CRISPR/Cas9 based gene editing in wheat and potato to enhance multiple disease resistance

Bikram Poudel

Plant Science Department

McGill University, Montreal

January 2024

A thesis submitted to the McGill University in partial fulfillment of the requirements of the degree of Doctor of Philosophy

Dedicated to my family and scientific community

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2,4-D	2,4-Dicholorophenoxyacetic acid
AAFC	Agriculture Agri-Food Canada
$AgNO_3$	Silver nitrate
AL-PCD	Apoptotic-like programmed cell death
Apaf-1	Apoptotic-protease activating factor 1
AS	Acetosyringone
AtMC	*Arabidopsis thaliana* metacaspase
ATP	Adenosine triphosphate
AUDPC	Area under disease progress curve
BBM	Baby boom
BCAs	Biological control agents
BLAST	Basic local alignment search tool
bp	Base pair
CaM	Calmodulin
CaMC9	*Capsicum annum* metacaspase 9
CaMV	Cauliflower mosaic virus
Cas	CRISPR associated protein
CBL	Calcineurin B-like
cDNA	Complementary deoxyribonucleic acid
$CuSO_4$	Copper sulphate
CCaMK	Ca^{2+}/CaM-independent protein kinase
CC-NBS-LRR	Coiled coils – nuclear binding site- leucine rich repeats
CDPK	Ca^{2+} -dependent protein kinases
CIM	Callus induction media
CNGCs	Cyclic nucleotide-gated channels
CRISPR	Clustered regularly interspaced short palindromic repeats
CRN	Crinkling necrosis
C_T	Cycle threshold

CTAB	Cetyl trimethyl ammonium bromide
ddH_2O	Double distilled water
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
Dpi	days post inoculation
EB	Early blight
ESR	Enhanced shoot regeneration
ETI	Effector triggered immunity
FAO	Food and Agriculture Organization
FHB	Fusarium head blight
GIF	Growth initiating factor
gPCR	Genomic polymerase chain reaction
GRF	Growth regulating factor
gRNA	Guide ribonucleic acid
HDR	Homology directed repair
Hpi	Hours post-inoculation
HRC	Histidine-rich calcium-binding protein
HR-PCD	Hypersensitive response programmed cell death
IBIS	Institute of Integrative Biology and Systems
IE	Immature embryos
InDels	Insertions and deletions
kDa	Kilo dalton
LB	Late blight
MS	Murashige and Skoog
MAMP	Microbe-associated molecular patterns
MAPAQ	Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec
MDR	Multiple disease resistance
ME	Mature embryos

MES	Morpholinoethanesulfonic acid
NAA	Naphthalene acetic acid
NaOCl	Sodium hypochlorite
NCBI	National Centre for Biotechnology Information
NHEJ	Non-homologous end joining
NIL-S	Near isogenic line - susceptible
NIL-R	Near isogenic line - resistant
OD	Optical density
PAM	Protospacer adjacent motif
PAMP	Pathogen-associated molecular pattern
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PEI	Prince Edward Island
PEP1	Plant elicitor peptide
PROPEP1	Precursor complex PEP1
PT	Permeability transition
PTI	Pattern-triggered immunity
qPCR	Quantitative polymerase chain reaction
QTL	Quantitative trait loci
RB	Russet Burbank
RCBD	Randomized complete block design
RM	Regeneration media
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Reverse transcriptase-quantitative polymerase chain reaction
SCRs	Small cysteine-rich proteins
sgRNA	Single guide ribonucleic acid
SIM	Shoot induction media

SlDMR6	*Solanum lycopersicum* Downy Mildew Resistance 6
StCAN2	*Solanum tuberosum* Ca^{2+} - dependent endonuclease 2
StDND1	*Solanum tuberosum* Defense Not Dead 1
StHRC	*Solanum tuberosum* Histidine-rich calcium-binding protein
StMC7	*Solanum tuberosum* Metacaspase 7
TaEm	*Tricticum aestivum* Early maturing
TaHRC	*Triticum aestivum* Histidine-rich calcium-binding protein
TALEN	Transcription activator-like effector nucleases
TaMC	*Triticum aestivum* metacaspases
TaMs2	*Triticum aestivum* male sterility 2
TDNA	Transfer deoxy ribonucleic acid
TKC	Transgene killer CRISPR
VDAC	Voltage-dependent anion channel
VIGS	Virus-induced gene silencing
WCIM	Wheat callus regeneration media
WIND1	Wound induced dedifferentiation 1
ZFN	Zinc-finger nucleases

ABSTRACT

Wheat and potato are two of the major economically important Canadian crops. They are among the most important crops worldwide, acting as a staple food for a combined 4 billion people. Wheat is one of the first domesticated food crops and remains one of the most important sources of calories and proteins. Potato is the most grown staple crop after wheat, rice and maize. Wheat and potato suffer from biotic and abiotic stresses, resulting in lower yield and quality. Fusarium Head Blight (FHB) caused by the fungal pathogen *Fusarium graminearum* is one of the most significant diseases in wheat causing severe yield and yield quality loss through the accumulation of mycotoxins such as deoxynivalenol (DON) and nivalenol (NIV). In potato, late blight (LB) disease caused by *Phytophthora infestans* and early blight (EB), caused by *Alternaria solani* can have devastating impact on potato yield, even capable of wiping out the entire crop. As response to the pathogen attack, the plants carry out programmed cell death (PCD) to restrict the pathogen to the point of infection. However, hemibiotrophic and necrotrophic pathogens feed on these dead cells for their further growth. Gene editing of PCD pathway genes using CRISPR/Cas9 can improve host resistance to pathogen attack in plant cultivars in a short time with minimum cost and effort. Wheat is one of the recalcitrant crops, requiring improvisation and optimization for different cultivars. This study focused on optimization of tissue culture protocol in wheat for delivering CRISPR/Cas9 components and genome editing of potato through CRISPR/Cas9 tool to enhance resistance to hemibiotrophic and necrotrophic pathogens. The first study optimizes tissue culture for successful transgenic wheat regeneration and delivery of CRISPR/Cas9, targeting *TaHRC* gene which when non-functional, enhances resistance to FHB in cultivar ‘Pasteur’. Upon optimization of tissue culture and selection of GRF-GIF based vector as the most efficient, one gRNA was inserted into the vector

system and delivered through *Agrobacterium*-mediated transformation and biolistic gene delivery into immature embryo derived calli. *Agrobacterium*-mediated transformation failed to regenerate any transgenic plant in ‘Pasteur’. Three transgenic wheat plants with successful insertion of CRISPR vector components were generated through biolistic delivery. In the second study, *StMC7*, a metacaspase responsible for conduction PCD in potato, was silenced for enhancing disease resistance to *P. infestans* and *A. solani*. Three gRNAs were designed to edit the two exons of *StMC7*. Eight transgenic plants were regenerated through tissue culture. Upon restriction digestion assay and sanger sequencing, 6 plants from 5 independent lines were found to be edited. Disease severity and pathogen biomass was significantly decreased in the *Stmc7* mutants compared to the RB plants for both early and late blight of potato. The study displays the potential of metacaspases in enhancing multiple disease resistance.

RÉSUMÉ

Le blé et la pomme de terre sont deux des cultures canadiennes les plus importantes sur le plan économique. Ils comptent parmi les cultures les plus importantes au monde et constituent des aliments de base pour plus de 4 milliards de personnes. Le blé a été l'une des premières cultures vivrières domestiquées et reste l'une des plus importantes sources de calories et de protéines alimentaires. La pomme de terre est la culture de base la plus cultivée après le blé, le riz et le maïs. Le blé et la pomme de terre souffrent de stress biotiques et abiotiques, ce qui entraîne une baisse du rendement et de la qualité. La fusariose de l'épi causée par l'agent pathogène fongique *Fusarium graminearum* est l'une des maladies les plus importantes du blé, entraînant une grave perte de rendement et de qualité du rendement en raison de l'accumulation de mycotoxines telles que le déoxynivalénol (DON) et le nivalénol (NIV). Chez la pomme de terre, la brûlure tardive causée par *Phytophthora infestans* et la brûlure hâtive causée par *Alternaria solani* peuvent avoir des effets dévastateurs sur le rendement des pommes de terre, allant jusqu'à anéantir des récoltes entières. En réponse à l'attaque d'un agent pathogène, les plantes procèdent à une mort cellulaire programmée pour limiter l'agent pathogène au point d'infection. Cependant, les agents pathogènes hémibiotrophes et nécrotrophes se nourrissent de ces cellules mortes pour leur croissance. L'édition génétique des gènes de la voie de la mort cellulaire programmée à l'aide de CRISPR/Cas9 peut améliorer la résistance de l'hôte aux attaques d'agents pathogènes en peu de temps avec un minimum de coûts et d'efforts. Le blé est une culture récalcitrante à la transformation génétique, nécessitant de l'improvisation et de l'optimisation pour des cultivars comme « Pasteur ». Cette étude s'est concentrée sur l'optimisation du protocole de culture tissulaire du blé pour la livraison des composants CRISPR/Cas9 et l'édition du génome de la pomme de terre via l'outil CRISPR/Cas9 pour

améliorer la résistance aux agents pathogènes hémibiotrophes et nécrotrophes. La première étude optimise la culture tissulaire pour une régénération réussie du blé transgénique et l'administration de CRISPR/Cas9, ciblant le gène *TaHRC* qui, lorsqu'il est non-fonctionnel, améliore la résistance du blé à la fusariose de l'épi. Après optimisation de la culture tissulaire et sélection du vecteur d'édition génique basé sur GRF-GIF comme étant le plus efficace, un ARNg ciblant le gène *TaHRC* a été introduit et livré par transformation médiée par *Agrobacterium* et par transfert biolistique dans des cals dérivés d'embryons immatures. La transformation médiée par Agrobacterium n'a réussi à régénérer aucune plante transgénique chez « Pasteur ». Trois plants de blé transgéniques avec insertion réussie des composants du vecteur CRISPR ont été générés par transfert biolistique. Dans la deuxième étude, *StMC7*, une métacaspase responsable de la régulation de la mort cellulaire programmée chez la pomme de terre, a été rendu silencieux pour améliorer la résistance aux maladies causées par *P. infestans* et *A. solani*. Trois ARNg ont été conçus pour modifier les deux exons de StMC7. Huit plantes transgéniques ont été régénérées par culture tissulaire. Un test de digestion par restriction et le séquençage Sanger ont démontrés que 6 plantes provenant de 5 lignées indépendantes ont été modifiées. La gravité de la maladie et la biomasse des agents pathogènes ont diminuées chez les mutants Stmc7 par rapport aux plantes sauvages RB, pour la brûlure hâtive et la brûlure tardive de la pomme de terre. L'étude montre le potentiel des métacaspases pour améliorer la résistance à de multiples maladies.

ACKNOWLEDGEMENT

I feel extremely grateful and privileged to be under the guidance of my Supervisors **Prof. Ajjamada C. Kushalappa** and **Prof. Jacqueline Bede**. Prof. Kushalappa has provided constant support, guidance, and encouragement during my time as a Ph.D. student. He has not been just my supervisor but a mentor and a guardian as well. I am indebted to him for coming up with solutions when the times were tough. The research projects would not have been finished without his guidance and support. **Prof. Jacqueline Bede** has been a constant source of encouragement, suggestions and guidance, which made my research and writing better and scientifically robust. She has been extremely helpful towards my research and always tried to motivate me to complete my research on time. I am extremely grateful for all she has done for me.

I would like to thank my thesis committee members: **Prof. Donald Smith** and **Prof. Mark Lefsrud** for their suggestions and help, without which this thesis could not have been completed. Their insightful comments, suggestions and questions have hugely contributed, especially when the research projects had to be modified. I would like to thank my Comprehensive Exam Committee: **Prof. Philippe Seguin (Chair)** and **Prof. Jennifer Ronholm** for their insightful comments, questions, and encouragement.

I am very thankful to **Prof. Valerie Gravel** for her suggestions and encouragement in carrying out the research work. She was extremely valuable by providing me fresh insights on what to include in my thesis and encouraging me to complete my research work. I will always be very thankful for all she has done as Graduate Program Director – Plant Science, especially during challenging phases of my research.

I am sincerely thankful to all the faculty of Plant Science Department for their direct and indirect support and guidance. I am very grateful to the Plant Science Department staff, **Mr. Serge Dernovici**, **Ms. Sarah-Ann Persechino**, **Mr. Micheal Gossage-Bleho**, **Mr. Ian Ritchie (Retired)**, **Mr. Guy Rimmer (Retired)**, **Mr. Wadih Tadros** for their technical support in greenhouse, phytorium and for allowing me to work as Greenhouse Assistant. **Mr. Serge Dernovici** has been very helpful in providing suggestions and guidance and I am extremely grateful to him for everything he has done for me and for the Plant Science Department.

I am deeply thankful to the administrative support staff **Ms. Erin Minnett**, **Ms. Diane Chan-Hum**, **Ms. Lindsay Flood**, **Ms. Susan Gregus** and **Ms. Cinthya Horvath** for their support and help in administration related issues. Thank you very much for all you have done for me and all of us.

My labmates have been a constant source of meaningful scientific discussions and support. I am heartly grateful to **Russiachand Singh Heikham**, **Sripad Joshi**, **Niranjan Hedge**, **Atul Sathe**, **Nancy Soni**, **Krishna Kumar** and **Chaithra Nagaraju** for their excellent support and great coordination during my research. I would also like to acknowledge my friends from the Plant Science Department, **Mohammad Antar**, **Irfan Iqbal**, **Ximena Florez Buitrago, Meha Sharma**, **Dikshya Bhola**, **Mehtab Singh** for their support. I am grateful to my friends **Mohit Jain**, **Ashutosh Kushwaha**, **Harsh Banal**, **Neeraj Neeraj**, **Manish Ojha**, **Maheshwor Bastakoti**, **Gaurav Khatiwada**, **Gaurab Pradhan**, **Mohammad Jafar Sami** for their constant support, help in good and bad times and for keeping the fun alive during my Ph.D. study. I am grateful to **Amelia Redl** for her help with French abstract.

Nothing would have been possible without the support of my family. I am extremely grateful to my father, **Buddi Prasad Upadhyaya Poudel**, my mother **Kamala Poudel**, for all the love,

support, guidance and understanding before and during my Ph.D. journey. I am extremely thankful and grateful to my beautiful and loving wife, **Susmita Bhandari** for her constant support, understanding, guidance, encouragement, motivation and for pushing me to complete my Ph.D. I am thankful to my younger brother, **Bishal Paudel**, for his support and encouragement.

Finally, I am very thankful to **McGill University** for accepting me as a Ph.D. Student, for constant support and help, both academically, and financially, through awarding Schulich Graduate Fellowship, Graduate Excellence Award, Research Assistantship. I am thankful to the **Macdonald Campus** for being so welcoming, for all the friends I made. I acknowledge the research project funding by **Natural Science and Engineering Research Council of Canada (NSERC)** and **Weston-Loblaw, Seeding Food Innovation**.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

Preface

The following thesis was prepared in manuscript form as per the "Thesis Guidelines" of McGill University. Two chapters represent two separate research manuscripts (Chapter 3-4). In the first study, tissue culture protocol was optimized for efficient transformation and regeneration of wheat cultivar 'Pasteur' to produce CRISPR/Cas9-edited plants. *TaHRC*, a programmed cell death-related gene conferring resistance to Fusarium head blight when non-functional, was targeted for a knockout mutation. In the second study, CRISPR/Cas9-mediated gene editing was conducted in an elite potato cultivar 'Russet Burbank' for improving disease resistance to hemibiotrophic pathogen *Phytophthora infestans* and necrotrophic pathogen *Alternaria solani*. *StMC7*, a metacaspase gene involved in programmed cell death pathway was targeted for gene editing.

Contributions to knowledge

- Tissue culture, transformation and regeneration was optimized for the highly recalcitrant wheat cultivar 'Pasteur'. Optimization of media components such as 2,4-D, copper sulphate and silver nitrate and addition of osmotic treatment before and after bombardment resulted in the regeneration of transgenic plants. The optimized protocol can be applied to other wheat cultivars, especially those derived from 'Pasteur' and 'Cadenza'.
- The ideal source material (immature embryo-derived callus), transformation method (biolistic delivery) and CRISPR vector system (GRF-GIF based vector) was identified as the best method for CRISPR construct delivery in 'Pasteur'.

- A transgene killer CRISPR (TKC) vector was designed for wheat, which can be evaluated for obtaining transgene-free CRISPR-edited plants. TKC vectors can also be designed for other crop species based on the wheat construct.
- The *StMC7* gene was sequenced, and protein structure predicted. CRISPR-edited Russet Burbank potato with enhanced disease resistance to multiple hemibiotrophic and necrotrophic pathogens was developed by silencing metacaspase *StMC7*. This study is one of the few studies with successful CRISPR editing in potato and can serve as a template for developing CRISPR-edited potato.
- Silencing type II metacaspase *StMC7* led to disease-resistant potato plants against multiple pathogens. Metacaspases serve as good targets for improving disease resistance in plants against hemibiotrophic and necrotrophic pathogens.
- Putative activation mechanism was suggested for *StMC7* gene, which can be validated to identify the mechanisms of apoptotic-like programmed cell death (AL-PCD).

CONTRIBUTION OF AUTHORS

Research activities carried out under this thesis work were designed by BP (myself) under the guidance of Dr. Ajjamada C. Kushalappa.

In chapter III, all experiments (both laboratory and greenhouse) were conducted by BP. This includes, but not limited to, construction of vectors, formulation and preparation of medias, analysis of the results and writing of the manuscript. All activities were supervised by Dr. Ajjamada C. Kushalappa. Dr. Jacqueline Bede edited the manuscript.

Chapter IV was co-authored by Dr. Atul P. Sathe, who sequenced the *StMC7* gene and performed the initial phase of tissue culture. Dr. Ajjamada C. Kushalappa supervised the project and edited the manuscript. Dr. Jacqueline Bede edited the manuscript.

CHAPTER 1: INTRODUCTION

As the fifth largest agricultural exporter behind US, China, Brazil and EU countries, Canada produces many economically important crops such as canola, wheat, soybean, barley, oat, potato and pulses (AAFC, 2022). Agriculture and agri-food system employed 2.3 million people in 2022, generating $143.8 billion of Canadian GDP, which makes up 7% of total GDP. Major crops grown and exported are wheat, pulses, potato (fresh and processed), canola. Among them, wheat and potato are considered among the big four crops worldwide, acting as staple foods for over 4 billion people (Bentley et al., 2022; Devaux et al., 2021).

Wheat was one of the first domesticated food crops among all the agricultural food crop and remains one of the most important dietary sources of calories and proteins. It is cultivated in nearly all regions of the world ranging from 67^{o}N in Scandinavia and Russia to 45^{o}S in Argentina and up to an altitude of 3,000 m above sea level (Shewry, 2009). Canada produced 22.29 million tons of wheat harvested from an area of 9.2 million hectares in 2021, making it the 8th largest producer worldwide (FAOSTAT, 2023). Major cultivated wheat species are hexaploid bread wheat (*T. aestivum*), tetraploid durum wheat (*T. durum*) and Emmer wheat (*T. dicoccum*) and diploid Einkorn wheat (*T. monococcum*). About 95% of the wheat grown worldwide is bread wheat and most of the remaining 5% is durum wheat. Bread wheat (*Triticum aestivum*; AABBDD subgenome) is an allopolyploid derived from the hybridization between three distinct diploid species.

Potato (*Solanum tuberosum L.*) is a non-cereal staple food crop grown around the world. It is the most grown staple crop after wheat, rice and maize (Birch et al., 2012). It is a preferred crop in developing countries due to its nutritional value, its wide adaptability to diverse environments and yield potential (Salmensuu, 2021). The total production of potato worldwide

was 376 million tonnes in 2021 with China as the largest producer, followed by India and Ukraine (FAOSTAT, 2023). Canada is the 12th largest producer of potato with an annual yield of approximately 6.3 million tonnes (FAOSTAT, 2023).

Wheat and potato suffer from biotic and abiotic stresses, resulting in lower yield and quality (Han et al. 2023). Biotic stresses are caused by several organisms such as fungi, bacteria, viruses, insects, nematodes and weeds. Among them, fungi and oomycete pathogens have presented a challenge to production quality and quantity. In wheat, Fusarium Head Blight (FHB) caused by a fungal pathogen *Fusarium graminearum* (Teleomorph: *Gibberella zeae*) is one of the most important diseases, causing severe losses in yield and yield quality through the accumulation of mycotoxins such as deoxynivalenol (DON) and nivalenol (NIV), which are harmful to animal and human health (Jennings, 2007). Losses due to FHB were estimated to be $2 billion USD in the US between 1993 and 2001, and $520 million CAD between 1990 and 2000 in Canada (Xia et al., 2020). Annual losses due to FHB in Canada is estimated to be $50-300 million CAD since the 1990s (Alberta, 2012). In potato, late blight of potato, caused by the oomycete *Phytophthora infestans* has had devastating impact on potato yield, even capable of wiping out the entire crop. This pathogen lead to Irish famine in 1845, leaving 1.5 million people dead and 1.5 million people emigrating from Ireland in the 10 years between 1841-1851 (Powderly, 2019). Late blight of potato remains a food security threat, costing up to 3-10 billion USD per year worldwide in yield loss and management cost (Haverkort et al. 2009). Early blight of potato, caused by necrotrophic fungi *Alternaria solani* is another important disease spread globally. It causes premature defoliation with significant impact on tuber yield quantity and quality.

Various measures have been implemented to combat these fungal diseases in wheat and potato because of the serious economic damage caused by these pathogens. Chemical control through pesticide application remains the most followed management practice. However, several rounds of fungicide application are required per season for effective control of these pathogens, which accounts for up to 25% of production cost (Dong & Zhou, 2022). Disease-resistant population of *F. graminearum* and *P. infestans* have evolved, lowering the effectiveness of chemical control. This, then, requires a higher pesticide dosage and frequency of fungicides for disease control, often resulting in a vicious cycle. Fungicide application can also adversely affect human health as well as the environment (Fisher et al. 2012). Thus, enhancing genetic resistance of plant varieties is considered the most sustainable, cost-effective, and environment-friendly approach.

Since wheat (2n = 6x = 42) and potato (2n = 4x = 48) are polyploids, they are highly heterozygous, making genetic improvement challenging. Multiple alleles are associated with a single locus, making segregation more complex (Schaart et al., 2021). More rounds of backcrossing are required to introgress a novel trait-of-interest, which makes crop improvement laborious and time-consuming. Therefore, the introduction of new potato cultivar through traditional breeding can take 15-20 years (Halterman et al. 2016). Genome editing may serve as a good alternative to traditional breeding because knocking out deleterious genes or correcting genes can be achieved through simultaneous mutations in multiple genome sites with less cost, effort and time. Though the challenges of multiple alleles in polyploid plants remains, overall, it is a better alternative to traditional breeding owing to the benefits of genome editing such as simultaneous editing of multiple alleles.

Genome editing is a collection of precise, efficient molecular biology techniques aimed at targeted modifications at genomic loci (Zhang et al., 2018). Host genome engineering approaches include sequence-specific nucleases (SSNs), such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the clustered regularly interspaced short palindromic repeats (CRISPR) nuclease system. Genetic engineering through SSNs relies on the introduction of DNA double-strand breaks (DSBs) at targeted sites which triggers DNA repair mechanisms. Breaks generated by engineered nucleases are repaired by either non-homologous end-joining (NHEJ) or homologous recombination (HR) (Wyman and Kannar, 2006). These mechanisms of repair generate gene knockouts, replacements, insertions and rearrangements. While NHEJ is error-prone and introduces small deletions and insertions, HR is precise and used to replace or correct genes (Gao, 2014). CRISPR/Cas is the most preferred gene editing tool in plants owing to its simplicity (in requiring only a single gRNA to target a sequence), versatility (can be reprogrammed to target different sites by changing the gRNA), multiplexity (the ability to target multiple sites simultaneously), efficiency (higher mutation efficiency compared to ZFNs and TALENs), compatibility (can be delivered through existing delivery methods), simplicity (simple to design and execute) and cost effectiveness (Cardi et al., 2023). CRISPR/Cas9 allows researchers to make targeted modifications to specific genes by using guide RNA to cut the genomic DNA at precise locations and then relying on the cell's natural repair mechanisms to introduce desired changes.

Initially recognized as part of the bacterial immune system, CRISPR has become a preferred gene editing technology over its predecessors, such as ZFNs and TALENs, due to its simplicity, versatility and efficiency (Hossain, 2021). CRISPR consists of three components: an endonuclease such as Cas9, a guide RNA and a protospacer adjacent motif (PAM) adjacent to

the target gene sequence (Figure 1.1). The Cas9 protein and gRNA form a Cas9-gRNA complex. This complex binds to the PAM region of the target sequence and induces DSBs at 3-5 base pairs adjacent to PAM region. The availability of the repair template determines the repair mechanism. The repair template, if provided, is inserted into the DSB site creating a knock-in mutation, which repairs the gene by insertion. In the absence of repair template, InDel mutations are created, silencing the gene.

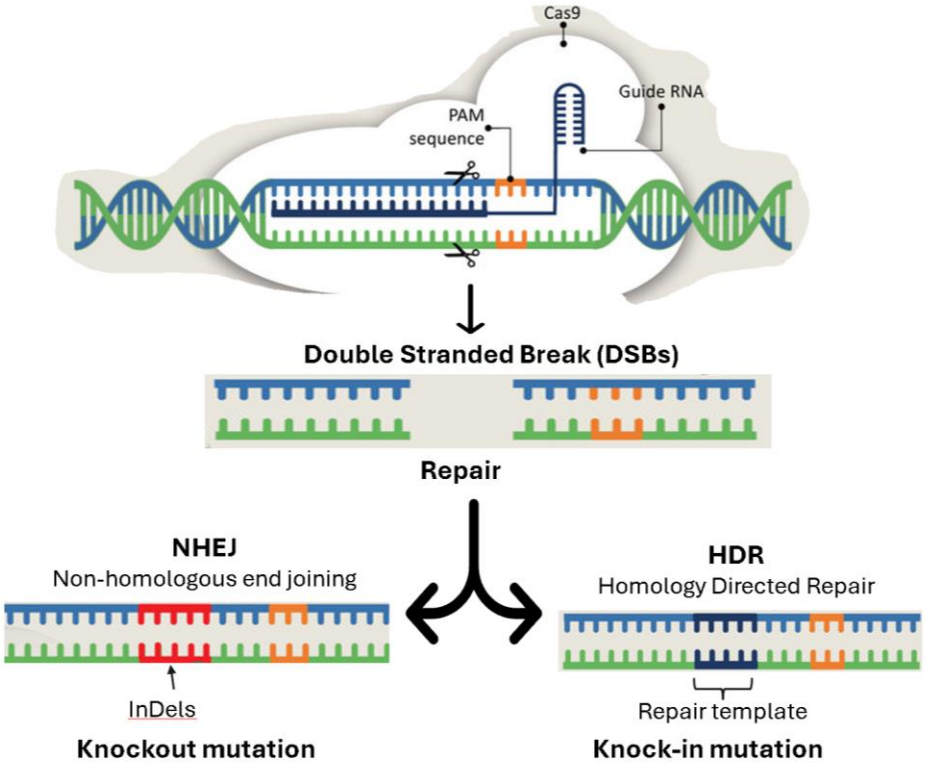


Figure 1.1 CRISPR/Cas9 general working mechanism. Once in the cell, the Cas9 and guide RNA form a complex and bind to the PAM region adjacent to the target site. The Cas9 cuts the DNA at 3-4 bases prior to PAM region, creating a double stranded break (DSB). This break is repaired through Homology Directed Repair (HDR) in presence of repair template and through non-homologous end joining pathway (NHEJ), introducing insertion and deletion (InDels) in the absence of repair template.

CRISPR/Cas is a versatile technology with the ability to achieve mutations at multiple target sites simultaneously (Xu et al., 2022). This makes CRISPR suitable for polyploid crops, such as wheat and potato since the guide RNAs can be designed to target multiple. CRISPR/Cas has been successfully applied to obtain desired phenotypes in wheat (Elsharawy et al. 2023), potato (Hegde et al. 2021), sugarcane (Oz et al. 2021), banana (Ntui et al. 2021) and peanut (Neelakandan et al. 2022).

Losses due to fungal diseases such as FHB in wheat, late and early blight in potato can be reduced through genome editing (Kieu et al., 2021; Munoz et al., 2019). Bauer et al. (2020) increased FHB resistance upon silencing the wheat *TaNFXL1* gene using CRISPR. Kieu et al. (2021) generated potatoes with reduced late blight through CRISPR-mediated editing of genes, *StDND1* and *StCHL1*. Thus, genome editing can greatly contribute to cultivar improvement, especially by introducing or knocking out specific genes (Kushalappa et al., 2022).

Several QTLs which confer FHB resistance have been identified in wheat. Among them, QTL-*Fhb1*, present in chromosome 3B, has a major contribution to FHB resistance. Sequencing this QTL revealed more than 30 genes that may contribute to FHB resistance (Soni et al., 2021). Among them, histidine-rich calcium-binding protein gene (*TaHRC*) was a key determinant of Fhb1-mediated FHB resistance (Su et al., 2019). In potato, *StHRC* was functional and induced apoptotic-like PCD (AL-PCD) upon pathogen infection. Silencing of this gene leads to increased resistance to multiple diseases (Kushalappa et al., 2022). AL-PCD is a form of PCD induced upon pathogen attack, exhibiting morphological and biochemical features similar to apoptosis in animal cells. It plays an important role in development as well as during biotic and abiotic stress (Watanabe & Lam, 2011). Metacaspases, such as *StMC7*, mediate AL-PCD triggered by biotic stress. Since hemibiotrophic and necrotrophic pathogens require dead cells to feed on, silencing

the functional *StMC7* could enhance multiple disease resistance by preventing the induction of AL-PCD.

1.1 General Hypothesis

Wheat (allopolyploid) and potato (autopolyploid) are two important staple food crops worldwide. Several pathogens attack these crops and reduce the yield quality and quantity. Gene editing can sustainably enhance genetic host resistance. Silencing *TaHRC* gene in wheat can provide durable resistance against FHB by suppressing AL-PCD. Gene editing requires the explant to undergo tissue culture and transformation. Wheat is a recalcitrant crop and optimizing regeneration in cultivar such as 'Pasteur' through tissue culture is essential for obtaining genome-edited wheat. *Agrobacterium*-mediated and biolistic transformation method of wheat transformation are two major techniques to transform wheat. Suitable vector selection for delivery of CRISPR components is important for genome editing in wheat. Silencing other genes in PCD pathway, such as *StMC7* in potato, could also suppress apoptotic-like AL-PCD, thereby leading to increased disease resistance against early and late blight caused by *Alternaria solani* and *Phytophthora infestans*.

1.2 Objectives

1. To optimize tissue culture and edit *TaHRC* in wheat cultivar 'Pasteur' through CRISPR/Cas technology.
    a. To identify the best vector system for CRISPR/Cas9 application in wheat
    b. To regenerate CRISPR-edited wheat plants through optimization of tissue culture parameters after *Agrobacterium*-mediated or biolistic transformation.

