Genetic Engineering, Efficacy and Environmental Biosafety of Transgenic Pea (*Pisum sativum* L.)

by

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Abstract

Canada is the world's largest producer and exporter of field peas (*Pisum sativum* L.). Plant productivity can be greatly reduced by biotic and abiotic stresses, including fungal diseases and drought. Biotechnological tools are available for enhancing tolerance against these stresses. We developed a putative drought tolerant transgenic pea AC EARLY STAR coexpressing *PR10a* from potato (*Solanum tuberosum* L.) and transcription factor *DREB2A* from rice (*Oryza sativa* L.) using a dicistronic vector through *Agrobacterium*-mediated gene transformation. Gene expression analysis using RT-PCR was conducted on the PCR positive transgenic plants (with a transformation efficiency of 3.65%). A preliminary drought bioassay under laboratory conditions showed enhanced drought tolerance of the developed transgenic lines compared to non-transgenic lines. T3 generation has been obtained and imported to Canada. In addition, antifungal genes were previously transformed in pea in Germany and tested for their field efficacy in Canada. Transgenic lines with four antifungal genes (*1-3 β-1,3 glucanase*, *endochitinase*, *polygalacturonase inhibiting proteins* and *stilbene),* were tested for their efficacy against Fusarium root rot and Mycosphaerella blight in two different confined trials over three years (2013 to 2015) and in comparedison with two parental German lines and three Canadian lines, in Edmonton, AB. In both field trials conducted in Alberta over three years, no consistent differences in pattern of superior emergence, higher fresh weight or yield benefit, lower disease ratings between of transgenic lines in presence of pathogen inoculum wereas observed when compared to the parental and Canadian lines in the presence of pathogen inoculum. No indication of an advantage of stacked genes over single genes was observed. Most transgenic lines had lower relative gene expression in

roots than leaves, suggesting the role of promoters chosen or silencing of genes. Due to concerns about unintended consequences on non-target organisms including beneficial soil associates, pea transformed with four antifungal were tested in confined field trials in 2013, these transgenic to determine the impact of disease tolerant pea or gene products on colonization by non-lines target did not show differences in root colonization by arbuscular mycorrhizae fungius, (AMF) and nodulation by Rhizobium. Transgene insertion, as single gene or stacked genes, did not alter root colonization by AMF or root nodulation by Rhizobium inoculation in the field. We found no effects of transgenes on the plant growth performance were noted, although having a dual inoculant with both AMF and Rhizobium yielded higher shoot-to-root ratio in all the lines tested. Field trials are crucial in testing agronomic and ecological relevance of engineered traits of interest from laboratory studies.

Preface

This document is the original work of the candidate with editorial assistance by supervisors and committee members. Chapter 3 was co-authored by the candidate, Dr. Linda Hall, Dr. Alemayehu Teressa Negawo, Dr. Hans Jacobsen and Dr. Fathi Hassan. The candidate was responsible for carrying out preparations of media, reagents, reactions and plant material for *Agrobacterium*-mediated gene transfer experiments, data collection for transgenic survival, selection and maintaining the potential transgenic plants and manuscript preparation. Dr. Fathi was responsible for Agrobacterium preparation, gene cloning and designing primers. Dr. Jacobsen hosted the candidate at the Institute for Plant Genetics, Section of Plant Biotechnology, Gottfried Wilhelm Leibniz Universitat Hannover, Germany and Dr. Hassan supervised the experiments. For molecular confirmation of transgenics, Dr. Negawo carried out gene expression analysis. All co-authors provided writing and editing input. The candidate was responsible for permits from CFIA for import of plant material and cataloguing for inventory.

Chapter 4 was co-authored by the candidate, Dr. Linda Hall, Dr. Hans-Jörg Jacobsen, Dr. Syama Chatterton, Dr. Fathi Hassan and Ms. Robyne Bowness. The candidate was responsible for co-designing the trial design with suggestions from Dr. Linda Hall. The candidate was responsible for conducting the trials and all associated data collection with support from Dr. Hall's technical staff. Dr. Chatterton and her technical staff provided the pathogen characterization data. The candidate was responsible for trial and data collection for field trials for 2015 from April- June (due to maternity leave) and then led by Dr. Linda Hall. Ms. Bowness's team provided the pathogen inoculum and

training for disease severity rating to the candidate. The candidate was responsible for statistical analysis, manuscript preparation with editing and suggestions from co-authors.

Chapter 5 was co-authored by the candidate, Dr. Linda Hall, Dr. Hans Jacosben, Dr. Deng-Jin Bing and Ms. Lisa Raatz. The candidate was responsible for co-designing, execution of field trials, data collection with support from Dr. Hall and her technical staff. The candidate was responsible for setting up and data collection for field trials for 2015 from April- June (due to maternity leave) and afterwards by Dr. Linda Hall. The candidate carried out the processing of samples. Dr. Bing provided the comparator line seed for this experiment as well as for Chapter 5. The candidate was also responsible for statistical analysis and manuscript preparation with suggestions and reviews from all co-authors.

Chapter 6 was co-authored by the candidate, Dr. Linda Hall, Dr. J C Cahill Jr. and Dr. Hans Jacobsen. The candidate was responsible for designing and executing the field and lab experiments. Dr. Hall provided guidance and support. Dr. Cahill provided training for mycorrhizal colonization estimation and hosted the candidate in his lab. The candidate was responsible for manuscript preparation, data analysis with editing and guidance from all co-authors. A version of this chapter was published in Mycorrhiza: Kahlon JG, Jacobsen HJ, Cahill JF, Hall LM. 2017. Antifungal genes expressed in transgenic pea (*Pisum sativum* L.) do not affect root colonization of arbuscular mycorrhizae fungi. Mycorrhiza 27:683-694. Gene expression experiments included in Chapters 4, 5 and 6 were chiefly carried out by Dr. Hafiz Ahmed, the candidate assisted on some experiments and grew plant tissues in greenhouse. Dr. Linda Hall is listed as a co-author on each chapter and was also candidate's supervisor for the PhD program and Dr. Syama

Chatterton (listed as co-author for chapter 5) is also member of the candidate's Supervisory committee.

Dedicated to my parents Gurdial and Gurjit Gill

For their tireless efforts towards

my education and life endeavours,

and who have inspired me to become the person I am today.

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List of abbreviations

AMF	arbuscular mycorrhizae fungus
bp	base pair
C	celsius
CaMV	cauliflower mosaic virus
CFIA	Canadian Food Inspection Agency
cm	centimeter
DAI	days after inoculum
DAP	days after planting
DNA	deoxyribonucleic acid
DREB	dehyrdration responsive element binding
ERF	ethylene responsive binding element binding factors family
g	grams
GM	genetically modified
LEA	late embryogenesis abundant protein
LTA	long term average
PCR	polymerase chain reaction
PNT	plants with Novel Traits

PR	pathogenesis related
R-genes	resistance genes
RNA	ribonucleic acid
ROS	reactive oxygen species
Spp.	species

Chapter One: Introduction

1. 1. Background

Grain legumes, next to cereals, are valuable worldwide for their health and nutritional benefits and their role in sustainable cropping systems (Araújo et al. 2015). Pulses are defined as the edible seeds from grain legumes such as dry pea, lentil, chickpeas and beans (MacWilliam et al. 2014). Members of the Fabaceae family were domesticated as grain legumes at a similar time as cereals and were an important dietary component of early civilizations. Pea was among the first grain legumes domesticated in the Fertile Crescent (Smýkal et al. 2014; Smýkal et al. 2015). To date, grain legumes are integrated in cropping systems of temperate regions worldwide as sources of agro-biodiversity, protein dietary resource, phytosanitary effects in crop rotation and as a source of nitrogen for succeeding crops (Döring 2015; Peoples et al. 2009). The United Nations declared 2016 as the "International Year of Pulses" to simulate research. Neglecting legumes has compromised human health and sustainable food production (Foyer et al. 2016).

Among grain legumes, pea (*Pisum sativum* L.) stands next to soybeans and beans in economic importance worldwide (FAOSTAT 2015). Pea is also the largest pulse crop in Canada and accounts for most of our pulse exports in the multibillion-dollar industry (AAFC 2016). Canadian pulse growers have identified improved yield, seed quality and better abiotic and biotic stress tolerance as their major research priorities (Bueckert et al. 2015b; Tayeh et al. 2015; Warkentin et al. 2015). However, researchers, agronomists, end users and producers have several challenges to overcome, two being the two major fungal endemic disease complexes including Fusarium root rot (*Fusarium* spp.) and

Mycosphaerella/Aschochyta blight complex (*Peyronellaea pinodes*) which affects pea yield (Xue et al. 1997). Fusarium root rot control is reliant on crop rotation although the pathogen species involved in the complex may survive for several years in soil (Bailey 2003; Feng et al. 2010). Mycosphaerella blight complex can also survive in the soil as sclerotia and chlamydospores for several years (Bretag et al. 2006) and control strategies include crop rotation and seed treatment (Gossen et al. 2011). For both theses complexes, fungicides are either partially effective and/or not cost effective for farmers to adopt. No complete genetic resistance has been identified in the pea germplasm (Bodah et al. 2016; Conner et al. 2012; Khan et al. 2013).

Disease resistance in pea would have a benefit for the pulse industry. An alternative to conventional breeding of resistant cultivars is genetic transformation (Dita et al. 2006; Khan et al. 2013; Rubiales et al. 2015; Warkentin et al. 2015). If an effective gene conferring disease resistance could be identified and stably incorporated into the genome, the economic benefits a GM (genetically modified) crop will need to surpass the regulatory costs, and not cause disruption to Canadian markets. The traits may not be transferred to native or weed species through pollen. Additionally, GM pea may not cause non-target effects that may inadvertent harm other organisms.

Recent developments of transgenic pea in European cultivars in Germany, expressing four antifungal genes, namely 1-3 β glucanase, endochitinase (belonging to PR (pathogenesis-related) family), *polygalacturonases-inhibiting proteins* (PGIPs) and *stilbene synthase* singly and stacked, with enhanced tolerance to fungal growth *in vitro*, suggests disease resistance could be conferred via genetic modification. However, field-testing is imperative to establish trait efficacy, agronomic capacity and pertinence,

especially with soil pathogens, due to the complexity and high degree of temporal and spatial variation in soil-based ecosystems (Birch et al. 2007b), and to test the traits in intended environments (Wozniak and McHughen 2012). These GM plants could not be tested in confined field trials in Germany because of the non-transparent GM crop legislation (Nelissen et al. 2014), whereas Canada regulates products derived from genetic transformations as "novel products" known as Plants with Novel Traits (PNTs) under the auspices of Canadian Food Inspection Agency (CFIA), Health Canada and Environment Canada (CFIA 2017a). However, as with any PNT, effects of these genes or gene products on non-target species must be quantified prior to the release of crop (CFIA 2017a).

Genetic improvement of pea with abiotic stress tolerant cultivars is desired to enhance production (Sudheesh et al. 2015). Recurring droughts in the Canadian prairies have been one of the principal reasons for pea production declines in 2000's and 2010's (Agriculture and Agri-Food Canada 2016; Hickling 2003; Masud et al. 2016; Ross et al. 2015; Sun et al. 2012) and enhancing drought tolerance may increase crop productivity (Daryanto et al. 2015; Dita et al. 2006). Amongst various approaches used for improvement of drought stress tolerance, expression of PR proteins and transcription factors hold promise (El-Banna et al. 2010; Jain et al. 2012; Liu and Ekramoddoullah 2006a; Mizoi et al. 2012; Pellegrineschi et al. 2004; Yamaguchi-Shinozaki and Shinozaki 2006); however, Canadian pulse industry is yet to evaluate the option of creating drought tolerant pea cultivar with success. Drought tolerance has been successfully released as a GM trait in corn (Genuity® DroughtGard®) in the USA (Castiglioni et al. 2008; Kreimeyer et al. 2015).

1.2. Research objectives

1.2.1 Conduct genetic transformation of Canadian pea cultivars using drought tolerant genes

As a novel approach for creating drought tolerant pea, Canadian pea cultivar 'AC Early Star' was used for *Agrobacterium* mediated genetic transformation, using coexpression of PR10a from potato (*Solanum tuberosom* L.) and transcription factor DREB2A from rice (*Oryza sativa* L.) at an experienced German lab with established protocols. Focus was on quantification of stable genomic integration and inheritance of introduced genes while maintaining transformation efficacy and is covered in Chapter 3.

1.2.2. Quantify disease resistance of transgenic pea lines to Fusarium root rot (*Fusarium spp.*) under field conditions

To determine the viability of antifungal genes as an option for disease tolerance, field efficacy trials depicting the tolerance of PNT lines to *Fusarium* spp. under field conditions, are necessary. These genes are publically available for use and are not patent protected. However, the trials need to be conducted with utmost care to confine the PNT seed movement in order to keep Canadian pulse industry from exposure to PNT. To address this, three years of confined field trials were established and PNT lines were challenged with *Fusarium* spp. (Chapter 4) and following hypotheses were made:

i) The transgenic pea lines will have enhanced disease resistance in comparison to conventional pea to Fusarium root rot under field conditions;

ii) Transgenic lines with stacked genes will have an advantage over single gene insertions in response to Fusarium root rot in field.

1.2.3. Quantify disease resistance of transgenic pea lines to Mycosphaerella blight (*P. pinodes*) under field conditions

Similar to the above research objective, in order to determine the efficacy of antifungal genes against Mycosphaerella blight, field trials are necessary. Confined field trials for three years under auspices of CFIA were established and PNT lines were challenged with *P. pinodes* (Chapter 5) and following hypotheses were made:

i) The transgenic pea lines will have enhanced disease resistance in comparison to conventional pea to Mycosphaerella blight under field conditions;

ii) Transgenic lines with stacked genes will have advantages over single gene insertions in response to Mycosphaerella blight in field.

1.2.4. Quantify potential non target effects of the anti-fungal genes to beneficial soil associates

CFIA regulates the environmental release of PNTs in Canada. Hence, these PNTs (disease tolerant pea lines) must be quantified prior to release for their effects of gene products on non-target species in the field as part of environmental risk assessment (CFIA 2017a). Confined field trial was established in 2013 to determine the effect of disease tolerant peas on beneficial soil associates arbuscular mycorrhiza and Rhizobium (Chapter 6) and the following hypotheses were made:

i) The transgenic pea lines will have no adverse effects on root colonization by arbuscular mycorrhiza and root nodulation by Rhizobium in the field.

ii) Transgenic lines with stacked and single genes will have different responses to root colonization and nodulation.

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Chapter Two: Literature Review

2.1 Pea

Pea (*Pisum sativum* L.) is a cool season annual legume that belongs to the Fabaceae family (formerly known as Leguminosae family) and is among the first domesticated crops (Harlan 1992). Pea is grown worldwide because of its exceptional nutritional benefits (reviewed by Burstin et al. (2011); Dahl et al. (2012), capacity to fix atmospheric nitrogen, leave soil nutrient benefits for succeeding crops (Xie et al. 2017) and a role as a break crop for reducing pest pressure (MacWilliam et al. 2014); hence, peas are an essential part of sustainable cropping systems (Jensen et al. 2012). Particularly for human nutrition, peas can be used in diverse forms: fresh seedlings, immature pods and dry seeds cooked in various dishes (Tayeh et al. 2015). The pea genome is organized into 7 pairs of chromosomes (2n=14) and the mature seed phenotype for field peas is round (genetically, *RR*) and yellow, green and red cotyledon varieties exist (Warkentin et al. 2015). Amongst its various benefits, pea also served as the basis for Mendel's principles of heredity that forms the basis of modern day plant genetics (Bateson and Mendel 2013).

2.1.1 Pea production in Canada

Canada is the largest producer and exporter of field peas (3.4 MT), and production has been consistently increasing in the prairie provinces of Saskatchewan, Alberta and Manitoba for the past two decades (Raghunathan et al. 2017; Statistics Canada 2016; Taheri et al. 2016). Since prairies play an important role in Canadian agriculture (85% of Canadian farmland), agronomic benefits of including pulse crops in rotations in otherwise

monoculture cereal and canola cropping system, are improved returns to farmers, reduced inputs and optimized crop management (MacWilliam et al. 2014).

2.1.2 Major uses

Peas is a valuable crop, its seeds are an exceptional diet with a diverse nutrient profile: major constituents are starch (from 18.6 to 54.1%) and proteins (15.8–32.1%), followed by fiber (5.9–12.7%), sucrose (1.3–2.1%), and oil (0.6–5.5%) and minerals, vitamins and micronutrients which makes them an excellent source for human consumption and livestock feed (Tayeh et al. 2015). Their capacity to fix atmospheric nitrogen promotes better environmental stewardship leading to: 1) reduction input costs such as fertilizers 2) reduced greenhouse gas emissions and 3) increased nitrogen availability for subsequent crops (Luce et al. 2015; O'Donovan et al. 2014). When integrated in rotation, pea crop is highly desired, as it provides fertility (Beckie et al. 1997), soil tilth improvement (Grant and Lafond 1993; McPhee 2003), impedes disease cycles (Kirkegaard et al. 2008), decreases fungal diversity and composition of soil fungal community (Bainard et al. 2017), and can improve yield and quality of succeeding crops (Ross et al. 2015). Canada primarily grows yellow and green peas and smaller quantities of maple, marrowfat and Austrian winter pea are also grown (www.pulsecanada.com). Pea production in western Canada is limited by various factors, including biotic stresses including fungal diseases, insects, weeds and abiotic stresses such as drought.

2.2. Diseases of pea in Western Canada

Pea production has been increasing in the three prairie provinces consistently and disease pressure has increased (Taheri et al. 2016). The two major endemic fungal disease

complexes of pea have been identified as major threats to pea industry by Canadian pea growers - these are Fusarium root rot (*Fusarium avenaceum*) (Fr.) Sacc., (syn. *Gibberella avenacea* R. J. Cook) (Xue 2003) and Mycosphaerella/Ascochyta blight complex (*Peyronella pinodes*) (Berk. & A. Bloxam) Aveskamp, Gruyter & Verkley (syn. *Mycosphaerella pinodes* (Berk. & A. Bloxam) Vestergr.) (Xue et al. 1997). Some other minor fungal or oomycete caused diseases also prevail in pea crops grown in the prairies; for example, powdery mildew (*Erysiphe pisi* Syd.) and bacterial blight (*Pseudomonas syringae* pv. *pisi* (Sackett) Young, Dye & Wilkie (Gossen et al. 2011) and downy mildew (*Peronospora viciae* f. sp. *pisi*) (Chang et al. 2013). The two major disease complexes are discussed below.

2.2.1. Fusarium root rot complex

Fusarium is a cosmopolitan genus of filamentous fungi belonging to the Ascomycota (Sordariomyctes: Hypocreales: Nectriaceae). *Fusarium* spp. includes many toxin-producing plant pathogens of agricultural importance (Chelkowski 2014; Ma et al. 2013), whose relative significance can vary by soil type, host plant and management practices (Taheri et al. 2016; Xue 2003). *Fusarium* pathogens can cause symptoms acting individually or synergistically, such as seed rot, root rot, foot rot, seedling blight, wilt, cankers, blights, reduction in stand establishment, reduced nitrogen fixation, in horticultural, field, ornamental and forest crops of agricultural and natural ecosystems (Hwang et al. 2001; Ma et al. 2013; Xue 2003). Fusaria can infect healthy host tissue employing various strategies. They are often classified as hemibiotrophs, as the infection process resembles that of a pathogen relying on living hosts (biotrophic) but eventually growing into and killing and consuming host cells (necrotrophic) (Ma et al. 2013).

Fusarium spp. often have extended host ranges and many are found in natural habitats as competitive saprotrophs on crop debris or resting spores, hence, growing several crops in rotation may not be able to reduce the disease potential of the fungus (Dixon and Tilston 2010). On disintegration of infected tissue, hyphae and conidia become long living chlamydospores, which can survive in soil for several years (Feng et al. 2010; Gossen et al. 2016). Also, host specificity varies among *Fusarium* species; for example, *F. oxysporum* species complex can cause wilt diseases in over a hundred agronomic plant species. Furthermore, the genome of *Fusarium* is compartmentalized and there are specified regions for critical functions and for host specialization and pathogen virulence, making it very adaptable to various niches (eg., virulence on specific hosts, growth in various environments, etc.) (Ma et al. 2013).

The Fusarium root rot complex on peas is often associated with various causal organisms such as *Fusarium solani*, *F. avenaceum*, *F.oxysporum*, *F.redolens*, *F. culmorum*, *Alternaria* spp., *Phoma* spp., *Sclerotinia sclerotiorum*, *Pythium* spp., and most recently, *Aphanomyces euteiches* (Chatterton et al. 2015; Esmaeili Taheri et al. 2011; Esmaeili Taheri et al. 2017; Feng et al. 2010; Xu et al. 2012a; Xue 2003). However, *Fusarium* species and especially *F. avenaceum* has been identified as chief causal agent of pea root rot in Alberta (Chang et al. 2007; Feng et al. 2010; Xue 2003). Yield losses of up to 30-57% of pea crops have been reported in commercially and manually inoculated plots in Canada (Basu et al. 1976). Symptoms appear as brown to blackish lesions with red discoloration of the vascular system of roots. Fine roots can be completely demolished resulting in lower root nodulation. *F. avenaceum* is a generalist necrotrophic fungus whose isolates have shown great genetic and ecological plasticity and hence can

affect root tissues of legumes, head and root tissues of cereals, etc. (Abdellatif et al. 2010) leading to root rots associated with common prairie crops like canola (Chen et al. 2014), wheat (Tyburski et al. 2014), faba bean (Chang et al. 2014) and lentil (Hwang et al. 2000). The fungus can produce toxins such as fusarin C, moniliformin and beauvercin; however, because the fungus is usually limited to roots (underground parts), it is not considered as a threat to human or livestock consumption (Feng et al. 2010). Current management strategies are reliant majorly on crop rotation as no fungicides are effective and only partial disease resistance exists in pea germplasm (Bodah et al. 2016).

2.2.2. Mycosphaerella blight complex

Under cool and wet conditions, this disease complex can cause an average of 50% yield losses in commercial peas (Xue et al. 1997). Often, three species: *Ascochyta pisi*, *Phoma medicaginis* var. *pinodella* and *Peyronella pinodes* are associated with Mycosphaerella blight complex (Bretag et al. 2006; Kumar and Banniza 2017); however, *Peyronella pinodes* is the most widely distributed and damaging pathogen of all in Canada (Gossen et al. 2011; Khan et al. 2013; Liu et al. 2013; Tivoli et al. 1996). In recent surveys in Canadian prairies, 99% of tested isolates, belonged to *P. pinodes* in recent surveys in Canadian prairies and their aggressiveness is on an increasing (Ahmed et al. 2015). Other species of *Peyronella* are known to cause Ascochyta blight of chickpea and lentil (Barilli et al. 2016; Owati et al. 2017). This foliar hemibiotrophic fungus penetrates the host epidermal cells directly (Suzuki et al. 2017), attacking all plant parts i.e. leaves, stems, flowers and pods. Symptoms include small characteristic purplish black lesions with irregular margins, which later coalesce to form bigger lesions and eventually blighting the whole plant starting from foot rot hence lowering the quality (Gossen et al. 2011),

harvestability and lodging. There is a significant reduction in seed number and size affecting the overall yield (Tivoli et al. 1996; Xue et al. 1997). The chlamydospores can survive for years, making the control even harder (Bretag et al. 2006). Western Canadian farmers rely on cultural management strategies like crop rotation and seed treatment (Gossen et al. 2011). Most available fungicides are partially effective or not cost effective (Khan et al. 2013), however, the quest for chemical option continues (Bowness et al. 2016). So far, only partial resistance has been found in the pea germplasm (Carrillo et al. 2013; Conner et al. 2012; Fondevilla et al. 2008; Zhang et al. 2007), although breeding efforts utilizing quantitative trait loci are currently in progress (Jha et al. 2017). Repetitive growing of pea crops in same fields (Bainard et al. 2017) and variation in virulence in *P. pinodes* populations in prairies are contributing to the complex nature of selection for resistance trait (Khan et al. 2013).

2.3 Drought response in pea

Pea is considered a cool season crop, but its production has seen a steady increase in dry warm regions of Canada (Huang et al. 2017). Pea is highly sensitive to climatic conditions during its growing cycle (Bénézit et al. 2017) and yield can easily be affected by abiotic stresses such as drought (Daryanto et al. 2015; Iglesias-García et al. 2015). In Canadian prairies, drought has been a recurring phenomenon (Sun et al. 2012) and caused pea production declines in 2000's and 2010's in western Canada (Agriculture and Agri-Food Canada 2016; Hickling 2003; Ross et al. 2015). The occurrence of frequent drought and heat waves especially during reproductive phases has led to consideration of drought tolerance into pea improvement programs (Warkentin et al. 2015). Under drought stress pea can decrease photosynthesis, reduce the plant root/shoot ratio, reduce transpiration,

increase epicular waxes and reduce transpiration rate, produce reactive oxygen species (ROS) and accumulate osmotically active metabolites, such as amino acids like proline, valine and sugars (for a review see Araújo et al. 2015)). Many stress-induced genes and gene products are activated in the plant as a response to drought, such as activation/crosstalk of ROS detoxification enzymes, phytohormones, transcription factors, protein kinases, pathogenesis related (PR)10 proteins, etc. (for a review see Rejeb et al. 2014). Hence, overexpression of stress induced genes and transcription factors etc. (Kumar et al. 2003) can enhance drought tolerance in pea. However, owing to the narrow gene of pool of pulse crops (Kumar et al. 2003), such intricate characteristics such as abiotic stress tolerance are difficult to select for and incorporate (Dita et al. 2006; Moran et al. 1994; Pellegrineschi et al. 2004). Enhancing pea for drought tolerance can positively affect crop productivity (Daryanto et al. 2015; Dita et al. 2006; Magyar-Tábori et al. 2011); however, most efforts are currently restricted to evaluations of molecular markers to select tolerant genotypes (Iglesias-García et al. 2015) and exploring possibilities in wild germplasm (Muñoz et al. 2017; Naim-Feil et al. 2017).

2.4 Biotechnological approaches

Although breeding efforts for biotic and abiotic stresses in pea are in place (Warkentin et al. 2015), genetic transformation could aid in classical breeding program. Despite the advent of transgenic technology in the 1980's and a first report of the development of a GM pulse crop i.e. moth bean (*Vigna aconitifolia* L. Jacq*)* merely 7 years later (Eapen et al. 1987; Köhler et al. 1987a; Köhler et al. 1987b) the advancement in case of legumes are not as remarkable as that of cereals (Eapen 2008). The scarcity of efficient reproducible genetic transformation methods (Carlos Popelka et al. 2004), unavailability

of complete nuclear genome (Kosterin 2016) and cost of regulatory process that needs to be amortized for a relatively minor crop (Kalaitzandonakes et al. 2007) are major reasons for a lag in GM pulse crop development.

2.4.1 Transformation Methods in Pulse crops

The process of genetic transformation involves choosing a trait, identifying and isolating the gene(s) and eventual transfer of those gene(s) into the plant. The DNA sequences introduced into the plant must integrate, express and maintain themselves in the genome for subsequent generations and be competent of regeneration in the whole plant (Atif et al. 2013). Amongst various gene delivery systems used in pulses, direct gene transfer methods (mediated by physical and chemical force to be delivered into the target tissues, such as electroporation (Puonti-Kaerlas et al. 1992; Warkentin et al. 1992) electroporation and PEG mediated transformation together (Köhler et al. 1987a; Köhler et al. 1987b)) and *Agrobacterium* mediated gene transfer (utilizing the inherent capacity of vector bacterium *Agrobacterium tumefaciens* to transfect genes into plant (Schroeder et al. 1993; Schroeder et al. 1995), are commonly used. Particularly for pea, *A. tumefaciens* mediated gene transfer system (because of higher transformation efficacy) has been historically utilized because of higher transformation efficacy (Eapen 2008; Somers et al. 2003). Since most of the fabaceae family regenerates from young embryonic tissues, embryonic axes (Hassan et al. 2009; Krishnamurthy et al. 2000) and cotyledonary nodes (Kumar et al. 2004)are most favored explants for transformation.

Three pathways of *de novo* organogenesis, somatic embryogenesis and reproduction of shoot meristems from vicinity of shoot buds, are generally employed for plant regeneration in pulses (Jaiwal and Singh 2013). Among these, meristematic areas of

cotyledonary nodes are the most reliable explant source in *P. sativum* (Pniewski and Kapusta 2005). Major selectable marker genes employed in pea, for selection of transgenic cells and plants are, neomycin phosphotransferase (*npt*II) gene (conferring resistance to kanamycin) (Puonti-Kaerlas et al. 1990; Schroeder et al. 1993), hygromycin phosphotransferase (*hpt*) gene (resistant to hygromycin B)(Puonti-Kaerlas et al. 1990) and *bar* gene (resistant to herbicide) (Richter et al. 2006c).

2.4.2 Successes in pulse crops

Genes of economic importance have been introduced to various pulse crops via *Agrobacterium* mediated gene transfer method (reviewed by (Atif et al. 2013; Dita et al. 2006; Eapen 2008; Somers et al. 2003). Despite the recalcitrant nature (Nguyen et al. 2016; Polowick et al. 2000b), some pulses are easier to transform than others (Somers et al. 2003). The major focuses have been herbicide resistance and insect resistance but to date,no registered GM pea is available to farmers (ISAAA 2016). Generation of insect resistant genetically modified pea (Schroeder et al. 1995; Shade et al. 1994; Teressa Negawo et al. 2016), transgenic pea with improved source-sink partitioning (Zhang et al. 2015) and drought tolerant pea (Kahlon et al., 2017 unpublished, discussed in chapter 3) have been reported, but no field trials to test the efficacy of traits are in pipeline. However, the oilseed legume, transgenic glyphosate resistant transgenic soybean (Padgette et al. 1995) is to date, the most successful genetic modification in the Fabaceae family (Arruda et al. 2013). Another recent success is the release of Bean Golden Mosaic Virus resistant transgenic bean cultivars in Brazil (Aragão and Faria 2009; Aragão et al. 2013), and is indeed encouraging.

2.5 Traits of interest

Continued enhancement of grain yield is important for peas to remain a lucrative option in comparison to cereals in crop rotations for farmers (Duc et al. 2015; Foyer et al. 2016). Meanwhile, fungal diseases of peas are the major biotic stress, followed by insects and viruses, whereas main abiotic stresses are drought and heat stress especially during flowering, frost and salinity (Tayeh et al. 2015). Canadian pulse growers have identified improving biotic and abiotic stress tolerance and enhancing yields components and seed quality as the priority research areas for pea crop improvement (Bueckert et al. 2015b; Tayeh et al. 2015; Warkentin et al. 2015). There is also a lot of interest in optimization of pea interactions with beneficial organisms, for example rhizobia and mycorrhiza as well as crop stresses, modification of plant morphology and phenology to novel cropping systems, adaption of seed composition for novel end uses (Duc et al. 2015; Tayeh et al. 2015). Two major Canadian programs are pivotal in pea improvement, namely University of Saskatchewan at Saskatoon, SK and Agriculture and Agri-food Canada at Lacombe, AB and are currently working on targets of early maturing, high yielding pea cultivars with resistance to powdery mildew, Mycosphaerella blight and lodging, and with exceptional quality for export and domestic ventures. More focus is on yellow and green cotyledon pea (60% and 30% of activity focus respectively), whereas specialty field pea markets (for example, marrowfat, dun, maple and forage) make up only 10% (Warkentin et al. 2015). In Canada and worldwide, amidst the issues of food security and climate change, abiotic and biotic stress tolerance remains the most desired trait to incorporate in pea (Considine et al. 2017).

2.5.1 Disease tolerance

Acquiring disease resistance/tolerance is deemed as the most effective strategy of controlling diseases in a plant. However, with many pathogens when no source of effective resistance or tolerance exists, genetic engineering may provide a solution (Collinge et al. 2016). Among several genetic engineering strategies to combat fungal plant pathogens, three are commonly utilized in the plant world:

1) Direct interference with pathogen physiology

1a) constitutive expression of anti-microbial factors (for example, oxalate oxidase)

1b) pathogen induced gene expression in transgenic plant (for example, stilbene synthase)

2) Regulation of innate induced host defenses

2a) altering the recognition of the pathogen (for example, R genes)

2b) downstream regulatory pathways (for example, SAR), including transcription factors

Pathogen mimicry (often utilized in viral resistance) (Collinge et al. 2008).

The most commercially important traits of interest, their biological functions and anti-pathogen activity are discussed below:

2.5.1.1 Pathogenesis related proteins

Compounds that inhibit fungal growth are abundantly present and are a great source of natural resistance in plants (Hegedüs and Marx 2013; van der Weerden, Nicole L et al. 2013). Genes encoding for such compounds are often screened and inserted in plants to enhance fungal disease resistance. Among these, pathogenesis related proteins (PR) are proteins produced in host plant as a result of specific pathological situation and are induced systemically, often associated with systemic acquired resistance (SAR) against

pathogen infection (Van Loon and Van Strien 1999). Chitinase and glucanase are the two most pivotal enzymes in area of plant and fungal populations that belong to the PR protein family. Both catalyze the hydrolysis of two major structural components of fungal cell walls, chitin and glucan, respectively (Saharan et al. 2016).

Chitinases have been identified from many plants and microorganisms and are broadly known as PR-3, PR-4, PR-8, PR-11 class of proteins. They cleave internal β-1,4-glycoside bonds of chitin (a homopolymer of β-(1 to 4)-linked *N*-acetylglucosamine (GlcNAc) units present abundantly in cell walls of living organisms) and chitooligomers (Collinge et al. 1993; Neuhaus et al. 1991). Chitinases perform various physiological and ecological roles in living organisms (Grover 2012). For instance, they digest chitin for usage as carbon and nitrogen sources in bacteria, modify cell wall and aid in daughter cell separation in yeast, and aid in conjugation with proteases to help insects molt (Cletus et al. 2013). When expressed constitutively, they participate in cell division, flower development, somatic embryogenesis, seed development and programmed cell death. Induced expression of chitinases in response to abiotic stresses (osmotic pressure, drought, salinity, heavy metals or wounding) and biotic stresses (bacteria, fungi or virus attacks) have been observed (Cletus et al. 2013; Collinge et al. 1993; Van Loon et al. 2006). Chitinases recognize benign microbes such as root nodulating bacteria (Rhizobium spp.) and mycorrhizal fungi and allow symbiotic relationships with host plant (Cletus et al. 2013). Since chitin is not present in mammals and plants, chitin metabolism could be targeted with chitinases against agriculturally important fungi and insects (Nagpure et al. 2014). Various *in vitro* studies with purified chitinases and *in vivo* studies with transgenic plants overexpressing chitinases have shown that these enzymes

play an antifungal role by directly hydrolyzing chitin and indirectly by activation of SAR (Arlorio et al. 1992; Collinge et al. 1993; Sandhu et al. 2017). Chitinases are also known for their antifungal effect on phytopathogenic fungi belonging to Phyla Ascomycota and Basidiomycota (Punja 2004).

