understand the mechanisms of DLMM genes that could be utilized to enhance disease resistance, which depending on the disease can contribute to both yield and quality. One of the prevalent DLMM genes utilized in barley breeding is the Mildew resistance Locus O (*Mlo*), which confers resistance to powdery mildew (*Blumeria graminis*) in barley. The *mlo* mutant expresses a DLMM phenotype and has been deployed as a non-race-specific resistance gene that has remained remarkably durable in barley. Barley carrying *mlo* resistance have a recessively inherited loss-of-function mutation that confers broad-spectrum powdery mildew resistance (Acevedo-Garcia et al., 2014). Aist et al. (1987) observed that the host-pathogen genetic interaction/s occurring between *Blumeria graminis* and *mlo* containing barley arrested the infection process at the penetration stage. Mutant 66 was the first *mlo* mutant line induced by X-ray irradiation in the German variety Haisa in 1942 (Jorgensen, 1992). *Mlo* mutants were subsequently induced in other barley backgrounds utilizing different mutagens. Mutations vary based on the chemical or radiation used, some mutagens like high energy radiation (i.e. fast neutron) induce large deletions whereas lower energy radiation sources like X-rays and γ-rays induce relatively smaller deletions. X-rays, γ-rays and the chemical mutagen ethyl methanesulfonate (EMS) were all used to generate independent *mlo* mutants. Mutagenesis tools will be discussed in detail later in this chapter to clarify the similarities and differences among them. Many studies have been undertaken to identify and characterize the function of the *mlo* gene (Büschges et al., 1997). *Mlo* encodes a transmembrane receptor like protein that

consists of seven transmembrane (TM) helices, and the location of both N- and C- terminuses are extracellular and intracellular, respectively (Devoto et al., 1999).

Mutagenesis in Plant Sciences

An important breakthrough in plant genetics was inducing mutations (Muller, 1930; Stadler, 1932) which contributed significantly to gene discovery efforts. It was an especially important tool for the identification of conserved genes because of the ability to create genetic variation that was otherwise not present in the primary germplasm pool (McCallum et al., 2000). Different mutagenic agents are utilized to induce mutants including chemicals and radiation. The most commonly used physical mutagenic agents are X-rays, γ-irradiation, and fast neutron. X-rays have been used extensively in plants as well as medical research (Mba et al., 2012). Roentgen discovered X-rays in 1895 and were subsequently utilized for the induction of mutations in plants by Städler in 1928.

Fast neutron irradiation was discovered in 1932 by James Chadwick (Chadwick, 1932) as a small particle of matter without electric charge that caused large deletion in chromosomes or induced chromosomal rearrangements (Sikora et al., 2011). Fast neutron mutagenesis has been utilized in both forward and reverse genetics with various doses used in plants such as 60 Gy being used in *Arabidopsis thaliana*, and 20 Gy in rice (Belfield et al., 2012; Bolon et al., 2011). The large deletions can be both beneficial and a hindrance in gene discovery as it is typically easier to identify the large deletions (Kumawat et al., 2019). However, as in the case with the FN396 deletion described in this research the large MB sized deletions can contain multiple candidate genes which can complicate the validation of the gene responsible for the mutant phenotype.

Although, irradiation induced mutants that typically contain deletions are a powerful gene discovery and validation tool, chemical mutagenic agents such as ethyl methanesulfonate (EMS), ethyleneimine (EI), dimethyl sulfate (DMS), and diethyl sulfate (DES) typically induce base substitutions which can be more useful depending upon the application. Ethyl MethaneSulfonate (EMS) is a popular mutagenic agent in plants due to the fact that it is easy to use, requires no special equipment, and induces a high frequency of mutations (Sikora et al., 2011). EMS has been used in various plants, including Arabidopsis (Qu & Qin, 2014), wheat (Hussain et al., 2018), and barley (Schreiber et al., 2019). Chemical mutagens usually cause single base-pair (bp) changes or single-nucleotide polymorphisms (SNPs), while irradiation causes small to large deletion, and translocations (Sikora et al., 2011). EMS mutation results in silent, missense, or nonsense nucleotide substitutions whereas, in noncoding regions, EMS may also affect and change either promoter sequences or other regulatory regions, which lead to up- or downregulation of gene expression (Sikora et al., 2011).

Forward Genetics

Using the forward genetics strategy requires that individuals with the phenotype/s of interest are identified in order to isolate the gene(s) controlling the phenotype, which can require a major time and resource investment (Alonso and Ecker, 2006). Historically, utilizing this strategy for gene discovery involved several steps, including the development of a mutant population, and screening thousands of individuals from the mutant population to identify independent mutants with the phenotype of interest. Next, a biparental population must be developed to generate a genetic map to localize the causal mutated gene by crossing the mutant individual with a second parent that has adequate sequence polymorphism to generate polymorphic markers across the genome. Both the segregation of the phenotypic and genotypic

scores are used to identify the mutant locus. At this stage, different methods can be applied for further studies such as fine mapping or comparative genomics for candidate gene identification. To identify candidate mutant genes, genomic sequencing approaches such as RNAseq and exome capture sequencing are powerful next generation tools for comparative analysis that when coupled with a high quality reference genome sequence allows for the rapid discovery of mutated genes (Sarin et al., 2008). Forward genetics is a powerful approach for gene discovery with positive attributes including the ability to characterize a wide range of mutations in many biological pathways and functions and the ability to identify allelic series depending upon the extent of mutant population screening (Vidaurre & Bonetta, 2012).

Reverse Genetics

In this approach, knowledge of genes is the crucial step to study the phenotype. Previous knowledge will help researchers to link a genotype to a phenotype. For example, the sequences of many plants including Arabidopsis, barley and wheat are known yet the function of the majority of these genes are unknown. Thus, reverse genetic approaches represent tactics to discover the function of these genes (Sessions et al., 2002). The reverse genetics approach helps to detect and select only valuable candidate alleles in a gene to discover a phenotype (Jankowicz-Cieslak et al., 2011). The standard method that has been used to identify the function is gene silencing, which knocks down the targeted gene in the genome to observe the result compared to wild type plants. A limitation of this strategy is that a single phenotype may require several genes to be silenced (Jankowicz-Cieslak et al., 2011). There are several techniques of mutagenesis or gene silencing used to validate genes discovered by reverse genetics, and the technique will be utilized in future research is virus-induced gene silencing (VIGS), which has been successfully used in both monocot and dicot plants.

Tilling

Targeting Induced Local Lesions in Genomes (TILLING) populations is a powerful reverse genetics tool to discover gene function. In the TILLING approach, all mutant individuals from a mutant population are tested at the DNA level for a specific gene (Sikora et al., 2011), thus it is a powerful tool in reverse genetics to get from the genetic variation or mutant allelic series to the mutant phenotype. The TILLING approach is utilized in many crops where priori knowledge of a gene is used to get to the function of the gene by characterizing null mutants as well as mutants with amino acid substitutions which. allows for the identification of critical residues in protein motifs or domains that are required for function. TILLING mutants have been discovered by various methods, including direct sequencing, Li-Cor, high-performance liquid chromatography (HPLC), electrophoresis, and next-generation sequencing (Sikora et al., 2011). Next-generation sequencing (NGS) is one of the more popular methods that significantly accelerated the discovery of mutations at the whole-genome level due to its high accuracy, high throughput, and increased capacity (Sikora et al., 2011). TILLING has been utilized in many crops such as maize (Till et al., 2004), wheat (Slade et al., 2005), rice (Till et al., 2007), and tomato (Menda et al., 2004; Saito et al., 2009).

A TILLING advantage is that it can be utilized in any species regardless of the size of the genome (Kurowska, 2011). High efficacy is achieved due the high-throughput screening capacity to detect mutants from large mutant populations (De-Kai et al., 2006). In general, TILLING involves three steps: (1) a mutagenized population needs to be generated by either physical or chemical mutagenic agents like EMS, (2) collecting DNA mutagenized individuals and detecting mutant genes (3) interpretation and analysis of the mutant's phenotype/s (Kurowska et al., 2011).

References

Acevedo-Garcia, J., Kusch, S., & Panstruga, R. (2014). Magical mystery tour: MLO proteins in plant immunity and beyond. *New Phytologist, 204*(2), 273–281.

Adachi, H., & Tsuda, K. (2019). Convergence of cell-surface and intracellular immune receptor signalling. *New Phytologist, 221*(4), 1676–1678.

Aist JR, Gold RE, Bayles CJ. (1987). Evidence for the involvement of molecular components of papillae in ml-o resistance to barley powdery mildew. *Phytopathology 77*, 17–32.

Alonso, J. M., & Ecker, J. R. (2006). Moving forward in reverse: genetic technologies to enable genome-wide phenomic screens in Arabidopsis. Nature Reviews Genetics, 7(7), 524-536.

AMBA. (2017). National Barley Improvement Committee: Economic Importance of Barley.

Amezrou, R., Gyawali, S., Belqadi, L., Chao, S., Arbaoui, M., Mamidi, S., ... & Verma, R. P. S. (2018). Molecular and phenotypic diversity of ICARDA spring barley (Hordeum vulgare L.) collection. *Genetic resources and crop evolution, 65*(1), 255–269.

Ariyadasa, R., Mascher, M., Nussbaumer, T., Schulte, D., Frenkel, Z., Poursarebani, N., ... & Felder, M. (2014). A sequence-ready physical map of barley anchored genetically by two million single-nucleotide polymorphisms. *Plant physiology*, *164*(1), 412–423.

Armstrong, W. (1979). Aeration in higher plants. *Advances in Botanical Research, 7*, 225–332.

Atkinson, M. M., Midland, S. L., Sims, J. J., & Keen, N. T. (1996). Syringolide 1 triggers Ca2+ influx, K+ efflux, and extracellular alkalization in soybean cells carrying the disease-resistance gene Rpg4. *Plant Physiology, 112*(1), 297–302.

Badr, A., Rabey, H. E., Effgen, S., Ibrahim, H. H., Pozzi, C., Rohde, W., & Salamini, F. (2000). On the origin and domestication history of barley (Hordeum vulgare). *Molecular biology and evolution, 17*(4), 499–510.

Balagué, C., Lin, B., Alcon, C., Flottes, G., Malmström, S., Köhler, C., ... & Roby, D. (2003). HLM1, an essential signaling component in the hypersensitive response, is a member of the cyclic nucleotide–gated channel ion channel family. *The Plant Cell, 15*(2), 365–379.

Balint-Kurti, P. (2019). The plant hypersensitive response: concepts, control and consequences. *Molecular plant pathology, 20*(8), 1163–1178.

Bayer, M. M., Rapazote-Flores, P., Ganal, M., Hedley, P. E., Macaulay, M., Plieske, J., ... & Waugh, R. (2017). Development and evaluation of a barley 50k iSelect SNP array. *Frontiers in Plant Science*, *8*, 1792.

Beier, S., Himmelbach, A., Colmsee, C., Zhang, X. Q., Barrero, R. A., Zhang, Q., ... & Groth, M. (2017). Construction of a map-based reference genome sequence for barley, Hordeum vulgare L. *Scientific Data*, *4*(1), 1–24.

Belfield, E. J., Gan, X., Mithani, A., Brown, C., Jiang, C., Franklin, K., ... & Kalwani, S. (2012). Genome-wide analysis of mutations in mutant lineages selected following fast-neutron irradiation mutagenesis of Arabidopsis thaliana. *Genome Research*, *22*(7), 1306–1315.

Birkenbihl, R. P., & Somssich, I. E. (2011). Transcriptional plant responses critical for resistance towards necrotrophic pathogens. *Frontiers in Plant Science*, *2*, 76.

Bolon, Y. T., Haun, W. J., Xu, W. W., Grant, D., Stacey, M. G., Nelson, R. T., ... & Orf, J. H. (2011). Phenotypic and genomic analyses of a fast neutron mutant population resource in soybean. *Plant physiology, 156*(1), 240–253.

Bouajila, A., Zoghlami, N., Ahmed, M. A., Baum, M., & Nazari, K. (2012). Pathogenicity spectra and screening for resistance in barley against Tunisian Pyrenophora teres f. teres. *Plant Disease*, *96*(10), 1569–1575.

Brodersen, P., Petersen, M., Pike, H. M., Olszak, B., Skov, S., Ødum, N., ... & Mundy, J. (2002). Knockout of Arabidopsis accelerated-cell-death11 encoding a sphingosine transfer protein causes activation of programmed cell death and defense. *Genes & Development, 16*(4), 490–502.

von Bothmer, R., Jacobsen, N., Baden, C., Jorgensen, R. B., & Linde-Laursen, I. (1995). *An ecogeographical study of the genus Hordeum*.

Büschges, R., Hollricher, K., Panstruga, R., Simons, G., Wolter, M., Frijters, A., ... & Töpsch, S. (1997). The barley Mlo gene: a novel control element of plant pathogen resistance. *Cell*, *88*(5), 695–705.

Century, K. S., Shapiro, A. D., Repetti, P. P., Dahlbeck, D., Holub, E., & Staskawicz, B. J. (1997). NDR1, a pathogen-induced component required for Arabidopsis disease resistance. *Science, 278*(5345), 1963–1965.

Chadwick, J. (1932). Possible existence of a neutron. Nature, 129(3252), 312-312.

Chen, F. Q., & Hayes, P. M. (1989). A comparison of Hordeum bulbosum-mediated haploid production efficiency in barley using *in vitro* floret and tiller culture. *Theoretical and applied genetics*, *77*(5), 701–704.

Chiang, Y. H., & Coaker, G. (2015). Effector triggered immunity: NLR immune perception and downstream defense responses. *The Arabidopsis Book, 2015*(13).

Civáň, P., & Brown, T. A. (2017). A novel mutation conferring the nonbrittle phenotype of cultivated barley. *New Phytologist*, *214*(1), 468–472.

Close, T. J., Bhat, P. R., Lonardi, S., Wu, Y., Rostoks, N., Ramsay, L., ... & Bozdag, S. (2009). Development and implementation of high-throughput SNP genotyping in barley. *BMC genomics*, *10*(1), 582.

Clough, S. J., Fengler, K. A., Yu, I. C., Lippok, B., Smith, R. K., & Bent, A. F. (2000). The Arabidopsis dnd1 "defense, no death" gene encodes a mutated cyclic nucleotide-gated ion channel. *Proceedings of the National Academy of Sciences, 97*(16), 9323–9328.

Coll, N. S., Epple, P., & Dangl, J. L. (2011). Programmed cell death in the plant immune system. Cell Death & Differentiation, 18(8), 1247-1256.

Comadran, J., Kilian, B., Russell, J., Ramsay, L., Stein, N., Ganal, M., ... & Hedley, P. (2012). Natural variation in a homolog of Antirrhinum CENTRORADIALIS contributed to spring growth habit and environmental adaptation in cultivated barley. *Nature genetics*, *44*(12), 1388–1392.

Cotton, J. L., Wysocki, W. P., Clark, L. G., Kelchner, S. A., Pires, J. C., Edger, P. P., ... & Duvall, M. R. (2015). Resolving deep relationships of PACMAD grasses: a phylogenomic approach. *BMC plant biology*, *15*(1), 178.

Cuesta-Marcos, A., Szűcs, P., Close, T. J., Filichkin, T., Muehlbauer, G. J., Smith, K. P., & Hayes, P. M. (2010). Genome-wide SNPs and re-sequencing of growth habit and inflorescence genes in barley: implications for association mapping in germplasm arrays varying in size and structure. BMC genomics, 11(1), 707.

Dai, F., Nevo, E., Wu, D., Comadran, J., Zhou, M., Qiu, L., ... & Zhang, G. (2012). Tibet is one of the centers of domestication of cultivated barley. *Proceedings of the National Academy of Sciences*, *109*(42), 16969–16973.

Daneva, A., Gao, Z., Van Durme, M., & Nowack, M. K. (2016). Functions and regulation of programmed cell death in plant development. *Annual Review of Cell and Developmental Biology*, *32*, 441–468.

De-Kai, W. A. N. G., Zong-Xiu, S. U. N., & Yue-Zhi, T. A. O. (2006). Application of TILLING in plant improvement. *Acta Genetica Sinica, 33*(11), 957–964.

Devoto, A., Piffanelli, P., Nilsson, I., Wallin, E., Panstruga, R., von Heijne, G., & Schulze-Lefert, P. (1999). Topology, subcellular localization, and sequence diversity of the Mlo family in plants. *Journal of Biological Chemistry*, *274*(49), 34993–35004.

Distelfeld, A., Li, C., & Dubcovsky, J. (2009). Regulation of flowering in temperate cereals. Current opinion in plant biology, 12(2), 178-184.

Dodds, P. N., Lawrence, G. J., Catanzariti, A. M., Teh, T., Wang, C. I., Ayliffe, M. A., ... & Ellis, J. G. (2006). Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. *Proceedings of the National Academy of Sciences, 103*(23), 8888–8893.

Doebley, J. F., Gaut, B. S., & Smith, B. D. (2006). The molecular genetics of crop domestication. *Cell*, *127*(7), 1309–1321.

Duxbury, Z., Ma, Y., Furzer, O. J., Huh, S. U., Cevik, V., Jones, J. D., & Sarris, P. F. (2016). Pathogen perception by NLRs in plants and animals: *Parallel worlds. BioEssays, 38*(8), 769–781.

Ellis, R. P., Forster, B. P., Robinson, D., Handley, L. L., Gordon, D. C., Russell, J. R., & Powell, W. (2000). Wild barley: a source of genes for crop improvement in the 21st century? *Journal of Experimental Botany*, *51*(342), 9–17.

Faris JD, et al. (2010) A unique wheat disease resistance-like gene governs effector-triggered susceptibility to necrotrophic pathogens. *Proceedings of the National Academy of Sciences of the United States of America, 107*(30):13544–13549.

Felix, G., Duran, J. D., Volko, S., & Boller, T. (1999). Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *The Plant Journal, 18*(3), 265–276.

Ferrari, S., Savatin, D. V., Sicilia, F., Gramegna, G., Cervone, F., & De Lorenzo, G. (2013). Oligogalacturonides: plant damage-associated molecular patterns and regulators of growth and development. *Frontiers in plant science, 4*, 49.

Fukuda, H. (1997). Programmed cell death during vascular system formation. *Cell Death & Differentiation, 4*(8), 684–688.

Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology, 43*, 205–227.

Gomez-Gomez, L., and Boller, T. (2000). FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. *Molecular Cell, 5*, 1003–1011.

Gong, X., Li, C., Zhou, M., Bonnardeaux, Y., & Yan, G. (2014). Seed dormancy in barley is dictated by genetics, environments and their interactions. *Euphytica, 197*(3), 355–368.

Graner, A., Jahoor, A., Schondelmaier, J., Siedler, H., Pillen, K., Fischbeck, G., ... & Herrmann, R. G. (1991). Construction of an RFLP map of barley. *Theoretical and Applied Genetics*, *83*(2), 250–256.

Grant, M., Brown, I., Adams, S., Knight, M., Ainslie, A., & Mansfield, J. (2000). The RPM1 plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. *The Plant Journal, 23*(4), 441–450.

Greenberg, J. T. (1996). Programmed cell death: a way of life for plants. *Proceedings of the National Academy of Sciences, 93*(22), 12094–12097.

Guzman, P., & Ecker, J. R. (1990). Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. The Plant Cell, 2(6), 513-523.

Halterman, D., Zhou, F., Wei, F., Wise, R. P., & Schulze-Lefert, P. (2001). The MLA6 coiled-coil, NBS-LRR protein confers AvrMla6-dependent resistance specificity to Blumeria graminis f. sp. hordei in barley and wheat. The Plant Journal, 25(3), 335-348.

Hamblin, M. T., Close, T. J., Bhat, P. R., Chao, S., Kling, J. G., Abraham, K. J., ... & Hayes, P. M. (2010). Population structure and linkage disequilibrium in US barley germplasm: implications for association mapping. *Crop Science*, *50*(2), 556–566.

Heese, A., Hann, D.R., Gimenez-Ibanez, S., Jones, A.M., He, K., Li, J., Schroeder, J.I., Peck, S.C., and Rathjen, J.P. (2007). The receptor- like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. *Proceedings of the National Academy of Sciences, 104*, 12217–12222.

Herzig, P., Maurer, A., Draba, V., Sharma, R., Draicchio, F., Bull, H., ... & Pillen, K. (2018). Contrasting genetic regulation of plant development in wild barley grown in two European environments revealed by nested association mapping. *Journal of Experimental Botany*, *69*(7), 1517–1531.

Horsley, R. D., Schwarz, P. B., & Hammond, J. J. (1995). Genetic diversity in malt quality of North American six-rowed spring barley. *Crop science*, *35*(1), 113–118.

Hua, J., Grisafi, P., Cheng, S. H., & Fink, G. R. (2001). Plant growth homeostasis is controlled by the Arabidopsis BON1 and BAP1 genes. *Genes & Development, 15*(17), 2263–2272.

Hubbard, A., Lewis, C. M., Yoshida, K., Ramirez-Gonzalez, R. H., de Vallavieille-Pope, C., Thomas, J., ... & Saunders, D. G. (2015). Field pathogenomics reveals the emergence of a diverse wheat yellow rust population. *Genome biology*, *16*(1), 23.

Hussain, M., Iqbal, M. A., Till, B. J., & Rahman, M. U. (2018). Identification of induced mutations in hexaploid wheat genome using exome capture assay. *PloS one*, *13*(8), e0201918.

Huysmans, M., Coll, N. S., & Nowack, M. K. (2017). Dying two deaths—programmed cell death regulation in development and disease. Current opinion in plant biology, 35, 37-44.

IBGSC (2012) A physical, genetic and functional sequence assembly of the barley genome. *Nature 491*, 711–716

Iriondo, J. M., Milla, R., Volis, S., & Rubio de Casas, R. (2018). Reproductive traits and evolutionary divergence between Mediterranean crops and their wild relatives. *Plant Biology*, *20*, 78–88.

Jacob, F., Vernaldi, S., and Maekawa, T. (2013). Evolution and conservation of plant NLR functions. *Frontiers in Immunology, 4*:297. doi: 10.3389/fimmu.2013.00297

Jambunathan, N., Siani, J. M., & McNellis, T. W. (2001). A humidity–sensitive Arabidopsis copine mutant exhibits precocious cell death and increased disease resistance. *The Plant Cell, 13*(10), 2225–2240.

Jankowicz-Cieslak, J., Huynh, O. A., Bado, S., Matijevic, M., & Till, B. J. (2011). Reverse-Genetics by Tilling Expands Through the Plant Kingdom. *Emirates Journal of Food and Agriculture*, 290–300.

Johal, G. S., Hulbert, S. H., & Briggs, S. P. (1995). Disease lesion mimics of maize: a model for cell death in plants. *Bioessays, 17*(8), 685–692.

Jones, G., Jones, H., Charles, M. P., Jones, M. K., Colledge, S., Leigh, F. J., ... & Brown, T. A. (2012). Phylogeographic analysis of barley DNA as evidence for the spread of Neolithic agriculture through Europe. *Journal of Archaeological Science*, *39*(10), 3230–3238.

Jones, H., Civáň, P., Cockram, J., Leigh, F. J., Smith, L. M., Jones, M. K., ... & Brown, T. A. (2011). Evolutionary history of barley cultivation in Europe revealed by genetic analysis of extant landraces. *BMC Evolutionary Biology*, *11*(1), 320.

Jones, J. D., & Dangl, J. L. (2006). The plant immune system. nature, 444(7117), 323-329.

Jørgensen, I. H. (1992). Discovery, characterization and exploitation of Mlo powdery mildew resistance in barley. Euphytica, 63(1-2), 141-152.

Jurkowski, G. I., Smith Jr, R. K., Yu, I. C., Ham, J. H., Sharma, S. B., Klessig, D. F., ... & Bent, A. F. (2004). Arabidopsis DND2, a second cyclic nucleotide-gated ion channel gene for which mutation causes the "defense, no death" phenotype. *Molecular Plant-Microbe Interactions, 17*(5), 511–520.

Karsai, I., Szűcs, P., Mészáros, K., Filichkina, T., Hayes, P. M., Skinner, J. S., ... & Bedő, Z. (2005). The Vrn-H2 locus is a major determinant of flowering time in a facultative× winter growth habit barley (Hordeum vulgare L.) mapping population. Theoretical and Applied Genetics, 110(8), 1458-1466.

Kato, K. (2003). Genetic analysis of two genes for vernalization response, the former Vrn2 and Vrn4, by using PCR based molecular markers. In Proc. 10th Int. Wheat Genet. Symp., 2003 (Vol. 3, pp. 971-973).

Kazan, K., & Lyons, R. (2014). Intervention of phytohormone pathways by pathogen effectors. *The Plant Cell*, *26*(6), 2285–2309.

Kellogg, E. A. (1998). Relationships of cereal crops and other grasses. *Proceedings of the National Academy of Sciences*, *95*(5), 2005–2010.

Kippes, N., Debernardi, J. M., Vasquez-Gross, H. A., Akpinar, B. A., Budak, H., Kato, K., ... & Dubcovsky, J. (2015). Identification of the VERNALIZATION 4 gene reveals the origin of spring growth habit in ancient wheats from South Asia. Proceedings of the National Academy of Sciences, 112(39), E5401-E5410.

Kleinhofs, A., Kilian, A., Maroof, M. S., Biyashev, R. M., Hayes, P., Chen, F. Q., ... & Ananiev, E. (1993). A molecular, isozyme and morphological map of the barley (Hordeum vulgare) genome. *Theoretical and Applied Genetics*, *86*(6), 705–712.

Komatsuda, T., Maxim, P., Senthil, N., & Mano, Y. (2004). High-density AFLP map of nonbrittle rachis 1 (btr1) and 2 (btr2) genes in barley (Hordeum vulgare L.). *Theoretical and Applied Genetics*, *109*(5), 986–995.

Komatsuda, T., Pourkheirandish, M., He, C., Azhaguvel, P., Kanamori, H., Perovic, D., ... & Lundqvist, U. (2007). Six-rowed barley originated from a mutation in a homeodomain-leucine zipper I-class homeobox gene. *Proceedings of the National Academy of Sciences*, *104*(4), 1424–1429.

Kumawat, S., Rana, N., Bansal, R., Vishwakarma, G., Mehetre, S., Das, B. K., ... & Deshmukh, R. (2019). Fast Neutron Mutagenesis in Plants: Advances, Applicability and Challenges.

Kurowska, M., Daszkowska-Golec, A., Gruszka, D., Marzec, M., Szurman, M., Szarejko, I., & Maluszynski, M. (2011). TILLING-a shortcut in functional genomics. *Journal of applied genetics*, *52*(4), 371.

Laurie, D. A., Pratchett, N., Snape, J. W., & Bezant, J. H. (1995). RFLP mapping of five major genes and eight quantitative trait loci controlling flowering time in a winter× spring barley (Hordeum vulgare L.) cross. *Genome, 38*(3), 575–585.