2. To enhance multiple disease resistance in the elite potato cultivar ‘Russet Burbank’ through CRISPR/Cas9-based editing of *StMC7* gene.

    a. To develop CRISPR-edited transgenic potato plants by introducing CRISPR vector through *Agrobacterium*-mediated transformation and tissue culture.

    b. To show multiple disease resistance by disease severity and pathogen biomass and gene expression assays against both early and late blight of potato.

CHAPTER II: LITERATURE REVIEW

2.1 Wheat

Wheat (*Triticum aestivum* L.) is one of the most important cereal crops extensively grown worldwide. Since its first cultivation as a part of the 'Neolithic Revolution' about 10,000 years ago in the Fertile Crescent area (present day Middle East), it is now cultivated in most parts of the world (Schiermeier, 2015). The earliest cultivated forms of wheat were diploid einkorn and tetraploid emmer wheat (Shewry, 2009). Wheat is grown worldwide in an area of 220 million hectares with a total production of 770.8 million tons. Major wheat producing countries are China India, Russia, USA, Ukraine, Pakistan, EU, Australia. Canada is the 8th largest producer of wheat, producing 22.29 million tons of wheat from 9.2 million hectares in 2021 (FAOSTAT, 2023). Today, about 95% of the wheat grown worldwide is hexaploid bread wheat and most of the remaining 5% is tetraploid durum wheat. Wheat is a major source of dietary carbohydrates in the form of starch and protein. About 85% of the present global population is dependent on wheat as a basic calorie source and 60% on wheat as primary source of protein (Nazia et al., 2022).

2.2 Potato

Potato (*Solanum tuberosum* L.) is the most important non-cereal crop grown in more than 178 countries with a worldwide production of 376 million tons in 18 million hectares (FAOSTAT, 2023). Despite its origin in the Andes of southern Peru and northern Bolivia, it is presently grown in different climatic zones under various agroecological conditions in diverse socioeconomic environments throughout the world. It is cultivated between the altitude of 2,000 – 4,000 m above sea level and between the latitude of 47^{o}S to 65^{o}N. Major potato producing

countries are China, India, Ukraine, USA, Russia, EU, Bangladesh, and Egypt. Canada is the 12th largest producer, producing 6.3 million tons in 2021 (FAOSTAT, 2023).

Potato is a major source of carbohydrates, protein, vitamin C, iron and dietary fiber though content varies among the cultivars. A 100 g of baked potato contains 21.44 g carbohydrate, 2.63 g protein, 2.3 g of total dietary fiber, 0.55 g potassium and several other major micronutrients such as vitamin B (0.35 mg), vitamin C (8.3 mg), calcium (18 mg), magnesium (30 mg), phosphorus (71 mg), iron (1 mg) (McGill et al., 2013).

2.3 Necrotrophic and hemibiotrophic fungal pathogens of wheat and potato

Wheat and potato suffer significant reduction in yield quality and quantity due to hemibiotrophic and necrotrophic pathogen attacks. Pathogens are often characterized by their feeding mode since it is often closely related to plant resistance strategies. Biotrophic pathogens feed on the living cells for their survival. Necrotrophic pathogens feed on the dead and dying cells for their growth and development. Hemibiotrophs feed on the living plant tissue similar to biotrophs during the initial infection phase and then switch to necrotrophy. These pathogens secrete enzymes and toxins to kill the host tissue.

Hemibiotrophic and necrotrophic pathogens use the defense response of plants, such as the hypersensitive response (HR) and associated programmed cell death (PCD) of host tissue to enhance infection. HR deprives the food supply for biotrophs and confines them to the initial site of infection. However, hemibiotrophs and necrotrophs trigger the HR in plants by inducing effector-triggered immunity (ETI), allowing the plant defense mechanism to kill its cells, which is then utilized by the pathogen for survival and growth (Govrin & Levine, 2000).

2.3.1 Fusarium head blight (FHB)

Fusarium head blight (FHB) is a devastating disease of wheat, barley, oats, corn and other cereals that hinders grain development and leads to significant yield losses. It produces trichothecene mycotoxins, such as deoxynivalenol (DON) and zearalenone, which presents a serious risk for human and animal health (Alisaac & Mahlein, 2023). In wheat, *Fusarium graminearum* (teleomorph: *Gibberella zeae*) is the most predominant species causing FHB, although at least 16 other species in different areas of the world with diverse climatic conditions are also associated with FHB such as *F. culmorum, F. avenaceum, F. poae, F. tricinctum, Microdochium nivale* (Aoki et al., 2012). The initial sources of inoculum are contaminated crop residues, especially when maize is the preceding crop as well as spores dispersed through wind, rain splash or arthropods (Karlsson et al., 2021). When the spores reach the wheat spikelet, they germinate under favorable conditions, such as high humidity and continuous wetness, and colonize spikelet tissues. Initially, the pathogen is a biotroph for 48-72 hours post inoculation (hpi) and then shifts to the necrotrophic phase. These pathogens spread both intra-cellularly and inter-cellularly, reaching rachilla node and spreading throughout the spike through vascular bundles. The colonized spikelets bleach within 5 days post inoculation (dpi) due to host cell death (Figure 2.1a). The bleaching symptoms expand to rachis 12 dpi (Brown et al., 2010).

2.3.2 Late blight of potato

Late blight of potato is caused by the oomycete *Phytophthora infestans*, one of the most devastating pathogens in human history. It was first identified around early mid-1800s just before it caused the infamous Irish Potato Famine around 1845 (Powderly, 2019). Since then, it remains the most destructive disease in potato, resulting in annual crop losses enough to feed more than

80 million people (Yoshida et al., 2013). It can destroy the entire potato canopy within 10-15 days and result in total yield loss (Rakotonindraina et al., 2012). Sporangia and zoospores on the aerial plants parts of infected potato are the primary source of dispersal. Upon landing on a new host, they germinate within hours, entering through natural openings or penetrate host epidermal cells through appressorium-like germ tube (Whisson et al., 2016). An intracellular biotrophic infection vesicle is formed in epidermal cells and transforms into intercellular biotrophic finger-like haustoria. Similar to *F. graminearum*, the mode of interaction between *P. infestans* and host cell becomes necrotrophic after 36-48 hpi. Infected leaf areas are necrotic at 72-96 hpi and sporulating hyphae release sporangia to facilitate further infection (Avrova et al., 2008). The symptoms of late blight infection in potato first emerge as dark grey to brown water-soaked spots on leaf tissues (Figure 2.1b). The infection spreads rapidly within and between plants under high humidity (>90%) and low temperature (10-23^{o}C) through infectious asexual spores leading to total necrosis of infected plants. The underground tuber infection initiates from physical weak spots, such as the eyes, lenticels and existing wounds, if any. The pathogen grows within the tuber, leading to color change and rotting through secondary infection by soft rot bacteria, making the tuber unfit for consumption (Dong & Zhou, 2022).

2.3.3 Early blight of potato

Early blight is another important disease in potato and has spread worldwide in all potato growing areas (Ivanovic et al., 2022). It primarily affects leaves and stems of Solanaceous crops, such as potato and tomato, resulting in considerable defoliation and yield loss. Four *Alternaria* species are reported as the causal pathogens of early blight of potato: *Alternaria solani, A. grandis, A. protenta and A. linariae* (Landschoot et al., 2017). The fungus overwinters in crop residues, infected tubers or on the soil surface. Primary infection takes place in the lower leaves

through conidia formed on crop debris on soil. These conidia are dispersed by wind, water, or arthropods to neighboring plants. Upon landing on a new host, conidia germinate through germ tubes in presence of water and penetrate the host cells with appressorium, either directly through stomata or indirectly through wounds. Early blight infection starts from the bottom of the plants and progresses upwards. The pathogen invades the host cells through hyphae by 8 hpi. By 24 hpi, the host cell is infected and destroyed by 72 hpi. Necrotic lesions begin to form 2-3 days post initial infection (Figure 2.1c). High temperature (24-29^{o}C), high humidity or frequent prolonged wetness (at least 3 h) increase the possibility of early blight outbreak (Jindo et al., 2021).

Figure 2.1. Plant infection by hemibiotrophic and necrotrophic pathogens (a) Fusarium head blight of wheat, showing the characteristic bleached spikelets, (b) Late blight of potato, showing necrotic lesion in the infected leaf and (c) Early blight of potato, showing the characteristic round necrotic lesions.

2.4 Resistance mechanism in plants

Though plants lack adaptive immunity, they have an innate cellular immune system and produce signals from infection sites systematically activate the plant defense system to increase resistance throughout the plant. Resistance in plants is controlled by a hierarchy of R genes that encode regulatory proteins and resistance related (RR) metabolites and proteins. These RR metabolites and proteins provide resistance, because of their antimicrobial or cell wall reinforcement properties (Kushalappa et al., 2016).

In general, fungal pathogens of plants attack in four main stages; adhesion to the host surface, forming an infection structure, invade the host, colonization of tissues and expansion within the host. Upon infection, microbe-associated molecular patterns (MAMPs), such as chitin and cell-wall derivative glucan from fungi, and β-glucan and glutamine aminotransferase from oomycetes, are recognized by pattern recognition receptors (PRRs) such as receptor-like kinases (RLKs) and proteins (RLPs) on plant cell membrane surfaces (Burkart & Stahl, 2017). Upon recognition, a plant defense response, termed as pattern-triggered immunity (PTI), is triggered to limit pathogen invasion, (Li et al., 2022). This immunity includes calcium influx, a reactive oxygen species (ROS) burst, and expression of induced genes. The pathogen, then, produce effector proteins to avoid host recognition and inhibit the host's immune response. These effectors may be recognized by membrane and/or cytoplasmically localized receptors, called effector recognition receptors (ELRRs), triggering downstream regulatory and metabolite biosynthetic genes to confer resistance, resulting in effector-triggered immunity (ETI) (Ngou et al., 2022). Plant receptor R genes have been extensively used for developing resistant genotypes due to their simple inheritance.

2.4.1. R genes with confirmed disease resistance function in wheat and potato

Several R genes have been identified and confirmed for their role in wheat resistance to FHB in wheat. Six major QTLs (namely *Fhb1, Fhb2, Fhb3*, *Fhb4*, *Fhb5* and *Fhb6*) have been found to be crucial for FHB resistance in wheat (Ma et al., 2020). These QTLs confer one of the five types of FHB resistance, type I resistance (resistance to initial infection), type II (resistance to fungal spread within the spike after initial infection), type III (resistance to kernel infection) and type IV (resistance to toxin accumulation). Type II is the most studied type of resistance because of its importance in reducing yield loss and grain toxin accumulation (Paudel et al., 2020). Among the QTLs conferring type II resistance, *Fhb1* is the most stable region with the largest effect on type II resistance among diverse backgrounds (Suzuki et al., 2012). Among the genes associated with *Fhb1, TaHRC,* which encodes a reticulum histidine-rich calcium-binding protein, was expressed in near isogenic lines (NILs) carrying susceptible *Fhb1* alleles (NIL-S) while non-functional in resistant NILs (NIL-R) (Gunnaiah, 2013). *TaHRC* promotes FHB susceptibility during *Fusarium* infection in all FHB-susceptible cultivars (Su et al., 2021). *TaHRC* seems to suppress pattern-triggered immunity (PTI), a plant immune response activated upon chitin detection (Chen et al., 2022). In FHB-resistant cultivars, a large deletion in the start codon is found, silencing *TaHRC* and imparting FHB resistance (Su et al., 2019).

Several race-specific resistance genes against *P. infestans* (*Rpi*) have been discovered in wild potato species (Rodewald & Trognitz, 2013). More than 70 Rpi genes have been identified and mapped in 32 *Solanum* species across the world, primarily in Mexico and South America (Peru, Bolivia, Argentina) (Paluchowska et al., 2022). Many of these genes belong to the coiled coils – nuclear binding site- leucine rich repeats (CC-NBS-LRR) protein family and recognize *P. infestans* avirulence effectors (Avr) of class RxLR (Ballvora et al., 2002). Some of these *Rpi*

genes are *R1, R2, R3a, R3b, R8* and *R9a* from *Solanum demissum, Rpi-blb1* and *Rpi-blb12* from *Solanum bulbocastanum, Rpi-vnt1* from *Solanum venturii, Rpi-amr1* from *Solanum americanum, Rpi-mcq1* from *Solanum mochiquense* (Monino-Lopez et al., 2021). Loss-of -function mutation of susceptibility genes (S-gene), such as the downy mildew resistance gene in tomato (*SlDMR6*) conferred broad spectrum disease resistance against *Phytophthora* spp. Knocking out *StDMR6*, Defense not dead (*StDND1*) and *StCIB1*/HBI1-like1 (*StCHL1*) increased resistance against potato late blight (Kieu et al., 2021).

Resistance against *A. solani* is suggested to be quantitative in nature and controlled by polygenes (Sajeevan et al., 2023). Resistance appears to be linked to cultivar maturity time; early maturing cultivars are often more susceptible and late maturing cultivars more resistant to *A. solani* (Duarte et al., 2014). No specific *R* genes have been identified for controlling early blight (EB) (Brouwer et al., 2021). Several quantitative trait loci (QTLs) have been identified for foliar EB on chromosomes 1,5,6,7,11 and 12 based on tetraploid potato segregating population (Odilbekov et al., 2020).

2.4.2. Multiple disease resistance in plants

Multiple disease resistance (MDR) in plants is a highly desirable trait and can be conferred through a nonspecific component of disease resistance. Variation in constitutive defense, such as physical barriers or antimicrobial compounds, may hinder pathogen and affect multiple resistance. Several mechanisms of MDR have been suggested such as recognition of conserved signals in effectors, crosstalk between various hormone signaling pathways, defense response through sugar concentration, broad antimicrobial peptide production, oxidative burst

and chemical stress to detoxify ROS and/or PCD (Wiesner-Hanks & Nelson, 2016). In this research, we focused on MDR related to PCD.

2.4.3. Programmed cell death for multiple disease resistance

PCD in plants is a regulated and controlled cell suicide process which occurs during normal growth (xylem formation, seed maturation), development (plant reproductive process) and biotic (plant-pathogen interactions) and abiotic stresses (drought, cold, hypersalinity, heat) (Dickman et al., 2017). PCD involves a complex network of molecular signaling pathways such as calcium signaling, ROS and reactive nitrogen species (RNS) (Locato & Gara, 2018). During plant pathogen response, it is initially initiated through the hypersensitive response. HR is a genetically controlled organized cell suicide activated at the initial infection site to restrict the growth of biotrophic pathogens (Heath, 2000). Another form of PCD, apoptosis-like PCD (AL-PCD) is present which is morphologically and biochemically similar to apoptosis in animals. AL-PCD occurs at high Ca^{2+} concentration in response to pathogen attack. It is characterized by distinct morphological markers such as cell shrinkage, mitochondrial membrane rupture, chromatin condensation, plasma membrane blebbing, and a characteristic DNA fragmentation (Locato & Gara, 2018). Though HR-PCD also involves cytoplasmic shrinkage, chromatin condensation, mitochondrial swelling and rupture and DNA fragmentation, the plasma membrane blebbing and a characteristic DNA fragmentation is unique to AL-PCD (Dickman et al., 2017).

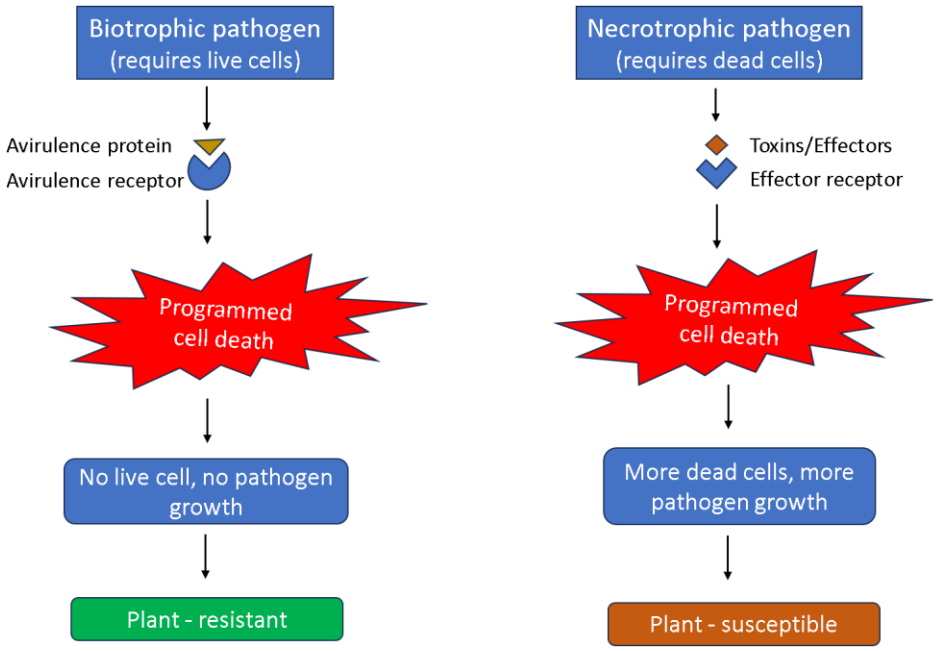


Figure 2.2. Schematic representation of programmed cell death as unfavorable cell mechanism for biotrophic pathogens and favorable mechanism for necrotrophic pathogens.

Upon recognition of pathogen effectors or avirulence proteins from biotrophs by plant immune receptors, the plant triggers HR-PCD signaling cascade, restricting the pathogen spread (Salguero-Linares & Coll, 2019). However, PCD acts as a pathway to infection for hemibiotrophic and necrotrophic pathogens since they feed on dead cells for their growth. Hemibiotrophs such as *F. graminearum* and *P. infestans* undergo transition from biotrophy to necrotrophy after defense mechanism such as HR are activated, which further increases the pathogen infection (Figure 2.2). Necrotrophic pathogens produce necrosis-inducing enzymes and effectors to induce PCD and feed on the dead cells (Malvestiti et al., 2022). Reducing PCD could confer MDR through suppressing the availability of dead and dying cells to these pathogens and lead to enhanced resistance.

2.5. Histidine-rich calcium-binding protein (HRC) gene

Upon pathogen infection, there is an influx of Ca^{2+} into cytosol. Ca^{2+} signals as a stress response are perceived by calcium sensors such as calmodulin (CaM), CaM-like proteins, Ca^{2+} - dependent protein kinases (CPRKs) and Ca^{2+}- and Ca^{2+}/CaM-independent protein kinase (CCaMK) and calcineurin B-like proteins (CBLs) (Galon et al., 2010). The Ca^{2+} - CaM complex binds to the NSCaTE motif of HRC proteins. In wheat, HRC regulates Ca^{2+} uptake and release to maintain Ca^{2+} homeostasis (Chen et al., 2022). HRC moves to the nucleus and releasing Ca^{2+} , increasing the nuclear concentration, which triggers endonucleases such as StCaN2 of Clade-I to induce AL-PCD (Kushalappa et al., 2022). In plants, HRC was first reported in wheat as a part of the *Fhb1* QTL and caused PCD in susceptible near isogenic lines (S-NIL) but was absent in resistant near isogenic line (R-NIL) (Gunnaiah, 2013). *TaHRC* was mutated in FHB-resistant wheat cultivars and functional in FHB-susceptible wheat cultivars (Su et al., 2019). HRC induced AL-PCD in wheat and potato, inducing DNA fragmentation (Kushalappa et al., 2022). Thus, this presents TaHRC as an excellent target for gene knockout by CRISPR/Cas9 to generate FHB resistant wheat lines.

2.6. Metacaspase

Apoptosis in animals is executed by caspases, cysteine-containing, aspartate-specific protease. Plants lack caspases but contain caspase-like proteases, known as metacaspases, to execute AL-PCD. Metacaspases are found in eukaryotes lacking true caspases, such as fungi and plants (Carmona-Gutierrez et al., 2010). The rise in cytosolic Ca^{2+} in response to pathogen recognition opens the mitochondrial permeability transition pore and release cytochrome c, which induces transcription of PCD genes, including metacaspases, during the progression of

HR. (Rantong & Gunawardena, 2015). Structurally, metacaspases are similar to animal caspases and consists of two domains, a catalytic p20 domain and a regulatory p10 domains. Based on the architecture of the two domains, metacaspases in plants can be divided into type I and type II metacaspases. Type I metacaspases consists of an N-terminal extension and a zinc-finger motif in a p20-p10 arrangement. Type II metacaspases contain a linker domain, separating the p10 and p20 domains in p20-linker-p10 arrangement (Minina et al., 2020). Metacaspases are involved during plant-pathogen interaction regulating cell death in the activation of HR. *AtMC1* knockout mutants suppress bacterial and oomycete-triggered cell death through HR. *AtMC2* acts as a negative regulator of HR and cell death (Coll et al., 2011). Knockout mutants of another type II metacaspases, *AtMC2d,* exhibit reduced sensitivity to PCD-inducing mycotoxin fumonisin and oxidative stress inducers (Watanabe & Lam, 2011). *NbMC1* was overexpressed following infection with necrotrophic fungus *Colletotrichum destructivum* (Hao et al., 2007). In potato, CRISPR-mediated silencing of *StHRC* led to highly reduced expression of *StMC7* upon infection with *P. infestans* and *A. solani*, suggesting its involvement in the PCD. Silencing *StMC7* through gene editing could further enhance multiple disease resistance in potato (Kushalappa et al., 2022).

2.7 Integrated pathogen management strategies

Pathogen management approaches against fungal and oomycete pathogens, such as cultural practices, water management, chemical control and genetic resistance approaches have been adopted to minimize crop loss. Cultural practices, such as clean cultivation, crop rotation, weed removal and proper water management, can help in reducing the amount of initial inoculation for infection and dispersal. Deep ploughing can also reduce the primary inoculum by removing the deeply lodged crop residues. Crop rotation by avoiding alternate hosts such as

maize, oat or barley for FHB, or tomato for blights of potato can decrease the incidence of fungal pathogens. Hilling of the soil can avoid excessive moisture, which facilitates the incidence of blights in potatoes. Early sowing or growing the short duration cultivars can also help escape pathogens (De et al., 2021).

Chemical management is the most popular management strategy for fungal and oomycete pathogens. The effectiveness of fungicide varies depending on several factors such as time of application, the proper combination of fungicides with different modes of action. The most commonly used fungicides to control FHB are prothioconazole, tebuconazole and their combination. However, the fungicide efficiency under field conditions can be variable and mostly insufficient for controlling FHB in infected fields (Mesterházy et al., 2011). The most used fungicides against late blight of potato are chlorothalonil, dithiocarbamates, triphenyl tin hydroxide, metalaxyl/mefenoxam, aliphatic nitrogen fungicides such as cymoxanil and morpholines such as dimethomorph (Nowicki et al., 2012). Several fungicides such as mancozeb, chlorothalonil, difenoconazole and azoxystrobin are used against early blight of potato. Fungicide application effectiveness increases when complemented with the weather conditions (wet conditions, cloudy) and can reduce the number of fungicidal treatments needed by 50-60% (Hijmans et al., 2000). Though chemical control is currently the most used strategy, it is not safe for the environment and human health as pesticides. In addition, several fungal strains have evolved resistance against specific fungicides (Rosenzweig et al., 2008). This compels farmers to apply more than the recommended concentration more frequently, which increases the input cost for farmers and further harms human health and environment.

Biological management of hemibiotrophic and necrotrophic pathogens has been suggested as a safe alternative to the chemical control method. Biological control agents (BCAs)

are natural antagonists of the pathogen and can parasitize or outcompete the pathogen through antibiosis or nutrient competition (Gao et al., 2017). Various BCAs, including bacteria such *Bacillus* spp., *Pseudomonas fluorescens*, *Paenibacillus fluorescens*; yeasts such as *Sporobolomyces roseus* and *Cryptococcus*; and filamentous fungi such as *Trichoderma harzianum* and *T. viren*, have been found effective in controlling FHB (Xu & Nicholson, 2009). Bacterial (*Bacillus* spp., *Pseudomonas* spp, *Streptomyces*, *Acinetobacter*), fungal (*Chaetomium spp., Rhizophagus irregularis*) and yeasts (*Aureobasidium*, *Metschnikowia*, *Curvibasidium*) have been reported as successful BCAs of *P. infestans* (Volynchikova & Kim, 2022). BCAs such as *Pythium oligandrum*, *B. subtilis*, *T. harzianum* were found to be effective against *A. solani* in small-scale greenhouse trial. However, they were not effective in full-scale field trials (Stridh et al., 2022).

Integrated disease management approaches combining resistant cultivars with crop rotation, soil tillage, biological control and timely application of fungicides is the best strategy to manage fungal pathogens. The integrated approach beginning with the use of resistant crop cultivars and their clean or treated seeds is important for managing pathogen attack. Development and use of resistant varieties is the most sustainable, economical, and effective approach to combat necrotrophic pathogens. Improvement in host plant resistance through plant breeding and gene editing can minimize disease incidence and severity in a cost-effective and environmentally friendly way.

2.8. Plant breeding and gene editing for host plant resistance

Plant breeding has contributed to the development of high disease resistant cultivars since the Green Revolution in the 1960s (Saville et al., 2011). Breeders and geneticists have

successfully identified sources of disease resistance from the wild and relative species and incorporated them to produce hybrids with desirable traits (Hasan et al., 2021). Standard breeding strategies for progeny selection involve identifying and developing DNA markers linked to the desirable trait, selection of quantitative trait loci (QTLs) or specific genes-of-interest related to the trait, genotyping progenies using molecular markers and selection of individuals with desired traits based on marker information (Collard & Mackill, 2008). However, marker-assisted breeding success depends on several factors such as marker quality, genetic basis of traits, linkage-related genes and their effects (Guo-Liang, 2013). On average, it takes one to two decades to develop a new commercial cultivar through conventional breeding depending on the crop and trait genetics (Voss-Fels et al., 2019).

In comparison, genome editing techniques, such as TALENs, ZFNs and CRISPR, can generate new cultivars with improved disease resistance at low cost and effort in a short time through precise editing of target regions (Wolter et al., 2019). Trait-responsive genes can be directly edited in commercial cultivars or elite breeding lines, eliminating linkage drag and the time-consuming, effort-intensive backcrossing (Pixley et al., 2022).

2.9. Genome editing

Genome editing is a revolutionary technology based on site-directed nucleases including mega nucleases, ZFNs, TALENs and CRISPR/Cas (Gaj et al., 2013). Among them, CRISPR is the most popular due to its simplicity, precision, easy manipulation and cost-effectiveness. CRISPR/Cas is made up of two components, a guide RNA (gRNA) which can be programmed to bind to the target DNA and a Cas nuclease, such as Cas9, Cas12a, Cas3, to perform the double stranded break (DSB). The Cas9:gRNA complex searches for the appropriate protospacer

adjacent motif (PAM) located adjacent to the target site. Upon PAM site recognition, the Cas9 protein unwinds the DNA for sgRNA to hybridize with the DNA strand. If the gRNA sequence matches with the exposed DNA, catalytic domains of Cas9 initiate double-strand break. This break is repaired through non-homologous end joining (NHEJ) pathway or homologous directed repair (HDR). Though the process is simple, various factors such as genome size, composition can influence the success in generating CRISPR edited plants. A major bottleneck for gene editing is the plant species ability to generate callus culture, ease of transformation and regeneration ability.

2.10. Optimizing wheat for CRISPR/Cas9

Wheat is considered highly recalcitrant in tissue culture compared to other cereal crops due to low regeneration efficiency and genotype dependency (Li et al., 2012). Different wheat genotypes show varying response to tissue culture and transformation protocol because of genotype-specific endogenous level of growth regulators, soluble sugar and phenol content (Alikina et al., 2016). Though several protocols have been developed demonstrating successful transformation and regeneration, they remain ineffective for numerous commercial wheat cultivars (Yu et al., 2023). Two major strategies have been implemented to overcome wheat recalcitrance: application of regulatory genes and transcription factors in vector system and optimization of tissue culture steps.

2.10.1. Regulatory genes and transcription factors to enhance plant regeneration.

Various developmental regulatory genes and transcription factors involved in regulating somatic embryogenesis and embryonic growth have been overexpressed to obtain successful regeneration in recalcitrant crops such as wheat. The expression of Baby Boom (BBM), Leafy

Cotyledon1 (LEC1), LEC2, Wuschel (WUS), Clavata3 (CLV3), Enhanced Shoot Regeneration (ESR) and Wound Induced Dedifferentiation (WIND1) have improved plant regeneration in wheat by promoting shoot regeneration (Bekalu et al., 2023). However, the continued overexpression of these regulatory genes causes severe growth abnormalities such as abnormal vegetative and reproductive organs and infertility (Kong et al., 2020). Recently, the expression of a chimera composed of Growth Regulating Factor 4 (GRF4) transcription factor and its Growth Initiation Co-Factor (GIF1) (GRF4-GIF1) technology increased the regeneration efficiency in monocotyledonous and dicotyledonous plant species without any reported defects or abnormalities by enhancing embryogenesis, and cell proliferation in the stem (Debernardi et al., 2020; Bull et al., 2023). GRF genes belong to a plant-specific transcription factor, forming a transcriptional complex with GIF and regulate plant growth by initiating primordial vegetative and reproductive organ cells (Kong et al., 2020).

2.10.2. Optimizing the tissue culture parameters

Several factors determine the success of wheat genotypes for tissue culture regeneration such as the starting material used, plant growth regulators, their concentration and the combination and parameters of the transformation method used (Miroshnichenko et al., 2017). Tissues harboring totipotent cells, such as calluses and somatic embryos, are used as starting material. In wheat, immature embryos are the most used source material, though mature embryos have also been successful for some cultivars. Growth regulators application, their timing and intensity of exposure should be determined for each species and cultivars (Altpeter et al., 2016). *In vitro* regeneration is mainly mediated by auxin to cytokinin ratio, with an optimal ratio inducing callus formation, a lower ratio promoting shoot regeneration while a high ratio inducing root formation (Fehér, 2015). However, the ratio differs among genotypes, requiring optimization

in every step for successful regeneration. As the wheat cultivar ‘Pasteur’ used in our study has a parentage of Cadenza X (Palermo x KS91WGC11), the protocols applied for Cadenza were used as a reference.

In contrast, potato can be readily transformed through *Agrobacterium*-mediated transformation using internodes and leaves as starting explant, making it easier for genetic modifications. Tissue culture parameters were followed for ‘Russet Burbank’ according to previous research conducted in the Kushalappa lab (Hegde et al., 2021).

CONNECTING STATEMENT FOR CHAPTER III

Chapter III is a manuscript titled "**CRISPR/Cas9 delivery and tissue culture optimization for successful regeneration in wheat cultivar 'Pasteur'**" authored by Bikram Poudel, Jacqueline Bede and Ajjamada C. Kushalappa. In this manuscript, I (BP) optimized tissue culture parameters, transformation protocol to support CRISPR construct delivery.