Another PR family (PR-2 class) member glucanase are inducible and expressed under stresses such as during pathogen attack, wounding and other physiochemical changes (Van Loon et al. 1994). Glucanases have direct antifungal activities directly by catalyzing the hydrolytic breakdown of β 1,3/1,6-glucans of fungal cell walls and indirectly by partially digesting glucans and chitin, leading to cell lysis and death (Balasubramanian et al. 2012; Yan et al. 2015). Apart from antifungal activity, they have important roles in cell division, trafficking of materials through plasmodesmata, flower formation, seed maturation, fruit ripening and combating abiotic stresses (Balasubramanian et al. 2012). In addition, the two PR-protein families (chitinase and glucanase) indirectly induce plant defense mechanism by release of β 1,3-glucan and chitin oligosaccharides (elicitors) that are recognized and transduced by plant's localized and systemic defense systems (Somssich and Hahlbrock 1998).

Together, chitinases and glucanases are probably the most frequently researched PR proteins (Moosa et al. 2017). Synergistic effects of chitinase and glucanase against fungal pathogens are well demonstrated (Mauch et al. 1988) and utilized for improving plant defense (Anand et al. 2003; Ceasar and Ignacimuthu 2012; Zhu et al. 1994). Lack of chitin in plants and associated induction of chitinase, glucanase and fungal resistance in plants suggests the role of these hydrolytic enzymes in systemically induced resistance (Mauch and Staehelin 1989; Sela-Buurlage et al. 1993). However, the effectiveness of

one hydrolase is suggested to be dependent on concurrent activity of another to hydrolyze chitin-glucan polymers of fungal cell walls (Stintzi et al. 1993). Table 1 summarizes the transgenic legume crops developed for disease resistance against various pathogens utilizing chitinases, glucanases and combinations of both. For usage as antifungal strategy in other crops, please see latest review by Sandhu et al. (2017).

Defensins and thionins are small, cysteine rich PR antifungal microbial peptides belonging to PR-12 family and PR13 family, respectively, and cause membrane disruption by pore formation in cell membranes, ultimately leading to cell death (Moosa et al. 2017). For example, Drr23oa (a defensin) isolated from pea, exhibited in vitro sctiviity against fungal growth and germination of the soybean pathogens *Fusarium tucumaniae* and *Colletotrichum gossypii* var. *cephalosporioides* (Lacerda et al. 2016). Another class of PR-5 family's thaumatin-like proteins or osmotin-like proteins have also shown promises of anti fungal activity in various crops (for example, in potato (Acharya et al. 2013) and wild peanut (Singh et al. 2013). PR proteins can behave as food allergens, latex allergens and pollen allergens, which can be a concern for public acceptance when transgenic crops expressing PR proteins become commercially available (Sinha et al. 2014; Van Loon et al. 2006). Apart from this, lower expression levels of chitinase and glucanase transgenes rely on host internal system (for example, intracellular pH, cellular localization and environmental stress (Sela-Buurlage et al. 1993), and hence isolation and selection processes for different chitinases and glucanases becomes very important for expression in the target crop (Saharan et al. 2016). The continuous interest in PR protein expressed singly or synergistically with more than one gene, for their antifungal activities distinguishes them as a promising and plausible

strategy for combating phytopathogens (reviewed by (Moosa et al. 2017; Sandhu et al. 2017)).

2.5.1.2 Phytoalexins

Phytoalexins are antimicrobial, low molecular weight compounds that are induced in plants after infection or stress. The greatest number of phytoalexins has been derived from Leguminosae (now known as Fabaceace) (130) (Kuc 1995). Most phytoalexins inhibit phytopathogenic fungal growth but some are also toxic to bacteria, nematodes and other organisms and often considered markers for plant disease resistance (Jeandet et al. 2002; Singh and Chandrawat 2017). Biochemically, they are derived from the shikimic-polymalonic acid pathway and different plant families are known to produce different type of phytoalexins. For example, Poaceae forms mostly diterpenoids, Leguminosae forms isoflavones and Vitaceae produce stilbenes (Dercks et al. 1995; Singh and Chandrawat 2017). Even though most phytoalexins are less toxic than commercial fungicides, they can accumulate in huge quantities within plant tissues, with concentrations more than necessary to inhibit fungal growth (Jeandet et al. 2002). Various groups of phytoalexins are available, yet the Vitaceae family's stilbenes are amongst the most studied for transgenic disease resistance (Bavaresco et al. 2009).

Even with a huge chemical diversity, phytoalexins from Vitaceae family constitute a rather restricted group of molecules belonging to stilbene family and that have a trans-resveratrol based structure (Jeandet et al. 2002; Sotheeswaran and Pasupathy 1993). The ability of grapevine to induce its defense mechanisms against some pathogens is often connected to the synthesis of trans-resveratrol and its oligomers such as inducible viniferins (Bavaresco et al. 2016). Biologically, stilbenes are formed in the

phenylalanine/polymalonate pathway. The last step of this biosynthesis pathway is catalyzed by stilbene synthase, which produces the simple stilbene phytoalexins in one enzymatic reaction from coenzyme A-esters of cinnamic acid derivatives (p-coumaroyl-CoA in the case of resveratrol synthesis (detail: (Jeandet et al. 2002)). Resveratrol inhibits or reduces conidial germination of various fungi; however, the mechanism leading to stilbene toxicity in fungal cells is not well understood (Ahuja et al. 2012; Chong et al. 2009). Apart from fungal attack, abiotic stress, ultra violet (UV)-irradiation is known as a trigger for stilbene synthase (Bavaresco et al. 2009). Stilbene synthase is closely related to chalcone synthase, the key enzyme in flavonoid-type compound biosynthesis (Schröder 1990), as they share a 70 to 75% identity at the protein level and use the same substrates and catalyze the same condensing-type of enzyme reaction, but form two different products- chalcone and stilbenes, respectively (Schroder et al. 1988).

Stilbene synthase is naturally synthesized in grape berries (Jeandet et al. 1991), where cis- and trans- isomers of resveratrol are produced during all stages of development in the skin but none in the flesh (Versari et al. 2001). Different monomers, oligomers and tetramers of resveratrol are constitutively present in healthy grapevine stem and roots (Chong et al. 2009). In addition to their role in plant pathogen interactions, constitutive stilbenes can also act as allelochemicals (Fiorentino et al. 2008) and have antioxidant activities (Privat et al. 2002). Transgenic plants have been created using the anti-fungal properties of stilbene genes in numerous crops, however so far, they have been utilized only in transgenic pea development from the Fabaceae family (Amian A et al. 2011; Richter et al. 2006c).

2.5.1.3 PGIPs

Cell wall degrading enzymes such as endo and exo-polygalacturonases (PGs) are secreted by fungus at the penetration stage by phytopathogenic fungi and this forms an important step in pathogenesis (De Lorenzo et al. 2001). These PGs cleave the α-(1–4) linkages between the main element of pectin ; the D-galacturonic acid residues of homogalacturonan, causing cell partition and maceration of the plant host tissue (Kalunke et al. 2015). PGs can be induced by pectin and polygalacturonic acid (Kubicek et al. 2014) and are highly variable in their primary structure, specific activity, pH and mode of action (D'Ovidio et al. 2004). To retard fungal growth and colonization, a plant may produce inhibitors of these cell wall degrading fungal enzymes to retard fungal growth and colonization, such as Polygalacturonase-inhibiting proteins (PGIPs) that inhibit the pectin-depolymerizing activity of PGs (Kalunke et al. 2015). The occurrence of PGIPs has been reported in many plants, including dicotyledon and moncotyledon crops (Kubicek et al. 2014). PGIPs are ubiquitous leucine rich repeat (LRR) glycoproteins that counteract the action of fungal PGs but are ineffective against other microbial or plant derived pectic enzymes (De Lorenzo and Ferrari 2002). PGIPs (depending on each member in a gene family), may express in response to stress stimuli, wounding, elicitor treatment and/or pathogen attack. Apart from their role in plant protection, PGIPs aid with signal transduction, cell adhesion, DNA repair, recombination, transcription and RNA processing (D'Ovidio et al. 2004; Protsenko et al. 2008). Also, the interaction between PGs and PGIPs enhances the formation of oligogalacturonides that are elicitors of varied defense activities (Ferrari et al. 2013). The usage of PGIPs for crop protection requires deciphering the inhibitors with broad specificities against the various PGs of fungus and/or the construction of novel PGIPs with stronger and broader antifungal

activity. A review by (Kalunke et al. 2015) highlights the range of transgenic crops expressing PGIPs from various sources in various crops. Particularly for legume crops, PGIPs have been transformed only in two crops: pea (Richter et al. 2006a) and chickpea (Senthil et al. 2004). Bean PGIP, however, has been expressed transgenically in Canola *Brassica napus* (Akhgari et al. 2012) and tobacco (Borras-Hidalgo et al. 2012) against *Rhizoctonia solani* and in wheat against *Bipolaris sorokiniana* (Janni et al. 2008)

2.5.2 Drought tolerance

Long-term efforts in developing legume crops with enhanced drought tolerance through conventional breeding have had limited success mainly because of inadequate knowledge of the underlying physiological mechanisms and genetic basis of drought resistance and lack of sufficient polymorphism and/or appropriate genes to confer drought tolerance trait (Bhatnagar-Mathur et al. 2009; Mitra 2001). Often, genes responsible for low molecular weight metabolites are utilized in stress tolerance strategies, because such metabolic traits are more compliant to changes than structural and developmental traits (Bhatnagar-Mathur et al. 2009). Amongst various strategies used in genetic engineering for stress tolerance include expression of genes encoding for enzymes required for biosynthesis of osmoprotectants (Vinocur and Altman 2005) and modification of membrane lipids (Vinocur and Altman 2005), late embryogenesis abundant (LEA) (Bartels and Sunkar 2005), ROS detoxification enzymes (Umezawa et al. 2006), protein kinases and enzymes involved in phosphoinositide metabolism (Xu et al. 2014), cis- and trans-acting elements (Dubouzet et al. 2003), transcription factors (Agarwal and Jha 2010; Hussain et al. 2011) and PR-10 proteins (Dubos and Plomion 2001; El-Banna et al. 2010; Jain et al. 2012; Liu and Ekramoddoullah 2006a). It is difficult to achieve drought tolerance, as it is a complex

phenomenon controlled by multiple genes and regulatory pathways. Hence, many genes involved in stress response, are often simultaneously expressed rather than using a single gene (Bhatnagar-Mathur et al. 2009; Deikman et al. 2012). The traits relevant to this study are discussed in detail below:

2.5.2.1 Pathogenesis related proteins

PR proteins are induced de novo in plants in response to stress, pathogen attack or abiotic stimuli, or are developmentally regulated, giving abiotic and biotic stress tolerance, thus play a crucial role in plant's defense system (Jain et al. 2012; Van Loon et al. 2006). Among various PR families, PR10 is the largest with more than 100 members across 70 plant species, including pea (Lebel et al. 2010; Liu and Ekramoddoullah 2006a). Although commonly identified as allergens (Midoro-Horiuti et al. 2001), PR10 proteins have various functions in plants. For instance, they have a role in regulation of plant architecture and development (Srivastava et al. 2007), sexual reproduction (Lebel et al. 2010) and storage proteins with antibacterial and antifungal properties (Flores et al. 2002). They have also been employed as promoters of other genes in various transgenic crops (for example, in bean (Walter et al. 1996). PR10 proteins have been detected in various parts of plants and at various growth stages, for example, in flowering organs, pollen grains, fruit, seed, root and leaves (Liu and Ekramoddoullah 2006a).

It is now well established that PR10 protects the plant during pathogen infection (Liu and Ekramoddoullah 2006a), drought (Dubos and Plomion 2001), salinity and cold stress (Kav et al. 2004a), high temperature (Bahramnejad et al. 2010), herbicides (Castro et al. 2005), ultraviolet radiation and heavy metals (Rakwal et al. 1999). Although they have a well-described role against abiotic and biotic stresses, their mechanism of action is

sparsely described (Jain and Kumar 2015). It has been postulated that PR10 protein mediates stress tolerance through ABA and/or JA- mediated signaling cascades or by virtue of their ability to bind with cytokinins which has a role of protecting plants during drought stress (Jain et al. 2012). Various PR10 proteins were induced following drought *in vitro* in potato (El-Banna et al. 2010) and rice (Hashimoto et al. 2004). PR10 from peanut, when expressed transgenically, in banana (Rustagi et al. 2015), from potato in faba bean (El-Banna et al. 2010) and from peanut to tobacco (Jain et al. 2012) led to enhanced drought and salt tolerance (for a review see (Jain and Kumar 2015).

2.5.2.2 DREB

Transcription factors are important regulators of gene expression and stress responses in the environment. To cater the multigenicity of plant response to stress, transcription factors are attractive targets as they tend to target multiple pathways, and participate in manipulation of regulatory elements (Hussain et al. 2011). Many genes induced during osmotic stress, have a conserved drought responsive element (DRE) in their promoters (Shinozaki and Yamaguchi-Shinozaki 2000). Transcription factors belonging to ERF/AP2 family, which are specific to plants and that bind to dehydration responsive element/C-RepeaT (DRE/CRT) elements are called DREB1 (induced by cold stress) and DREB2 (induced by dehydration stress) and their products may activate other genes involved in drought stress tolerance (Liu et al. 1998; Yamaguchi-Shinozaki and Shinozaki 2006). The DREB2 protein is expressed during normal growth conditions and activated by osmotic stress through post-translational modification in the early stages of osmotic stress, for example, drought (Shinozaki and Yamaguchi-Shinozaki 2007). However, not much is known about their tissue specific expression (Agarwal and Jha

2010). Transgenic *Arabidopsis* expressing DREB2A (Liu et al. 1998; Sakuma et al. 2006) and VrDREB2A (Chen et al. 2016a) and GmDREB2 (Chen et al.) resulted in significant drought stress tolerance. Similarly, DREB2A in rice, led to drought tolerance (Dubouzet et al. 2003). DREB2 being the ethylene responsive element binding factors (ERF) family, binds to GCC box DNA motif (AGCCGCC) which is also present in several PR genes conferring ethylene responsiveness and the DRE/CRT motif-also is involved in the expression of osmotic stress responsive genes (Agarwal and Jha 2010; Sakuma et al. 2006).

Co-expression of several transcription factors along with PR proteins suggests enhanced expression of PR genes can confer osmotic stress tolerance. For example, basic domain/Leu zipper bZIP family (Johnson et al. 2003; Kesarwani et al. 2007) and WRKY protein family (Robatzek and Somssich 2002) enhance PR genes in *Arabidopsis*. Co-expression of transcription factor and PR protein in plants could have an additive effect in conferring drought tolerance. Although very few GM crops with drought tolerance traits have been approved (according to ISAAA 2017, only two events Verdeca HB4 soybean and Genuity® DroughtGard™ maize have been approved till date), immense progress is happening in transgenic approaches for drought tolerant crops. Also, current focus is on translating results from models in laboratories to crops in field (Deikman et al. 2012).

2.5.3 Stacking of traits

The term gene stacking (often used as a synonym to gene pyramiding) is used in agricultural research to describe breeding and/or genetic modification techniques to achieve multiple targets at the same time. For example, gene stacking could be a way to

identify and introduce multiple genes (acting on multiple pathways, protein complexes and quantitative traits) in a plant that confers resistance to an independent insect/pest/weed or a combination of abiotic/biotic stress (Taverniers et al. 2008). This can be done by a) introduction of several genes of interest simultaneously b) iterative procedures such as retransformation or conventional crossing of GM plants with different genes of interest, or a combination of these two (Halpin 2005; Taverniers et al. 2008). Many GM crops with stacked traits are commercialized and currently in use by farmers field (most recent list at ISAAA 2017; reviewed by Taverniers et al. 2008). However, there are bottlenecks of developing multiple genes or traits such as: i) genes are stacked using iterative procedures that are not linked and can get inserted at different loci in plant genome, and segregate again in subsequent generations ii) need of multiple selectable markers for each gene inserted or in case of co-transformation, transgenes could be incorporated in high copy numbers leading to gene silencing and iii) hinders regulatory approval process (Halpin 2005). With newer technologies being tested for the multiple gene delivery systems, gene stacking may come into more usage in the future (Que et al. 2010).

2.5.4 Role of promoters

Promoters used to drive and regulate transgenes form an important part of successfully depoloying transgenic technology. Hence, the strength and inducibility (stress induced or constitutive developmental stage or tissue specific) of the promoter are crucial for gene expression and plant response (Bajaj et al. 1999; Qu and Takaiwa 2004). Some genes need stronger promoters as they are required in large quantities (such as LEA3), whereas some genes/gene products (such as enzymes for polyamine synthesis) may require

moderate strength and inducible promoter (Bhatnagar-Mathur et al. 2009) while transiently needed traits, such as herbicide resistance may function best with chemically induced promoters (Daniell 2002). Some strong promoters, for example the Cauliflower mosaic virus (CaMV), have also been known to retard plant growth under normal growth conditions (Kasuga et al. 1999b), although doubling of this promoter also shows evidence of enhanced activity (Kay et al. 1987). Inducible promoters, especially when used pathogen resistance, can limit harmful or toxic protein accumulation in plants as well as in the environment (Gonzales-Salazar et al. 2017). A constitutive promoter's activity, on the other hand, can differ substantially depending on plant developmental stage, species and target organ (Samac et al. 2004). Hence, the appropriate choices of promoters used in creating transgenics is crucial for the maximum expression of desired trait and selection of promoter is case-to-case dependent.

2.5.5 Field efficacy trials

Under laboratory conditions or controlled environment conditions, differences amongst the transgenic lines being evaluated may be easier to discern as a single challenge can be imposed and tested (for example one pathogen or drought stress), whereas in field trials, the number of pathogens, weather, soil conditions and/or number of stresses are not controllable. Hence, field-testing could better depict real world effects of transgenes. Long term field-testing is key to test the agronomic performance and ecological relevance of the previously determined positive effects under laboratory/controlled conditions (Wozniak and McHughen 2012). Tactical field experiments can identify genotype x environment interactions. New genes when evaluated in a local environment will help to

evaluate yield compromise (if any) (Cohen 2005), issues around stability, pleotropic effects (Pons et al. 2012) and satisfy regulatory agencies (several location and actual area of cultivation testing is encouraged) (Romeis et al. 2008). Nevertheless ecological risks and benefits of transgenic crops need to be evaluated by regulatory agencies before their unconfined release is warranted (Wolfenbarger and Phifer 2000).

2.6 Environmental risk assessments

Genetically modified (GM) crops, classified as plants with novel traits (PNTs) in Canada, are evaluated for food, feed and environmental biosafety before being approved for unconfined release in Canada. A PNT is defined as "a new variety of a species that has one or more traits that are novel to that species in Canada". For a trait to be "novel" it has to be new to otherwise stably cultivated populations of the plant species in Canada and has capacity to cause an environmental effect (CFIA 2017a). Two government agencies share the responsibility of regulating PNT crops: Canadian Food Inspection Agency (CFIA) and Health Canada. The Plant Biosafety Office (PBO) of CFIA and animal feed division of CFIA carries the environmental and feed safety testing, respectively. PBO also manages post commercialization and monitoring activities of PNTs. Decision documents are published (publically) after CFIA has made regulatory decisions. Canada has a unique product-based regulatory system for PNTs where irrespective of the method used (for example, PNT may be developed using mutagenesis, somaclonal variation, intraspecific and interspecific breeding, recombinant DNA technology etc.). Any plant with a novel trait will be a subject to notification and authorization requirements (CFIA 2016). The five major categories under which PNTs are assessed for environmental safety assessment by CFIA are:

- Altered weediness potential of the PNT, in agricultural land or be invasive of natural habitat
- The PNT's potential for causing gene flow to sexually compatible plants
- The PNT's potential to become an plant pest
- Impact of the PNT or its gene products on non-target species
- The PNT's impact on biodiversity

Before a PNT is released into the environment, determination of the risk to environment is required. These risk assessments cannot guarantee absence of risk but can assess relative risk in the situation. Risk assessment is defined as a "structured, reasoned approach to identify a GM crop's potential to cause adverse effects and to characterize the seriousness and likelihood of the potential harm" (Keese et al. 2014). Regulatory decisions are made following comparative risk assessments to quantify the risk and defining the ground for comparison. The PBO is responsible for authorization of release of PNT as confined (release for research purposes with conditions such as reproductive isolation, harvested material restrictions and monitoring of field plots in following field seasons) or unconfined (with no restrictions with to potential commercialization) (CFIA 2016).

Risk assessments are analytical tools which help making science based regulatory decisions (Garcia-Alonso et al. 2014) and each risk assessment is carried out on a case-by-case basis (Conner et al. 2003a). Risk assessments must examine not only the potential risk of PNT under consideration, but also the likelihood that harm will occur. Risk is hence, defined as a function (f) of hazard and its probability of exposure:

Risk = f (hazard, exposure)

If formulated accurately, a risk assessment can provide high confidence with minimal risk (Raybould 2006); however, its reliability can only be guaranteed by empirical validation, determined by comparing predictions with observed outcomes (Keese et al. 2014).

2.6.1 Tiered risk assessments

Tiered tests are designed and executed to be time and input efficient and limit the unnecessary collection of excess data (Mallory-Smith et al. 2015). Tiered tests begin with artificial 'worst case' scenarios followed by more environmentally realistic approaches (Raybould and Cooper 2005; Romeis et al. 2008; Wilkinson et al. 2003). A key principle for conducting tiered test approach is that particular studies are conducted only when they work to reduce uncertainty of risk assessment. Hence, in the case of no hazard (or risk) detection, tiered tests prevent costly and superfluous data collection (Romeis et al. 2008). In general, lower tiers are based off of conservative assumptions and worst-case conditions and if risks determined at each step are deemed negligible or acceptable within reason, or insufficient information is available to make a regulatory decision, the assessment can be halted. However, in cases where uncertainty remains or initial tier testing identifies risks, further tiers can be investigated (Garcia-Alonso et al. 2006).

The first tier (Tier 0) is a "problem formulation step", which focuses on organism, trait of interest, the receiving environment and hazards associated with the introduction and assures that conclusions will be appropriate to guide the decision making process (Garcia-Alonso et al. 2006). Information gathered from this tier is kept for synthesis and interpretation of further tiers. The first experimental tier (Tier I) is the analytical phase of any risk assessment, which is conducted under 'worst case' conditions (Raybould and

Cooper 2005). It is designed to conservatively address broader questions using simple experimental tools (Wolt 2009). The aim of tier I is to maximize the chance for detection of hazard occurrence and minimize the chance of committing false negatives (Type II errors). Laboratory or greenhouse conditions are generally preferred over field studies in tier I, as they eradicate environmental factors that may confound/complicate observations and results. If this stage's testing still warrants for unacceptable risk, higher tiers are recommended to refine the risk assessment (Garcia-Alonso et al. 2006).

Tier II experiments may include additional laboratory, controlled environment or greenhouse experiments, or progress to small-plot field experiments (Raybould and Cooper 2005; Romeis et al. 2008). However, if results from Tier II are shown to be a potential risk, higher tiered tests will be continued, otherwise, the assessment can stop. Tier III tests are designed to incorporate more natural conditions, and may be conducted as medium to large-scale field experiments or elaborate laboratory tests (Raybould and Cooper 2005). These studies are often data heavy and labor intensive, and the results yielded can be difficult to interpret without the aid of the previous tiers' data. As with other tiers, if the assessments from Tier III indicate the risk level to be acceptable, assessments could be deemed complete. However, if the results from Tier III confirm a potential risk exists, further refinement may be required, or a decision of unacceptable risk may be made (Garcia-Alonso et al. 2006). One can always return to lower tiers to conduct additional studies or use alternative designs (Romeis et al. 2008).

The tiered approach is iterative, systematic, scientific and flexible, where knowledge obtained in lower tiers directs data collection at higher tiers and is designed to provide information to support a regulatory decision as rigorously as possible (Garcia-

Alonso et al. 2006; Romeis et al. 2008; Wilkinson et al. 2003). A tiered approach to risk assessment provides a scientific rationale for environmental risk assessment and assists with subsequent regulatory decision-making (Romeis et al. 2008). Risk assessment considerations specific for peas are discussed below.

2.6.2 Gene flow in peas

Gene flow is defined as alterations in population due to the movement of gametes, extranuclear segments of DNA, such as mitochondria, plasmids and viruses, individuals, or group of individuals from one place to another (Slarkin 1985). Pea is predominantly a self-pollinating crop (Fehr 1987) and has cleistogamous flowers that tend to open only for 24 h post-pollination (Cousin 1997). The rate of outcrossing in pea has been reported as around 1% (Gritton 1980), but with the advent of transgenic peas and the potential for future commercialization, there are concerns about gene flow between transgenics and commercial, wild relatives (Bhowmik and Basu 2008). In Canada, transgenic pea (PLP1) was studied for transgene movement (using normal leaf form and overexpressed gusA gene as markers for pollen migration) to three non-transgenic lines (Carneval, Montana and Tipu) in 1997 and 1999. Only 0.06% of plants sampled from a population of over 9,000 plants scored positive for both markers and an outcrossing rate of 0.07% was recorded in trap plants growing subsequent to transgenic lines with no outcrossing reported in plots at 5 m from the outcrossing plots in presence of wind and pollinators (Polowick et al. 2002). Based on this study, the possibility of transgene flow from transgenic pea lines to related crops and non-transgenic pea is relatively low (Bhowmik and Basu 2008). Yet another study using non-GM pea cv. Zekon (trap cv.) and Arvika (pollen donor), after screening 40,000 F1 plants for two years, it was concluded that the

probability of outcrossing in commercial pea varieties was extremely low (Dostálová et al. 2005). In another study, about 2% fertile ovules were obtained in the field and 8% in greenhouse between *Pisum sativum* x *Vicia faba* cross in comparison to 26% and 48% for *Pisum sativum* x *Pisum sativum* in the field and greenhouse, respectively (Gritton and Wierzbicka 1975). However, the *Pisum sativum* x *Vicia faba* cross eventually had collapsed embryos and did not develop properly. Also, the wild relatives in primary and secondary gene pools can be crossed with pea but their hybrids, due to crossability barriers, may be partially sterile and depict limited genetic recombination (Gaur 2010; Ladizinsky et al. 1988).

The soil seed bank is a total of all viable seeds at or below the soil surface at a given time and place. Seed bank formation aids plant populations to buffer against harsh and/or unfavorable environmental events and persist over time. Mechanisms of dormancy and seed germination impacts seed bank composition (Thompson and Grime 1979). Pea is a weak competitor with weeds (Lemerle et al. 2006; Neil Harker 2001) and displays indehiscent pods and little seed dormancy, which are among the major traits pea was bred for (Warkentin et al. 2015). Along with a large seed size, little dormancy and a transient seed bank, lower outcrossing makes pea an easy crop in terms of isolation and containment of gene movement for risk assessments pertaining to transgenic pea.

2.6.3 Effect on non-target organisms

In Canada, CFIA deems 'the impact of PNT or its gene products on non-target species' as one of the five environmental safety assessment criteria (CFIA 2016a). Consequences of growing GM crops in the field have consequences to ecosystem and environment (Conner et al. 2003b), particularly their environmental impact on non-target organisms

(NTOs) remains pending (Devos et al. 2016). Like pesticides, they can lead to a direct effect on NTOs (for example, toxicity due to gene or gene product), indirect effect (such as trophic interactions) and cause changes in soil ecosystem (effecting soil organisms (NTOs)) (Birch et al. 2007a; Meyer et al. 2013). Hence, for a complete risk assessment, it becomes crucial that investigator has developed and tested a risk hypothesis addressing negative effects of GM and GM products on NTOs (Dale et al. 2002; Devos et al. 2016).

Particularly in the case of pea, two symbiotic relationships i.e. nitrogen fixing bacteria belonging to genus *Rhizobium* and arbuscular mycorrhiza fungus (AMF) belonging to genus *Glomus* (Long 1989; Smith and Read 2010) form an important part in the environmental risk assessment focusing on effects of NTOs. Most of the biological nitrogen fixation in agri-ecosystems comes from symbiotic relationships of nitrogen fixing bacteria with legume crops (Yang et al. 2017). AMF on other hand facilitates phosphorus uptake and improved resistance to various biotic stresses (Parniske 2008; Smith and Read 2010). In return, they receive carbohydrates from the plant and hence are excellent indicators of any changes occurring in the host plant (Hannula et al. 2014). Pea producers in Canada commonly use AMF and Rhizobium as inoculants for their crop (Lupwayi et al. 2006). Because AMF, pathogenic fungi and Rhizobium share common genetic and signaling pathways in plants (Albrecht et al. 1999; Vierheilig et al. 1994), it's apparent that they might be influenced with transgenic addition of antifungal genes into the plant. Hence, it is crucial to identify (if any) adverse effects of the transgenic genes on beneficial soil associations of pea.

2.6.4 Process of conducting field trials of PNT pea in Canada

In Canada, " A confined research field trial of PNTs is the release of a PNT in the environment, for research purposes, under terms and conditions of confinement" (CFIA 2016b). Confined field trials of a PNT crop allow developers and researchers to asses the field performance of the PNT and to gather science based information to address the environmental safety criteria for an application for unconfined release or for academic research or analytical needs. The confined research field trial program is conducted under auspices of CFIA's Plant Biosafety Office (PBO). Researchers have an opportunity to grow PNT for research purposes under strict terms and conditions of confinement, which are in place to minimize the exposure of PNT to the environment. They include but are not restricted to reproductive isolation, monitoring during and after the growing season and post harvest land use restrictions. Many components can lead to a breach in confinement from species/trait under test itself, methods of isolation and confinement chosen by the proponent, or when the PNT in confinement poses an unacceptable risk to the environment, which may cause the refusal or restriction for the researcher. Each confined field trial application submission is also subjected to restrictions on size and total number of sites per province to limit the exposure of PNT material to environment (CFIA 2011).

The application procedure for PNT pea confined field trials can be found under Directive 2009-09: Plants with novel trait regulated under Part V of the seeds regulations and Directive 2000-07: Conducting Research field trials of Plants with Novel Traits in Canada and at http://www.inspection.gc.ca/plants/plants-with-novel-traits/applicants/eng/1300208718953/1300208874046. The applicant has to be a permanent resident of Canada and applications are sent to CFIA's Pre-Market

Application Submissions Office (PASO), Ottawa, ON. The application must be received 30 days before the expected planting date (CFIA 2017b). Brfiely, the application must contain applicant's information (address, affiliation etc.,), with background information with supporting literature for the origin of the transgene, plant species, history, trait introduction and selection methods, name of the plasmid with genetic map, gene construct, regulatory elements, gene products, non-translated DNA sequences and affected metabolic pathways. Apart from this, the applicant also needs to provide the following information: spatial and temporal trait expression for each of the transgene inserted in pea, any known toxicity and allergencity of the novel trait, altered plant characterstics (known and expected) for example, dormancy, weediness, seed/pollen dispersal etc. The application must also provide information on the trail protocol-it's title and purpose. Reproduction isolation, method of seeding and harvest, anticipated chemical usage need to be presented in details. Proponent must list the contingency plans in case of unexpected spread of PNT material, extent and frequency of trial monitoring, during course of field trial and post trial. In the particular case of PNT pea, terms and conditions for confined field trials of pea are summarized by CFIA (2017c). Briefly, following points need to be ensured by the proponent for carrying out successful PNT pea confined field trials:

1) Safe transportation and cleaning of equipment

Plant material (PNT pea seed and non-PNT pea seed to be used as controls in experiment) to be transported in clearly identifiable 'secure' containers and kept isolated from other seed material. To prevent dispersal of novel plant material, all machinery and equipment used during the trial, for example for seeding, site maintenance and

harvesting, must be well cleaned well for removal of all residual plant material at the trial site before being moved.

2) Reproductive isolation

Seeded pea plants at the confined field trial must be reproductively isolated from other *Pisum sativum* plants as well as prohibited species *Vicia faba* (faba beans), by a minimum distance of 10 metres. Pea and prohibited species must be removed before seed set from the 10metres isolation distance. In the trial site and 10 metre zone (50 metres if a commercial-scale combine was for harvest) perhiphering the trial site, *Pisum sativum* or *Vicia faba* should not be grown for one year following harvest of the trial. All volunteers, if found, during the post-trial growing season must be removed from the trial site before flowering.

3) Field trial site location

Clearly identifiable markers (e.g. corner posts) should be placed at each corner of the trial site to identify the boundaries during growing season and post-harvest restriction period. Fences with trial site protocol information and details for person of contact can be put around the trial site. Global Positioning System (GPS) coordinates must be recorded precisely at all corners of the site and submitted to the PBO within seven days of seeding. In case chemical treatment is used on the trial site a sign must be posted at the entry to the trial with the date and time of spraying as well as the time/date until safe entry.

4) Harvest and disposal

Pea plants should be harvested before full maturity to ensure minimum shattering and seed dispersal. All seed and other propagable material including non-PNT material must be harvested. Any remaining plant material (PNT or non-PNT) after harvest or the

propagable material from PNT trial that is not be retained, is to be destroyed/disposed off (by burning, autoclaving or burial at a depth of one metre). All non-propagable plant material after harvest, remaining on the confined field trial site must be either incorporated in the soil or destroyed by incineration to ash.

5) Post-Harvest storage

Containers labeled “PNT MATERIAL DO NOT MIX” should be used for storing the PNT and other seed material from the trial site and should be kept separate from other seed and plant material. Triple containment is required offering three layers of protection.