Levine, A., Pennell, R. I., Alvarez, M. E., Palmer, R., & Lamb, C. (1996). Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. *Current Biology*, *6*(4), 427–437.

Li, H., Chen, G., & Yan, W. (2015). Molecular characterization of barley 3H semi-dwarf genes. *PLoS One*, *10*(3), e0120558.

Li, K., Hegarty, J., Zhang, C., Wan, A., Wu, J., Guedira, G. B., ... & Dubcovsky, J. (2016). Fine mapping of barley locus Rps6 conferring resistance to wheat stripe rust. *Theoretical and applied genetics*, *129*(4), 845–859.

Li, T., & Bai, G. (2009). Lesion mimic associates with adult plant resistance to leaf rust infection in wheat. *Theoretical and applied genetics, 119*(1), 13–21.

Liang, H., Yao, N., Song, J. T., Luo, S., Lu, H., & Greenberg, J. T. (2003). Ceramides modulate programmed cell death in plants. *Genes & development, 17*(21), 2636–2641.

Liller, C. B., Walla, A., Boer, M. P., Hedley, P., Macaulay, M., Effgen, S., ... & Koornneef, M. (2017). Fine mapping of a major QTL for awn length in barley using a multiparent mapping population. *Theoretical and Applied Genetics*, *130*(2), 269–281.

Liu, H., Bayer, M., Druka, A., Russell, J. R., Hackett, C. A., Poland, J., ... & Waugh, R. (2014). An evaluation of genotyping by sequencing (GBS) to map the Breviaristatum-e (ari-e) locus in cultivated barley. *BMC genomics*, *15*(1), 1–11.

Liu, Y., & He, C. (2017). A review of redox signaling and the control of MAP kinase pathway in plants. *Redox biology, 11*, 192–204.

Liu, Z. W., Biyashev, R. M., & Maroof, M. S. (1996). Development of simple sequence repeat DNA markers and their integration into a barley linkage map. *Theoretical and Applied Genetics*, *93*(5-6), 869–876.

Lorrain, S., Vailleau, F., Balagué, C., & Roby, D. (2003). Lesion mimic mutants: keys for deciphering cell death and defense pathways in plants? *Trends in plant science, 8*(6), 263–271.

Lu, D., Wu, S., Gao, X., Zhang, Y., Shan, L., & He, P. (2010). A receptor-like cytoplasmic kinase, BIK1, associates with a flagellin receptor complex to initiate plant innate immunity. *Proceedings of the National Academy of Sciences, 107*(1), 496–501.

Macho, A.P., and Zipfel, C. (2014). Plant PRRs and the activation of innate immune signaling. *Molecular Cell, 54*, 263–272.

Martis, M. M., Zhou, R., Haseneyer, G., Schmutzer, T., Vrána, J., Kubaláková, M., ... & Korzun, V. (2013). Reticulate evolution of the rye genome. The Plant Cell, 25(10), 3685-3698.

Mascher, M., Gundlach, H., Himmelbach, A., Beier, S., Twardziok, S. O., Wicker, T., ... & Bayer, M. (2017). A chromosome conformation capture ordered sequence of the barley genome. *Nature*, *544*(7651), 427–433.

Mascher, M., Muehlbauer, G. J., Rokhsar, D. S., Chapman, J., Schmutz, J., Barry, K., ... & Himmelbach, A. (2013). Anchoring and ordering NGS contig assemblies by population sequencing (POPSEQ). *The Plant Journal*, *76*(4), 718–727.

Mascher, M., Richmond, T. A., Gerhardt, D. J., Himmelbach, A., Clissold, L., Sampath, D., ... & Akhunov, E. D. (2013). Barley whole exome capture: a tool for genomic research in the genus Hordeum and beyond. *The Plant Journal, 76*(3), 494–505.

Mascher, M., Schuenemann, V. J., Davidovich, U., Marom, N., Himmelbach, A., Hübner, S., ... & Schreiber, M. (2016). Genomic analysis of 6,000-year-old cultivated grain illuminates the domestication history of barley. *Nature Genetics*, *48*(9), 1089–1093.

Mba, C., Afza, R., & Shu, Q. Y. (2012). Mutagenic radiations: X-rays, ionizing particles and ultraviolet. *Plant Mutation Breeding and Biotechnology*, 83–90.

McCallum, C. M., Comai, L., Greene, E. A., & Henikoff, S. (2000). Targeting induced locallesions in genomes (TILLING) for plant functional genomics. *Plant physiology, 123*(2), 439–442.

Mehdy, M.C. (1994). Active oxygen species in plant defense against pathogens. *Plant Physiol. 105*, 467–472.

Menda, N., Semel, Y., Peled, D., Eshed, Y., & Zamir, D. (2004). In silico screening of a saturated mutation library of tomato. The Plant Journal, 38(5), 861-872.

Meng, X., & Zhang, S. (2013). MAPK cascades in plant disease resistance signaling. *Annual Review of Phytopathology, 51*, 245–266.

Meyers, B. C., Kozik, A., Griego, A., Kuang, H., & Michelmore, R. W. (2003). Genome-wide analysis of NBS-LRR–encoding genes in Arabidopsis. *The Plant Cell, 15*(4), 809–834.

Middleton, C. P., Senerchia, N., Stein, N., Akhunov, E. D., Keller, B., Wicker, T., & Kilian, B. (2014). Sequencing of chloroplast genomes from wheat, barley, rye and their relatives provides a detailed insight into the evolution of the Triticeae tribe. *PLoS One*, *9*(3), e85761.

Mikel, M. A., & Kolb, F. L. (2008). Genetic diversity of contemporary North American barley. *Crop science*, *48*(4), 1399–1407.

Moeder, W., & Yoshioka, K. (2008). Lesion mimic mutants: a classical, yet still fundamental approach to study programmed cell death. *Plant signaling & behavior*, *3*(10), 764–767.

Molnár-Láng, M., Linc, G., & Szakács, É. (2014). Wheat–barley hybridization: the last 40 years. Euphytica, 195(3), 315-329.

Monaghan, J., and Zipfel, C. (2012). Plant pattern recognition receptor complexes at the plasma membrane. *Curr*. *Opin*. *Plant Biology, 15*, 349–357. doi: 10.1016/j.pbi.2012.05.006

Moore, G., Devos, K. M., Wang, Z., & Gale, M. D. (1995). Cereal genome evolution: grasses, line up and form a circle. *Current Biology*, *5*(7), 737–739.

Morrell, P. L., & Clegg, M. T. (2007). Genetic evidence for a second domestication of barley (Hordeum vulgare) east of the Fertile Crescent. *Proceedings of The National Academy of Sciences*, *104*(9), 3289–3294.

Muir, C. E., and Nilan, N. A. (1973). Registration of Steptoe barley. *Crop Science, 13*, 770.

Muller, H. J., & Muller, H. J. (1930). Types of visible variations induced by X-rays in Drosophila. *Journal of Genetics, 22*(03).

Müller, K. J., Romano, N., Gerstner, O., Garcia-Marotot, F., Pozzi, C., Salamini, F., & Rohde, W. (1995). The barley Hooded mutation caused by a duplication in a homeobox gene intron. *Nature*, *374*(6524), 727–730.

Muñoz-Amatriaín, M., Cuesta-Marcos, A., Endelman, J. B., Comadran, J., Bonman, J. M., Bockelman, H. E., ... & Muehlbauer, G. J. (2014). The USDA barley core collection: genetic diversity, population structure, and potential for genome-wide association studies. *PloS one*, *9*(4), e94688.

Muñoz-Amatriaín, M., Moscou, M. J., Bhat, P. R., Svensson, J. T., Bartoš, J., Suchánková, P., ... & Castillo, A. M. (2011). An improved consensus linkage map of barley based on flow-sorted chromosomes and single nucleotide polymorphism markers. *The Plant Genome*, *4*(3), 238–249.

Muñoz-Amatriaín, M., Lonardi, S., Luo, M., Madishetty, K., Svensson, J. T., Moscou, M. J., ... & Wise, R. P. (2015). Sequencing of 15 622 gene-bearing BAC s clarifies the gene-dense regions of the barley genome. *The Plant Journal*, *84*(1), 216–227.

Neale, D. B., Saghai–Maroof, M. A., Allard, R. W., Zhang, Q., & Jorgensen, R. A. (1988). Chloroplast DNA diversity in populations of wild and cultivated barley. *Genetics*, *120*(4), 1105–1110.

Newman, C. W., & Newman, R. K. (2006). A brief history of barley foods. *Cereal foods world*, *51*(1), 4–7.

Oliver, R.P., & Solomon, P.S. (2010). New developments in pathogenicity and virulence of necrotrophs. *Current Opinion in Plant Biology, 13*(4), 415–419.

Olvera-Carrillo, Y., Van Bel, M., Van Hautegem, T., Fendrych, M., Huysmans, M., Simaskova, M., ... & Coppens, F. (2015). A conserved core of programmed cell death indicator genes discriminates developmentally and environmentally induced programmed cell death in plants. *Plant physiology, 169*(4), 2684–2699.

Opanowicz, M., Vain, P., Draper, J., Parker, D., & Doonan, J. H. (2008). Brachypodium distachyon: making hay with a wild grass. *Trends in plant science*, *13*(4), 172–177.

Orabi, J., Backes, G., Wolday, A., Yahyaoui, A., & Jahoor, A. (2007). The Horn of Africa as a centre of barley diversification and a potential domestication site. *Theoretical and Applied Genetics*, *114*(6), 1117–1127.

Pandelova I, et al. (2009) Analysis of transcriptome changes induced by Ptr ToxA in wheat provides insights into the mechanisms of plant susceptibility. *Molecular Plant 2*(5):1067–1083.

Pandey, P., Leary, A. Y., Tümtas, Y., Savage, Z., Dagvadorj, B., Tan, E., ... & Mirkin, F. G. (2020). The Irish potato famine pathogen subverts host vesicle trafficking to channel starvation-induced autophagy to the pathogen interface. *bioRxiv*.

Paterson, A. H., Bowers, J. E., Peterson, D. G., Estill, J. C., & Chapman, B. A. (2003). Structure and evolution of cereal genomes. *Current Opinion in Genetics & Development*, *13*(6), 644–650.

Pennell, R.I., and Lamb, C. (1997). Programmed Cell Death in Plants. *Plant Cell, 9*, 1157–1168.

Pieterse, C. M., Leon-Reyes, A., Van der Ent, S., & Van Wees, S. C. (2009). Networking by small-molecule hormones in plant immunity. *Nature chemical biology*, *5*(5), 308–316.

Pieterse, C. M., Van der Does, D., Zamioudis, C., Leon-Reyes, A., & Van Wees, S. C. (2012). Hormonal modulation of plant immunity. *Annual Review of Cell and Developmental Biology, 28*.

Poets, A. M., Fang, Z., Clegg, M. T., & Morrell, P. L. (2015). Barley landraces are characterized by geographically heterogeneous genomic origins. *Genome biology*, *16*(1), 1–11.

Pourkheirandish, M., & Komatsuda, T. (2007). The importance of barley genetics and domestication in a global perspective. *Annals of Botany*, *100*(5), 999–1008.

Pourkheirandish, M., Hensel, G., Kilian, B., Senthil, N., Chen, G., Sameri, M., ... & Mascher, M. (2015). Evolution of the grain dispersal system in barley. *Cell, 162*(3), 527–539. Available at: http://dx.doi.org/10.1016/j.cell.2015.07.002.

Purugganan, M. D., & Fuller, D. Q. (2009). The nature of selection during plant domestication. *Nature*, *457*(7231), 843–848.

Qi, D., & Innes, R. W. (2013). Recent advances in plant NLR structure, function, localization, and signaling. *Frontiers in immunology, 4*, 348.

Qi, X., Stam, P., & Lindhout, P. (1998). Use of locus specific AFLP markers to construct a high-density molecular map in barley. *Theoretical and Applied Genetics*, *96*(3-4), 376–384.

Qu, L. J., & Qin, G. (2014). Generation and identification of Arabidopsis EMS mutants. In *Arabidopsis Protocols* (pp. 225–239). Humana Press, Totowa, NJ.

Ramsay, L., Macaulay, M., Degli Ivanissevich, S., MacLean, K., Cardle, L., Fuller, J., ... & Maestri, E. (2000). A simple sequence repeat-based linkage map of barley. *Genetics*, *156*(4), 1997–2005.

Raz, V., & Fluhr, R. (1992). Calcium requirement for ethylene-dependent responses. The Plant Cell, 4(9), 1123-1130.

Raz, V., & Fluhr, R. (1993). Ethylene signal is transduced via protein phosphorylation events in plants. The Plant Cell, 5(5), 523-530.

Richards, J. K., Friesen, T. L., & Brueggeman, R. S. (2017). Association mapping utilizing diverse barley lines reveals net form net blotch seedling resistance/susceptibility loci. *Theoretical and Applied Genetics, 130*(5), 915–927.

Rostoks, N., Schmierer, D., Mudie, S., Drader, T., Brueggeman, R., Caldwell, D. G., ... & Kleinhofs, A. (2006). Barley necrotic locus nec1 encodes the cyclic nucleotide-gated ion channel 4 homologous to the Arabidopsis HLM1. *Molecular Genetics and Genomics, 275*(2), 159–168.

Russell, J., Mascher, M., Dawson, I. K., Kyriakidis, S., Calixto, C., Freund, F., ... & Hofstad, A. (2016). Exome sequencing of geographically diverse barley landraces and wild relatives gives insights into environmental adaptation. *Nature Genetics*, *48*(9), 1024–1030.

Saito, T., Asamizu, E., Mizoguchi, T., Fukuda, N., Matsukura, C., & Ezura, H. (2009). Mutant resources for the miniature tomato (Solanum lycopersicum L.)'Micro-Tom'. Journal of the Japanese Society for Horticultural Science, 78(1), 6-13.

Salamini, F., Özkan, H., Brandolini, A., Schäfer-Pregl, R., & Martin, W. (2002). Genetics and geography of wild cereal domestication in the near east. *Nature Reviews Genetics*, *3*(6), 429–441.

Sang, T. (2009). Genes and mutations underlying domestication transitions in grasses. *Plant Physiology*, *149*(1), 63–70.

Sarin, S., Prabhu, S., O'Meara, M. M., Pe'er, I., and Hobert, O. (2008). Caenorhabditis elegans mutant allele identification by whole- genome sequencing. *Natural Methods, 5*, 865–867.

Sasani, S., Hemming, M. N., Oliver, S. N., Greenup, A., Tavakkol-Afshari, R., Mahfoozi, S., ... & Trevaskis, B. (2009). The influence of vernalization and daylength on expression of flowering-time genes in the shoot apex and leaves of barley (Hordeum vulgare). Journal of Experimental Botany, 60(7), 2169-2178.

Schindler, T., Bergfeld, R., & Schopfer, P. (1995). Arabinogalactan proteins in maize coleoptiles: developmental relationship to cell death during xylem differentiation but not to extension growth. *The Plant Journal, 7*(1), 25–36.

Schreiber, M., Barakate, A., Uzrek, N., Macaulay, M., Sourdille, A., Morris, J., ... & Waugh, R. (2019). A highly mutagenised barley (cv. Golden Promise) TILLING population coupled with strategies for screening-by-sequencing. *Plant Methods*, *15*(1), 99.

Schreiber, M., Mascher, M., Wright, J., Padmarasu, S., Himmelbach, A., Heavens, D., ... & Waugh, R. (2020). A genome assembly of the barley 'transformation reference'cultivar Golden Promise. *G3: Genes, Genomes, Genetics, 10*(6), 1823–1827.

Schulte, D., Close, T. J., Graner, A., Langridge, P., Matsumoto, T., Muehlbauer, G., ... & Stein, N. (2009). The international barley sequencing consortium—at the threshold of efficient access to the barley genome. *Plant Physiology*, *149*(1), 142–147.

Schwarz, P., Horsley, R., and Scott, H. (2011). History of barley production in the USA. Proc. Master Brewer Association of the Americas, Minneapolis, MN.

Sessions, A., Burke, E., Presting, G., Aux, G., McElver, J., Patton, D., Dietrich, B., Ho, P., Bacwaden, J., Ko, C., Clarke, J.D., Cotton, D., Bullis, D., Snell, J., Miguel, T., Hutchison, D., Kimmerly, B., Mitzel, T., Katagiri, F., Glazebrook, J., Law, M., and Goff, S.A. (2002). A high throughput arabidopsis reverse genetics system. *Plant Cell, 14*, 2985–2994.

Shinya, T., Nakagawa, T., Kaku, H., & Shibuya, N. (2015). Chitin-mediated plant–fungal interactions: catching, hiding and handshaking. *Current Opinion in Plant Biology, 26*, 64–71.

Sikora, P., Chawade, A., Larsson, M., Olsson, J., & Olsson, O. (2011). Mutagenesis as a tool in plant genetics, functional genomics, and breeding. *International Journal of Plant Genomics*.

Slade, A. J., Fuerstenberg, S. I., Loeffler, D., Steine, M. N., & Facciotti, D. (2005). A reverse genetic, nontransgenic approach to wheat crop improvement by TILLING. Nature biotechnology, 23(1), 75-81.

Smetana, O. (2010). Molecular and cellular aspects of programmed cell death in response to genotoxics in plants.

Smith, K. P., Rasmusson, D. C., Schiefelbein, E., Wiersma, J. J., Wiersma, J. V., Budde, A., ... & Steffenson, B. (2010). Registration of 'Rasmusson'barley. *Journal of Plant Registrations*, *4*(3), 167–170.

Staal, J., & Dixelius, C. (2009). Plant innate immunity. In: Encylopedia of Life Sciences (ELS). Wiley, Chichester.

Stadler, L. J. (1932). On the genetic nature of induced mutation in plants. Proc. 6th int. Congr. Genet. (Ithaca),1, 274–94.

Taibi, K., Bentata, F., Rehman, S., Labhilili, M., El Aissami, A., Verma, R. P. S., & Gyawali, S. (2016). Virulence of Moroccan Pyrenophora teres f. teres revealed by international differential barley genotypes. *Cereal Research Communications*, *44*(2), 263–271.

Takahashi, R., & Yasuda, S. (1956). Genetic studies of spring and winter habit of growth in barley. Berichte des Ohara Instituts für Landwirtschaftliche Biologie, Okayama Universität, 10(4), 245-308.

Tamang, P., Neupane, A., Mamidi, S., Friesen, T., & Brueggeman, R. (2015). Association mapping of seedling resistance to spot form net blotch in a worldwide collection of barley. *Phytopathology, 105*(4), 500–508.

Till, B. J., Cooper, J., Tai, T. H., Colowit, P., Greene, E. A., Henikoff, S., & Comai, L. (2007). Discovery of chemically induced mutations in rice by TILLING. BMC plant biology, 7(1), 19.

Till, B. J., Reynolds, S. H., Weil, C., Springer, N., Burtner, C., Young, K., ... & Greene, E. A. (2004). Discovery of induced point mutations in maize genes by TILLING. BMC plant biology, 4(1), 12.

Tornero, P., Merritt, P., Sadanandom, A., Shirasu, K., Innes, R. W., & Dangl, J. L. (2002). RAR1 and NDR1 contribute quantitatively to disease resistance in Arabidopsis, and their relative contributions are dependent on the R gene assayed. *The Plant Cell, 14*(5), 1005–1015.

Trevaskis, B., Bagnall, D. J., Ellis, M. H., Peacock, W. J., & Dennis, E. S. (2003). MADS box genes control vernalization-induced flowering in cereals. Proceedings of the National Academy of Sciences, 100(22), 13099-13104.

Uauy, C., Paraiso, F., Colasuonno, P., Tran, R. K., Tsai, H., Berardi, S., ... & Dubcovsky, J. (2009). A modified TILLING approach to detect induced mutations in tetraploid and hexaploid wheat. *BMC Plant Biology, 9*(1), 115.

Ullrich, S. E. (2010). *Barley: Production, improvement, and uses (Vol. 12)*. John Wiley & Sons.

Van Doorn, W. G., Beers, E. P., Dangl, J. L., Franklin-Tong, V. E., Gallois, P., Hara-Nishimura, I., ... & Mur, L. A. J. (2011). Morphological classification of plant cell deaths. *Cell Death & Differentiation*, *18*(8), 1241–1246.

Vidaurre, D., & Bonetta, D. (2012). Accelerating forward genetics for cell wall deconstruction. *Frontiers in Plant Science, 3*, 119.

Visioni, A. Rehman, S., Viash, S. S., Singh, S. P., Vishwakarma, R., Gyawali, S., … &Verma R. P. S. (2020). Genome Wide Association Mapping of Spot Blotch Resistance at Seedling and Adult Plant Stages in Barley.Frontiers in plant science, 11, 642.

Vleeshouwers, V. G., & Oliver, R. P. (2015). Effectors as Tools in Disease Resistance Breeding Against Biotrophic, Hemibiotrophic, and Necrotrophic Plant Pathogens. Molecular plant-microbe interactions: MPMI, 2015(1), 40-50.

Wang, J., Ye, B., Yin, J., Yuan, C., Zhou, X., Li, W., ... & Ma, B. (2015). Characterization and fine mapping of a light-dependent leaf lesion mimic mutant 1 in rice. *Plant Physiology and Biochemistry, 97*, 44–51.

Wang, X., Richards, J., Gross, T., Druka, A., Kleinhofs, A., Steffenson, B., ... & Brueggeman, R. (2013). The rpg4-mediated resistance to wheat stem rust (Puccinia graminis) in barley (Hordeum vulgare) requires Rpg5, a second NBS-LRR gene, and an actin depolymerization factor. *Molecular Plant-Microbe Interactions, 26*(4), 407–418.

Wang, Y., Ren, X., Sun, D., & Sun, G. (2015). Origin of worldwide cultivated barley revealed by NAM-1 gene and grain protein content. *Frontiers in Plant Science*, *6*, 803.

Wang, Y., Ren, X., Sun, D., & Sun, G. (2016). Molecular evidence of RNA polymerase II gene reveals the origin of worldwide cultivated barley. *Scientific Reports*, *6*, 36122.

Xingquan, Z., Xu, T., Zhihao, L., Wang, Y., Li, X., Xu, S., ... & Jiabu, D. (2020). An improved high-quality genome assembly and annotation of Tibetan hulless barley. *Scientific Data, 7*(1).

Xu, Y., Jia, Q., Zhou, G., Zhang, X. Q., Angessa, T., Broughton, S., ... & Li, C. (2017). Characterization of the sdw1 semi-dwarf gene in barley. *BMC Plant Biology*, *17*(1), 11.

Yu, Y., Tomkins, J. P., Waugh, R., Frisch, D. A., Kudrna, D., Kleinhofs, A., ... & Wing, R. A. (2000). A bacterial artificial chromosome library for barley (Hordeum vulgare L.) and the identification of clones containing putative resistance genes. *Theoretical and Applied Genetics*, *101*(7), 1093–1099.

Zeng, L. (2005). A novel mechanism underlying programmed cell death in plant defense signaling (Doctoral dissertation, The Ohio State University).

Zeng, X., Long, H., Wang, Z., Zhao, S., Tang, Y., Huang, Z., ... & Yao, X. (2015). The draft genome of Tibetan hulless barley reveals adaptive patterns to the high stressful Tibetan Plateau. Proceedings of the National Academy of Sciences, 112(4), 1095-1100.

Zhang, Y., Lubberstedt, T., & Xu, M. (2013). The genetic and molecular basis of plant resistance to pathogens. *Journal of Genetics and Genomics, 40*(1), 23–35.

Zhang, Z., Lenk, A., Andersson, M. X., Gjetting, T., Pedersen, C., Nielsen, M. E., ... & Thordal-Christensen, H. (2008). A lesion-mimic syntaxin double mutant in Arabidopsis reveals novel complexity of pathogen defense signaling. *Molecular Plant, 1*(3), 510–527.

Zhou, F., Kurth, J., Wei, F., Elliott, C., Valè, G., Yahiaoui, N., ... & Schulze-Lefert, P. (2001). Cell-autonomous expression of barley Mla1 confers race-specific resistance to the powdery mildew fungus via a Rar1-independent signaling pathway. *The Plant Cell, 13*(2), 337–350.

Zhou, M. X. (2009). Barley production and consumption. In *Genetics and improvement of barley malt quality* (pp. 1-17). Springer, Berlin, Heidelberg.

Zohary, D., & Hopf, M. (2000). *Domestication of plants in the Old World: The origin and spread of cultivated plants in West Asia, Europe and the Nile Valley (No. Ed. 3).* Oxford University Press.

CHAPTER 2: UNDERSTANDING PROGRAMMED CELL DEATH PATHWAYS BY CHARACTERIZING BARLEY DISEASE LESION MIMIC MUTANTS

Abstract

Programmed cell death (PCD) in plants refers to localized cell death resulting from normal biological processes, including development. However, PCD has also evolved as a significant component of the plant's innate immune system, which is effective against a broad taxonomy of plant pathogens, including bacteria, fungi, viruses and invertebrates. Upon pathogen perception, typically through cell surface or cytosolically localized immunity receptors, salicylic acid and reactive oxygen intermediate (ROI) signaling responses are triggered that contribute to the outcome of disease resistance or susceptibility. This outcome is highly dependent upon the spatial and temporal expression of PCD. Efforts to identify the conserved genes that regulate PCD pathways have included the generation and characterization of disease lesion mimic mutants (DLMM) utilizing forward genetic approaches. The DLMMs can identify negative regulators of PCD as the spontaneous expression may result from the deletion of gene/s that directly or indirectly suppress PCD when it is not needed. Five non-allelic fast neutrons generated barley DLMMs in the variety Steptoe background (fast neutron (FN)360, FN361, FN365, FN370, and FN396) were genetically mapped by crossing each with the variety Morex followed by mutant screening from ~400 F_2 individuals of each population. Allele frequency mapping of the homozygous mutant individuals from the five populations was used to map each of the mutation/s to distinct chromosomal locations: 1) FN360 (chromosomes 1H and 6H), 2) FN362 (chromosome 6H), 3) FN365 (chromosome 1H and 5H), FN370 (chromosome 1H) and FN396 (chromosome 2H). Genetic to physical mapping using the cultivar (cv) Morex genome sequence was utilized to identify flanking markers for each DLMM gene locus. Thus, the mutant

mapping reported is the groundwork towards identifying and characterizing these genes, which will ultimately further the understanding of the molecular mechanisms governing PCD in barley and other plant species filling critical knowledge gaps in regard to plant development and disease resistance.