Pasteur is a high yielding special purpose wheat cultivated in the eastern Prairies. Fusarium head blight is one of the most destructive pathogens in wheat. Though many major quantitative trait loci (QTLs) are involved in imparting host plant resistance, incorporating all these QTLs in susceptible cultivars remains a challenge. QTL-*Fhb1*, located in chromosome 3B, is the most stable QTL responsible for FHB resistance. *TaHRC* was identified as a major gene which, when naturally mutated or upon silencing in susceptible cultivars, imparts FHB resistance. *TaHRC* seems to be involved in Ca^{2+} ion transport and causes apoptotic-like PCD, though the exact mechanism is unknown. Genome editing of *TaHRC* has been found to enhance FHB resistance in the model cultivar 'Bobwhite'. However, enhancing the resistance level of cultivated cultivars such as 'Pasteur' through genetic engineering is a challenge owing to the recalcitrant nature of this cultivar, making transformation and regeneration through tissue culture challenging. In this study, I optimized the specific conditions for transformation and tissue culture regeneration in the cultivated cultivar 'Pasteur' to conduct gene editing in plants. For genome editing, I delivered three types of vectors through both *Agrobacterium*-mediated and biolistic transformation using different explants such as mature embryos, immature embryos and embryos derived from both types of embryos.

CHAPTER III

CRISPR/Cas9 construct delivery and tissue culture optimization for successful transformation and regeneration of wheat cultivar 'Pasteur'

Bikram Poudel, Jacqueline C. Bede, Ajjamada C. Kushalappa

Plant Science Department. McGill University, Ste.-Anne-de-Bellevue, QC, Canada

3.1 Abstract

Fusarium head blight (FHB) is a major pathogen of wheat, one of the most important food crops in the world and can lead to significant yield quantity and quality loss. Enhancing host plant resistance using gene editing techniques such as CRISPR/Cas9 is an economic, effective and environmentally friendly approach to tackle pathogens, such as FHB. However, regeneration of cultivars through tissue culture is a huge bottleneck due to the recalcitrant nature of wheat. Optimizing wheat tissue culture is essential for generating CRISPR-edited wheat seedlings. This study aimed to optimize tissue culture in wheat cultivar 'Pasteur' for CRISPR-mediated gene editing. The *TaHRC* gene, which provides FHB resistance when non-functional, was targeted. A gRNA was designed and inserted into two vectors, the modular vector and the GRF-GIF vector, to silence the functional exon of *TaHRC*. Both *Agrobacterium* and biolistic-mediated transformation to deliver the CRISPR construct to immature or mature embryos or callus derived from both embryos were compared. Wheat seedlings with successful transformation with the CRISPR construct were regenerated with transformation efficiency of 0.8% through the biolistic-mediated transformation using the GRF-GIF vector on immature embryo-derived-calli after optimizing several steps of tissue culture protocol. Other wheat cultivars may also be regenerated upon editing with CRISPR/Cas9 technology using the GRF-GIF chimera vector inspired by the methods described in this study.

3.2 Introduction

Wheat (*Triticum aestivum* L.) is one of the main grain crops grown worldwide, contributing 18% of total calories and 19% of dietary proteins (Erenstein et al., 2022). It is cultivated in nearly all agroecological regions from 67^{o}N in Scandinavia to 45^{o}S in Argentina and up to an altitude of 3,000 m above sea level (Shewry, 2009). The global production of wheat is 770.8 million tonnes from an area of 220.7 million ha (FAOSTAT, 2023). However, biotic and abiotic stresses reduce yield quality and quantity in wheat. Among them, Fusarium head blight (FHB), caused by the hemibiotrophic fungal pathogen *Fusarium graminearum* (Teleomorph: *Gibberellae zeae*), is one of the most devastating fungal wheat diseases. Genetic engineering with gene editing technology, such as CRISPR/Cas9, can improve host resistance to FHB.

CRISPR/Cas9 allows efficient, precise targeted gene modification, improving the speed, accuracy and reducing cost and effort for producing transgene-free, genome-edited plants (Zhou et al., 2023). In agriculture, CRISPR has been used to increase yield, improve the quality of produce, enhance resistance to diseases and confer herbicide resistance (Zhu et al., 2020). CRISPR/Cas9 has been successfully used in wheat cultivars such as Bobwhite, which is recognized for their high transformability (Pellegrineschi et al., 2002; Su et al., 2019). However, wheat is one of the recalcitrant crop species. Commercially cultivated wheat cultivars such as ‘Pasteur’ are more recalcitrant for transformation and regeneration and thus, requires optimization. ‘Pasteur’ is a general purpose, high yielding, spring wheat adapted to the Prairies region and eastern Canada. It was selected based on its widespread adaptation to different environments in Canada and its susceptibility to FHB. Major strategies to overcome wheat recalcitrancy in cultivated cultivars are using genes encoding regulatory genes and transcription

factors that promote somatic embryogenesis or developmental processes such as shoot regeneration as well as optimization of tissue culture procedures.

Incorporation of GRF-GIF1 complex encoding wheat growth regulating factor (GRF4) and its GRF-interacting factor (GIF1) into CRISPR vector have improved transformation efficiency in triticale, wheat, rice, citrus and watermelon (Debernardi et al., 2020; Feng et al., 2021). GRF transcription factors are associated with prolific cell division and regulate multiple plant organ development while GIFs enhance the function of GRFs (Bull et al., 2023). Other strategies to optimize wheat transformation involve increasing T-DNA availability by using geminiviral replicons or a transgene killer CRISPR (TKC) vector (Čermák et al., 2017; Yubing et al., 2019). The TKC vector consists of two cassettes, one to induce pollen sterility through the expression of male sterility gene, and another cassette with bacterial BARNASE gene guided by a strong embryonic tissue promoter to kill the transgenic embryonic tissues so that only transgene-free embryos survive. Successful application of TKC vector could produce transgenic plants in the T1 generation, saving time and effort in screening wheat seedlings. These vectors contribute to transformation and tissue culture for regeneration of CRISPR-edited plants.

Transformation and tissue culture of different cultivars in wheat is a challenge to genome editing in wheat. Optimization of steps such as selection of the target tissue for transformation, conditions for callus production, transformation, root and shoot regeneration vary with genotypes. Immature embryos (IEs) are the most preferred target tissue for transformation in wheat (Harwood, 2011). However, several studies have demonstrated successful transformation with mature embryos (MEs) (Chauhan & Khurana, 2017). Most widely used methods of wheat transformation are biolistic delivery and *Agrobacterium tumefaciens*-mediated transformation. Though *Agrobacterium*-mediated transformation produces high quality transformation events, its

application is limited to specific wheat genotypes unlike the biolistic method and requires more optimization (Jones & Shewry, 2009). Several factors limit the application of *Agrobacterium*-mediated transformation such as the absence of virulence genes in plasmids, *Agrobacterium* strains, suspension density, transformation parameters such as temperature, time, selection marker agents used (Hayta et al., 2019).

The parameters and media compositions are another important part of tissue culture optimization. Harvesting immature embryos at the right time (11-14 days post anthesis) is an important factor in the success of tissue culture and regeneration (Karthikeyan et al., 2023). Callus growth depends on the concentration of synthetic auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D), picloram or dicamba (Kumar et al., 2017). Different cultivars require different concentrations ranging from 0.5 – 10 mg/L for immature embryos (Perez-Pineiro et al., 2011). Several studies have reported increased transformation efficiency when IEs were exposed to high concentration of sucrose prior to bombardment (Javier et al., 2011; Kim et al., 2021). Phenolic inducers such as acetosyringone (100 µM concentration) with surfactants such as silwet (0.01%) improves transformation efficiency when used during *Agrobacterium*-mediated transformation (He et al., 2010). Integration of stress inducers such as $CuSO_4$ and $AgNO_3$ in shoot regeneration enhance regeneration rate (Yu et al., 2008). These parameters improve tissue culture, transformation and regeneration for applying CRISPR for enhancing wheat resistance to FHB and other pathogens.

FHB causes yield quantity loss through damaged kernels and low test weight of harvested grains and yield quality loss through contamination through mycotoxin such as deoxynivalenol (DON) and zearalenone, that are a serious risk to human and animal health (Hay et al., 2022). Management strategies such as cultural practices (crop rotation, clean cultivation, chemical

control through pesticides, such as prothioconazole, tebuconazole and their combination, biological control agents, such as *Pseudomonas fluorescens, Paenibacillus fluorescens, Trichoderma harzianum, T. viren* have been implemented to control and restrict FHB. However, genetic resistance through resistant varieties acts as a basis for future management practices.

Six types of resistance contribute to wheat resistance against FHB, Type I (resistance to primary infection), type II (resistance to disease spread through the spike), type III (resistance against kernel infection), type IV (resistance to yield loss), type V (resistance to trichothecene mycotoxin accumulation in the grain) and type VI (resistance to alteration of grain composition) (Mesterházy, 1997). Type II is the most extensively studied type of resistance because it appears to be more stable and less affected by nongenetic factors (Bai & Shaner, 2004). Quantitative trait loci (QTLs) associated with these resistance types are distributed throughout the wheat genome and confer resistance to FHB. Among them, *Fhb1*, present on chromosome 3B and derived from Chinese landrace Sumai3, is the most consistent QTL with the largest and most stable effect on type II resistance in different wheat genetic background against *Fusarium* spp. (Buerstmayr et al., 2009). This QTL can reduce initial infection, disease spread, kernel infection and DON accumulation (Suzuki et al., 2012). Among the genes within the *Fhb1* region, *TaHRC*, encoding a histidine-rich calcium-binding protein, is highly expressed in susceptible near isogenic lines (S-NIL) as compared to resistant NILs (R-NILs) (Gunnaiah, 2013). Further studies found the gene is functional in all FHB-susceptible cultivars and non-functional in resistant cultivars (Su et al., 2018). HRC regulates Ca^{2+} uptake and release and induces apoptotic-like programmed cell death (AL-PCD) in wheat, conferring susceptibility to hemibiotrophic and necrotrophic pathogens (Chen et al., 2022; Kushalappa et al., 2022). Knockout mutants of *TaHRC* in susceptible wheat cultivars have increased FHB resistance (Su et al., 2019).

This study focuses on optimizing tissue culture, transformation and regeneration of cultivated cultivar ‘Pasteur’. Three vector systems, modular based vector, GRF-GIF-based vector and the TKC system were designed. All of them used the same gRNA for targeting exon of *TaHRC*, a susceptibility gene for FHB resistance. prepared and one additional vector system (TKC system) designed after selecting one gRNA. The TKC vector was not continued after repeated attempts to produce final transformation vector failed. The modular-based and GRF-GIF-based vectors were introduced into ‘Pasteur’ wheat using *Agrobacterium* and biolistic-mediated transformation to identify the best method for transformation. Tissue culture regeneration was optimized prioritizing published protocols for ‘Cadenza’, the parental line for ‘Pasteur’.

3.3 Materials and Methods

3.3.1 Plant growth and DNA extraction

The cultivated wheat cultivar ‘Pasteur’ was obtained from SeCan. Seeds were planted in a 6-inch pot filled with Fafard Agro Mix G6. Pots were maintained under greenhouse condition at 23 ± 3 ℃ and a 16 hr photoperiod (intensity of ~1,500 μmol/m^2/s) with (70 ± 10%) relative humidity. DNA was extracted from leaves using the modified cetyl trimethyl ammonium bromide (CTAB) method as described by Dreisigacker et al. (2016). Briefly, 100 mg of young wheat leaves were crushed using a mortar and pestle in liquid nitrogen. CTAB extraction buffer (500 µL) was added and incubated for 20-30 min at 65ºC for cell lysis and separation of DNA from other cellular components (Carey et al., 2023). Chloroform-isoamyl alcohol (24:1) (500 µL) was added and centrifuged for 15 min at 12,000 rpm to separate the aqueous and chloroform phases. The top phase was transferred into a fresh 1.5 ml test tube and DNA was precipitated using ice-cold isopropanol (700 µL) followed by centrifuging for 5 min at 12,000 rpm. After

removal of solution, the pellet was rinsed with ice-cold 70% ethanol (500 µL), dried in room temperature, and resuspended in sterile ddH_2O (50 µL).

3.3.2 Sequencing of *TaHRC* gene and gRNA selection

The *TaHRC* gene sequence was retrieved as TraesCS3B03G0045000 from the reference genome sequence of Chinese Spring IWGSC 2.1. The partial gene sequence of *TaHRC* in the B genome covering the third exon was amplified with specific primers (Table 3.1) using a high fidelity Q5 Polymerase (New England Biolabs, Ipswich, MA, USA). The amplified PCR product was purified using Biobasic PCR Clean Up (Bio Basic INC., Markham, ON, CA) and cloned into the pGEMT-Easy plasmid (Promega Corp., Madison, WI, USA) using T4 DNA Ligase (NEB, MA, USA). The final plasmid was transformed into chemically competent *E. coli* DH5α cells (New England Biolabs, Ipswich, MA, USA) and sequenced using M13-forward and reverse primers by Sanger Sequencing (Génome Québec Innovation Centre). As *TaHRC* in the B genome was found to have a more critical role for FHB susceptibility (Su et al,. 2019), one gRNA with the highest overall score targeting the cds region, was selected using the WheatCRISPR website https://crispr.bioinfo.nrc.ca/WheatCrispr/ (Cram et al., 2019).

3.3.3 Vector selection and construction

3.3.3.1 Modular vector

Modular vectors were selected for insertion of the gRNA. Further gRNA-based primers were designed using webtool http://crispr-multiplex.cbs.umn.edu/ . The sgRNA along with its spacer was cloned into the SapI site replacing the ccDB gene of the vector pMOD_B2103 and sequenced with Sanger sequencing to confirm the integration of gRNA. The intermediate module plasmids pMOD_A1510 consisting of *TaCas9*, pMOD_B2103 containing the gRNA and

pMOD_C000 as placeholder were assembled into the AarI site of transformation backbone vector pTRANS_200 through Golden Gate assembly (Cermak et al., 2017). The final construct was transformed into chemically competent *E. coli* DH5α cells (NEB, USA) and sequenced using TC430 and TC211F as forward and reverse primers, respectively, to confirm the vector by Sanger Sequencing (Génome Québec Innovation Centre).

3.3.3.2 Transgene killer CRISPR (TKC) vector

The transgene killer CRISPR (TKC) vector with hygromycin as selection into the vector pUC57-Kan was inserted in place of the placeholder vector pMOD_C000. These components, if successfully integrated into the final vector and delivered to the plant tissue, were designed to self-eliminate the CRISPR construct and produce transgene free plants within a single generation (Yubing et al., 2019). Two cassettes were designed separately, one to induce male sterility in pollens and another to induce transgenic embryo death. The male sterility cassette was controlled by the Cauliflower Mosaic Virus (CaMV) 35S promoter and consisted of male sterility gene (*TaMs2)* (NCBI GenBank Accession Number KX533929.1), conferring genetically dominant 100% male sterility in hexaploid and tetraploid wheat, (Ni et al., 2017). As a second cassette, bacterial BARNASE encoding a toxic protein with nuclease activity which kills plant cells, was expressed under an early-maturing wheat (*TaEm*) promoter, a strong promoter for embryonic tissues in wheat and barley so that the BARNASE gene expressed only in the embryonic tissues (Furtado et al., 2009). A single gene fragment combining these two cassettes was synthesized by Bio Basic Gene Services (Bio Basic, Markham, ON, CA) and cloned into the pUC57 plasmid. The final pUC57 plasmid was used as intermediate module plasmid replacing pMOD_C000 in the transformation backbone vector pTRANS_200 in Golden Gate reaction. It failed to integrate into the transformation vector upon repeated attempts and was abandoned.

3.3.3.3 GRF-GIF based vector

The GRF-GIF based final vector was constructed through introduction of gRNA into the plasmid pGTR using two primer sets (Table 3.1), L5AD-F and HRC-gRNA-B-R, HRC-gRNA-B-F and L3AD-R (Xie et al., 2015). In brief, the two PCR fragments from the two primer sets had 5' -end tails with BsaI cut site and FokI ligation site. After FokI ligation to combine the two fragments, another PCR was conducted to amplify the ligated fragments using primers S5AD-F and S3AD-R (Table 3.1). Primers L5AD-F and S5AD-F were modified from Wang et al. (2016) to suit the JD633 vector. The PCR product was purified, digested with BsaI and ligated into the AarI digested final transformation backbone plasmid JD633 using T4 DNA Ligase (NEB, Ipswich, MA, USA). The final construct was transformed into a chemically competent *E. coli* DH5α (ThermoFisher Scientific, Waltham, MA, USA) and validated the gRNA in the final construct by Sanger Sequencing using primers TC430 and M13-F (Table 3.1). The validated plasmid was transformed into *Agrobacterium* AGL1 strain competent cells for plant tissue culture.

3.3.4 *Agrobacterium*-mediated transformation using immature embryos

Immature Embryos (IEs) were harvested from greenhouse-grown 'Pasteur' wheat spikes, two weeks after anthesis. After surface sterilization in 70% ethanol for 30s, the solvent was removed and added 1.2% sodium hypochlorite solution, followed by shaking for 15 min. The bleach was removed and seeds were washed five times with sterilized water, the IEs were excised and incubated at 24^{o}C in modified wheat pre-cultivation media (WPCM) (Hensel et al., 2009). The WPCM media was composed of 4.3 g/L Murashige and Skook (MS) mineral salts with vitamins (PhytoTech Labs, USA), 0.5 ml/L $CuSO_4.5H_2O$ stock, 0.5 ml/L L-glutamine, 0.1 g/L casein hydrolysate, 1 mg/L 2,4-Dichlorophenoxyacetic acid (2,4-D) and 2.5g/L phytagel for

media solidification. After 5 days, the IEs were immersed in freshly prepared liquid wheat inoculation media (WIM) (4.3 g/L MS media with vitamins, 0.5 ml/l $CuSO_4 \cdot 5H_2O$ stock, 0.5 ml/l L-glutamine, 0.1 g/L casein hydrolysate, 10 mg/L 2,4-D, 2g/L morpholinoethanesulfonic acid (MES), 60 g/L maltose and filter-sterilized 0.5 ml/L acetosyringone (AS), pH (5.8). After incubating for 5 minutes at 42^{o}C, the WIM was removed and replaced by *Agrobacterium* culture (600 µl) - (OD_{600}=0.5 - 3) along with freshly prepared AS (100 µM). The culture was incubated for different time periods (10 min - 3 hrs) in the dark and then washed with liquid WIM (1.5 ml). The IEs were placed on top of sterile filter paper disk into which WIM (300 µl) was added and placed on top of WIM solid media (2.5 g/L phytagel added to WIM liquid media). The plate was incubated in dark at 23^{o}C. After 3 days, the IEs were transferred to solid wheat callus induction media (WCIM). WCIM media was composed of 4.3 g/L MS with vitamins, 0.5 mg/L $CuSO_4 \cdot 5H_2O$ stock, 0.5 g/L L-glutamine, 0.1 g/L casein hydrolysate, 30 g/L maltose, 10 mg/L 2,4-D, 0.5 g/L MES, and 2.5 g/L phytagel. 50 mg/L hygromycin and 200 mg/L timentin were added after autoclaving was completed. The plates were sealed and incubated at 24^{o}C in the dark for 10-14 days..

3.3.5 *Agrobacterium* transformation using mature embryos

Mature embryos (MEs) were also used to generate callus as per Medvecká and Harwood (2015) and Chauhan and Khurana (2017) with slight modifications. The major modification was the addition of 10 mg/L 2,4-D in the wheat callus regeneration media (WCIM). MEs were dissected from wheat seeds germinated overnight by cutting the tip of the embryos with scalpel and placing them in WCIM to induce callus and prevent germination. After 2 weeks, callus from the MEs were subjected to *Agrobacterium* transformation, kept in co-cultivation for 3 days in 23^{o}C, and kept on WCIM with timentin (160 mg/L).

3.3.6 Biolistic transformation

Transformation of 'Pasteur' was conducted using particle bombardment following established protocols (Sparks & Jones, 2014; Vasil & Vasil, 2006). Briefly, 40 mg of 0.6 µm gold particles were coated with final construct plasmid of concentration 1000 µg and bombarded into IEs or MEs as well as ME and IE-derived calli using PDS-1000 Helium particle gun (Bio-Rad, Hercules, USA). The embryos were incubated in osmotic media (4.3 mg/L MS, 36.44 g of sorbitol, 36.44 g/L mannitol, solidified with 3 g/L phytagel) for osmotic treatment 4 h before and 16 h after bombardment. The bombarded embryos were incubated on callus induction media (CIM) (4.3 g/L MS, 100 mg/L *myo*-inositol, 100 mg/L D-glutamine, 500 mg/L casein hydrolysate, 10 mg/L $AgNO_3$, 3 mg/L 2,4-D, 3 g/L phytagel) at 23^{o}C for 14 days in dark and then on CIM media containing hygromycin (20 mg/L) as selective antibiotic for another 14 days in light. Responsive calli were incubated at 23^{o}C in regeneration media (RM) (4.3 g/L MS media with vitamins, 0.5 mg/L 2,4-D, 100 mg/L *myo*-inositol, 25 mg/L $CuSO_4.5H_2O$, 5 mg/L zeatin, 3 g/L phytagel) with hygromycin (30 mg/L) for shoot regeneration. Regenerated seedlings were sterilely transferred to a magenta box with half-MS agar and hygromycin (50 mg/L) for root generation. After root growth was observed, the transgenic seedlings were transferred to soil in greenhouse. Transformation efficiency was calculated by dividing the total number of regenerated seedlings by the number of callus subjected to transformation.

3.3.7 Identification of transgenic plants

The transgenic T_0 plants were screened for the presence of GRF-GIF construct by polymerase chain reaction (PCR) using GoTaq® Green Master Mix (Promega, Madison, WI, USA) and primers directed against Cas9 (Table 3.1). DNA was extracted from Cas9-positive

plants by CTAB method and used to amplify the *TaHRC* gene containing the target gRNA using HRC-B primers (Table 3.1). For PCR reaction, 10 µL reaction volume was used, which contained 5 µL of GoTaq® Green Master Mix, 3 µL sterile H_2O, 0.5 µL of each primer and 1 µL of genomic DNA. PCR reaction cycle was initial denaturation at 95^oC for 2 min, followed by 35 cycles at 95^oC for 30s, 56^oC (annealing temperature) for 30s, 72^oC (extension temperature) for 1 min followed by final extension at 72^oC for 5 min. The PCR product was purified and sent for Sanger sequencing (Genomic Analysis Platform, Laval University).

3.4 Results

3.4.1 *Agrobacterium*-mediated transformation is not suitable for 'Pasteur'.

Agrobacterium-mediated transformation of wheat cultivar 'Pasteur' was carried out using ME- and IE– derived calli for tissue culture but did not produce transgenic plants (Table 3.2). Regeneration of plants was not obtained even after exposing IE, ME and embryo-derived calli to various concentration of *Agrobacterium* suspension (OD_{600}: 0.5, 1, 1.5, 2, 2.5 3) for various exposure time (10 min, 30 min, 1 hr, 1.5 hr, 2 hr, 2,5 hr, 3 hours) and modifying media components before and after *Agrobacterium* exposure. For all explants, further callus growth was not observed after exposure to *Agrobacterium* culture at all OD_{600} values and exposure times. Reducing the concentration of timentin below 250 mg/L increased the *Agrobacterium* contamination, killing the infected callus. Delaying the exposure of *Agrobacterium* infected callus to hygromycin did not induce growth even after a month in CIM.

3.4.2 Optimization of particle bombardment parameters in immature embryos

This study optimized the particle bombardment parameters for a successful biolistic delivery in 'Pasteur'. Sterilized IEs were excised from the sterile seeds and maintained in callus

media for 5 days to induce callus growth followed by the introduction of genetic material by biolistic delivery. IEs were transferred to an osmoticum media with a high sucrose concentration (50 g/L sucrose) for 4 hr prior to bombardment. Both IEs and MEs showed no callus growth even after exposing them to osmotic treatment pre- and post-bombardment. For this reason, further transformation by particle bombardment was conducted on embryo-derived callus from both IEs and MEs. The bombarded calli were kept osmoticum media for another 24 hr for recovery and then transferred to CIM to stimulate further callus growth. This treatment is an important step as skipping this step resulted in no further callus growth after bombardment. A transformation efficiency of 0.8% was obtained upon delivering GRF-GIF vector through biolistic delivery (Table 3.2). Among the two constructs introduced, pTRANS200 and JD630 construct with GRF-GIF chimera, only JD630 construct successfully produced transgenic plants. Hence, transformation efficiency was calculated by dividing the number of regenerated plants by the number of callus transformed only using GRF-GIF based vector through biolistic delivery. Based on the success of plant regeneration in this study, biolistic delivery was found to be efficient for regeneration of transgenic events in wheat cultivar 'Pasteur'.

3.4.3 Confirmation of transgene in T_0 and T_1 plants

Ten T_0 plants were regenerated from particle bombardment-mediated DNA delivery of wheat cultivar 'Pasteur'. DNA was extracted from these plants and amplified by a genomic PCR using Cas9 primers to confirm the presence of the genome editing construct (Table 3.1). The insertion of the Cas9 gene in the genome indicated the success of transgene delivery (Figure 3.4). There was no mutation in the *TaHRC* gene at the B genome target site. All T_1 plants showed the presence of Cas9, validating stable transgene integration, thus demonstrating the stable inheritance of the CRISPR construct in wheat cultivar 'Pasteur'.

3.5 Discussion

Fusarium head blight is one of the most important wheat pathogen stresses causing yield loss and yield quality loss through mycotoxin contamination, making wheat grains unfit for human and animal consumption. Genetic engineering of wheat resistance genes such as *TaHRC* presents the possibility of enhancing plant resistance. However, wheat has lagged other major crops in terms of genetic engineering owing to its recalcitrant nature and genotype-dependent tissue culture requirement. Transformation and tissue culture regeneration have been optimized only for a few wheat cultivars. Thus, optimizing these factors as well as the delivery of CRISPR components is of upmost importance for genome editing to improve cultivated wheat cultivars to increase production and reduce losses due to biotic and abiotic stress.

We report the successful regeneration of transgenic plants from the commercially cultivated wheat variety 'Pasteur' through biolistic particle bombardment. We showed stable transgene integration into the wheat genome with a transformation efficiency of 0.8% with IE-derived calli. Upon application of both *Agrobacterium*- and biolistic mediated delivery on IE and ME embryos as the starting tissue, only IE-derived calli transformed with biolistic delivery was able to regenerate, producing wheat seedlings (Figure 3.3). Despite stable integration of gRNA and Cas9 in the wheat genome, a mutation in the target sequence *TaHRC* was not found. Some of the reasons behind the absence of this mutation may be incomplete or insufficient expression of Cas9 or gRNA scaffold, incomplete editing of homologs leading to masking of edited copies with unedited copies from other homologs or delayed Cas9 activity in the generations. Previous studies have reported increased mutation efficiency in the progenies, obtaining mutation at T_1, T_2 or T_3 generation (Zhang et al., 2019; Morineau et al., 2017). Thus, further optimizations are necessary to obtain successful CRISPR-mediated gene editing in wheat 'Pasteur'.

3.5.1 *Agrobacterium*-mediated transformation failed to regenerate ‘Pasteur’ seedlings.

Despite using a hypervirulent AGL1 strain (Lazo et al., 1991) for transformation, which has been successfully used in broad wheat genotype tissue culture studies, regeneration was not observed after *Agrobacterium*-mediated transformation (Hayta et al., 2021; Kim et al., 2021). Successful transformation of ‘Pasteur’ with *Agrobacterium* requires success in two critical elements, the tissue must be susceptible to *Agrobacterium* infection and the tissue’s capacity for callus induction and regeneration must be optimized (Wijerathna-Yapa et al., 2022). Both these elements must be optimized for different wheat cultivars. For example, *Agrobacterium*-mediated transformation and regeneration of ‘Fielder’ achieved a success rate of 77%, compared to only 4% for ‘Cadenza’, the parent lineage cultivar of ‘Pasteur’ (Hayta et al., 2021)

Agrobacterium infection affect plant processes, modifying the defense-related gene expression, affecting tissue susceptibility to external and internal stresses, producing ROS, inducing browning and cell death of target tissues (Khanna et al., 2007; Pitzschke, 2013). These are possible reasons behind the unsuccessful attempt to regenerate wheat ‘Pasteur’ after *Agrobacterium*-mediated transformation. Our results show that it is better to use particle bombardment when establishing protocols for the transformation of new wheat cultivar.

3.5.2 Genotype-specific parameters for tissue culture

Wheat cultivars pose challenges for genetic engineering due to inherent difficulties associated with transgene delivery and the recovery of transgenic wheat plants (Kim et al., 2021). Thus, those steps need to be optimized for genetic transformation. Narrow optimal windows involved in wheat transformation due to factors such as IE harvest period and its effect on embryo quality, higher recalcitrancy of ME, excising embryo axes after co-cultivation, pre-

treatment requirement for some cultivars may explain low transformation frequency and low reproducibility of a protocol in different laboratories (Ishida et al., 2015). IEs, MEs and the calli generated from IEs and MEs were used as bombardment material. Most studies reported successful regeneration with the excised IEs as source material for biolistic bombardment (Kim et al., 2021). Most studies have reported successful transformation and regeneration with excised IEs as a source material. However, callus growth was not observed in our study from the bombarded IEs and MEs from 'Pasteur'. Further experimentation with IEs is necessary to optimize this step. Osmotic treatment through the use of 0.4 M mannitol and 500 mM sucrose was applied through media 4h pre and 24h post bombardment to induce elevated osmotic pressure and reduce the damage to the target tissue during microparticle penetration. Osmotic treatment protects the cells from damage by reducing the extrusion of the protoplasm in the bombarded cells (Vain et al., 1993). Similar treatments improved regeneration in other wheat cultivars (Hashem et al., 2018). These optimizations in media and transformation may have contributed to successful regeneration of wheat 'Pasteur'.

Optimization at different stages of tissue culture, transformation and regeneration resulted in successful transgenic wheat 'Pasteur' plants with stable transgene inheritance in T_1 generation. (Table 3.3). Different parameters related to biolistic bombardment and regeneration process were manipulated for obtaining successful delivery in cultivar 'Pasteur' for this study (Table 3.3). Application of auxins such as 2,4-D was optimized at 3 mg/L for 'Pasteur' IEs and 10 mg/L for MEs to generate callus. Silver nitrate ($AgNO_3$) (10 mg/L) was added in the CIM as a stress-inducing agent to improve callus growth and frequency in wheat (Wu et al., 2006). Osmotic treatment, pre- and post- bombardment, increases transformation and regeneration frequency in our study (Jaclyn et al., 2022). In the RM, copper sulphate ($CuSO_4$) (25 mg/L) was

added as a stress-inducing agent to promote shoot and root regeneration (Malik et al., 2021; Purnhauser, 1991). Applying high concentration of antibiotics, such as hygromycin, had a negative effect on shoot regeneration. To overcome the negative effects of hygromycin, a gradual increase in hygromycin concentration was applied, initial callus induction after bombardment did not contain antibiotics. Hygromycin concentration was increased to 20 mg/L at the second CIM step, 30 mg/L at the RM step and 50 mg/L in half-MS media during initial plantlet development. However, such a gradual increase in antibiotic concentration also increased the regeneration of non-transgenic plants. Only 3 out of 10 regenerated plants were transgenic. Further studies for the improvement and optimization of steps is necessary for achieving better efficiency and establish effective protocols for other cultivated cultivars.