6) Monitoring of trial site

Monitoring of trial site and the reproductive isolation distance (10m or 50m in case of combine usage) should be ensured once every two weeks to ensure that all pea plants and prohibited species are removed before seed set.

7) Record keeping and reporting

A detailed trial log book should be maintained with records of the confined research field trial, including protocols, maps of the site, seeding and in season activites, seed transport, current season and post-harvest site monitoring, cleaning of machinery, disposition and storage of all seed must be maintained by the applicant and made available to the CFIA upon request. In case of accidental release of seed in environment, PBO should be notified immediately and the site should be marked and monitored. Within 15 working days after harvest of trial site, the applicant has to report to the PBO about the date of harvest, quantity and storage location of seed and plant material harvested, quantity of seed disposed (location, method, date and quantity of disposal).

2.7 Developing a GM crop

Bringing a new GM crop as a commercialized product is a long term, resource and time intensive and requires a lot of capital investment (Kalaitzandonakes et al. 2007). Various steps/ activity stages are recognized for biotech crop discovery and development; for example, the discovery phase (many genes/ideas; 1-3 years), proof of concept (construct optimization, early efficacy trials; 2+years), early development (commercial events; 2+years), advanced development and pre launch introgression, breeding, wide area testing and regulatory approval 7+years) (McDougall 2011). Confined field trials are designed to screen/select events, test even efficacy, increase seed/GM product for safety or compositional testing and gather data for an environmental risk assessment. Confined field trials put the proponent and crop industry at risk; hence the chief goal remains to minimize the gene flow. Therefore, the receiving market has to be large and valuable enough to warrant the investment and risk involved in product discovery, development and a satisfactory regulatory approval (Prado et al. 2014). Overall, from conceptualizing the idea to product development and regulatory approvals, a GM crop can cost up to USD $136 million and take 13 years (McDougall 2011). The widely successful and commercialized Roundup Ready (glyphosate tolerant) soybean serves as an excellent example of when the market for such a trait was large enough to deploy the investment and a viable technical solution was available, the GM soybeans were an instant hit (Kishore et al. 1992). The high cost involved in development and achieving regulatory approval for GM crops has been a limiting factor in crop improvement in minor or staple crops in developing countries (Prado et al. 2014).

Table 2-1. Transgenic expression of PR proteins (chitinase and glucanase) in legumes.

Transgenic legume	Source gene	Target pathogen	Reference
Peanut (*Arachis hypogea* L.)	Rice chitinase, *RCG3*	*Cercospora arachidicola*, early leaf spot	(Iqbal et al. 2012a)
Peanut (*Arachis hypogea* L.)	Rice chitinase, *Rchit*	*Aspergillus flavus*, aflotoxin *Cercosporidium personatum*, late leaf spot and *Puccinia arachidis*, rust	(Prasad et al. 2013)
Peanut (*Arachis hypogea* L.)	Tobacco chitinase, *Chi-V*	*Cercospora arachidicola*, leaf spot	(Rohini and Rao 2001)
Black gram (*Vigna mungo* L.)	Barley chitinase (AAA56786)	*Corynespora cassicola* (leaf spot)	(Chopra and Saini 2014)
Pigeonpea (*Cajanus cajan* L.)	Rice chitinase, *Rchit*	*Fusarium oxysporum*	(Kumar et al. 2004)
Soybean (*Glycine max* L.)	Bean chitinase, *Chi*	Sheath blight	(Li et al. 2004)
Peanut (*Arachis hypogea* L.)	Rice chitinase	*Aspergillus flavus*	(Sharma 2006)
Pea (*Pisum sativum* L.)	*Streptomyces olivaceoviridis, Chit30*	*T. harzianum*	(Hassan et al. 2009)
Alfalfa (*Medicago sativa* L.)	*T. harzianum chitinase ech42*	*Phoma medicaginis*, *Colletotrichum trifolii*	(Tesfaye et al. 2005)
Peanut (*Arachis hypogea* L.)	Tobacco glucanase, β 1,3-glucanase	*Cercospora personanta*	(Qiao et al. 2014)
Peanut (*Arachis hypogea* L.)	Tobacco glucanase, 1,3-glucanase	*C.arachidicola*, *Aflotoxin, A. Flavus*	(Sundaresha et al. 2010)
Pea (*Pisum sativum* L.)	Barley β-1,3-glucanase (*gluc*), chitinase (*Chit30*)	*T. harzianum, C. acutatum, B. cinerea & Ascochyta pisi*	(Amian et al. 2011)

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Chapter Three: Drought tolerant transgenic pea development by co-expressing *DREB2A* from *Oryza sativa* L. and *PR10a* from *Solanum tuberosum* L.

3.1 Introduction

Grain legumes are valuable worldwide for nutritional and health benefits and their impact on agricultural sustainability (Araújo et al. 2015) and are only second to cereals in ensuring food security (Akibode and Maredia 2011). Among grain legumes, pea is economically important next only to soybeans and beans in production worldwide (FAOSTAT 2015), mostly grown in temperate regions (Smýkal et al. 2012). Canada is the world's largest producer and exporter of field pea (*Pisum sativum* L.) (FAOSTAT 2015). Pea production is severely affected by abiotic stresses such as drought (Daryanto et al. 2015, Iglesias-García et al. 2015), whose occurrence is more frequent owing to climatic fluctuations (Schmidhuber and Tubiello 2007). Drought has been a recurring phenomenon in the Canadian prairies (Sun et al. 2012), (Masud et al. 2016) and the principal reason for pea yield declines in 2001-2002 (Hickling 2003), 2008 (Ross et al. 2015) and in 2015 (Agriculture and Agri-Food Canada 2016) in western Canada. Crop productivity may be enhanced by improving drought tolerance (Daryanto et al. 2015; Dita et al. 2006; Magyar-Tábori et al. 2011).

Drought stress causes lower stomatal conductance, CO_2 fixation and photosynthetic rates, increases photorespiration and induces oxidative stress (Moran et al. 1994), consequently reducing yield especially during flowering (Bueckert et al. 2015a; Saskatchewan Ministry of Agriculture 2015). Plants respond to drought stress at the

physio-biochemical level by induction of stomatal closure, repression of cell growth and photosynthesis (Iglesias-García et al. 2015) or at molecular and cellular levels by expression of stress induced genes (Ingram and Bartels 1996; Liu et al. 1998; Shinozaki and Yamaguchi-Shinozaki 1997; Stockinger et al. 1997) encoding for protein and gene products that can play an important role in protecting plant cells from drought. Similarly, peas respond to drought stress by decreased photosynthesis, reduced root/shoot ratio, reduced transpiration, increased epicuticular waxes and reduced residual transpiration rate, production of ROS (reactive oxygen species) and accumulation of osmotically active metabolites, such as amino acids like proline, valine and sugars (reviewed by Araújo et al. 2015).

Stress-induced genes and gene products including late-embryogenesis-abundant (LEA) proteins, ROS detoxification enzymes (Umezawa et al. 2006), transcription factors, protein kinases and enzymes involved in phosphoinositide metabolism(Xu et al. 2014), cis- and trans-acting elements (Dubouzet et al. 2003), and *PR10* proteins (Dubos and Plomion 2001; El-Banna et al. 2010; Jain et al. 2012; Liu and Ekramoddoullah 2006b) play a role in protecting plants from drought. Among these, *PR10* proteins belong to widely distributed PR (pathogenesis related proteins) family which are induced due to stress (biotic and abiotic) (El-Banna et al. 2010), as well as expressed constitutively (33,34) and are regulated developmentally and environmentally (Liu and Ekramoddoullah 2006b). They have been isolated from both mono and dicots (Hashimoto et al. 2004) including pea (Srivastava et al. 2004; Tewari et al. 2003), and have a possible role in plant protection (Liu and Ekramoddoullah 2006b) against biotic and abiotic stresses including drought tolerance (Hanafy et al. 2013; Rustagi et al. 2015), salt

tolerance (El-Banna et al. 2010; Jain et al. 2012), cold (Lee et al. 2012), osmotic tolerance (Vaas et al. 2012) and pathogen infection (McGee et al. 2001). Transcription factors are the main regulatory elements that bind to specific cis regulatory elements in the promoter region of abiotic stress related genes, individually or in gene networks, and activate the expression of secondary genes resulting in stress tolerance (Agarwal and Jha 2010; Naika et al. 2013). Particularly, transcription factors such as *DREB* (dehydration responsive element binding) proteins, belonging to AP2/ERF family, have rapid and transient gene expression amidst drought, salinity and cold stress (Mizoi et al. 2012; Yamaguchi-Shinozaki and Shinozaki 2006). The strategic overexpression of constitutive active *DREB2A* resulted in significant drought stress tolerance but relatively less freezing tolerance (Sakuma et al. 2006) and *VrDREB2A* activated the expression of downstream genes, resulting in enhanced tolerance to drought and high-salt stresses (Chen et al. 2016b) in transgenic *Arabidopsis.*

Various strategies have been utilized for improvement of drought stress tolerance in plants. For instance, in maize, successes with modulating ethylene which regulates plants under stress as in the development of Droughtgard**™** maize utilizing expression of a cold shock protein CspB encoding gene from bacteria *Bacillus subtilis* (Castiglioni et al. 2008; Kreimeyer et al. 2015) and transgenic gene silencing approach to down regulate ACC synthase to decrease ethylene biosynthesis (Habben et al. 2014) has been used. Similar targeting of ethylene pathway by overexpressing Hahb4—a homeodomain-leucine zipper (HD-zip) transcription factor from sunflower - along with an Hahb4 native promoter, has led to the development of drought tolerant soybean (Manavella et al. 2006). In case of wheat, *DREB1A* transcription factor under the control of rd29A promoter

(Kasuga et al. 1999a) and *HVA1* (member of late embryogenesis abundant (LEA) protein) gene from barley (Bahieldin et al. 2005) have been introduced. Development of drought tolerant tomato using overexpressing osmotin-encoding genes under the control of 35S CaMV (cauliflower mosaic virus) promoter is in development (Goel et al. 2010). Over-expression of stress induced genes and transcription factors can be utilized in pea crop improvement for enhancing tolerance to drought and other abiotic stresses (Dita et al. 2006; Moran et al. 1994; Pellegrineschi et al. 2004). Methods for genetic transformation of pea have been developed (Polowick et al. 2000a; Schroeder et al. 1993); however, the efforts have mainly transgeted pea development efforts have been targeting mainly disease resistance (Amian A et al. 2011; Hassan et al. 2009; Richter et al. 2006c) or insect resistance (Negawo et al. 2013). In the present study, we report the development of putative drought tolerant transgenic pea expressing *PR10a* from potato (*Solanum tuberosum* L.) and transcription factor *DREB2A* from rice (*Oryza sativa* L.) using *Agrobacterium*-mediated gene transfer method. The genomic integration and inheritance of *PR10a* and *DREB2A* in pea was been validated by molecular analyses. The preliminary drought bioassay under laboratory conditions showed better tolerance of the developed transgenic lines as compared to non-transgenic lines.

3.2. Materials and methods

3.2.1 Plant materials and transformation vector

Embryo axis excised from mature seed of Canadian pea cv. AC Early Star (*P. sativum* L. cv. AC Early Star) were used as explants for *Agrobacterium*-mediated transformation using *Agrobacterium* strain EHA105 harboring a helper plasmid pSoup and transformation vector pGII*PR10a*cp148*DREB2A* (Figure 6.1). The vector harbors a

codon optimized for drought tolerant *PR10a* gene from potato (*Solanum tuberosum* L.) (El-Banna et al. 2010), transcription factor *DREB2A* gene from rice (*Oryza sativa* L.) (Dubouzet et al. 2003) and herbicide resistant *bar* gene (selectable marker) from *Streptomyces hygroscopicus* (Murakami et al. 1986; Thompson et al. 1987). Dr. Fathi Hassan designed the vector. The *PR10a* and *DREB2A* genes were connected by cp148 IRES from TMV(Ivanov et al. 1997; Skulachev et al. 1999) which enables their co-expression under the control of the single promoter (mannopine synthase promoter from *Agrobacterium tumefaciens*) and terminator (35S from cauliflower mosaic virus). The *bar* gene is under the control of nopaline synthase (*nos*) promoter and terminator from *Agrobacterium tumefaciens*.

3.2.2. *Agrobacterium*-mediated transformation of explants and recovery of putative transgenic shoots

The putative transgenic plants were developed at the Plant Biotechnology Department (Institute of Plant genetics, Leibniz University of Hannover, Germany) using transformation protocols of Schroeder et al. (1995) with modification according to Richter et al. (2006c). Mature pea seeds (*Pisum sativum* L. cv. AC Early Star) were surface sterilized in 70% (v/v) ethanol for 1 min followed by 6% sodium hypochlorite for 15 min and thoroughly washed with sterile distilled water 3-4 times to remove the sodium hypochlorite and imbibed overnight in sterile distilled water. Then, embryos were extracted, longitudinally sliced and inoculated with *Agrobacterium* suspension (OD_{600} ~ 1.0) for 60 min. After three days of co-cultivation in the dark, explants were washed thoroughly in sterile distilled water followed by a final wash for 15 minutes in antibiotic solution (100 mg/L ticarcillin) to eliminate *Agrobacterium* cells. Explants were then placed on shoot induction medium for 10 days and finally transferred to selective

regeneration medium supplemented with increased concentrations of PPT (phosphinothricin) (2.5, 5, 7.5 and 10 mg/L) every three weeks (the mediums used have been described in Schroeder et al 1993). *In vitro* putative transgenic shoots were recovered by micro grafting (Pickardt et al. 1995) onto seedling rootstock grown on soil substrate and leaf samples were collected for PCR (polymerase chain reaction) analysis of the T-DNA region integration when they were two-three node stage. PCR positive putative transgenic shoots were maintained to collect T1 seeds which were then grown in the greenhouse for further PCR analysis and leaf paint assay of transgene inheritance and expression.

3.2.3. Genomic DNA isolation and PCR analysis

Genomic DNA was extracted using CTAB (cetryltrimethyl ammonium bromide) method (Doyle and Doyle 1990) from young leaves and used for PCR analysis using transgene specific primers. The PCR program included the initial denaturation step of 94°C for five min followed by 30 cycles of 94°C for one min denaturation step, primers specific temperature with one min annealing step and 72°C for one min extension step and the final extension steps at 72°C for 10 min.

The following primers were used for *PR10a* gene (PR10-For: 5'-ATGGGTGTCACTAGCTATACACATG-3' and *PR10a*-Rev: 5'-TTAAGCGTAGACAGAAGGATTGGC-3', with 480 bp expected PCR product size) annealing temperature was 57°C and for *DREB2A* gene (Dreb-For: 5'-AGGGGAGATTGCTCCGTGC-3' and Dreb-Rev: 5'- CCCATCATCTCCCTCTTGG-3', with 780 bp expected PCR product) and bar gene (Bar-For: 5'-CTACCATGAGCCCAGAACGACG-3' and Bar-Rev: 5'-

CTGCCAGAAACCCACGTCATGCCAGTTC-3‘, with 499 bp expected PCR product) annealing temperature was 62°C. (HMG-For: 5’-ATGGCAACAAGAGAGGTTAA-3’ and HMG-Rev: 5’-TGGTGCATTAGGATCCTTAG-3’) were the primers used for pea housekeeping gene (high mobility group *hmg*-I/Y) (Gupta et al. 1997) and was used as internal control to check the quality of DNA and amplifying a PCR product of (570 bp from genomic DNA and 370 bp from cDNA), which helped to monitor the contamination of RNA by genomic DNA during expression analysis. For monitoring the persistence of *Agrobacterium* cells in the tissue of putative transgenic shoots, *Agrobacterium* chromosomal DNA specific primers (PicA-For: -ATGCGGATGAGGCTCGTCTTCGAG-3' and PicA-Rev: 5'-GACGCAACGCATCCTCGATCAGCT-3', with 550 bp expected PCR product) were used at 63°C annealing temperature. Agarose gel (1 %, w/v) was prepared in 1X TAE buffer and used for separation of PCR products. Redsafe™ Nucleic Acid staining solution (iNtRON Biotechnology) was used as a DNA stain to help gel documentation under ultra-violet illumination.

3.2.4. Leaf paint functional assay

In addition to PCR analysis, leaf paint functional assay was conducted on T1 plants to verify the expression of *bar* gene (plant selectable marker gene) according to Schroeder et al. (1993). A BASTA® (Aventis GmbH) herbicide solution (600 mg/L, with a drop of Tween 20) was prepared and applied thoroughly onto the upper surface of a four week old leaflet while another opposite leaflet was marked as control. The effect of herbicide on the leaflet was evaluated one week after application. The plants were classified as tolerant when the leaf did not show wilting and susceptible when the leaf showed a sign

of wilting. Non-transgenic plants were also treated with the herbicide solution as a negative control.

3.2.5. Preliminary water stress experiment

In order to assess potential drought tolerance of *PR10a*-expressing plants, a preliminary water stress experiment was conducted. Transgenic and non-transgenic (control) pea seeds were germinated in perlite (Perligran G, Knauf) in a greenhouse (16/8 h day/night photoperiod) at 20 ±2°C. Transgenic (T2 generation) and control plants were grown in pots and watered every 2 days for 4 weeks. For drought stress, at week 5, the control and transgenic plants were watered once and subjected to 3 weeks of water withholding followed by resuming of regular watering. The responses of the plants to drought stress were observed. Leaf samples were collected from the transgenic and control plants prior to water withholding and again after three weeks of water withholding for *PR10a* gene expression analysis. Further information on number of plants used in experiment is unavailable.

3.2.6. RNA isolation and RT-PCR analysis of gene expression

For expression analysis, total RNA was isolated from young leaves before and after water withholding as described above, using NucleoSpin® RNA plant (Macherely-Nagel) kit according to the manufacturer's instruction. The isolated RNA was used to synthesize cDNA using RevertAid^{TM} H Minus cDNA synthesis kit (MBI Fermentas). The cDNA was used as a template in PCR detection of the transgene expression using gene specific primers. The expression level of *PR10a* gene was determined using Quantitative real time PCR (qPCR) using primers rPR10-1F 5'-ATGGGTGTCACTAGCTATAC-3' and rPR10-2R123 5'-CAACATTCTTAACATTTGGC-3' as described in (Hanafy et al.

2013). Primers HMGIII-For: 5‘-AGGGGTAGGCCGAAGAAGAT-3’ and HMGIII-Rev: 5’-TGAGGCTTCACCTTAGGAGG -3’ for pea housekeeping gene (*HMG-I/Y*) (Gupta et al. 1997) were used as internal reference to normalize the expression of *PR10a* gene.

3.3 Results

3.3.1. Genomic integration of T-DNA region

Transgenic pea lines co-expressing *PR10a* and *DREB2A* genes using dicistronic vector were developed through *Agrobacterium*-mediated transformation. The result of PCR analysis demonstrated the stable genomic integration of the introduced transgene in the developed transgenic pea lines (Figure 3.2-5). Based on the PCR analysis, 25 transgenic shoots were obtained from a total of 684 explants giving a transformation efficiency of 3.65 %. PCR analysis using primers specific to *Agrobacterium* chromosomal DNA and vector backbone indicated absence of *Agrobacterium* persistence and backbone integration in the regenerated putative transgenic shoots.

3.3.2. Inheritance of the T-DNA region

To study the inheritance of the introduced transgene, first generation (T1) seeds collected from PCR positive T0 transgenic shoots were grown in the greenhouse. The grown plants were characterized by PCR analysis for T-DNA presence and leaf paint assay for herbicide tolerance. The results indicated the inheritance of the T-DNA region to the next generation. Accordingly, 21 transgenic T1 plants from nine different clones were obtained. Figure 3.6 shows the PCR result for eight T1 plants. Confirmed transgenic T1 plants were maintained to produce T2 seeds. PCR analysis of T2 plants of selected lines showed the presence of the transgenes in some of the randomly analyzed plants (Figure 3.7). Using data from PCR and leaf paint assay, Chi-square (X^2) analysis of T2 plants

showed Mendelian segregation of the introduced T-DNA region (Table 3.1). The lines with stable expression in T2 as well were advanced to T3.

3.3.3 Leaf paint (LP) functional characterization of segregating progenies

Segregating progenies of transgenic plants were characterized by LP assay using Basta® (glufosinate) herbicide solution. The result of LP assay was in line with expectation (Figure 3.8 and Table 3.1) where both herbicide resistant (partial or complete) and susceptible plants were observed in the progenies of transgenic plants.

3.3.4. Expression of the T-DNA region in transgenic lines

RT-PCR expression analysis of the transgenes (*PR10a* 480bp; *DREB2A* 780bp) and housekeeping gene (370 bp) on selected PCR positive transgenic plants led to detection of both transgenes in cDNA of the T2 transgenic plants (lines 2: PR10-4-12, 3: PR111-2-4; 4: PR112-2-6, 5: PR113-1-6, 6: PR122-1-5, 7: PR*-2-1,8: PR123-1-2, 9: b-1-2, 10: b5-1-1, 11: PR10-2-3), while no amplification was observed in the negative (12-C: gDNA of non-transgenic pea plants) and water controls (Figure 3.9).

Preliminary water stress assessment was conducted on T2 plants by withholding water for three weeks and the expression level of *PR10a* gene was estimated using qRT-PCR before and after the water stress. The effect of water withholding was more pronounced on the control plants as compared to the developed transgenic lines which showed fewer signs of wilting (Figure 3.10) and remained green. The expression level of *PR10a* gene was very low (less than one-fold for nine plants and 1.26 fold for one plant) when the plants were grown under sufficient watering condition (Figure 3.11). The expression level of *PR10a* gene increased up to 4.5 fold after withholding water for three

weeks (Figure 3.11) in transgenic lines, suggesting that the *PR10a* gene was being expressed and conferring drought tolerance.

3.4. Discussion

We report the first successful integration of drought tolerant genes *PR10a* and *DREB2A* into pea cv. AC Early Star, using cultivar independent *Agrobacterium*-mediated genetic transformation system with a transformation efficiency of 3.65%, which is a significant improvement over the earlier gene transfer methods for legumes which had transformation efficiencies ranging from 1.1% (Bean et al. 1997) to 2.5% (Schroeder et al. 1993). We followed their performance for stable integration, inheritance and expression using PCR, leaf paint assay and RT-PCR and found all of the transgenic pea lines expressing *PR10a* and *DREB2A* were fertile and advanced to the T3 generation without any visible detrimental effect on plant growth. Expression of *PR10a* and improved performance of transgenic pea plants in a three-week water withholding experiment was demonstrated. Increases from 1-3 fold of expression level of *PR10a* gene (normalized to pea housekeeping gene), were observed among different lines upon withholding water for three weeks. The gene expression analysis of transgenic lines with *PR10a* gene in the current study as well as previous work with *PR10* genes (El-Banna et al. 2010; Hanafy et al. 2013; Hashimoto et al. 2004; Jain et al. 2012; Rustagi et al. 2015; Vaas et al. 2012) suggests that its levels are increased by drought conditions and support the hypothesis that this protein has a plausible role in protection of plant cellular components from detrimental effects of drought (Dubos and Plomion 2001; Kav et al. 2004b). Similar effect of *PR10a* expression in enhancing tolerance to salinity and drought

as compared to non-transgenic lines has been observed in faba beans (Hanafy et al. 2013) and overexpression of *PR10a* enhanced survival under salt and osmotic stress (El-Banna et al. 2010) and during cryopreservation of potato cell culture (Vaas et al. 2012).

Expression level of *DREB2A* before and after water withholding was not quantified in this particular experiment. However, previos research showed that DREB2A was expressed following dehydration in *Arabidopsis* (Liu et al. 1998), after dehydration and high-salinity in rice (Dubouzet et al. 2003) and following dehydration, high salt concentration and abscisic acid treatment in mung bean (Chen et al. 2016a) indicating its role in stress tolerance. *DREB2* transcription factors belong to the ERF (ethylene responsive element binding factors family). These ERF proteins are known to bind to the GCC box DNA motif (AGCCGCC) which is also found in promoters of several PR (*pathogenesis-related*) genes conferring ethylene responsiveness, and the C-repeat (CRT)/dehydration-responsive element (DRE) motif, which is involved in the expression of dehydration and low temperature responsive genes (Agarwal and Jha 2010; Sakuma et al. 2002). In *Arabidopsis*, TGA transcription factors from basic domain/Leu zipper (bZIP) family have been found to have positive contributions to expression of PR genes (Johnson et al. 2003; Kesarwani et al. 2007), AtWRKY18, a transcription factor from WRKY proteins family in moderate levels resulted in enhanced expression of *PR* genes (Robatzek and Somssich 2002) and, a novel transcriptional factor, Whirly (StWhy1), binding to a single-stranded DNA element (GTCAAAA) of the *PR-10a* promoter, has been implicated in the regulation of potato *PR-10a* gene (Desveaux et al. 2004) which indicate an additive effect of *PR10a* and *DREB2A* in conferring drought tolerance.

Transgenic technologies have not been commercialized in minor crop grain legumes like pea, despite their environmental and socio-economic importance (James 2011). Owing to the narrow gene pool of pulses, intricate characteristics such as abiotic stress tolerance are difficult to select for (Kumar et al. 2003). Genetic improvement by incorporation of traits that can contribute to yield under drought into well-adapted genotypes is suggested to be a viable alternative (Wasson et al. 2012). However, progress in testing of genetically engineered drought tolerant rice (Todaka et al. 2015), tomato, wheat (Waltz 2014) and acceptance of drought tolerant DroughtGard™ maize in USA as reflected by 15 fold increase in planting area since it's introduction in 2013 (also expected to be released in Africa in 2017) (James 2015), and the recent regulatory approval of drought tolerant soybean (Waltz 2015) in Argentina, is encouraging. The lag in case of legume crops is a combination of lack of efficient and reproducible genetic transformation methods (Carlos Popelka et al. 2004), concentration of efforts in creating a specialty crop to mostly developing countries (Parisi et al. 2016) and the costs of the regulatory process that must be amortized over a relatively minor crop (Kalaitzandonakes et al. 2007).

Our results suggest that coexpressing *DREB2A* and *PR10a* gene in pea plants could be a promising approach to improve drought tolerance and this technology could be used for improvement of related legumes as well. However, for demonstrating commercial pertinence of drought tolerance, validation of field efficacy of such traits is critical (Liang 2016) and commercial adoption of drought tolerant peas will depend on adequate safety assessment and public acceptance.

Table 3-1. Chi-square (X^2) segregation analysis of T2 plants (against test ratio of 3:1)

Code of T1 plants	LP* result for T2 plants								T2 plants PCR** for PR10 gene						PCR for *DREB2A* gene					
	+	±	∑(+&±)	-	Total	E∑(+& ±)	E(-)	X^2	+	-	Total	E(+)	E(-)	X^2	+	-	Total	E(+)	E(-)	X^2
PR10-2	**3**	1	4	2	6	4.5	1.5	0.637	5	1	6	4.5	1.5	0.637	5	**1**	6	4.5	1.5	0.637
PR10-4	**7**	13	20	0	20	15	5.0	0.010	20	0	20	15.0	5.0	0.010	20	**0**	20	15.0	5.0	0.010
PR111-2	**1**	4	5	5	10	7.5	2.5	0.068	5	5	10	7.5	2.5	0.068	5	**5**	10	7.5	2.5	0.068
PR112-2	**2**	7	9	2	11	8.2	2.8	0.602	9	2	11	8.2	2.8	0.602	9	**2**	11	8.3	2.3	0.602
PR113-1	**2**	12	14	0	14	10.5	3.5	0.031	14	0	14	10.5	3.5	0.031	14	**0**	14	10.5	3.5	0.031
PR122-1	**3**	7	10	0	10	7.5	2.5	0.068	10	0	10	7.5	2.5	0.068	10	**0**	10	7.5	2.5	0.068
PR123-1	**6**	17	23	9	32	24	8.0	0.683	23	9	32	24.0	8.0	0.683	23	**9**	32	24.0	8.0	0.683
PR*-2	**7**	7	14	0	14	10.5	3.5	0.031	14	0	14	10.5	3.5	0.031	14	**0**	14	10.5	3.5	0.031
b-1	**0**	6	6	3	9	6.8	2.3	0.564	9	0	9	6.8	2.3	0.083	9	**0**	9	6.8	2.3	0.083
b5-1	**6**	8	14	4	18	13.5	4.5	0.785	14	4	18	13.5	4.5	0.785	14	**4**	18	13.5	4.5	0.785

∑: sum: E: expected. Test ratio = 3:1 and X^2 at df 1= 3.841;
*LP assay: - susceptible, ± partially resistant & + resistant;
**PCR result: + positive for the gene of interest (GOI) & - negative for the GOI

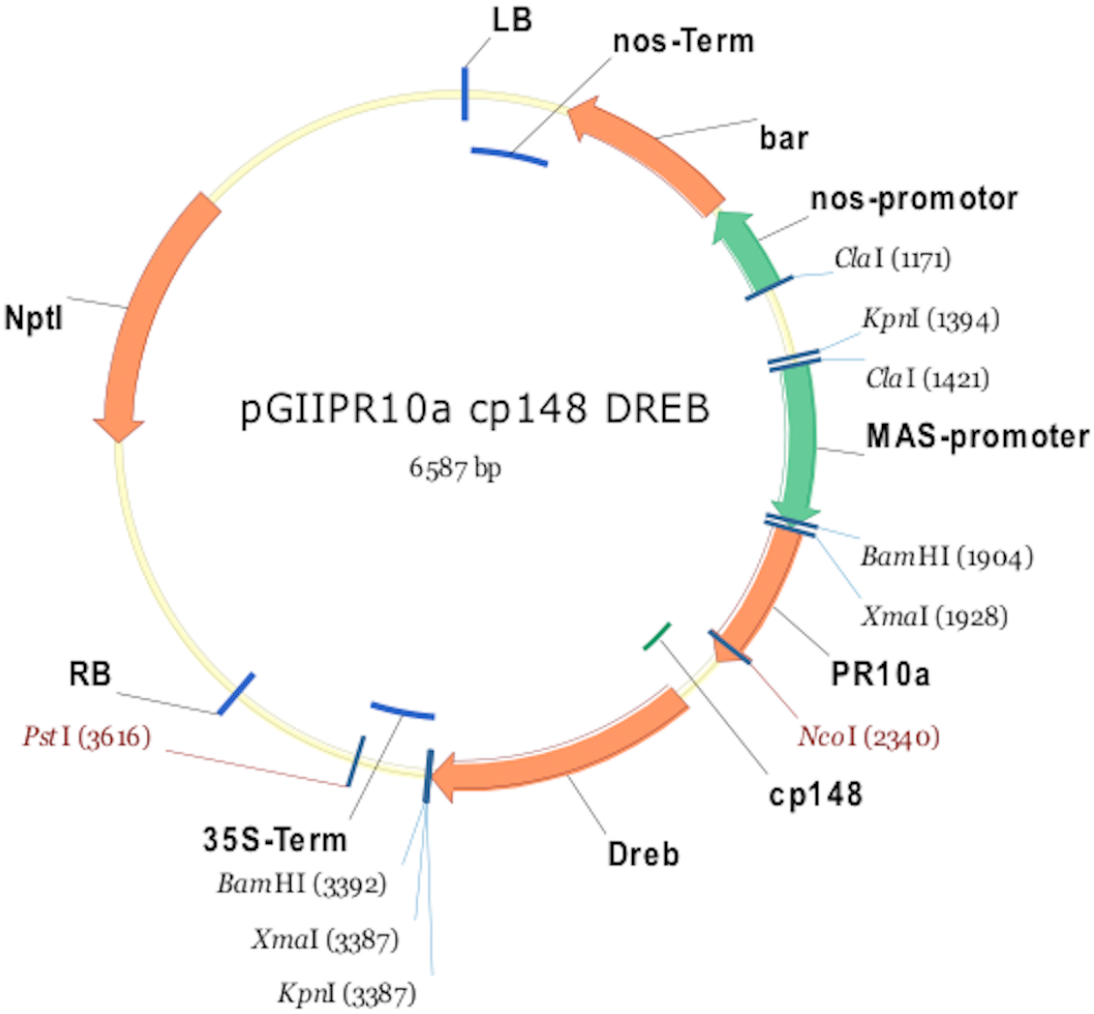

Figure 3-1. Functional map of the transformation construct.

LB: left border, T-nos: terminator sequence of the nopaline synthase gene, bar: bar gene, P-nos: promoter sequence of nopaline synthase, P-MAS: manopine synthase promoter, PR10a: pathogenesis-related proteins from potato, cp148: internal ribosome entry site (IRES) from tobacco mosaic virus, DREB2a: dehydration responsive element binding sequence from rice, T-35S: terminator sequence of the cauliflower mosaic virus and RB: right border.

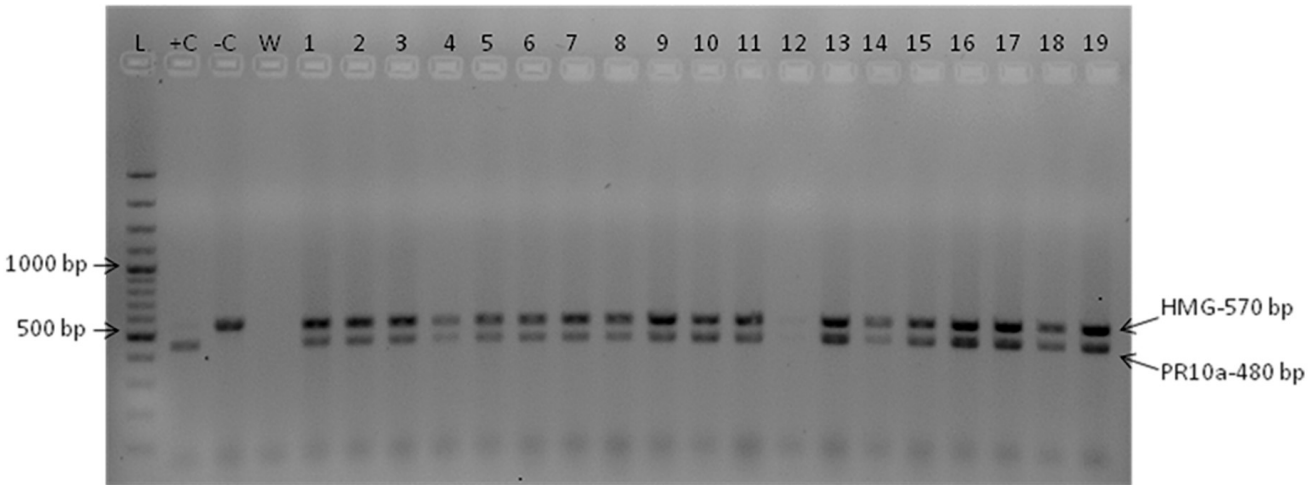

Figure 3-2. Multiplex PCR analysis for detection of *PR10a* transgene and HMG housekeeping gene in the putative transgenic shoots.