Introduction

Plant growth and development result from cell division and cell specialization that results in differentiated tissues and plant morphology. Plant cells also undergo programmed cell death (PCD) for specific outcomes such as leaf morphology (Greenberg, 1996), formation of the vascular system (Daneva et al., 2016), sex determination (Greenberg, 1996), protection from pathogen attack (Mittler et al., 1997) and pollen tube growth and arrest (Wang et al., 1996). Environmental conditions that result in abiotic stress conditions can also trigger PCD responses that evolved to deal with the threat to a plant's survival and reproduction. For example, plants develop schintia to prevent waterlogging during flooding; thus, PCD pathways evolved to adapt the plant to stressful abiotic or environmental conditions. These pathways also evolved or were coopted as a significant component of the plant's innate immune system effective against a range of phytopathogens to protect against constant challenge by a diverse array of opportunistic microbes which represent potential and specialized pathogens.

The programmed cell death process eliminates unwanted cells during developmental stages in plants. For example, PCD occurs in parenchyma cells to differentiate into xylem surrounding wound site (Greenberg, 1996). There are also proteins involve in PCD that loosen the cell wall to modify the cell as PCD progresses, such as arabinogalactan proteins in maize coleoptiles (Schindler et al., 1995). Also, PCD occurs in response to biotic or abiotic cues, or stress and have been characterized as two types. The PCD that can occur due to injury or

pathogen detection, that is not confined to a single cell and expands to foci containing several neighboring cells is known as cell necrosis (Palavan-Unsal et al., 2005). Cell necrosis is also induced by phytotoxins that result in the second type known as apoptosis, which is defined as a tightly regulated PCD process that occurs with little or no damage to neighboring cells (Palavan-Unsal et al., 2005). Apoptosis is induced in a cell by several proteases that contains a cysteine amino acid at their active site and degrades their target proteins at specific aspartic acids (Palavan-Unsal et al., 2005).

The genes controlling developmental and stress-induced PCD responses are presumed to be evolutionarily conserved among plant species, yet, many have not been identified, and their underlying mechanisms represent a vital knowledge gap (Olvera-Carrillo, 2015). A significant reason for the lack of genetic and subsequent functional analysis of the genes underlying PCD is that they are conserved, thus, have limited genetic and phenotypic polymorphism. An effective way to identify genes involved in these conserved PCD pathways is inducing mutations in these genes. Plants containing these mutations can be identified by forwarding genetics, which first requires phenotyping mutant populations for physiological disorders that are presumed to be in these pathways. Some of these mutants display spontaneous disease lesion mimic mutant (DLMM) phenotypes (Figure 1). Genetic mapping and identification of the genes underlying these DLMMs will fill knowledge gaps concerning the mechanisms of PCD that plants utilize for innate immunity responses.

The generation of DLMMs in the plant requires mutagenic agents, including physical or chemical agents. First, seed (M_0) are treated with the mutagenic agent; in this study, the barley seed were exposed to fast neutron radiation and the M_0 seed were planted to advance to the M_1 generation. Due to the heterozygous nature of the mutated genes at the M_1 generation the

recessive mutations typically do not result in observable mutant phenotypes. However, visual observation of the M_2 individuals allowed for the identification of homozygous mutant individuals containing phenotypes of interest. Similar methods have been utilized in diverse crop species for gene functional characterization, analysis and validation. The Targeting Induced Local Lesions in Genome (TILLING) strategy is applied to create a mutant population to study genes of interest and connect them to a phenotype via forward genetics approaches. Additionally, TILLING populations are excellent resources to identify mutationally induced alleles for known genes to analyze and identify important functional domains and motifs within proteins of known or unknown function (Szarejko et al., 2017). The traditional methods utilized the detection of PCR products with mismatches sites in heteroduplexes with either the celery endonuclease enzyme (CEL I) (Oleykowski et al., 1998), or enzymatic mixes (Till et al., 2004). The alternative method utilizes DNA sequencing followed by comparative analysis with a reference sequence to identify the new mutant allele/s (Tsai et al., 2011).

Disease lesion mimic mutants develop necrotic lesions that are indistinguishable from those formed by some necrotrophic pathogens yet spontaneously develop in the absence of pathogen attack, abiotic stress, or mechanical damage. The genetic and functional characterization of these DLMMs will contribute to the understanding of PCD pathways in plants and the different pathways and crosstalk between them that result in spatially and temporally distinct PCD responses (Greenberg et al., 2000).

Many different mutagens have been used to create DLMMs in plants such as ethyl methane-sulfonate (EMS) in Arabidopsis (Zhang et al.,2008), x-rays in wheat (Kinane & Jones, 2001), and fast neutron in barley (Rostoks et al., 2003). Some of these DLMMs have been further studied in Arabidopsis (Bouchez, 2007), rice (Yin, 2000), and barley (Wolter, 1993).

Many DLMM genes in Arabidopsis, such as constitutive expresser of PR 5 (*cpr5*), constitutive expresser of PR 22 (*cpr22*), suppressor of SA insensitivity 1 (*ssi1*), and hypersensitive response-like lesions 1 (*hrl1*) show high level of plant defensin gene (*Pdf1.2*) expression, a hallmark of the activation of the ethylene and JA pathways (Penninckx et al., 1998). *Pdf1.2* is an antifungal plant defensin gene that stops fungal growth and is induced systemically upon pathogen infection (Penninckx et al., 1996). In rice, the limited number of genes identified that underlie DLMMs encode proteins that fall under different protein families such as spotted leaf (*spl*) that result in DLMM phenotype and shows resistance to both *Magnaporthe grisea* and *Xanthomonas oryza* pv. *oryza* (*Xoo*) pathogens, and cell death and resistance (*cdr*) mutant that show resistance to the blast fungus *M. grisea* (Takahashi et al., 1999; Yamanouchi, 2002; Zeng, 2005). *Spl* encodes a U-box protein that is involved in the ubiquitination of substrate proteins and the ubiquitination pathway is related to PCD and defense activation. At the same time, *cdr* generates high levels of H_2O_2 in cells, and result in PCD (Takahashi et al., 1999; Tsunezuka et al., 2005; Zeng, 2005).

The characterization of DLMMs has had some utility in plant breeding with a limited number of DLMMs showing resistance to phytopathogens and DLMM genes deployed as disease resistance genes. For example, *Mlo* (Mildew Locus O) is a DLMM where the recessive allele confers resistance to the biotrophic powdery mildew fungus *Blumeria graminis* f. sp. *hordei* in barley (Wolter et al, 1993). The *mlo* allele shows a highly resistant reaction to the fungus, and the first variety containing *mlo* was released in 1979 (Lyngkjær et al., 2000) and was deployed into spring barley varieties in Europe by 1980 (Jørgensen, 1992). The *Mlo* gene was cloned shown to encode a conserved ROP like G-protein that suppresses PCD and is found in other species (Wolter et al., 1993). In wheat, the *Lr34* gene shows resistance to leaf rust (*Puccinia recondita*) and was identified and named by Dyck and Samborski (1979) (German &

Kolmer, 1992; Dyck & Samborski,1979; Vanegas et al., 2008). Many varieties carry the *Lr34* gene including Chinese, Italian, and South American wheat varieties. Interestingly, *Lr34* is associated with the DLMM phenotype that results in leaf tip necrosis that can be used as a marker (Ellis et al., 2014; Singh, 1992). The *Lr34* gene encodes a full-length ATP-binding cassette (ABC) transporter protein localized to wheat chromosome 7D (Ellis et al., 2014) that confers race nonspecific resistance against multiple fungal pathogens (Krattinger et al., 2016). The resistance reaction is conferred by delaying the pathogen development by extending the latency period and reducing infection frequency (Rubiales & Niks, 1995). Two gain-of-function mutations, a deletion of a phenylalanine residue and a conversion of a highly conserved tyrosine to histidine contribute to the PCD phenotype and resistance (Dakouri et al., 2010; Krattinger et al., 2011, 2016). The Light-Induced Lesion Mimic Mutant 1 (LIL1) identified from an ethylmethane sulfonate mutagenized population of indica rice (*Oryza sativa* L. ssp. *indica*) 93-11 was identified and cloned (Zhou et al., 2017). LIL1 was mapped to chromosome 7, and encodes a 687 amino acid putative cysteine-rich receptor-like kinase (CRK) protein that contains a signal-peptide domain (Zhou et al., 2017). The barley lesion mimic mutant bspl (barley spotted leaf 1) was identified by Zhang et al. (2011), and the lesions are the result of the accumulation of ROS (Sun et al., 2014). It was generated by ethylmethane sulfonate mutagenesis and is controlled by a single recessive gene (Sun et al., 2014; Zhang et al., 2019;). Another DLMM mutant is spotted leaf 11 (spl11), which was identified by Kinoshita (1995) and shows resistance to multiple blast and bacterial blight isolates. Also, in some cases, DLMM genes function as a negative regulator for PCD, such as LSD1 in Arabidopsis (Dietrich et al., 1997).

Mapping genes controlling DLMM phenotypes is a crucial step in the understanding and eventual identification and characterization of genes involved in PCD pathways. We

hypothesized that the five non-allelic barley DLMMs (FN360, FN362, FN365, FN370, and FN396) resulted from mutations in different suppressors of PCD or PCD pathway genes. Thus, the causal mutated genes underlying these DLMM would map to distinct loci. In this study, the five barley DLMMs generated by fast neutron irradiation were genetically mapped to distinct loci with FN360 mapping to two loci on chromosomes 1H and 6H, FN362 on chromosome 6H, FN365 on chromosome 1H and 5H, FN370 on chromosome 1H, and FN396 on chromosome 2H. A genetic to the physical map was generated utilizing the cv Morex genome sequence to identify flanking markers for each DLMM loci.

Material and Methods

Plant Material, Genetics Population and Phenotype Evaluation

Five barley DLMMs designated FN360, FN362, FN365, FN370, and FN396 were generated by fast neutron mutagenesis in the variety Steptoe, which is a six-row, spring feed barley developed and released by Washington State University in 1973 (Muir & Nilan, 1973). Steptoe was selected from a Washington 3564 x Unitan cross (Muir & Nilan, 1973). The DLMMs in the Steptoe background were provided by Dr. Andris Kleinhofs from Washington State University. The Steptoe mutant population from which all the mutants were identified was generated by seed exposure to fast neutron irradiation at 3.5 and 4 Gy at the FAO/IAEA Seibersdorf SNIF facility (Rostoks, 2003). The five DLMMs were crossed with the variety Morex, a six-row malting barley released in 1978 from the University of Minnesota (Rasmusson & Wilcoxson, 1979). The Steptoe x Morex cross was chosen for the genetic analysis because previous research has shown that these two varieties have a high level of polymorphism for genetic markers across the genome (Marcel et al., 2007). To screen the bi-parental populations for homozygous mutant progeny, 392 F_2 individuals of each cross were planted in containers and

grown in the greenhouse to the adult plant stage (~ 3 months). Due to limited seed and poor germination, only 310 F_2 individuals were evaluated for the FN365 x Morex population. All the populations segregated in 1 mutant: 3 wild type ratios (Table 1) except the FN 360 x Morex population exhibited a 12 wildtype :3 dark lesion:1 tan lesion (Table 1.1).

The progeny from each population that expressed the DLMM phenotypes were selected by visual observation to score as typical mutant -vs- wildtype phenotypes for each mutant (Figure 2). Leaf tissue from each individual expressing the mutant phenotypes and parents were collected from 3-5-week-old plants and stored at 80oC until further processing for DNA isolation.

DNA Extraction, PCR-GBS Library Preparation and Ion Torrent Sequencing

Genomic DNA was extracted from the leaf tissue samples collected from recombinant progeny showing the mutant phenotypes as described by Richard et al. (2016). A PCR genotyping-by-sequencing (PCR-GBS) amplicon sequencing library was constructed for each population utilizing a panel of 365 polymorphic markers (www.triticeaetoolbox.org/barley). All markers were mined from the 9K iSelect array (www.triticeaetoolbox.org/barley) and selected based on their level of polymorphism and. even distribution throughout the barley genome (~1 marker per 5 cM). The protocol for DNA extraction, PCR cycle parameters, library preparation, and sequencing on the Ion Torrent PGM was followed as. Described in. Richards et al. (2016).

SNP Calling

The raw reads generated on the Ion Torrent sequencer were processed in CLC genomics v8.4 (CLC bio, Aarhus, Denmark) before the downstream variant calling. A 22 bp long PCR adapters attached to the 5’ and 3’ ends of sequencing reads were removed (Richards et al., 2016) and quality trimming accomplished using the. default parameters for Ion torrent sequence data in

CLC genomics. The high-quality trimmed reads were aligned to a reference FASTA file consisting of previously identified amplicon sequences from the T3 database (https://triticeaetoolbox.org/barley/), using the BWA-MEM algorithm with default settings (Li, 2013). The alignments were converted to BAM files using SAMtools (Li et al. 2009). SNP calling was performed using the. Genome Analysis Toolkit's Unified Genotyper (GATK) tool with default setting (Van der Auwera et al., 2013). VCFtools was used to remove individual calls with read depth of less than six and genotype quality less than ten (Danecek et al., 2011). To generate a genetic map, MapDisto v1.7.7.0.1.1 was utilized with a default minimum LOD of 3.0, rmax of 0.3, and Kosambi mapping function (Kosambi, 1943).

Qualitative Trait Loci Analysis

The iSelect consensus genetics map developed by Muñoz-Amatriaín et al. (2014) was utilized as a reference for our markers in the study to validate chromosome positions. For QTL analysis, the de novo loci position for non-co-segregating loci was used. The mutant phenotyping trait and genotyping data were assigned for analysis to manually construct .qdf file and analyze the association between markers and mutant phenotype in QTL mapping software QGene v.4.3.10 (Joehanes and Nelson, 2008). QTL analysis was done using the composite interval mapping algorithm (CIM) (Jansen and Stam, 1994; Zeng, 1994) at a scanning interval of 5. A forward cofactor selection method was used to select marker as cofactors with options, 'A maximum number of cofactor' and 'F to add' set at auto to control the background variation. To determine a LOD threshold at the $p \leq 0.05$ significance level, a permutation test containing of 1000 iterations was used.

Results

Phenotype Observations in Greenhouse

The barley mutants and the homozygous mutant progeny seedlings expressed the DLMM phenotypes on all mature leaves. For FN360 and FN361 both tan and dark lesions (Figure 2.1 and 2.2) began to appear as small necrotic spots that expanded over time on the primary leaf at 3-4 weeks post germination under greenhouse conditions. FN365 and FN396 began to express necrotic lesions on leaves at the fifth week post germination. However, the FN370 mutant exhibited the lesions earlier with lesion formation on primary leaves by the second week post germination.

Table 2.1: Chi square analysis for mutant populations showing the ratio between wild type and mutant phenotypes among the bi-parental populations developed by crossing the FN mutants with the variety Morex.

Population	Wildtype	Mutant	Ratio	Chi Square	Critical Value
FN360 x Morex	273	72 dark :17 Tan	12: 3: 1	1.66	5.99
FN361 x Morex	267	71	3: 1	3.25	3.84
FN365 x Morex	228	82	3: 1	0.35	3.84
FN370 x Morex	281	101	3: 1	0.422	3.84
FN396 x Morex	281	80	3: 1	3.56	3.84

All the populations except FN360 x Morex segregated in a 1 mutant : 3 wild type ratio indicating that the DLMM phenotypes were controlled by a single recessive mutant gene. However, this was observed for the FN360 x Morex F_2 population which did not fit the expected 3:1 ratio (Table 2.1) and appeared to contain two independent recessive mutations. The homozygous mutant F_2 individuals from the DLMM populations showed consistent phenotypes with each developing unique necrotic lesion. However, the FN361 x Morex and FN365 x Morex homozygous mutant F_2 individuals were very similar in appearance yet as previously described

had different timing of phenotypic expression (Figure 2.2). The one exception of consistent expression of phenotypes in the F_2 populations was the FN360 x Morex population which exhibited both tan or dark lesion phenotypes (Figure 2.1). The tan lesions had a more expanded lesion size compared to the dark lesions within the progeny derived from the cross. The FN361 x Morex homozygous F_2 individuals showed more prominent dark lesions with both large and smaller pinpoint lesions that differed from the FN360 dark lesions (Figure 2.2). However, in the greenhouse, both FN360 dark lesions and FN361 lines started exhibiting lesions on the primary and secondary leaves with similar timing. The FN365 x Morex F_2 homozygous mutant individuals developed larger lesions compared to FN361 x Morex F_2 homozygous mutant individuals without the pinpoint lesion (Figure 2.3). The FN370 x Morex homozygous F_2 individuals only developed pinpoint lesions that first appear at the leaf tip early in the second week, then continue to develop downward until they were present across the entire leaf. Although, the FN370 lesions are present on the entire leaf the individual lesions do not expand and remain as individual pinpoint spots (Figure 2.4). The FN396 x Morex F_2 homozygous mutant individuals developed lesion that are very similar to those induced by the necrotrophic fungal pathogen *Pyrenophora teres* f. *maculata* the causal agent of the disease spot form net blotch. From these descriptions it is apparent that these mutants exhibit differential DLMM phenotypes and have mutations in genes that affect the temporal and spatial occurrence of the PCD.

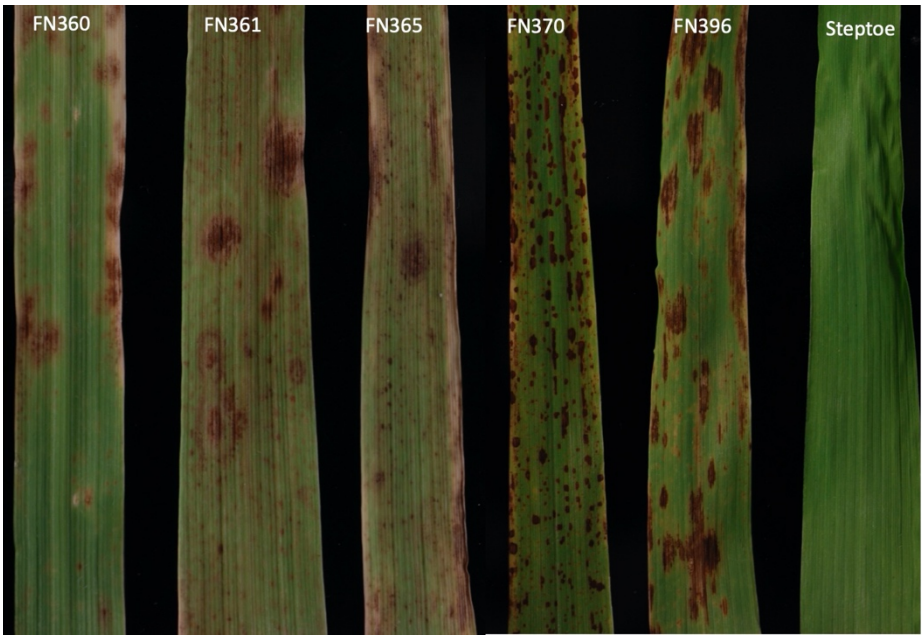

Figure 2.1: Photos of the typical lesion development on the original Steptoe mutants. The FN360 mutant has dark phenotype. FN361 mutant with bigger dark lesion and pinpoint phenotype. FN365 mutant is similar to FN361. FN370 develops pinpoint dark lesions. FN396 mutant develops dark lesion with chlorotic halos similar to the disease spot form net blotch.

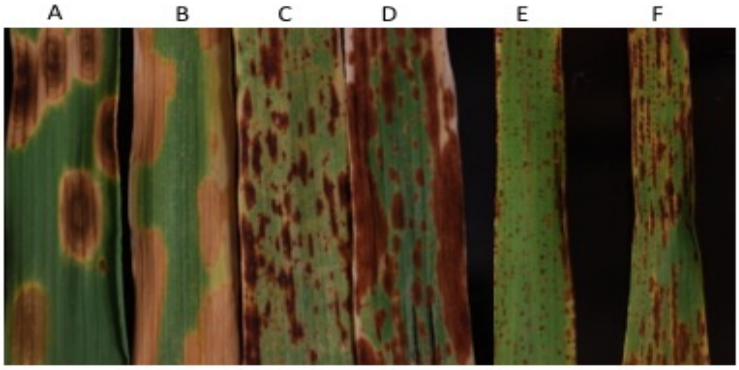

Figure 2.2: Photos of the typical lesions that developed on the FN mutants x Morex F2 homozygous mutant individuals. (A) FN360 x Morex mutant progeny with the dark lesion phenotype. (B) FN360 x Morex mutant progeny with the tan lesion phenotype. (C) FN360 X Morex mutant progeny with mixture of large and pinpoint dark lesions. (D) FN365 X Morex mutant progeny with the larger dark lesion lacking pinpoint lesion. (E) FN370 X Morex mutant progeny develop only pinpoint lesions. (F) FN396 X Morex mutant progeny with a phenotype similar to the spot disease form net blotch.

Genetics Mapping of All Mutant

The genetic mapping of all five DLMMs to distinct loci supported the previous allelism tests that determined that these mutants were not allelic. However, the FN360 x Morex F_2 progeny produced two distinct phenotypes, dark lesions with presumed phenolics build up and tan lesion lacking phenolics buildup (Figure 2.2). Each of these DLMM phenotypes mapped to two different loci on chromosomes 1H (dark lesions) and 6H (tan lesions), suggesting that the FN360 mutant contained two recessive mutations contributing to the original DLMM phenotype. The single trait composite interval mapping analysis showed that the QTL contributing to the dark lesion phenotype was within an ~11.92 cM interval on chromosome 1H translating to ~6.1 Mb of physical sequence. The QTL was flanked by the iSelect markers 12_31144 at position 4,653,990 bp and 12_30588 at position 10,747,464 bp mapping to POPSEQ positions 4.98 cM and 15.9 cM, respectively. The most significant marker in this interval was 11_21174 with a LOD score of 57 (Figure 2.3). The FN360 tan lesion locus fell within an interval size of 9.89 cM (86 Mb) between iSelect SNP markers 12_10758 at position 41,7827,179 bp and 11_20892 at position 503,880,223 bp with genetic positions of 64.29 cM and 74.18 cM, respectively. The most significant marker within the tan lesion interval was 11_11483 with a LOD score of 63.7 (Figure 2.4).

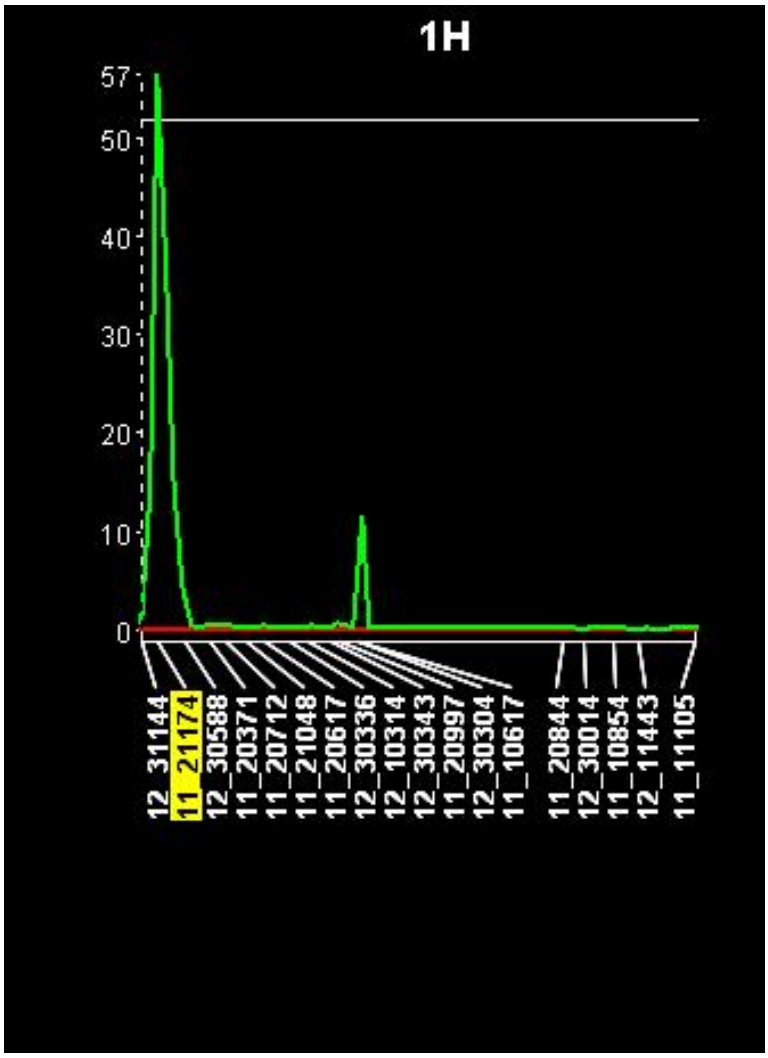

Figure 2.3: Genetic mapping of the FN360 dark necrotic lesion phenotype using composite interval mapping algorithm analysis for a single trait. The SNP markers shown in the figure are polymorphic between Steptoe and Morex and are located on chromosome 1H. The FN360 dark phenotype locus is flanked by the SNP markers 12_31144 and 12_30588 with the most significant marker being 11_21174.

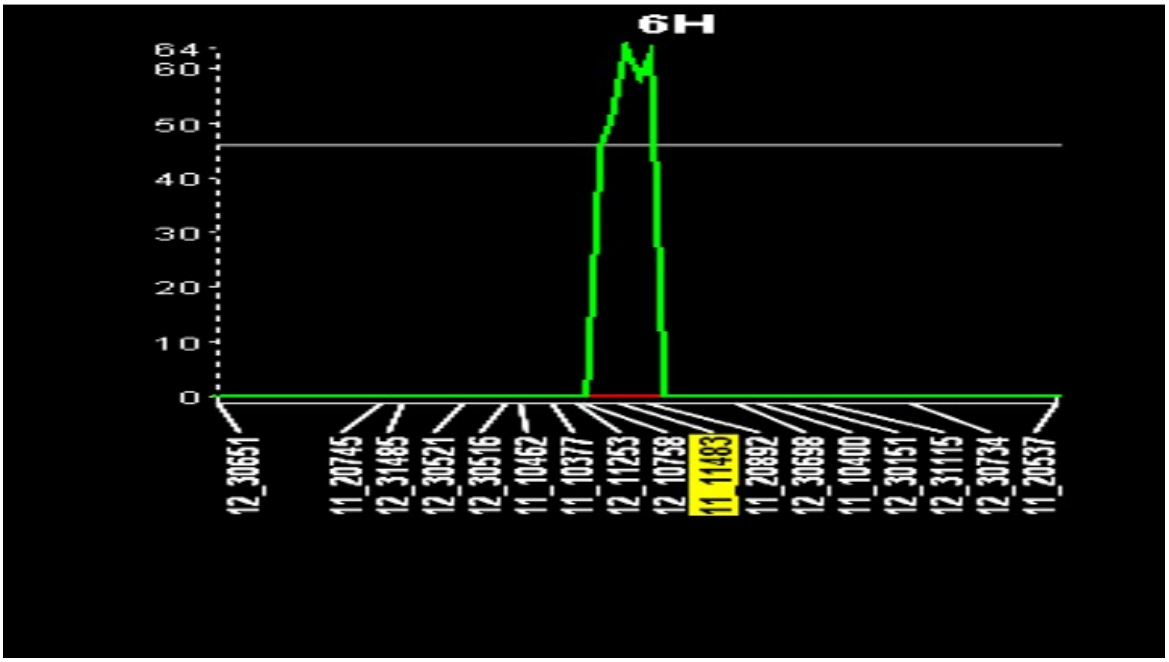

Figure 2.4: Genetic mapping of the FN360 tan necrotic lesion phenotype using composite interval mapping algorithm analysis for a single trait. The SNP markers shown in the figure are polymorphic between Steptoe and Morex and are located on chromosome 5H. The FN360 tan phenotype locus is flanked by the SNP markers 12_10758 and 11_20892 with the most significant marker being 11_11_11483.