3.5.3 GRF-GIF vector contributes to regeneration.

Initial attempts to introduce the pTRANS200 vector in Pasteur were unsuccessful (Čermák et al., 2017). We were able to regenerate transformed wheat with the vector containing the GRF-GIF chimera (Debernardi et al., 2020). GRF transcription factors are strongly expressed in actively growing tissues, such as immature leaves, stem tips, flower buds, and play an important role in early plant growth and development (Huang et al., 2021). Chen et al. (2022) improved the regeneration of maize 7-fold upon the application of vector with the GRF-GIF chimera and BBM transcription factors (Chen et al., 2022). Vectors containing both GRF-GIFs and morphogenic regulators, such as BBM and WUS2 often result in higher transformation efficiencies in wheat cultivars. Such development regulators may enable the transformation of more wheat cultivars (Hayta et al., 2021). The combination of the GRF-GIF chimera with these developmental regulators should be used for improved wheat regeneration in the future.

3.6 Conclusion

Genetic engineering is important for improving wheat cultivars such as ‘Pasteur’ for better resistance to diseases such as FHB. Optimization of tissue culture, transformation and regeneration is essential for each cultivar. *Agrobacterium*-mediated transformation and particle bombardment remain the two most used methods of genetic transformation in wheat. Numerous studies have reported successful transformation through both methods. However, different wheat cultivars require optimization at all stages. Attempts to develop generalized protocols feasible for many wheat genotypes, consistent, successful wheat regeneration still remains elusive. Our protocol was optimized for wheat cultivar ‘Pasteur’ after following the combination and optimization of transformation and regeneration steps that have been successfully used in different studies (Hayta et al., 2021; Ishida et al., 2015). This study can serve as a template for developing transformation and regeneration protocols for other wheat cultivars.

Table 3.1 Primer sequences used in this study.

Primer name	**Primer sequence (5' – 3')**
TaHRC-F	ATTCCTACTAGCCGCCTGGT
TaHRC-R	ACTGGGGCAAGCAAACATTG
M13-F	CAGGAAACAGCTATGAC
M13-R	GCGCCTCCGTATTTGCTGACG
L5AD-F-mod	CGGGTCTCAGGCAGGATGGGCAGTCTGACTTACAAAGCACCAGTGG
L3AD-R	TAGGTCTCCAAACGGATGAGCGACAGCAAACAAAAAAAAAAAGCACCGACTCG
S5-AD-F-mod	CGGGTCTCAGGCAGGATGGGCAGTCTGACTT
S3-AD-R	TAGGTCTCCAAACGGATGAGCGACAGCAAAC
TC430-F	GTTGGATCTCTTCTGCAGCA
TC211-F	AACACATTGCGGACGTTTTT
Cas9-F	GGCAGGGATATGTACGTGGA
Cas9-R	CGCTCTTGAGCTTCTTGGAC
HRC-gRNA-F	TAGGTCTCGTCTGAGGACCGGGTTTTAGAGCTAGAA
HRC-gRNA-R	CGGGTCTCGCAGACGATGAATTGCACCAGCCGGG

Table 3.2 Transformation efficiency using various source material in cultivated wheat cultivar 'Pasteur.'

Source material	**Transformed source material with:**				**Successful regeneration**	**Transformation efficiency (%)**
	Agrobacterium		**Biolistic delivery**			
	Modular vector	**GRF-GIF vector**	**Modular vector**	**GRF-GIF vector**		
Mature embryos	146	112	238	207	-	-
Mature embryo-derived calli	220	146	766	216	-	-
Immature embryos	422	228	992	680	-	-
Immature embryo-derived calli	422	248	1152	1216	10*	0.8

(*) obtained from transformation of immature embryo-derived calli with GRF-GIF vector through biolistic delivery.

Table 3.3 Summary of optimization steps that contributed to successful tissue culture regeneration in 'Pasteur'. The treatment in bold represents the ideal treatment for that step.

Step optimized	**Stage of optimization**	**Reason**	**Treatments tested**	**Replicates**
2,4-D concentration in callus induction media (CIM)	Callus generation	Induces callus production	1 mg/L 2 mg/L **3 mg/L (For immature embryos)** 5 mg/L 7 mg/L **10 mg/L (Mature Embryos)**	50 immature (IEs) and mature embryos (MEs) for all concentrations
Osmotic treatment	Pre and post-bombardment	Reduce tissue damage during biolistic transformation	2 hr pre bombardment **4 hr pre bombardment** 6 hr pre bombardment 12 hr post bombardment **24 hr post bombardment** 48 hr post bombardment	30 IEs, MEs and callus derived from IEs and MEs for each treatment
Silver nitrate in callus induction media (CIM)	Callus proliferation after bombardment	Improve callus induction	5 mg/ L **10 mg/L** 15 mg/L	25 IEs, MEs and callus derived from IEs and MEs for each treatment
Copper sulphate in regeneration media (RM)	Shoot regeneration	Improve shoot generation	10 mg/L 20 mg/L **25 mg/L** 30 mg/L	20 calli per treatment

Figure 3.1 *TaHRC* gene sequence. **(a)** Alignment of *TaHRC* gene sequence between 'Sumai3', a resistant cultivar and 'Pasteur', a susceptible cultivar. **(b)** target site of gRNA for CRISPR in 'Pasteur'.

(a)

```
                1       10        20        30        40        50        60        70        80        90       100       110       120       130
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
HRC-Pasteur     ATGGATGCCAAGAAGTTCCTGCAGATGGTCGAGACAAAGAAGAAGAGACTCCTCGAGAAGAAGGAAGCCCCTCTGAAATGGCAGCAGAAACTGGAAGCAGCAATCAAGGCCACTGAAGAAAAGGAGAAGA
HRC-Sumai3                          TGGTCGAGACAAAGAAGAAGAGACTCCTCGAGAAGAAGGAAGCCCCTCTGAAATGGCAGCAGAAACTGGAAGGGGCAATTAAGGCCACTGAAGAAAAGGAGAAGA
Consensus       ......................TGGTCGAGACAAAGAAGAAGAGACTCCTCGAGAAGAAGGAAGCCCCTCTGAAATGGCAGCAGAAACTGGAAGcaGCAATcAAGGCCACTGAAGAAAAGGAGAAGA

                131     140       150       160       170       180       190       200       210       220       230       240       250       260
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
HRC-Pasteur     AGCTCAAGTCGAAAAAGCACAGGAGACGAAGCTATTCTTCCTCAGAATCTGACAGTGAATCCGAGAGCGACAGTGATCGGAAACACAGGAAGAGGAAGGACCGCAAAAGGCACAAAAGACATGGCCATTC
HRC-Sumai3      AGCTCAAGTCGAAAAAGCACAGGAGGCGAAGCTATTCTTCCTCAGAATCCGACAGTGAATCCGAGAGCGACAGTGATCGGAAACGCAGGAAGAGGAAGGACCGCAAAAGGCACAAAAAACATGGCCACTC
Consensus       AGCTCAAGTCGAAAAAGCACAGGAGaCGAAGCTATTCTTCCTCAGAATCcGACAGTGAATCCGAGAGCGACAGTGATCGGAAACaCAGGAAGAGGAAGGACCGCAAAAGGCACAAAAaACATGGCCAcTC

                261     270       280       290       300       310       320       330       340       350       360       370       380       390
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
HRC-Pasteur     TGACTCTGATGGTGCCAGGAGGCGCAAGCACAGGTCAAAGAGGAGGAGCTCGGGCTCTAGCGACGAGAGCGACAGTGATGAATATGATGGCGAATCTGAAGAAGAGCGCCGAAGGAAGAAGCACTCGCAC
HRC-Sumai3      TGACTCTGATGGTGCCAGGAGGCGCAAGCACAGGTCAAAGAGGAGGAGCTCGGACTCTAGCGATGAGAGCGACAGTGATGAATATGATAGCGAATCTGAAGAGGATCGCCGAAGGAAGAAGCACTCGCAC
Consensus       TGACTCTGATGGTGCCAGGAGGCGCAAGCACAGGTCAAAGAGGAGGAGCTCGGaCTCTAGCGAcGAGAGCGACAGTGATGAATATGATaGCGAATCTGAAGAaGAgCGCCGAAGGAAGAAGCACTCGCAC

                391     400       410       420       430       440       450       460       470       480       490       500       510       520
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
HRC-Pasteur     AGGAGGAAGCATCGCCGACACTCTTCAAGGTCAGAGTCTGACGCCTCAGATTACAGCAGCGATGATGATGAGCGAAGGTCAACCAGGAAGGACCATTCTAGGAGCCGGAGGCGTCGCCACCGGTCCTCAG
HRC-Sumai3      AGGAGGAAGCATCGCCGGCACTCTTCAAGGTCAGAGTCTGATGCTTCAGATTACAGCAGCGATGATGATGAGCGGAGATCAACCAGGAAGGACCACACTAGGAGCCGCAGGCGTCGCCACCGATCCTCAG
Consensus       AGGAGGAAGCATCGCCGaCACTCTTCAAGGTCAGAGTCTGAcGCcTCAGATTACAGCAGCGATGATGATGAGCGaAGaTCAACCAGGAAGGACCAcaCTAGGAGCCGcAGGCGTCGCCACCGaTCCTCAG

                521     530       540       550       560       570       580       590       600       610       620       630       640       650
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
HRC-Pasteur     ACGATGAATCTGAGGAGAAGATCAGGTCGAGGCATAGGAAGCGTCATCACAGATCAAGTGACGAGGACAAGCCGTCAGATTCTGACAACCATAAGCGCCACAGGAGCCGCTCTATGTCCTTGGATGATGG
HRC-Sumai3      ACGATGAATCTGAGGAGAAGATCAGGTTGAGGCATAGGAAGCGTCA---CAGATCAAGTGACGAGGACAAGCCGTCAGATTCTGACAACCATAAGCGTCACAGGAGCCGCTCTATGTCCTTGGATGACGG
Consensus       ACGATGAATCTGAGGAGAAGATCAGGTcGAGGCATAGGAAGCGTCA...CAGATCAAGTGACGAGGACAAGCCGTCAGATTCTGACAACCATAAGCGcCACAGGAGCCGCTCTATGTCCTTGGATGAcGG

                651     660       670       680       690       700       710       720       730       740       750       760       770       780
                |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
HRC-Pasteur     TGCTGCTGGTGAGCCAGACAAGATGAATGATGGCAAGGGGTCTCACAAAAGCCGGCACCACCGCCGCCACCACCATCACCATCATGATCATCGTGCGAACTCTGCTGAACCCAGAGACGGGAAGCAACTC
HRC-Sumai3      TGCTGCTGGCGAGCCAGACAAGATGAATGATGGCAAGGGGTCTCACAAAAGCCGGCACCACCGCCGCCACCACCATCACCATCATGATCATCGTGCGAACTCTGCTGAACCCAGTGACGGGAAGCAACTC
Consensus       TGCTGCTGGcGAGCCAGACAAGATGAATGATGGCAAGGGGTCTCACAAAAGCCGGCACCACCGCCGCCACCACCATCACCATCATGATCATCGTGCGAACTCTGCTGAACCCAGaGACGGGAAGCAACTC

                781786
                |----|
HRC-Pasteur     GTGTAA
HRC-Sumai3      GTGTAA
Consensus       GTGTAA
```

(b)

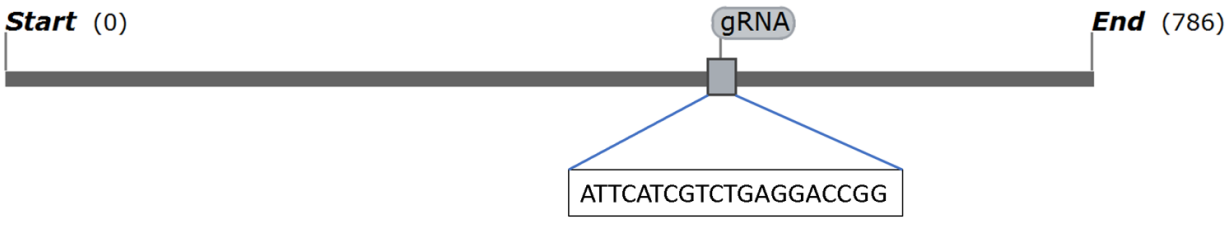
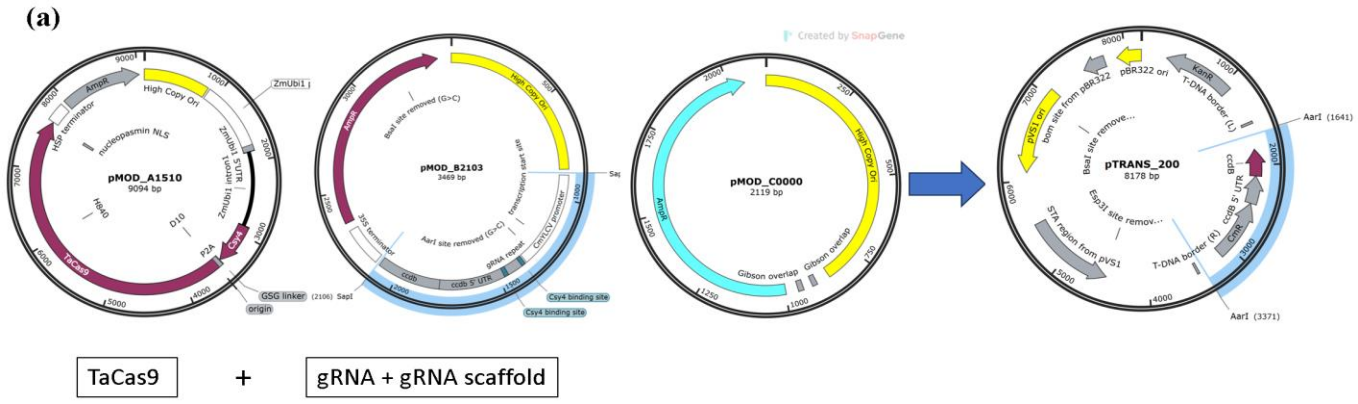


Figure 3.2: CRISPR-Cas9 vector map of vectors designed and used for transformation. **(a)** modular vector with A module for TaCas9 in pMOD_A1510, B module for gRNA insertion through SapI, replacing the highlighted ccdB region in pMOD_B2103, empty C module in pMOD_C0000 combined into the final transformation vector through Golden Gate reaction using AarI restriction enzyme, replacing the highlighted ccdB region in pTRANS_200. **(b)** Transgene killer CRISPR (TKC) vector designed to replace empty C module vector and insert into final transformation vector (not used for transformation purpose). **(c)** Growth regulating factor (GRF) and its GRF initiating co-factor (GIF) based final transformation vector (Debernardi et al., 2020).



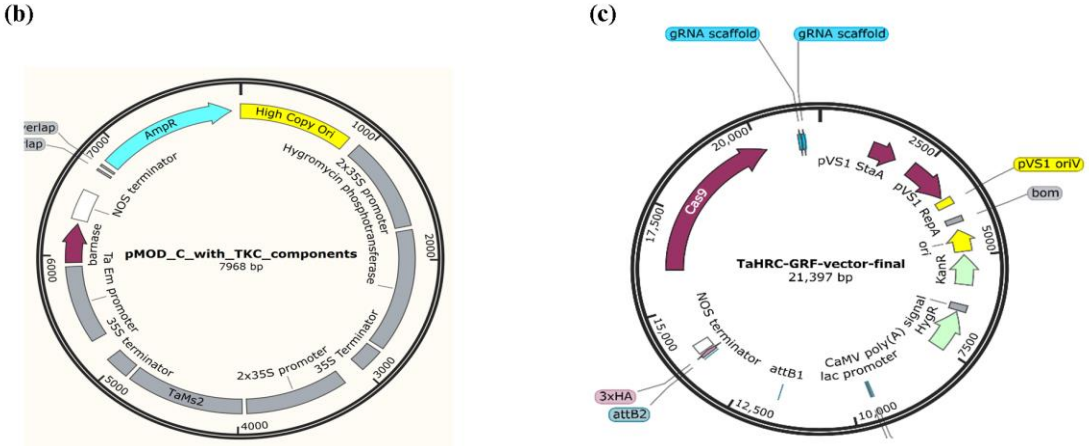


Figure 3.3: Pasteur wheat regeneration from immature embryo-derived calli. **(a)** Calli produced from immature embryos. **(b)** Regenerating calli after bombardment. **(c)** Emerging plantlets from calli shown using black arrows. **(d)** Regenerated seedling

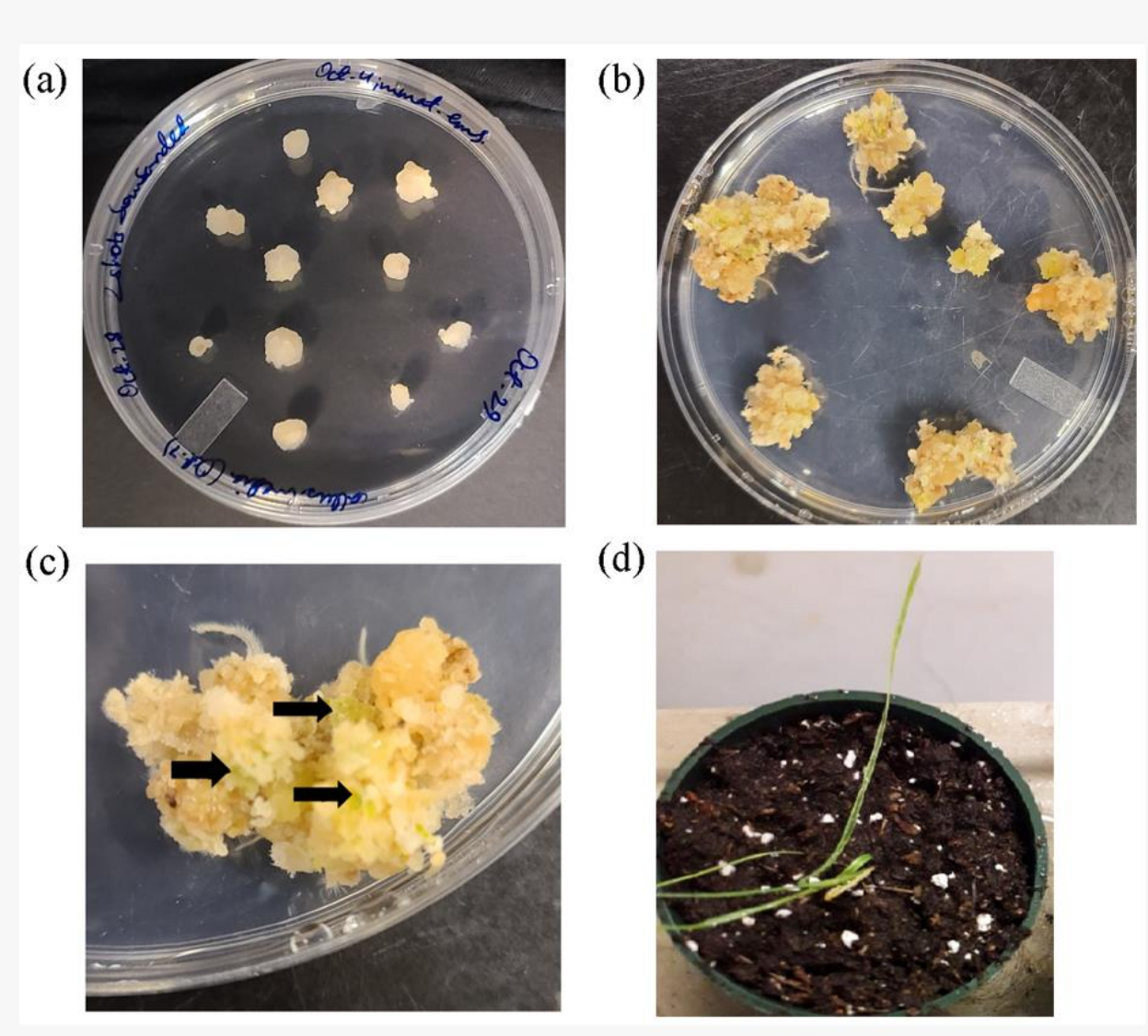
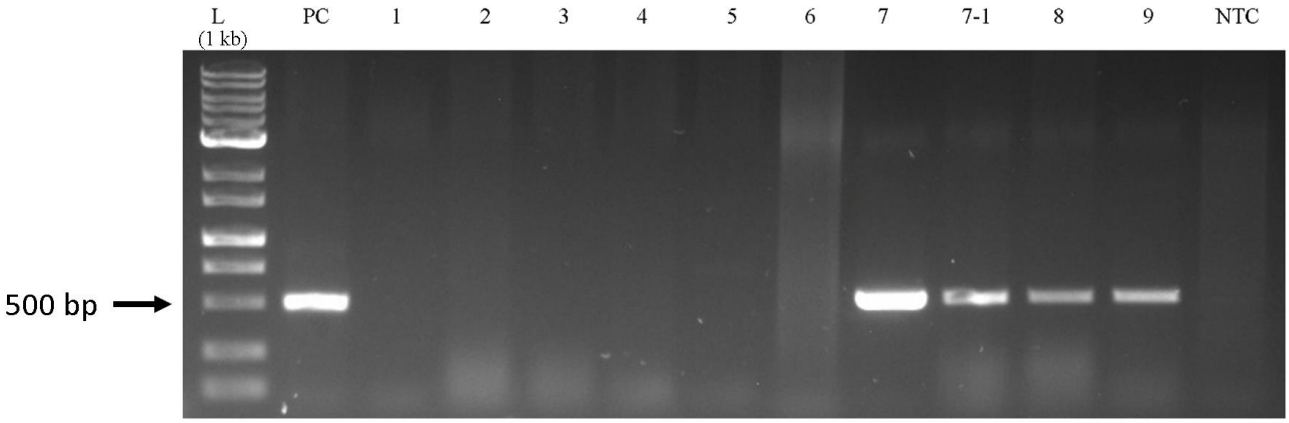


Figure 3.4: Detection of Cas9 positive plants through polymerase chain reaction (PCR). L: 1 Kb molecular weight ladder PC- positive control, 1-9 samples, NTC – non template control. The samples with bands represent Cas9 positive samples.



CONNECTING STATEMENTS TO CHAPTER IV

Chapter 4, entitled, "**Editing metacaspase (StMC7) gene enhances multiple disease resistance in Russet Burbank potato.**" authored by Bikram Poudel, Atul Sathe, Jacqueline C. Bede and Ajjamada C. Kushalappa, will be submitted to a peer-reviewed scientific journal for publication.

In Chapter III, tissue culture, transformation, regeneration and CRISPR construct delivery was optimized for the cultivated wheat cultivar 'Pasteur'. By delivering the construct to edit *TaHRC* gene, the expectation was to improve wheat resistance to Fusarium head blight (FHB). Since gene editing was not successful among the regenerated wheat plants, the research shifted to potato, where plant regeneration is well established. Previous studies on *TaHRC* in wheat and *StHRC* in potato demonstrated that HRC gene is involved in apoptotic-like programmed cell death (AL-PCD) induced during hemibiotrophic and necrotrophic pathogen infection. Since hemibiotrophic and necrotrophic pathogens gain nutrition from dead tissues, silencing *StHRC* reduces AL-PCD and improves multiple disease resistance in wheat and potato. However, the actual hierarchy of genes involved in AL-PCD is unknown. Metacaspases are cysteine-like proteases activated upon the Ca^{2+} spike after pathogen infection detection and regulate PCD. One of the metacaspases in potato, *StMC7* is highly expressed during pathogen infection but not in *StHRC* mutants, suggesting it lies downstream of *HRC* and plays a possible role in disease resistance.

In Chapter IV, the gene encoding metacaspase *StMC7* was knocked-out in elite potato cultivar Russet Burbank (RB) using CRISPR-Cas9 to elucidate its role in resistance to hemibiotrophic and necrotrophic pathogen infection in potato. The role of this metacaspases was assessed in plant resistance to both hemibiotrophic and necrotrophic pathogens (*P. infestans* and *A. solani* respectively), through disease severity assay and pathogen infection quantification.

Chapter IV

Editing metacaspase (*StMC7*) gene enhances multiple disease resistance in Russet Burbank potato

Bikram Poudel, Atul Sathe, Jacqueline C Bede, Ajjamada C Kushalappa

Plant Science Department. McGill University, Ste.-Anne-de-Bellevue, QC, Canada

4.1 Abstract

In plants, the host plant induces hypersensitive response programmed cell death (HR-PCD), upon pathogen perception, to contain pathogen to the point of infection. Apoptotic-like PCD (AL-PCD) has also been reported upon prolonged pathogen infection in potato. Hemibiotrophic and necrotrophic pathogens induce PCD in host plant to feed upon the dead cells for their growth. Metacaspases, a cysteine-protease that mediates PCD in plants, play an essential role during plant-pathogen interaction. In this study, a type II metacaspase in potato, *StMC7*, was silenced in potato cultivar 'Russet Burbank' using CRISPR-Cas9 to improve disease resistance against late and early blight of potato caused by *Phytophthora infestans* and *Alternaria solani* respectively. Upon pathogen infection, the *StMC7* gene expression was lower in *Stmc7* mutants as compared to wild type. Disease severity analysis and pathogen biomass study revealed enhanced disease resistance to both early and late blight of potato in the knockout mutants. Thus, silencing *StMC7* improved disease resistance against multiple pathogens in potato.

4.2 Introduction

Potato is one of the most widely consumed food crops grown around the world under various agroecological environments. However, potato cultivation is continually threatened by pathogens that destroy the quality and quantity of potato yield. Major pathogens in potato are the oomycete pathogen *Phytophthora infestans* causing late blight and the fungal pathogen *Alternaria solani* causing early blight. A major management strategy to counter these pathogens involves application of chemical pesticides, but this increases the cost of production and is also associated with human health and environmental hazards (Ivanov et al., 2021). Improving host genetic resistance through development of multiple disease resistant cultivars is the most sustainable and effective management strategy. Understanding plant defense response to pathogens is essential for improving genetic resistance.

Plants activates programmed cell death (PCD) as a defense response to plant-pathogen interaction initially in the form of hypersensitive response (HR), which is termed HR-PCD (Camagna & Takemoto, 2018). HR is a localized plant cell death at the infection point to restrict the pathogen spread to the point of infection. HR serves as the first line of defense during plant-pathogen interaction. True apoptosis is absent in plants but a mode of PCD is present which is morphologically and biochemically similar to apoptosis in animals, termed AL-PCD (Reape et al., 2008). AL-PCD is induced later than HR-PCD, but it is still a rapid process, initiating and often ending within 6 hr (Dickman et al., 2017). The DNA ladders, a characteristic feature of AL-PCD, appear by 6 h of pathogen detection (Balk et al., 2003).Though the HR-PCD and AL-PCD both are characterized by cytoplasmic shrinkage, mitochondrial swelling, cytochrome c release, chloroplast disruption, chromatin condensation and DNA fragmentation, they differ in presence of plasma membrane blebbing and a characteristic DNA fragmentation (Coll et al., 2011). Both

HR and AL-PCD is detrimental to biotrophic pathogens as PCD limits its access to living cells and cuts off the nutrient source. However, hemibiotrophic and necrotrophic pathogens actively promote PCD in their host cells and utilize it to derive nutrients from the dead cells. Delaying or preventing AL-PCD can stop cell death, limiting the nutrient source for hemibiotrophic and necrotrophic pathogen, resulting in enhanced plant resistance.

Pathogen-associated calcium ion (Ca^{2+}) influx signals are perceived by calcium sensors such as calmodulin (CaM), CaM-like proteins, Ca^{2+} -dependent protein kinases (CDPKs) and Ca^{2+}- and Ca^{2+}/CaM-independent protein kinase (CCaMK) and calcineurin B-like proteins (CBLs) (Galon et al., 2010). The pathogen-associated Ca^{2+} influx is perceived and transported to the nucleus by the sarcoplasmic/endoplasmic histidine-rich Ca^{2+} -binding protein (HRC) (Chen et al., 2022). *TaHRC* may suppress Ca^{2+} -mediated plant immune response and promote pathogen spread in wheat during infection (Chen et al., 2022). *TaHRC* was naturally mutated in FHB resistant cultivars such as Sumai3, Wangshuibai, Nyu Bai and functional in susceptible cultivars (Su et al., 2018). Mutation of *TaHRC* increased resistance to fusarium head blight (FHB) in resistant near-isogenic lines (NILs) by preventing PCD whereas the PCD was induced, leading to susceptibility in susceptible NILs (Gunniah, 2013).

Ca^{2+} influx stimulates the activity of metacaspases, a class of cysteine-proteases. Metacaspases mediate PCD in during development, abiotic and biotic stresses resistance (Suarez et al., 2004; Watanabe & Lam, 2011b). Two types of metacaspases are found in plants, type I metacaspase, which contains an N-terminal prodomain before a subunit p20, and type II metacaspases, which contains a linker joining two subunits, p20 and p10 (Garcia et al., 2022). Changes in Ca^{2+} concentration affect the activity of metacaspases and endonuclease. Upon increased Ca^{2+} influx, metacaspases undergo conformational changes and is activated by

multiple cleavage in the linker region of type II metacaspases (Zhu et al., 2020). *AtMC2d*, the most abundant type II metacaspases in Arabidopsis, exhibits Ca^{2+} dependency for activation (Watanabe & Lam, 2011a). Similarly, pathogen-associated Ca^{2+} influx activates *AtMC4* to initiate a defense response (Hander et al., 2019). Metacaspases then processes substates such as Propep1, producing Pep1 elicitors, which triggers downstream immune response (Hander et al., 2019). Among the 9 metacaspases in potato, *StMC7*, showing significant similarity to *AtMC4* in arabidopsis, is constitutively highly expressed in most plant tissues (Dubey et al., 2019). Silencing *StHRC* also reduced the expression of *StMC7* and increased the disease resistance to *Phytophthora infestans* and *Alternaria solani* (Kushalappa et al., 2022). Hence, silencing *StMC7* leading to the suppression of AL-PCD, may inhibit the infection process by hemibiotrophic and necrotrophic pathogens, due to unavailability of their nutritional source, leading to increased plant resistance.

Here, we report *Solanum tuberosum* metacaspase 7 (*StMC7*) as a susceptibility gene in plant-pathogen interaction with a hemibiotrophic pathogen *Phytophthora infestans* and a necrotrophic pathogen *Alternaria solani*. CRISPR/Cas9 mediated knockout of *StMC7* in Russet Burbank potato showed significant reduction in disease severity and pathogen biomass for both the pathogens. Based on these findings, we find that silencing of the functional *StMC7* leads to enhanced multiple disease resistance in potato.