L: GeneRuler^{TM} 100 bp plus DNA ladder; +C: plasmid (pGII*PR10a*-cp148-*DREB2A*) DNA as a positive control; -C: gDNA of non-transgenic pea plants as a negative control; W: water control and lane 1-19: gDNA from T0 shoots (1: PR1, 2: PR2, 3: PR3, 4: PR4, 5: PR5, 6: PR6, 7: PR7, 8: PR8, 9: PR9, 10: PR10, 11: PR111, 12: PR112, 13: PR113, 14: PR121, 15: PR122, 16: PR123, 17: b, 18: b5, 19: PR*)

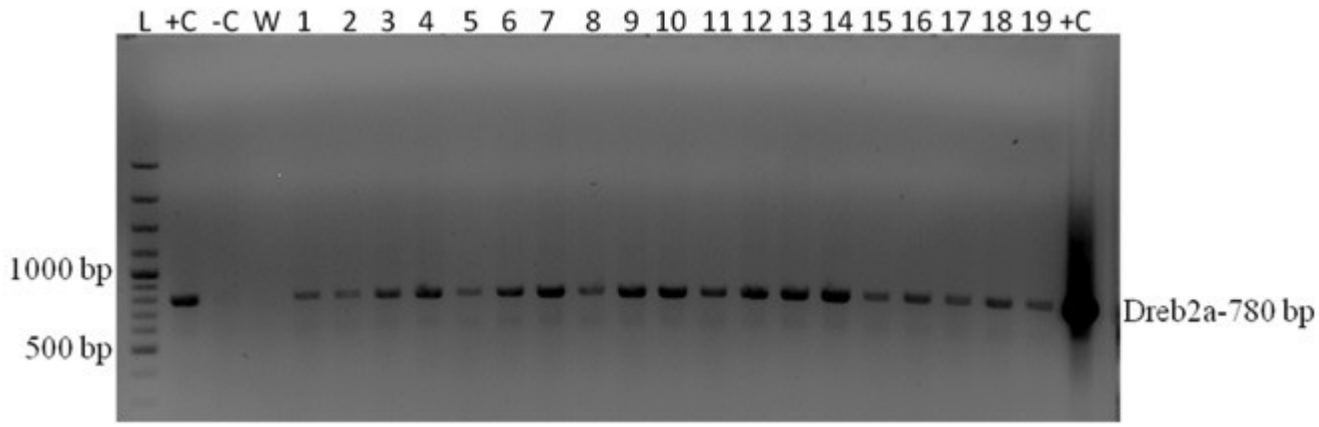

Figure 3-3. PCR analysis for detection of *DREB2A* gene in the putative transgenic shoots.

L: GeneRuler^{TM} 100 bp plus DNA ladder; +C: plasmid (pGII*PR10a*-cp148-*DREB2A*) DNA as a positive control; -C: gDNA of non-transgenic pea plants as a negative control; W: water control and lane 1-18: gDNA from T0 shoots (1: PR1, 2: PR2, 3: PR3, 4: PR4, 5: PR5, 6: PR6, 7: PR7, 8: PR8, 9: PR9, 10: PR10, 11: PR111, 12: PR112, 13: PR113, 14: PR121, 15: PR122, 16: PR123, 17: b, 18: b5).

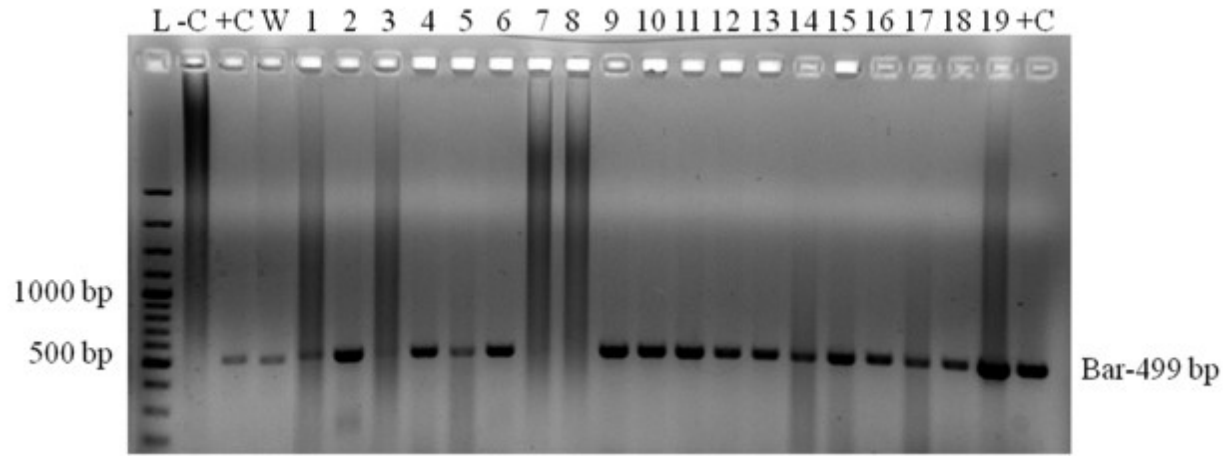

Figure 3-4. PCR analysis for detection of *bar* gene in the putative transgenic shoots.

L: GeneRuler^{TM} 100 bp plus DNA ladder; +C: plasmid (pGII*PR10a*-cp148-*DREB2A*) DNA as a positive control; -C: gDNA of non-transgenic pea plants as a negative control; W: water control and lane 1-18: gDNA from T0 shoots (1: PR1, 2: PR2, 3: PR3, 4: PR4, 5: PR5, 6: PR6, 7: PR7, 8: PR8, 9: PR9, 10: PR10, 11: PR111, 12: PR112, 13: PR113, 14: PR121, 15: PR122, 16: PR123, 17: b, 18: b5).

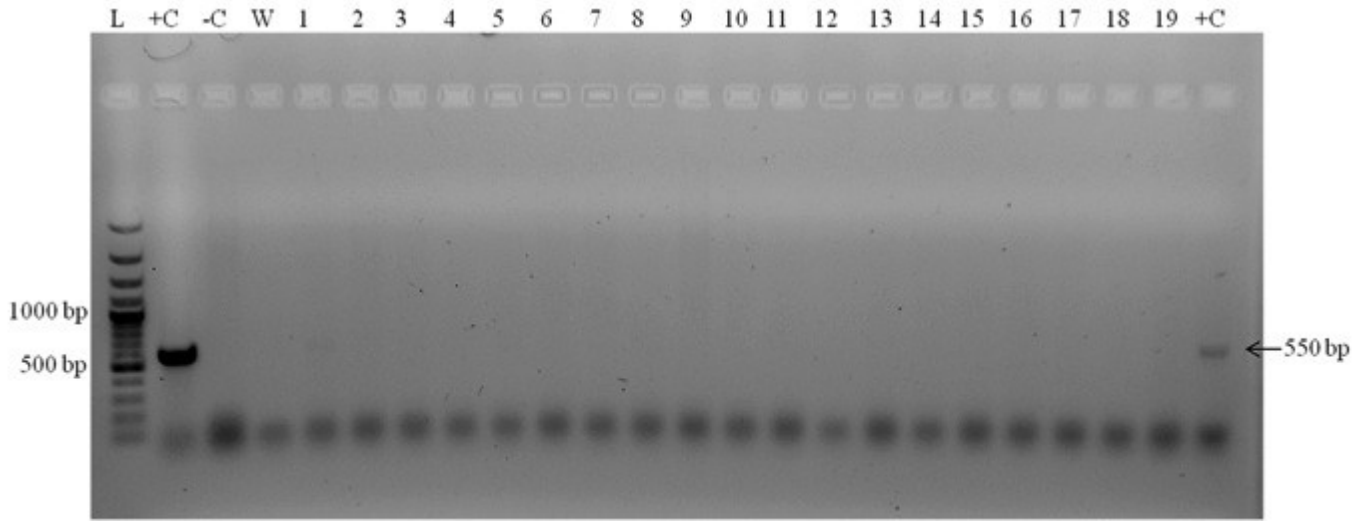

Figure 3-5. Detection of *Agrobacterium* cells persistence in the regenerated putative transgenic shoots using *Agrobacterium* chromosome specific primer set.

L: GeneRuler^{TM} 100 bp plus DNA ladder; +C: *Agrobacterium(*strain EHA105) DNA as a positive control; -C: gDNA of non-transgenic pea plants as a negative control; W: water control and lane 1-19:gDNA from T0 shoots (1: PR1, 2: PR2, 3: PR3, 4: PR4, 5: PR5, 6: PR6, 7: PR7, 8: PR8, 9: PR9, 10: PR10, 11: PR111, 12: PR112, 13: PR113, 14: PR121, 15: PR122, 16: PR123, 17: b, 18: b5. 19: PR*).

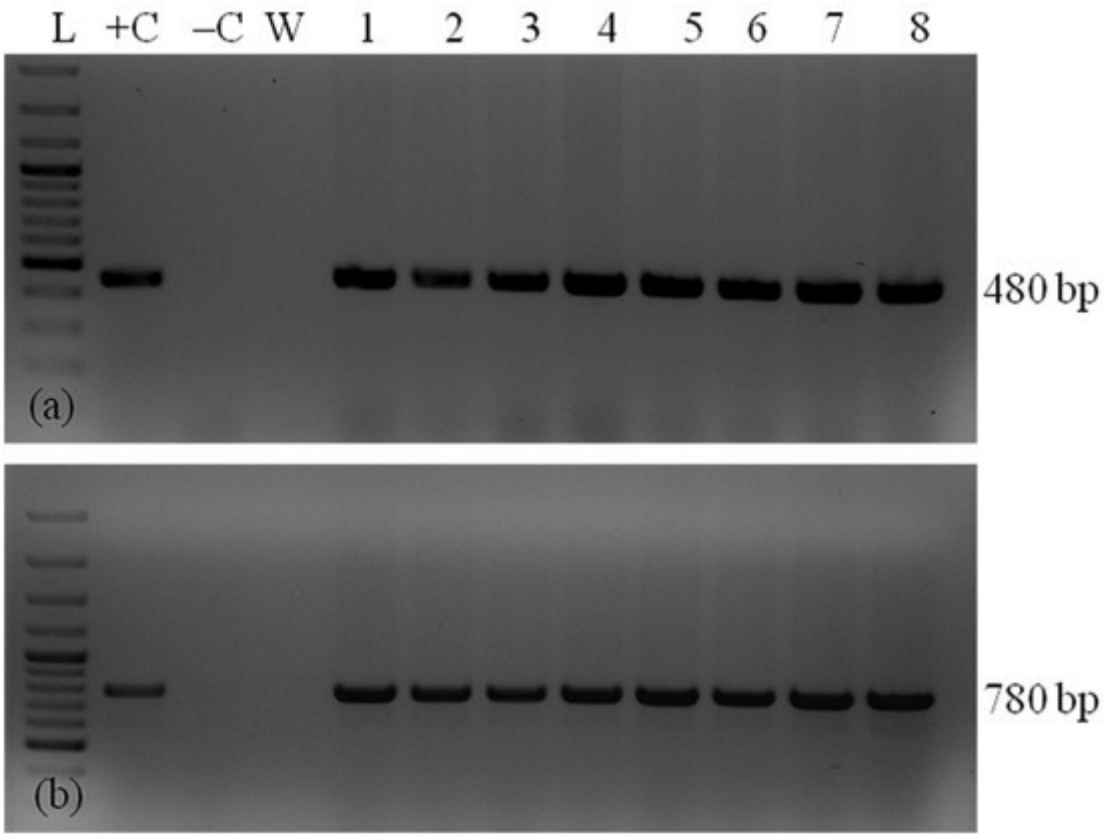

Figure 3-6. PCR analysis to confirm the inheritance of the T-DNA region using transgene specific primers in the genome of randomly selected T1 generation plants. (a) *PR10a* gene and (b) *DREB2A* gene.

L: GeneRuler^{TM} 100 bp plus DNA ladder; +C: plasmid (pGII*PR10a*-cp148-*DREB2A*) DNA as a positive control; -C: gDNA of non-transgenic pea plants as a negative control; W: water control; lane 1-8: gDNA from T1 plants of different clones (1: PR10-4, 2: PR111-6, 3: PR112-2, 4: PR113-1, 5: PR122-1, 6: PR123-1, 7: PR*-2, 8: b-1).

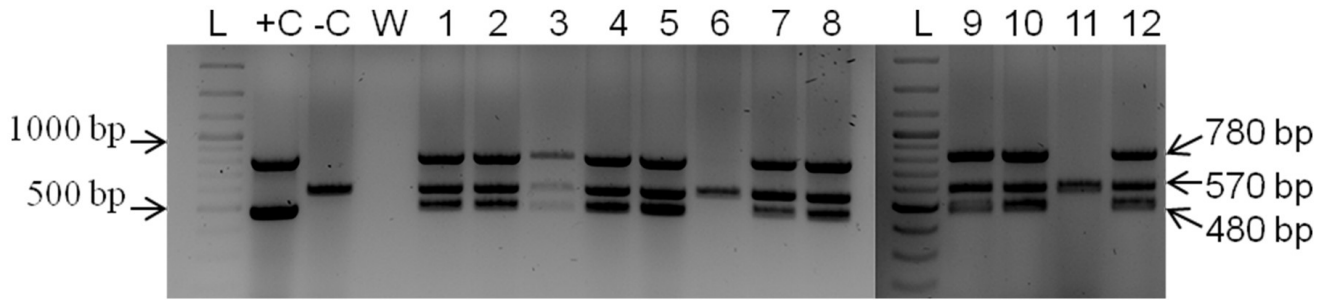

Figure 3-7. Multiplex PCR analysis for *PR10a* (480 bp), *DREB2A* (780 bp) and *HGM* (570 bp) genes in T2 transgenic generation.

L: GeneRuler^{TM} 100 bp plus DNA ladder; +C: plasmid (pGII*PR10a*-cp148-*DREB2A*) DNA as a positive control; -C: gDNA of non-transgenic pea plants as a negative control; W: water control and lane 1-12: gDNA from T2 plants of different clones (1: PR10-4-1, 2: PR111-2-5, 3: PR112-2-5, 4: PR113-1-7, 5: PR122-12, 6: PR123-1-15, 7: PR123-1-20, 8: PR*-2-7, 9: b-1-3, 10:b5-1-1, 11: PR10-2-1 and 12: PR10-2-3).

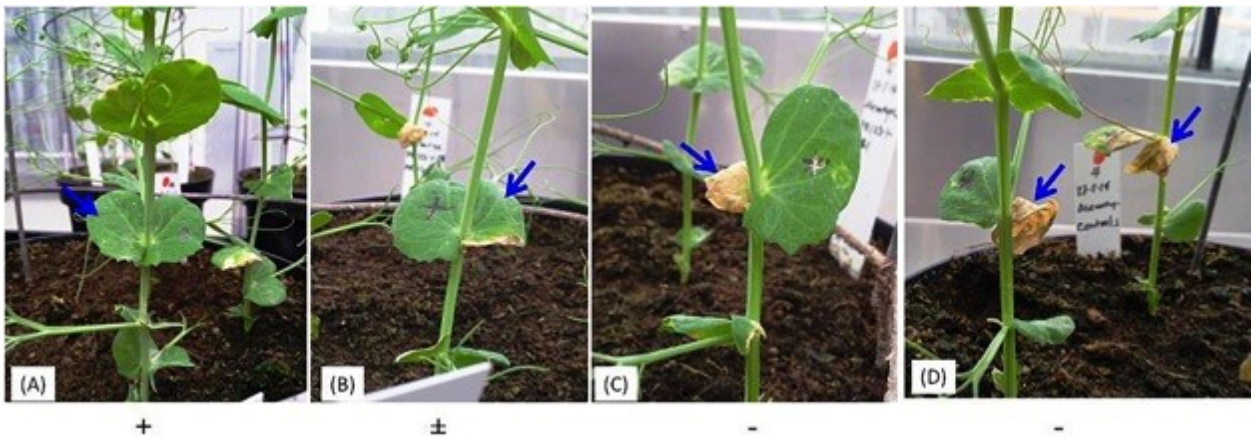

Figure 3-8. Herbicide tolerance of T2 transgenic pea plants.

(A) herbicide resistant (+), (B) partially herbicide resistant (±), (C) herbicide susceptible (-) and (D) herbicide susceptible (-) leaves of non-transgenic control plants

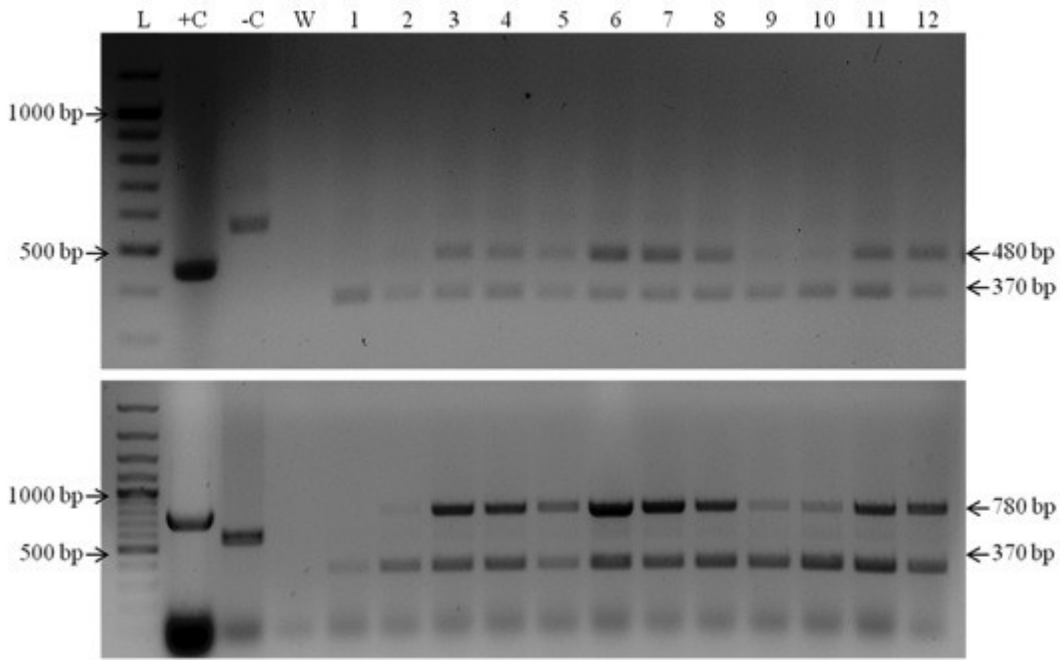

Figure 3-9. Expression analysis of Housekeeping gene (370 bp from cDNA and 570 bp from gDNA) *PR10a* gene (480 bp) and *DREB2A* gene (780 bp) in selected T2 transgenic plants.

L: GeneRuler^{TM} 100 bp plus DNA ladder; +C: plasmid (pGII*PR10a*-cp148-*DREB2A*) DNA as a positive control; -C: gDNA of non-transgenic pea plants as a negative control; W: water control; lane 1: cDNA from control plants; lane 2-12: cDNA from T2 transgenic plants (2: PR10-4-12, 3: PR111-2-4; 4: PR112-2-6, 5: PR113-1-6, 6: PR122-1-5, 7: PR*-2-1,8: PR123-1-2, 9: b-1-2, 10: b5-1-1, 11: PR10-2-3) and lane 12: cDNA from T0 plants of Navarro cultivar (non-transgenic pea cultivar).

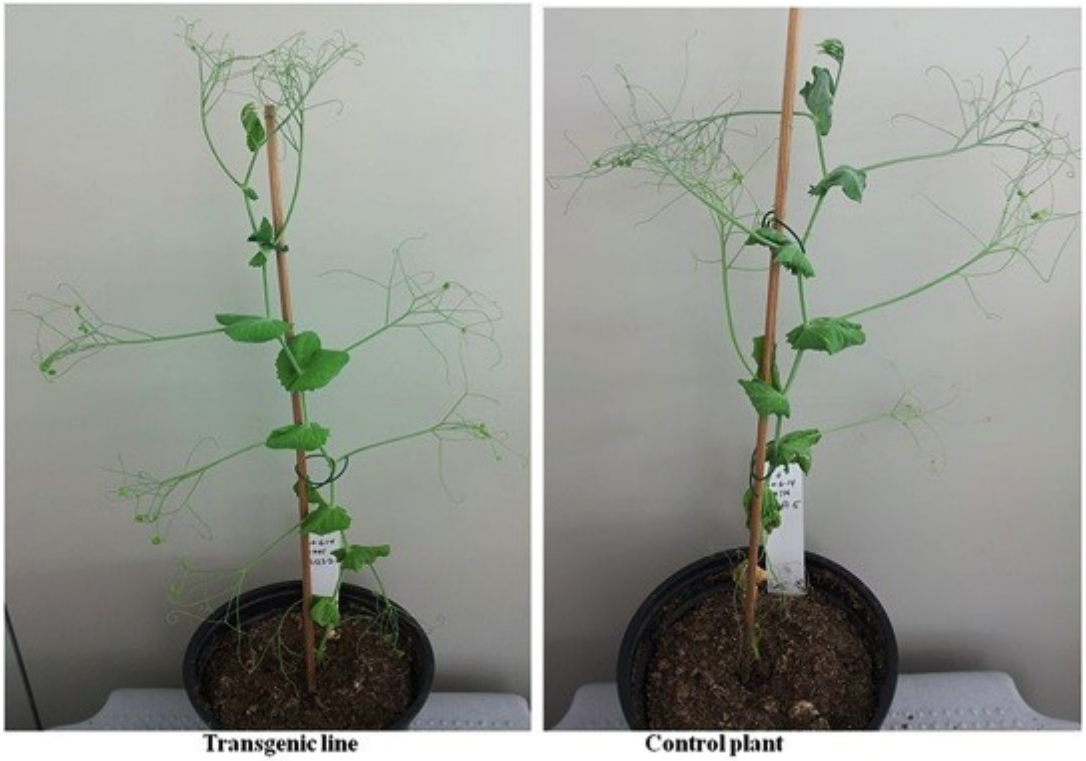

Figure 3-10. Effect of three weeks water withholding on transgenic and control plants. The wilting of leaves was more pronounced in the control plant compared to the transgenic plant.

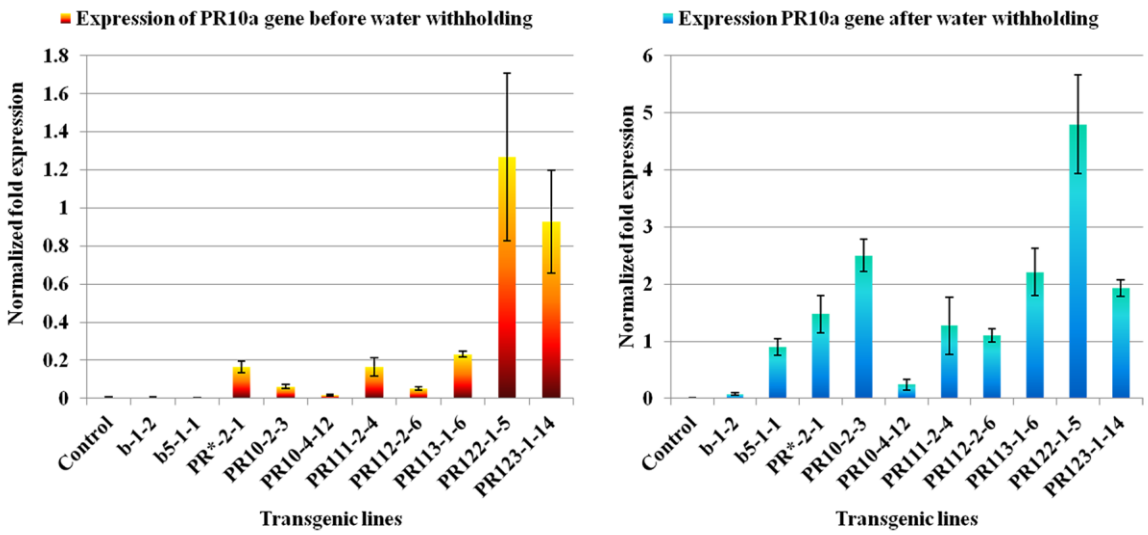

Figure 3-11. The expression level of *PR10a* gene normalized to pea housekeeping gene before and after withholding for three weeks. The expression of *PR10a* gene increased 1-3 fold upon withholding water for three weeks.

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Chapter Four: Efficacy of transgenic pea (*Pisum sativum* L.) stably expressing antifungal genes against *Fusarium* spp. in three years of confined field trials

4.1 Introduction

Grain legumes are valuable worldwide for their nutritional and health benefits and contribution towards agricultural sustainability (Araújo et al. 2015). Among grain legumes, pea (*Pisum sativum* L.) is economically important next only to soybean and bean worldwide (FAOSTAT 2015) and are mostly grown in temperate regions. Peas are the largest pulse crop in the multi-billion dollar Canadian pulse industry, grown primarily in the prairie provinces of Saskatchewan, Alberta and Manitoba, with a harvested area of 1.68 M ha in 2016 (Statistics Canada 2016). The ability of peas to fix nitrogen promotes environmental stewardship by decreasing fertilizer application, reducing greenhouse gas emissions while increasing nitrogen availability for subsequent crops. Development of field pea cultivars with improved yield, disease resistance, abiotic stress tolerance and seed quality have been identified as major research priorities by Canadian pulse growers (Warkentin et al. 2015). However, further improvement to yield and seed quality is severely limited by pea diseases, particularly the fungal endemic, Fusarium root rot (*Fusarium* spp.) caused by a pathogen complex (Xue 2003) which could be responsible for up to 60% yield losses in pea crops commercially in Canada (Basu et al. 1976).

Recent disease surveys in Alberta have pinpointed *Fusarium* species associated with pea root rot complex (Chang et al. 2007), with *F. avenaceum* as the primary causal agent, whose increasing soil populations may have been benefitted by the

crop rotations with canola (Feng et al. 2010) and frequent planting of pea (Bainard et al. 2017). *Fusarium avenaceum* isolates show genetic and ecological plasticity, occupying various ecological niches such as root tissues of legumes, head and root tissues of cereals (Abdellatif et al. 2010) and hence, is also the causal agent for root rots associated with other common crops in the prairies, including canola (Chen et al. 2014) and wheat (Tyburski et al. 2014). Currently, no fungicides are effective against Fusarium root rot of pea and only partial disease resistance has been identified (Bodah et al. 2016). Management strategies are reliant on crop rotation, although species involved in this fungal complex can survive for several years in soil (Feng et al. 2010). Disease resistance would have positive economic impacts on the pea industry in the prairies. Genetic transformation could aid classical breeding techniques, by overcoming sexual incompatibility of related species and lack of natural source of resistance in pea. Because of its self-pollinating nature, low degree of outcrossing and low allergenicity, pea is a good candidate for genetic modification (Rubiales et al. 2015; Warkentin et al. 2015). However, the economic benefits of genetically modified (GM) pea will need to surpass the regulatory costs, time and labor involved in bringing a GM crop to market (Smyth et al. 2017).

Despite the effort in crop improvement via transgenic technology, there are few examples of commercially successful transgenic legumes besides soybean (Eapen 2008). Particularly in the case of pea, biotechnological approaches have been restricted to development of insect resistance trait (Negawo et al. 2013; Schroeder et al. 1995) or drought tolerance (Kahlon et al. 2017 unpublished). Successful examples of commercial release of GM disease resistant crops, in general, are rare, currently limited to the

example of papaya genetically modified with the ringspot coat proteins from mild virus strains of the pathogen (Gonsalves and Ferreira 2003). The lack of GM disease resistant crops could be attributed to lower levels of disease resistance conferred (compared to other traits such as herbicide resistance), which is below economic threshold for producers or high level of resistance but only to a very specific pathogen (Wally and Punja 2010).

Several approaches have been used to engineer plants for fungal resistance (for reviews, see ref Punja 2001; Saharan et al. 2016; Wally and Punja 2010) such as introduction of resistance-genes (R-genes), utilizing plants basal defense responses (Gururani et al. 2012), detoxification of virulence factors (Wally and Punja 2010), expression of antimicrobial secondary metabolites like phytoalexins and pathogenesis related (PR) proteins (inhibiting the pathogen's capacity to degrade polysaccharides within cell wall or RNA) (Moosa et al. 2017; Wally and Punja 2010), and modification of plant signaling pathways including transcription factors. It is notable that for achieving enhanced disease tolerance, working on pathogens with a wide host range (particularly for seedling infecting pathogens), have been more successful (Punja 2001). *Fusarium* spp. are often classified as hemibiotrophs because their infection pattern initially resembles that of a biotrophic pathogen (relying on living host) and gradually transitions into a necrotropic pathogen (consumer of host cells after killing them) (Ma et al. 2013). Such pathogens invade living plant cells and subvert the metabolism in favor of their own growth; hence, with such specialized plant-hemibiotrophic pathogen interactions, even minor changes in either host or pathogen can upset the balance (Hammond-Kosack and Jones 1997). Achieving genetic resistance/tolerance becomes even more difficult when

pathogens are genetically variable, as has been reported in cases of *F. graminearum* (O'Donnell et al. 2004) and *F. avenaceum* (Holtz et al. 2011) and other *Fusarium* species, because variation facilitates rapid evolution of resistance (Feng et al. 2010). Often, disease resistance is a highly complex multigenic trait, and thus single gene transformations may be insufficient and/or offer limited spectrum disease resistance (Anand et al. 2003), or single gene resistance could be circumvented by a mutation reducing the function of the introduced gene (Gurr and Rushton 2005). Hence, stacking/pyramiding more than one gene decreases the risk of development of resistance (Halpin 2005). However, coordinated expression of several genes in one plant could pose an additional challenge (Halpin 2005; Que et al. 2010).

Recently, European field pea cultivars expressing four antifungal genes, *β 1-3 glucanase (G)*, *endochitinase* (C) (belonging to PR proteins family), *polygalacturonase inhibiting proteins* (PGIPs) (P) and *stilbene synthase* (V) have been transformed for disease tolerance (Amian A et al. 2011; Hassan et al. 2009; Richter et al. 2006a). The PR proteins (β -1,3 glucanase and endochitinase) degrade microbial cell wall components (i.e. glucan and chitin) (Van Loon et al. 2006) and PGIPs can inhibit fungal endopolygalacturonases, causing fungal wall degradation and plant tissue maceration (De Lorenzo et al. 2001); both are important components of quantitative plant defense responses. *Stilbene synthase* belongs to the phytoalexins class of secondary metabolites that possess biological activity against a wide range of pathogens (Jeandet et al. 2002). Transgenic plants showed enhanced tolerance to fungi in *in vitro* testing (inhibition of fungal spore germination of *Trichoderma harzianum* (T12 strain) (Amian A et al. 2011). However, field-testing is crucial to establish trait efficacy, especially with soil dwelling

pathogens because of the complexity and high degree of temporal and spatial variation in soil based ecosystems (Birch et al. 2007b). However, the field-testing of GM crops in Germany is complicated due to non-transparent legislation of GM crops (Nelissen et al. 2014). An experienced regulatory framework exists in Canada and Canada regulates products derived from biotechnology processes under "novel products". Hence, GM plants are known as Plant with Novel Trait (PNT) and regulated under the auspices of Canadian Food Inspection Agency (CFIA), Health Canada and Environment Canada (CFIA 2017a).

We report here our investigation of three years of confined field trials of transgenic pea stably expressing antifungal genes single and stacked against *Fusarium avenaceum* in comparison to parental lines and well adapted Canadian pea lines. Our hypothesis was that pea transformed with antifungal proteins would tolerate Fusarium root rot better than Canadian conventional pea lines and parental lines. We also hypothesize that transgenic lines with stacked genes will have an advantage over single gene insertions in response to Fusarium root rot in the field.

4.2. Materials and Methods

4.2.1. Plant material

Four antifungal genes V, P, G, C encoding for disease resistance were inserted into European pea cultivars "Baroness" and "Sponsor" at the Institute of Plant Genetics, Department of Plant Biotechnology, Leibniz University, Hannover, Germany. Embryo axis excised from mature seeds using the modified protocol (by Schroeder et al. 1993) served as explants for *Agrobacterium*-mediated transformation using *Agrobacterium* strain EHA105 (Hood et al. 1993). European pea cv. 'Baroness' (*P. sativum* L. cv.

'Baroness') was used for transformation with V, P, G genes (V had inducible Vst promoter, P, G had constitutive D35S promoter), and similarly, European pea cv. 'Sponsor' (*P. sativum* L. cv. 'Sponsor') was employed for transformation with C gene (promoter D35S) (see Chapter 3). Herbicide resistant *bar* gene from *Streptomyces hygroscopicus* was present along with the genes as a selectable marker (Murakami et al. 1986; Thompson et al. 1987) (for functional map of each transgene, details of choice of promoters, sources of genes, please refer to Kahlon et al. (2017). Two single c*hitinase* gene lines have either D35SP (double 35S promoter from CaMV {Cauliflower Mosaic Virus}) (line 18) or *Vst* (*Stilbene synthase*) from grape (line 20). Conventional breeding was employed to incorporate genes into a single line (V:P (Richter et al. 2006c) x G:C (Amian A et al. 2011) = V:P:G:C (Hassan et al. 2010).