The single trait composite interval mapping (CIM) analysis of the FN361 x Morex population identified a single major QTL on chromosome 5H within an interval size 34.89 cM translating to ~469.5 Mb of physical sequence. The FN361 QTL was flanked by the two iSelect SNP markers 11_20010 at position 6,354,602 bp and 12_30745 at position 475,851,929 bp mapping to POPSEQ positions 11.32 cM and 46.21 cM, respectively. The most significant marker within the locus was 11_21065 with a LOD score of 142.328 (Figure 2.5).

The CIM analysis of the FN365 x Morex population identified two minor QTL on chromosome 1H and a major QTL on chromosome 5H. The major QTL interval spanned 33.57 cM (~40 Mb) and is flanked by the two iSelect SNP markers 11_20375 and 11_10600 at POPSEQ position 132 cM (physical position 609,073,896 bp) and 165.57 cM (physical position 649,232,960 bp) on chromosome 5H. The most significant marker within the locus was

11_20375 with a LOD score of 25 (Figure 2.6). The minor QTL on chromosome 1H is flanked by the iSelect SNP markers 12_30343 and 11_20844 located at 52.08 cM (physical position 401,872,471 bp) and 109.53 cM (physical position 525,478,648 bp), respectively. Thus, the interval size is ~57.45 cM (123.6 Mb) (Figure 2.6).

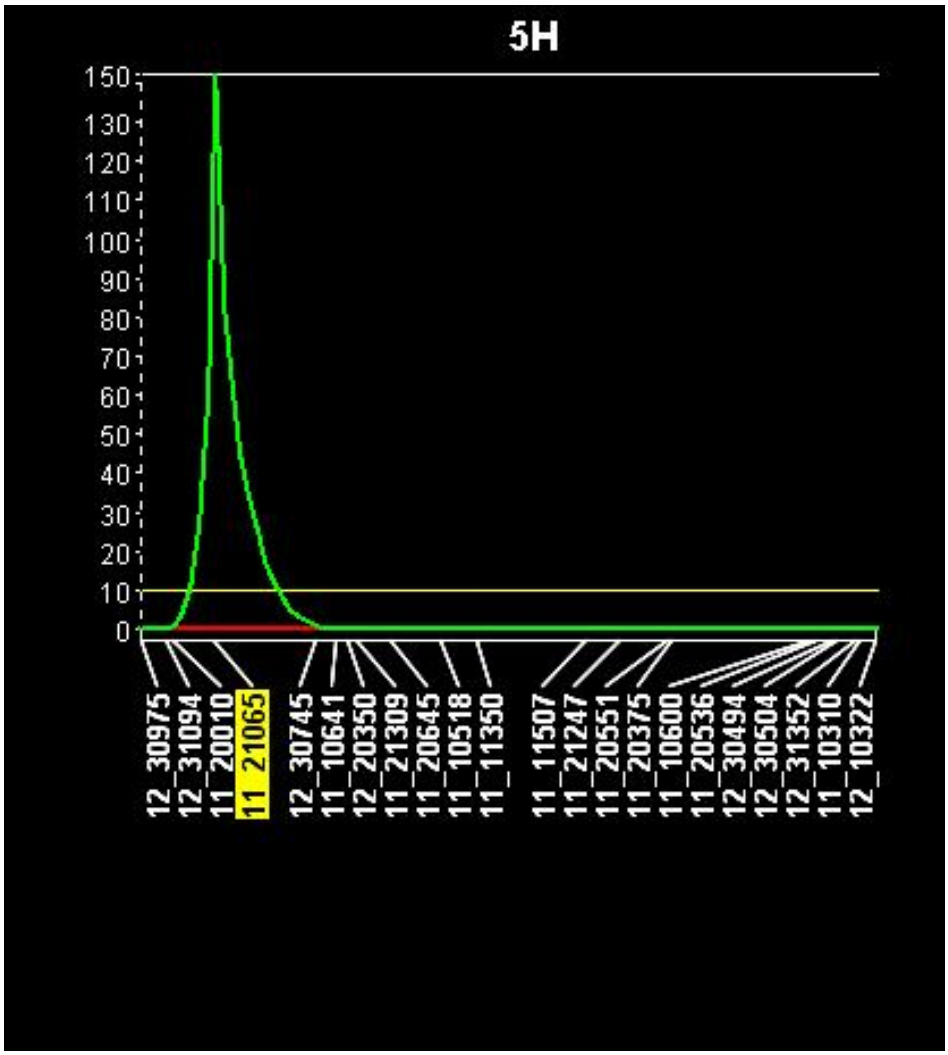

Figure 2.5: Genetic mapping of the FN361 mutant using composite interval mapping for a single trait. The SNP markers shown in the figure are polymorphic between Steptoe and Morex and are located on chromosome 5H.

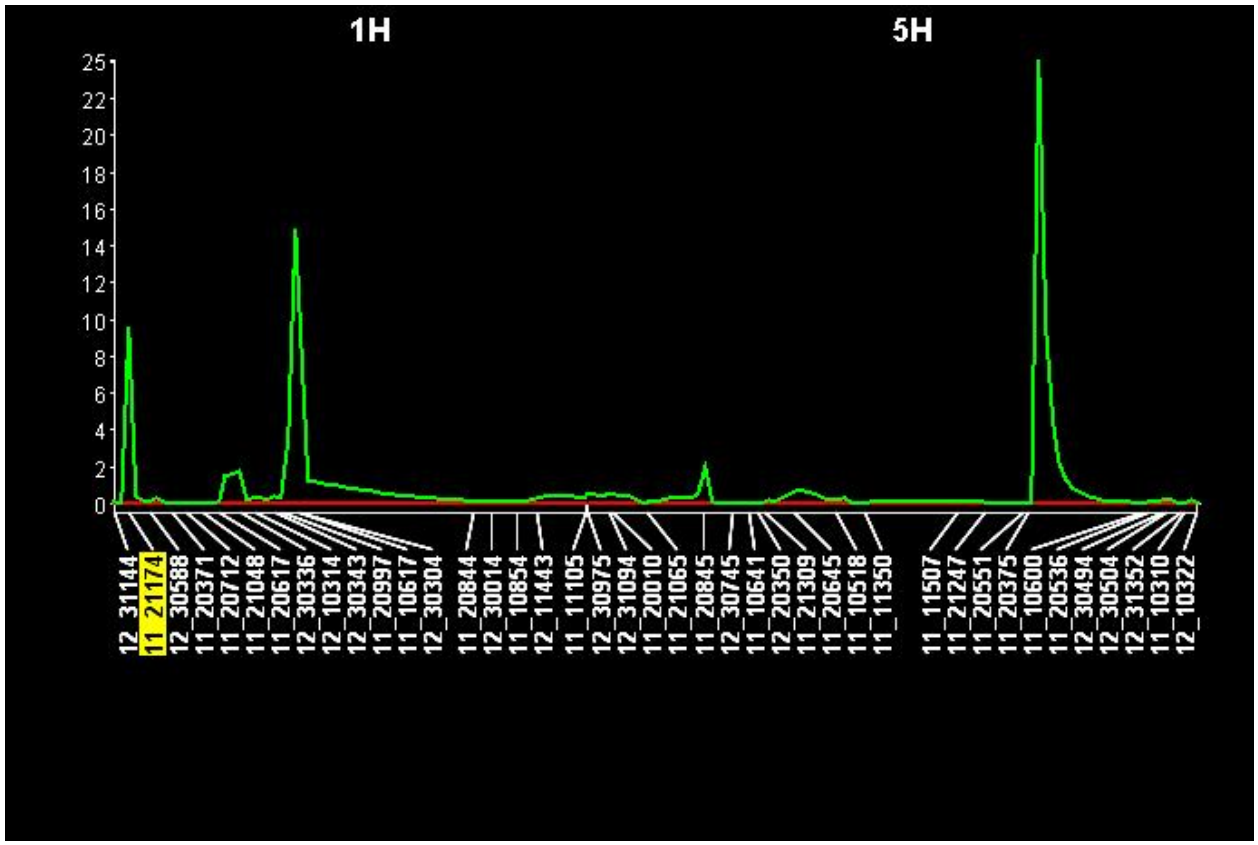

Figure 2.6: Genetic mapping of the FN365 mutant using composite interval mapping analysis for a single trait. The SNP markers shown in the figure are polymorphic between Steptoe and Morex and are located on chromosome 5H for the major QTL. The minor QTL are located on chromosome 1H.

The CIM analysis of the FN370 x Morex population which develops the pinpoint lesion phenotype mapped to a single locus on chromosome 1H. The QTL covered an interval size of 50.46 cM (~90.6 Mb) flanked by the iSelect SNP markers 11_10617 and 11_20844 at POPSEQ position 59.07 cM (physical position 434,823,728 bp) and 109.53 cM (physical position 525,478,648), respectively (Figure 2.7).

The final analysis of the FN396 x Morex population analyzed with CIM identified a single QTL located on chromosome 2H between iSelect SNP markers 12_10936 at position 659,264,767 bp and 11_10429 at position 704,365,928 bp with the most significant marker being

11_21175 with a LOD score of 6.6. The QTL was delimited by POPSEQ position 93.14 cM and 126.63 cM with an interval size is 33.49 cM (~45 Mb) (Figure 2.8).

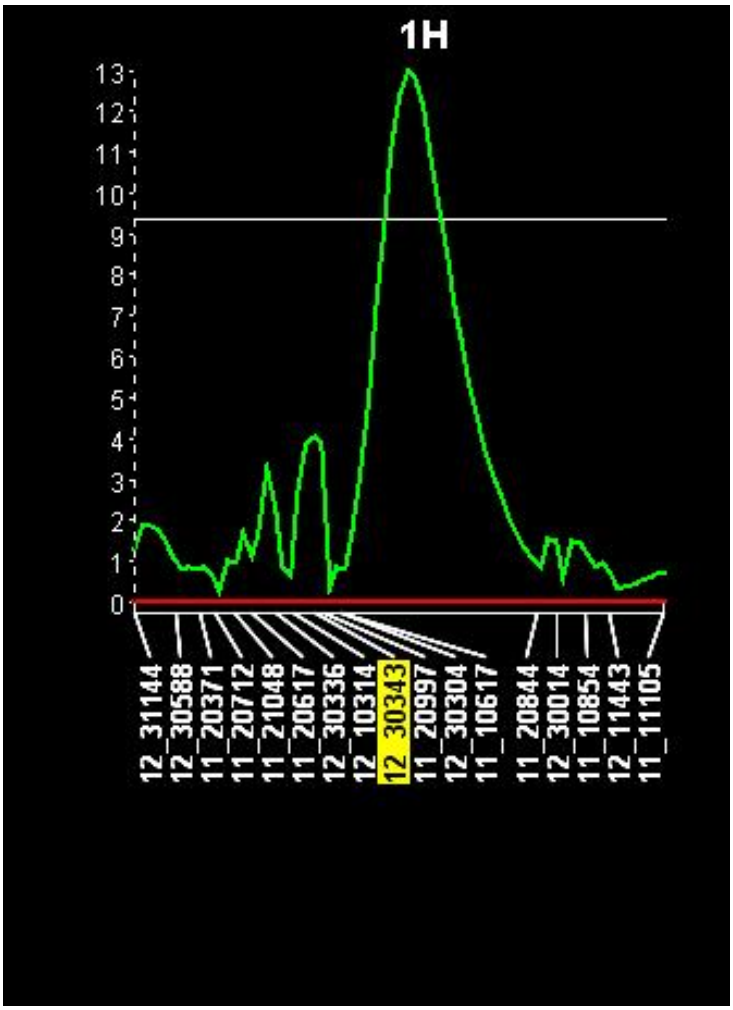

Figure 2.7: Genetic mapping of the FN370 mutant using composite interval mapping using Qgene software was used to generate QTL. The SNP markers shown in the figure are polymorphic between Steptoe and Morex and are located on chromosome 1H.

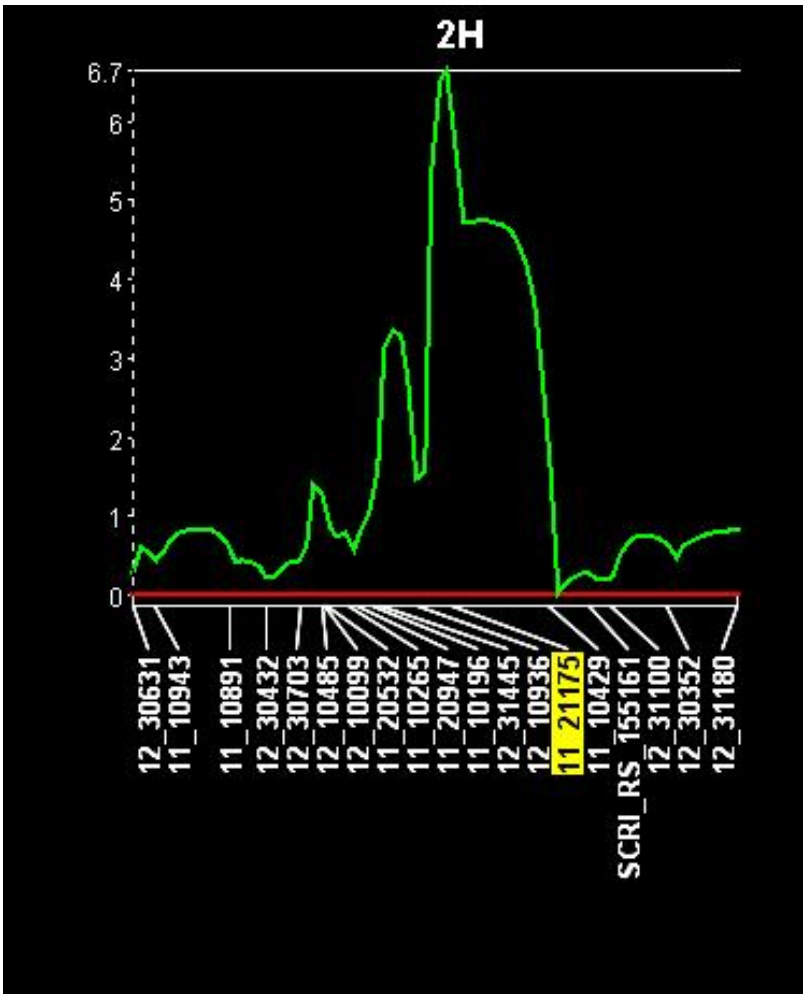

Figure 2.8: Genetic mapping of FN396 mutant using composite interval mapping analysis. The Steptoe and Morex genetic of chromosome 2H shows all the polymorphic SNP markers below and the QTL is flanked by the. SNP markers 12_10936 and 11_10429.

Table 2.2: QTL mapping on different chromosomes and region as supported by LOD and R^2 values for all mutants.

Mutant	QTL region	Centimorgan (cM)	LOD	R^2
FN 360 dark	1H	8.9 cM	56.3	0.998
FN 360 Tan	6H	69.79 cM	63.7	1
FN 361	5H	21.24 cM	142.328	1
FN 365	1H,5H	59.07 cM , 132 cM	14.915 and 24.073	0.567 and 0.741
FN 370	1H	76.9 cM	19.931	0.612
FN 396	2H	101.98 cM	6.648	0.318

Discussion

This objective of this research was to genetically characterize and map DLMMs that spontaneously develop necrotic lesion phenotypes that are similar to and sometimes indistinguishable from lesions induced by necrotrophic pathogens. Five distinct DLMMs generated by fast neutron mutagenesis in the barley variety Steptoe background (designated FN360, FN362, FN365, FN370, and FN396) were genetically characterized. Each of these five mutant lines developed lesions that visually varied in color and size, further supporting previous allelism tests (personal communications with Dr. Andris Kleinhofs) that showed each DLMM phenotype resulting from distinct mutated gene(s). In previous studies, many DLMMs have been identified by screening mutant populations of diverse plant species including barley, wheat, rice, and Arabidopsis. Only a limited number of the genes underlying DLMMs have been cloned leaving these genetic materials as an underutilized source to understand suppressors and signaling pathways that are utilized for PCD function in plant immunity and developmental pathways.

Programmed cell death is a normal physiological process found among plants and animals, but the underlying molecular mechanisms directing PCD mechanisms in plants is less understood when compared to the animal system. Studying PCD is difficult because identifying genes involved in PCD through genetic mapping is difficult or impossible because these are highly conserved genes with little or no phenotypic or genetic polymorphism. Thus, mutagenesis is utilized to introduce this polymorphism in genes required for normal PCD function and signaling to facilitate gene discovery. Many mutagenic agents have been used to generate mutants in plants including irradiation and chemicals. Irradiation using high energy fast neutrons (FN), the mutagenic agent utilized in this study, causes deletions that can range in size from 1 bp

to several Megabases. The large FN generated mutations can harbor multiple genes which can facilitate the ease of identifying the mutation yet can complicate gene validation due to the possibility of several candidate genes present within the single deletion. In this study, five barley DLMM generated in the line Steptoe background were genetically characterized utilizing bi-parental populations and genetic mapping which allowed for the localization of these DLMM mutants to distinct and defined positions within the barley genome.

Phenotype and Chi Square of Mutants

Five DLMMs (FN360, FN361, FN365, FN370 and FN396) were genetically characterized utilizing F_2 individuals from biparental populations derived from FN mutants (Steptoe background) crossed with the variety Morex. Most of the five mutants characterized displayed distinct phenotypes with different lesion shape and color. The homozygous mutant F_2 individuals characterized from the FN360 x Morex population showed two different phenotypes, both tan and dark necrotic lesions (Figure 2.2). The dark lesion phenotype is the predominant phenotype expressed in the population and developed sequestered smaller lesions compared to the tan to orange colored lesions that expand once induced (Figure 2.2). The segregation ratio for the FN360 x Morex F_2 individuals as seen in Table 2.1 is 12 wildtype: 3 dark lesion: 1 tan lesion, showing that the dark lesions segregated in a 3 wildtype: 1 mutant ratio. Thus, the data indicated that the dark DLMM phenotype was controlled by a single recessive gene. Interestingly, the tan lesion DLMM phenotype which resembled the unique barley *nec3* phenotype segregated in a 15 wildtype and dark lesion: 1 tan lesion, showing that the tan DLMM phenotype is controlled by two recessive genes that are independently assorting. It appears that both the dark locus on chromosome 1H that determines the dark DLMM phenotype as well as the *nec3* locus on chromosome 6H are required for the tan DLMM phenotype to develop.

FN361, FN365, FN370, and FN396 x Morex F_2 mutant progenies show the same lesion phenotype of the original parental mutants (Figure 1 and 2), and the mutant phenotypes in each F_2 population segregates in a 3 wild type: 1 mutant ratio indicating that a single recessive gene controls the DLMM phenotypes (Table 2.1). Many DLMMs in different plant species show single recessive gene segregation including wheat (Anand et al., 2003), rice (Matin et al., 2010) and Soybean (Al Amin et al.,2019). All the mutants characterized in this study express unique lesions on leaves in terms of morphology or timing under greenhouse conditions. Interestingly, under greenhouse condition the mutants also expressed the DLMM phenotypes at different times. For example, FN370 expresses lesion early compared to the other mutants while both FN365 and FN396 don't develop lesions until the fifth week after germination. These observations indicate the variation of PCD pathways among this set of barley DLMM indicating that different genes were mutated in each of the DLMMs characterized. Thus, supporting the hypothesis that each mutant has a different gene disrupted that alters the same or different PCD signaling pathways. The mapping of each DLMM mutant to a unique locus within the barley genome strongly validated this hypothesis.

Genetic Mapping of the Barley DLMMs

The mapping of the dark lesion phenotype in the FN360 x Morex F_2 population delimited the major locus with SNP iSelect markers 12_31144 at position 4.98 cM and 12_30588 located at position 15.9 cM on chromosome 1H (Figure 2.3) while the *nec3* (tan-orange necrotic lesions) was delimited by the SNP markers 12_10758 and to 11_20892 on chromosome 6H (Figure 2.4) validating that it most likely represented a new *nec3* DLMM mutant allele. FN360 dark lesion QTL region contains around 131 High confidence annotated genes. Under this QTL, there are many genes involved in PCD signaling pathways including Chymotrypsin inhibitors that are

homologs of the Arabidopsis Serine protease inhibitor involved in plant immunity pathways that involve PCD responses (Mishra et al, 2020: Neurath, 1986). The FN360 *nec3* lesion QTL region contains about 556 high confidence annotated genes including the Cytochrome P450 *nec3* gene that our lab recently cloned and validated. Interestingly, FN360 x Morex F_2 individuals developed two different phenotypes with the first dark lesion phenotype segregating as a single recessive gene that mapped to chromosome 1H. However, the *nec3* phenotype segregated in a 15;1 ratio suggesting that the tan-orange lesion phenotype was governed by two recessive mutant genes. Based on the QTL mapping and segregation analysis it appears that both the 1H and 6H mutations must be present to develop the tan-orange *nec3* phenotype.

The FN361mutant develops large dark necrotic lesions with interspersed pinpoint lesions, and the QTL mapping showed that the FN361 mutant locus is delimited by the iSelect SNP markers 11_20010 and 12_30745 at POPSEQ position 11.32 cM and 46.21 cM (Figure 2.5). As expected, within this interval, there are genes that are involved in plant immunity signaling that induce PCD responses such as the WRKY transcription factors (Sarris et al.,2015).

The FN365 DLMM develops larger spot lesion than FN361 with minimal pinpoint lesions, and this phenotype varies from *nec13.al* (FN365) as described by Kleinhofs (2013). *Nec13.al* shows infrequent small to medium-sized dark brown spots on the leaves often with limited chlorosis surrounding the necrotic lesions (Kleinhofs, 2013). However, the lesion phenotypes described by Kleinhofs (2013) were from field samples and they could be slightly different because greenhouse conditions may have lower light intensity. The FN365 x Morex F_2 population produced one major and two minor QTL. However, the statistical analysis show that the ratio fit 3 wild type :1 mutant indicating a single recessive gene, thus, we are confidence that the major QTL contained the candidate mutant gene of interest. The explanation for this result is

that the minor QTL arise from the genomic backgrounds contain loci on chromosome 1H that interact with the mutation on chromosome 5H in the F_2 population. Thus, the contribute to the DLMM phenotype or these QTL were the result of false association in this population due to segregation distortion. The major QTL mapped to chromosome 5H and was flanked by the two SNP markers 11_20375 and 11_10600 at POPSEQ position 132 cM and 165.57 cM (Figure 2.6). The delimited QTL contains 844 high confidence annotated genes with many having putative functions that are involved in PCD pathways.

The FN370 DLMM develops pinpoint lesions similar to *nec1* described by Fedak (1972) and Jensen (1971) that were described as small black-brown spots that developed on all light-exposed plant structures starting near the leaf tip. In our experience FN370 started to develop necrotic spots near the leaf tip early at the second leaf stage and develop lesions on the leaves only. The FN370 QTL was delimited by the iSelect SNP markers 11_10617 and 11_20844 at POPSEQ position 59.07 cM and 109.53 cM on chromosome 1H. Druka et al. (2011) mapped nec1.i to chromosome 1H flanked by the SNP markers 2_1072 and 2_0625 (Druka, 2011) at positions 36.71 to 108.4 cM. Based on the iSelect SNP synonym both mutants fall in same region. The FN370 QTL region is ~ 50 cM region containing 1119 high confidence annotated genes with many involve in PCD pathways. This large region on a chromosome 1H contains many genes such as the receptor-like kinase 902 that is involved in the immunity signaling pathways (Mendy et al, 2017). Also, the RING/U-box superfamily protein results in PCD in rice when the gene is knocked out (Zeng et al., 2008). Further, comparative analysis at these regions via exome capture or RNAseq analysis needs to be performed to refine the candidate gene list.

The last DLMM genetically characterized was FN396, which shows a phenotype similar to the disease spot form net blotch. The FN396 QTL was located on chromosome 2H delimited

by the flanking markers 12_10936 at position 659,264,767 bp and 11_10429 at position 704,365,928 bp with POPSEQ positions of 93.14 cM and 126.63 cM, respectively. The QTL contains around 644 High confidence annotated genes that are involved in PCD in different organisms. These genes are the RING/U-box superfamily protein (Zeng et al., 2008), superoxide dismutase (Vacca et al., 2004)., and Thioesterase superfamily (Tillander et al., 2017).

QTL analyses were performed by composite interval mapping with a threshold of 3 LOD in our study, and all QTL were associated with the mutant's phenotypes based on high LOD values (6.7 – 143) (Table 2.2). Based on our results, all five mutants have different genes involved in PCD pathways that develop different lesions. All mutants in our studies are unique and most had not been previously mapped or characterized. Mapping these mutants to the different chromosomal locations is the first step in understanding the PCD pathways.

Conclusion

Programmed cell death is a crucial physiological process that occurs in plants to adapt the plant to environmental conditions. In this study, we evaluated six lesion mimic mutants in barley obtained from Fast Neutron mutagenesis. These mutants have unique shapes that express on a different stage, and all of them, based on genotype and phenotype data, have been mapped on different chromosomes and regions. Fast neutron radiation results in large deletion of genes or translocation of chromosomes; studying the QTLs for each mutant to determine the deletion needs to be done through Exome capture or RNAseq to compare between Steptoe and mutants. Finding deletion underlying the QTLs will help in understanding the PCD pathways in plant cells. Also, these results need to be studied further to find genes govern the phenotypes and test their reaction to both necrotrophic and biotrophic pathogens to find which pathway are hijacked

easily by pathogens resulting in developing plants with high adaptability likely against both biotic and abiotic stresses (Valandro et al., 2020).

References

Al Amin, G. M., Kong, K., Sharmin, R. A., Kong, J., Bhat, J. A., & Zhao, T. (2019). Characterization and Rapid Gene-Mapping of Leaf Lesion Mimic Phenotype of spl-1 Mutant in Soybean (Glycine max (L.) Merr.). *International Journal of Molecular Sciences, 20*(9), 2193.

Ameen, G. (2019). *Cloning and Characterization of rcs5, Spot Blotch Resistance Gene and Pathogen Induced NEC3 Gene Involved in Programmed Cell Death in Barley* (Doctoral dissertation, North Dakota State University, Fargo, North Dakota).