4.3 Materials and methods

4.3.1 Plant production

Tissue cultured plantlets of the susceptible potato genotype Russet Burbank (RB) was obtained from the New Brunswick Plant Propagation Center (Potato Research Centre,

Agriculture and Agri-Food Canada, New Brunswick, Canada). The tissue culture plantlets were further multiplied in the lab and then grown in the greenhouse in pots with Fafard AGRO MIX® G6 under greenhouse condition (20 ± 3ºC temperature, 70 ± 10% relative humidity, 16 hr photoperiod, 1500 µmol/m^2/s light intensity). The tissue cultured plantlets were grown in sterile half strength M516 media, supplemented with 3% sucrose and 2g/L Phytagel™. Plantlets were incubated in a Percival growth chamber at 22ºC with 16 h light cycle. Internodal segments and leaf segments from 3-week-old sterile plants were used for *Agrobacterium*-mediated transformation.

4.3.2 DNA extraction and *StMC7* sequencing

DNA was extracted from RB potato seedlings using a modified CTAB method (Borges et al., 2009). The full gene sequence of *StMC7* covering both exons was amplified with specific primers using a high fidelity Q5 Polymerase (New England Biolabs, Ipswich, MA, USA) (Table 4.1). The amplified PCR product was purified using BioBasic PCR Clean Up (Bio Basic INC., Markham, ON, CA). The purified PCR product was cloned into pGEMT-Easy plasmid (Promega Corp., Madison, WI, USA) using T4 DNA Ligase (NEB, MA, USA). The final plasmid was transformed into competent *E. coli* DH5α cells (New England Biolabs, Ipswich, MA, USA) and sequenced using M13-F and R primers by Sanger sequencing (Institute of Integrative Biology and Systems (IBIS), Laval University) sequences were translated with Expasy (https://www.expasy.org/) to obtain protein sequence (Gasteiger et al., 2003). The protein structure of *StMC7* was predicted using RoseTTAFold (Baek et al., 2021).

4.3.3 Vector construction and *Agrobacterium*-mediated transformation

Three guide RNAs (gRNAs) were designed based on the *StMC7* gene sequence, targeting both exons, one targeting the first exon and two targeting the second exon, using CRISPR-P 2.0 (http://crispr.hzau.edu.cn/CRISPR2/) (Liu et al., 2017). The three gRNAs were individually cloned into three separate pDIRECT21A vector through Golden Gate Assembly using AarI restriction enzyme (Čermák et al., 2017). The final vectors were transformed into *E. coli* DH5α competent cells and sequenced (IBIS, Laval University). The clones with successfully inserted gRNA were transformed using the standard freeze-thaw method into *Agrobacterium tumefaciens* (GV3101) for explant transformation.

Transformation was carried out according to Duan et al. (2012) with slight modifications. Briefly, internodes and leaves were sterilized using 70% ethanol for 30s followed by 50% NaOCl solution for 10 min for internodes and 5 min for leaves. The explants were subjected to a mix of three *Agrobacterium* suspension cultures harboring the three different constructs in pDIRECT_21A construct. The explants were transferred to co-cultivation media (1/10 MS salts, 3 % sucrose, pH 5.7, 6g/L agar) at 22^{o}C for 2 days and then transferred to callus induction media (CIM) (4.3 g/L MS salts, 2.5 mg/L zeatin, 0.1 mg/L naphthalene acetic acid (NAA), 3 % sucrose. 6 g/L agar) with timentin (150 mg/L) and hygromycin (50 mg/L) and incubated at 22^{o}C. After 1 month, growing calli were transferred to shoot induction media (SIM) (4.3 g/L MS media, 2.5 mg/L zeatin, 0.3 mg/L gibberellic acid, 6 g/L sugar, 3 % sucrose) with timentin (150 mg/L) and hygromycin (50 mg/L). Calli with emerging shoots were transferred to MS media supplemented with 3% sucrose, 6 g/L agar, supplemented with previous dose of timentin and hygromycin.

4.3.4. Detection of putative CRISPR-Cas9 potato mutants

A 526-bp surrounding the *StMC7* gRNA1 region was amplified using Phusion Green Hot Start II High-Fidelity PCD Master Mix (ThermoFisher Scientific, MA, USA) and purified using the BioBasic PCR Purification Kit (Biobasic, ON, CA). The purified sample were subjected to restriction digestion assay using HindIII, which cut at a unique site within the gRNA 1, as per the manufacturer's protocol. The purified sample was sent for sequencing by Illumina NextSeq PE300-500K at Genome Quebec, Montreal. Results from amplicon sequencing were analyzed using CRISPResso2 (Clement et al., 2019).

4.3.5 Disease severity and pathogen biomass assay

Phytophthora infestans isolate US-8, A2 mating type, a highly virulent, aggressive strain, (received from Dr. H. Platt, AAFC, Charlestown, PEI, CA) was maintained on potato dextrose agar (PDA). Spores were produced by inoculating a thin potato tuber slice with *P. infestans* and incubating the sealed and moist Petri dish in 18^{o}C for sporulation. Sporangia were harvested and spore concentration was adjusted to 10^5 sporangia per ml. *Alternaria solani* (obtained from Dr. A. Dionne, MAPAQ, QC) was maintained on PDA. For spore production, the plate was incubated at room temperature with 12 hr light and dark photoperiod.

The experiment for pathogen biomass and disease severity was conducted in a Randomized Complete Block Design (RCBD) with two genotypes (wild RB and *StMC7* mutants), two inoculations (mock and pathogen), and three temporal replicates. Each experimental unit consisted of five pots with two plants and ten leaves inoculated in each pot. Young leaves of 5-6 weeks old plants were point inoculated on the either side of midrib on the lower surface with 10 µl sporangial suspension. The plants were covered with plastic bags upon

inoculation for 72 h and 48 h for *P. infestans* and *A. solani*, respectively. Disease severity was quantified by measuring the lesion diameter (in mm) at 3 days interval until 9 post-inoculation (dpi) and calculating the area under the disease progress curve (AUDPC). Relative biomass for both *P. infestans* and *A. solani* from infected samples were quantified based on quantitative PCR (qPCR) to determine the biomass of the pathogens in infected samples. Genomic DNA was isolated from the infected leaves 6 dpi using the CTAB method. Specific primers for each pathogens as well as against the potato genome were used in qPCR performed using a Luna Universal qPCR Mastermix (NEB, MA, USA) using Mic qPCR Cycler (Bio Molecular Systems, Queensland, Australia) (Table 4.1) (Table 4.1) (Hegde et al., 2021; Leiminger et al., 2015). Statistical analysis was conducted using SPSS (IBM SPSS Statistics 29.0).

4.3.6 RNA extraction and gene expression by RT-qPCR.

NCBI BLAST was used to design RT-qPCR primers (Altschul et al., 1990). RNA was extracted from the leaves of RB genotype and *Stmc7* knockout plants 6 days post inoculation (dpi) following *P. infestans* or *A. solani* and mock (water) inoculation using RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands). cDNA was synthesized using Maxima H Minus First Strand cDNA Synthesis Kit (ThermoFisher Scientific, MA, USA). qPCR was conducted with Luna Universal qPCR Mastermix (NEB, MA, USA). Two genes, elongation factor alpha (*StEf1α)* and *tubulin* (*Sttubulin*)were used as reference genes. The relative gene expression levels were analyzed based on delta-delta C_T (cycle threshold) method ($2^{-\Delta\Delta Ct}$) (Livak & Schmittgen, 2001). All the gene expression values are presented as gene expression ratio, obtained through comparison with RB mock inoculation.

4.4 Results

4.4.1 Characterization of *StMC7*

The *StMC7* gene is 3061 bp containing two exons, 346 bp and 905 bp. Protein sequence analysis revealed the presence of a peptidase C14 caspase domain, supporting the role of *StMC7* as a metacaspase (Figure 4.1b). Upon comparison with arabidopsis *AtMC4* sequence, the exact positions for conserved caspase-like catalytic domains such as the p-20 subunit (20 kDa), p-10 subunit (10 kDa) and linker between them were identified. The catalytic histidine was found within the sequence HYSGHG and the catalytic cysteine was found within DSCHS, similar to *AtMC4* (Figure 4.1) (Vercammen et al., 2004). The protein structure of *StMC7* was predicted using RoseTTAFold, revealing its catalytically inactive state (Figure 4.1c).

4.4.2 *StMC7* knockout based on CRISPR-Cas9

Plants with InDel mutations were generated targeting the *StMC7* exon 1 through *Agrobacterium*-mediated transformation of the CRISPR-Cas9 construct (Figure 4.2) in the late blight susceptible cultivar Russet Burbank (RB). Transformed internodes and leaves successfully produced callus under the selection of hygromycin, resulting in putative transgenic potato plants (Figure 4.3). Eight plants were obtained after hygromycin selection through every stage of tissue culture. Restriction digestion assay showed InDel mutations in 6 plants at gRNA 1 (Figure 4.4a). No mutations were observed upon Sanger sequencing for gRNA 2 and 3. Amplicon-sequencing followed by CRISPResso2 analysis determined five heterozygous mutants in exon 1 of *StMC7* (Figure 4.4b).

4.4.3 Silencing *StMC7* reduced disease severity and pathogen biomass in *StMC7* mutants.

StMC7 was upregulated in pathogen-infected RB wildtype plants compared to the *Stmc7* mutants for both *P. infestans* and *A. solani* inoculated leaves (Figure 4.5d. 4.6d). The area under disease progress curve (AUDPC), calculated based on the lesion diameter, was seven times lower in *Stmc7* mutants (36.25) as compared to RB (249) and twice lower than *Sthrc* mutants (77.25). Pathogen biomass in the infected leaves with *P. infestans* specific primers quantified based on qPCR at 6 dpi were higher in RB compared to the *Stmc7* mutants (Figure 4.5), which reflects lower *P. infestans* levels in *Stmc7* mutants compared to the RB wildtype (Figure 4.5c). In *A. solani* – infected leaves, the AUDPC, calculated based on lesion diameter, was twice lower in *Stmc7* mutants (19.5) compared to RB (43.5) and the pathogen biomass lower (49 times) in *Stmc7* mutants, compared to the wildtype RB.

4.4.4 Gene expression of *StMC7*

StMC7 gene expression was lower in *Stmc7* mutants for both *P. infestans* (3.5 fold) and *A. solani* (4 fold) inoculated leaves compared to pathogen inoculated RB plants (Figure 4.5d, 4.6d).

4.5 Discussion

Programmed cell death (PCD) is an important defense strategy against biotrophic pathogens in plants. Hemibiotrophic oomycetes, such as *Phytophthora infestans* and necrotrophic fungus, such as *Alternaria solani* have a short, sometimes cryptic, biotrophic phase before switching to necrotrophic phase (Rajarammohan, 2021). These pathogens secrete toxins and enzymes to feed on nutrients from the dead cells. *Phytophthora infestans* produce fatty acids, such as Arachidonic acid, apoplastic effectors, such as necrotizing toxins Nep1-like proteins (NLPs), PcF-like (*Phytophthora cactorum* in *Fragaria*-like) small cysteine-rich proteins

(SCRs)), and cytoplasmic effectors, such as CRN (crinkling, necrosis), to induce AL-PCD (Haas et al., 2009; Knight et al., 2001). *Alternaria* spp. produces non-host specific toxins such as alternariol, zinniol, tentoxin and host-specific toxin families to elicit host defense response suppression and stimulate cell death (Meena et al., 2017). Thus, inducing cell death is a behavior important for necrotrophic and hemibiotrophic pathogens, leading to the supposition that, suppressing host PCD mechanism can confer broad spectrum disease resistance to such pathogens (Kushalappa et al., 2022).

Metacaspases have been reported to be involved in plant pathogen resistance in Arabidopsis, tomato, wheat, and pepper (Garcia et al., 2022). In Arabidopsis, mutations in type 2 metacaspases, *AtMC2* to *AtMC6* resulted in reduced susceptibility to *Botrytis cinera* and *B. tulipae* (VAN Baarlen et al., 2007). In tomato, *LeMC1* was rapidly induced when infected by *B. cinera* (Hoeberichts et al., 2003). In wheat, the expression of *TaMC4* was significantly upregulated when challenged with *Puccinia striiformis* f.sp. *tritici*. Virus-induced gene silencing (VIGS) of *TaMC4* resulted in limited fungal growth (Wang et al., 2012). Another metacaspase, *TaMC1* was significantly upregulated upon infection by *Puccinia striiformis* and increased disease resistance upon knockdown of *TaMC1* expression through VIGS (Hao et al., 2016). VIGS of metacaspase 9 in pepper (*CaMC9*) lead to delayed cell death symptoms and reduced cell death induced by *Xanthomonas campestris* pv. *vesicatoria* while the overexpression of *CaMC9* enhanced cell death and increased disease susceptibility to *Pseudomonas syringae* pv. *tabaci* (Kim et al., 2013). In potato, *StMC7* was significantly downregulated upon silencing histidine-rich calcium-binding protein coding gene (*StHRC*) indicating its prominent role in inducing AL-PCD (Kushalappa et al., 2022).

Using CRISPR-mediated genome editing technology, *Stmc7* mutants were generated with InDel mutations in exon 1. *StMC7* was highly expressed in RB wildtype plants compared to the *Stmc7* mutants upon pathogen infection by either *P. infestans* or *A. solani*. Following the infection of *Stmc7* mutants with either of *P. infestans* and *A. solani*, the disease progression and pathogen biomass in the diseased leaf area was reduced relative to the RB wildtype plants. This reduction was observed in all transgenic lines, irrespective of the percentage of mutation according to the amplicon sequencing (data not shown). The reduction in the AUDPC for both early and late blight possibly indicate successful suppression of pathogen attack in the mutants. For both *P. infestans* and *A. solani*, there was lower pathogen growth in *Stmc7* mutants compared to control RB plants (Figure 4.5c, 4.6c). *StMC7* was highly expressed in RB plants compared to *Stmc7* mutants upon both pathogen infection, indicating the important role of *StMC7* in imparting susceptibility to hemibiotrophic and necrotrophic pathogens. This study indicates successful multiple disease resistance upon silencing *StMC7* in potato. Further experiments in field condition are necessary to understand the *Stmc7* mutants' response to *P. infestans* and *A. solani* spores. The inoculation suspension used during the experiment contains pathogen-produced toxins, which might have induced PCD more quickly than in field condition. Thus, disease resistance in the field condition might be even more than tested in the greenhouse.

Though AL-PCD is induced during plant-pathogen interaction, the exact mechanism of AL-PCD is not clear. An essential condition for metacaspases activation is an increase in Ca^{2+}. A proposed model of AL-PCD in potato is illustrated in Figure 4.8. In general, an influx of intracellular Ca^{2+} following fungal pathogen is sensed by various Ca^{2+} influx protein sensors responsible for maintaining cytosolic Ca^{2+} homeostasis (Figure 4.8) (Köster et al., 2022). Ca^{2+} -transporters such as Ca^{2+} -ATPases, two-pore Ca^{2+} channels and cyclic nucleotide gated channels

(CNGCs), transport Ca^{2+} out of cytosol, across various cellular membranes (Park & Shin, 2022). When Ca^{2+} ion concentration increases in mitochondria, cytochrome c is released via the formation of permeability transition (PT) pore or through the large cytochrome c-conducting channel formed by voltage-dependent anion channel (VDAC), increasing the reactive oxygen species (ROS) (Jones, 2000). Ca^{2+} - binding proteins such as *StHRC* regulate the downstream response processes (Lee & Seo, 2021). HRC transports the Ca^{2+} into nucleus, where the Ca^{2+} triggers Ca^{2+}- dependent endonucleases such as *StCaN2*, to degrade DNA (Kushalappa et al., 2022; Sui et al., 2019). Ca^{2+} triggers the activation of *StMC7* in the cytosol through multiple cleavage at 6 sites in the linker region releasing the linker region and making the active site available for substrate processing (Zhu et al., 2020). The activated *MC7* cleaves the plant elicitor peptide (PEP1) from its precursor complex (PROPEP1) (Hander et al., 2019). The released Pep1 signals surrounding cells to activate defense response. However, in plants, the exact mechanism of metacaspases activation and its action is not well understood.

In animals, changes in mitochondrial morphology is one of the the first visible indicator of apoptosis. This morphology change releases mitochondrial molecules, such as cytochrome c, into cytosol, which activates apoptotic-protease activating factor 1 (Apaf-1) assembles to form the caspase-activating complex, apoptosome (Bao & Shi, 2007). The apoptosome is a heptameric wheel-like structure, composed of 7 Apaf1 molecules bound to 7 cytochrome c molecules (Bao & Shi, 2006). This complex triggers the activation of caspases that causes cell death. Though such a pathway has yet to be reported during PCD induction in plants, plant NLRs (nucleotide-binding domain leucine-rich repeat (LRR)-containing gene family) were found to oligomerize into a supramolecular structure, resistosome, and likely execute cell death or regulate downstream immune response during HR-PCD in Arabidopsis (Wang et al., 2019). Cryo-electron

microscopy may elucidate if similar structures may form before AL-PCD. Silencing other candidate genes involved in AL-PCD, endonucleases such as *StCaN2*, could also reduce the DNA fragmentation, induction of AL-PCD and provide further multiple disease resistance.

4.6 Conclusion

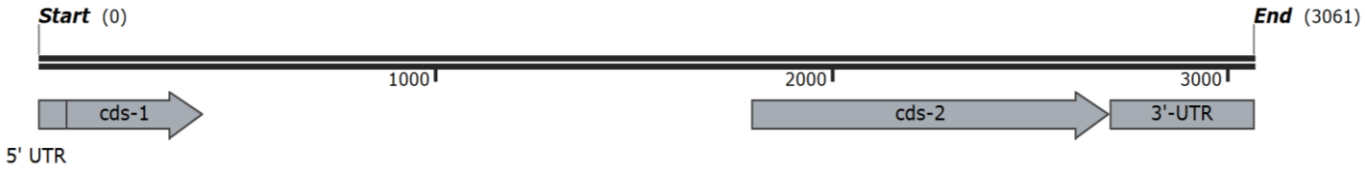
Potato cultivars with multiple disease resistance is the desired outcome for all potato breeding programs. PCD is an important pathway in plant-pathogen interactions, which when suppressed can increase resistance to multiple pathogens. Upon pathogen- or damage-associated molecular pattern recognition, the plant activates the HR-PCD pathway. Apoptotic-like PCD has also been observed in plants, which involves plasma membrane blebbing and characteristic DNA fragmentation. These different PCD generate nutrient sources for hemibiotrophic and necrotrophic pathogens. By silencing one of the important gene that mediate PCD, a metacaspase *StMC7*, we have shown increased resistance against two important potato pathogens, *Phythophthora infestans* and *Alternaria solani*, causing late blight and early blight in potato respectively. Further experiments with other hemibiotrophic and necrotrophic pathogens on *StMC7* mutants can show increased multiple disease resistance.

Table 4.1 Primers used for gRNA synthesis, amplicon sequencing, pathogen biomass quantification, qRT-PCR.

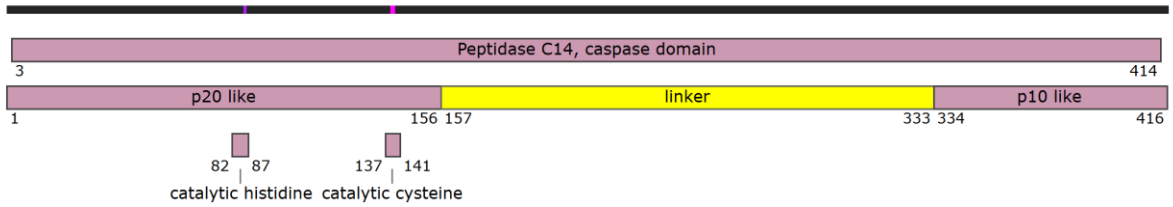
Gene name	Primer sequence
sgRNA-1	F: 5'- GATTGAATATACGTAAAGCTTTAT-3' R: 5'-AAACATAAAGCTTTACGTATATTC-3'
sgRNA-2	F: 5'- GATTGAGCTATGCGAAACCAGCCA-3' R: 5'-AAACTGGCTGGTTTCGCATAGCTC-3
sgRNA-3	F: 5'- GATTGCAGATGCCACCCCTGCAGG-3' R: 5'-AAACCCTGCAGGGGTGGCATCTGC-3'
StMC7-gRNA-1	F: 5'- GCGAAAAAGGCAGTGTTAATTGG-3' R: 5'- CCCCCAACAAATTCAACAAACAC-3'
StMC7-gRNA-2-3	F: 5'- TGGGAAACTTAGGCCAACAC-3' R: 5'- AGGCATAGCACAGCAGAATC-3'
StMC7-amplicon	F:5'- ACACTCTTTCCCTACACGACGCTCTTCCGATCTGCGAAAAAG GCAGTGTTAATTGG-3' F:5'- GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTCCCCCAAC AAATTCAACAAACAC-3'
StEf1-α	F: 5'- ATTGGAAACGGATATGCTCCA -3' R: 5'- TCCTTACCTGAACGCCTGTCA -3'
Stβ-tubulin	F: 5'- ATGTTCAGGCGCAAGGCTT-3' R: 5'- TCTGCAACCGGGTCATTCAT-3'
O-8(Phytophthora infestans)	F: 5'- TGGGAAACTTAGGCCAACAC-3' R: 5'- TAACCGACCAAGTAGT AAA -3'
ITS (*Alternaria* sp.)	F: 5'-TCCGTAGCTGAACCTGCGG -3' R: 5'-TGGGTTGGTCCTTGTGGTG-3'
StMC7-RT	F: 5'- GCCTTCTCGAGTAGCTGTTGA -3' R: 5'- TCACATGATGGAGAATGGTT-3'

Figure 4.1 Characterization of the potato metacaspase StMC7 **(a)** gene structure showing the two coding sequence regions, corresponding to the p20 and p10 domain associated with type II metacaspases. **(b)** StMC7 protein structure with the domains, p20 and p10 region, linker region and the conserved catalytic histidine and cysteine within the p20 region. **(c)** Predicted protein structure of StMC7 obtained using RoseTTAFold.

(a)



(b)



(c)

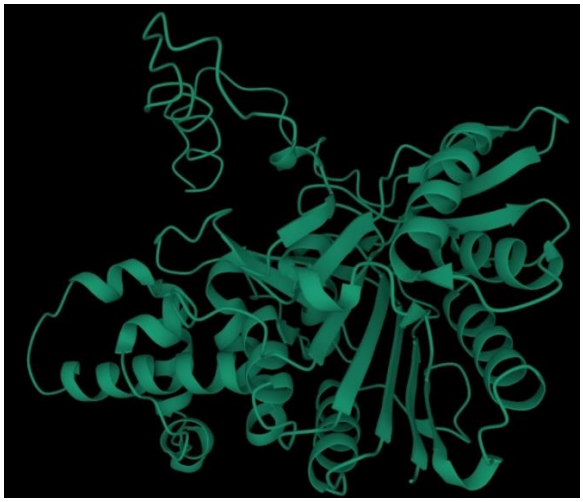


Figure 4.2 CRISPR design and construct preparation. gRNA was selected from the Exon 1 of *StMC7* and introduced into the pDIRECT_21A vector using Golden Gate Assembly at the AarI position of the vector to prepare CRISPR construct.

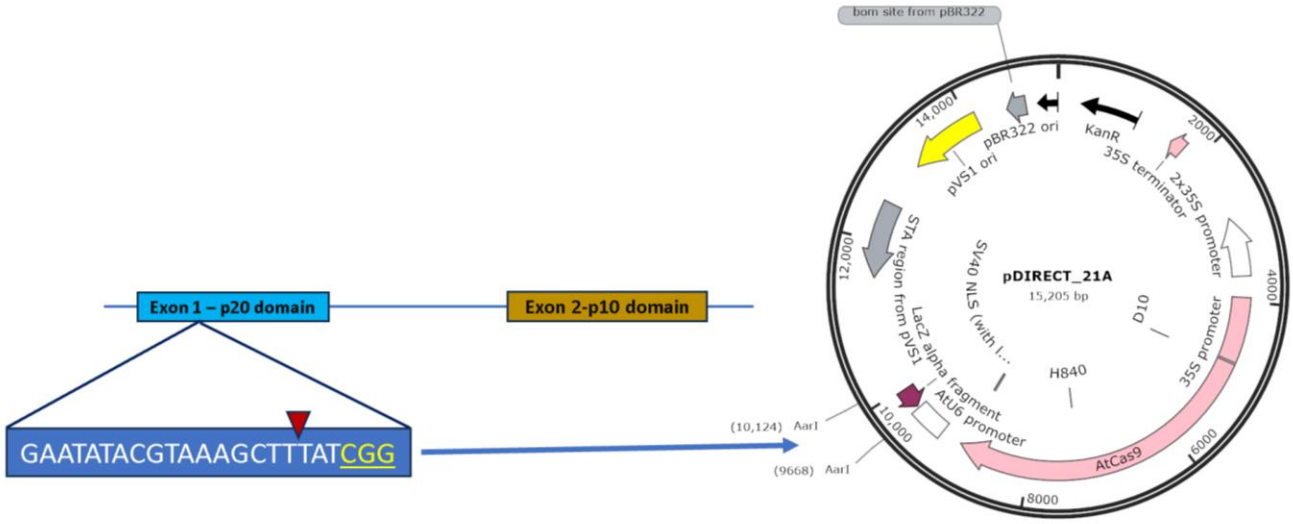


Figure 4.3 *Stmc7* mutant regeneration from callus regeneration of internodes and leaves of Russet Burbank potato using *Agrobacterium*-mediated transformation for CRISPR construct delivery. **(a)** Regenerating plantlets after *Agrobacterium*-mediated transformation of infected leaves and internodal segments. **(b)** Transgenic seedlings growing in shoot regeneration media **(c)** *Stmc7* mutant in greenhouse pots.

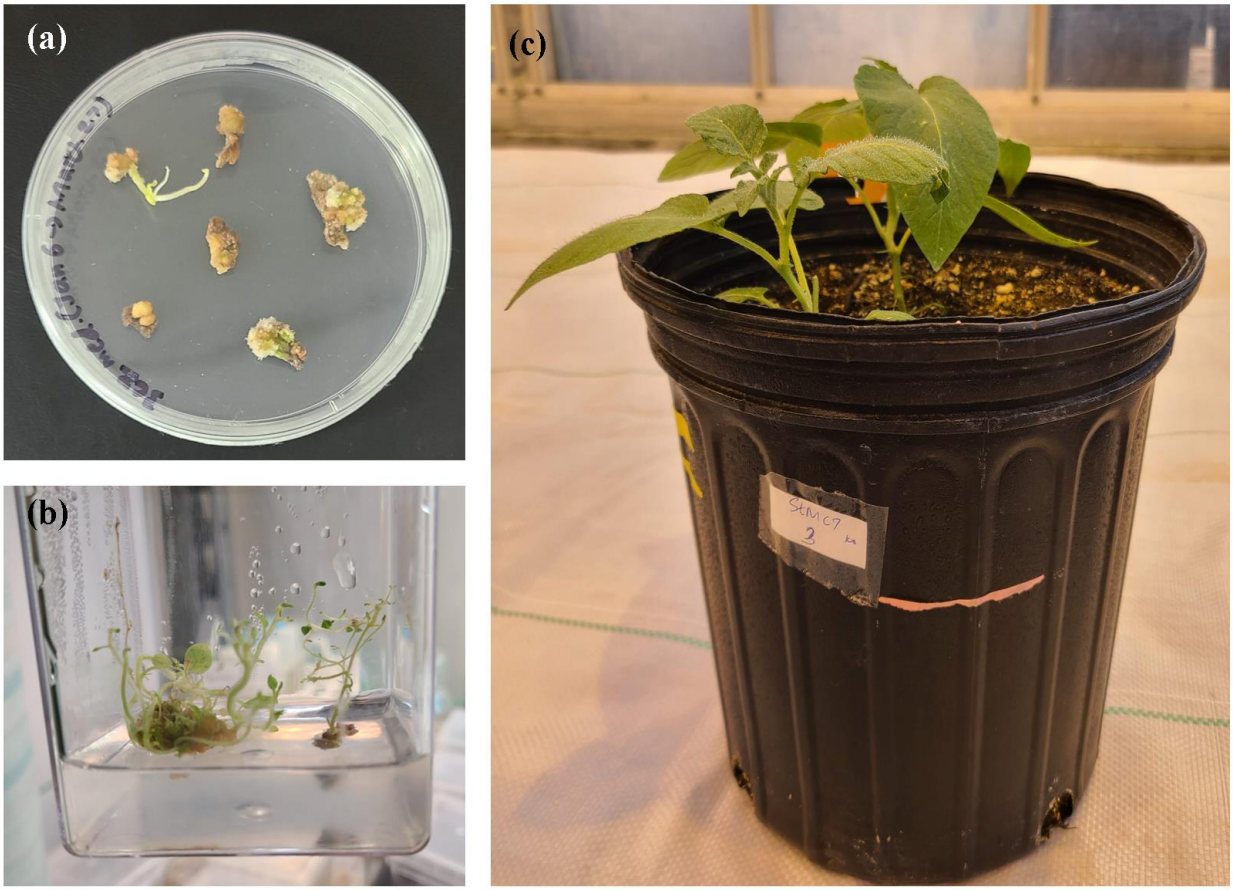
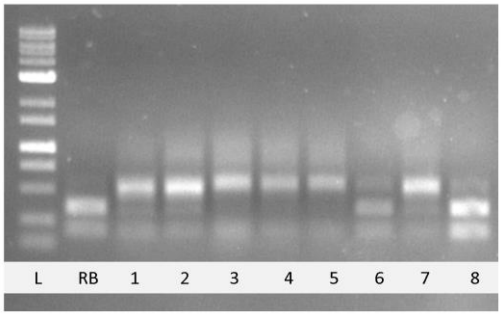
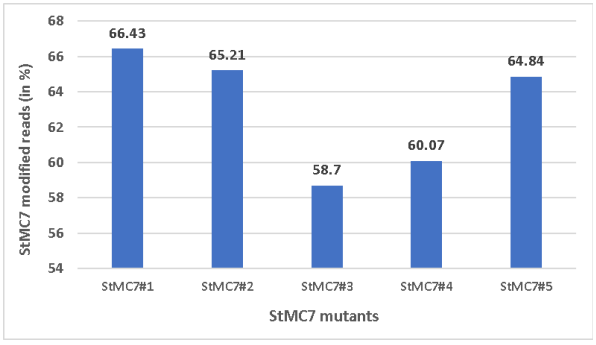


Figure 4.4 CRISPR-Cas9-mediated mutation analysis of *Stmc7* mutants. **(a)** *HindIII* digestion assay to identify *Stmc7* mutants. L: 1 kb plus ladder, RB: Positive Control, 1-8: *Stmc7* transgenic samples. Wild type RB (lane RB) or non-edited samples (lane 6,8) gives two bands. **(b)** Percentage of total modified reads for each *Stmc7* mutant line. The value at the top of the bar represents the exact percentage of modified reads. **(c)** Indels detected in *Stmc7* mutants by targeted amplicon sequencing mapped to wild type sequence. The percentage of reads for each sequence and no. of reads with that sequence are shown on the right. Sequences below 2% are not shown. The asterisk (*) indicates non-edited reads. Blue vertical dash line indicates predicted cleavage position, black horizontal dash indicates base deletion.