4.2.2. Gene expression

To determine if gene expression in transgenic and non-transgenic lines used in the field experiment was maintained in subsequent generations, they were grown in a greenhouse in a separate experiment. Plants (10 plants line^{-1}) were seeded in pre-autoclaved vermiculite-perlite mixture (Sunshine Mix®#4, Sun Gro Horticulture, Canada) and retained at 25 ± 2 °C with a 16/8-h light/dark photoperiod for four weeks after which root and leaf samples were removed and cleaned thrice with RNAse free water. According to Qiagen (Canada)'s recommended protocol, total RNA was extracted from tissues using the Qiagen RNeasy Plant Mini Kit. Briefly, frozen tissues were ground to a fine powder with liquid nitrogen in baked (250 °C, 3 hr) and chilled (-80 °C) mortar and pestle and 100 mg of the powder was taken and mixed with 450 µl of buffer RLT (containing β-ME) and vortexed to obtain a slurry, incubated at 56 °C for 3 min, passed through the

QIAshredder column and centrifuged at 21,000 x *g* for 2 min. In a new tube (with flow through), 200 µl of absolute ethanol was added and the mix applied to RNeasy column and centrifuged at 10,000 x *g* for 30 sec. The flow-through was dispensed off and column washed by adding 350 ul RW1 buffer, centrifuged again and subjected to on-column DNA digestion using RNase free DNase set (Qiagen) by adding 27.27U DNase in 80 µl RDD buffer to the column and incubated at room temperature for 15 min. This was followed by additions of 700 µl RW1, 500 µl RPE and 50 µl RPE and centrifugation at 10,000 x *g* and discarding of the flow-through to the column at each step. In a new 2 ml collection tube, the column was centrifuged at 12,000 x *g* for 2 min and finally, transferred to a 1.5 ml microfuge tube, 50 µl of nuclease free water was added to the centre of the column, and the RNA eluted by centrifugation at 12,0000 x *g* for 30 sec. NanoDrop™ spectrophotometer (Thermo Fisher Scientific™) was used to quantify the extracted RNA and stored at -80 °C until further analysis.

cDNA synthesis

In a 20 µl volume, complimentary DNA (cDNA) was synthesized using 1ug of total RNA from RevertAid RT kit (Thermo Fisher Scientific™) as per the recommended protocol. Briefly, 1 µg of total RNA was used as the template in a 20 µl reaction containing 100 nmole of random hexamer primer, 20U/µl of RiboLock RNase inhibitor, 10 nmole of dNTP and 200U/µl of RevertAid reverse transcriptase in 1x reaction buffer. The contents were mixed, centrifuged and incubated at 25 °C for 5 min, followed by 60 min at 42 °C for cDNA synthesis and then heating the tubes to 70 °C for 5 min. Products were stored at -80 °C.

Quantitation of gene expression by Real-Time PCR

Gene expression levels (of V, P, G, and C) in transgenic pea tissues (from root and leaf), were quantified using SYBR Green based q-RT-PCR on a StepOnePlus™ instrument (Applied Biosystems®, Canada) with quantitation employing $\Delta\Delta C_T$ method with melt curve. A 10 µl reaction contained 5 µl of 2 x KAPA SYBR® Fast Master Mix (Kapa Biosystems, Boston, MA, USA), 1 µl of 1:15 diluted cDNA, and 5 pmol of each (forward and reverse) gene specific primers which were designed using either Primer Express 3.0 (Applied Biosystems) or PrimerQuest (Integrated DNA Technologies, Coralville, Iowa) with Tm of 60 °C and amplicon sizes between 100-140 bp. Elongation factor 1a was used as endogenous control. Primers used are "P" forward: 5'-CTTCGAAATCAAGACAGCCTTCA-3'; reverse: 5'-GGGATCACACTCGACGCAGTA-3'; "V" forward: 5-AGAAATGCCCGGTGCAGAT-3', reverse: 5'-TTCCACCTGCATAGCAACCTT-3'; "G" forward: 5'- AAC GCG CGG AAC TAC AA -3', reverse: 5'- CTC GTT GAA CAT GGC GAA TAT G -3'; "C" forward: 5'- GAA CCG GAA CTC CTT CTA CAG -3', reverse: 5'- TCC TGC TTC TTG GTG GTG -3' and endogenous control forward: 5'-GATGGATGCTACCACCCCTAAG-3', reverse: 5'-GAGATGGGAACGAAGGGAATT-3'). Every reaction was carried out in triplicate, using 6 cDNA samples from individual plants from each line, and the average C_T values were used for calculating gene expression. The detection limit for the plasmid copies was obtained with a dilution series between 10^7 and 10^1 copies per reaction, and linear range of detection was established and were added to the German parental lines ('Sponsor' and 'Baroness')'s cDNA sample to serve as the baseline for estimating relative expression. These methods are previously published (Kahlon et al. 2017).

4.2.3. Field trials

A confined field trial was established at a secure field site located at the Crop Diversification Center (CDC) North, Alberta Agriculture and Forestry (AAF), north east of Edmonton, AB (lat. 53°38'N, long.113°22'W), on a black chernozemic sandy loam soil in spring of 2013, 2014 and 2015 following the guidelines for field testing of PNTs outlined by the CFIA. Seventeen treatments comprised of nine transgenic lines (five lines with single gene insertions {5(G), 18(C), 20(C), 21(V) and 23(P)} one line with double gene insertion {4(V:P)}, one line with triple gene insertion {11(P:C:G)}, two lines with four gene insertions {8(V:P:G:C), 10(V:P:G:C)} and as comparator, four lines including two German parental lines, 'Sponsor' and 'Baroness' and three Canadian lines with/without pathogen inoculum : 'Agassiz' (resistant to powdery mildew (*Erysiphe pisi* Syd.) and moderately susceptible to Mycosphaerella blight (*Mycosphaerella pinodes)* (CFIA (Canadian Food Inspection Agency 2017a), 'AC Earlystar' (resistant to powdery mildew, moderately resistant to Mycosphaerella blight and Fusarium wilt (*Fusarium oxysporum*)} (CFIA (Canadian Food Inspection Agency 2017b)) and AAC Royce (resistant to powdery mildew, moderately susceptible to Mycosphaerella blight and Fusarium wilt (Bing et al. 2016). Seeds were individually planted by hand at 30 seeds $plot^{-1}$ (1 x 0.5m) at 5 cm depth. Each plot was separated by seeded rows of conventional AC Ultima triticale to better delineate one genetic composition from another and limit tangling of pea lines between plots. All plots were inoculated with *Rhizobium leguminosarum* bv. viciae (1.6×10^{9} viable cells g^{-1})(Galloway Seeds Ltd., Fort Saskatchewan, AB) at a rate of 291.58 g ha^{-1}. @ 0.004g for 2.5 g seeds for promoting root nodulation. All transgenic lines, German parental controls and three Canadian lines were also treated with *Fusarium*

avenaceum inoculum @ 5gm^{-1} row, ground into fine power from previous year's infected wheat plants and was applied in contact with the seed at the time of seeding just under the seed row, to promote disease establishment. After soil testing each year, appropriate fertilizers were added and plots were hand weeded throughout the growing season. The plots were arranged in randomized complete block design with pea lines as treatment randomly arranged in blocks, with six replicates per treatment.

4.2.4. Confirmation of pathogen presence

Pea roots, the *Fusarium avenaceum* inoculum and random soil samples from the field site for 2013, 2014 and 2015 trials were used for characterization of the pathogen using agar plating and polymerase chain reaction (PCR).

Inoculum plating

Approximately 1 mg of ground *Fusarium avenaceum* inoculum used in the field experiment each year was plated onto potato dextrose agar (PDA) with antibiotics on petri dishes. Plates were incubated for 7-10 days at room temperature, and resulting cultures were sub cultured and confirmed as *F. avenaceum* based on the colony morphology using culture identification techniques outlined in Taheri et al. (2017).

Root sample plating

Five random roots plot^{-1} were selected and tap roots showing necrosis were cut into 1 cm pieces and 3 pieces plot^{-1} were randomly selected each field season, transferred into a 15 ml culture tube and surface sterilized using 70% ethanol for 30 s followed by 0.5% NaOCl (10% bleach) for 2 min, rinsed thrice in distilled water and blotted dry on sterile filter paper. Three root pieces plot^{-1} were plated on acidified potato dextrose agar (APDA) in 90 mm Petri dishes and incubated on the laboratory benchtop for 7 -10 days

(8 h light, 16 h dark, 22C). Colonies growing from the root were transferred to a new APDA plate, using hyphal tip transfer under a dissecting scope. Presumptive identification of *F. avenaceum*, *F. solani*, *F. redolens* and *F. oxysporum* were then made based on the distinct morphological characteristics of these species (pink/yellow/dark red/or purple pigmentation and shape of macroconidia under microscope) when possible, and in comparison to stock cultures as per Taheri et al. (2017), and confirmed with PCR. Number of roots plate^{-1} yielding a *Fusarium* culture was recorded. In 2014 and 2015, the symptomatic areas of roots were also used in a series of multiplex PCR reactions to assess for presence of 10 *Fusarium* species and *Aphanomyces euteiches* (another pathogen commonly associated with root rots in prairies). Briefly, root samples were lyophilized for 48 h (4.5L FreeZone, LabConco, Kansas City, Missouri, USA) and then 30 mg of tissue transferred to collection microtubes in a 96-well plate format. Samples were then ground using a TissueLyzer II (Qiagen, Carlsbad, California, USA) and DNA extracted using the PlantDNABiosprint kit according to manufacturer's instructions (Qiagen). The multiplex reactions were performed using the Qiagen Multiplex PCR Kit according to manufacturer's directions with 2 □L of DNA and 0.2 □M of each primer (details of primers sequences and expected amplicon sizes used for pathogen detection from root were obtained from the literature as follows: *F. avenaceum (*Doohan et al., 1998; Turner et al., 1998*); F. graminearum* and *F. culmorum* (Nicholson et al., 1998), *F. sporotrichoides* (Demeke et al., 2005), *F. equisiti* and *F. oxysporum* (Mishra et al., 2003); *F. poae* (Parry and Nicholson, 1996); *F. acuminatum* (Williams et al., 2002); *F. redolens* (Bogale et al., 2007); and *A. euteiches* (Gangnuex et al., 2014). Multiplex combinations were as follows: 1) *F. graminearum*, *F. poae*, *F. oxysporum* and *A. euteiches*; 2) *F.*

acuminatum, *F. solani* and *F. equisiti*; 3) *F. culmorum*, *F. redolens*, *F. sporotrichoides* and *A. euteiches*; and 4) *F. avenaceum* (2 primer pairs). A positive DNA standard from stock cultures of all species was included with all multiplex reactions to ensure there was no cross-reactivity between primer pairs. There was cross-reactivity between the *F. avenaceum* primer pairs with *F. acuminatum* DNA. A combination of the two *F. avenaceum* and *F. acuminatum* primers could usually differentiate between these two species, but in some cases the reaction was scored as mixed for *avenaceum/acuminatum* when results were not clear.

Soil sample plating

Soil samples were collected from randomly chosen plots immediately prior to seeding of the trials each year. Soils were diluted 1:50 in sterile distilled water, and 1 ml was plated onto potato dextrose agar (PDA) with antibiotics on petri dishes. Plates were incubated for 7-10 days at room temperature, and the resulting mixture of cultures were scraped from the agar surface and subjected to DNA extractions using a multiplex PCR, as described above.

4.2.5. Plant growth assessment and disease ratings

Data was collected for seedling emergence (14 days after planting, DAP), plant height and root diameter (28, 42 DAP, 5 plants plot^{-1}). The disease symptoms and severity for above-ground symptoms (Infantino et al. 2006) and below-ground symptoms (Bilgi et al. 2008) (Table 4.1) was recorded on all plants in each plot after destructive sampling at 8 weeks DAP. Fresh weight (gm plot^{-1}) was recorded as an indicator of the potential yield, because of the destructive nature of sampling for disease severity ratings.

4.2.6. Statistical analysis

Data generated from field experiments was analyzed using analysis of variance (ANOVA) using PROC MIXED in SAS 9.4 (SAS Institute Inc. 2014), where lines and years were used as fixed effects and blocks as random effect. LSmeans were compared using pre-planned orthogonal contrasts statements.

4.3. Results

4.3.1. Gene expression analysis

The relative gene expression was higher in leaf tissues than root tissues in all of the transgenic pea lines except C, where expression was similar in leaf and root tissues (Table 3.2, previously published, in part (Kahlon et al. 2017). Gene expression for different genes and lines was highly variable. Relative V expression was significantly higher ($p < 0.05$) in leaves compared to roots for line 21(V) ($p=0.0011$) and line 10 (V:P:G:C) ($p=<0.0001$). It was highest in line 10 (V:P:G:C) followed by line 21 and line 4, and negligible in line 8 (V:P:G:C). Interestingly, P had significantly lower relative root expression than in leaf, for line 10 (V:P:G:C) ($p=<0.0001$). Highest relative P expression was observed in leaf tissues of line 10 (V:P:G:C) followed by line 4 (V:P), 11(P:C:G) but lower in roots of these lines and negligible in root tissues of line 8 (V:P:G:C). Relative G expression in root tissues of line 10 (V:P:G:C) was significantly lower than leaf tissues ($p=<0.0001$) which is a four-gene line, but was not significantly different in line 8, another other four-gene stacked line (V:P:G:C) and in line 11(P:C:G), a three-gene stacked line, as well as single gene line 5 (G). All lines tested for relative expression levels of C were not significantly different between leaf and root tissues; however, line 18 (C) had some low, relative gene expression (14.62±4.01 in leaf and 18.01±2.56 in

root) in comparison to negligible expression in stacked lines like 8 (V:P:G:C), 10 (V:P:G:C) and 11 (P:C:G) and same gene but a different promoter line 20 (C). Variable relative gene expression among various genetic compositions of pea lines suggests gene silencing or possibly unequal efficacy of promoters. All transgenic lines were confirmed for homozygosity (data not shown).

4.3.2. Confirmation of pathogen

In 2013, the majority of cultures originating from the root samples were identified and confirmed by PCR as *Fusarium* spp. The most prevalent *Fusarium* species identified were *F. solani*, *F. avenaceum* and *F. redolens,* while other *Fusarium* species, for example, *F. acuminatum,* were relatively less abundant. Presence of other common soil inhabiting fungi like *Rhizoctonia* spp., *Rhizopus* spp., *Trichoderma* spp. and *Clonostachys rosea* were also recorded. The soil plating followed similar recovery trends and *F. redolens, F. solani* and *F. avenaceum* were the predominant *Fusarium* species present in the soil. In the 2014 and 2015, *F. redolens* and *F. solani* were the most abundant in soil samples, with lesser *F. avenaceum* than in 2013. The root plating and PCR confirmations for 2014 and 2015 resulted in fewer *Fusarium* spp. identified from all samples than in 2013. For 2014 samples, major *Fusarium* spp. identified and confirmed were *F. solani*, *F. equisiti*, *F. oxysporum* and *F. avenaceum*. For 2015, pathogens present were characterized as *Fusarium* spp. and were not found in all the samples tested.

4.3.3. Plant growth assessment

The rainfall accumulated during the growing period for three years at the trial location was 58.43%, 75.59% and 60.17% of the long term average (LTA) for 2013, 2014 and

2015 (Table 3.3), respectively. The percentage emergence of plants plot^{-1} 14 DAP are summarized in Figure 4.1. The line*year interaction was significant ($p<0.0001$) at 0.05% level of significance and hence data is presented per year. Overall, all transgenic, parental and Canadian lines which received *F. avenaceum* inoculum showed reduced emergence (%) plot^{-1} than the Canadian lines that did not receive the inoculum, which indicates that pathogen inoculum challenged growth of pea seedlings.

In 2013, transgenic lines were significantly different from German parental lines 24 (Sponsor) and 25 (Baroness) ($p=0.0095$) and Canadian lines (with inoculum)- 26 (Agassiz), 28 (AC Earlystar) and 30 (AAC Royce) ($p=<0.0001$); the highest emergence (%) being for lines 23 (P) & 21 (V) of the transgenic lines. However, this trend did not continue in 2014 and 2015, where transgenic lines were not significantly different than the parental lines 24 (Sponsor) and 25 (Baroness) and Canadian lines (with inoculum), 26 (Agassiz), 28 (AC Earlystar) and 30 (AAC Royce). We observed significant differences among some genes in 2014; for instance, two genes stacked line 4 (V:P) had significantly lower percent emergence than three genes stacked line 11 (P:C:G) ($p=0.0054$) and four genes stacked lines 8 and 10 (V:P:G:C) ($p=0.0007$). In 2015, the highest percent emergence in presence of disease was recorded with transgenic line 8 (V:P:G:C) but it was not significantly different from parental lines 24 (Sponsor) and 25 (Baroness) and Canadian lines (with inoculum), 26 (Agassiz), 28 (AC Earlystar) and 30 (AAC Royce). In conclusion, we did not observe a consistent pattern of superior emergence of transgenic line(s) in the presence of disease in the three years of field experiments.

Pea plants were tallest (height in cm) in 2013 followed by 2014 and 2015, but not statistically significant and no stunting or dwarfing was observed in plants that received

inoculum compared to those that did not (data not shown). Because of the destructive nature of the experiment and as an indication of yield, fresh weight (gm plot^{-1}) was recorded in three years and all lines, years and line*year interaction were significant (p=<0.001) at 0.05% level of significance; hence, data is presented separately for each year. Fresh weight (gm plot^{-1}) production was highest for lines 21 (V) and 23 (P), respectively, for 2013 and 2014; however, this pattern wasn't consistent for 2015 where line 5 (G) had the highest fresh weight (Figure 4.2). All transgenic lines had significantly higher fresh weight than their parents in 2013 (p=0.007), but not in 2014 and 2015, and higher than Canadian lines that received pathogen inoculation in 2013 (p=<0.0001) and 2015 (p=0.0396) but not in 2014. We observed that in the presence of disease, single gene lines 5 (G), 18 (C), 20 (C), 21 (V) and 23 (P) had significantly higher fresh weight than four genes stacked lines 8, 10 (V:P:G:C) in 2013 (p=0.0222) and 2014 (p=0.0304) but not in 2015. Transgenic lines were significantly different in all three years (2013 and 2015 (p=<0.0001), 2014 (p=0.014)) from the Canadian lines that did not receive inoculum (lines 27, 29 and 30), asserting that pathogen inoculum was effective. In general, fresh weight measurements among the three years did not identify any transgenic line producing a significantly higher fresh weight among others or compared to parents and Canadian lines in presence of disease, or indicating an advantage of stacked genes over single genes. Interestingly, expressing the same chitinase gene with two different promoters (line 18(C) (D35S) and 20 (C) (Vst), did not yield any significant differences among the two transgenic lines for emergence (%) and fresh weight (gm plot^{-1}) throughout the three field seasons. Ample precipitation in 2014 possibly explains the

higher emergence percent plot $^{-1}$ and higher fresh weight production plot $^{-1}$ in comparison to 2013 and 2015 which received lesser precipitation.

4.3.4. Disease severity ratings

The line*year interaction for disease severity ratings was significant ($p<0.0001$) at 0.05% and hence data is presented as each year (Figure 4.3 (above ground) and Figure 4.4 (below ground)). Transgenic lines had significantly lower above ground disease ratings than German parental lines (24 (Sponsor), 25 (Baroness)) in 2013 ($p=0.0016$) but not in 2014 or 2015 and Canadian lines that received pathogen inoculum (26 (Agassiz), 28 (AC Earlystar, 30 (AAC Royce)) in 2013 and 2015 ($p=<0.0001$) but not in 2014. No significant differences were observed among transgenic lines expressing a single or two, three or four genes stacked in 2013, but there were some significant differences between single or multiple gene lines in 2014 and 2015. For instance, single gene lines (5 (G), 18 (C), 20 (C) and 21 (V)) had significantly lower above ground disease rating vs. two gene line 4 (V:P) in 2014 ($p=0.0016$) and two gene line 4 (V:P) ($p=0.0308$) and three gene line 11 (P:C:G) ($p=0.0334$) in 2015.

Similar results were recorded for disease ratings below ground. Transgenic lines had significantly lower below ground disease severity ratings than German parental lines (24 (Sponsor), 25 (Baroness)) in 2013 ($p=0.0311$), but not in 2014 and 2015 and lower than Canadian lines that received pathogen inoculum (26 (Agassiz), 28 (AC Earlystar, 30 (AAC Royce)) in 2013 ($p=<0.0001$), 2014 ($p=0.0165$) and 2015 ($p=0.0076$). In 2013, no significant advantage of single vs multiple genes in transgenic lines for disease severity ratings below ground was recorded; however, differences were observed in 2014 and 2015. For example, in 2014, two gene line 4 (V:P) had significantly higher below ground

disease ratings than single gene (line 5 (G), 18 (C), 20 (C), 21(V) and 23 (P)) ($p=0.0273$) and four gene lines 8, 10 (V:P:G:C) ($p=0.0254$) and single gene lines 5 (G), 18 (C), 20 (C), 21 (V) and 23 (P) had significantly lower disease severity ratings than two gene line 4 (V:P) ($p=0.0238$) and four gene lines 8, 10 (V:P:G:C) ($p=0.0324$) in 2015. Interestingly, throughout the three trial years (except above ground disease ratings in 2014), disease severity ratings above and below ground were significantly lower than in the Canadian lines (26 (Agassiz), 28 (AC Earlystar, 30 (AAC Royce)) that received pathogen inoculum and had some genetic advantage of partial disease resistance. It is notable that the Canadian lines have been selected in the presence (inadvertently) of similar strains of these pathogens, whereas the German breeding efforts would have occurred under different selection pressure. As observed for growth parameters, lines with chitinases genes with two different promoters (line 18 (C)(D35S) and 20 (C) (Vst), did not show significant differences for disease severity ratings (above and below) throughout the three field seasons. Overall, contrary to our expectation, we did not observe any transgenic line or gene combination that performed better than parental lines for disease tolerance performance in three consecutive years of field trials.

4.4. Discussion

In this study, confined field trials were conducted for three consecutive years to test nine transgenic pea lines with four antifungal genes, singly or stacked, against Fusarium root rot and were compared to their parental lines as well as Canadian pea lines in presence/absence of pathogen inoculum. The variability found in the transgenic lines throughout the three years of field trial revealed the complexity of the disease tolerance traits and their interaction with variable environmental conditions and multiple

pathogens. Consequently, we were not able to identify transgenic pea lines that outperformed parental lines or well adapted Canadian lines in presence of disease over the course of three consequent field seasons. Although some transgenic lines (for example, lines 21(V) and 23 (P) in 2013 and 2014) did demonstrate better performance (higher emergence, more biomass production and lower disease ratings) in the presence of the pathogen than other transgenic lines, that did not translate into consistent performance or statistically significant differences vs comparators over the three trial years. No advantage of gene pyramiding over individual genes was recorded, contrary to our initial hypothesis. Our results are consistent with other researchers who found that transgene insertions can have variable or no effect on disease tolerance or resistance. For example, high-level expression of tobacco chitinases gene in *Nicotiana sylvestris* did not increase resistance to *Cercospora nicotiana* (Neuhaus et al. 1991). Gene PGIP2 expressed in transgenic wheat did not reduce *Claviceps purpurea* symptoms (Volpi et al. 2013) and β-1,3 glucanase constitutively expressed in alfalfa did not decrease root severity of fungi containing chitin (Masoud et al. 1996a). However, contrary to what we observed, many successful transgenic lines, particularly in legumes, have been reported to enhance antifungal activity. For example, rice chitinases under control of CaMV35S promoter improved resistance of peanut (*Arachis hypogaea* L.) against leaf spot (*Cercospora arachidicola*) (Iqbal et al. 2012b) and late leaf spot (*Phaeoisariopsis personata*), rust (*Puccinia arachidis* Speg.) and *Aspergillus flavus* (Prasad et al. 2013). Similarly, barley chitinases (AAA56786) improved resistance to Corynespora leaf spot disease (*Corynespora cassiicola*) in blackgram (*Vigna mungo* L. Hepper) (Chopra and Saini 2014). Resveratrol synthase (a stilbene) from peanut decreased black stem and leaf

spot disease (*Phoma medicaginis*) in alfalfa (*Medicago sativa* L.) (Hipskind and Paiva 2000). Overexpression of tobacco β-1,3 glucanase in transgenic peanut led to enhanced protection against *Cercospora arachidicola* and *Aspergillus flavus* (Sundaresha et al. 2010) and *Cercospora personanta* (Qiao et al. 2014). All of these reports, however, were conducted under laboratory conditions and against a single pathogen. Under laboratory conditions, differences between tested lines may be easier to differentiate as a single pathogen challenge could be imposed whereas in field trials, the number of pathogens, differences in soil moisture and weather variance more accurately represent the true agronomic effect of transgenes. Also, having multiple pathogens acting on the plant at the same time (as observed in the field trial) can increase occurrence of disease symptoms (Willsey et al. 2016).

Gene pyramiding did not consistently confer resistance advantages in trials conducted in the field as suggested by other researchers. For instance, Moravčíková et al. (2004) observed no enhanced resistance to *Rhizoctonia solani* in a hyphal extension assay using extracts from transgenic potato with chitinases and glucanase genes. Transgene combinations often result in successful inhibition of fungal growth *in vitro,* but fail to translate the same success under greenhouse or field conditions, as was the case for our transgenes. Oilseed rape transformed with double gene construct of chitinase and β-1,3-glucanase genes from barley both driven by CaMV 35S promoter did not increase fungal resistance against *Alternaria brassicae*, *A. brassicola*, *Verticillium longisporum* and *L. maculans* when assayed in the greenhouse whereas purified chitinases and β-1,3-glucanase did reduce fungal grown in vitro (Melander et al. 2006). This can be attributed to PR protein's differential activity against fungal cell wall targets, providing very

specific resistance against some pathogens yet completely ineffective against others in respect to same crop, thus not providing continuous and sustainable resistance (Moosa et al. 2017). For example, no known PGIP is able to inhibit polygalacturonases produced by the fungal pathogen *F. verticillioides* (Kalunke et al. 2015) and PGIP1 which is unable to inhibit *F. moniliforme*, partially inhibits *F. oxysporum* f. sp. *lycopersici* polygalacturonases in bean (*Phaseolus vulgaris*) (Desiderio et al. 1997) and chitinases obtained from *Trichoderma* sp. are considered more effective in conferring disease resistance (Sandhu et al. 2017). The capacity of many fungal pathogens such as *Fusarium* spp., to change their genetic structure in the face of selection forces such as resistance genes and environmental factors (Punja 2001), when they possess high inherent genetic variation (Feng et al. 2010), coupled with their hemibiotrophic nature (Ma et al. 2013), can also contribute to difficulties to achieve disease resistance. Environmental variation has been attributed as the most important factor contributing to disease progression and resistance responses in Fusarium root rot, making this trait highly challenging to accomplish (Foroud et al. 2014).

Genes used in this research had variable relative expression levels in transgenic lines and a lower relative gene expression in the roots as compared to the shoots in general. In particular, C had very low gene expression in both root and shoot tissues, which might have contributed to lack of resistance. No significant differences amongst single or stacked genes, or choice of promoters for same gene, were recorded. The major constraint in co-expression of different transgenes is that the gene expression remains uncoordinated even with physically linked genes (Maqbool and Christou 1999) and transcriptional silencing of transgenes may occur (Matzke and Matzke 1998). Other

factors, for instance choice of promoters, which can affect the strength, tissue specificity, timing (Qu and Takaiwa 2004) or unexpected gene silencing (Daxinger et al. 2008) or transgene copy number and epigenetic effects (Dietz-Pfeilstetter 2010; Finnegan and McElroy 1994; Jaenisch and Bird 2003) can contribute to varied transgene expression. However, using two different promoters to drive the same chitinases gene (line 18 with Vst from *Stilbene synthase* and line 20 with D35SP from CamV) in the current experiment did not yield any different responses in antifungal activity.

Many genes encoding for antifungal proteins have been isolated, cloned, sequenced and expressed in plants against different phytopathogenic fungi, with some success (for a latest review, see (Moosa et al. 2017). Examples of a synergistic effect of pyramiding genes on combating fungal diseases are reported (Amian et al. 2011; Anand et al. 2003; Richter et al. 2006a; Ziaei et al. 2016) However, most of these results were obtained under *in vitro* and/or greenhouse testing and few crops have been commercially released employing this strategy (ISAAA 2017). Long term field testing is required to test the agronomic performance and ecological relevance of the promising transgene effects detected under laboratory conditions in the complex environment of the field especially in the local environment (Wozniak and McHughen 2012). Field studies can help to evaluate any yield reduction which can occur due to introduction of new genes (Cohen 2005) or to study the interactions and interplay between various biotic and abiotic stresses in their natural form (Bostock et al. 2014), to identify and rectify issues with stability and resultant pleotropic effects (Pons et al. 2012) and lastly, to satisfy regulatory agencies who rely on results from field trials conducted at several locations and are representative of the actual target area of crop cultivation (Romeis et al. 2008). Since the

goal of such research programs is improving grower yield and productivity (Godfray et al. 2010), strategic field trial experiments allowing realistic evaluations of genotype x environment interactions, become crucial.

Research efforts in legume crop improvement should continue since public acceptance and high costs incurred in developing and deregulating transgenic crops (Kalaitzandonakes et al. 2007) make it difficult to fit them into a farmer's diversification strategy (Rubiales et al. 2015). Ideally, for addressing efficacy of transgenic disease resistant plants, multiyear, multi-location field trials are desired. However, the limited seed availability and cost of developing a transgenic crop such as peas is beyond the budgetary scope of most public institutions (Parisi et al. 2016). Often, research data without significant differences between treatments are not published, yet are very important for the scientific community (Knight 2003), especially considering the resources used to conduct confined release trials.

Table 4-1. Above ground (adapted from (Infantino et al. 2006)) and below ground disease rating scale (adapted from (Bilgi et al. 2008))

Tissue used	**Observations recorded**			**Rating**
Above ground	Healthy plant			1
	Slight yellowing of lower leaves			2
	Yellowing of lower leaves upto the 3rd or 4th node, some stunting			3
	Necrosis of at least half or more of the plants with some stunting			4
	All plants dead or nearly so			5
Below ground	***Lesions***	***(%) Root discoloration***	***Root mass reduction***	
	0	0	0	1
	0.1-0.2cm, small reddish brown at hypocotyl base	0	0	2
	Coalescing of localized root/hypocotyl lesions from 0.5-1cm, around the stem	10-20%	0	3
	Lesions extending and completely encircling the stem	95%	5-10%	4
	Increasingly discolored and extended hypocotyl lesions	100%	20-50%	5
	Hypocotyl lesions encircling the stem extending up to 2 cm	100%	50-80%	6
	Pithy or hollow hypocotyl with very extended lesions	Dead	Dead	7

Table 4-2. Relative gene expression ± standard error (SE) of each gene in roots and leaves, for each transgenic line in field experiments

Gene	Line	Relative gene expression	
		Root ± SE	**Leaf ± SE**
V	4(V:P)	16.87±2.45	44.42±4.40
	21(V)	32.85±2.36	558.48±84.99
	8(V:P:G:C)	0.02±0.00	1.17±0.35
	10(V:P:G:C)	40.47±12.54	699.14±220.76
P	4(V:P)	268.79±159.53	32963±14166.04
	23(P)	*	*
	11(P:C:G)	122.66±69.40	11892.53±6171.69
	8(V:P:G:C)	3.58±0.47	469.48±93.35
	10(V:P:G:C)	614.05±72.30	111577.50±27728.24
G	5(G)	127.21±71.77	452.07±214.25
	11(P:C:G)	1.53±0.40	4.26±1.03
	8(V:P:G:C)	0.32±0.20	1.77±0.32
	10(V:P:G:C)	94.94±31.61	1566.15±462.75
C	18(C)	18.01±2.56	14.62±4.01
	20(C)	0.10±0.02	0.19±0.03
	11(P:C:G)	0.25±0.04	0.08±0.01
	8(V:P:G:C)	0.24±0.03	0.10±0.01
	10(V:P:G:C)	0.08±0.01	0.09±0.01

***** data not available

Table 4-3. Precipitation at field trial location in three sequential years

Location	Accumulated from May-October	Long term Average (LTA)	% of LTA
	----------mm----------		--%--
2013	201.3	344.5	58.43
2014	260.4	344.5	75.59
2015	207.3	344.5	60.17

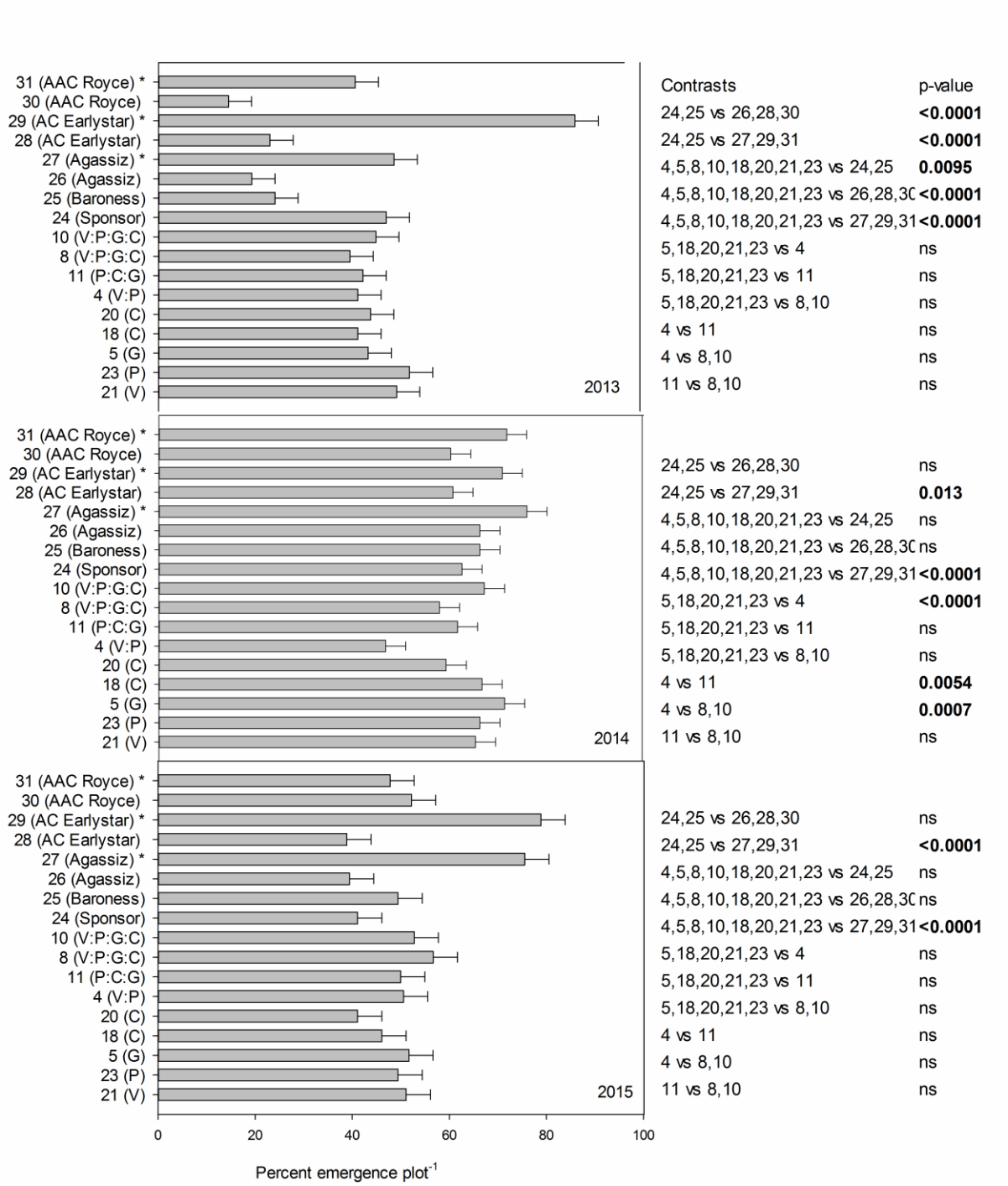

Figure 4-1 Emergence percent (plot^{-1}) in presence or absence of pathogen inoculum, of transgenic lines and conventional pea lines (transgenic lines: 21(V), 23(P), 5(G), 18(C), 20(C), 4(V:P), 11(P:C:G), 8(V:P:G:C), 10(V:P:G:C), 24(Sponsor), 25(Baroness), 26(Agassiz), 27(Agassiz)*, 28(AC Earlystar), 29(AC Earlystar)*, 30(AAC Royce), 31(AAC Royce)* in three year confined field trials
* Pathogen inoculum was not provided.