Bouchez, O., Huard, C., Lorrain, S., Roby, D., & Balagué, C. (2007). Ethylene is one of the key elements for cell death and defense response control in the Arabidopsis lesion mimic mutant vad1. *Plant physiology*, *145*(2), 465–477.

Brueggeman, R., Drader, T., & Kleinhofs, A. (2006). The barley serine/threonine kinase gene Rpg1 providing resistance to stem rust belongs to a gene family with five other members encoding kinase domains. *Theoretical and Applied Genetics, 113*(6), 1147–1158.

Dakouri, A., McCallum, B. D., Walichnowski, A. Z., & Cloutier, S. (2010). Fine-mapping of the leaf rust Lr34 locus in Triticum aestivum (L.) and characterization of large germplasm collections support the ABC transporter as essential for gene function. Theoretical and applied genetics, 121(2), 373-384.

Danecek, P., Auton, A., Abecasis, G., Albers, C. A., Banks, E., DePristo, M. A., ... & McVean, G. (2011). The variant call format and VCFtools. *Bioinformatics*, *27*(15), 2156–2158.

Daneva, A., Gao, Z., Van Durme, M., & Nowack, M. K. (2016). Functions and regulation of programmed cell death in plant development. *Annual Review of Cell and Developmental Biology, 32*, 441–468.

Dietrich, R. A., Richberg, M. H., Schmidt, R., Dean, C., & Dangl, J. L. (1997). A novel zinc finger protein is encoded by the Arabidopsis LSD1 gene and functions as a negative regulator of plant cell death. *Cell*, *88*(5), 685–694.

Druka, A., Franckowiak, J., Lundqvist, U., Bonar, N., Alexander, J., Houston, K., ... & Stein, N. (2011). Genetic dissection of barley morphology and development. *Plant Physiology*, *155*(2), 617–627.

Dyck, P., and Samborski, D. (1979). Adult-plant leaf rust resistance in PI 250413, an introduction of common wheat. *Canadian Journal of Plant Sciences, 59*, 329–332. doi: 10.4141/cjps79-053.

Ellis, J. G., Lagudah, E. S., Spielmeyer, W., & Dodds, P. N. (2014). The past, present and future of breeding rust resistant wheat. *Frontiers in Plant Science, 5*, 641.

Fedak, G., Tsuchiya, T., & Helgason, S. B. (1972). Use of monotelotrisomics for linkage mapping in barley. *Canadian Journal of Genetics and Cytology*, *14*(4), 949–957.

Freialdenhoven, A., Scherag, B., Hollricher, K., Collinge, D. B., Thordal-Christensen, H., & Schulze-Lefert, P. (1994). Nar-1 and Nar-2, two loci required for Mla12-specified race-specific resistance to powdery mildew in barley. *The Plant Cell, 6*(7), 983–994.

German, S. E., and Kolmer, J. A. (1992). Effect of gene Lr34 in the enhance- ment of resistance to leaf rust of wheat. *Theoretical and Applied Genetics, 84*, 97–105. doi: 10.1007/BF00223987

Greenberg, J. T. (1996). Programmed cell death: a way of life for plants. *Proceedings of the National Academy of Sciences, 93*(22), 12094–12097.

Greenberg, J. T., Silverman, F. P., & Liang, H. (2000). Uncoupling salicylic acid-dependent cell death and defense-related responses from disease resistance in the Arabidopsis mutant acd5. *Genetics*, *156*(1), 341–350.

Hinze, K., Thompson, R. D., Ritter, E., Salamini, F., & Schulze-Lefert, P. (1991). Restriction fragment length polymorphism-mediated targeting of the ml-o resistance locus in barley (Hordeum vulgare). *Proceedings of the National Academy of Sciences, 88*(9), 3691–3695.

Jansen, R. C., & Stam, P. (1994). High resolution of quantitative traits into multiple loci via interval mapping. *Genetics, 136*(4), 1447–1455.

Jensen, J. (1971). Mapping of 10 mutant genes for necrotic spotting in barley by means of translocations. In *International Barley Genetics Symposium*.

Jiao, Y., Cai, C., Kermany, M. H., Yan, J., Cai, Q., Miller, D., ... & Gu, W. (2009). ENU induced single mutation locus on chr 16 leads to high-frequency hearing loss in mice. *Genes & Genetic Systems, 84*(3), 219–224.

Joehanes, R. and Nelson, J.C., (2008). QGene 4.0, an extensible Java QTL-analysis platform. *Bioinformatics, 24*(23), pp.2788–2789.

Jørgensen, I. H. (1992). Discovery, characterization and exploitation of Mlo powdery mildew resistance in barley. *Euphytica, 63*(1-2), 141–152.

Kinane, J. T., & Jones, P. W. (2001). Isolation of wheat mutants with increased resistance to powdery mildew from small induced variant populations. *Euphytica, 117*(3), 251–260.

Kinoshita, T. (1995). Report of committee on gene symbolization, nomenclature, and linkage groups. *Rice Genetics Newsletter*, *12*, 9–153.

Kleinhofs, A. (2013). Barley Genet. Newsl. 43:187.

Kleinhofs, A. (2013). Barley Genet. Newsl. 43:199.

Kosambi, D. D. (2016). The estimation of map distances from recombination values. In *DD Kosambi* (pp. 125–130). Springer, New Delhi.

Krattinger, S. G., Sucher, J., Selter, L. L., Chauhan, H., Zhou, B., Tang, M., ... & Schaffrath, U. (2016). The wheat durable, multipathogen resistance gene Lr34 confers partial blast resistance in rice. *Plant Biotechnology Journal, 14*(5), 1261–1268.

Krattinger, S.G., Lagudah, E.S., Wicker, T., Risk, J.M., Ashton, A.R., Selter, L.L., Matsumoto, T. et al. (2011) Lr34 multi-pathogen resistance ABC transporter: molecular analysis of homoeologous and orthologous genes in hexaploid wheat and other grass species. *Plant Journal, 65*, 392–403.

Li, H. (2013). Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *arXiv preprint arXiv:1303.3997*.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., ... & Durbin, R. (2009). The sequence alignment/map format and SAMtools. Bioinformatics, 25(16), 2078-2079.

Locato, V., & De Gara, L. (2018). Programmed cell death in plants: an overview. In Plant Programmed Cell Death (pp. 1–8). Humana Press, New York, NY.

Lyngkjær, M., Newton, A., Atzema, J., & Baker, S. (2000). The barley mlo-gene: an important powdery mildew resistance source.

Marcel, T. C., Varshney, R. K., Barbieri, M., Jafary, H., De Kock, M. J. D., Graner, A., & Niks, R. E. (2007). A high-density consensus map of barley to compare the distribution of QTLs for partial resistance to Puccinia hordei and of defence gene homologues. *Theoretical and applied Genetics, 114*(3), 487–500.

Matin, M. N., Saief, S. A., Rahman, M. M., Lee, D. H., Kang, H., Lee, D. S., & Kang, S. G. (2010). Comparative phenotypic and physiological characteristics of spotted leaf 6 (spl6) and brown leaf spot2 (bl2) lesion mimic mutants (LMM) in rice. *Molecules and cells, 30*(6), 533–543.

Mendy, B., Wang'ombe, M. W., Radakovic, Z. S., Holbein, J., Ilyas, M., Chopra, D., ... & Siddique, S. (2017). Arabidopsis leucine-rich repeat receptor–like kinase NILR1 is required for induction of innate immunity to parasitic nematodes. *PLoS Pathogens, 13*(4).

Mishra, U. N., Reddy, M. V., & Prasad, D. T. (2020). Plant serine protease inhibitor (SPI): A potent player with bactericidal, fungicidal, nematicidal and antiviral properties. *IJCS, 8*(1), 2985–2993.

Mittler, R., Del Pozo, O., Meisel, L., & Lam, E. (1997). Pathogen-induced programmed cell death in plants, a possible defense mechanism. *Developmental genetics, 21*(4), 279–289.

Moeder, W., & Yoshioka, K. (2008). Lesion mimic mutants: a classical, yet still fundamental approach to study programmed cell death. *Plant Signaling & Behavior, 3*(10), 764–767.

Muir, C. E., & Nilan, R. A. (1973). Registration of Steptoe Barley1 (Reg. No. 134). *Crop Science, 13*(6), 770–770.

Neurath, H. (1986). The versatility of proteolytic enzymes. *Journal of Cellular Biochemistry, 32*(1), 35–49.

Oleykowski, C. A., Bronson Mullins, C. R., Godwin, A. K., & Yeung, A. T. (1998). Mutation detection using a novel plant endonuclease. *Nucleic Acids Research, 26*(20), 4597–4602.

Olvera-Carrillo, Y., Van Bel, M., Van Hautegem, T., Fendrych, M., Huysmans, M., Simaskova, M., ... & Coppens, F. (2015). A conserved core of programmed cell death indicator genes discriminates developmentally and environmentally induced programmed cell death in plants. *Plant Physiology*, *169*(4), 2684–2699.

Palavan-Unsal, N., Buyuktuncer, E. D., & Tufekci, M. A. (2005). Programmed cell death in plants. *Cellular & Molecular Biology, 4*, 9–23.

Parrott, D. L., Huang, L., & Fischer, A. M. (2016). Downregulation of a barley (Hordeum vulgare) leucine-rich repeat, non-arginine-aspartate receptor-like protein kinase reduces expression of numerous genes involved in plant pathogen defense. *Plant Physiology and Biochemistry, 100*, 130–140.

Pennell, R. I., & Lamb, C. (1997). Programmed cell death in plants. *The Plant Cell*, *9*(7), 1157.

Penninckx, I. A., Eggermont, K., Terras, F. R., Thomma, B. P., De Samblanx, G. W., Buchala, A., ... & Broekaert, W. F. (1996). Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid-independent pathway. *The Plant Cell, 8*(12), 2309–2323.

Penninckx, I. A., Thomma, B. P., Buchala, A., Métraux, J. P., & Broekaert, W. F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. *The Plant Cell*, *10*(12), 2103–2113.

Rasmusson, D. C., & Wilcoxson, R. W. (1979). Registration of Morex Barley1 (Reg. No. 158). *Crop Science, 19*(2), 293–293.

Richards, J., Chao, S., Friesen, T., & Brueggeman, R. (2016). Fine mapping of the barley chromosome 6H net form net blotch susceptibility locus. *G3: Genes, Genomes, Genetics*, *6*(7), 1809–1818.

Rostoks, N., Schmierer, D., Kudrna, D., & Kleinhofs, A. (2003). Barley putative hypersensitive induced reaction genes: genetic mapping, sequence analyses and differential expression in disease lesion mimic mutants. *Theoretical and Applied Genetics*, *107*(6), 1094–1101.

Rostoks, N., Schmierer, D., Mudie, S., Drader, T., Brueggeman, R., Caldwell, D. G., ... & Kleinhofs, A. (2006). Barley necrotic locus nec1 encodes the cyclic nucleotide-gated ion channel 4 homologous to the Arabidopsis HLM1. *Molecular Genetics and Genomics*, *275*(2), 159–168.

Rubiales, D., & Niks, R. E. (1995). Characterization of Lr34, a major gene conferring nonhypersensitive resistance to wheat leaf rust. *Plant disease (USA)*.

Sarris, P. F., Duxbury, Z., Huh, S. U., Ma, Y., Segonzac, C., Sklenar, J., ... & Wirthmueller, L. (2015). A plant immune receptor detects pathogen effectors that target WRKY transcription factors. *Cell, 161*(5), 1089–1100.

Sathe, A. P., Su, X., Chen, Z., Chen, T., Wei, X., Tang, S., ... & Wu, J. L. (2019). Identification and characterization of a spotted-leaf mutant spl40 with enhanced bacterial blight resistance in rice. *Rice, 12*(1), 68.

Schindler, T., Bergfeld, R., & Schopfer, P. (1995). Arabinogalactan proteins in maize coleoptiles: developmental relationship to cell death during xylem differentiation but not to extension growth. The Plant Journal, 7(1), 25-36.

Singh, R. P. (1992). Association between gene Lr34 for leaf rust resistance and leaf tip necrosis in wheat. *Crop Science, 32*(4), 874–878.

Sun, Y., Lu, W., Zhang, Y., Wang, G., Sun, J., Xue, D., & Zhang, X. (2014). Genetic and physiological analyses of barley lesion mimic mutant bspl1. *Journal of Hangzhou Normal University (Natural Science Edition), 6*, 8.

Szarejko, I., Szurman-Zubrzycka, M., Nawrot, M., Marzec, M., Gruszka, D., Kurowska, M., ... & Maluszynski, M. (2017). Creation of a TILLING population in barley after chemical mutagenesis with sodium azide and MNU. In *Biotechnologies for Plant Mutation Breeding* (pp. 91-111). Springer, Cham.

Takahashi, A., Kawasaki, T., Henmi, K., Shii, K., Kodama, O., Satoh, H., & Shimamoto, K. (1999). Lesion mimic mutants of rice with alterations in early signaling events of defense. *The Plant Journal, 17*(5), 535–545.

Till, B. J., Burtner, C., Comai, L., & Henikoff, S. (2004). Mismatch cleavage by single-strand specific nucleases. *Nucleic acids research, 32*(8), 2632–2641.

Tillander, V., Alexson, S. E., & Cohen, D. E. (2017). Deactivating fatty acids: acyl-CoA thioesterase-mediated control of lipid metabolism. *Trends in Endocrinology & Metabolism, 28*(7), 473–484.

Tsai, H., Howell, T., Nitcher, R., Missirian, V., Watson, B., Ngo, K. J., ... & Khan, A. A. (2011). Discovery of rare mutations in populations: TILLING by sequencing. Plant physiology, 156(3), 1257–1268.

Tsunezuka, H., Fujiwara, M., Kawasaki, T., and Shimamoto, K. (2005). Proteome analysis of programmed cell death and defense signaling using the rice lesion mimic mutant cdr2. Mol. *Plant-Microbe Interact, 18*, 52–59.

Vacca, R. A., de Pinto, M. C., Valenti, D., Passarella, S., Marra, E., & De Gara, L. (2004). Production of reactive oxygen species, alteration of cytosolic ascorbate peroxidase, and impairment of mitochondrial metabolism are early events in heat shock-induced programmed cell death in tobacco Bright-Yellow 2 cells. *Plant physiology, 134*(3), 1100–1112.

Valandro, F., Menguer, P. K., Cabreira-Cagliari, C., Margis-Pinheiro, M., & Cagliari, A. (2020). Programmed Cell Death (PCD) control in Plants: New insights from the Arabidopsis thaliana deathosome. *Plant Science,* 110603.

Van der Auwera, G. A., Carneiro, M. O., Hartl, C., Poplin, R., Del Angel, G., Levy-Moonshine, A., ... & Banks, E. (2013). From FastQ data to high-confidence variant calls: the genome analysis toolkit best practices pipeline. *Current Protocols in Bioinformatics, 43*(1), 11–10.

Vanegas, C., Garvin, D., and Kolmer, J. (2008). Genetics of stem rust resis- tance in the spring wheat cultivar Thatcher and the enhancement of stem rust resistance by Lr34. *Euphytica 159*, 391–401. doi: 10.1007/s10681-007- 9541-0

Wang, H., Wu, H. M., & Cheung, A. Y. (1996). Pollination induces mRNA poly (A) tail-shortening and cell deterioration in flower transmitting tissue. *The Plant Journal, 9*(5), 715–727.

Wolter, M., Hollricher, K., Salamini, F., & Schulze-Lefert, P. (1993). The mlo resistance alleles to powdery mildew infection in barley trigger a developmentally controlled defence mimic phenotype. *Molecular and General Genetics MGG*, *239*(1-2), 122–128.

Yamanouchi, U., Yano, M., Lin, H., Ashikari, M., & Yamada, K. (2002). A rice spotted leaf gene, Spl7, encodes a heat stress transcription factor protein. *Proceedings of the National Academy of Sciences*, *99*(11), 7530–7535.

Yan, W., Chen, Z., Lu, J., Xu, C., Xie, G., Li, Y., ... & Tang, X. (2017). Simultaneous identification of multiple causal mutations in rice. *Frontiers in plant science, 7*, 2055.

Zeng, L. (2005). *A novel mechanism underlying programmed cell death in plant defense signaling*. [Doctoral dissertation, The Ohio State University].

Zeng, Z. B. (1994). Precision mapping of quantitative trait loci. *Genetics, 136*(4), 1457–1468.

Zhang, X. Q., Xue, D. W., Zhou, W. H., Wu, F. B., & Zhang, G. P. (2011). Screening and identification of the mutants from two□ row barley cultivar ZJU3 induced by ethyl methane sulfonate. *Journal of Zhejiang University (Agriculture and Life Sciences), 37*(2), 169–174.

Zhang, X., Tian, B., Fang, Y., Tong, T., Zheng, J., & Xue, D. (2019). Proteome analysis and phenotypic characterization of the lesion mimic mutant bspl in barley. *Plant Growth Regulation, 87*(2), 329–339.

Zhang, Z., Lenk, A., Andersson, M. X., Gjetting, T., Pedersen, C., Nielsen, M. E., ... & Thordal-Christensen, H. (2008). A lesion-mimic syntaxin double mutant in Arabidopsis reveals novel complexity of pathogen defense signaling. *Molecular plant, 1*(3), 510–527.

Zhou, Q., Zhang, Z., Liu, T., Gao, B., & Xiong, X. (2017). Identification and map-based cloning of the Light-Induced Lesion Mimic Mutant 1 (LIL1) gene in rice. *Frontiers in Plant Science, 8*, 2122.

CHAPTER 3: IDENTIFYING CANDIDATE GENES UNDERLYING DISEASE LESION MIMIC MUTANTS OF BARLEY USING EXOME CAPTURE AND RNASEQ

Abstract

Programmed cell death (PCD) is an important physiological process that occurs in both plants and animals during development and in respond to biotic and abiotic stresses. The plant innate immune system evolved to rely on PCD to eliminate cells that have been colonized by phytopathogens via tightly regulated mechanisms. Thus, research efforts have focused on these processes in plants to understand disease resistance mechanisms that are important for plant breeding and crop productivity. However, identifying the genes underlying PCD mechanisms is difficult because they are highly conserved, limiting genetic analysis due to the lack of polymorphism. To begin filling this knowledge gap we identified candidate genes underlying genetically characterized barley disease lesion mimic mutants (DLMMs) using forward genetics via the genomic analysis techniques, exome capture and RNA sequencing (RNAseq). Fast neutron irradiation was utilized to generate five DLMMs (FN360, FN361, FN365, FN370, and FN396) in the barley line Steptoe background. Fast neutron irradiation can result in single nucleotide to megabase sized deletions. The Nimblegen barley exome capture array and RNAseq analysis were utilized to capture the coding regions from wildtype (WT) Steptoe and the five independent DLMM mutants for comparative analysis and for differential gene expression utilizing the genetic mapping of each mutant to focus the analyses to the delimited regions. The exome capture analysis identified a large ~2.3 Mb deletion in the FN396 mutant line at the genetically delimited region containing 26 deleted genes. The list of deleted candidate genes from the FN396 mutant contained six genes that encoded putative peptidoglycan-binding LysM domain-containing protein, three RING/U-box superfamily proteins and two Cytochrome P450

superfamily proteins with functions that are known to play a role in PCD pathways representing strong FN396 candidate genes. The exome capture analyses did not identify candidate genes for the other four DLMM mutants possibly due to the lack of exome capture probes or mutations that occur in the promoter region of the mutated genes. Thus, RNAseq analysis was utilized for further analysis confirming the FN396 deletion and identifying candidate genes for the other five DLMMs.

Introduction

Core physiological processes employed by plants during development and to respond to biotic and abiotic stresses rely on programmed cell death (PCD) responses and pathways. Thus, understanding the underlying molecular mechanisms of PCD elicitation is important. To elucidate the signaling pathways that lead to the tightly regulated PCD mechanisms utilized by plants to respond to biotic stresses (i.e. elicitation of plant innate immunity post pathogen perception) will require a comprehensive knowledge of the genes and proteins that suppress PCD when it is not needed. However, filling this knowledge gap is difficult as these pathways are conserved making it impossible to identify genes via natural genetic variation and genetic mapping. The generation of irradiation induced mutant populations, identification and genetic characterization of DLMMs and identification of candidate suppressors of PCD and important signaling components via forward genetic is the strategy reported here. This process was expedited by genomics techniques such as RNAseq and exome capture and the genes identified will provide the gene discovery foundation in order to begin filling important knowledge gaps concerning the molecular mechanisms underlying PCD elicitation and or suppression during development and immunity responses.

Programmed cell death is a biological process that is referred to as cellular suicide in organisms (Raff, 1998), thus requires tight regulation when not needed. The efficient utilization of PCD by plants for normal biological processes necessitates suppression of these signaling mechanisms, which appear to be quite complex due to crosstalk between signaling pathways that interconnect multiple transcription factors (activators and suppressors), enzymes, and other molecular components. The PCD responses that evolved for plant development and reproduction were also coopted as a major component of plant innate immune systems (Vaux & Korsmeyer, 1999). Pathogen perception via immunity receptors in plants trigger defense signaling cascades that typically result in high amplitude PCD responses referred to as the hypersensitive response (HR). The HR is somewhat complex and its spatial and temporal occurrence as well as magnitude relies on signaling mechanisms involving the plant hormones salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and the reactive oxygen species (ROS) and nitric oxide (NO) molecules (Dong, 1998; Laloi et al., 2004; Thomma et al., 2001). Plants contain two different classes of receptors that detect pathogens to elicit the two levels of resistance that are known as Pathogen Associated Molecular Pattern (PAMP) triggered immunity (PTI) and effector triggered immunity (ETI). The two levels of resistance although considered distinct still rely on some common signaling pathways and components.

Transmembrane cell surface receptors referred to as pattern recognition receptors (PRRs) that are typically localized to the plasma membrane in heterologous complexes (Monaghan & Zipfel, 2012) elicit PTI responses through the activation of underlying cytosolic signaling cascades including mitogen activated protein kinase (MAPK) pathways (Macho & Zipfel, 2014). PRRs typically contain extracellular receptor domains, a transmembrane domain and are grouped into two classes based on the presence of an intracellular kinase signaling domain known as the

receptor-like kinases (RLKs), or are missing the kinase signaling domain and are known as receptor-like proteins (RLPs) (Zipfel, 2008). The PRR complexes detect conserved PAMPs that are essential for the fitness of the pathogen, such as chitin a major component of fungal cell walls and flagella used for bacteria motility. For example, the RLKs FLS2 and BAK1 create complexes that work together to detect the flagella subunit flg22 to activate signal transduction pathways that regulate PTI responses. Pathogens on the other hand counter evolved effectors to stop PTI responses either by hiding their PAMPs from the plants innate immune system (Toruño et al., 2016) or suppressing the signaling mechanisms resulting in effector triggered susceptibility as proposed in the zig-zag model of the plant host-microbial pathogen molecular arms race (Jones & Dangl, 2006).

An important evolutionary process studied in the field of phytopathology is the evolution of virulence effectors that suppress early PTI responses known as non-host resistance mechanisms giving rise to specialized pathogens. This selection pressure applied on the first line of induced defense responses in plants, dictated the counter evolution of cytoplasmically localized immune receptors; a continuation of events depicted in the zig-zag model (Jones & Dangl, 2006). The cytoplasmically localized immune receptors that fall into the class of NOD-like receptors (NLR), also known as nucleotide binding-leucine rich repeat resistance-proteins (R-proteins), elicit HR at the sight of infection effectively stopping biotrophic pathogen colonization. NLRs interact with and detect pathogen effectors either directly or more commonly indirectly. Several models have been proposed to illustrate these different ETI interaction at the functional or protein level. The direct interaction model, which is typically referred to as the functional model for gene-for-gene interactions, are nicely exemplified by the direct interaction between the flax NLR R-protein *L5* with the flax rust Avr567 protein in the flax-flax rust

pathosystem (Flor, 1971; Ravensdale et al, 2011). The guardee model represents interactions where a host protein is targeted by a virulence effector and the action of the effector on the host virulence target protein is monitored by an NLR R-protein, which elicits the ETI defense responses (Qi & Innes, 2013). An excellent example is the *Pseudomonas syringae* pv. *maculicola* virulence effector AvrRPM1 that targets the RIN4 (RPM1 interacting protein 4) protein in *Arabidopsis* to suppress basal resistance responses that include stomate closure and seizure post pathogen recognition to prevent host entry. RPM1 encodes an NLR R-protein that detects the AvrRPM1-mediated modification of RIN4 eliciting ETI responses that result in HR (Mackey et al., 2002). The decoy model is similar to the guard model except that the plant host evolved a mimic of the guardee that does not have its original biological function and possibly evolved a higher affinity for the avirulence effector for enhanced effector recognition and defense elicitation. A nice example of the decoy model is the RCR3 (a secreted papain-like cysteine protease) decoy for the operative target PIP1, which is a cysteine protease that is secreted in abundance during defense responses in tomato. RCR3 is guarded by Cf-2 an R-protein that interacts with RCR3 to detect the manipulation by the *Cladosporium fulvum* effector Avr2 (Shabab et al., 2008). The integrated decoy or integrated sensory domain model is based on the hypothesis that effector targets can be translocated to dual NLR loci where one NLR considered the receptor NLR has the integrated sensory domain (ISD) fused as part of the NLR immunity receptor forming an NLR-ISD immunity receptor. The other NLR known as the signaling NLR forms an interactive duplex with the NLR-ISD receptor NLR and is kept in its inactive state. Once the pathogen effector or avirulence protein effector interacts with the NLR-ISD receptor the signaling NLR disassociates from the NLR-ID becoming activated eliciting the resistance responses that typically results in HR-mediated immunity. An excellent example is the

RRS1 and RPS4 duel NLRs, where RRS1 contains a WRKY transcription factor domain that is targeted by the *P. syringae* AvrRps4 and PopP2 effectors eliciting the resistance responses (Cesari et al., 2014). This new NLR-ISD genome architecture, which results in an immunity receptor-Avr protein direct interaction provides effective resistance responses and/or stabilize the resistance locus by preventing recombination events that may lead to the separation of the NLRs and ISD virulence target tha could result in the loss of immunity function (Bomblies et al., 2007; Cesari et al. 2014). These models show how ETI evolved in plants to detect biotrophic pathogens that elicit HR immunity responses to sequester the biotrophic pathogens in a foci of dead cells. This immunity response restricts the pathogen from nutrient access effectively stopping colonization and disease progress.

Immunity responses that lead to PCD/HR conferring effective resistance against biotrophic pathogens can be hijacked by necrotrophic pathogens that produce necrotrophic effectors to purposefully elicit immunity responses through R-protein recognition via the inverse gene-for-gene model (Friesen & Faris, 2010). This elicitation of the immunity responses leads to PCD, which the necrotrophic pathogen utilizes to extract nutrient to facilitate further disease development through the process known as necrotrophic effector triggered susceptibility (NETS; Liu et al., 2015). Biotrophic and necrotrophic pathogens elicit immunity responses that rely on PCD/HR with vastly different results, incompatibility (resistance responses) or compatibility (susceptibility responses), respectively, that are determined by the pathogen's lifestyle. Thus, understanding PCD pathways, which is the main goal of this research, is important to understand how both classes of pathogens interact with crop plants to elicit resistance and susceptibility responses.