(a)



(b)



(c)

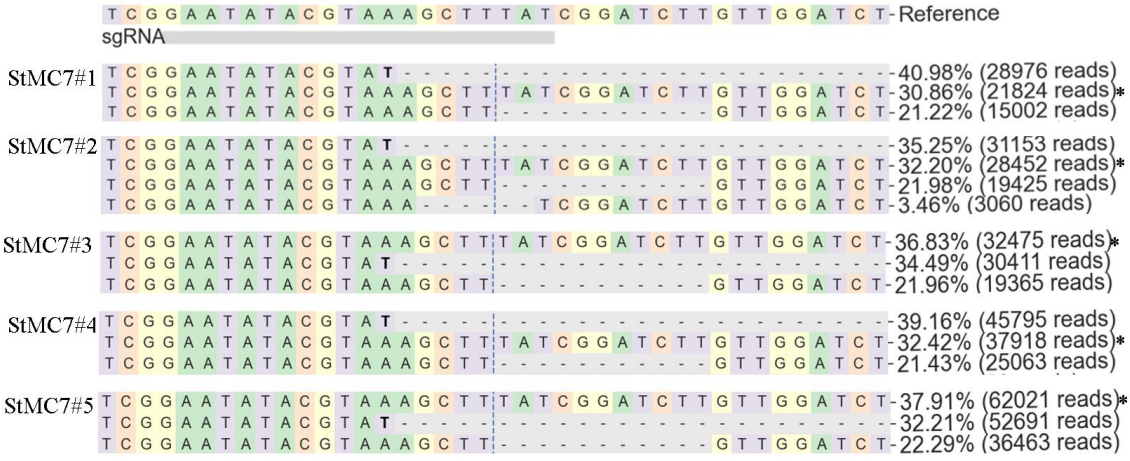


Figure 4.5 Russet Burbank (RB) potato leaves, with functional and mutated metacaspase *Stmc7*, inoculated with *Phytophthora infestans*. **(a)** Disease progression based on the lesion diameter (mm) at 3-, 6- and 9-days post inoculation (dpi) **(b)** Late blight symptoms showing significant differences between RB and *Stmc7* mutants, considerable differences between *StHRC* and *StMC7* mutants. **(c)** Pathogen biomass quantification at 6 days post-inoculation, quantified as relative P. infestans specific (O-8) gene expression **(d)** Relative gene expression of *StMC7* in RB and *Stmc7* mutants compared to the reference genes *StEf1* and tubulin, relative to wildtype mock inoculation, following *Phytophthora infestans* inoculation. Significance determined by Student's t-test, P value: **p<0.01, *p<0.05, or multiple comparison test (Tukey's HSD), significant differences shown by lowercase letters ($p < 0.05$). Error bars represent standard error (SEM) and asterisks denote values significantly different to wild type ($p < 0.05$).

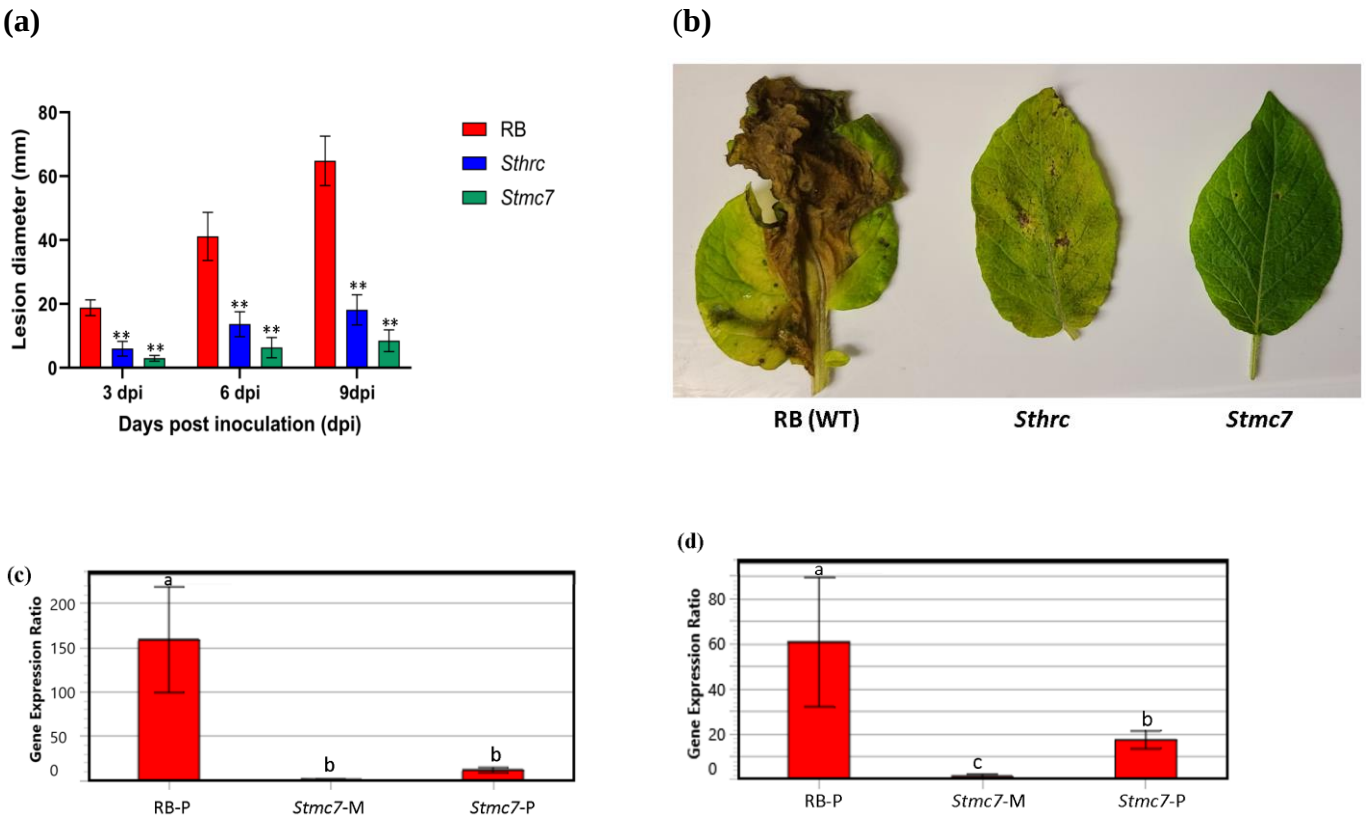


Figure 4.6 Russet Burbank potato leaves, with functional and mutated metacaspase *Stmc7*, infected with *Alternaria. solani*. **(a)** Disease progress based on the lesion diameter (mm) at 3-, 6- and 9-days post inoculation **(b)** Early Blight symptoms showing differences between RB and *Stmc7* mutants **(c)** Pathogen biomass quantification at 6 days post-inoculation, quantified as relative *A. solani* specific (*ITS*) gene expression **(d)** Relative gene expression of *StMC7* in RB and *Stmc7* mutants compared to the reference genes *StEf1* and tubulin, relative to wildtype mock inoculation, following *Phytophthora infestans* inoculation. Significance determined by Student's t-test, P value: **p<0.01, *p<0.05, or multiple comparison test (Tukey's HSD), significant differences shown by lowercase letters ($p < 0.05$). Error bars represent standard error (SEM) and asterisks denote values significantly different to wild type ($p < 0.05$).

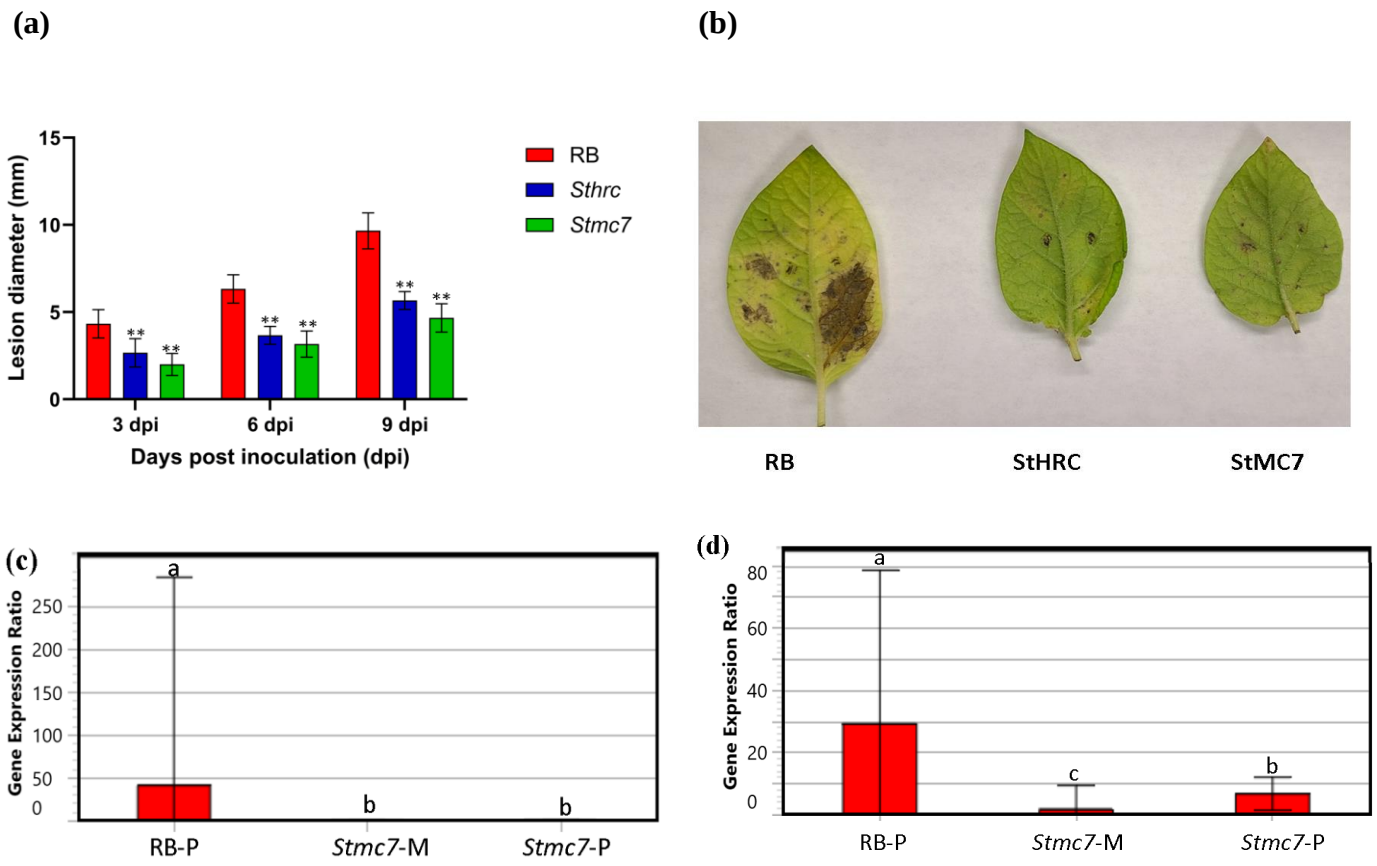
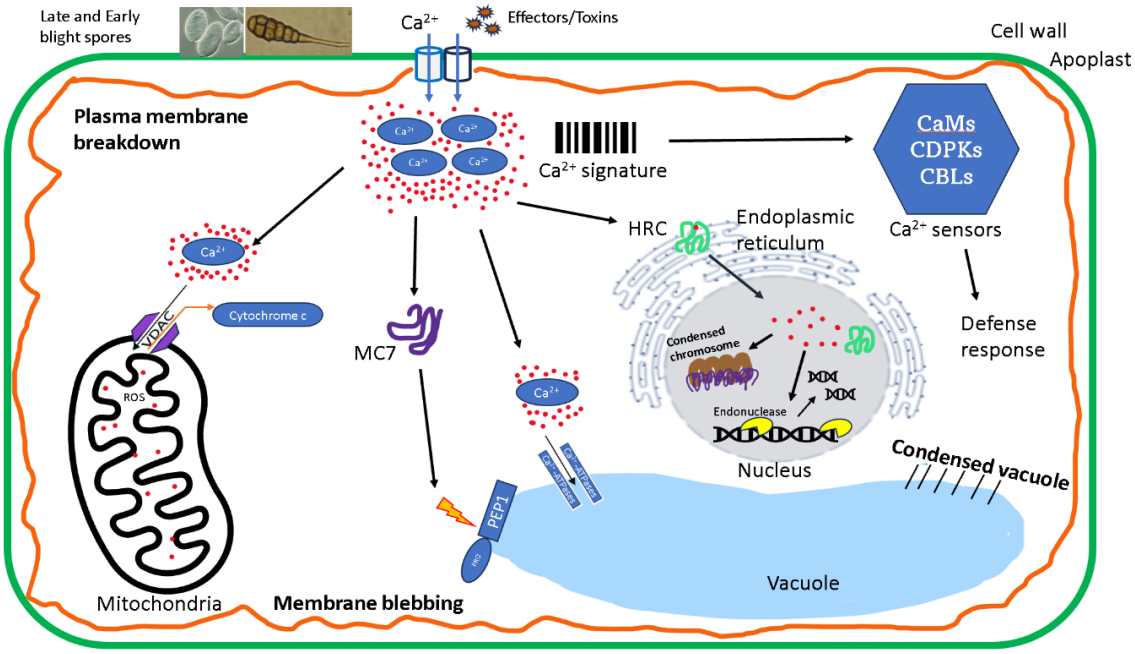


Figure 4.7 Proposed model of apoptotic-like programmed cell death (AL-PCD) in plants. Upon Ca^{2+} entry associated with pathogen attack, Ca^{2+} sensors decode the calcium signal for downstream activation. *StHRC* (histidine-rich calcium-binding protein) binds with Ca^{2+} and moves into the nucleus, increasing the calcium concentration in nucleus. Sustained Ca^{2+} increase in nucleus activates endonucleases, which causes DNA fragmentation. Ca^{2+} also enters the mitochondria through VDAC (voltage-dependent anion channel), which produces ROS in mitochondria. Ca^{2+} activates metacaspases *StMC7* present in cytosol. The *StMC7* then cleaves the PROPEP1, releasing PEP1, which signals nearby cells to initiate damage response and leads to condensed vacuole and other organelles upon prolonged PEP1 accumulation. The major characteristics of AL-PCD are shown in bold letters: Plasma membrane breakdown, condensed organelles such as vacuole, mitochondria, condensed chromosome, membrane blebbing.



Abbreviations: CaMs: calmodulins, CDPKs: Calcium-dependent protein kinases, CBLs: calcineurin B-like proteins

CHAPTER V: SUMMARY, GENERAL DISCUSSION, AND SUGGESTIONS FOR FUTURE RESEARCH

5.1 General discussion and summary

5.1.1 Importance of gene editing for crop improvement

Wheat and potato are the two most important staple foods contributing to food security and nutritional challenges throughout the world. However, several pathogens attack these crops resulting in yield quality and quantity loss. Integrated management strategies involving various approaches such as cultural practices, biological control approach, chemical approach are followed to reduce the yield quality and quantity loss. Currently, fungicide application is the most widely used preventive and curative measure, which adds to the environmental issues faced by Earth (Zubrod et al., 2019). Repeated application of fungicides with same modes of action also allows the fungicide-resistant strains to thrive and cause more yield quality and quantity loss in the long term (Yin et al., 2023). Improving host plant resistance is the most efficient, cost effective and environment-friendly method for tackling pathogen attacks in plants (Corkley et al., 2021). Marker assisted breeding has resulted in huge improvement in developing disease resistant cultivars over the past decades. However, it takes at least 6-12 years for developing new cultivars through classical plant breeding (Ahmar et al., 2020). It is much more challenging to develop cultivars with multiple disease resistance through conventional breeding. Gene editing through CRISPR-Cas9 is time effective, efficient, precise tool for crop improvement through editing resistance mechanism related genes to enhance disease resistance (Ali et al., 2022). Regulatory approval of CRISPR edited plants would also save time and money, compared to conventional breeding or genetically modified plants involving the incorporation of foreign genes. For example, a company would require at least six years and $30-50 million to generate

data for regulatory approval of genetically engineered plants through USDA. CRISPR-edited plants takes, on average, two years to develop and two months for regulatory approval without spending millions (Waltz, 2018).

5.1.2 Genome editing in wheat

Fusarium head blight (FHB) (caused by *Fusarium graminearum*) is one of the most important pathogens for wheat, affecting the yield quantity through shriveled grains and quality through mycotoxin (such as DON, NIV) contamination in grain, which makes it unfit for human or animal consumption (Alisaac & Mahlein, 2023). *TaHRC* is mutated in resistant cultivars and knocking out the gene can improve resistance in susceptible wheat cultivars (Su et al., 2019). However, most studies conduct gene editing in model wheat cultivar 'Bobwhite' or 'Fielder' and not in cultivated cultivars. It is imperative that gene editing technology is applied to cultivated cultivars (Ali et al., 2022). In Study III, *TaHRC* was sequenced in 'Pasteur', a special purpose wheat grown for feed in western Canada, through Sanger sequencing. A gRNA was designed targeting the cds region of *TaHRC* and inserted into three types of vectors to identify the best vector for gene editing in wheat. Though the modular vector approach allows multiple gRNA insertion for multiplex editing, GRF-GIF based vector was found to be the most efficient for plant regeneration. One of the main bottlenecks to application of CRISPR in cultivated cultivars is regeneration through tissue culture due to genotype dependency and various genetic factors. Tissue culture was optimized through biolistic transformation of immature embryo-derived callus. Application of expression factor GRF-GIF chimera in transformation vector may have contributed to successful regeneration. This study optimized the tissue culture and CRIPSR-Cas9 construct delivery in cultivated cultivar 'Pasteur'. The optimized steps for 'Pasteur' regeneration can serve as a guide to attempt gene editing in other Canadian cultivars. From this study, biolistic

delivery of GRF-GIF vector into immature embryo-derived calli was found to be the best method for CRISPR construct delivery and subsequent transgene seedling regeneration.

Though the Cas9 was integrated in 'Pasteur' T_0 regenerated seedlings, no editing was observed in the target region. No mutation was observed in the T_1 progenies as well. Though further sequencing was not done for T_2 and T_3 generations due to time limitation, it would be worthwhile to sequence the T_2 and T_3 progenies for targeted mutation. Application of other gene editing tools such as base editing might enhance mutation efficiency in Pasteur. Base editors allow precise conversion of a targeted base without introducing a double stranded break in DNA and achieve higher editing efficiency in plants. Li et al. (2018) obtained up to 59.1% mutation frequency in wheat. Thus, base editing has the potential to increase editing efficiency in Pasteur. Further, the transformation efficiency was only 0.8% in Pasteur. This hints at the need for more optimization to obtain a better transformation efficiency through parameters optimization or application of vectors with GRF-GIF and other transcription factor genes such as BABYBOOM or WUSCHEL to improve regeneration efficiency when introduced into the tissue culture protocol.

5.1.3 Gene editing in potato

Hemibiotrophic *Phytophthora infestans* and necrotrophic *Alternaria solani* are economically important pathogens in potato. Late blight was responsible for Irish Famine (1845-1852), which killed millions and forced the immigration of another million people in Ireland and remains one of the most destructive diseases (Turner, 2005). Early blight of potato is a serious disease in potato found in all potato growing areas worldwide (Ivanović et al., 2022). Plants respond to both hemibiotrophic and necrotrophic pathogens similarly by inducing programmed cell death (PCD) in infected cells (Zhou et al., 2023). Hypersensitive response PCD (HR-PCD) is

activated initially as a part of pattern triggered immunity (PTI) when the Ca^{2+} concentration spike due to pathogen attack is low. AL-PCD is activated upon higher Ca^{2+} concentration detection and mediates cell suicide through metacaspases. Though this restricts biotrophic pathogens to the point of infection, hemibiotrophic and necrotrophic pathogens utilize the dead cells for further infection. Thus, manipulating the PCD pathway can provide multiple disease resistance. Metacaspases are cysteine proteases involved in PCD, which reduce the cell death upon silencing (Tsiatsiani et al., 2011). In chapter IV, *StMC7* gene was sequenced, characterized, predicted protein structure and silenced using one gRNA through CRISPR-Cas9. *Agrobacterium* mediated transformation was carried out in RB leaves and internodes, regenerating 8 plants. Restriction digestion assay confirmed six plants were edited and confirmed by targeted amplicon sequencing using Illumina MiSeq. Upon analysis with webtool CRISPResso2 (Clement et al., 2019), heterozygous mutation was observed in all edited lines. Gene expression confirmed reduced *StMC7* expression in the mutants. Disease severity and pathogen biomass assay demonstrated enhanced disease resistance compared to the wild plants upon pathogen infection with both *P. infestans* and *A. solani*. This study demonstrates that manipulating metacaspase *StMC7* can enhance multiple disease resistance against hemibiotrophic and necrotrophic pathogens.

Metacaspases are important part of programmed cell death pathway, activating upon Ca^{2+} flux during abiotic stresses as well and initiating self-cleavage as per the calcium influx to mediate appropriate immune response (Zhu et al., 2020). Among the 9 metacaspases in potato, *StMC7* is a key metacaspase which was found to be highly expressed under biotic stress and differentially expressed under abiotic stress conditions (Dubey et al., 2019). Silencing *StMC7* may alter the response of potato plant to various abiotic stress conditions as well. Future studies

elucidating the effects of abiotic stress response on *Stmc7* mutants could contribute to understanding the role of *StMC7*.

Though resistance to *P. infestans* and *A. solani* was achieved in *Stmc7* mutants, it is necessary to test the mutants for other strains not used in our study. For example, *P. infestans* strain US-8 was inoculated in our study. It is necessary to observe the mutants' response upon inoculation by the more prevalent and more aggressive strains in Canada, such as US-23 in Manitoba, Ontario and New Brunswick, US-22 in southern Ontario, US-24 in Quebec (Kalischuk et al., 2012). Our study achieved heterozygous mutation in *Stmc7* target site for all the alleles. Future studies can be directed to produce more mutants and obtain homozygous mutations at the target site.

5.2 Suggested future studies

- **Application of developed tissue culture protocol for biotic stress resistance through CRISPR-Cas9 in wheat cultivar 'Pasteur'**

  According to the technical bulletin published by SeCan, 'Pasteur' demonstrate intermediate resistance to leaf spots such as tan spot (caused by *Pyrenophora tritici-repentis*, glume blotch (caused by *Parastagonospora nodorum*) and speckled leaf blotch (STB) (caused by *Zymoseptoria tritici*). Pasteur is susceptible to common bunt (caused by *Tilletia caries*) and loose smut (caused by *Ustilago tritici*). Tissue culture protocol developed in Chapter III can be applied to improve disease resistance to these pathogens through CRISPR/Cas9 or other gene editing technologies in the future.

- **Application of findings from study III to improve related Canadian cultivars**

  Findings from chapter III can further improve tissue culture and transgenic wheat regeneration in other related Canadian wheat cultivars, especially those sharing lineage to

Pasteur or its parent Cadenza. Cadenza is widely used as a research tool for molecular genetics studies and for developing mutant populations. Cadenza has also been used in gene-edited wheat trials in Europe (Raffan et al., 2023). Findings from study III can improve regeneration in other cultivars derived from Cadenza.

- **Further improvements for successful regeneration through *Agrobacterium*-mediated transformation**.

  Agrobacterium tumefaciens transfers low copies (mostly single copy) of transgenes and fewer genetic rearrangements (due to defined ends of T-DNA), resulting in more stable inheritance as compared to the random integration from biolistic delivery (Karthikeyan et al., 2023). However, different varieties and cultivars respond differently to *Agrobacterium*-mediated transformation, requiring protocol optimization. Elite cultivars such as 'Pasteur' are more recalcitrant to genetic transformation (Luo et al., 2021). Despite varying media compositions and parameters, no growth was observed in chapter III after exposing Pasteur callus to *Agrobacterium* suspension. However, several studies have successfully obtained regeneration in elite wheat cultivars through *Agrobacterium*-mediated transformation (Wang et al., 2018). In chapter III, a hypervirulent AGL-1 strain of *A. tumefaciens* was used but no regeneration was obtained, which indicates AGL1 is incompatible with Pasteur. Other strains of *A. tumefaciens* such as C58C1, EHA105, GV3101 have also been used for successful transformation of wheat (Wang et al., 2018; Richardson et al., 2014). Using other strains may result in successful regeneration through *Agrobacterium*-mediated transformation in Pasteur.

- **Assessment of biotrophic pathogens in *Stmc7* mutants**

  Biotrophic pathogens present a significant threat to potato production throughout the world. PCD is an important arsenal during plant-pathogen response, especially for biotrophic

pathogens. Hemibiotrophic and necrotrophic pathogens induce PCD in plant cells, which provides them with dead cells essential for their growth and development. Reducing PCD by silencing PCD pathway genes such as *StMC7* can improve host response to hemibiotrophic and necrotrophic pathogens, as shown in potato in study IV. However, reducing PCD may negatively affect the plant response to biotrophic pathogens such as *Synchytrium endobioticum* (causing potato wart), *Blumeria graminis* (causing powdery mildew) or *Ralstonia solanacearum* (causing bacterial wilt). PCD limits the infection caused by biotrophic pathogens through cell suicide (HR-PCD) and restricts the pathogen to the point of infection. Further pathogen attack leads to higher Ca^{2+} concentration, activating apoptotic-like PCD (PCD). Reducing PCD may delay or prevent cell suicide and increase the disease severity and pathogen biomass during biotrophic pathogen infection in *Stmc7* mutants. Further studies involving biotrophic pathogens are required to assess the impact of biotrophic pathogen attack in *Stmc7* mutants.

- **Assessment of all strains of all hemibiotrophic and necrotrophic pathogens**

  Only one strain of the hemibiotrophic pathogen *P. infestans* was investigated for disease severity in study IV. Though US-8 is one of the strains causing late blight in Canada, other strains such as US-23, US-22, US-17, US-11 are more prevalent at present due to their aggressiveness and fungicide resistance. US-23 is the dominant strain in western Canadian provinces, US-24 in Manitoba, US-17 in British Colombia, US-11 in Alberta, US-22 in Ontario and US-8 in eastern Canada (Babarinde et al., 2024). All these strains can be evaluated to investigate their response to mutation in *StMC7*. In addition to the different strains, it is important to evaluate other necrotrophic pathogens such as *Rhizoctonia solani* (causing stem canker and black scurf of potato), *Pectobacterium atrosepticum* (causing

backleg of potato) in *Stmc7* mutants to assess disease resistance to hemibiotrophic and necrotrophic pathogens in potato.

- **Assessment of *Stmc7* mutants during abiotic stress**

  Metacaspases play a key role in response to abiotic stresses such as oxidative stress, salinity, heat stress and drought. Metacaspases induce programmed cell death as a response to abiotic stress after the detection of Ca^{2+} signals (Watanabe & Lam, 2011). Reducing PCD can have negative effects on the ability of plants to tolerate abiotic stress. In wheat, silencing metacaspase *TaMCA-1d* reduced PCD under salt stress, which made seedlings more sensitive to salt stress (Yue et al., 2022). Silencing type II metacaspase *AtMC8* reduced cell death triggered by oxidative stress agents such as UV-C radiation and hydrogen peroxide and made the plant more susceptible to oxidative stress (He et al., 2008). *StMC7* was found to be differentially expressed when exposed to various abiotic stresses such as salt, hormones, heat (Dubey et al., 2019). It is essential to understand the impact of silencing *StMC7* on the plant response abiotic stress.

- **Genome editing tools for better editing of *StMC7* in potato**

  Heterozygous mutation at the target site was observed in chapter IV upon CRISPR-mediated silencing. Other more precise and efficient gene editing techniques with extremely low off-target effect such as prime editing can be applied to achieve homozygous mutation in all alleles and increase disease resistance in Russet Burbank. Another advantage of prime editing would be the possibility of editing target gene sequence without any limitation. The CRISPR system requires PAM sequence region with specific sequence (-NGG for Cas9) to bind to the target DNA region. Prime editing uses prime editing guide RNA (pegRNA), which can flexibly bind to the target sequence. This increases the

availability of large areas of target genome sequence for editing, which were previously unavailable due to lack of PAM near the target sequence.

- **Understanding the actual metacaspase activation process and action**

  Though it is known that metacaspases activate upon the Ca^{2+} surge in cytoplasm, the actual mechanism is yet to be fully understood. Apoptosome have been detected in animal on the onset of apoptosis, which act as activation platform for caspases (Bao & Shi, 2007). Resistosomes have been found in arabidopsis, which triggers immune responses such as hypersensitive response (Hander et al., 2019). However, it is unknown if similar complex structures are formed as a part of AL-PCD. Cryo-electron microscopy (cryo-EM) can elucidate the actual mechanism of metacaspases activation and downstream plant cell response. Cryo-EM enables visualization of three-dimensional structures of biological macromolecular complexes at near-atomic resolution (Carroni & Saibil, 2016). Cryo-EM has been applied to understand the mechanism of Cas9 binding on DNA after the formation of Cas9-gRNA-DNA product complex (Zhu et al., 2019). So, cryo-EM could detect the presence or absence of apoptosome or similar complex structure before the onset of AL-PCD.