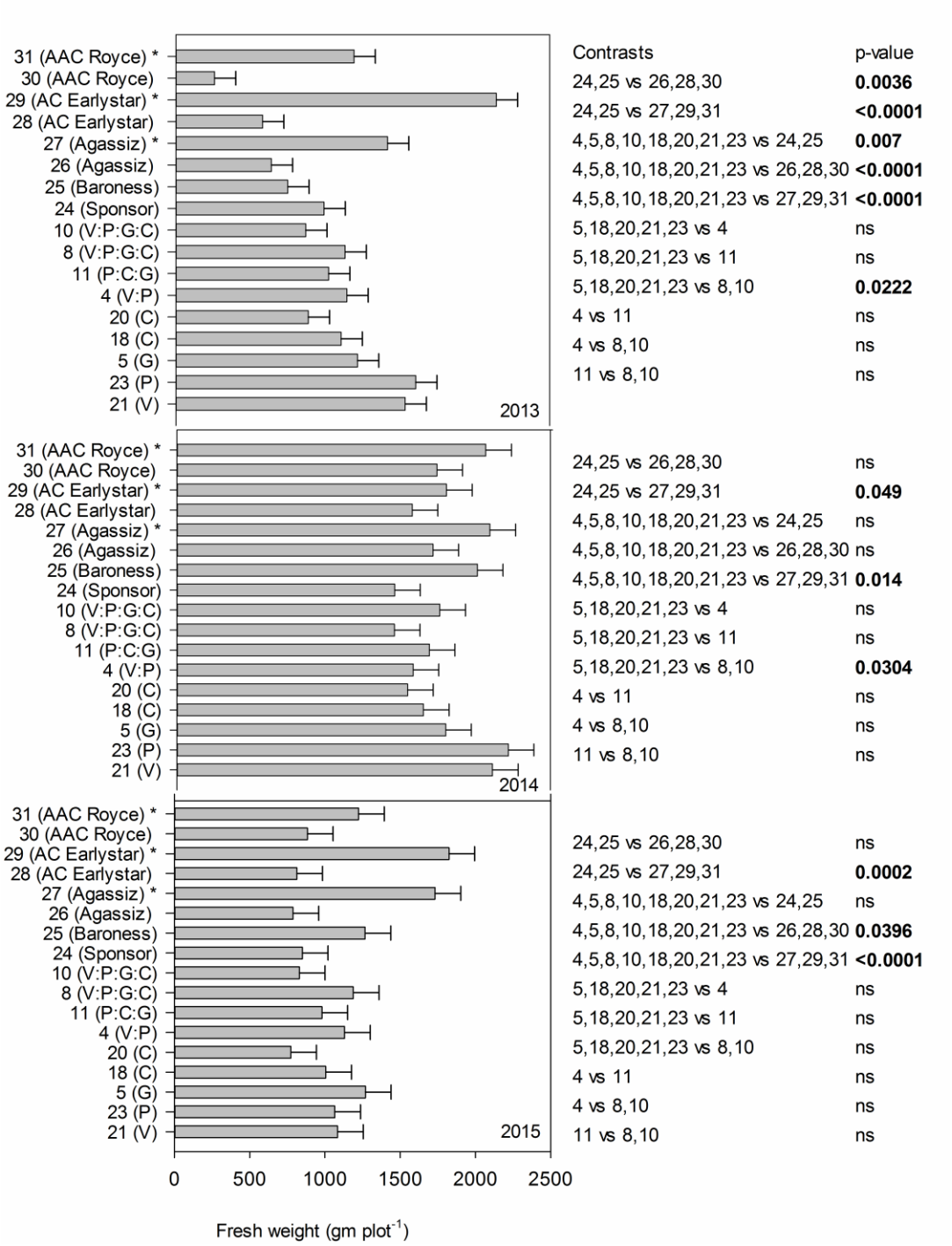

Figure 4-2 Fresh weight biomass plot^{-1} (gm) in presence or absence of pathogen inoculum, of transgenic lines and conventional pea lines (transgenic lines: 21(V), 23(P), 5(G), 18(C), 20(C), 4(V:P), 11(P:C:G), 8(V:P:G:C), 10(V:P:G:C), 24(Sponsor), 25(Baroness), 26(Agassiz), 27(Agassiz)*, 28(AC Earlystar), 29(AC Earlystar)*, 30(AAC Royce), 31(AAC Royce)* in three year confined field trials
* Pathogen inoculum was not provided.

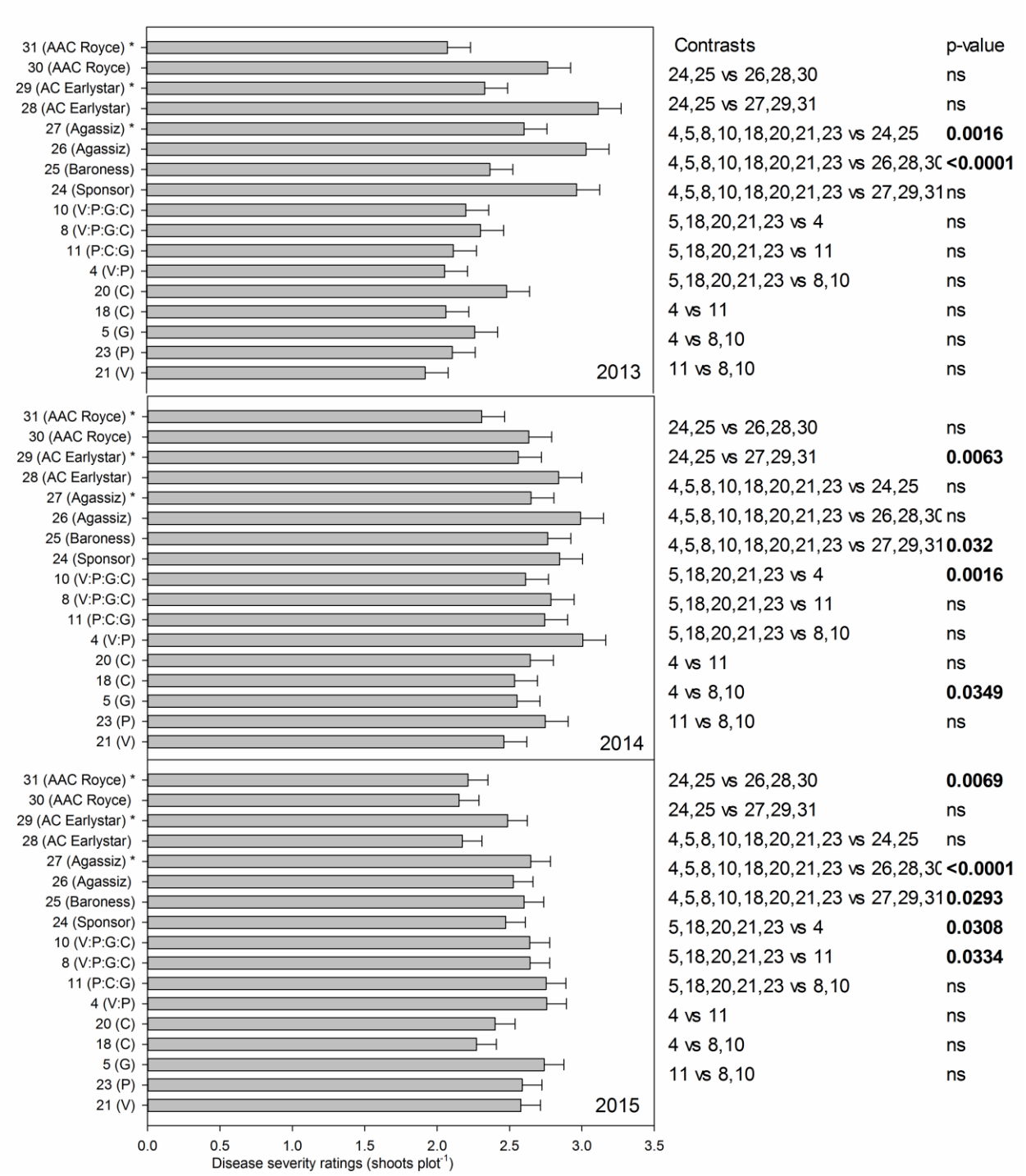

Figure 4-3 Disease severity ratings above ground (plot^{-1}) in presence or absence of pathogen inoculum, of transgenic lines and conventional pea lines (transgenic lines: 21(V), 23(P), 5(G), 18(C), 20(C), 4(V:P), 11(P:C:G), 8(V:P:G:C), 10(V:P:G:C), 24(Sponsor), 25(Baroness), 26(Agassiz), 27(Agassiz)*, 28(AC Earlystar), 29(AC Earlystar)*, 30(AAC Royce), 31(AAC Royce)* in three year confined field trials
*Pathogen inoculum was not provided.

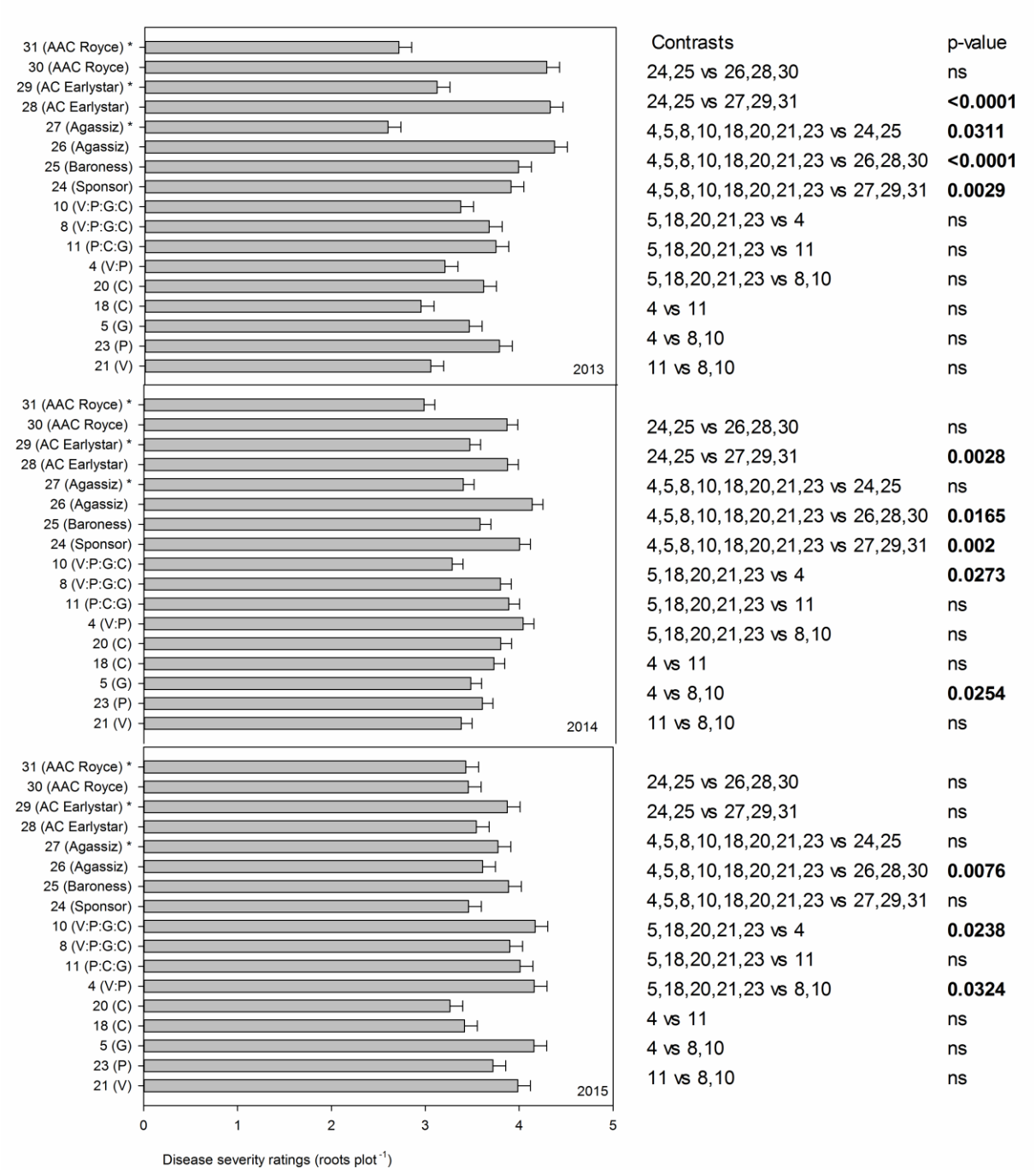

Figure 4-4 Disease severity ratings below ground (plot^{-1}) in presence or absence of pathogen inoculum, of transgenic lines and conventional pea lines (transgenic lines: 21(V), 23(P), 5(G), 18(C), 20(C), 4(V:P), 11(P:C:G), 8(V:P:G:C), 10(V:P:G:C), 24(Sponsor), 25(Baroness), 26(Agassiz), 27(Agassiz)*, 28(AC Earlystar), 29(AC Earlystar)*, 30(AAC Royce), 31(AAC Royce)* in three year confined field trials
* Pathogen inoculum was not provided.

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Chapter Five: Performance of antifungal genes expressed in transgenic pea (*Pisum sativum* L.) against Mycosphaerella blight in three years of confined field trials

5.1. Introduction

The legume family includes 41 domesticated species known for their nutritional and health benefits and contribution towards agricultural sustainability (Araújo et al. 2015). Among these, peas (*Pisum sativum* L.) rank second economically only to soybeans and beans worldwide (FAOSTAT 2015). The western Canadian prairie provinces are the largest producers of field pea (3.4 million tonnes (MT)) and production has been consistently increasing over the past two decades (Raghunathan et al. 2017; Taheri et al. 2016). Canadian pulse growers have established field pea cultivar research priorities aimed at improving yields, seed quality and tolerance to abiotic and biotic stresses (Bueckert et al. 2015b; Tayeh et al. 2015; Warkentin et al. 2015). However, pea diseases, particularly Mycosphaerella/Ascochyta blight complex caused by *Peyronellaea pinodes* (Berk. & A. Bloxam) Aveskamp, Gruyter & Verkley (syn. *Mycosphaerella pinodes* (Berk. & A. Bloxam) Vestergr.) (teleomorph: *Didymella pinodes*, also known as *Ascochyta pinodes*) severely reduces yield under wet conditions in most pea producing regions in the world (Xue et al. 1997).

Although Mycopshaerella Blight Complex of peas has several associated fungal pathogens, *P. pinodes* is the most widespread and damaging in Canada (Gossen et al. 2011; Khan et al. 2013; Liu et al. 2013; Tivoli and Banniza 2007) and worldwide (Bretag et al. 2006; Skoglund et al. 2011). Recent surveys in the Canadian prairies have reinforced that *P. pinodes* is the most prevalent pathogen in Mycopshaerella Blight

Complex (99% of the tested isolates) and its aggressiveness on the host plants has increased over time (Ahmed et al. 2015). The Mycopshaerella Blight Complex is present in almost every pea field annually (McLaren et al. 2017) and disease is influenced by pathogen frequency, distribution and environment (May et al. 2005) and is known to reduce seed yield up to 50% (Xue et al 1997). This foliar necrotrophic fungus attacks almost all parts of the plant i.e. leaves, stems, flowers, and pods of field pea. Initially, small distinct purplish black lesions with irregular margins develop on leaves, stems and pods which enlarge and coalesce as the season progresses. These lesions cause extensive blight and root rot, weaken stems, increase lodging and cause shrunken or discolored seeds, ultimately lowering quality (Gossen et al. 2011). Severe disease on leaves and internodes of the basal part of the plants reduces both the number of seeds per stem and seed size, contributing to lower yield (Garry et al. 1998; Tivoli et al. 1996; Xue et al. 1997). *P. pinodes* can survive as mycelium on diseased pea trash, or in the soil as sclerotia and chlamydospores for several years and moist conditions are required for infection and spread (Bretag et al. 2006).

The majority of disease management strategies in Western Canada rely on crop rotation and seed treatment (Gossen et al. 2011) because of the lack of effective and/or cost-effective fungicides (Khan et al. 2013). Although partially resistant cultivars are available in pea germplasm, they confer only incomplete or partial resistance (Carrillo et al. 2013; Conner et al. 2012; Fondevilla et al. 2008; Zhang et al. 2007). Variation in the virulence of *P. pinodes* populations in western Canada contributes substantially to the complexity of selection for resistance (Su et al. 2006). As suggested by Khan et al. (2013), stacking of broad antifungal genes on current moderately resistant varieties using

biotechnological tools and/or GM technologies may provide effective control of *D. pinodes*.

Pea is an excellent candidate for genetic transformation because of its self-pollinating nature, low allergenicity and lower degree of outcrossing, and transformation could aid in classical breeding techniques by overcoming sexual incompatibility between species of interest (Dita et al. 2006; Rubiales et al. 2015; Warkentin et al. 2015). However, the economic advantages of disease resistant peas will need to be weighed over the costs, time and labor involved in the regulatory procedures required to bring a genetically modified (GM) crop to market. Biotechnological approaches have been used on legume crops and successfully implemented in soybean (Eapen 2008), but have been restricted to the development of insect resistance (Morton et al. 2000; Schroeder et al. 1995; Shade et al. 1994; Timmerman-Vaughan et al. 2001) or drought tolerance (Kahlon et al., 2017 unpublished) in field pea. Commercially released GM disease resistant crops, in general, are few (ISAAA 2017). This low success rate could be attributed to the transgenes not being sufficiently effective, or they confer high levels of resistance but only to a very specific pathogen (Wally and Punja 2010).

Approaches used to engineer plants for fungal resistance mainly include insertions of resistance-genes (R-genes) (that utilize plants basal defense responses), detoxification of virulence factors, antimicrobial peptides such as protein and pathogenesis-related (PR) protein expression (which inhibit the fungus's capacity to degrade polysaccharides within the cell wall or RNA) and plant signaling pathway modifications (Gururani et al. 2012; Moosa et al. 2017; Pandolfi et al. 2017; Saharan et al. 2016; Sree and Rajam 2015; Wally and Punja 2010). Disease resistance is a very

complex multigenic trait; hence, single gene transformations may not be sufficient to deliver broad spectrum disease resistance (Anand et al. 2003), or could be lost by a single mutation causing the introduced gene to be ineffective (Gurr and Rushton 2005). Therefore, stacking/pyramiding more than one gene of a desired trait (Halpin 2005; Mundt 2014) through coordinated expression of many genes in one plant at a time could be challenging and issues with durability of genes remain the same as breeding for resistance (Halpin 2005; Mundt 2014; Que et al. 2010). Especially in the case of PR proteins, their ectopic expression can cause defense responses in the absence of pathogens that could lead to reductions in yield (Moosa et al. 2017) or a fitness penalty (Tian et al. 2003).

Recently, four antifungal genes, representing PR proteins *β-1,3 glucanase (G)*, *endochitinase* (C), *polygalacturonase inhibiting proteins* (PGIPs) (P) and *stilbene synthase* (V) were transformed and expressed in European pea (*Pisum sativum* L.) cultivars for disease tolerance (Amian A et al. 2011; Hassan et al. 2009; Richter et al. 2006a) (Table 1) as individual insertions and stacked as two, three or four genes in one line (stacked using conventional breeding (V:P (Richter et al. 2006a) x G:C (Amian A et al. 2011)= V:P:G:C (Hassan et al. 2010)). These genes provide resistance against fungal pathogens using different strategies; for example, the PR proteins (*β-1,3 glucanase* and *endochitinase*) degrade microbial cell wall components (Van Loon et al. 2006), PGIPs inhibit fungal endopolygalacturonases to reduce plant tissue maceration and fungal cell wall degradation (De Lorenzo et al. 2001), and *Stilbene synthase*, a phytoalexin class of secondary metabolites that exhibits biological activity against a range of pathogens (Jeandet et al. 2002). *In vitro* testing showed enhanced tolerance to fungi in transgenic

pea plants (Amian A et al. 2011; Selatsa et al. 2008); however, field testing is pivotal to establish trait efficacy under complex and varying environments. Due to non-transparent legislation of GM crops (Nelissen et al. 2014), field-testing of GM crops in Germany is not possible. Canada is a good location to test these genes in the field as products derived from biotechnological processes known as Plants with Novel Traits (PNT) are regulated under the auspices of the Canadian Food Inspection Agency (CFIA), Health Canada and Environment Canada (CFIA 2017a) just the same as conventionally bred products.

We report here three years of confined field trials evaluating transgenic disease tolerant pea stably expressing antifungal genes as single and stacked against *P. pinodes* in comparison to parental lines and well adapted Canadian pea lines (bred in and adapted to Alberta growing conditions). Our *a priori* hypotheses are: 1) antifungal genes expressed in transgenic pea plants will exhibit improved tolerance to Mycosphaerella blight complex than Canadian conventional pea lines and parental lines; 2) transgenic lines with stacked genes will have an advantage over single gene insertions in response to Mycosphaerella blight complex in field trials.

5.2. Materials and Methods

5.2.1. Plant material

European pea cultivars “Baroness” and “Sponsor” were transformed with four antifungal genes V, P, G, C at the Institute of Plant Genetics, Department of Plant Biotechnology, Leibniz University Hannover, Germany. Using a modified protocol by Schroeder *et al.* (1993), embryo axis excised from mature seeds of European pea ‘Baroness’ were used as explants for *Agrobacterium*-mediated transformation using strain EHA105 (Hood et al. 1993) for insertion of V,P,G genes (V had inducible Vst promoter, P, G had constitutive

D35S promoter), and similarly, European pea 'Sponsor' was used for transformation with C gene (driven by D35S promoter) (see Chapter 3). Herbicide resistant *bar* gene (selectable marker) from *Streptomyces hygroscopicus* was inserted along with the genes (Murakami et al. 1986; Thompson et al. 1987) (for details on functional maps of the genes used, see Kahlon et al. 2017).

5.2.2. Gene expression

Transgenic and non-transgenic lines were grown in the greenhouse at the Department of Agricultural, Food and Nutritional Sciences, University of Alberta, Edmonton, AB, Canada, in pre-autoclaved vermiculite-perlite soilless medium (Sunshine Mix®#4, Sun Gro Horticulture, Canada). Each plant (10 plants $line^{-1}$) were seeded in pots and kept at 25 ± 2 °C with a 16/8-h light/dark photoperiod for four weeks. Root and leaf samples were removed and cleaned three times with RNAse free water. The methods for quantitation and statistical analysis of gene expression have been described in Kahlon et al. 2017.

5.2.3. Field trials

A secure field site located at the Crop Diversification Center (CDC) North, Alberta Agriculture and Forestry (AAF), in north-east of Edmonton, AB (lat. 53°38'N, long.113°22'W), was used for establishing confined field trials in the spring of 2013, 2014 and 2015 under the auspices of CFIA, following the guidelines for field testing of PNTs (CFIA authorization numbers 13-UOA1-257-PEA, 14- UOA1-257-PEA and 15-UOA1-257-PEA). The site has a Black Chernozemic sandy loam soil and annual soil tests were conducted to determine the amount of phosphorus to be added at seeding. Seventeen treatments, comprised of nine transgenic lines (five lines with single gene

insertions {5(G), 18(C), 20(C), 21(V) and 23(P)}, one line with double gene insertion {4(V:P)}, one line with triple gene insertion {11(P:C:G)}, two lines with four gene insertions {8 (V:P:G:C), 10 (V:P:G:C)}, and as comparators, five lines including the two German parental lines, 'Sponsor' and 'Baroness' and three Canadian-bred lines 'Agassiz' (resistant to powdery mildew (*Erysiphe pisi* Syd.) and moderately susceptible to Mycosphaerella blight (*P. pinodes)* (Canadian Food Inspection Agency 2017a), 'AC Earlystar' (resistant to powdery mildew, moderately resistant to Mycosphaerella blight and Fusarium wilt (*Fusarium oxysporum*) (Canadian Food Inspection Agency 2017b), and 'AAC Royce' (resistant to powdery mildew, moderately susceptible to Mycosphaerella blight and Fusarium wilt (Bing et al. 2016). All lines tested, except one each of the three Canadian lines, received seed treatment with Apron Maxx RTA® Syngenta, Canada (@0.01 mL per 30 seeds). Seeds were individually planted at 30 seeds $plot^{-1}$ (1 m x 0.5 m) at a 5 cm depth. Each plot was separated by conventional 'AC Ultima' triticale. All plots were inoculated with *Rhizobium leguminosarum* bv. viciae for root nodulation promotion (1.6 x 109 viable cells g^{-1}) at a rate of 292g ha^{-1} (@0.004g for 2.5 g seeds) (CellTech® Novozymes). All transgenic lines, German parental controls and three Canadian lines were provided with *P. pinodes* infected pea vines from the previous years' confirmed diseased pea vines from research trials from Alberta Agriculture and Forestry, Lacombe, AB, as pathogen inoculum. Diseased vines were spread by hand on the plot at the 6-10 node stage. The plots were arranged in a randomized complete block design with six replicates per treatment.

5.2.4. Plant growth assessment

Data was collected for seedling emergence plot^{-1} (14 DAP, days after planting), plant heights (28, 42 DAP, 5 plants plot^{-1}) to ensure uniformity of growth among transgenic lines.

5.2.5. Disease severity ratings

Plots were assessed weekly for Mycosphaerella blight severity after the application of diseased vines each year and continued until pea physiological maturity. Visual disease estimates were taken on foliage in a plot, dividing the plant into three sections: upper, middle and lower, using a scale of 0 (no disease) to 9 (whole plants severely blighted) as described by Xu et al. (1996).

5.2.6. Yield

Plants were hand harvested and threshed using a single plant thresher (SPT-1A, Agriculex Inc., Guelph, ON) on the site at harvest. Lines were carefully kept separate and the thresher thoroughly cleaned using a hand held air blower after harvest of each line to avoid mixing genotypes. Seed yield per plot was recorded on site using an electronic scale and samples were triple packaged and brought to a secure room at the Laird W. McElroy Environmental and Metabolism Research Centre, University of Alberta, Edmonton, AB (to confine the transgenic seed movement). Seed number plot^{-1} was recorded using an electronic seed counter (Model#945, Key-Mat equipment Co., Inc, St. Charles, IL, USA), which was also cleaned in a similar manner as described above to avoid mixing genotypes

5.2.7. Statistical analysis

Data acquired from the field experiments were analyzed using analysis of variance (ANOVA) with PROC MIXED in SAS 9.4 (SAS Institute Inc. 2014) at 0.05 probability. Lines and years were fixed effects and blocks were random. Pre-planned orthogonal contrasts statements were used to compare LSmeans for lines of interest.

5.3. Results

5.3.1. Gene expression analysis

Overall, the relative gene expression was higher in leaf tissues compared to root tissues in all of the transgenic pea lines except C (single gene which had similar expression levels in leaf and root tissues) (Table 4-1). For details, see chapter 4 section 4.3.1.

5.3.2. Plant growth assessment

Plant emergence (percent plants plot^{-1} 14 DAP) are summarized in Figure 5.1. The line*year interaction was significant ($p<0.0001$) at $\alpha < 0.05$ level of significance and therefore data is presented by year. In 2013, all transgenic lines had significantly more emergence (%) plot^{-1}, than the untreated Canadian lines (27, 29, 31) ($p<0.0001$) but not significantly different than the Canadian lines which received seed treatment at the time of seeding (26, 28, 30). This trend continued in 2014 but not in 2015 when the transgenic lines had significantly more emergence (%) plot^{-1} than both treated and untreated lines ($p<0.0001$). The emergence (%) plot^{-1} for the transgenic lines ranged from 72 to 95% in 2013, 67 to 93% in 2014 and 72 to 90% in 2015, which suggests similar seedling emergence in all three years. Also, we observed that with seed treatment, Canadian lines (26, 28, 30) had more emergence (%) plot^{-1} than their counterparts, which did not receive seed treatment in 2013, 2014 and 2015 (with the exception of lines 26 and 27 in 2015). We did not observe any transgenic line or a single vs. stacked line, having superior

emergence in the three years of field experiments. All pea plants had uniform heights in 2013, 2014, and 2015 and no stunting or dwarfing was observed in any plants (data not presented).

Disease rating observations consistently increased over the three years of field trials from application of inoculum until maturity, which reinforces that artificial inoculation was resulting in disease, which challenged the pea lines. Characteristic purplish lesions coalescing to form larger lesions were observed on the entire plant, starting at the bottom and progressing upwards and were observed on all plant parts (leaves, stems, stipules, tendrils, flowers). However, transgenic lines were not significantly less diseased than the Canadian lines which received seed treatment (26, 28, 30) in 2013 and 2014 when assessed at 15 and 21 days after inoculation (DAI) but were significantly different than parental lines (24, 25) ($p=0.0167$ at 15 DAI and $p=0.0059$ at 21 DAI), as well as Canadian lines with seed treatment (26, 28, 30)($p<0.0001$ at 15 DAI and at 21 DAI) in 2015 (Figure 5.2). It is unclear why 2015 was different, although the amount of moisture in the pea canopy at the time of disease spread may have been higher and therefore may have contributed to differences observed among lines and genes. However, no consistent pattern was observed in three years of study in single vs. stacked genes and there was no particular transgenic line that tolerated the disease better compared to the Canadian or parental lines.

The transgenic lines had significantly higher seed yields (gm $plot^{-1}$) in the presence of disease than Canadian lines that were seed-treated (26, 28,30) ($p=<0.0001$) in 2013 and 2014 ($p=<0.0001$) but not in 2015 (Figure 5.3) in the presence of disease. However, they were not significantly different from their parental lines (24, 25)

consistently in all three years. There was no significant advantage of having two, three or four genes over a single gene, as was the initial expectation. Similarly, while seed numbers plot^{-1} showed some differences among various gene combinations in three years, they were not significantly different from their parents (24, 25) (Figure 5.4) consistently across three years, which was counter to our initial expectations. The seed from all three field trials had very low lesion numbers, but some seed shrinkage was observed in the transgenic lines, as well as the parental and Canadian lines without seed treatment, although this was not consistent in all replications. Growing season precipitation was consistently below the long term average (LTA) precipitation for the region in all three years (58, 76 and 60% of the LTA precipitation, respectively for 2013, 2014 and 2015) (Table 4-2). This might have affected how the disease progressed each year and reduced our ability to observe differences between treatments. Although 2014 had reasonable precipitation (76% of LTA), the previous year (2013), had only 58% of LTA, which indicates the possibility that moisture reserves in the soil were depleted. In years with higher moisture availability, larger differences may have been observed but disease was sufficiently present to reduce yields in pathogen-inoculated plants.

5.4. Discussion

We established confined field trials for three consecutive years to test the efficacy of nine transgenic pea lines transformed with four antifungal genes, present singly or stacked against *P. pinodes* in comparison to their parental lines and Canadian pea lines in the presence/absence of seed treatment. We were not able to identify transgenic pea lines that surpassed the parental lines or partially disease tolerant Canadian lines in the presence of disease over the course of three different field seasons when evaluating their growth,

disease tolerance and yield. The variability observed within transgenic lines over the course of three consecutive years of field-testing reinforces the complexity of disease tolerance traits. We did not observe a benefit of antifungal gene stacking over individual genes, refuting our initial hypothesis. Our results corroborate the findings of other researchers, where transgene insertions have inconsistent or no effect on disease tolerance or resistance. For example, expression of PGIP2 gene in transgenic wheat from bean (*Phaseolus vulgaris*) did not reduce *Claviceps purpurea* symptoms (Volpi et al. 2013), chitinase genes expressed in *Nicotiana sylvestris* did not confer resistance to *Cercospora nicotiana* (Neuhaus et al. 1991), and constitutive expression of β-1,3 glucanase did not decrease fungal growth severity in alfalfa roots (Masoud et al. 1996a). However, contrary to our results, many transgenics expressing antifungal genes, particularly in legumes, exhibited successful disease resistance. For example, in rice, chitinases under CaMV35S improved resistance against leaf spot (*Cercospora arachidicola*) (Iqbal et al. 2012b) and in peanut (*Arachis hypogaea* L.), against late leaf spot (*Phaeoisariopsis personata*), rust (*Puccinia arachidis* Speg.) and *Aspergillus flavus* (Prasad et al. 2013). When β-1,3 glucanase from tobacco was overexpressed in transgenic peanut, there was improved protection against *Cercospora arachidicola* and *Aspergillus flavus* (Sundaresha et al. 2010) and *Cercospora personanta* (Qiao et al. 2014). Similarly, improved resistance to Corynespora leaf spot disease (*Corynespora cassiicola*) in blackgram (*Vigna mungo* L. Hepper) was achieved by expressing barley chitinases (AAA56786) (Chopra and Saini 2014). A peanut resveratrol synthase (a stilbene) decreased severity of black stem and leaf spot disease (*Phoma medicaginis*) in alfalfa (*Medicago sativa* L.) (Hipskind and Paiva 2000). However, all of these results are from research on single pathogens tested

under laboratory conditions, where it is relatively easier to differentiate susceptibility and resistance than under field conditions where the number of pathogens, soil moisture and climate more accurately represent how the crop will be grown and realistically test the efficacy of the transgenes.