Plant mutant populations are induced using radiation or chemical mutagen agents that delete or alter the nucleotide sequences of important developmental genes. Radiation and chemically induced mutant populations that contained DLMMs have been available for close to 100 years in diverse crop plants (Stadler, 1928). The DLMM mutants typically arise by altering the function of suppressors, signaling components or enzyme involved in PCD pathways (Lorrain et al., 2003; Mou et al., 2000). Disease lesion mimic mutants produce spontaneous lesion that resemble diseases caused by necrotrophic pathogens like *Bipolaris sorokiniana* or *Pyrenophora teres* that are expressed in the absence of pathogen infection. However, it wasn't until the last two decades that the advancement of molecular techniques allowed for the identification and functional analysis of genes and signaling pathways underlying interesting developmental mutants including the DLMMs (Druka *et al.*, 2010; Greenberg & Ausubel, 1993; Lorrain et al., 2003; Penmetsa and Cook, 2000). Utilizing forward genetics to characterize the genes underlying DLMM phenotypes will answer important questions concerning the regulation of PCD pathways that are important in immunity responses and developmental processes (Lorrain et al., 2003). Thus, identifying and characterizing the genes underlying these mutant loci can be facilitated by comparative genomic analysis of wild type plants and mutants in large grass genomes like the 5000 Mb genome of barley utilizing RNAseq and exom capture approaches (Brodersen et al., 2002). However, few DLMMs have been characterized leaving them as an underutilized resource.

Several barley DLMMs have been described (Lorrain et al., 2003), but only three genes underling this class of barley mutants have been identified, *Hvnec1*, *mlo* and the recent identification of the *nec3* gene in our lab. *Nec1* encodes a cyclic-gated ion channel protein (Rostoks et al., 2003, 2006) with high sequence similarity to the *HLM1* gene of *Arabidopsis*

(Lundqvist et al., 1997). *HLM1*, similar to *nec1* shows high pathogenesis-related (PR) protein expression and displays a DLMM phenotype, which includes spontaneous leaf tip necrosis. HLM1 also has increased susceptibility to necrotrophic pathogens (Balagué et al., 2003; Stintzi et al., 1993;). The *mlo* mutants of barley confer increased resistance to the fungal pathogen *Erysiphe graminis* f. sp. *hordei,* the causal agent of powdery mildew. The *mlo* mutant has been widely deployed in Northern European malting barley varieties providing durable powdery mildew resistance for nearly 40 years (Lyngkjær & Carver, 2000). However, the deployment of *mlo* confers susceptibility to necrotrophic pathogens including *Bipolaris sorokiniana* the spot blotch pathogen (Kumar et al., 2001), *Fusarium graminearum* the cause of fusarium head blight (Jansen et al., 2005), *Ramularia collo-cygni* the Ramularia leaf spot pathogen (McGrann et al., 2014) and *Magnaporthe oryzae* the rice blast pathogen (Jarosh et al., 1999). Based on the inverse gene-for-gene and NETS models this inverse interaction of the *mlo* mutants is not surprising. The cloning of the *mlo* genes showed that *Mlo* encodes a conserved ROP like G-protein that suppresses PCD and is found in other species (Wolter et al., 1993).

The barley *nec3* mutants have distinctive cream to orange necrotic lesions lacking the typical dark pigmented lesions indicative of serotonin/phenolics deposition. The *nec3* gene was recently shown to be elicited by a diverse taxonomy of pathogens and was delimited genetically to a 0.14 cM region on chromosome 6H representing ~16.5 Mb of physical sequence. This physical region contained 149 annotated high confidence genes. RNAseq was utilized on five independent *nec3* mutants and comparative analysis against the wild type background genotypes identified a cytochrome P450 gene as *nec3*. Four of the five *nec3* mutants contained deletions or nucleotide substitutions that resulted in premature stop codons resulting in predicted truncated non-functional proteins. The fifth *nec3* mutant had a nucleotide substitution that resulted in an

A308P amino acid substitution of a conserved residue of the oxygen binding motif (Ameen et al., 2018; Ameen et al., under review). *Nec3* is an ortholog of the Sekiguchi lesion (*sl*) mutant gene of rice that catalyzes the conversion of tryptamine to serotonin, thus it was shown that this distinctive tan-orange lesion phenotype was primarily due to the lack of serotonin build up in the necrotic lesions. The *sl* mutant of rice expresses a similar orange-tan DLMM phenotype as the barley *nec3* mutants (Fujiwara et al., 2010; Ishihara et al., 2008). The rice *sl* gene encodes a CYP71P1 cytochrome P450 monooxygenase gene that encodes a Tryptamine 5-Hydroxylase enzyme that converts tryptamine to serotonin (Ishihara et al., 2008) and shares 87% amino acid identity with the barley *Nec3* protein. The *sl* mutant was susceptible to the rice brown spot fungus (*Bipolaris oryzae*) and susceptibility was eliminated by the exogenous application of serotonin (Fujiwara et al., 2010). The exogenous serotonin deposited into the cell wall of the *sl* lesions restored the dark pigmentation and resistance to the necrotrophic pathogen *B. oryzae* (Ishihara et al., 2008). Thus, it is hypothesized that plants evolved oxidative polymerization of serotonin in cell walls as a physical barrier and phenolics deposition at the infection site during pathogen infection to inhibit pathogen growth and sequester them in the foci of dead cells. This mechanism may also act as a signaling mechanism to sequester lesion expansion to preserve the leaf's photosynthetic capability after reacting to pathogen challenge. Interestingly in this study the FN360 mutant may have uncovered an allele of the *nec3* mutant that was present in this DLMM with another disease lesion mimic mutation on chromosome 2H that independently assorted.

In this study, we hypothesized that we may identify suppressor of PCD that keep these pathways inactive when not needed to defend against biotrophic pathogens. Thus, keeping these conserved immunity responses in check with tight regulation but ready to spring into action

when challenged by a pathogen. Each of the five DLMMs characterized (FN360, FN361, FN365, FN370, and FN396) demonstrated different lesion mimic phenotypes that mapped to different chromosomal locations (presented in chapter 2), indicating that each contains distinct mutations in genes that function in PCD pathways. Both the exome capture and RNAseq techniques were utilized to identify gene/s underlying mutant phenotypes in a forward genetics approach. Identifying these genes and the function of the proteins they encode will allow us to begin filling important knowledge gaps concerning the molecular pathways and components of PCD in plants.

The exome capture tool developed by Mascher et al (2013) provided the barley research community with an excellent genomic resource to reduce genome sequencing down to the transcribed gene space. We expected that the exome capture would allow for the detection of at least some of the deleted genes within the delimited regions compared to reference line 'Morex' and WT Steptoe background. However, the analyses are only as robust as the probe coverage for each region delimited by the genetic mapping and since we did not have probes for some genes within these regions and some of the mutants could represent promoter mutations there was the possibility that we would not identify the causal mutation underlying each DLMM analyzed. Thus, we used RNAseq as a backup to the exome capture sequencing. Exome capture was able to identify strong candidate genes for the FN396 DLMM and RNAseq confirmed this deletion as well as identified some candidate genes for the other DLMMs.

Materials and Methods

Plant Material

Exome capture and RNAseq analyses were performed on the DLMM FN360, FN361, FN365, FN370, and FN396. all the mutants used in these analyses were generated by seed

exposure to fast neutron irradiation at 3.5 and 4 Gy at the FAO/IAEA Seibersdorf SNIF facility (Rostoks, 2003), in the Steptoe background, which is six-row, spring feed barley developed and released by Washington State University in 1973 (Muir & Nilan, 1973). Steptoe is derived from Washington 3564 x Unitan cross (Muir & Nilan, 1973). The Steptoe accession number is CIho 15229. All the mutants utilized develop different phenotypes based on lesion morphology or timing of development and were previously described in Chapter 2. For the exome capture experiment, three to five seed of each genotype were used to extract genomic DNA from germinated seed as described below. For the RNAseq analysis three different biological replicates of each genotype were grown in controlled environmental conditions for four weeks prior to sample collection as described below.

Exome Capture and Bioinformatics Analysis

Genomic DNA (gDNA) was isolated from 3-5 embryos extracted from wildtype (WT) Steptoe, FN360, FN361, FN365, FN370, and FN396 mutant seed pregerminated overnight in 100x15mm petri dishes containing a disk of Watman filter paper and ~3mls of H_2O. DNA extractions were performed using the PowerPlant Pro DNA isolation kit (MoBIO Laboratories Inc., QIAGEN Carlsbad CA) following the manufactures standard protocol. To check the quality of extracted DNA, an aliquot of 1 µL of gDNA was separated on a 1% agarose gel supplemented with GelRED (Biotium) fluorescent nucleic acid dye. The DNA was considered high quality and of adequate integrity if a high molecular weight band at ~15-20 kb was visualized with minimal low molecular weight smearing indicative of intact genomic DNA with minimal degradation. The DNA was quantified utilizing the Qubit Fluorometer using the Qubit Broad Range DNA Quantification kit (Thermo Scientific) following the manufacturers standard protocol. The gDNA was fragmented utilizing reactions containing 1.5 µg of gDNA in a 20 µl reaction with 1

µl of NEB dsDNA Fragmentase enzyme, 1x Fragmentase reaction buffer and 10mM $MgCl_2$ (New England Biolabs, Ipswich MA), which was experimentally shown to produce a majority of DNA fragments in the size range between 250-450 bp. The digested DNA was analyzed for size distribution on an Agilent 2100 Bioanalyzer (Agilent Technologies) using a DNA 1000 kit (Agilent Technologies) following the manufacturers standard protocols for the chip loading and data analysis. The enzymatic digestion for 25 minutes produced the optimal fragment size between 250-450 base pairs, which was used to develop the exome capture libraries from WT Steptoe and all five mutants.

The exome capture sequencing experiments for all the fragmented gDNA samples was accomplished using the Roche NimbleGen SeqCap EZ Developer probe pool barley exome design 120426_Barley_BEC_D04, which contains a total capture design size of 88.6 Mb. This exome capture effectively reduces the barley genome sequence load by 98%. The Illumina sequencing library preparation was done using the KAPA HTP gDNA library preparation kit following the manufacturers standard protocol, except the Pippin Prep gel purification system (Sage Science) was used for size selection utilizing a precast 2% gel cassette set to collect fragments between 250-450 bp. The barcoded barley whole exome capture multiplexed library was prepared from the gDNA following seqCAP EZ Library SR user guide 4.1 protocol. To determine both the quality and size distribution of the final capture library the Agilent 2100 Bioanalyzer (Agilent Technologies) was utilized using the DNA 1000 kit (Agilent Technologies) following the manufacturers standard protocols for the chip loading and data analysis. The final DNA quantification of the exome capture libraries for dilution and subsequent sequencing on an Illumina NextSeq flow cell was performed on a Qubit fluorometer. For the Illumina sequencing a Nextseq 500 kit was used set to generate 150 base pair single end reads. The Illumina raw

sequencing reads for WT Steptoe and all the mutants were parsed by their specific barcodes using CLC Genomics Workbench v8. The Quality scores for the raw reads were determined by the FQC dashboard (Brown et al., 2017) and CLC Genomics Workbench v8 was used to trim the adapter sequences from the raw Illumina NextSeq sequences in the FASTQ format. The BWA 'mem' algorithm with default settings was used to align all samples to the barley reference genome (IBGSC, 2012; Li & Durbin, 2010). In order to identify the deleted regions, the alignments were analyzed utilizing two separate pipelines, where SAMtools 'mpileup' with default settings was used to identify small deletions (less than 100 bp) (Li et al., 2009). A minimum read depth of 3 and a minimum individual genotype quality of 10 were used to filter the identified variants using VCFtools (Danecek et al., 2011). As fast neutron mutagenesis may induce large chromosomal deletions, BEDTools 'genomecov' was utilized to identify full gene deletions by calculating the sequencing coverage across all exome capture targets (Quinlan & Hall, 2010; Solanki et al., 2019).

RNAseq and Bioinformatics Analysis

Three biological samples were collected from WT Steptoe and the mutants FN360, FN361, FN365, FN370, and FN396 as mentioned above, and three pieces of leaves, ~3 cm in length, were used for each sample (the total of nine leaves from each genotype divided into three sample tubes). The samples were collected after four weeks from the germination. An Illumina platform RNAseq library was prepared using the TruSeq RNA library prep kit v2 (Illumina) following the manufacturers standard protocol. The RNAseq library was sequenced on the Illumina NextSeq 500 flowcell. The read quality was checked using the FastQC v0.11.5 software. The CLC Genomics workbench 8.0.3 software (QIAGEN Bioinformatics, CA) was used to run data analysis and comparisons. The reads from all 21 samples were aligned using the

barley concatenated reference high confidence and low confidence gene list provided in the barley IBSC IPK 2016 database. Alignment was performed as follows; the mismatch cost was 2, while both the insertion and deletion costs were 3, the length and similarity fraction were 0.9, reads were aligned for both strand specificity, and the maximum number of hits for a read was set at 10. RPKM and was used to calculate gene expression. For each comparison between Steptoe and other mutants', an Empirical analysis of DGE (EDGE) test was used to run an "Exact test". The criteria were used as FDR corrected $P< 0.05$ and EDGE ≥3-fold regulation values to identify the differentially expressed genes between Steptoe and the mutant samples. The data generated determined the differential gene expression between Steptoe and the five mutants (FN360, FN361, FN365, FN370, and FN396) that were generated in the Steptoe background. The RNAseq data was utilized via expression analysis and comparative sequence analysis to identify candidate mutant genes within the genetically defined mutant regions for each independent mutant.

Results

Exome Capture

Sequence reads obtained from the Illumina Nextseq 500 sequencing of the barley exome capture libraries of FN360, FN361, FN365, FN370, FN396 and WT Steptoe were analyzed to identify potential deletions or nucleotide differences within the genomic regions delimited by the genetic mapping of each DLMM (the genetic mapping was described thoroughly in Chapter 2). The physical genomic regions analyzed were delimited by the genetic markers described in chapter 2 for each of the mutants and anchored to the WGA Morex sequence released in 2019 (Monat et al., 2019). The data was utilized for comparative analysis between WT Steptoe and all five mutants. Based on the comparative analysis of FN360, FN361, FN365, and FN370 exome

capture sequences against WT Steptoe, no mutations within the regions delimited by genetic analysis were identified. However, the comparative analysis of FN396 identified a large deletion of ~ 2.3 Mbp between the flanking SNP markers 12_10936 on the distal side at position 659264767 bp and 11_10429 on the proximal side at position 704365928 bp. The ~ 2.3 Mbp deletion contained fifteen high confidence deleted genes (Table 3.1) that were all in the positive orientation (Figure 3.1). Based on gene annotations of the deleted genes, six were considered strong candidates based on their previously characterized function in PCD signaling mechanisms. These annotated high confidence barley genes included a putative RING/U-box superfamily protein, a superoxide dismutase-like protein, peptidoglycan-binding LysM domain-containing protein, a Thioesterase superfamily protein and a Cytochrome P450 protein. These classes of proteins have been shown to function in PCD responses and signaling in diverse plant and animal species including tobacco, rice, and humans (Tillander et al., 2017; Vacca et al, 2004; Zeng et al, 2004).

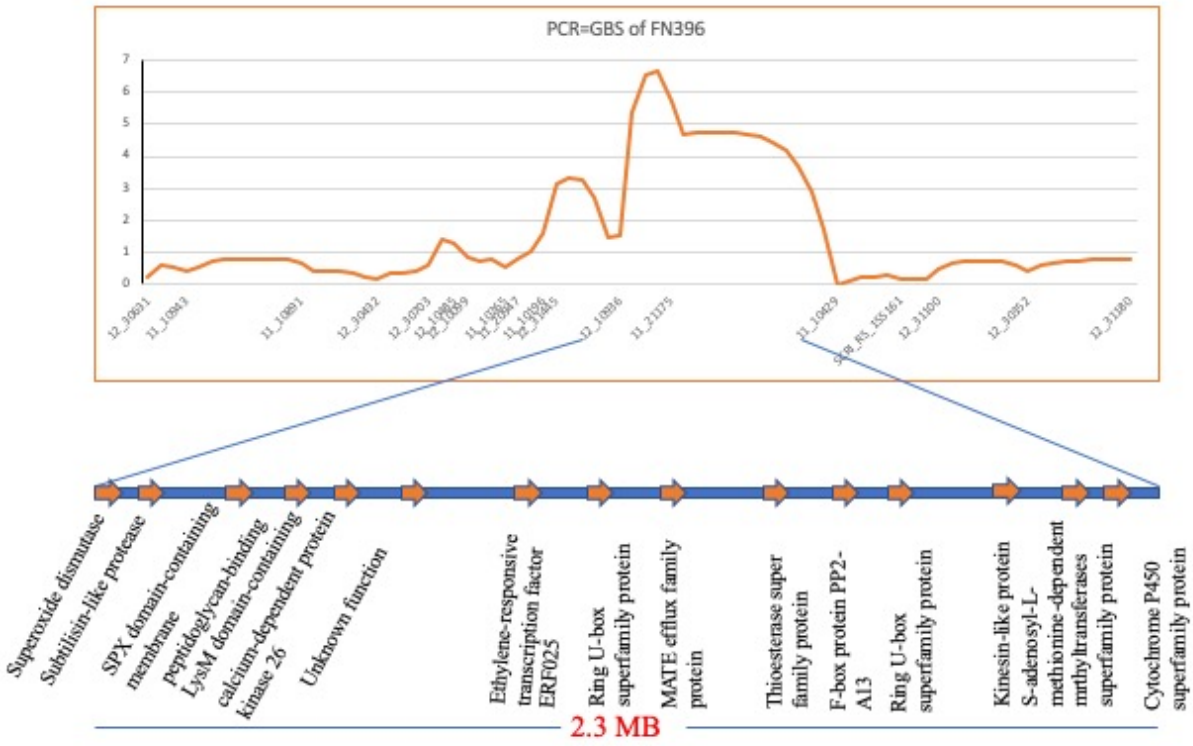

Figure 3.1: The genetic mapping for FN396 shows the deleted genes underlying the FN396 QTL. The arrows represented the direction of all deleted genes (forward direction)

Table 3.1: The complete list of annotated genes presents in the FN396 2.3 Mb deletion underlying QTL defined by genetic mapping.

Gene_ID	chromosome	confidence_class	Barleyanno
HORVU2Hr1G094650	chr2H	HC_G	Superoxide dismutase [Cu-Zn]
HORVU2Hr1G094660	chr2H	HC_G	Subtilisin-like protease
HORVU2Hr1G094670	chr2H	HC_G	Subtilisin-like protease
HORVU2Hr1G094680	chr2H	HC_G	Oxygen-dependent choline dehydrogenase
HORVU2Hr1G094690	chr2H	HC_G	SPX domain-containing membrane protein
HORVU2Hr1G094730	chr2H	HC_G	peptidoglycan-binding LysM domain-containing protein
HORVU2Hr1G094740	chr2H	HC_G	calcium-dependent protein kinase 26
HORVU2Hr1G094760	chr2H	HC_U	unknown function
HORVU2Hr1G094770	chr2H	HC_u	undescribed protein
HORVU2Hr1G094780	chr2H	HC_G	Dehydration-responsive element-binding protein 1E
HORVU2Hr1G094790	chr2H	HC_G	INO80 complex subunit C
HORVU2Hr1G094810	chr2H	HC_G	Ethylene-responsive transcription factor 1
HORVU2Hr1G094840	chr2H	HC_G	RING/U-box superfamily protein
HORVU2Hr1G094860	chr2H	HC_G	RING/U-box superfamily protein
HORVU2Hr1G094870	chr2H	HC_G	MATE efflux family protein
HORVU2Hr1G094890	chr2H	HC_G	Thioesterase superfamily protein
HORVU2Hr1G094910	chr2H	HC_TE?	unknown function
HORVU2Hr1G094930	chr2H	HC_u	undescribed protein
HORVU2Hr1G094960	chr2H	HC_G	F-box protein PP2-A13
HORVU2Hr1G094980	chr2H	HC_G	RING/U-box superfamily protein
HORVU2Hr1G095010	chr2H	HC_TE?	SWIM zinc finger family protein
HORVU2Hr1G095030	chr2H	HC_G	Cytochrome P450 superfamily protein
HORVU2Hr1G095050	chr2H	HC_G	Kinesin-like protein
HORVU2Hr1G095070	chr2H	HC_G	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein
HORVU2Hr1G095080	chr2H	HC_G	Cytochrome P450 superfamily protein
HORVU2Hr1G095090	chr2H	HC_TE?	zinc ion binding
HORVU2Hr1G095100	chr2H	HC_G	Carboxypeptidase Y
HORVU2Hr1G095130	chr2H	HC_G	Cytochrome P450 superfamily protein

RNAseq Differential Gene Expression

Exome capture sequencing identified candidate genes for FN396, but failed to identify any deletions or mutations for the other four mutants characterized. The reason deletions or nucleotide substitution mutations within the genetically delimited regions were not identified for 4 of the 5 DLMM mutants was most likely due to mutations occurring outside the primary

coding sequences including promoter regions that are not captured from the gDNA by the Roche NimbleGen barley exome capture array. The exome capture probes contain a total capture design size of 88.6 Mb and based on the newest barley genome assembly and annotation (Roche NimbleGen, Roche, Indianapolis, IN, USA) the exome capture contain ~85% of the high confidence genes. In order to circumvent the missing genes and the possibility of mutations in regions not targeted by the exome capture we utilized RNAseq for further analyses of the DLMM mutants. These RNAseq analyses utilized the recently released cv Morex genome sequence annotation (Mascher et al, 2017) that relied on RNAseq from multiple tissues and treatments to predict high confidence (HC) genes. For each of the mutants there was an extensive list of differentially expressed genes but similar to the exome capture data the genes within the genetically delimited regions were the focus of these analyses for candidate gene identification.

For the RNAseq analysis of the FN360 DLMM we focused on both regions associated with the segregating mutant phenotypes (tan-orange and dark lesions) that genetically mapped to two distinct regions when analyzing the FN360 x Morex F_2 population (Chapter 2). The distinctive tan-orange *nec3*-like phenotype that segregated in the FN360 x Morex population mapped to the *nec3* locus (Ameen et al., 2018; Kesia et al., 2010) at the centromeric region of chromosome 6H, and the dark DLMM phenotype genetically mapped to the short arm of chromosome 1H. The genetic mapping of the *nec3* phenotype delimited the region to ~102 Mb flanked distally by the SNP marker 12_10758 at position 403,737,244 bp and proximally by the SNP marker 12_30698 at position 505,428,378 encompassing the previously defined *nec3* locus (Ameen et al., 2019: Kesia et al., 2010). Based on the Morex_2016 barley genome assembly that was originally utilized for the RNAseq analyses two differentially expressed genes (DEGs) that were both significantly upregulated were identified within the genetically defined *nec3* region

(Table 3.2). However, since these analyses were completed a new version of the barley genome assembly had been released and the *nec3* gene identified via positional cloning and RNAseq analyses. Interestingly, the cytochrome P450 *nec3* gene (HORVU.MOREX.r2.6HG0460850) was not present in the exome capture array and was also misassembled and placed in the wrong genomic position, thus was not identified by the original analyses utilized in this study. Therefore, the RNAseq data was realigned to the HORVU.MOREX.r2.6HG0460850 HC gene model showing that the FN360 mutant utilized in these analyses did not identify the *nec3* gene because it was only induced after pathogen inoculation and this treatment was not utilized in this study.

The RNAseq analysis was also utilized to identify DEGs within the genetically defined region for the dark necrotic lesions genetically defined on chromosome 1H in the FN360 x Morex F_2 population. Utilizing the Morex_2016 barley genome assembly the genetic mapping determined that the FN360 locus was flanked by the distal marker 12_31144 located at position 4,653,990 bp and the proximal marker 12_30588 located at position 10,747,464 bp representing a physical region of ~ 6 Mb. Within this genetically defined region one DEG was identified that was significantly downregulated (Table). This gene represented a strong candidate gene as it was predicted to encode a Chymotrypsin inhibitor and it is homolog of *Arabidopsis* serine protease inhibitor gene, which are known to be negative regulators of PCD in plant and pathogen interaction (Li et al., 2008).

Table 3.2: Differentially expressed genes identified by RNAseq analysis between Steptoe and FN360 within the genetically defined *nec3* region at the centromeric region of chromosome 6H.

Feature ID	Barley annotation	Arabidopsis Homology	Steptoe Vs FN360 tan	
			Fold change	Corrected P-value
HORVU6Hr1G064620	Dehydrin COR410	AT1G20440.1	3.70021	9.66E-08
HORVU6Hr1G065210	transcription factor-related	AT2G27230.2	5.92769	0.0000855

Table 3.3: Differentially expressed genes identified by RNAseq analysis between Steptoe and FN360 within the genetically defined region on chromosome 1H containing the mutation that resuled in the dark necrotic lesion phenotype.

Feature ID	Barley annotation	Arabidopsis Homology	Steptoe Vs FN360 Dark	
			Fold change	Corrected P-value
HORVU1Hr1G004150	Chymotrypsin inhibitor	AT2G38870.1	-11.88892667	1.61866E-23

The RNAseq analysis of the FN361 DLMM was genetically delimited to a region of the short arm of chromosome 5H. Utilizing the Morex_2016 barley genome assembly the region was defined by the flanking SNP markers 11_20010 at position 6,354,602 bp and 12_30745 at position 475,851,929 bp representing a large physical region of ~469 Mb. The RNAseq analysis of FN361 identified a single downregulated gene in the region and five upregulated genes (Table 3.4). The downregulated gene is a WRKY transcription factor, which is a class of genes that plays a major role in defense against pathogens. WRKY transcription factors are activated by defense signaling cascades and function to activate pathogen related proteins and signaling mechanisms that trigger PCD responses, thus is considered a strong candidate gene.

Table 3.4: Differentially expressed genes identified by RNAseq analysis between Steptoe and FN361 within the genetically defined region on chromosome 5H.