Literature cited

AAFC. (2022) Overview of Canada's agriculture and agri-food sector - agriculture.canada.ca. Retrieved January 22, 2024 from https://agriculture.canada.ca/en/sector/overview

Abdallah, N. A., Prakash, C. S., & McHughen, A. G. (2015). Genome editing for crop improvement: Challenges and opportunities. *GM Crops & Food*, *6*, 183–205. https://doi.org/10.1080/21645698.2015.1129937

Abid, N., Maqbool, A., & Mlaik, K. (2014). Screening commercial wheat (*Triticum aestivum* L.) varieties for *Agrobacterium*-mediated transformation ability. *Pakistan Journal of Agricultural Sciences*, *51*, 83-89. http://inis.iaea.org/search/search.aspx?orig_q=RN:45082667

Ahmar, S., Gill, R. A., Jung, K. H., Faheem, A., Qasim, M. U., Mubeen, M., & Zhou, W. (2020). Conventional and molecular techniques from Simple Breeding to Speed Breeding in crop plants: Recent advances and future outlook. *International Journal of Molecular Sciences*, *21*, 2590-2590. https://doi.org/10.3390/ijms21072590

Alberta *Fusarium graminearum* Management Plan. (2012). Alberta Fusarium Action Committee. Retrieved March 21, 2022, from https://www1.agric.gov.ab.ca/$department/deptdocs.nsf/all/agdex5210/$file/110_632-3.pdf?OpenElement

Ali, Q., Yu, C., Hussain, A., Ali, M., Ahmar, S., Muhammad Aamir Sohail, Riaz, M., Muhammad Furqan Ashraf, Dyaaaldin Abdalmegeed, Wang, X., Imran, M., Hakim Manghwar, & Zhou, L. (2022). Genome engineering technology for durable disease resistance: Recent progress and future outlooks for sustainable agriculture. *Frontiers in Plant Science*, *13*. https://doi.org/10.3389/fpls.2022.860281

Alikina, O., Chernobrovkina, M., Dolgov, S., & Miroshnichenko, D. (2016). Tissue culture efficiency of wheat species with different genomic formulas. *Crop Breeding and Applied Biotechnology*, *16*, 307-314. https://doi.org/10.1590/1984-70332016v16n4a46

Alisaac, E., & Mahlein, A. K. (2023). Fusarium head blight in wheat: Biology, modern detection and diagnosis and integrated disease management. *Toxins (Basel)*, *15*, 192-192. https://doi.org/10.3390/toxins15030192

Altpeter, F., Springer, N. M., Bartley, L. E., Blechl, A. E., Brutnell, T. P., Citovsky, V., Conrad, L. J., Gelvin, S. B., Jackson, D. P., Kausch, A. P., Lemaux, P. G., Medford, J. I., Orozco-Cárdenas, M. L., Tricoli, D. M., Van Eck, J., Voytas, D. F., Walbot, V., Wang, K., Zhang, Z. J., & Stewart, C. N., Jr. (2016). Advancing crop transformation in the era of genome editing. *Plant Cell*, *28*, 1510-1520. https://doi.org/10.1105/tpc.16.00196

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*, 403-410. https://doi.org/10.1016/s0022-2836(05)80360-2

Aoki, T., Ward, T. J., Kistler, H. C., & O'Donnell, K. (2012). Systematics, Phylogeny and Trichothecene mycotoxin potential of Fusarium head blight cereal pathogens. *JSM Mycotoxins*, *62*, 91-102 https://doi.org/10.2520/myco.62.91

Avrova, A. O., Boevink, P. C., Young, V., Grenville-Briggs, L. J., Van West, P., Birch, P. R. J., & Whisson, S. C. (2008). A novel *Phytophthora infestans* haustorium-specific membrane protein is required for infection of potato. *Cellular Microbiology*, *10*, 227-2284. https://doi.org/10.1111/j.1462-5822.2008.01206.x

Baek, M., DiMaio, F., Anishchenko, I., Dauparas, J., Ovchinnikov, S., Lee, G. R., Wang, J., Cong, Q., Kinch, L. N., Schaeffer, R. D., Millán, C., Park, H., Adams, C., Glassman, C.

R., DeGiovanni, A., Pereira, J. H., Rodrigues, A. V., van Dijk, A. A., Ebrecht, A. C., . . . Baker, D. (2021). Accurate prediction of protein structures and interactions using a three-track neural network. *Science*, *373*, 871-876. https://doi.org/10.1126/science.abj8754

Bai, G. H., Shaner, G., & Ohm, H. (2000). Inheritance of resistance to *Fusarium graminearum* in wheat. *Theoretical and Applied Genetics*, *100*, 1-8. https://doi.org/10.1007/PL00002902

Bai, G., & Shaner, G. (2004). Management and resistance in wheat and barley to Fusarium head blight. *Annual Review of Phytopathology*, *42*, 135-161. https://doi.org/10.1146/annurev.phyto.42.040803.140340

Babarinde, S., Burlakoti, R. R., Peters, R. D., Al-Mughrabi, K., Novinscak, A., Sapkota, S., & Prithiviraj, B. (2024). Genetic structure and population diversity of *Phytophthora infestans* strains in Pacific western Canada. *Applied Microbiology and Biotechnology*, *108*, 237. https://doi.org/10.1007/s00253-024-13040-6

Ballvora, A., Ercolano, M. R., Weiß, J., Meksem, K., Bormann, C. A., Oberhagemann, P., Salamini, F., & Gebhardt, C. (2002). The R1 gene for potato resistance to late blight (*Phytophthora infestans*) belongs to the leucine zipper/NBS/LRR class of plant resistance genes. *Plant Journal*, *30*, 361-371. https://doi.org/10.1046/j.1365-313X.2001.01292.x

Bao, Q., & Shi, Y. (2007). Apoptosome: a platform for the activation of initiator caspases. *Cell Death & Differentiation*, *14*, 56-65 https://doi.org/10.1038/sj.cdd.4402028

Bartlett, J. G., Alves, S. C., Smedley, M., Snape, J. W., & Harwood, W. A. (2008). High-throughput *Agrobacterium*-mediated barley transformation. *Plant Methods*, *4*, 22. https://doi.org/10.1186/1746-4811-4-22

Bekalu, Z. E., Panting, M., Bæksted Holme, I., & Brinch-Pedersen, H. (2023). Opportunities and challenges of *in vitro* tissue culture systems in the era of crop genome editing.

International Journal of Molecular Sciences, *24*, 11920. https://www.mdpi.com/1422-0067/24/15/11920

Bentley, A. R., Donovan, J., Sonder, K., Baudron, F., Lewis, J. M., Voss, R., . . . Govaerts, B. (2022). Near- to long-term measures to stabilize global wheat supplies and food security. *Nature Food, 3,* 483-486. https://doi.org/10.1038/s43016-022-00559-y

Borges, A., Rosa, M. c., Gustavo Henrique, R., Queiroz-Silva, J. R., & Elizabeth Ann, V. (2009). CTAB methods for DNA extraction of sweetpotato for microsatellite analysis. *Scientia Agricola*, *66*, 529-534. https://doi.org/10.1590/s0103-90162009000400015

Brouwer, S. M., Brus-Szkalej, M., Saripella, G. V., Liang, D., Liljeroth, E., & Grenville-Briggs, L. J. (2021). Transcriptome analysis of potato infected with the necrotrophic pathogen *Alternaria solani*. *Plants*, *10*, 2212. https://www.mdpi.com/2223-7747/10/10/2212

Brown, N. A., Urban, M., van de Meene, A. M. L., & Hammond-Kosack, K. E. (2010). The infection biology of *Fusarium graminearum*: Defining the pathways of spikelet to spikelet colonisation in wheat ears. *Fungal Biology*, *114*, 555-571. https://doi.org/10.1016/j.funbio.2010.04.006

Buerstmayr, H., Ban, T., & Anderson, J. A. (2009). QTL mapping and marker-assisted selection for Fusarium head blight resistance in wheat: a review. *Plant Breeding*, *128*, 1-26. https://doi.org/10.1111/j.1439-0523.2008.01550.x

Bull, T., Debernardi, J., Reeves, M., Hill, T., Bertier, L., Van Deynze, A., & Michelmore, R. (2023). GRF–GIF chimeric proteins enhance in vitro regeneration and Agrobacterium-mediated transformation efficiencies of lettuce (*Lactuca* spp.). *Plant Cell Reports*, *42*. 629-643. https://doi.org/10.1007/s00299-023-02980-4

Burkart, R. C., & Stahl, Y. (2017). Dynamic complexity: plant receptor complexes at the plasma membrane. *Current Opinion in Plant Biology*, *40*, 15–21. https://doi.org/10.1016/j.pbi.2017.06.016

Cardi, T., Murovec, J., Bakhsh, A., Boniecka, J., Bruegmann, T., Bull, S. E., Eeckhaut, T., Fladung, M., Galovic, V., Linkiewicz, A., Lukan, T., Mafra, I., Michalski, K., Kavas, M., Nicolia, A., Nowakowska, J., Sági, L., Sarmiento, C., Yıldırım, K., . . . Van Laere, K. (2023). CRISPR/Cas-mediated plant genome editing: outstanding challenges a decade after implementation. *Trends in Plant Science*, *28*, 1144-1165. https://doi.org/10.1016/j.tplants.2023.05.012

Carey, S. J., Becklund, L. E., Fabre, P. P., & Schenk, J. J. (2023). Optimizing the lysis step in CTAB DNA extractions of silica-dried and herbarium leaf tissues. *Applications in plant sciences*, *11*, e11522. https://doi.org/10.1002/aps3.11522

Carmona-Gutierrez, D., Fröhlich, K. U., Kroemer, G., & Madeo, F. (2010). Metacaspases are caspases. Doubt no more. *Cell Death & Differentiation*, *17*, 377-378. https://doi.org/10.1038/cdd.2009.198

Carroni, M., & Saibil, H. R. (2016). Cryo electron microscopy to determine the structure of macromolecular complexes. *Methods*, *95*, 78-85. https://doi.org/10.1016/j.ymeth.2015.11.023

Čermák, T., Curtin, S. J., Gil-Humanes, J., Čegan, R., Kono, T. J. Y., Konečná, E., Belanto, J. J., Starker, C. G., Mathre, J. W., Greenstein, R. L., & Voytas, D. F. (2017). A multipurpose toolkit to enable advanced genome engineering in plants. *Plant Cell*, *29*, 1196-1217. https://doi.org/10.1105/tpc.16.00922

Chauhan, H., & Khurana, P. (2017). Wheat genetic transformation using mature embryos as explants. In P. L. Bhalla & M. B. Singh (Eds.), *Wheat Biotechnology: Methods and*

Protocols, 1679, 153-167. Springer New York. https://doi.org/10.1007/978-1-4939-7337-8_10

Chen, H., Su, Z., Tian, B., Hao, G., Trick, H. N., & Bai, G. (2022). *TaHRC* suppresses the calcium-mediated immune response and triggers wheat Fusarium head blight susceptibility. *Plant Physiology*, *190*, 1566-1569. https://doi.org/10.1093/plphys/kiac352

Chen, Z., Debernardi, J. M., Dubcovsky, J., & Gallavotti, A. (2022). The combination of morphogenic regulators BABY BOOM and GRF-GIF improves maize transformation efficiency. *bioRxiv*, 2022.2009.2002.506370. https://doi.org/10.1101/2022.09.02.506370

Clement, K., Rees, H., Canver, M. C., Gehrke, J. M., Farouni, R., Hsu, J. Y., Cole, M. A., Liu, D. R., Joung, J. K., Bauer, D. E., & Pinello, L. (2019). CRISPResso2 provides accurate and rapid genome editing sequence analysis. *Nature Biotechnology*, *37*, 224-226. https://doi.org/10.1038/s41587-019-0032-3

Coll, N. S., Epple, P., & Dangl, J. L. (2011). Programmed cell death in the plant immune system. *Cell Death & Differentiation, 18* 1247-1256. https://doi.org/10.1038/cdd.2011.37

Collard, B. C., & Mackill, D. J. (2008). Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *363*, 557-572. https://doi.org/10.1098/rstb.2007.2170

Corkley, I., Fraaije, B., & Hawkins, N. (2021). Fungicide resistance management: Maximizing the effective life of plant protection products. *Plant Pathology*, *71*, 150-169. https://doi.org/10.1111/ppa.13467

Cram, D., Kulkarni, M., Buchwaldt, M., Rajagopalan, N., Bhowmik, P., Rozwadowski, K., Parkin, I. A. P., Sharpe, A. G., & Kagale, S. (2019). WheatCRISPR: a web-based guide RNA design tool for CRISPR/Cas9-mediated genome editing in wheat. *BMC Plant Biology*, *19*, 474. https://doi.org/10.1186/s12870-019-2097-z

De, L. C., De, T., Biswas, S. S., & Kalaivanan, N. S. (2021). Organic plant nutrient, protection and production management. In V.S. Meena, S. K. Mena, A. Rakshit, J. Stanley, & C. Srinivasarao (Eds.), *Advances in Organic Farming.* Woodhead Publishing. *115-131*. https://doi.org/10.1016/B978-0-12-822358-1.00010-9

Debernardi, J. M., Tricoli, D. M., Ercoli, M. F., Hayta, S., Ronald, P., Palatnik, J. F., & Dubcovsky, J. (2020). A GRF–GIF chimeric protein improves the regeneration efficiency of transgenic plants. *Nature Biotechnology, 38*, 1274-1279. https://doi.org/10.1038/s41587-020-0703-0

Devaux, A., Goffart, J.-P., Kromann, P., Andrade-Piedra, J., Polar, V., & Hareau, G. (2021). The potato of the future: Opportunities and challenges in sustainable agri-food systems. *Potato Research, 64*, 681-720. https://doi.org/10.1007/s11540-021-09501-4

Dickman, M., Williams, B., Li, Y., Paul de Figueiredo, & Wolpert, T. (2017). Reassessing apoptosis in plants. *Nature Plants*, *3*, 773-779. https://doi.org/10.1038/s41477-017-0020-x

Dong, S., & Zhou, S. (2022). Potato late blight caused by *Phytophthora infestans*: From molecular interactions to integrated management strategies. *Journal of Integrative Agriculture, 21,* 3456-3466. https://doi.org/10.1016/j.jia.2022.08.060

Dreisigacker, S., Sehgal, D., Reyes-Jaimez, A., Luna-Garrido, B., Muñoz-Zavala, S., Núñez-Ríos, C., Mollins, J., & Mall, S. (2016). CIMMYT wheat molecular genetics: laboratory protocols and applications to wheat breeding.

Duan, H., Richael, C., & Rommens, C. M. (2012). Overexpression of the wild potato eIF4E-1 variant Eva1 elicits Potato virus Y resistance in plants silenced for native eIF4E-1. *Transgenic Research, 21,* 929-938. https://doi.org/10.1007/s11248-011-9576-9

Henrique, Laércio Zambolim, Rodrigues, F. A., Paul, P. A., Pádua, J. G., Ribeiro, I., Antonio, & Rosado, C. (2014). Field resistance of potato cultivars to foliar early blight and its

relationship with foliage maturity and tuber skin types. *Tropical Plant Pathology*, *39*, 294-306. https://doi.org/10.1590/s1982-56762014000400004

Dubey, N., Trivedi, M., Suresh Varsani, Vyas, V., Manisha Farsodia, & Sunil Kumar Singh. (2019). Genome-wide characterization, molecular evolution and expression profiling of the metacaspases in potato (*Solanum tuberosum* L.). *Heliyon*, *5*, e011162. https://doi.org/10.1016/j.heliyon.2019.e01162

Elsharawy, H., & Refat, M. (2023). CRISPR/Cas9 genome editing in wheat: enhancing quality and productivity for global food security—a review. *Functional & Integrative Genomics, 23*, 265. https://doi.org/10.1007/s10142-023-01190-1

Erenstein, O., Jaleta, M., Mottaleb, K. A., Sonder, K., Donovan, J., & Braun, H.-J. (2022). Global trends in wheat production, consumption and trade. In M. P. Reynolds & H.-J. Braun (Eds.), *Wheat Improvement: Food Security in a Changing Climate* (pp. 47-66). Springer International Publishing. https://doi.org/10.1007/978-3-030-90673-3_4

FAOSTAT. (2023). Retrieved January 21, 2024, https://www.fao.org/faostat/en/#data/QCL

Fehér, A. (2015). Somatic embryogenesis — Stress-induced remodeling of plant cell fate. *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, *1849*, 385-402. https://doi.org/10.1016/j.bbagrm.2014.07.005

Feng, Q., Xiao, L., He, Y., Liu, M., Wang, J., Tian, S., Zhang, X., & Yuan, L. (2021). Highly efficient, genotype-independent transformation and gene editing in watermelon (*Citrullus lanatus*) using a chimeric *ClGRF4-GIF1* gene. *Journal of Integrative Plant Biology*, *63*, 2038-2042. https://doi.org/10.1111/jipb.13199

Furtado, A., Henry, R. J., & Pellegrineschi, A. (2009). Analysis of promoters in transgenic barley and wheat. *Plant Biotechnology Journal*, *7*, 240-253. https://doi.org/https://doi.org/10.1111/j.1467-7652.2008.00394.x

Gaj, T., Gersbach, C. A., & Barbas, C. F. (2013). ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends in Biotechnology*, *31*. 397-405. https://doi.org/10.1016/j.tibtech.2013.04.004

Galon, Y., Finkler, A., & Fromm, H. (2010). Calcium-regulated transcription in plants. *Molecular Plant*, *3*, 653-669. https://doi.org/10.1093/mp/ssq019

Gao, C. (2014). Genome editing in crops: from bench to field. *National Science Review*, *2*, 13-15. https://doi.org/10.1093/nsr/nwu054

Gao, Z., Zhang, B., Liu, H., Han, J., & Zhang, Y. (2017). Identification of endophytic *Bacillus velezensis* ZSY-1 strain and antifungal activity of its volatile compounds against *Alternaria solani* and *Botrytis cinerea*. *Biological Control*, *105*, 27-39. https://doi.org/https://doi.org/10.1016/j.biocontrol.2016.11.007

Garcia, N., Kalicharan, R. E., Kinch, L., & Fernandez, J. (2022). Regulating death and disease: Exploring the roles of metacaspases in plants and fungi. *International Journal of Molecular Sciences*, *24*, 312. https://doi.org/10.3390/ijms24010312

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R. D., & Bairoch, A. (2003). ExPASy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Research*, *31*, 3784-3788. https://doi.org/10.1093/nar/gkg563

Gil-Humanes, J., Victoria, C., Marin, S., Leon, E., Barro, F., & Pisto, F. (2011). Genetic transformation of wheat: Advances in the transformation method and applications for obtaining lines with improved bread-making quality and low toxicity in relation to celiac

disease. In A. María (Ed.), *Genetic Transformation* (pp. Ch. 7). IntechOpen. https://doi.org/10.5772/22246

Govrin, E. M., & Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Current Biology, 10*, 751-757. https://doi.org/10.1016/S0960-9822(00)00560-1

Gunnaiah, R. (2013). Functional characterization of wheat, fusarium head blight resistance (QTL) «Fhb1» based on non-target metabolomics and proteomics. McGill University. https://escholarship.mcgill.ca/concern/theses/k643b439k

Guo-Liang, J. (2013). Molecular markers and marker-assisted breeding in plants. In A. Sven Bode (Ed.), *Plant Breeding from Laboratories to Fields* (pp. Ch. 3). IntechOpen. https://doi.org/10.5772/52583

Haas, B. J., Kamoun, S., Zody, M. C., Jiang, R. H. Y., Handsaker, R. E., Cano, L. M., Grabherr, M., Kodira, C. D., Raffaele, S., Torto-Alalibo, T., Bozkurt, T. O., Ah-Fong, A. M. V., Alvarado, L., Anderson, V. L., Armstrong, M. R., Avrova, A., Baxter, L., Beynon, J., Boevink, P. C., . . . Nusbaum, C. (2009). Genome sequence and analysis of the Irish potato famine pathogen *Phytophthora infestans*. *Nature*, *461*, 393-398. https://doi.org/10.1038/nature08358

Halterman, D., Guenthner, J., Collinge, S., Butler, N., & Douches, D. (2016). Biotech potatoes in the 21st century: 20 years since the first biotech potato. *American Journal of Potato Research, 93*, 1-20. doi:10.1007/s12230-015-9485-1

Han, X., Yang, R., Zhang, L., Wei, Q., Zhang, Y., Wang, Y., & Shi, Y. (2023). A Review of potato salt tolerance. *International Journal of Molecular Sciences, 24*, 10726. https://doi.org/10.3390/ijms241310726

Hander, T., Fernández-Fernández Á, D., Kumpf, R. P., Willems, P., Schatowitz, H., Rombaut, D., Staes, A., Nolf, J., Pottie, R., Yao, P., Gonçalves, A., Pavie, B., Boller, T., Gevaert, K., Van Breusegem, F., Bartels, S., & Stael, S. (2019). Damage on plants activates Ca^{2+} -dependent metacaspases for release of immunomodulatory peptides. *Science*, *363*, 6433. https://doi.org/10.1126/science.aar7486

Hao, L., Goodwin, P. H., & Hsiang, T. (2007). Expression of a metacaspase gene of *Nicotiana benthamiana* after inoculation with *Colletotrichum destructivum* or *Pseudomonas syringae* pv. tomato, and the effect of silencing the gene on the host response. *Plant Cell Reports*, *26*, 1879-1888. https://doi.org/10.1007/s00299-007-0387-7

Hao, Y., Wang, X., Wang, K., Li, H., Duan, X., Tang, C., & Kang, Z. (2016). *TaMCA1*, a regulator of cell death, is important for the interaction between wheat and *Puccinia striiformis*. *Scientific Reports*, *6*, 26946. https://doi.org/10.1038/srep26946

Harwood, W. A. (2011). Advances and remaining challenges in the transformation of barley and wheat. *Journal of Experimental Botany*, *63*, 1791-1798. https://doi.org/10.1093/jxb/err380

Hasan, N., Choudhary, S., Naaz, N., Sharma, N., & Laskar, R. A. (2021). Recent advancements in molecular marker-assisted selection and applications in plant breeding programmes. *Journal of Genetic Engineering and Biotechnology*, *19*, 128. https://doi.org/10.1186/s43141-021-00231-1

Hashem, H. A., Hassanein, R. A., Fahmy, A. H., Ibrahim, A. S., El Shihyh, O. M., & Qaid, E. A. (2018). Particle bombardment-mediated co-transformation of the Cht-2 gene in wheat and the associated changes in defense mechanisms in transgenic plants infected with

Fusarium graminearum. *Data in Brief*, *21*, 1111-1118. https://doi.org/10.1016/j.dib.2018.09.130

Haverkort, A. J., Struik, P. C., Visser, R. G. F., & Jacobsen, E. (2009). Applied biotechnology to combat late blight in potato caused by *Phytophthora infestans*. *Potato Research*, *52*, 249-264. https://doi.org/10.1007/s11540-009-9136-3

Hay, W. T., Anderson, J. A., McCormick, S. P., Hojilla-Evangelista, M. P., Selling, G. W., Utt, K. D., Bowman, M. J., Doll, K. M., Ascherl, K. L., Berhow, M. A., & Vaughan, M. M. (2022). Fusarium head blight resistance exacerbates nutritional loss of wheat grain at elevated CO_2. *Scientific Reports*, *12*, 15. https://doi.org/10.1038/s41598-021-03890-9

Hayta, S., Smedley, M. A., Clarke, M., Forner, M., & Harwood, W. A. (2021). An efficient *Agrobacterium*-mediated transformation protocol for hexaploid and tetraploid wheat. *Current Protocols*, *1*, e58. https://doi.org/10.1002/cpz1.58

Heath, M. C. (2000). Hypersensitive response-related death. *Plant Molecular Biology*, *44*, 321-334. https://doi.org/10.1023/a:1026592509060

He, R., Drury, G. E., Rotari, V. I., Gordon, A., Willer, M., Farzaneh, T., Woltering, E. J., & Gallois, P. (2008). Metacaspase-8 modulates programmed cell death induced by ultraviolet light and H_2O_2 in arabidopsis. *Journal of Biological Chemistry*, *283*, 774-783. https://doi.org/10.1074/jbc.m704185200

He, Y., Jones, H. D., Chen, S., Chen, X. M., Wang, D. W., Li, K. X., Wang, D. S., & Xia, L. Q. (2010). *Agrobacterium*-mediated transformation of durum wheat (*Triticum turgidum* L. var. *durum* cv Stewart) with improved efficiency. *Journal of Experimental Botany*, *61*, 1567-1581. https://doi.org/10.1093/jxb/erq035

Hegde, N., Joshi, S., Soni, N., & Kushalappa, A. C. (2021). The caffeoyl-CoA O-methyltransferase gene SNP replacement in Russet Burbank potato variety enhances late blight resistance through cell wall reinforcement. *Plant Cell Reports, 40*, 237-254. https://doi.org/10.1007/s00299-020-02629-6

Hensel, G., Kastner, C., Oleszczuk, S., Riechen, J., & Jochen Kumlehn. (2009). *Agrobacterium*-mediated gene transfer to cereal crop plants: current protocols for barley, wheat, triticale, and maize. *International Journal of Plant Genomics, 2009*, 835608. https://doi.org/10.1155/2009/835608

Hijmans, R. J., Forbes, G. A., & Walker, T. S. (2000). Estimating the global severity of potato late blight with GIS-linked disease forecast models. *Plant Pathology*, *49*, 697-705. https://doi.org/10.1046/j.1365-3059.2000.00511.x

Hoeberichts, F. A., Arjen ten Have, & Woltering, E. J. (2003). A tomato metacaspase gene is upregulated during programmed cell death in *Botrytis cinerea*-infected leaves. *Planta, 217*, 517-522. https://doi.org/10.1007/s00425-003-1049-9

Hossain, M. A. (2021). CRISPR-Cas9: A fascinating journey from bacterial immune system to human gene editing. *Progress in Molecular Biology and Translational Science*, *178*, 63-83. https://doi.org/10.1016/bs.pmbts.2021.01.001

Huang, W., He, Y., Yang, L., Lu, C., Zhu, Y., Sun, C., Ma, D., & Yin, J. (2021). Genome-wide analysis of growth-regulating factors (GRFs) in *Triticum aestivum*. *PeerJ, 9*, e10701. https://doi.org/10.7717/peerj.10701

Ishida, Y., Yukoh Hiei, & Toshihiko Komari. (2015). High efficiency wheat transformation mediated by *Agrobacterium tumefaciens*. *Springer EBooks*, 167-173. https://doi.org/10.1007/978-4-431-55675-6_18

Ivanov, A. A., Ukladov, E. O., & Golubeva, T. S. (2021). *Phytophthora infestans*: An Overview of methods and attempts to combat Late Blight. *Journal of Fungi*, *7*, 1071. https://doi.org/10.3390/jof7121071

Jindo, K., Evenhuis, A., Kempenaar, C., Pombo Sudré, C., Zhan, X., Goitom Teklu, M., & Kessel, G. (2021). Review: Holistic pest management against early blight disease towards sustainable agriculture. *Pest Management Science*, *77*, 3871-3880. https://doi.org/10.1002/ps.6320

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., & Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science*, *337*, 816-821. https://doi:10.1126/science.1225829

Jones, A. (2000). Does the plant mitochondrion integrate cellular stress and regulate programmed cell death? *Trends in Plant Science*, *5*, 225-230. https://doi.org/10.1016/s1360-1385(00)01605-8

Kalischuk, M., Harding, M., Howard, H., Peters, R., Wijekoon, C. , LeBoeuf, J., Sabaratnam, S., Waterer, D., Bizimungu, B., Al-Mughrabi, K., & Kawchuk, L. (2016). *Characterization of Phytophthora infestans strains causing late blight provides new disease prevention strategies.* "Pathology", 100th Annual Meeting of The Potato Association of America, Amway Grand Plaza Hotel, Grand Rapids, Michigan, USA. https://profils-profiles.science.gc.ca/en/publication/characterization-phytophthora-infestans-strains-causing-late-blight-provides-new-disease

Karlsson, I., Persson, P., & Friberg, H. (2021). Fusarium head blight from a microbiome perspective. *Frontiers in Microbiology*, *12*, 2021. https://doi.org/10.3389/fmicb.2021.628373

Khanna, H. K., Paul, J.-Y., Harding, R. M., Dickman, M. B., & Dale, J. L. (2007). Inhibition of *Agrobacterium*-induced cell death by antiapoptotic gene expression leads to very high transformation efficiency of banana. *Molecular Plant-Microbe Interactions®*, *20*, 1048-1054. https://doi.org/10.1094/mpmi-20-9-1048

Kieu, N. P., Lenman, M., Wang, E. S., Petersen, B. L., & Andreasson, E. (2021). Mutations introduced in susceptibility genes through CRISPR/Cas9 genome editing confer increased late blight resistance in potatoes. *Scientific Reports, 11*, 4487. https://doi.org/10.1038/s41598-021-83972-w

Kim, S., Bae, C., Oh, S., & Choi, D. (2013). A pepper (*Capsicum annuum* L.) metacaspase 9 (*Camc9*) plays a role in pathogen-induced cell death in plants. *Molecular Plant Pathology*, *14*, 557-566. https://doi.org/10.1111/mpp.12027

Kim, S., Sim, J.-R., Lee, S.-B., Kim, B.-G., Goo, T.-W., Lim, S.-H., & Lee, J.-Y. (2021). Establishment of a stable particle bombardment transformation method in Korean commercial wheat (*Triticum aestivum* L.) varieties. *Plant Biotechnology Reports*, *15*, 151-161. https://doi.org/10.1007/s11816-021-00670-9

Knight, V. I., Wang, H., Lincoln, J. E., Lulai, E. C., Gilchrist, D. G., & Bostock, R. M. (2001). Hydroperoxides of fatty acids induce programmed cell death in tomato protoplasts. *Physiological and Molecular Plant Pathology*, *59*, 277-286. https://doi.org/https://doi.org/10.1006/pmpp.2001.0366

Kong, J., Martin-Ortigosa, S., Finer, J., Orchard, N., Andika Gunadi, Lou Ann Batts, Dhiraj Thakare, Rush, B., Schmitz, O., Maarten Stuiver, Olhoft, P., & Pacheco-Villalobos, D. (2020). Overexpression of the transcription factor GROWTH-REGULATING FACTOR5 improves transformation of dicot and monocot species. *Frontiers in Plant Science*, *11*. https://doi.org/10.3389/fpls.2020.572319

Köster, P., DeFalco, T. A., & Zipfel, C. (2022). Ca^{2+} signals in plant immunity. *The EMBO Journal*, *41*, e110741. https://doi.org/10.15252/embj.2022110741

Kumar, R., Harohalli, M. M., Kaur, A., Venkatesh, K., Grewal, A., Kumar, R., & Tiwari, V. (2017). Development of an efficient and reproducible regeneration system in wheat (*Triticum aestivum* L.). *Physiology and Molecular Biology of Plants*, *23*, 945-954. https://doi.org/10.1007/s12298-017-0463-6

Kushalappa, A. C., Hegde, N. G., Gunnaiah, R., Sathe, A., Yogendra, K. N., & Ajjamada, L. (2022). Apoptotic-like PCD inducing HRC gene when silenced enhances multiple disease resistance in plants. *Scientific Reports*, *12*, 20402. https://doi.org/10.1038/s41598-022-24831-0

Kushalappa, A. C., Yogendra, K. N., & Karre, S. (2016). Plant innate immune response: qualitative and quantitative resistance. *Critical Reviews in Plant Sciences*, *35*, 38-55. https://doi.org/10.1080/07352689.2016.1148980

Landschoot, S., Vandecasteele, M., De Baets, B., Höfte, M., Audenaert, K., & Haesaert, G. (2017). Identification of *A. arborescens*, *A. grandis*, and *A. protenta* as new members of the European *Alternaria* population on potato. *Fungal Biology*, *121*, 172–188. https://doi.org/10.1016/j.funbio.2016.11.005

Lazo, G. R., Stein, P. A., & Ludwig, R. A. (1991). A DNA transformation–competent arabidopsis genomic library in *Agrobacterium*. *Nature Biotechnology*, *9*, 963-967. https://doi.org/10.1038/nbt1091-963

Lee, H.-J., & Seo, P. J. (2021). Ca^{2+}talyzing initial responses to environmental stresses. *Trends in Plant Science*, *26*, 849-870. https://doi.org/10.1016/j.tplants.2021.02.007