Recent modeling experiments with gene deployment strategies have indicated that gene stacking can provide the most long-lasting solution (Lof and van der Werf 2017). However, gene stacking does not always have advantages against pathogens as we observed in our research and has been suggested by other researchers. For example, no resistance to *Rhizoctonia solani* was observed in a hyphal extension assay using extracts from transgenic potato with chitinase and glucanase genes (Moravčíková et al. 2004). Transgene insertions often result in inhibition of fungal growth *in vitro* but often these do not to translate to the same result when studied under greenhouse or field conditions, as was the case in our research. For instance, purified chitinase and β-1,3-glucanase reduced fungal growth *in vitro;* however, when transformed with a double gene construct of chitinases and β-1,3-glucanase genes from barley (both driven by CaMV 35S promoter), and tested in the greenhouse, they were not able to impart fungal resistance in oilseed rape against *Alternaria brassicae*, *A. brassicola*. *Verticillium longisporum* and *L. maculans* (Melander et al. 2006). PR proteins's differential activity can result from their specificity against a few pathogens, but be completely ineffective against some others pathogens even within the same crop, which poses a challenge for continuous and sustainable resistance (Moosa et al. 2017). Alternately, the source of gene itself could be a challenge; for example, chitinases obtained from *Trichoderma* spp. are considered more effective in conferring disease resistance (Sandhu et al. 2017). As with breeding efforts in

P. pinodes resistance, it is suggested that resistance to leaf and stem infection may be controlled by different genes (Xue and Warkentin 2001) and less is known about the role of soil, rhizosphere, and root fungal communities in disease development in pea roots (Xu et al. 2012b). More research is required in these areas to reveal pathogen-targeted strategies rather than expressing broad anti-fungal genes.

Co-expression of different transgenes can also cause many problems. Some issues include the uncoordinated expression even with physically linked genes (Maqbool and Christou 1999) and transcriptional silencing of transgenes (Matzke and Matzke 1998). The choice of promoters (affecting the strength, tissue specificity, timing (Qu and Takaiwa 2004)), unexpected gene silencing (Daxinger et al. 2008), transgene copy number and epigenetic effects (Dietz-Pfeilstetter 2010; Finnegan and McElroy 1994; Jaenisch and Bird 2003) can contribute to uneven gene expression, as well. We found that genes used in this experiment, with two different promoters driving the same chitinases gene (line 18 with Vst from *Stilbene synthase* and line 20 with D35SP from CamV) showed no difference in antifungal activity against *P. pinodes.* Additionally, they exhibited variable gene expression in transgenic lines and a lower gene expression in the roots in general, as compared to the shoots, which may explain why they did not exhibit differences in disease response against *P. pinodes* in field.

The amount of chitinase enzyme produced and the proportion of chitin present in phytopathogenic fungal cell walls may also contribute to the lower disease reduction (Punja and Raharjo 1996). Necrotrophs such as *P. pinodes* usually secrete a large amount of cell wall-degrading enzymes to degrade cell wall polymers and hence cause significant cell damage it is possible that expression of two specific cell degrading genes such as

chitinase and glucanase may not pose a solution (Horbach et al. 2011). In our experiment, higher relative gene expression in leaves did not lead to disease resistance against the foliar *P. pinodes*. We postulate the pathogen was perhaps, well equipped with various plant cell wall degrading enzymes to deal with our two specific cell wall degrading genes. Also, acidic chitinases (such as Chit30 as used in transformations of our antifungal genes), which aim the cell wall are less effective at decreasing disease caused by necrotrophic pathogens such as *P. pinodes* than intracellular basic chitinase isoforms (Ferreira et al. 2007; Prasad et al. 2013; Punja and Raharjo 1996; Wally et al 2009).

There are many examples of success where antifungal proteins as transgenes in various plant species express resistance against large numbers of fungal pathogens (for latest reviews, see (Babar et al. 2014; Eapen 2008; Jacob et al. 2016; Moosa et al. 2017). Stacked genes have been reported to be successful in combating fungal diseases(Amian A et al. 2011; Anand et al. 2003; Richter et al. 2006a; Rivero et al. 2012; Szankowski et al. 2003; Ziaei et al. 2016). Nevertheless, the majority of these results were acquired via testing *in vitro* and/or under greenhouse conditions rather than under field testing. It is crucial to test the transgene efficacy and the agronomic performance of the transformed crop in order to determine the relevance for a particular trait-crop combination, particularly under the local environment (Wozniak and McHughen 2012). Field trials that target evaluation of genotype x environment interactions and efficacy of tested traits like the ones evaluated here are imperative for the ultimate goal of improving yield and productivity (Godfray et al. 2010). It is even more important to test these traits under local environmental conditions and with local pathogen strains to examine the interactions between abiotic and biotic stresses in the field (Bostock et al. 2014). Only

under field settings can researchers gauge any negative implications from this technology, for example yield reductions (Cohen 2005) or pleotropic effects (Pons et al. 2012), and satisfy regulatory requirements for testing trait efficacy in target environments (Romeis et al. 2008).

Multiyear, multi-location field trials are ideal for challenging efficacy of transgenic disease tolerant plants, however, limited seed amounts from greenhouse increases and the high cost of bringing a transgenic crop such as pea to market is beyond the budgetary scope of public institutions (Parisi et al. 2016). Reporting negative results contributes critical information to the scientific community to provide perspective on single gene insertions or modifications (Fanelli 2011; Knight 2003). Transgenic crops are more difficult for a grower to fit into their crop rotation (Rubiales et al. 2015) because of their low level of public approval and the high cost associated with developing them (gene identification, transformation process, trait efficacy experiments, establishment of their environmental biosafety and deregulating transgenic crops) (Kalaitzandonakes et al. 2007). However, research efforts for pea disease tolerance to Mycosphaerella blight should be encouraged since there is inadequacy in natural genetic source of resistance available (Fondevilla et al. 2008).

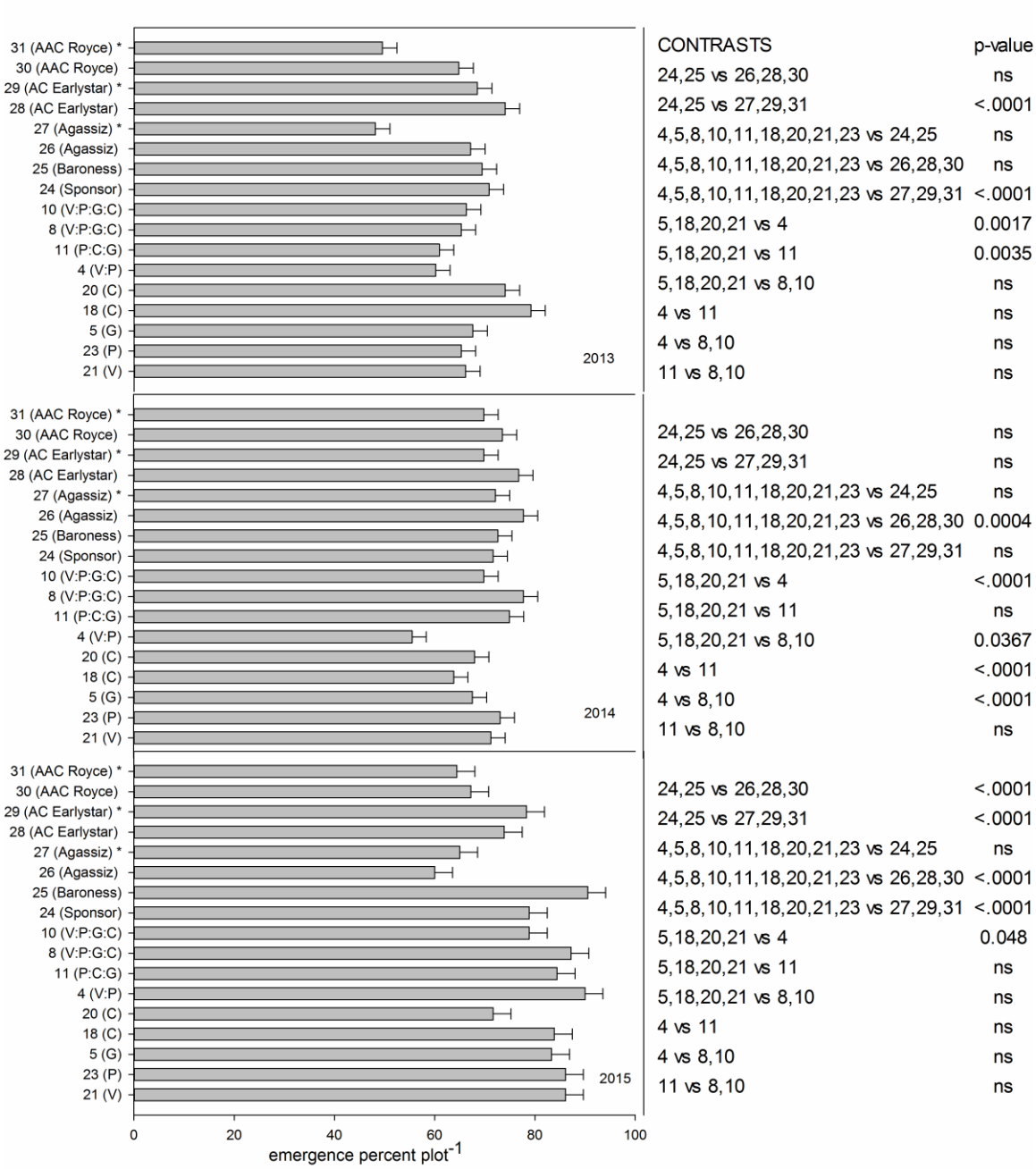
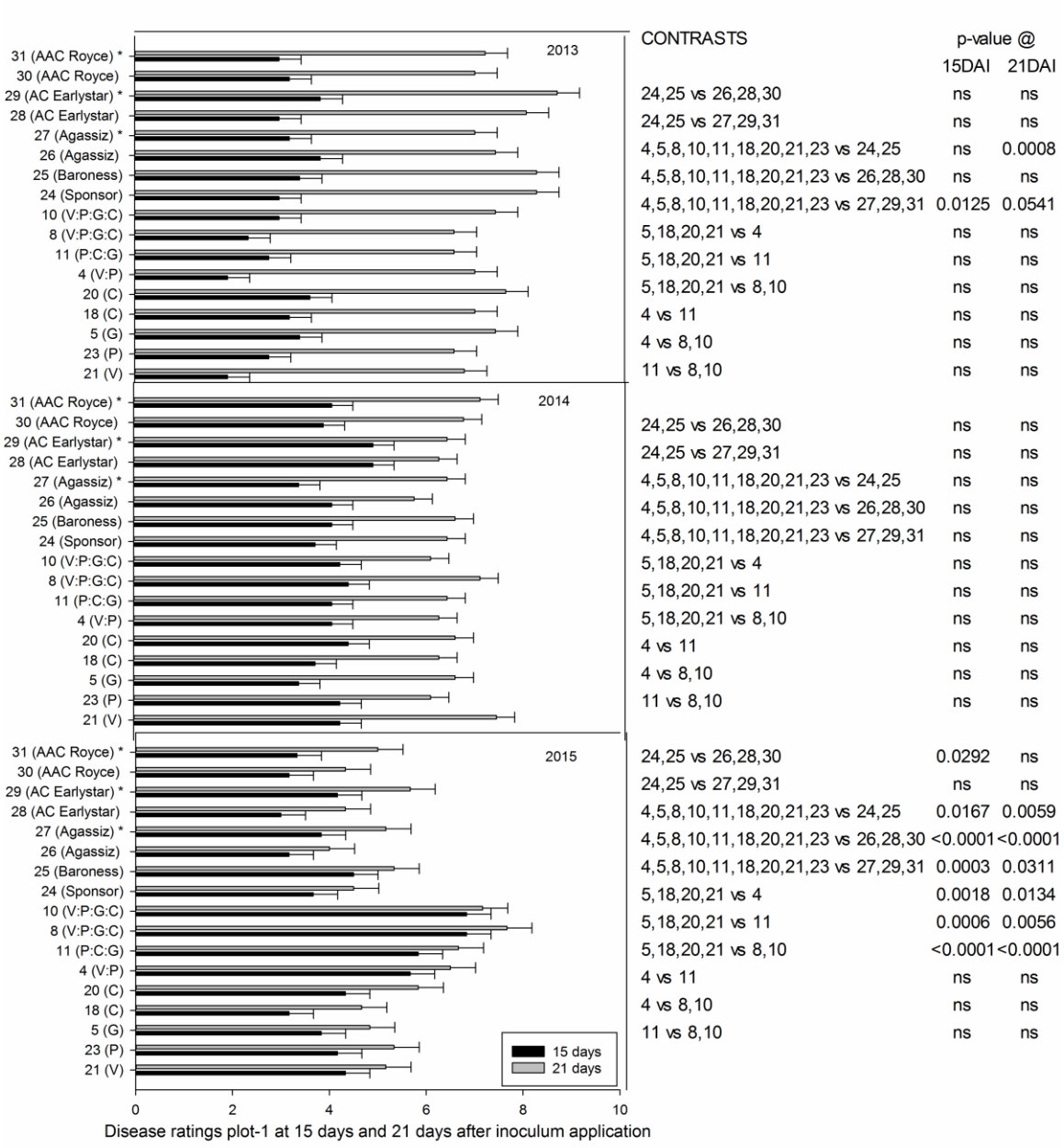


Figure 5-1. Emergence percent (plot^{-1}), of transgenic lines and conventional pea lines (transgenic lines: 21(V), 23(P), 5(G), 18(C), 20(C), 4(V:P), 11(P:C:G), 8(V:P:G:C), 10(V:P:G:C), 24(Sponsor), 25(Baroness), 26(Agassiz), 27(Agassiz)*, 28(AC Earlystar), 29(AC Earlystar)*, 30(AAC Royce), 31(AAC Royce)*.
* Seed treatment was not provided.



CONTRASTS	p-value @ 15DAI	21DAI
24,25 vs 26,28,30	ns	ns
24,25 vs 27,29,31	ns	ns
4,5,8,10,11,18,20,21,23 vs 24,25	ns	0.0008
4,5,8,10,11,18,20,21,23 vs 26,28,30	ns	ns
4,5,8,10,11,18,20,21,23 vs 27,29,31	0.0125	0.0541
5,18,20,21 vs 4	ns	ns
5,18,20,21 vs 11	ns	ns
5,18,20,21 vs 8,10	ns	ns
4 vs 11	ns	ns
4 vs 8,10	ns	ns
11 vs 8,10	ns	ns
24,25 vs 26,28,30	ns	ns
24,25 vs 27,29,31	ns	ns
4,5,8,10,11,18,20,21,23 vs 24,25	ns	ns
4,5,8,10,11,18,20,21,23 vs 26,28,30	ns	ns
4,5,8,10,11,18,20,21,23 vs 27,29,31	ns	ns
5,18,20,21 vs 4	ns	ns
5,18,20,21 vs 11	ns	ns
5,18,20,21 vs 8,10	ns	ns
4 vs 11	ns	ns
4 vs 8,10	ns	ns
11 vs 8,10	ns	ns
24,25 vs 26,28,30	0.0292	ns
24,25 vs 27,29,31	ns	ns
4,5,8,10,11,18,20,21,23 vs 24,25	0.0167	0.0059
4,5,8,10,11,18,20,21,23 vs 26,28,30	<0.0001	<0.0001
4,5,8,10,11,18,20,21,23 vs 27,29,31	0.0003	0.0311
5,18,20,21 vs 4	0.0018	0.0134
5,18,20,21 vs 11	0.0006	0.0056
5,18,20,21 vs 8,10	<0.0001	<0.0001
4 vs 11	ns	ns
4 vs 8,10	ns	ns
11 vs 8,10	ns	ns

Figure 5-2. Disease severity ratings (plot^{-1}) in presence of pathogen inoculum, of transgenic lines and conventional pea lines (transgenic lines: 21(V), 23(P), 5(G), 18(C), 20(C), 4(V:P), 11(P:C:G), 8(V:P:G:C), 10(V:P:G:C), 24(Sponsor), 25(Baroness), 26(Agassiz), 27(Agassiz)*, 28(AC Earlystar), 29(AC Earlystar)*, 30(AAC Royce), 31(AAC Royce)*.
*Seed treatment was not provided.

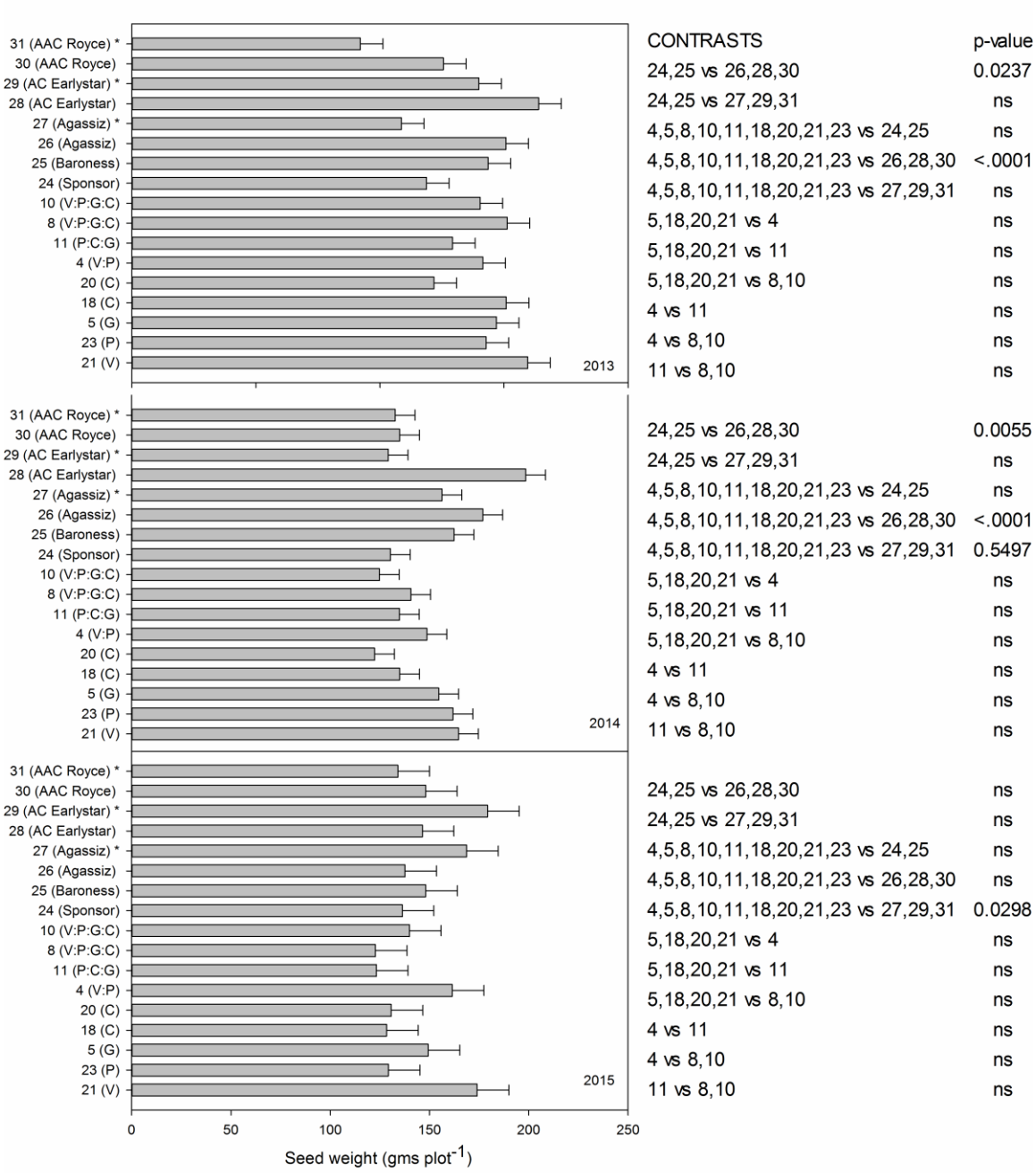


Figure 5-3. Seed weight plot^{-1} (gm) in presence of pathogen inoculum, of transgenic lines and conventional pea lines (transgenic lines: 21(V), 23(P), 5(G), 18(C), 20(C), 4(V:P), 11(P:C:G), 8(V:P:G:C), 10(V:P:G:C), 24(Sponsor), 25(Baroness), 26(Agassiz), 27(Agassiz)*, 28(AC Earlystar), 29(AC Earlystar)*, 30(AAC Royce), 31(AAC Royce)*
* Seed treatment was not provided.

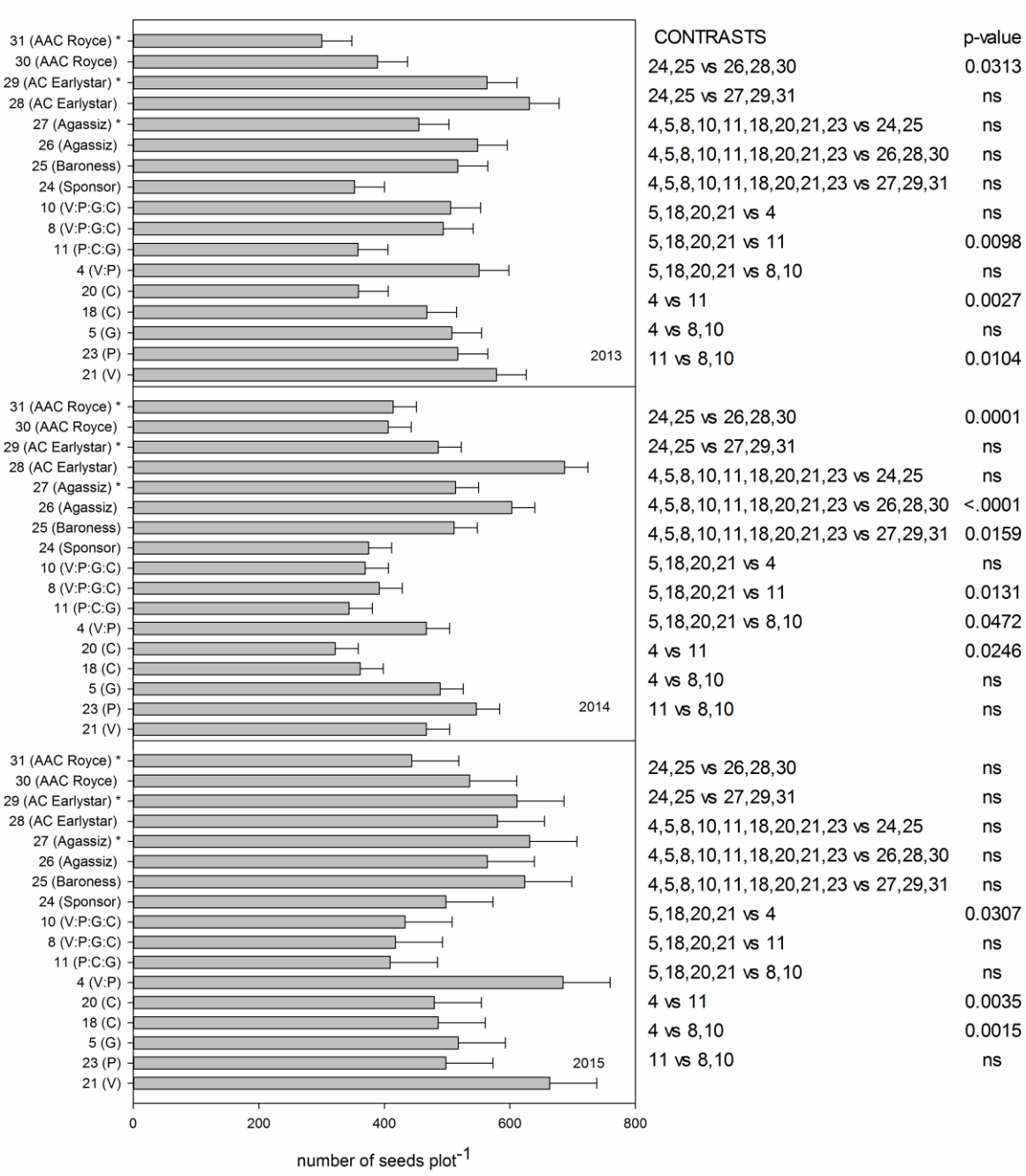


Figure 5-4. Seed number plot^{-1} (gm) in presence of pathogen inoculum, of transgenic lines and conventional pea lines (transgenic lines: 21(V), 23(P), 5(G), 18(C), 20(C), 4(V:P), 11(P:C:G), 8(V:P:G:C), 10(V:P:G:C), 24(Sponsor), 25(Baroness), 26(Agassiz), 27(Agassiz)*, 28(AC Earlystar), 29(AC Earlystar)*, 30(AAC Royce), 31(AAC Royce)*
*Seed treatment was not provided.

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Chapter Six: Antifungal genes expressed in transgenic pea do not affect root colonization of arbuscular mycorrhizae fungi

6.1. Introduction

Genetically modified (GM) crops are now planted in 28 countries globally and the cumulative planted area has increased more than 100-fold from 1.7 million hectares in 1996 to 2.1 billion hectares in 2016, making GM crops the fastest accepted crop technology (ISAAA 2016). Of the 617 commercial GM traits registered for crops, most are for herbicide tolerance (256), followed by insect resistance (conferred by expression of cry genes from *Bacillus thuringiensis* (BT)) (209), while a few are for disease resistance (28), of which only three are registered for fungal disease resistance (ISAAA 2017). Concerns over unintended consequences of growing GM crops in the field, to the ecosystem and environment (Conner et al. 2003b; Wolfenbarger and Phifer 2000), and the paucity of information on their potential environmental impact, particularly on non-target organisms (NTOs), remain unresolved (Devos et al. 2016; Turrini et al. 2015). GM crops may influence soil ecosystems beneficially, adversely or neutrally, impacting productivity and sustainability of cropping system above and below ground (Birch et al. 2007a; Oger et al. 1997). Like pesticides themselves, they can potentially cause a direct effect on NTOs (e.g., toxicity due to product of gene or gene product to a non-target species), indirect (i.e. via trophic interactions), metabolic (i.e changed rhizodeposition

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due to unintended changes in plant metabolism), management (changes due to unique GM crop cultivation practices) and/or changes to the soil ecosystem, where beneficial soil microorganisms (NTOs) dwell (Birch et al. 2007a; Meyer et al. 2013). Hence, during the environmental risk assessment of GM plants intended for cultivation, a testable risk hypothesis addressing adverse effects on NTOs, is pivotal (Dale et al. 2002; Devos et al. 2016).

Soil microorganisms are crucial components for many ecosystem processes, including nitrogen and carbon cycling, plant nutrient acquisition and improvement of agricultural soil fertility (Hayat et al. 2010; van der Heijden et al. 2015). Two major plant health and growth promoting microorganisms in soil that form mutualistic, symbiotic relationships with agricultural plants, including legume crops (e.g., pea (*Pisum sativum L.*)) are the nitrogen-fixing bacteria belonging to genus *Rhizobium* and arbuscular mycorrhiza fungus (AMF) (Long 1989; Smith and Read 2008). Reduction in shoot to root ratio is observed in plants when growth is restricted by mineral nutrients (Marschner et al.1996) especially N or P supply (Andrews 1993; Andrews et al. 1999). *Rhizobium* fixes atmospheric nitrogen for plants (Mylona et al. 1995), whereas AMF facilitates nutrient uptake, especially phosphorous, and enhances resistance to various biotic stresses, including diseases (Jin et al. 2013; Parniske 2008; Smith and Read 2010). In return, AMF receive carbohydrates from plants and hence are more sensitive to changes in the host plant than free-living soil fungi (Hannula et al. 2014). This makes them excellent indicators of unintended effects of genetically modified crops with antifungal genes (Liu 2010; Turrini et al. 2015).

Recently, European pea (*Pisum sativum* L.) cultivars expressing four antifungal genes singly or stacked - *β-1,3 glucanase* (G), *endochitinase* (C) (belonging to pathogenesis related (PR) proteins family), *polygalacturonase inhibiting proteins* (PGIPs) (P) under the constitutive d35S promoter from cauliflower mosaic virus (CAMV) and anti-microbial grape resveratrol synthase gene (Vst-1) (*Stilbene synthase*) (V) under its own inducible promoter *Stilbene synthase* from grape (*Vitis vinifera*) which can be induced by UV light, pathogen attack and stress (Figure 6.1 and Table 6.1) have been transformed for disease tolerance (Amian A et al. 2011; Hassan et al. 2009; Richter et al. 2006a). The PR proteins (*β- 1,3 glucanase* and *endochitinase*) degrade microbial cell wall components (Van Loon et al. 2006), while PGIPs can inhibit fungal endopolygalacturonases that cause fungal wall degradation and plant tissue maceration (De Lorenzo et al. 2001), hence providing resistance against fungal pathogens. Resveratrol (the product of chemical synthesized by *Stilbene synthase*) belongs to phytoalexin class of secondary metabolites that possess biological activity against a wide range of pathogens (Jeandet et al. 2002). Transgenic plants show enhanced tolerance to fungi in *in vitro* testing (Amian A et al. 2011; Selatsa et al. 2008) and are currently undergoing field trials for efficacy against fungal diseases in Canada. The Canadian Food Inspection Agency (CFIA) regulates the environmental release of a Plant with Novel Trait (PNT) in Canada. As with any PNT, effects of the transgenic disease tolerant peas or their gene products on non-target species must be quantified prior to release of the transgenic crop (CFIA 2017a). Similar assessment of unintended effects is required by other regulatory authorities for safety determination of GM crops.

Recent surveys indicate the Glomeraceae family is the most abundant type of arbuscular mycorrhizal fungi in agricultural areas of the Canadian prairies (Dai et al. 2013). Pea producers in Canada commonly inoculate seeds with AMF and *Rhizobium* (Lupwayi et al. 2006). Particularly, *Glomus intraradices* (now *Rhizophagus intraradices* (Schübler and Walker 2010)) and *Rhizobium leguminosarum* bv *viceae* are commercially available as inoculants and known to increase phosphorus uptake and nodulation, respectively, in pea (Bødker et al. 1998; Geneva et al. 2006). The molecular similarity between legumes/rhizobia and legumes/AMF symbiosis and a possible sharing of common signal transduction pathways, has been an important concept in plant/microorganism interaction studies (Balestrini and Bonfante 2005; Hirsch and Kapulnik 1998). The infection process by AMF, pathogenic fungi and Rhizobium in plants, share some common genetic and signaling elements (Albrecht et al. 1999; Vierheilig et al. 1995) and may be influenced by the transgenic addition of anti-fungal genes. AMF have cell walls that contain chitin and β 1,3 glucan which may be susceptible to damage by the chitinases and β -1,3 glucanases as would saprophytic and pathogenic fungi (Vierheilig et al. 1995). Similarly, some chitinases are known to show lysozyme activity in hydrolyzing the peptidoglycan of bacterial cell walls although root nodule chitinases might symbiotically protect roots from external pathogen infection (Minic et al. 1998; Zhang et al. 2016). Experimental evidence also suggests that AMF have a significant impact on host plant gene expression eg. stress and defense response, in the roots, vicinity of colonized cells as well as elsewhere in the plant (Balestrini and Bonfante 2005; Liu et al. 2007), which could pose difficulty in predicting the effect of introduced transgenes.

The majority of research on GM impact on NTOs have been conducted on maize, potatoes, tobacco and cotton and the changes in diversity of rhizosphere-associated fungal and bacterial communities were reported to show minor or no effect of GM plant on NTOs examined (Hannula et al. 2014; Stefani and Hamelin 2010; Weinert et al. 2010). Responses monitored included mycorrhizal growth and rate of colonization by AMF (Stefani and Hamelin 2010) or number of nodules formed by *Rhizobium* spp. (Powell et al. 2007). Testing each GM crop of commercial interest for its effect on NTOs is required to complete risk assessment (Ladics et al. 2015). It has not been established if antifungal genes expressed in transgenic pea can affect non-target AMF and Rhizobium.

Our *a priori* expectation is that antifungal proteins, when expressed in transgenic pea plants, will have an adverse effect on root colonization by arbuscular mycorrhiza and root nodulation by Rhizobium in the field. A reduced fresh weight shoot-to-root ratio may be observed if transgenes affect the associations of AMF and Rhizobium. We also hypothesize that stacked genes and single gene insertions in transgenic plants will have different responses and in particular, glucanases and chitinases may have a more pronounced effect on AMF colonization because their cell walls contain both chitin and β -1,3 glucan. Our testable risk hypothesis was: antifungal genes will have not have an effect on root colonization and nodulation by AMF fungi or Rhizobium in peas and on the growth of peas in the absence of fungal pathogen infection. We report our investigation of gene expression levels in roots and shoots of transgenic pea lines in the greenhouse and the effect of four antifungal genes stably expressed in transgenic pea expressing on arbuscular mycorrhizal colonization and Rhizobium nodulation in comparison to non-transgenic Canadian pea lines and German parental lines in the field.