Feature ID	Barley annotation	Arabidopsis Homology	Steptoe Vs FN361	
			Fold change	Corrected P-value
HORVU5Hr1G032980	fructose-bisphosphate aldolase 2	AT4G38970.1	3.258240052	0.000223051
HORVU5Hr1G034830	WRKY family transcription factor	AT4G11070.2	-5.461054541	4.4532E-05
HORVU5Hr1G046480	Chalcone--flavonone isomerase	AT5G05270.2	145.9448418	4.94378E-16
HORVU5Hr1G053480	Protein FLUORESCENT IN BLUE LIGHT, chloroplastic	AT3G14110.2	3.412932561	0.001760904
HORVU5Hr1G055950	Cysteine-rich venom protein	AT4G33720.1	3.862460136	0.004041019
HORVU5Hr1G056040	Cysteine-rich venom protein	AT4G33720.1	3.908531219	0.011920746

The genetic mapping of the FN365 DLMM in the FN365 x Morex F_2 population resulted in three significant QTL contributing to the phenotype. These three regions were classified as two minor QTL, of less significance on chromosome 1H and the major QTL, which mapped to the long arm of chromosome 5H. One explanation for the two minor QTL is that in the FN365 x Morex population had segregating genes that interact with the FN365 mutation and downstream signaling that influence the PCD responses. However, since this F_2 population and previous allelism test populations segregated as a single recessive gene it can be posited that the mutation lies at the major significant QTL on chromosome 5H. The 5H locus is flanked by the markers, 11_20375 proximally at position 609,073,896 bp and 11_10600 distally at position 649,232,960 bp representing a physical region of ~40 Mb. The RNAseq analysis identified twelve DEGs, two genes that were significantly downregulated and ten genes that were upregulated (Table 3.5). The RNAseq analysis was also used to identify DEGs within the other two significant loci. The

first minor QTL on chromosome 1H was delimited by the SNP markers, 12_31144 distally at position 4,653,990 bp and 12_30588 proximally at position 10,747,464 bp for a total physical region of ~6Mb. Within this delimited physical region there were five DEGs that were all upregulated (Table 3.6). The second minor QTL was flanked with the markers, 12_30343 proximally at position 401,872,471 bp and 11_20844 distally at position 525,478,648 bp representing a physical region of ~88 Mb. Within this genetically delimited region there were six high confidence genes downregulated and fifteen genes upregulated (Table 3.7).

Table 3.5: Differentially expressed genes identified by RNAseq analysis between Steptoe and FN365 within the genetically defined region of chromosome 5H.

Feature ID	Barley annotation	Arabidopsis Homology	Steptoe Vs FN365	
			Fold change	Corrected P-value
HORVU5Hr1G099390	NAC domain containing protein 42	AT2G43000.1	37.7045353	0.03930764
HORVU5Hr1G100150	Calmodulin-binding protein	AT2G18750.3	8.11076286	0.00024937
HORVU5Hr1G100180	unknown function	AT4G25800.2	28.0900488	0.00016757
HORVU5Hr1G101990	undescribed protein	N/A	-3.8865261	0.03876718
HORVU5Hr1G102900	Cysteine-rich receptor-like protein kinase 25	AT5G48540.1	4.5464897	0.00035933
HORVU5Hr1G103990	early nodulin-like protein 14	AT2G26720.1	6.54653298	0.00302929
HORVU5Hr1G104620	Type IV inositol polyphosphate 5-phosphatase 7	AT2G32010.4	17.3742551	0.03930764
HORVU5Hr1G104670	Glutathione S-transferase family protein	AT3G62760.1	29.3101921	6.6349E-11
HORVU5Hr1G104790	Protein kinase	AT2G41170.3	8.62013021	4.8187E-08
HORVU5Hr1G105930	CASP-like protein 1U3	AT4G15630.1	25.37123	1.657E-05
HORVU5Hr1G106010	Cysteine-rich secretory protein 3	AT3G19690.1	687.349828	2.0169E-57
HORVU5Hr1G106120	Opaque-2-2 protein	AT4G02640.4	-3.1192393	0.00038936
HORVU5Hr1G106550	histone H1-3	AT2G30620.2	3.32504671	0.00825703

Table 3.5: Differentially expressed genes identified by RNAseq analysis between Steptoe and FN365 within the genetically defined region of chromosome 5H (Continued).

Feature ID	Barley annotation	Arabidopsis Homology	Steptoe Vs FN365	
			Fold change	Corrected P-value
HORVU5Hr1G109190	germin-like protein 4	AT1G18980.1	18.8853922	6.425E-20
HORVU5Hr1G110180	phosphate transporter 1;7	AT3G54700.2	5.592062437	8.56321E-10
HORVU5Hr1G110220	phosphate transporter 1;7	AT3G54700.2	3.129020643	0.001123666
HORVU5Hr1G110900	calcium-dependent protein kinase 19	AT5G12480.1	3.193709602	0.004545262
HORVU5Hr1G111520	EF hand calcium-binding protein family	AT5G44460.1	42.02410375	0.015486439
HORVU5Hr1G112610	rRNA N-glycosidase	AT3G59490.3	36.08917335	6.68124E-07
HORVU5Hr1G114230	Bowman-Birk type trypsin inhibitor	AT4G21550.3	-3.403839931	0.006709552
HORVU5Hr1G114700	UDP-Glycosyltransferase superfamily protein	AT3G11340.1	12.21268717	3.69E-07
HORVU5Hr1G115230	non-specific phospholipase C1	AT1G07230.1	3.559585849	5.56138E-05
HORVU5Hr1G115250	unknown function	AT1G19530.2	-20.26449462	0.001298501
HORVU5Hr1G115750	PLANT CADMIUM RESISTANCE 2	AT1G14870.1	5.524989372	0.00304653
HORVU5Hr1G115870	PLANT CADMIUM RESISTANCE 2	AT1G14870.1	120.1792845	4.40974E-49
HORVU5Hr1G115880	PLANT CADMIUM RESISTANCE 2	AT1G14870.1	14.36225191	0.039307635

Table 3.6: Differentially expressed genes identified by RNAseq analysis between Steptoe and FN365 within the minor QTL identified at the genetically defined region of chromosome 1H (4.7 to 10.7 Mb).

Feature ID	Barley annotation	Arabidopsis Homology	Steptoe Vs FN365	
			Fold change	Corrected P-value
HORVU1Hr1G002320	unknown protein	AT2G43780.3	3.17099251	0.03515764
HORVU1Hr1G002460	receptor-like protein kinase 4	AT1G66910.1	15.7204366	8.2274E-17
HORVU1Hr1G002470	receptor-like protein kinase 4	AT1G70250.1	17.4643138	1.673E-05
HORVU1Hr1G002600	receptor-like protein kinase 4	AT1G70250.1	19.8421963	0.00016757
HORVU1Hr1G002900	unknown function	AT3G14470.2	17.6887683	0.00016757

Table 3.7: Differentially expressed genes identified by RNAseq analysis between Steptoe and FN365 within the minor QTL identified at the genetically defined region of chromosome 1H (437 to 525 Mb).

Feature ID	Barley annotation	Arabidopsis Homology	Steptoe Vs FN365	
			Fold change	Corrected P-value
HORVU1Hr1G062030	Chitinase family protein	AT3G12500.1	26.1143819	1.657E-05
HORVU1Hr1G062250	UDP-Glycosyltransferase superfamily protein	AT3G16520.3	4.00500653	0.00115691
HORVU1Hr1G062450	unknown protein	AT3G06070.1	-3.2262013	0.00410387
HORVU1Hr1G063010	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	AT3G60910.1	-3.1350527	0.01453652
HORVU1Hr1G063390	AAA-ATPase 1	AT5G40010.1	3.8596875	0.01453652
HORVU1Hr1G065150	undescribed protein	AT2G43550.1	14.5112589	8.7236E-19
HORVU1Hr1G067690	Nuclear transport factor 2 (NTF2) family protein	AT3G04890.1	6.37708508	0.00294574
HORVU1Hr1G067970	Whole genome shotgun assembly, reference scaffold set, scaffold scaffold_156	AT2G26790.2	4.55284856	1.6448E-08
HORVU1Hr1G068010	Cyclin family protein	AT5G06150.1	-5.4824174	0.00970691
HORVU1Hr1G069800	Non-specific lipid-transfer protein	AT5G01870.1	10.7205395	1.1174E-14
HORVU1Hr1G070190	Nodulin-like / Major Facilitator Superfamily protein	AT2G30300.1	-5.2060935	1.732E-05
HORVU1Hr1G070640	RING/U-box superfamily protein	AT3G11110.1	7.0576908	0.0003903
HORVU1Hr1G072250	Chitinase 2	AT4G20095.1	3.24798167	0.00726578
HORVU1Hr1G072720	unknown protein	AT5G02090.1	7.23861489	4.3367E-09
HORVU1Hr1G073010	unknown function	AT3G54000.3	-3.8221277	0.01753871
HORVU1Hr1G073040	Phosphatidylinositol:ceramide inositolphosphotransferase	AT3G54020.1	3.0709085	0.00803778
HORVU1Hr1G074310	receptor kinase 1	AT4G23180.1	4.38323175	0.00050577
HORVU1Hr1G074660	Subtilisin-like protease SBT3.6	AT1G71950.1	3.13681844	0.00045849
HORVU1Hr1G074840	unknown function	AT5G17210.1	7.9374523	1.5311E-06
HORVU1Hr1G076120	Protein of unknown function (DUF506)	AT4G32480.1	-3.6673607	0.01729317
HORVU1Hr1G079570	Cytokinin riboside 5'-monophosphate phosphoribohydrolase	AT5G11950.3	14.2869219	0.00300194

The genetic mapping of the FN370 DLMM using the FN370 x Morex F_2 population resulted in a single significant locus on chromosome 1H delimited by the flanking markers, 11_10617 proximally at position 434,823,728 bp and 11_20844 distally at position 525,478,648

bp (Figure 2.7). The RNAseq analysis of FN370 identified nine DEGs and all nine were upregulated in the FN370 mutant (Table 3.8).

Table 3.8: Differentially expressed genes identified by RNAseq analysis between Steptoe and FN370 within the genetically defined region of chromosome 1H.

Feature ID	Barley annotation	Arabidopsis Homology	Steptoe Vs FN370	
			Fold change	Corrected P-value
HORVU1Hr1G065150	undescribed protein	AT2G43550.1	10.58936573	9.0503E-21
HORVU1Hr1G065580	undescribed protein	AT2G13690.1	3.14308139	0.012352771
HORVU1Hr1G065670	undescribed protein	AT5G47910.1	4.402807057	3.22142E-12
HORVU1Hr1G067690	Nuclear transport factor 2 (NTF2) family protein	AT3G04890.1	5.609201229	0.009748057
HORVU1Hr1G069800	Non-specific lipid-transfer protein	AT5G01870.1	5.684243408	3.2759E-10
HORVU1Hr1G070580	receptor-like kinase 902	AT4G18640.1	3.115902743	0.032171256
HORVU1Hr1G070640	RING/U-box superfamily protein	AT3G11110.1	5.712356246	0.001770526
HORVU1Hr1G074840	unknown function	AT5G17210.1	3.698819445	0.023755472
HORVU1Hr1G079570	Cytokinin riboside 5'-monophosphate phosphoribohydrolase	AT5G11950.3	15.40310059	0.000795099

The last DLMM mutant FN396 was the only mutant in the study where we identified candidate genes via exome capture. Thus, it would be expected that these genes within the ~2.3 Mb deletion would be confirmed via the RNAseq analysis. The RNAseq analysis may also identify other genes that could have been missed by the exome capture. The genetic mapping of the FN396 DLMM using the FN396 x Morex F_2 population resulted in a single significant locus on chromosome 2H delimited by the flanking markers, 12_10936 proximally at position 659,264,767 bp and 11_10429 distally at position 704,365,928 representing a physical region of ~2.3 Mb (Figures 2.8 & 3.1). The RNAseq analysis identified 22 DEGs within the genetically defined region compares to wildtype, 15 downregulated and 7 upregulated genes (Table 3.9). The downregulated genes that fall within the ~2.3 Mb deletion identified by both the exome capture and RNAseq analysis represent our strongest candidate genes.

Table 3.9: Differentially expressed genes identified by RNAseq analysis between Steptoe and FN396 within the genetically defined region of chromosome 2H.

Feature ID	Barley annotation	Arabidopsis Homology	Steptoe Vs FN396	
			Fold change	Corrected P-value
HORVU2Hr1G094650	Superoxide dismutase [Cu-Zn]	AT1G12520.1	-24.8004077	8.13039E-06
HORVU2Hr1G094690	SPX domain-containing membrane protein	AT1G63010.7	-87.8812218	8.14243E-14
HORVU2Hr1G094730	peptidoglycan-binding LysM domain-containing protein	AT5G62150.1	-87.0259111	1.9845E-07
HORVU2Hr1G094780	Dehydration-responsive element-binding protein 1E	AT1G12610.1	-48.5375014	1.56291E-06
HORVU2Hr1G094840	RING/U-box superfamily protein	AT3G16720.1	-31.0767963	0.005352056
HORVU2Hr1G094870	MATE efflux family protein	AT4G23030.1	-59.6111765	6.06E-08
HORVU2Hr1G094890	Thioesterase superfamily protein	AT3G61200.1	-23.7083802	0.023197706
HORVU2Hr1G094960	F-box protein PP2-A13	AT3G61060.2	-72.2325783	1.43617E-09
HORVU2Hr1G094980	RING/U-box superfamily protein	AT3G63530.2	-36.1763486	0.00128434
HORVU2Hr1G095070	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	AT4G00750.1	-97.1978218	1.93963E-19
HORVU2Hr1G095080	Cytochrome P450 superfamily protein	AT1G12740.1	-78.2872534	2.08701E-05
HORVU2Hr1G096960	glutathione peroxidase 6	AT4G11600.1	-3.9457643	0.000297959
HORVU2Hr1G099470	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	AT3G22142.1	-14.0135212	0.003824472
HORVU2Hr1G100360	SAUR-like auxin-responsive protein family	AT2G24400.1	25.2705172	0.002075924
HORVU2Hr1G100450	HKT14-1	AT4G10310.1	18.2696924	1.37628E-07
HORVU2Hr1G101040	calcium-transporting ATPase, putative	AT5G57110.3	-3.2981926	0.005352056
HORVU2Hr1G101100	WRKY DNA-binding protein 11	AT4G31550.1	3.0441072	0.001331427
HORVU2Hr1G101920	Ubiquinol oxidase	AT3G22370.1	23.9148362	2.75932E-09
HORVU2Hr1G101980	Ubiquinol oxidase	AT3G22370.1	6.2409586	0.000196154
HORVU2Hr1G102710	Protein NRT1/ PTR FAMILY 8.3	AT2G02040.1	75.7276600	5.6354E-10
HORVU2Hr1G102720	Protein NRT1/ PTR FAMILY 8.3	AT2G02040.1	51.4419334	4.63312E-11
HORVU2Hr1G104030	Receptor-like protein kinase 2	AT5G46330.2	-23.0823823	8.99831E-05

Discussion

Plant PCD and mammalian apoptosis share some common underlying molecular mechanisms indicating a process that relies on highly conserved genes and pathway that are difficult to study genetically due to the lack of genetic and phenotypic polymorphism (Bacete et al, 2018; Bruggeman et al, 2015). Identifying DLMMs from barley mutant populations for forward genetic analysis to identify the genes underlying these mutations will help fill knowledge gaps concerning PCD signaling and expression in barley and other plant species. To date barley DLMM have contributed to the understanding of PCD pathways through the discovery of the three genes *nec1* (a cyclic gate ion channel protein), *mlo* (a transmembrane G protein) and *nec3* (a cytochrome p450 family protein). The diversity of these three characterized genes shows that there are a lot of signaling mechanisms that play roles in PCD responses and that there are significant knowledge gaps concerning how these mechanisms interact for the regulation of PCD in plant innate immunity responses and developmental pathways. In the past we hypothesized that many of the DLMMs represent suppressors of PCD to keep these processes in check when not needed. However, it is becoming apparent that these are complex pathways involving many classes of proteins, and crosstalk between signaling mechanisms.

Interestingly, the original FN360 mutant generated in the Steptoe background apparently contained two independent mutations that resulted in its DLMM phenotype, which was described as large dark necrotic lesions. However, after crossing FN360 with WT Morex the F_2 progeny independently segregated for two DLMM phenotypes, which were dark necrotic lesions that mapped to chromosome 1H and the typical tan to orange phenotype lacking serotonin/phenolics buildup distinctive of the *nec3* mutants that mapped to the *nec3* locus at the centromeric region of chromosome 6H. Interestingly, the RNAseq analysis only identified a single candidate gene

(HORVU1Hr1G004150) within the region delimited genetically for the FN360 dark lesion phenotype on chromosome 1H (Table 3.3). The HORVU1Hr1G004150 gene model is predicted to encode a Chymotrypsin inhibitor, a homolog of an *Arabidopsis* serine protease inhibitor. This family of proteases has a wide distribution across diverse plant and animal species (Mishra et al, 2020) and in plants this class of protease inhibitors have diverse roles in cellular signaling, wound healing (Howe et al., 2000), insect defense mechanisms (Neurath, 1986; Ryan, 2000) and serine protease inhibitor in plant disease defenses that involve negative regulatory roles in pathogen-triggered cell death (Li et al., 2008). Thus, the HORVU1Hr1G004150 Chymotrypsin inhibitor gene is a strong candidate for the FN360 DLMM on chromosome 1H that results in the dark lesion phenotype.

The *nec3* gene was recently cloned via positional cloning and subsequently validated by comparative analysis of five independent *nec3* mutants (Ameen, 2019; Ameen et al., 2020; Sager-Bittara, 2015). Thus, the FN360 mutant phenotype that has the distinctive *nec3* phenotype and colocalized with *nec3* in the genetic mapping should represent a sixth independent *nec3* allele. Thus, for the RNAseq analysis for the FN360 *nec3* region we primarily focused on the *nec3* gene (HORVU.MOREX.r2.6HG0460850; cytochrome P450) that was recently identified. However, zero RNAseq reads aligned to the *nec3* gene from the FN360 or Steptoe WT RNAseq data using single sequence files from each of the three replicates or with a file of all three replicates combined. However, this result was consistent with the results obtained for the RNAseq and qPCR data in the *nec3* cloning work as the *Nec3* expression for WT at 0 hrs before inoculation with the pathogen *Bipolaris sorokiniana* was an average of ~5 reads (nearly zero expression) and after inoculation it was upregulated ~120 fold. However, for the *nec3* mutant at 0 hr the expression was an average of ~6 reads (also nearly zero expression) and after inoculation

was upregulated ~1200 fold. In this study FN360 and WT Steptoe were not inoculated, therefore it was not surprising that we were unable to detect any reads for FN360 or WT Steptoe.

The RNAseq analysis of the FN361 mutant identified a WRKY transcription factor (TF) (HORVU5Hr1G034830) as the only downregulated gene underlying the delimited FN361 locus (Table 3.4). Programmed cell death signaling pathways consist of protein activators or repressors, which could be represented by DNA binding WRKY TFs. The WRKY TFs are a large group of plant transcription regulators (Bakshi & Oelmüller, 2014; Eulgem, 2007; Eulgem et al., 2000). WRKY TFs bind W-box elements at the promotor regions resulting in gene activation or repression (Agarwal et al., 2011; Eulgem et al., 2000; Rushton et al., 2010; Yu et al., 2001). The WRKYs are important for many physiological functions including pathogen defense, abiotic stress, nutrient deficiency, salt stress (Cai et al., 2017; Chen et al., 2010; Hichri et al., 2017; Kasajima et al., 2010; Li et al., 2009), and developmental processes like senescence, and root growth (Robatzek and Somssich, 2001, 2002; Grunewald et al., 2012).

There is a long list of WRKY TFs mediating defense responses related to PCD. The *AtWRKY6* TF regulates plant defenses against *P. syringae* pv. *tomato* involving PCD-mediated resistance responses and PCD regulated senescence in *Arabidopsis* (Robatzek & Somssich, 2002). Li et al. (2004) reported enhanced resistance to the biotrophic fungal pathogen *Erysiphe cichoracearum,* whereas an increase in susceptibility to the bacterial necrotroph *Erwinia carotovora* subsp. *carotovora* occurred upon the upregulation of *WRKY70* in *Arabidopsis* suggesting a positive role in PCD related responses that are effective against biotrophs but may promote disease when encountering a necrotroph. The WRKY TFs also negatively regulate defenses (Eulgem and Somssich, 2007; Robatzek & Somssich, 2002). The over expression of *WRKY38* and *WRKY62* compromised resistance to *P. syringae* and was a negative regulator of

plant basal defense responses (Kim et al., 2008; Mao et al., 2007). The WRKY TFs are known to play both a positive and negative regulatory role in processes involving PCD, thus represents a strong candidate gene for FN361.

Another DEG within the FN361 locus is a Chalcone-flavonone isomerase (HORVU5Hr1G046480) that was upregulated 145.9 fold in the mutant. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induces apoptosis a form of PCD in mammalian cancer cells. The ligand binds to the death receptors, TRAIL-R1 and TRAIL-R2 expressed in cancer cells and activates the PCD pathways. Interestingly, Chalcones can sensitize cancer cells to TRAIL by inducing the upregulation of TRAIL-R2 which in turn induced apoptosis (Szilszka et al., 2012). Thus, the large increase in the Chalcone-flavonone isomerase gene expression could have an effect on the induction of PCD in barley resulting in the DLMM phenotype thus was considered as a candidate gene. However, it could be argued that upregulated DEGs is against the current dogma that the majority of candidate DLMM mutants would represent deleted or down regulated genes at each genetically delimited region. Although, this is arguably correct, the upregulated genes cannot be ruled out as candidates. It has been shown that fast neutron irradiation can cause upregulation in gene expression, as was shown for the AUX/IAA7 gene in Arabidopsis (Fortunati et al., 2010). The most likely cause of such an effect on the transcriptional regulation of a candidate DLMM gene is the fast neutron irradiation induced a deletion within the promoter region of a gene that prevents a repressor from binding the DNA promoter, resulting in constitutive expression of a gene and the upregulation causes the activation of PCD and the DLMM phenotype. There are several upregulated genes within other mutants that are also considered as candidate genes.

The genetic analysis of the FN365 mutant identified one major locus on chromosome 5H and two minor loci contributing to the phenotype on chromosome 1H (Figure 2.6). Because the segregation analysis of the F_2 population and previous allelism tests determined that a single recessive gene was responsible for the FN365 DLMM phenotype it was posited that the major locus on chromosome 5H likely contained the mutant gene.

The RNAseq analysis of the FN365 chromosome 5H locus identified 26 DEGs with 4 significantly downregulated and 22 upregulated genes. The gene with the highest level of upregulation was a cysteine-rich secretory protein 3 (HORVU5Hr1G106010), which is a known antimicrobial peptide that is induced in response to pathogen challenge (Silverstein et al, 2007). The fold change was 687 (Table 3.5), thus, this gene may lead to a lesion phenotype in the FN365 DLMM and is a strong candidate gene. An EF hand calcium-binding-like protein (HORVU5Hr1G111520) was also upregulated in the mutant with a fold change of 42 (Table 3.5). In wheat, the expression of a homologous gene was similar between incompatible and compatible interactions in leaves, and the gene was designated as TaCab1 (*Triticum aestivum* calcium binding EF-hand protein 1), which was involved in many activities in plant cells, including pathogen recognition, symptom development, and basal tolerance to both biotic and abiotic stress mediating the SA signaling pathway, which are important in eliciting PCD (Feng et al., 2011). A Glutathione S-transferase family protein (GST) was also upregulated in the FN365 DLMM (Table 3.5), and it is involved in defense mechanisms (Gullner et al., 2018). The interaction leads to PCD and HR in fungal, bacteria and virus incompatible interactions (Gullner et al., 2018). In tomatoes, the resistance to the powdery mildew (*Oidium neolycopersici*) pathogen is associated with GST-mediated HR (Pei et al., 2011). Since GSTs play a crucial role

in plant pathogen defense mechanisms involving PCD, upregulation may cause the DLMM phenotype in the FN365.

The Calmodulin-binding protein (HORVU5Hr1G100150) was also upregulated 8.1fold in the FN365 mutant (Table 3.5). The Calmodulin-binding protein CAM gene family is known as a calcium sensor protein which controls and regulates cellular signaling cascades. The name came from Calcium Modulating proteins (Means & Dedman, 1980), and AtBAC6 (BCL2-associated athanogene) regulates Calcium in plants and is a CAM gene found in Arabidopsis (Kang et al., 2006). The overexpression of AtBAC6 results in cell death in leaves (Kang et al, 2006). Thus, upregulation on this class of genes could result in the FN365 DLMM phenotype and is also considered a candidate gene.

The FN370 mutant line shows upregulated genes in the QTL region that is flanked by the SNP markers 11_10617 at position 434,823,728 bp and 11_20844 at position 525,478,648 bp on barley chromosome 1H (Figure 2.7). The receptor-like kinase 902 gene (HORVU1Hr1G070580) was upregulated 3.1 fold in the FN370 DLMM (Table 3.8) and is an Arabidopsis homolog of the Leucine-rich repeat protein kinase family of RLK receptors that are typical immune receptors that can lead to PCD responses (Mendy et al., 2017). Another DEG within the region that has been shown to function as a negative regulator of PCD in rice (Zeng et al., 2008) is the RING/U-box superfamily protein (HORVU1Hr1G070640) that was upregulated 5.7 fold (Table 3.8).

In the FN 396 line, we found fifteen genes downregulated in the RNAseq data, while there were fifteen deleted genes identified in the exome capture data. The eleven extra genes represent genes that were not present or not captured by the exome capture array. The ~2.3 Mb deletion in the genetically delimited region contains twenty-six genes that may be involved in PCD pathways and result in DLMM phenotype. In other organisms and species such as fungi,

humans, and rice, genes have been shown to function as suppressors that delay or prevent PCD as negative regulators. Thus, we hypothesize that in the PCD pathways proteins act as suppressors of PCD when it is not needed to maintain plant fitness.

In rice, the RING/U-box superfamily protein is a family of many genes containing U-box protein that function as negative regulators of PCD signaling, and U-box proteins in rice and Arabidopsis share the same domain organization (Zeng et al., 2008). Thus, the RING/U-box superfamily proteins (HORVU2Hr1G094840 and HORVU2Hr1G094980) identified in the FN396 deletion are considered strong candidate genes (Table 3.9). The Superoxide dismutase [Cu-Zn] (HORVU2Hr1G094650) was also considered a strong candidate because in tobacco *(Nicotiana tabacum*) Bright-Yellow 2 (TBY-2) expresses cell death when heat shocked, but cell death was prevented when superoxide dismutase (SOD) substrate was added to media, showing that SOD can function as a negative regulator of cell death in tobacco (Vacca et al., 2004). The barley Peptidoglycan-binding LysM domain-containing protein (HORVU2Hr1G094730) was also considered a candidate because Peptidoglycan-binding LysM domain-containing protein found in plants are involved in the immunity responses that result in PCD acting as receptors of the fungal PAMP chitin (Spaink, 2004). The Thioesterase superfamily protein (HORVU2Hr1G094890) was also considered a candidate because the Thioesterase superfamily protein is found in different organisms and acts in humans as a negative regulator of the apoptosis process (Tillander et al., 2017). Also, cytochrome P450 gene is involved in regulating nec3 mutant in barley as describe previously. Thus, the genes of each of these classes identified in the FN396 deletion (Table 3.9) are considered strong candidate genes, yet there are several other genes within the deletion that cannot be excluded.