Leiminger, J., Bäßler, E., Knappe, C., Bahnweg, G., & Hausladen, H. (2015). Quantification of disease progression of *Alternaria* spp. on potato using real-time PCR. *European Journal of Plant Pathology*, *141*, 295-309. https://doi.org/10.1007/s10658-014-0542-2

Li, C., Zong, Y., Wang, Y., Jin, S., Zhang, D., Song, Q., Zhang, R., & Gao, C. (2018). Expanded base editing in rice and wheat using a Cas9-adenosine deaminase fusion. *Genome Biology*, *19*, 59. https://doi.org/10.1186/s13059-018-1443-z

Li, G., Zhou, J., Jia, H., Gao, Z., Fan, M., Luo, Y., . . . Ma, Z. (2019). Mutation of a histidine-rich calcium-binding-protein gene in wheat confers resistance to Fusarium head blight. *Nature Genetics*, *51*, 1106-1112. https://doi.org/10.1038/s41588-019-0426-7

Li, J., Ye, X., An, B., Du, L., & Xu, H. (2012). Genetic transformation of wheat: current status and future prospects. *Plant Biotechnology Reports*, *6*, 183-193. https://doi.org/10.1007/s11816-011-0213-0

Li, L., Zhu, X.-M., Zhang, Y.-R., Cai, Y.-Y., Wang, J.-Y., Liu, M.-Y., Wang, J.-Y., Bao, J.-D., & Lin, F.-C. (2022). Research on the molecular interaction mechanism between plants and pathogenic fungi. *International Journal of Molecular Sciences*, *23*, 4658. https://doi.org/10.3390/ijms23094658

Liu, H., Ding, Y., Zhou, Y., Jin, W., Xie, K., & Chen, L.-L. (2017). CRISPR-P 2.0: An improved CRISPR-Cas9 tool for genome editing in plants. *Molecular Plant*, *10*, 530-532. https://doi.org/10.1016/j.molp.2017.01.003

Liu, J., Han, P., Li, M., Yan, W., Liu, J., Liu, J., He, J., Tu, W., Xia, Y., Zhou, Z., Gong, J., Liu, M., Ding, Q., & Tian, D. (2015). The histidine-rich calcium binding protein (HRC) promotes tumor metastasis in hepatocellular carcinoma and is upregulated by SATB1. *Oncotarget*, *6*, 6811-6824. https://doi.org/10.18632/oncotarget.3049

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, *25*, 402-408. https://doi.org/10.1006/meth.2001.1262

Locato, V., & De Gara, L. (2018). Programmed cell death in plants: An overview. In L. De Gara & V. Locato (Eds.), *Plant Programmed Cell Death: Methods and Protocols* (pp. 1-8). Springer New York. https://doi.org/10.1007/978-1-4939-7668-3_1

Luo, J., Li, S., Xu, J., Yan, L., Ma, Y., & Xia, L. (2021). Pyramiding favorable alleles in an elite wheat variety in one generation by CRISPR-Cas9-mediated multiplex gene editing. *Molecular Plant*, *14*, 847-850. https://doi.org/10.1016/j.molp.2021.03.024

Ma, Z., Xie, Q., Li, G., Jia, H., Zhou, J., Kong, Z., Li, N., & Yuan, Y. (2020). Germplasms, genetics and genomics for better control of disastrous wheat Fusarium head blight. *Theoretical and Applied Genetics*, *133*, 1541–1568. https://doi.org/10.1007/s00122-019-03525-8

Malik, W. A., Mahmood, I., Razzaq, A., Afzal, M., Shah, G. A., Iqbal, A., Zain, M., Ditta, A., Asad, S. A., Ahmad, I., Mangi, N., & Ye, W. (2021). Exploring potential of copper and silver nano particles to establish efficient callogenesis and regeneration system for wheat (*Triticum aestivum* L.). *GM Crops Food*, *12*, 564-585. https://doi.org/10.1080/21645698.2021.1917975

Malvestiti, M. C., Maikel B. F. Steentjes, Beenen, H. G., Sjef Boeren, Jan, & Xiaoqian Shi-Kunne. (2022). Analysis of plant cell death-inducing proteins of the necrotrophic fungal pathogens *Botrytis squamosa* and *Botrytis elliptica*. *Frontiers in Plant Science*, *13*. https://doi.org/10.3389/fpls.2022.993325

McAinsh, M. R., & Pittman, J. K. (2009). Shaping the calcium signature. *New Phytologist*, *181*, 275-294. https://doi.org/10.1111/j.1469-8137.2008.02682.x

McGill, C. R., Kurilich, A. C., & Davignon, J. (2013). The role of potatoes and potato components in cardiometabolic health: A review. *Annals of Medicine*, *45*, 467-473. https://doi.org/10.3109/07853890.2013.813633

McMullen, M., Jones, R., & Gallenberg, D. (1997). Scab of wheat and barley: A re-emerging disease of devastating impact. *Plant Disease*, *81*, 1340-1348. https://doi.org/10.1094/PDIS.1997.81.12.1340

Medvecká, E., & Harwood, W. A. (2015). Wheat (*Triticum aestivum* L.) transformation using mature embryos. *Methods in Molecular Biology*, *1223*, 199-209. https://doi.org/10.1007/978-1-4939-1695-5_16

Meena, M., Gupta, S. K., Swapnil, P., Zehra, A., Dubey, M. K., & Upadhyay, R. S. (2017). *Alternaria* toxins: Potential virulence factors and genes related to pathogenesis [Review]. *Frontiers in Microbiology*, *8*, 01451. https://doi.org/10.3389/fmicb.2017.01451

Mesterházy, Á. (1997). Methodology of resistance testing and breeding against Fusarium head blight in wheat and results of the selection. *Cereal Research Communications*, *25*, 631-637. https://doi.org/10.1007/BF03543801

Mesterházy, A., Tóth, B., Varga, M., Bartók, T., Szabó-Hevér, A., Farády, L., & Lehoczki-Krsjak, S. (2011). Role of fungicides, application of nozzle types, and the resistance level of wheat varieties in the control of Fusarium head blight and deoxynivalenol. *Toxins (Basel)*, *3*, 1453-1483. https://doi.org/10.3390/toxins3111453

Minina, E. A., Staal, J., Alvarez, V. E., Berges, J. A., Berman-Frank, I., Beyaert, R., Bidle, K. D., Bornancin, F., Casanova, M., Cazzulo, J. J., Choi, C. J., Coll, N. S., Dixit, V. M., Dolinar, M., Fasel, N., Funk, C., Gallois, P., Gevaert, K., Gutierrez-Beltran, E., . . . Bozhkov, P. V. (2020). Classification and nomenclature of metacaspases and

paracaspases: No more confusion with caspases. *Molecular Cell, 77*, 927–929. https://doi.org/10.1016/j.molcel.2019.12.020

Miroshnichenko, D., Chaban, I., Chernobrovkina, M., & Dolgov, S. (2017). Protocol for efficient regulation of *in vitro* morphogenesis in einkorn (*Triticum monococcum* L.), a recalcitrant diploid wheat species. *PLOS ONE, 12*, e0173533. https://doi.org/10.1371/journal.pone.0173533

Mittler, R., Vanderauwera, S., Gollery, M., & Van Breusegem, F. (2004). Reactive oxygen gene network of plants. *Trends in Plant Science*, *9*, 490-498. https://doi.org/10.1016/j.tplants.2004.08.009

Monino-Lopez, D., Nijenhuis, M., Kodde, L., Kamoun, S., Salehian, H., Schentsnyi, K., Stam, R., Lokossou, A., Abd-El-Haliem, A., Visser, R. G. F., & Vossen, J. H. (2021). Allelic variants of the NLR protein Rpi-chc1 differentially recognize members of the *Phytophthora infestans* PexRD12/31 effector superfamily through the leucine-rich repeat domain. *Plant Journal*, *107*, 182-197. https://doi.org/10.1111/tpj.15284

Morineau, C., Bellec, Y., Tellier, F., Gissot, L., Kelemen, Z., Nogué, F., & Faure, J.-D. (2017). Selective gene dosage by CRISPR-Cas9 genome editing in hexaploid *Camelina sativa*. *Plant Biotechnology Journal*, *15*, 729-739. https://doi.org/https://doi.org/10.1111/pbi.12671

Nazia, N., Parwsha, Z., Tayyaba, S., Kanval, S., Akmaral, U. I., & Mahmood-ur-Rahman, A. (2022). Introductory Chapter: Current trends in wheat research. In A. Mahmood-ur-Rahman (Ed.), *Current Trends in Wheat Research* (pp. Ch. 1). IntechOpen. https://doi.org/10.5772/intechopen.103763

Neelakandan, A. K., Wright, D. A., Traore, S. M., Ma, X., Subedi, B., Veeramasu, S., . . . He, G. (2022). Application of CRISPR/Cas9 system for efficient gene editing in peanut. *Plants (Basel), 11*, 1361. https://doi.org/10.3390/plants11101361

Ngou, B. P. M., Ding, P., & Jones, J. D. G. (2022). Thirty years of resistance: Zig-zag through the plant immune system. *The Plant Cell, 34*, 1447-1478. https://doi.org/10.1093/plcell/koac041

Ni, F., Qi, J., Hao, Q., Lyu, B., Ming Cheng Luo, Wang, Y., Chen, F., Wang, S., Zhang, C., Epstein, L., Zhao, X., Wang, H., Zhang, X., Chen, C., Sun, L., & Fu, D. (2017). Wheat *Ms2* encodes for an orphan protein that confers male sterility in grass species. *Nature Communications, 8*, 15121. https://doi.org/10.1038/ncomms15121

Nowicki, M., Foolad, M. R., Nowakowska, M., & Kozik, E. U. (2012). Potato and tomato late blight caused by *Phytophthora infestans*: An overview of pathology and resistance breeding. *Plant Disease, 96*, 4-17. https://doi.org/10.1094/pdis-05-11-0458

Ntui, V. O., Tripathi, J. N., & Tripathi, L. (2020). Robust CRISPR/Cas9 mediated genome editing tool for banana and plantain (*Musa* spp.). *Current Plant Biology, 21*, 100128. https://doi.org/10.1016/j.cpb.2019.100128

Odilbekov, F., Selga, C., Ortiz, R., Chawade, A., & Liljeroth, E. (2020). QTL Mapping for resistance to early blight in a tetraploid potato population. *Agronomy, 10*, 728. https://www.mdpi.com/2073-4395/10/5/728

Oz, M. T., Altpeter, A., Karan, R., Merotto, A., & Altpeter, F. (2021). CRISPR/Cas9-mediated multi-allelic gene targeting in sugarcane confers herbicide tolerance. *Frontiers in Genome Editing, 3*, 673566. https://doi.org/10.3389/fgeed.2021.673566

Paluchowska, P., Śliwka, J., & Yin, Z. (2022). Late blight resistance genes in potato breeding. *Planta, 255*, 127. https://doi.org/10.1007/s00425-022-03910-6

Park, C.-J., & Shin, R. (2022). Calcium channels and transporters: Roles in response to biotic and abiotic stresses. *Frontiers in Plant Science*, *13*, 964059. https://doi.org/10.3389/fpls.2022.964059

Paudel, B., Zhuang, Y., Galla, A., Dahal, S., Qiu, Y., Ma, A., Raihan, T., & Yen, Y. (2020). WFhb1-1 plays an important role in resistance against Fusarium head blight in wheat. *Scientific Reports*, *10*, 7794. https://doi.org/10.1038/s41598-020-64777-9

Pérez-Piñeiro, P., Gago, J., Landín, M., & Pedro, P. G. (2012). *Agrobacterium*-mediated transformation. In Y. Ö. Çiftçi (Ed.), *Transgenic Plants* (pp. Ch. 1). IntechOpen. https://doi.org/10.5772/35232

Pellegrineschi, A., Noguera, L. M., Skovmand, B., Brito, R. M., Velazquez, L., Salgado, M. M., Hernandez, R., Warburton, M., & Hoisington, D. (2002). Identification of highly transformable wheat genotypes for mass production of fertile transgenic plants. *Genome*, *45*, 421-430. https://doi.org/10.1139/g01-154

Pinello, L., Canver, M. C., Hoban, M. D., Orkin, S. H., Kohn, D. B., Bauer, D. E., & Yuan, G.-C. (2016). Analyzing CRISPR genome-editing experiments with CRISPResso. *Nature Biotechnology*, *34*, 695-697. https://doi.org/10.1038/nbt.3583

Pitzschke, A. (2013). *Agrobacterium* infection and plant defense—transformation success hangs by a thread. *Frontiers in Plant Science*, *4*, 519. https://www.frontiersin.org/articles/10.3389/fpls.2013.00519

Pixley, K. V., Falck-Zepeda, J. B., Paarlberg, R. L., Phillips, P. W. B., Slamet-Loedin, I. H., Dhugga, K. S., Campos, H., & Gutterson, N. (2022). Genome-edited crops for improved food security of smallholder farmers. *Nature Genetics*, *54*, 364-367. https://doi.org/10.1038/s41588-022-01046-7

Powderly, W. G. (2019). How infection shaped history: Lessons from the Irish famine. *Transactions of the American Clinical and Climatological Association*, *130*, 127-135. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6735970/

Purnhauser, L. (1991). Stimulation of shoot and root regeneration in wheat *Triticum aestivum* callus cultures by copper. *Cereal Research Communications*, *19*, 419-423. http://www.jstor.org/stable/23784286

Raffan, S., Oddy, J., Mead, A., Barker, G., Curtis, T., Usher, S., Burt, C., & Halford, N. G. (2023). Field assessment of genome-edited, low asparagine wheat: Europe's first CRISPR wheat field trial. *Plant Biotechnology Journal*, *21*, 1097-1099. https://doi.org/10.1111/pbi.14026

Rajarammohan, S. (2021). Redefining plant-necrotroph interactions: The thin line between hemibiotrophs and necrotrophs. *Frontiers in Microbiology*, *12*, 673518. https://doi.org/10.3389/fmicb.2021.673518

Rakotonindraina, T., Chauvin, J.-É., Pellé, R., Faivre, R., Chatot, C., Savary, S., & Aubertot, J.-N. (2012). Modeling of yield losses caused by potato late blight on eight cultivars with different levels of resistance to *Phytophthora infestans*. *Plant Disease*, *96*, 935-942. https://doi.org/10.1094/pdis-09-11-0752

Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature Protocols, 8*, 2281-2308. https://doi.org/10.1038/nprot.2013.143

Rantong, G., & Gunawardena, A. H. L. A. N. (2015). Programmed cell death: genes involved in signaling, regulation, and execution in plants and animals. *Botany*, *93*, 193-210. https://doi.org/10.1139/cjb-2014-0152

Rodewald, J., & Bodo Trognitz. (2013). *Solanum* resistance genes against *Phytophthora infestans* and their corresponding avirulence genes. *Molecular Plant Pathology*, *14*, 740-757. https://doi.org/10.1111/mpp.12036

Richardson, T., Thistleton, J., Higgins, T. J., Howitt, C., & Ayliffe, M. (2014). Efficient *Agrobacterium* transformation of elite wheat germplasm without selection. *Plant Cell, Tissue and Organ Culture*, *119*, 647-659. https://doi.org/10.1007/s11240-014-0564-7

Rosenzweig, N., Olaya, G., Atallah, Z. K., Cleere, S., Stanger, C., & Stevenson, W. R. (2008). Monitoring and tracking changes in sensitivity to azoxystrobin fungicide in *Alternaria solani* in Wisconsin. *Plant Disease*, *92*, 555-560. https://doi.org/10.1094/pdis-92-4-0555

Sajeevan, R. S., Abdelmeguid, I., Saripella, G. V., Lenman, M., & Alexandersson, E. (2023). Comprehensive transcriptome analysis of different potato cultivars provides insight into early blight disease caused by *Alternaria solani*. *BMC Plant Biology*, *23*, 130. https://doi.org/10.1186/s12870-023-04135-9

Salguero-Linares, J., & Coll, N. S. (2019). Plant proteases in the control of the hypersensitive response. *Journal of Experimental Botany*, *70*, 2087-2095. https://doi.org/10.1093/jxb/erz030

Salmensuu, O. (2021). Potato importance for development focusing on prices. *Journal of Risk and Financial Management*, *14*, 137. https://doi.org/10.3390/jrfm14030137

Saville, R. J., Gosman, N., Burt, C. J., Makepeace, J., Steed, A., Corbitt, M., Chandler, E., Brown, M., Boulton, M. I., & Nicholson, P. (2011). The "Green Revolution" dwarfing genes play a role in disease resistance in *Triticum aestivum* and *Hordeum vulgare*. *Journal of Experimental Botany*, *63*, 1271-1283. https://doi.org/10.1093/jxb/err350

Schaart, J. G., van, & Marinus J. M. Smulders. (2021). Genome editing of polyploid crops: prospects, achievements and bottlenecks. *Transgenic Research*, *30*, 337-351. https://doi.org/10.1007/s11248-021-00251-0

Schiermeier, Q. (2015). Ancient DNA reveals how wheat came to prehistoric Britain. *Nature*. https://doi.org/10.1038/nature.2015.17010

Shewry, P. R. (2009). Wheat. *Journal of Experimental Botany*, *60*, 1537-1553. https://doi.org/10.1093/jxb/erp058

Smith, W. G. (1884). *Diseases of Field and Garden Crops*. MacMillian and Co. . https://books.google.ca/books?hl=en&lr=&id=0_sEAAAAQAAJ&oi=fnd&pg=PA1&ots=rALQD8QX_M&sig=HHXwWDikunUMhAcwOekGNR7tnu0&redir_esc=y#v=onepage&q&f=false

Soni, N. (2021). Identification and functional characterization of genes conferring resistance to Fusarium head blight, underlying QTL-Fhb1, based on forward and reverse genetics approach. McGill University Libraries.

Sparks, C. A., & Jones, H. D. (2014). Genetic transformation of wheat via particle bombardment. In R. J. Henry & A. Furtado (Eds.), *Cereal Genomics: Methods and Protocols* (pp. 201-218). Humana Press. https://doi.org/10.1007/978-1-62703-715-0_17

Stridh, L. J., Mostafanezhad, H., Andersen, C. B., Odilbekov, F., Grenville-Briggs, L., Lankinen, Å., & Liljeroth, E. (2022). Reduced efficacy of biocontrol agents and plant resistance inducers against potato early blight from greenhouse to field. *Journal of Plant Diseases and Protection*, *129*, 923-938. https://doi.org/10.1007/s41348-022-00633-4

Su, Z., Bernardo, A., Tian, B., Chen, H., Wang, S., Ma, H., Cai, S., Liu, D., Zhang, D., Li, T., Trick, H., St. Amand, P., Yu, J., Zhang, Z., & Bai, G. (2019). A deletion mutation in

TaHRC confers *Fhb1* resistance to Fusarium head blight in wheat. *Nature Genetics*, *51*, 1099-1105. https://doi.org/10.1038/s41588-019-0425-8

Su, Z., Jin, S., Zhang, D., & Bai, G. (2018). Development and validation of diagnostic markers for Fhb1 region, a major QTL for Fusarium head blight resistance in wheat. *Theoretical and Applied Genetics*, *131*, 2371-2380. https://doi.org/10.1007/s00122-018-3159-6

Suarez, M. F., Filonova, L. H., Smertenko, A., Savenkov, E. I., Clapham, D. H., von Arnold, S., Zhivotovsky, B., & Bozhkov, P. V. (2004). Metacaspase-dependent programmed cell death is essential for plant embryogenesis. *Current Biology*, *14*, R339-R340. https://doi.org/10.1016/j.cub.2004.04.019

Sui, W., Guo, K., Li, L., Liu, S., Takano, T., & Zhang, X. (2019). Arabidopsis Ca^{2+}-dependent nuclease *AtCaN2* plays a negative role in plant responses to salt stress. *Plant Science*, *281*, 213-222. https://doi.org/10.1016/j.plantsci.2018.12.007

Suzuki, T., Sato, M., & Takeuchi, T. (2012). Evaluation of the effects of five QTL regions on *Fusarium* head blight resistance and agronomic traits in spring wheat (*Triticum aestivum* L.). *Breeding Science*, *62*, 11-17. https://doi.org/10.1270/jsbbs.62.11

Tanaka, J., Bastian Minkenberg, Poddar, S., Staskawicz, B., & Cho, M.-J. (2022). Improvement of gene delivery and mutation efficiency in the CRISPR-Cas9 wheat (*Triticum aestivum* L.) genomics system via biolistics. *BioRxiv (Cold Spring Harbor Laboratory)*. https://doi.org/10.1101/2022.04.09.487748

Tsiatsiani, L., Van Breusegem, F., Gallois, P., Zavialov, A., Lam, E., & Bozhkov, P. V. (2011). Metacaspases. *Cell Death & Differentiation*, *18*, 1279-1288. https://doi.org/10.1038/cdd.2011.66

Turner, R. S. (2005). After the famine: plant pathology, *Phytophthora infestans*, and the late blight of potatoes, 1845–1960. *Historical Studies in the Physical and Biological Sciences, 35*, 341-370. https://doi.org/10.1525/hsps.2005.35.2.341

Van Baarlen, P., Woltering, E. J., Staats, M., & Van Kan, J. A. L. (2007). Histochemical and genetic analysis of host and non-host interactions of arabidopsis with three *Botrytis* species: an important role for cell death control. *Molecular Plant Pathology*, *8,* 41-54. https://doi.org/10.1111/j.1364-3703.2006.00367.x

Vasil, I. K., & Vasil, V. (2005). Transformation of wheat via particle bombardment. *Humana Press EBooks*, 273–284. https://doi.org/10.1385/1-59259-959-1:273

Volynchikova, E., & Kim, K. D. (2022). Biological control of oomycete soilborne diseases caused by *Phytophthora capsici*, *Phytophthora infestans*, and *Phytophthora nicotianae* in solanaceous Crops. *Mycobiology*, *50*, 269-293. https://doi.org/10.1080/12298093.2022.2136333

Voss-Fels, K. P., Stahl, A., & Hickey, L. T. (2019). Q&A: modern crop breeding for future food security. *BMC Biology*, *17*, 18. https://doi.org/10.1186/s12915-019-0638-4

Wang, J., Hu, M., Wang, J., Qi, J., Han, Z., Wang, G., Qi, Y., Wang, H.-W., Zhou, J.-M., & Chai, J. (2019). Reconstitution and structure of a plant NLR resistosome conferring immunity. *Science*, *364*, eaav5870. https://doi.org/10.1126/science.aav5870

Wang, K., Riaz, B., & Ye, X. (2018). Wheat genome editing expedited by efficient transformation techniques: Progress and perspectives. *The Crop Journal*, *6*, 22-31. https://doi.org/10.1016/j.cj.2017.09.009

Wang, X., Wang, X., Feng, H., Tang, C., Bai, P., Wei, G., Huang, L., & Kang, Z. (2012). TaMCA4, a novel wheat metacaspase gene functions in programmed cell death induced

by the fungal pathogen *Puccinia striiformis* f. sp. *tritici*. *Molecular Plant-Microbe Interactions®, 25*, 755-764. https://doi.org/10.1094/MPMI-11-11-0283-R

Waltz, E. (2018). With a free pass, CRISPR-edited plants reach market in record time. *Nature Biotechnology, 36*. https://doi.org/10.1038/nbt0118-6b

Watanabe, N., & Lam, E. (2011a). Arabidopsis metacaspase 2d is a positive mediator of cell death induced during biotic and abiotic stresses. *Plant Journal, 66*, 969-982. https://doi.org/10.1111/j.1365-313X.2011.04554.x

Watanabe, N., & Lam, E. (2011b). Calcium-dependent activation and autolysis of arabidopsis metacaspase 2d. *Journal of Biological Chemistry, 286*, 10027-10040. https://doi.org/https://doi.org/10.1074/jbc.M110.194340

Whisson, S. C., Boevink, P. C., Wang, S., & Birch, P. R. J. (2016). The cell biology of late blight disease. *Current Opinion in Microbiology, 34*, 127-135. https://doi.org/10.1016/j.mib.2016.09.002

Wiesner-Hanks, T., & Nelson, R. (2016). Multiple disease resistance in plants. *Annual Review of Phytopathology, 54*, 229-252. https://doi.org/10.1146/annurev-phyto-080615-100037

Wijerathna-Yapa, A., Ramtekey, V., Ranawaka, B., & Basnet, B. R. (2022). Applications of *in vitro* tissue culture technologies in breeding and genetic improvement of wheat. *Plants (Basel), 11*, 2273. https://doi.org/10.3390/plants11172273

Wolter, F., Schindele, P., & Puchta, H. (2019). Plant breeding at the speed of light: the power of CRISPR/Cas to generate directed genetic diversity at multiple sites. *BMC Plant Biology, 19*, 176. https://doi.org/10.1186/s12870-019-1775-1

Wu, L. M., Wei, Y. M., & Zheng, Y. L. (2006). Effects of silver nitrate on the tissue culture of immature wheat embryos. *Russian Journal of Plant Physiology, 53*, 530-534. https://doi.org/10.1134/S1021443706040157

Wyman, C., & Kanaar, R. (2006). DNA double-strand break repair: all's well that ends well. *Annual review of genetics, 40*, 363-383. https://doi.org/10.1146/annurev.genet.40.110405.090451

Xia, R., Schaafsma, A. W., Wu, F., & Hooker, D. C. (2020). Impact of the improvements inFusarium head blight and agronomic management on economics of winter wheat. *World Mycotoxin Journal*, *13*. https://doi.org/https://doi.org/10.3920/WMJ2019.2518

Xie, K., Bastian Minkenberg, & Yang, Y. (2015). Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. *Proceedings of the National Academy of Sciences of the United States of America*, *112*, 3570-3575. https://doi.org/10.1073/pnas.1420294112

Xu, X., & Nicholson, P. (2009). Community ecology of fungal pathogens causing wheat head blight. *Annual Review of Phytopathology*, *47*, 83–103. https://doi.org/10.1146/annurev-phyto-080508-081737

Xu, Y., Zhang, L., Lu, L., Liu, J., Yi, H., & Wu, J. (2022). An efficient CRISPR/Cas9 system for simultaneous editing two target sites in *Fortunella hindsii*. *Horticulture Research*, *9*, uhac064. https://doi.org/10.1093/hr/uhac064

Yin, Y., Miao, J., Shao, W., Liu, X., Zhao, Y., & Ma, Z. (2023). Fungicide resistance: Progress in understanding mechanism, monitoring, and management. *Phytopathology®*, *113*, 707-718. https://doi.org/10.1094/PHYTO-10-22-0370-KD

Yoshida, K., Schuenemann, V. J., Cano, L. M., Pais, M., Mishra, B., Sharma, R., Lanz, C., Martin, F. N., Kamoun, S., Krause, J., Thines, M., Weigel, D., & Burbano, H. A. (2013). The rise and fall of the *Phytophthora infestans* lineage that triggered the Irish potato famine. *eLife*, *2*, e00731. https://doi.org/10.7554/eLife.00731

Yu, Y., Yu, H., Peng, J., Yao, W. J., Wang, Y. P., Zhang, F. L., Wang, S. R., Zhao, Y., Zhao, X. Y., Zhang, X. S., & Su, Y. H. (2023). Enhancing wheat regeneration and genetic transformation through overexpression of *TaLAX1*. *Plant Communications, 5,* 100738. https://doi.org/10.1016/j.xplc.2023.100738

Yue, J., Wang, Y., Jiao, J., Wang, W., & Wang, H. (2022). The metacaspase TaMCA-Id negatively regulates salt-induced programmed cell death and functionally links with autophagy in wheat. *Frontiers in Plant Science*, *13*, 904933. https://doi.org/10.3389/fpls.2022.904933

Yubing, H., Min, Z., Lihao, W., Junhua, W., Qiaoyan, W., Rongchen, W., & Yunde, Z. (2019). Improvements of TKC Technology accelerate isolation of transgene-free CRISPR/Cas9-edited rice plants. *Rice Science*, *26*, 109-117. https://doi.org/https://doi.org/10.1016/j.rsci.2018.11.001

Žarko Ivanović, Jovana Blagojević, Gordana Jovanović, Borko Ivanović, & Žeželj, D. (2022). New insight in the occurrence of early blight disease on potato reveals high distribution of *Alternaria solani* and *Alternaria protenta* in Serbia. *Frontiers in Microbiology*, *13*, 856898. https://doi.org/10.3389/fmicb.2022.856898

Zhang, S., Zhang, R., Gao, J., Gu, T., Song, G., Li, W., Li, D., Li, Y., & Li, G. (2019). Highly Efficient and heritable targeted mutagenesis in wheat via the *Agrobacterium tumefaciens*-mediated CRISPR/Cas9 system. *International Journal of Molecular Sciences*, *20*, 4257. https://doi.org/10.3390/ijms20174257

Zhang, Y., Massel, K., Godwin, I., & Gao, C. (2018). Applications and potential of genome editing in crop improvement. *Genome Biology*, *19*, 210. https://doi.org/10.1186/s13059-018-1586-y

Zhou, D., Chen, X., Chen, X., Xia, Y., Liu, J., & Zhou, G. (2023). Plant immune receptors interact with hemibiotrophic pathogens to activate plant immunity. *Frontiers in Microbiology*, *14*, 1252039. https://doi.org/10.3389/fmicb.2023.1252039

Zhou, J., Luan, X., Liu, Y., Wang, L., Wang, J., Yang, S., Liu, S., Zhang, J., Liu, H., & Yao, D. (2023). Strategies and methods for improving the efficiency of CRISPR/Cas9 gene editing in plant molecular breeding. *Plants (Basel)*, *12*, 1478. https://doi.org/10.3390/plants12071478

Zhu, H., Li, C., & Gao, C. (2020). Applications of CRISPR–Cas in agriculture and plant biotechnology. *Nature Reviews Molecular Cell Biology*, *21*, 661-677. https://doi.org/10.1038/s41580-020-00288-9

Zhu, P., Yu, X.-H., Wang, C., Zhang, Q., Liu, W., McSweeney, S., Shanklin, J., Lam, E., & Liu, Q. (2020). Structural basis for Ca^{2+}-dependent activation of a plant metacaspase. *Nature Communications*, *11*, 2249. https://doi.org/10.1038/s41467-020-15830-8

Zhu, X., Clarke, R., Puppala, A. K., Sagar Chittori, Merk, A., Merrill, B. J., Miljan Simonović, & Subramaniam, S. (2019). Cryo-EM structures reveal coordinated domain motions that govern DNA cleavage by Cas9. *Nature Structural & Molecular Biology*, *26*, 679-685. https://doi.org/10.1038/s41594-019-0258-2

Zubrod, J. P., Bundschuh, M., Arts, G., Brühl, C. A., Imfeld, G., Knäbel, A., Payraudeau, S., Rasmussen, J. J., Rohr, J., Scharmüller, A., Smalling, K., Stehle, S., Schulz, R., & Schäfer, R. B. (2019). Fungicides: An overlooked pesticide class? *Environmental Science & Technology*, *53*, 3347-3365. https://doi.org/10.1021/acs.est.8b04392