Here, we reported on the use of two genomics methods, exome capture and RNAseq, that utilize next generation sequencing for comparative analysis between wild type and DLMMs at different genetically defined loci to identify candidate genes responsible for the phenotypes. Taken together, the DLMM mutants characterized and observations such as distinct morphologies, timing of expression, location of genes and candidate genes identified suggest that the mutant genes contributing to the DLMM phenotypes may play a role in different PCD pathways or have a temporally or spatially different function in the same or similar pathways that lead to differential timing or morphologically different lesion development. The explanation of upregulated and downregulated gene expression is typically the result of 1-Mb size deletions induced by fast neutron mutagenesis (Islam et al., 2019). Thus, deletions within genes that play a role in suppressing PCD pathways could result in the DLMM phenotypes. However, for some of the DLMM characterized no deletions were detected via exome capture and only DEGs with positive DEG were identified within the genetically defines regions for the DLMM mutants. However, these DLMM could be the result of a deletion in the promoter region that blocks the binding of a suppressor element thus the gene responsible for the DLMM phenotype is upregulated in the FN mutant. The exome capture did not detect deletion in the mutant lines except FN396 because the probes do not cover the entire barley transcriptome (Mascher et al., 2013) and some of the mutations may occur in non-transcribed regions of the genes including the promoter region.

The exome capture and RNAseq data generated and analyzed in this chapter was a well delineated effort to identify the genes underlying the five DLMMs identified from the Steptoe mutant population. In chapter 2 these mutants were utilized to develop genetic mapping populations that delimited the mutants to defined genetic intervals within the barley genome.

Utilizing the barley genome sequence available at the time that these analyses were completed (2016 Barley genome assembly) the physical regions and high confidence annotated genes underlying each mutant locus were identified as defined utilizing F_2 genetic mapping populations. Exome capture and RNAseq genomic analysis tools were utilized for forward genetics and identification of candidate genes for each of the DLMM mutants characterized in this study. This data lays the foundation for future functional analysis to validate candidate genes that will add to the knowledge base and understanding of programmed cell death pathways and signaling.

References

Ameen, G. (2019). Cloning and Characterization of *rcs5*, Spot Blotch Resistance Gene and Pathogen Induced *NEC3* Gene Involved in Programmed Cell Death in Barley (Doctoral dissertation, North Dakota State University, Fargo, North Dakota)

Ameen, G., Bittara, L., Richards, J., Solanki, S., Friesen, T. L., & Brueggeman, R. S. (2018, October). The Nec3 gene is a putative negative regulator of pathogen induced programmed cell death in barley. In PHYTOPATHOLOGY (Vol. 108, No. 10). 3340 PILOT KNOB ROAD, ST PAUL, MN 55121 USA: AMER PHYTOPATHOLOGICAL SOC.

Agarwal, P., Reddy, M. P., & Chikara, J. (2011). WRKY: its structure, evolutionary relationship, DNA-binding selectivity, role in stress tolerance and development of plants. Molecular biology reports, 38(6), 3883-3896.

Bacete, L., Mélida, H., Miedes, E., & Molina, A. (2018). Plant cell wall-mediated immunity: cell wall changes trigger disease resistance responses. *The Plant Journal*, *93*(4), 614–636.

Bakshi, M., & Oelmüller, R. (2014). WRKY transcription factors: Jack of many trades in plants. *Plant Signaling & Behavior*, *9*(2), e27700.

Balagué, C., Lin, B., Alcon, C., Flottes, G., Malmström, S., Köhler, C., ... & Roby, D. (2003). HLM1, an essential signaling component in the hypersensitive response, is a member of the cyclic nucleotide–gated channel ion channel family. The Plant Cell, 15(2), 365-379.

Bomblies, K., Lempe, J., Epple, P., Warthmann, N., Lanz, C., Dangl, J. L., & Weigel, D. (2007). Autoimmune response as a mechanism for a Dobzhansky-Muller-type incompatibility syndrome in plants. *PLoS Biology*, *5*(9).

Brodersen, P., Petersen, M., Pike, H.M., Olszak, B., Skov, S., Odum, N., Jorgensen, L.B., Brown, R.E., and Mundy, J. (2002). Knockout of *Arabidopsis accelerated-cell-death11* encoding a sphingosine transfer protein causes activation of programmed cell death and defense. *Genes & Development, 16*, 490–502.

Brown, J., Pirrung, M., & McCue, L. A. (2017). FQC Dashboard: integrates FastQC results into a web-based, interactive, and extensible FASTQ quality control tool. *Bioinformatics*, *33*(19), 3137–3139.

Bruggeman, Q., Raynaud, C., Benhamed, M., & Delarue, M. (2015). To die or not to die? Lessons from lesion mimic mutants. *Frontiers in Plant Science*, *6*, 24.

Cai, R., Dai, W., Zhang, C., Wang, Y., Wu, M., Zhao, Y., ... & Cheng, B. (2017). The maize WRKY transcription factor ZmWRKY17 negatively regulates salt stress tolerance in transgenic Arabidopsis plants. Planta, 246(6), 1215-1231.

Cesari, S., Bernoux, M., Moncuquet, P., Kroj, T., & Dodds, P. N. (2014). A novel conserved mechanism for plant NLR protein pairs: the 'integrated decoy hypothesis. *Frontiers in Plant Science*, *5*, 606.

Chen, L., Zhang, L., & Yu, D. (2010). Wounding-induced WRKY8 is involved in basal defense in Arabidopsis. Molecular Plant-Microbe Interactions, 23(5), 558-565.

Danecek, P., Auton, A., Abecasis, G., Albers, C. A., Banks, E., DePristo, M. A., ... & McVean, G. (2011). The variant call format and VCFtools. *Bioinformatics*, *27*(15), 2156–2158.

Dong X (1998) SA, JA, ethylene, and disease resistance in plants. *Current Opinion in Plant Biology, 1*, 316–323.

Druka, A., Franckowiak, J., Lundqvist, U., Bonar, N., Alexander, J., Guzy-Wrobelska, J., ... & Vendramin, V. (2010). Exploiting induced variation to dissect quantitative traits in barley.

Eulgem, T., Rushton, P. J., Robatzek, S., & Somssich, I. E. (2000). The WRKY superfamily of plant transcription factors. Trends in plant science, 5(5), 199-206.

Eulgem, T., & Somssich, I. E. (2007). Networks of WRKY transcription factors in defense signaling. Current opinion in plant biology, 10(4), 366-371.

Feng, H., Wang, X., Sun, Y., Wang, X., Chen, X., Guo, J., ... & Kang, Z. (2011). Cloning and characterization of a calcium binding EF-hand protein gene TaCab1 from wheat and its expression in response to Puccinia striiformis f. sp. tritici and abiotic stresses. *Molecular Biology Reports*, *38*(6), 3857–3866.

Flor H. H. (1971). Current status of the gene-for-gene concept. Annual Review of Phytopathology*, 9*, 275–296. 10.1146/annurev.py.09.090171.001423

Fortunati, A., Tassone, P., Damasso, M., & Migliaccio, F. (2010). Neutron irradiation affects the expression of genes involved in the response to auxin, senescence and oxidative stress in Arabidopsis. *Plant Signaling & Behavior*, *5*(8), 959–967.

Friesen, T. L., & Faris, J. D. (2010). Characterization of the wheat-Stagonospora nodorum disease system: what is the molecular basis of this quantitative necrotrophic disease interaction?. Canadian Journal of Plant Pathology, 32(1), 20-28.

Fujiwara, T., Maisonneuve, S., Isshiki, M., Mizutani, M., Chen, L., Wong, H. L., ... & Shimamoto, K. (2010). Sekiguchi lesion gene encodes a cytochrome P450 monooxygenase that catalyzes conversion of tryptamine to serotonin in rice. Journal of Biological Chemistry, 285(15), 11308-11313.

Greenberg, J. T., & Ausubel, F. M. (1993). Arabidopsis mutants compromised for the control of cellular damage during pathogenesis and aging. The Plant Journal, 4(2), 327-341.

Grunewald, W., De Smet, I., Lewis, D. R., Löfke, C., Jansen, L., Goeminne, G., ... & Teichmann, T. (2012). Transcription factor WRKY23 assists auxin distribution patterns during Arabidopsis root development through local control on flavonol biosynthesis. Proceedings of the National Academy of Sciences, 109(5), 1554-1559.

Gullner, G., Komives, T., Király, L., & Schröder, P. (2018). Glutathione S-transferase enzymes in plant-pathogen interactions. *Frontiers in Plant Science*, *9*, 1836.

Hichri, I., Muhovski, Y., Žižková, E., Dobrev, P. I., Gharbi, E., Franco-Zorrilla, J. M., ... & Motyka, V. (2017). The Solanum lycopersicum WRKY3 transcription factor SlWRKY3 is involved in salt stress tolerance in tomato. Frontiers in plant science, 8, 1343.

Howe, G. A., Lee, G. I., Itoh, A., Li, L., & DeRocher, A. E. (2000). Cytochrome P450-dependent metabolism of oxylipins in tomato. Cloning and expression of allene oxide synthase and fatty acid hydroperoxide lyase. *Plant Physiology*, *123*(2), 711–724.

Hyun, Y., Yun, H., Park, K., Ohr, H., Lee, O., Kim, D. H., ... & Choi, Y. (2013). The catalytic subunit of Arabidopsis DNA polymerase α ensures stable maintenance of histone modification. *Development*, *140*(1), 156–166.

International Barley Genome Sequencing Consortium. (2012). A physical, genetic and functional sequence assembly of the barley genome. *Nature*, *491*(7426), 711–716.

Ishihara, A., Hashimoto, Y., Tanaka, C., Dubouzet, J. G., Nakao, T., Matsuda, F., ... & Wakasa, K. (2008). The tryptophan pathway is involved in the defense responses of rice against pathogenic infection via serotonin production. The Plant Journal, 54(3), 481-495.

Islam, N., Stupar, R. M., Qijian, S., Luthria, D. L., Garrett, W., Stec, A. O., ... & Natarajan, S. S. (2019). Genomic changes and biochemical alterations of seed protein and oil content in a subset of fast neutron induced soybean mutants. *BMC Plant Biology*, *19*(1), 420.

Jansen, C., Von Wettstein, D., Schäfer, W., Kogel, K. H., Felk, A., & Maier, F. J. (2005). Infection patterns in barley and wheat spikes inoculated with wild-type and trichodiene synthase gene disrupted Fusarium graminearum. Proceedings of the National Academy of Sciences, 102(46), 16892-16897.

Jones, J. D., & Dangl, J. L. (2006). The plant immune system. nature, 444(7117), 323-329.

Kang, C. H., Jung, W. Y., Kang, Y. H., Kim, J. Y., Kim, D. G., Jeong, J. C., ... & Chung, W. S. (2006). AtBAG6, a novel calmodulin-binding protein, induces programmed cell death in yeast and plants. *Cell Death & Differentiation*, *13*(1), 84–95.

Kasajima, I., Ide, Y., Yokota Hirai, M., & Fujiwara, T. (2010). WRKY6 is involved in the response to boron deficiency in Arabidopsis thaliana. Physiologia plantarum, 139(1), 80-92.

Keiša, A., Brueggeman, R., Drader, T., Kleinhofs, A., & Rostoks, N. (2010). Transcriptome analysis of the barley nec3 mutant reveals a potential link with abiotic stress response related signaling pathways. Environ Exp Bot, 8, 1-16.

Kim, K. C., Lai, Z., Fan, B., & Chen, Z. (2008). Arabidopsis WRKY38 and WRKY62 transcription factors interact with histone deacetylase 19 in basal defense. The Plant Cell, 20(9), 2357-2371.

Kumar, J., Hückelhoven, R., Beckhove, U., Nagarajan, S., & Kogel, K. H. (2001). A compromised Mlo pathway affects the response of barley to the necrotrophic fungus Bipolaris sorokiniana (teleomorph: Cochliobolus sativus) and its toxins. Phytopathology, 91(2), 127-133.

Kunkel, T. A. and Burgers, P. M. (2008). Dividing the workload at a eukaryotic replication fork. *Trends Cell Biology, 18,* 521–527.

Laloi C., Apel K., Danon, A. (2004) Reactive oxygen signalling: The latest news. *Current Opinion in Plant Biology, 7*, 323–328

Li, H., & Durbin, R. (2010). Fast and accurate long-read alignment with Burrows–Wheeler transform. *Bioinformatics*, *26*(5), 589–595.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., ... & Durbin, R. (2009). The sequence alignment/map format and SAMtools. *Bioinformatics*, *25*(16), 2078–2079.

Li, J., Brader, G., & Palva, E. T. (2004). The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. The Plant Cell, 16(2), 319-331.

Li, J., Brader, G., & Palva, E. T. (2008). Kunitz trypsin inhibitor: an antagonist of cell death triggered by phytopathogens and fumonisin b1 in Arabidopsis. *Molecular Plant, 1*(3), 482–495.

Li, S., Fu, Q., Huang, W., & Yu, D. (2009). Functional analysis of an Arabidopsis transcription factor WRKY25 in heat stress. Plant cell reports, 28(4), 683-693.

Liu, Z., Holmes, D. J., Faris, J. D., Chao, S., Brueggeman, R. S., Edwards, M. C., & Friesen, T. L. (2015). Necrotrophic effector-triggered susceptibility (NETS) underlies the barley–P yrenophora teres f. teres interaction specific to chromosome 6H. Molecular plant pathology, 16(2), 188-200.

Lorrain, S., Vailleau, F., Balague, C., and Roby, D. (2003). Lesion mimic mutants: keys for deciphering cell death and defense pathways in plants? *Trends in Plant Science, 8*, 263–271.

Lundqvist, U. (1997). New and revised descriptions of barley genes. Barley Genet. Newslett., 26, 22-516.

Lyngkjær, M. F., & Carver, T. L. W. (2000). Conditioning of cellular defence responses to powdery mildew in cereal leaves by prior attack. Molecular Plant Pathology, 1(1), 41-49.

Macho, A. P., & Zipfel, C. (2014). Plant PRRs and the activation of innate immune signaling. Molecular cell, 54(2), 263-272.

Mackey, D., Holt III, B. F., Wiig, A., & Dangl, J. L. (2002). RIN4 interacts with Pseudomonas syringae type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis. *Cell*, *108*(6), 743–754.

Mao, P., Duan, M., Wei, C., & Li, Y. (2007). WRKY62 transcription factor acts downstream of cytosolic NPR1 and negatively regulates jasmonate-responsive gene expression.

Mascher, M., Richmond, T. A., Gerhardt, D. J., Himmelbach, A., Clissold, L., Sampath, D., ... & Akhunov, E. D. (2013). Barley whole exome capture: a tool for genomic research in the genus Hordeum and beyond. *The Plant Journal*, *76*(3), 494–505.

McGrann, G. R., Stavrinides, A., Russell, J., Corbitt, M. M., Booth, A., Chartrain, L., ... & Brown, J. K. (2014). A trade off between mlo resistance to powdery mildew and increased susceptibility of barley to a newly important disease, Ramularia leaf spot. Journal of experimental botany, 65(4), 1025-1037.

Means, A. R., & Dedman, J. R. (1980). Calmodulin—an intracellular calcium receptor. *Nature*, *285*(5760), 73–77.

Mendy, B., Wang'ombe, M. W., Radakovic, Z. S., Holbein, J., Ilyas, M., Chopra, D., ... & Siddique, S. (2017). Arabidopsis leucine-rich repeat receptor–like kinase NILR1 is required for induction of innate immunity to parasitic nematodes. *PLoS Pathogens*, *13*(4).

Mishra, U. N., Reddy, M. V., & Prasad, D. T. (2020). Plant serine protease inhibitor (SPI): A potent player with bactericidal, fungicidal, nematicidal and antiviral properties. *IJCS*, *8*(1), 2985–2993.

Monaghan, J., & Zipfel, C. (2012). Plant pattern recognition receptor complexes at the plasma membrane. Current opinion in plant biology, 15(4), 349-357.

Monat, C., Padmarasu, S., Lux, T., Wicker, T., Gundlach, H., Himmelbach, A., ... & Waugh, R. (2019). TRITEX: chromosome-scale sequence assembly of Triticeae genomes with open-source tools. *Genome Biology, 20*(1), 284.

Mou, Z., He, Y., Dai, Y., Liu, X., and Li, J. (2000). Deficiency in fatty acid synthase leads to premature cell death and dramatic alterations in plant morphology. *Plant Cell, 12*, 405–418.

Muir, C. E., & Nilan, R. A. (1973). Registration of Steptoe Barley1 (Reg. No. 134). Crop Science, 13(6), 770-770.

Neurath, H. (1986). The versatility of proteolytic enzymes. *Journal of Cellular Biochemistry*, *32*(1), 35–49.

Pei, D. L., Ma, H. Z., Zhang, Y., Ma, Y. S., Wang, W. J., Geng, H. X., et al. (2011). Virus-induced gene silencing of a putative glutathione S-transferase gene compromised *Ol-1*-mediated resistance against powdery mildew in tomato. *Plant Molecular Biology Reporter*, 29, 972–978. doi: 10.1007/s11105-011-0331-4

Penmetsa, R. V., & Cook, D. R. (2000). Production and characterization of diverse developmental mutants of Medicago truncatula. Plant Physiology, 123(4), 1387-1398.

Qi, D., & Innes, R. W. (2013). Recent advances in plant NLR structure, function, localization, and signaling. *Frontiers in Immunology, 4*, 348.

Quinlan, A. R., & Hall, I. M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics*, *26*(6), 841–842.

Raff, M. (1998). Cell suicide for beginners. *Nature*, *396*(6707), 119–119.

Ravensdale, M., Nemri, A., Thrall, P. H., Ellis, J. G., & Dodds, P. N. (2011). Co-evolutionary interactions between host resistance and pathogen effector genes in flax rust disease. *Molecular Plant Pathology*, *12*(1), 93–102.

Reig-Valiente, J. L., Borredá, C., Talón, M., & Domingo, C. (2020). The G123 rice mutant, carrying a mutation in SE13, presents alterations in the expression patterns of photosynthetic and major flowering regulatory genes. *Plos One*, *15*(5), e0233120.

Robatzek, S., & Somssich, I. E. (2001). A new member of the Arabidopsis WRKY transcription factor family, AtWRKY6, is associated with both senescence-and defence-related processes. The Plant Journal, 28(2), 123-133.

Robatzek, S., & Somssich, I. E. (2002). Targets of AtWRKY6 regulation during plant senescence and pathogen defense. Genes & development, 16(9), 1139-1149.

Rostoks, N., Schmierer, D., Kudrna, D., & Kleinhofs, A. (2003). Barley putative hypersensitive induced reaction genes: genetic mapping, sequence analyses and differential expression in disease lesion mimic mutants. *Theoretical and Applied Genetics*, *107*(6), 1094–1101.

Rostoks, N., Schmierer, D., Mudie, S., Drader, T., Brueggeman, R., Caldwell, D. G., ... & Kleinhofs, A. (2006). Barley necrotic locus nec1 encodes the cyclic nucleotide-gated ion channel 4 homologous to the Arabidopsis HLM1. *Molecular Genetics and Genomics*, *275*(2), 159–168.

Rushton, P. J., Somssich, I. E., Ringler, P., & Shen, Q. J. (2010). WRKY transcription factors. Trends in plant science, 15(5), 247-258.

Ryan, C. A. (2000). The systemin signaling pathway: differential activation of plant defensive genes. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, *1477*(1-2), 112–121.

Sager-Bittara, L. P. (2015). Characterization of Programmed Cell Death Responses Involved in Disease Resistance/Susceptibility Responses in Barley (Doctoral dissertation, North Dakota State University).

Serrano-Cartagena, J., Candela, H., Robles, P., Ponce, M. R., Pérez-Pérez, J. M., Piqueras, P. and Micol, J. L. (2000). Genetic analysis of incurvata mutants reveals three independent genetic operations at work in *Arabidopsis* leaf morphogenesis. *Genetics, 156*, 1363–1377.

Shabab, M., Shindo, T., Gu, C., Kaschani, F., Pansuriya, T., Chintha, R., ... & van der Hoorn, R. A. (2008). Fungal effector protein AVR2 targets diversifying defense-related cys proteases of tomato. *The Plant Cell*, 20(4), 1169-1183.

Silverstein, K. A., Moskal Jr, W. A., Wu, H. C., Underwood, B. A., Graham, M. A., Town, C. D., & VandenBosch, K. A. (2007). Small cysteine-rich peptides resembling antimicrobial peptides have been under-predicted in plants. *The Plant Journal*, *51*(2), 262–28

Szilszka, E., Jaworska, D., Malgorzata, K., Czuba, Z.P., and Krol, W. (2012) Targeting Death Receptor TRAIL-R2 by Chalcones for TRAIL-Induced Apoptosis in Cancer Cells. *Int J Mol Sci,* 13(11): 15343-15359.

Solanki, S., Richards, J., Ameen, G., Wang, X., Khan, A., Ali, H., ... & Fetch, T. G. (2019). Characterization of genes required for both Rpg1 and rpg4-mediated wheat stem rust resistance in barley. *BMC Genomics*, *20*(1), 495.

Spaink, H. P. (2004). Specific recognition of bacteria by plant LysM domain receptor kinases. *Trends in Microbiology*, *12*(5), 201–204.

Stadler, L. J. (1928). Genetic effects of X-rays in maize. Proceedings of the National Academy of Sciences of the United States of America, 14(1), 69.

Stintzi, A., Heitz, T., Prasad, V., Wiedemann-Merdinoglu, S., Kauffmann, S., Geoffroy, P., ... & Fritig, B. (1993). Plant 'pathogenesis-related'proteins and their role in defense against pathogens. Biochimie, 75(8), 687-706.

Tameling, W. I., Vossen, J. H., Albrecht, M., Lengauer, T., Berden, J. A., Haring, M. A., ... & Takken, F. L. (2006). Mutations in the NB-ARC domain of I-2 that impair ATP hydrolysis cause autoactivation. *Plant physiology*, *140*(4), 1233–1245.

Thomma BP, Penninckx IA, Broekaert WF, Cammue BP (2001) The complexity of disease signaling in Arabidopsis. *Current Opinions in Immunology, 13*: 63–68

Tillander, V., Alexson, S. E., & Cohen, D. E. (2017). Deactivating fatty acids: acyl-CoA thioesterase-mediated control of lipid metabolism. *Trends in Endocrinology & Metabolism*, *28*(7), 473–484.

Toruño, T. Y., Stergiopoulos, I., & Coaker, G. (2016). Plant-pathogen effectors: cellular probes interfering with plant defenses in spatial and temporal manners. *Annual review of phytopathology*, *54*, 419–441.

Vacca, R. A., de Pinto, M. C., Valenti, D., Passarella, S., Marra, E., & De Gara, L. (2004). Production of reactive oxygen species, alteration of cytosolic ascorbate peroxidase, and impairment of mitochondrial metabolism are early events in heat shock-induced programmed cell death in tobacco Bright-Yellow 2 cells. *Plant Physiology*, *134*(3), 1100–1112.

Vaux, D. L., and Korsmeyer, S.J. (1999). Cell death in development. *Cell, 96*, 245–254.

Wolter, M., Hollricher, K., Salamini, F., & Schulze-Lefert, P. (1993). The mlo resistance alleles to powdery mildew infection in barley trigger a developmentally controlled defence mimic phenotype. Molecular and General Genetics MGG, 239(1-2), 122-128.

Yu, D., Chen, C., & Chen, Z. (2001). Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression. The Plant Cell, 13(7), 1527-1540.

Zhang, Y., Yang, Y., Fang, B., Gannon, P., Ding, P., Li, X., & Zhang, Y. (2010). Arabidopsis snc2-1D activates receptor-like protein-mediated immunity transduced through WRKY70. *The Plant Cell*, *22*(9), 3153–3163.

Zeng, L. R., Qu, S., Bordeos, A., Yang, C., Baraoidan, M., Yan, H., ... & Wang, G. L. (2004). Spotted leaf11, a negative regulator of plant cell death and defense, encodes a U-box/armadillo repeat protein endowed with E3 ubiquitin ligase activity. The Plant Cell, 16(10), 2795-2808.

CHAPTER 4: CONCLUSIONS

This study addressed the gap in understanding of PCD signaling pathways by identifying genes in barley that are involved in these pathways through characterization of disease lesion mimic mutants (DLMMs). Each independent DLMM was mapped to a different chromosomal location validating that the five DLMM mutants characterized contain distinct genes involved in PCD pathways. The characterization of the physical sequence underlying the genetically defines DLMM loci identified many possible candidate genes with known function in PCD responses. Thus, exome capture and RNA seq analysis was utilized to further refine and identify candidate mutant genes underlying each locus. Thus, the research reported in this dissertation has made a significant contribution to identifying candidate genes that contribute to PCD signaling pathways.

This study identified several genes in the DLMM lines that may function as suppressors od PCD responses. The most promising candidate gene identified for the FN360 dark lesion mutant, encodes a chymotrypsin inhibitor gene, which was only gene shown to be downregulated within the FN360 region, indicating that the putative deletion or inactivation of this results in spontaneous PCD and the DLMM phenotype. Supporting this hypothesis is the previous findings showing that chymotrypsin inhibitors are a known negative regulator of PCD, which supports our finding.

The other unique finding is that the WRKY transcription factor (TF) gene was the only downregulated gene within the FN361 genetically defined locus. The WRKY TFs are known as susceptibility target genes of pathogen effectors and are involved in many biological activities in plant including defense responses that lead to PCD. Thus, the WRKY TFs could act as a suppressor of PCD and is a strong candidate gene underlying this DLMM phenotype.

In the QTL of FN396, many genes are known for their connection to PCD, and both exome capture and RNA seq data identified a large 2.3 Mb deletion containing 15 genes that underly this DLMM locus. One of these genes is a cytochrome P450, which is in the family of genes that we recently identified as a negative regulator of the *nec3* DLMM phenotype, thus is a strong candidate gene within the region. However, prior to us identifying nec3 several other genes present within the region were also known for their roles in PCD pathways and were used in the functional validation efforts. However, as we gain knowledge our hypotheses are adapted, and it now represents one of our strongest candidate gene.

The main goal of this research was to study and further understand PCD pathways by characterizing five DLMMs in barley for which we were highly successful. Five independent disease lesion mimic mutants with different lesion morphology were genetically mapped for the first time. All five mutants mapped to distinct loci and this data was utilized to target exome capture and RNA sequence analysis to identify candidate genes underlying these DLMM mutant phenotypes. VIGS will be utilized in an attempt to validate the candidate FN396 genes. Thus, although this research laid a foundation to identify the genes underlying these DLMMs future research efforts will still be needed to validate and functionally characterized the underlying genes.