6.2. Materials and methods

6.2.1. Plant material and transformation

Disease resistance genes encoding four antifungal genes V, P, G, C were inserted into European pea cultivars “Baroness” or “Sponsor” at the Institute of Plant Genetics, Department of Plant Biotechnology, Leibniz University Hannover, Germany. Embryo axis excised from mature seeds of European pea ‘Baroness’ were used as explants for *Agrobacterium*-mediated transformation using strain EHA105 (Hood et al. 1993) for transformation with V, P, G genes and similarly, European pea cv. ‘Sponsor’ was used for transformation with C gene, using a modified protocol of Schroeder *et al.* (1993) (see Chapter 3). Herbicide resistant *bar* gene (selectable marker) from *Streptomyces hygroscopicus* was inserted along with the genes (Murakami et al. 1986; Thompson et al. 1987) (Figure 1). Using conventional breeding, the four genes were stacked in pea lines as single, three and four genes (V:P (Richter et al. 2006a) x G:C (Amian A.A. et al. 2011) = V:P:G:C (Hassan et al. 2010).

6.2.2. Gene expression in root and leaf tissue

In a separate experiment, the transgenic lines and non-transgenic lines used in the field experiment were grown in a greenhouse in the Department of Agricultural, Food and Nutritional Sciences, University of Alberta, Edmonton, AB, Canada, in 2014. Each plant (10 plants line^{-1}) were seeded in autoclaved vermiculite-perlite mixture (Sunshine Mix®#4, Sun Gro Horticulture, Canada) and maintained at 25 ± 2 °C with a 16/8-h light/dark photoperiod in a greenhouse for four weeks, after which root and leaf samples were removed and triple cleaned in RNAse free water. Total RNA was extracted from tissues using the Qiagen RNeasy Plant Mini Kit (Qiagen, Canada) according to

manufacturer's recommended protocol. Briefly, frozen tissues were ground to a fine powder with liquid nitrogen in baked (250 °C, 3 hr) and chilled (-80 °C) mortar and pestle. To the frozen tissue powder (100mg), 450µl of buffer RLT (containing β-ME) was added to and vortexed to obtain slurry, incubated at 56 °C for 3 min, passed through the QIAshredder column and centrifuged at 21,000*xg* for 2 min. To a new tube containing the flow through, 200µl of absolute ethanol was added and the mix applied to RNeasy column and centrifuged at 10,000x*g* for 30 sec. The flow-through was discarded and the column washed by adding 350ul RW1 buffer, centrifuged again and subjected to on-column DNA digestion using RNase free DNase set (Qiagen) by adding 27.27U DNase in 80µl RDD buffer to the column and incubating at room temperature for 15 min. Subsequent additions of 700µl RW1, 500µl RPE and 50µl RPE with and centrifugation at 10,000*xg* and discarding of the flow-through to the column followed at each step. In a new 2ml collection tube, the column was centrifuged at 12,000*xg* for 2 min and finally, transferred to a 1.5ml microfuge tube, 50µl of nuclease free water was added to the centre of the column, and the RNA eluted by centrifugation at 12,0000x*g* for 30 sec. The extracted RNA was quantified with NanoDrop™ spectrophotometer (Thermo Fisher Scientific™) and then stored at -80 °C until further analysis.

6.2.3. cDNA synthesis

Complimentary DNA (cDNA) was synthesized in a 20 µl volume using 1 ug of total RNA employing RevertAid RT kit (Thermo Fisher Scientific™) according to manufacturer's recommended protocol. Briefly, 1 µg of total RNA was used as the template in a 20 µl reaction containing 100 nmole of random hexamer primer, 20U/µl of RiboLock RNase inhibitor, 10 nmole of dNTP and 200U/µl of RevertAid reverse

transcriptase in 1x reaction buffer. The components were gently mixed, briefly centrifuged and incubated at 25 °C for 5 min followed by 60 min at 42 °C for cDNA synthesis. The reaction was terminated by heating the tubes to 70 °C for 5min, and the products stored at -80 °C until further use.

6.2.4. Quantitation of gene expression by Real-Time PCR

Levels of gene expression (of V, P, G, and C) in transgenic pea line's root and leaf tissue, were determined using SYBR Green based q-RT-PCR on a StepOnePlus™ instrument (Applied Biosystems®, Canada) using comparative quantitation employing $\Delta\Delta C_T$ method with melt curve. A 10 µl reaction contained 5 µl of 2 x KAPA SYBR® Fast Master Mix (Kapa Biosystems, Boston, MA, USA), 1 µl of 1:15 diluted cDNA, and 5 pmol of each (forward and reverse) gene specific primers which were designed using either Primer Express 3.0 (Applied biosystems) or PrimerQuest (Integrated DNA technologies, Coralville, Iowa) with Tm of 60 °C and amplicon sizes between 100-140bp. Elongation factor 1a was used as endogenous control. Primers used are "P" forward: 5'-CTTCGAAATCAAGACAGCCTTCA-3'; reverse: 5'-GGGATCACACTCGACGCAGTA-3'; "V" forward: 5-AGAAATGCCCGGTGCAGAT-3', reverse: 5'-TTCCACCTGCATAGCAACCTT-3'; "G" forward: 5'- AAC GCG CGG AAC TAC AA -3', reverse: 5'- CTC GTT GAA CAT GGC GAA TAT G -3'; "C" forward: 5'- GAA CCG GAA CTC CTT CTA CAG -3', reverse: 5'- TCC TGC TTC TTG GTG GTG -3' and endogenous control forward: 5'-GATGGATGCTACCACCCCTAAG-3', reverse: 5'-GAGATGGGAACGAAGGGAATT-3'). All reactions were carried out in triplicate, employing 6 cDNA samples from individual plants from each line, and the average C_T

values were used for calculating gene expression. The detection limit for the plasmid copies was carried out with a dilution series between 10^7 and 10^1 copies per reaction, and linear range of detection was determined. These numbers of copies were added to the German parental lines (Sponsor and Baroness)'s cDNA sample to serve as the baseline for calculating relative expression.

6.2.5. Field trial

A confined field trial was established at a secure field site located at the Crop Diversification Center North, Alberta Agriculture and Forestry, in north east of Edmonton, AB (lat. 53°38'N, long.113°22'W), on a black chernozemic sandy loam soil in summer of 2013. Soil nutrient reports from 0-6" soil depth were obtained prior to seeding and indicated adequate nutrients, especially P. Ten treatments, comprised of seven transgenic lines (three lines with single gene insertions {5(G), 15(C), 21(V)} one line with triple gene insertion {11(P:C:G)}, two lines with four gene insertions {8 (V:P:G:C), 10 (V:P:G:C)} and as comparator four lines including two German parental lines (Sponsor and Baroness) and two Canadian lines (AC Early Star, Agassiz). Seeds were individually planted by hand at 10 seeds per plot (0.5m x 0.5m). Seed coated with peat based MYKE® PRO PS3+R by Premier Tech Technologies (Riviere-Du-Loup, QC) - a dual inoculant with endomycorrhizal fungi (*Glomus intraradices* (2750 viable spores g^{-1}) for the plots receiving AMF+Rhizobium inoculation and rest of plots were inoculated with only *Rhizobium leguminosarum* bv. viciae (1.6×10^9 viable cells g^{-1}) (Cell-Tech® Novozymes) at a rate of 291.58 g ha^{-1} (@0.004g for 2.5g seeds). No nitrogen was applied to alter the nodulation of pea plants and no disease was introduced to the plots. The plots

were arranged in split plot design with cultivars as main plot, inoculation as sub plot and four replicates per treatment.

6.2.6. Plant growth assessment

Seedling emergence 14 days after planting (DAP) and plant heights 28 and 42 DAP (5 plants $plot^{-1}$) was obtained to quantify uniformity in density and plant growth among plots. Plant roots and shoots were harvested 7 weeks after planting (the beginning of flowering) (Hassan et al. 2012). Plant roots were rinsed with deionized water three times to remove debris and dried on paper in the field. For fresh weight, 5 plants $plot^{-1}$ were divided into the part above the hypocotyl (shoot) and below (root) in the field.

6.2.7. Nodulation ratings

Whole roots from all 10 plants in a plot were cleaned and root nodulation was quantified by assessing nodules on roots randomly chosen from 5 plants $plot^{-1}$ according to the scale used by 20/20 Seed Labs Inc., (available at: http://www.2020seedlabs.ca/sites/default/files/Pulse%20Crop%20Nodulation%20Guide.pdf). Briefly, after cleaning with deionized water three times, roots were rated on three assessment criteria: plant growth and vigor, nodule color/number and nodule position and a total score (1-13) was obtained (Table 6.2).

6.2.8. Root colonization by arbuscular mycorrhizae

For estimation of root colonization by arbuscular mycorrhizae, arbuscules were more clearly visible and differentiable under the microscope than vesicles or fungal hyphae, at the stage of harvest. The root systems (5 plant roots (whole) $plot^{-1}$) were processed using the procedure outlined by Pitet et al. (Pitet et al. 2009). Five roots $plot^{-1}$ (from randomly

selected five plants) were cleaned thoroughly with deionized water and air dried on paper on a laboratory bench. Nodules and other debris were carefully removed, secondary and tertiary roots were excised in 1 cm pieces and stored in 70% v/v ethanol in glass vials (Fisher Scientific, PA, USA) until further processing. For clearing, roots were retrieved from vials and placed into three tissue cassettes (Fisherbrand™ tissue path™ IV tissue cassette, Fisher Scientific, PA, USA) per plot making three subsamples for each plot. Cassettes were put into 2% (w/v) KOH solution sufficient to cover roots and shaken for 24 hours at room temperature for clearing and submerged in 5% acetic acid solution for acidification for an hour. Acidified roots in cassettes were immersed 0.025% (v/v) trypan blue in acetoglycerol solution for 4 hr and then in acetoglycerol (glycerol 500ml:H_2O 450 ml and 5% (v/v) acetic acid solution 50 ml) for 48 hours at ambient room temperature for destaining. Cassettes were thoroughly washed with deionized water three times between each step. Roots were mounted in glycerin on a slide, covered with coverslips and viewed under a compound microscope using magnified intersection method (McGonigle et al. 1990) with the roots aligned parallel to the long axis of the slides at 400x magnification (higher than generally used for determining root-length colonization to reduce uncertainty in identification of fungal structures) and 100 intersections between roots and the vertical eyepiece crosshair per sample. Briefly, when the vertical eyepiece crosshair crossed a structure (for example, arbuscule) under the microscope, it was noted at each intersection. The same root length was used for each measurement and out of all structres identified under the eyepiece cross-section (in the 100 intersections) total arbuscules were expressed as percent.

6.2.9. Statistical analysis

Relative gene expression of each gene in roots and leaves for each transgenic line was analyzed using one-way analysis of variance ANOVA, lines as fixed effects, with PROC MIXED in SAS 9.4 (SAS Institute Inc. 2014). Tukey adjustment was used for comparisons between lines, within a gene group. Data generated from field experiment were analyzed using two-way ANOVA with PROC MIXED in SAS 9.4, where lines and inoculation were used as fixed effects and blocks as random effect. Normality and homogeneity of variance were assessed using Shapiro-Wilks and Levene's test in SAS and raw data was used. Least squares (LS) means estimates were compared using pre-planned orthogonal contrasts.

6.3. Results

6.3.1. Gene expression analysis

The relative gene expression after q-RT-PCR analysis was consistently higher in leaf tissues as compared to the root tissues in all of the transgenic pea lines except C, which was the only gene which was at comparable levels in leaf and root tissues (Table 6.3). For V, the relative gene expression was found highest in line 10 (V:P:G:C) (699.14±220.76 in leaf and 40.47±12.54 in root) followed by line 21 (558.48±84.99 in leaf and 32.84±2.36 in root) and negligible in line 8 (V:P:G:C). Relative V expression was significantly higher at 0.05% level of significance in leaves as compared to roots for line 21(V) ($p=0.0011$) and line 10(V:P:G:C) ($p=<0.0001$). Interestingly, P had highest relative expression in leaf tissues of line 10 (V:P:G:C) (111577.50±27728.24) followed by line 11 (P:C:G) (11892.53±6171.69) but lower in roots of both these lines as well as root tissue of line 8 (V:P:G:C) whose leaf had P relative expression at 469.48±93.35. Significantly

lower relative root P expression than in leaf, was recorded for line 10 (V: P:G:C) (p=<0.0001). Line 5(G) exhibited high levels of relative G expression (452.07±214.25 in leaf and 127.21± 71.77 in root) but lower than in line 10 (V:P:G:C) (1566.14±462.75 in leaf and 94.94±31.61 in root) a four gene line. Relative G expression of root tissue of line 10(V:P:G:C) was significantly lower than leaf tissue (p=<0.0001). However, the relative G expression was negligible in another four genes stacked line 8 (V:P:G:C) and three genes stacked line 11(P:C:G). Higher relative expression levels of C were found in line 15 (C)(59.72±5.88 in leaf and 76.75±9.83 in root) in comparison to negligible expression in stacked lines like 8 (V:P:G:C), 10 (V:P:G:C) and 11 (P:C:G). However, none of these had significantly different relative C expression between root and leaf tissue. This variable relative gene expression among various genetic compositions of pea lines suggests gene silencing or possible role of the choice of promoters.

6.3.2. Plant growth

Growth of transgenic pea lines was characterized on percent emergence, heights and shoot-to-root fresh weight ratios in comparison with non-transgenic pea lines in the presence of two inoculants. There was no significant difference recorded for the emergence and heights of pea plants in the presence of dual inoculant MYKE® PRO PS3+R and Rhizobium only inoculants, at $P < 0.05$ significance level (data not presented). Similarly, for fresh weight shoot-to-root ratio,'pea lines x inoculation' interaction was not significant, but pea lines (p=<0.0001) and inoculation (p=<0.0001) were significant. All transgenic lines had significantly more fresh weight shoot-to-root ratio overall than parents (contrasts p =0.0057) and Canadian lines (contrasts p=0.0243); however, the dual inoculant MYKE® PRO PS3+R's treatment yielded significantly more

fresh weight shoot-to-root ratio (p= < 0.0001) in all pea lines. In general, non transgenic Canadian lines had higher fresh weight shoot-to-root ratio than German parental lines (contrasts p = <0.0001). Although line 21 (V) had significantly higher fresh weight shoot-to-root ratio than other transgenic lines, it was not significantly different than its parents or Canadian lines (Figure 6.2). Interestingly, using contrasts, we observed line 11 with three genes (P:C:G) had significantly higher shoot-to-root ratio fresh weight (p= 0.0256), than lines 8, 10 with four genes (V:P:G:C) but was not significantly different than lines with single gene insertions, including line 5(G) and line 21(V) (p=0.1392), indicating that stacked genes lines did not enhance fresh weight shoot-to-root ratio more than the single gene lines.

6.3.3. Root nodulation

In general, transgenes did not affect the root nodulation among all transgenic lines in the presence of both the inoculants, as compared to the German parental lines and Canadian lines in the field. Higher nodulation ratings were observed when AMF was not provided as inoculant (p <0.0001). The genetic makeup of lines (p=0.0021) and inoculation (p<0.0001) were significantly different for nodulation ratings but the interaction of lines x inoculation was not significant. Overall, nodules looked pink and healthy in all lines. Nodulation ratings were not significantly different among transgenic lines containing single gene (line 5(G), 15(C), 21(V)) vs. three genes (line 11(P:C:G)) or four genes (line 8,10(V:P:G:C)); three genes (line 11(P:C:G)) vs four genes (line 8,10(V:P:G:C)); transgenic lines (5(G), 8(V:P:G:C), 10(V:P:G:C), 11(P:C:G), 15(C),21(V)) vs German parental lines (Sponsor, Baroness) or Canadian lines (Agassiz, AC Earlystar). Line 21 (V) alone had significantly lower root nodulation ratings when inoculated with

AMF+Rhizobium, in comparison to Canadian lines Agassiz and AC Earlystar (p=0.0007) (Figure 6.3).

6.3.4. Root colonization

We measured the percent colonization in roots of all lines for arbuscular, vesicular and hyphal colonization and did not identify any effect of transgenes in comparison to Canadian lines and German parental lines when inoculated with or without AMF. Well-developed *Arum* type arbuscular mycorrhizae, well connected with hyphae were observed in all AMF inoculated and non-AMF (Rhizobium only) inoculated plots as well. We observed more arbuscular colonization (percentage) when inoculated with dual inoculant MYKE® PRO PS3+R (ranging from 21.5%to 29.4%for transgenic lines; 25.58%to 27.83% German parental lines and 19.0% to 29.12% for Canadian lines) vs Rhizobium only inoculant (ranging from 14.5% to 20.5% for transgenic lines; 16.7% to 17.4% for German parental lines and 15.57% to 17.83% for Canadian lines ($p<0.0001$) (Figure 6.4). However, no significant differences were detected among the lines or the interaction between the lines and inoculation. There were no significant differences found between arbuscular colonization ratings in presence of inoculants among transgenic lines containing single gene (line 5(G), 15(C), 21(V)) vs three genes (line 11(P:C:G)) or four genes (line 8,10(V:P:G:C)); three genes (line 11(P:C:G)) vs four genes (line 8,10(V:P:G:C)); transgenic lines (5(G),8(V:P:G:C),10(V:P:G:C),11(P:C:G),15(C),21(V)) vs German parental lines (Sponsor, Baroness) or Canadian lines (Agassiz, AC Earlystar). Similar observations were recorded for vesicular and hyphal colonization percentage (data not presented). Comparing with the nodulation ratings (which were higher when

AMF was absent (Figure 6.3)), the arbuscular colonization was higher when AMF was included in inoculant (Figure 6.4).

6.4. Discussion

Here we report the results of a field study, aimed at comparing the impact of antifungal genes, expressed in GM pea, on plant colonization by beneficial AMF and nodulation by Rhizobium with the non-GM pea. Transgene insertion, whether as single gene or stacked genes, did not alter the root colonization by AMF or root nodulation by Rhizobium inoculation in the field. We did not observe any differences of the transgenes on plant growth and performance. Having a dual inoculant with both AMF and Rhizobium yielded higher fresh weight shoot-to-root ratio in all the lines. Line 21 (V) had significantly higher biomass than other transgenic lines but not Canadian lines or German parental lines. This gene is under a wound, pathogen or UV inducible promotor (vst-*Stilbene synthase*)(Langcake and Pryce 1976) and may not have been induced in root tissue. In greenhouse experiments, the relative gene expression of antifungal gene V, vst-*Stilbene synthase* in line 21 was found lower in roots (32.85±2.36) and stacked gene lines for example, in Line 10 (V:P:G:C)'s root (40.47±12.54). Lower level of antifungal gene expression in roots for all transgenic lines may have effected their activity rendering no adverse effect on AMF or Rhizobium's interactions with pea.

AMF are ubiquitous in the soil (Smith and Read 2010) and hence, it was not surprising that colonization was also observed in the non-AMF (Rhizobium only) inoculant as well, although the dual inoculant (AMF+ Rhizobium) treatment had significantly more arbuscular colonization than the Rhizobium only inoculant. The reverse was true with nodulation as we recorded significantly more root nodulation in all

lines when inoculated with Rhizobium only inoculant in comparison to the AMF+Rhizobium, which suggests a possible competition between these two organisms for photosynthases and reinforces the complexity of the interactions between pea, mycorrhizal and nodulation related symbiosis (Foo et al. 2016). However, it is known that the interaction between these two symbionts in legumes can depend on a number of factors, such as stage of development inside the host plant (Mortimer et al. 2008), light conditions (Ballhorn et al. 2016), abiotic stresses like drought (Franzini 2010) and the compatibility of strains of symbionts involved (Azcón et al. 1991; Redecker et al. 1997), that needs further exploration which is beyond the scope of this paper.

Our results also indicate a lower gene expression in roots as compared to leaves, in general. The transgenes C, G, P were under the same d35S cauliflower mosaic virus (CaMV) promoter; however, the relative gene expression was lower in leaves and roots for C and G as compared to the P. This promoter has enhanced transcriptional activity (Kay et al. 1987) but can also induce transgene rearrangements in some circumstances (Kohli et al., 2010). A number of factors contribute to the variation in expression level of genes in plants including choice of promoter which can contribute to strength, tissue specificity, timing (Qu and Takaiwa 2004), or unexpected silencing of genes (Daxinger et al. 2008) and other factors unrelated to transformation methods, including local chromatin structure and regulatory sequences at the site of integration (Iglesias et al. 1997; Thomson and Blechl 2015), transgene copy and epigenetic effects like transcriptional gene silencing and post-transcriptional gene silencing (Dietz-Pfeilstetter 2010; Finnegan and McElroy 1994; Jaenisch and Bird 2003; Matzke and Matzke 1998). The unbalanced gene expression among one or more genes expressed together can occur

even if transgenes are physically linked or unlinked and/or are driven by same promoter (Ferrer et al. 2016).

Many researchers examining unintended effects of transgenic crops to beneficial organisms have reported similar results, for instance, in case of transgenic disease resistant tomato plants expressing endochitinase and β -1,3 glucanase under the control of CaMV *35S* promoter had no statistically significant difference in mycorrhization frequency, intensity or quality in the root system of transgenic and non-transgenic controls (Girlanda et al. 2008). Similarly, wheat expressing *pm3b* mildew resistance transgene had minor differences in AMF colonization (Meyer et al. 2013), and flax conferring improved fibre quality and resistance to pathogens including *β-1,3 glucanase* transgene among others, did not negatively affect AMF colonization (Wróbel-Kwiatkowska et al. 2012). Reduced presymbiotic AM hyphal growth and development of appresoria was observed in defensin expressing transgenic aubergine (Turrini et al. 2004) and tobacco plants constitutively expressing the acidic isoform of tobacco pathogenesis related protein (PR)-2 had delayed root colonization with AMF (Vierheilig et al. 1995). In case of transgenic alfalfa and Rhizobium interaction, transgenic alfalfa expressing pea seed lectin nodulated well when inoculated with *Rhizobium leguminosarum* bv *viciae* (van Rhijn et al. 2001) and expression of rice basic chitinases did not negatively affect the *Rhizobium meliloti*/alfalfa interaction as depicted by number of nodules and plant vigour in transgenic vs control plants (Masoud et al. 1996b). Transgenes with broad acting antifungal properties (such as chitinases and β-1,3 glucanase) seem to have no deleterious effect on AMF perhaps because the mutualistic fungi adapts to their presence

as suggested by Vierheilig et al. (1995) and pathogen specific antifungal proteins may interact with positive soil microorganisms differently.

Whilst the antifungal genes were reported to have activity against pathogens in vitro (chitinase and glucanase had inhibitory effects on spore germination of *Trichoderma harzianum* and *Colletotrichum acutatum* and hyphal growth on *Botrytis cinerea* and *Ascochyta pisi* (Selatsa et al. 2008)), they did not affect the root-associated beneficial organisms in the field. The differential targeting of pathogenic fungus and beneficial organisms by the antifungal genes in pea lines may also be attributed to recognition specificity (Khan et al. 2010; Stefani and Hamelin 2010), genotype related functional specificity between beneficial soil organisms (Djordjevic et al. 1987; Gianinazzi-Pearson 1996; Saxena et al. 2006) and/or type of genetic modification and gene being expressed (Giovannetti et al. 2010). The limited expression of transgenes in roots may also have contributed to a lack of deleterious effect.

Our field study is unique as it substantiates an absence of unintended effects from single and multiple antifungal genes expressed in pea, with a direct comparison to their German parental lines and well-established non-transgenic Canadian pea lines, to two beneficial soil organisms AMF (*Glomus intraradices*) and Rhizobium (*Rhizobium leguminosarum* bv. *viciae*), crucial for pea production. Although our research adds to the pivotal risk assessment, we cannot extrapolate our findings to genetic modifications that may target other biological or chemical functions in plants or can be functional against other non-target organisms that form associations with pea, for example, *Burkholderia* spp. or *Pseudomonas* spp. The estimation of effect of novel transgenes expressed in GM crops on non-target organisms is a long–term, resource intensive process with the

monetary and regulatory constraints, but confined field trial studies are crucial for environmental risk assessment of GM crops (Garcia-Alonso et al. 2014).

Table 6-1. Antifungal genes, their sources, promoters and symbols used in the experiment

Symbol	Gene of interest	Promoter	Source	Reference
V	Vst-Stillbene synthase (Vst1)	Vst Stilbene synthase *	Grape (*Vitis vinifera*)	(Richter et al. 2006a)
P	Polygalacturonase inhibiting proteins (rPGIP)	d35S from CAMV**	Raspberry (*Rubus idaeus*)	(Richter et al. 2006a)
G	β 1,3 glucanase	d35S from CAMV	Barley (*Hordeum vulgare*)	(Richter et al. 2006b)
C	Chitinase	d35S from CAMV	*Streptomyces olivaceoviridis*	(Hassan et al. 2009)

* Inducible promoter (induced by UV light, pathogen attack and stress)
** Constitutive promoter
CAMV- Cauliflower Mosaic Virus

Table 6-2. Root nodulation assessment codes used to score roots

SNo	Criteria	Assessment	Score
1	Plant growth and vigour	Plants green and vigorous	5
		Plants green and relatively small	3
		Plants slightly chlorotic	2
		Plants very chlorotic	1
2	Nodule colour/number	Greater than 5 groups of pink pigmented roots	5
		3-5 groups of predominantly pink roots	3
		Less than 3 groups of nodules	1
		No nodules or white/green nodules	0
3	Nodule position	Crown and lateral nodulation	3
		Majority crown nodulation	2
		Generally lateral nodulation	1
	Total score	Effective nodulation	11-13
		Nodulation less effective	7-10
		Generally unsatisfactory nodulation	1-6

Table 6-3. Relative gene expression ± standard error (SE) of each gene in roots and leaves, for each transgenic line
* Indicates significant difference between root and leaf gene expression between lines within each gene at 0.05% level of significance

Gene	Line	Relative gene expression		Significance at 0.05%
		Root ± SE	Leaf ± SE	
V	21 (V)	32.85 ±2.36	558.48±84.99	* p=0.0011
	8 (V:P:G:C)	0.02±0.00	1.17±0.35	ns
	10 (V:P:G:C)	40.47±12.54	699.14±220.76	* p=<0.0001
P	11 (P:C:G)	122.66±69.40	11892.53±6171.69	ns
	8 (V:P:G:C)	3.58±0.47	469.48±93.35	ns
	10 (V:P:G:C)	614.05±72.30	111577.50±27728.24	* p=<0.0001
G	5 (G)	127.21±71.77	452.07±214.25	ns
	11 (P:C:G)	1.53±0.40	4.26±1.03	ns
	8 (V:P:G:C)	0.32±0.20	1.77±0.32	ns
	10 (V:P:G:C)	94.94±31.61	1566.15±462.75	* p=<0.0001
C	15 (C)	76.76±9.83	59.72±5.88	ns
	11 (P:C:G)	0.25±0.04	0.08±0.01	ns
	8 (V:P:G:C)	0.24±0.03	0.10±0.01	ns
	10 (V:P:G:C)	0.08±0.01	0.09±0.01	ns

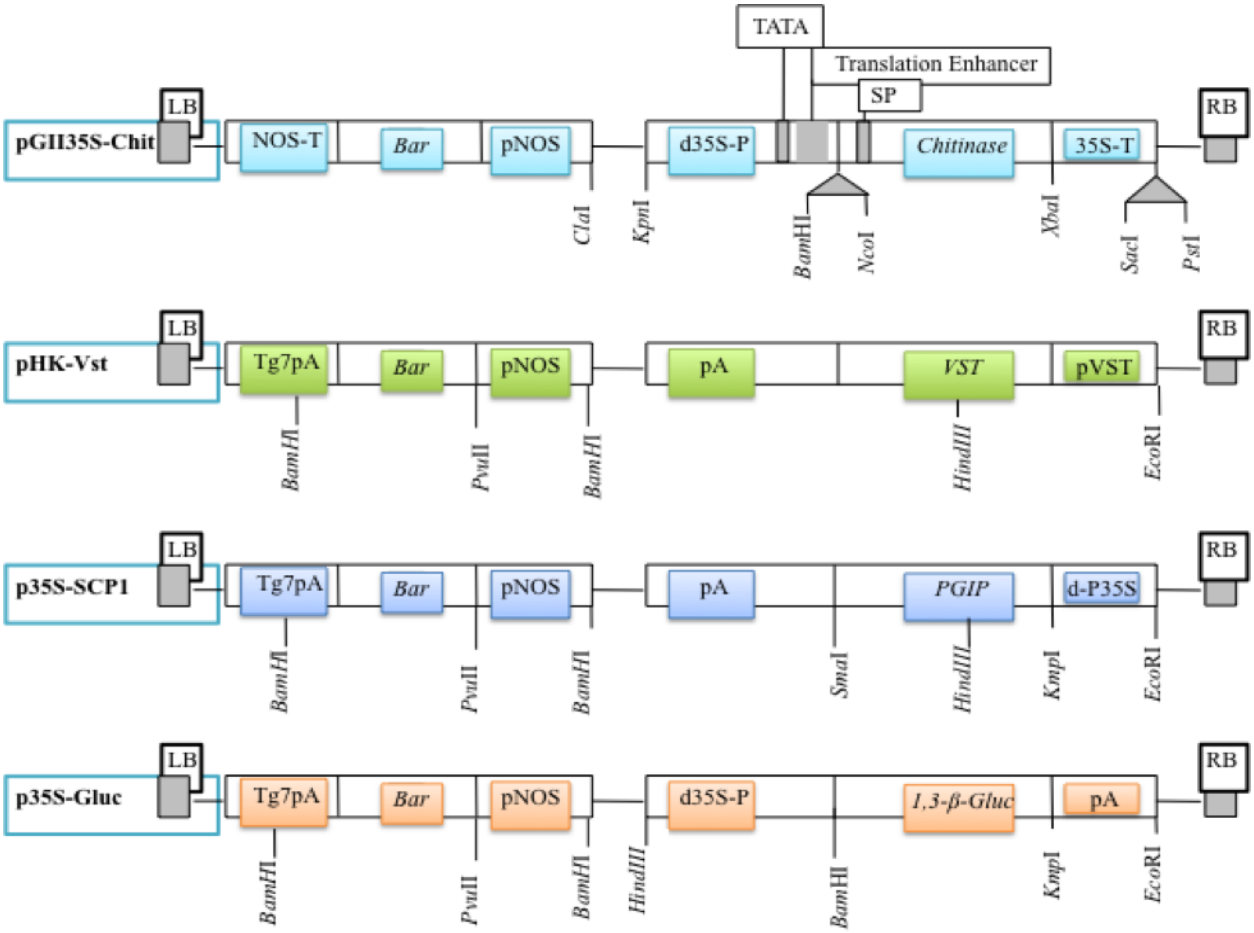

Figure 6-1. Physical maps of the binary vectors used for pea transformation from top to bottom: pGII35SChit, pHK-Vst, p35s-SCP1, p35S-Gluc : name of constructs used for *Chitinase*, *Stilbene synthase (VST)*, *PGIPs* and *Glucanase (β-1,3 glucanase)* insertions respectively, P: promoter, T: Terminator, NOS:Agobacterium nopaline synthase gene, Bar: herbicide resistance selectable marker from *Streptomyces hygroscopicus*, d35S:double 35S,TATA: TATA box, SP:Arabidopsis signal peptide, Tg7pA: poly A terminator sequence of T-DNA gene 7 "transcript 7" of *Agrobacterium tumefaciens* plasmid pTiAch5, pA: terminator poly Adenylation signal of the nopaline-synthase gene (NOS) from *Agrobacterium tumefaciens* plasmid pTiC58, (ClaI, Kpn1, BamHI, NcoI, XbaI, SacI, PstI, PvuII, HindIII, EcoRI, KmpI, SmaI): restriction enzymes, RB: right border, LB: left border

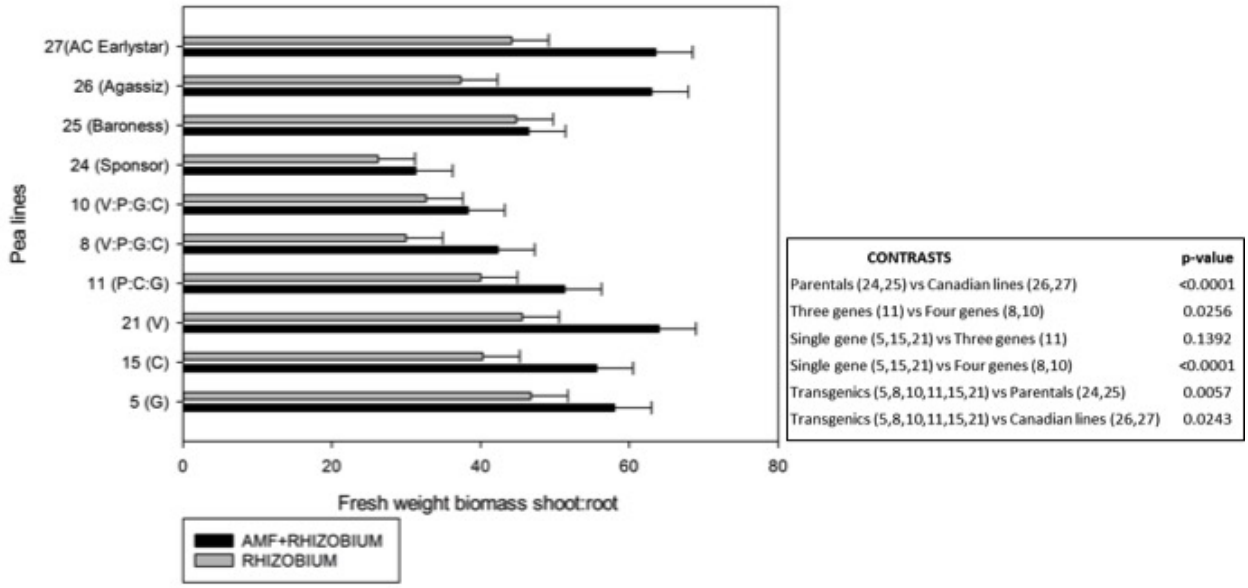

Figure 6-2. Fresh weight shoot-to-root ratio, in presence of inoculants AMF+rhizobium (MYKE® PRO PS3+R) or rhizobium only; of transgenic and conventional lines. Transgenic lines: 5 (G), 15(C), 21(V), 11(P:C:G), 8(V:P:G:C), 10(V:P:G:C); German parental lines 24 (Sponsor), 25 (Baroness) and Canadian lines 26 (Agassiz), 27 (AC Earlystar). Values are presented as LSmeans±SE and for contrasts, the p values are listed

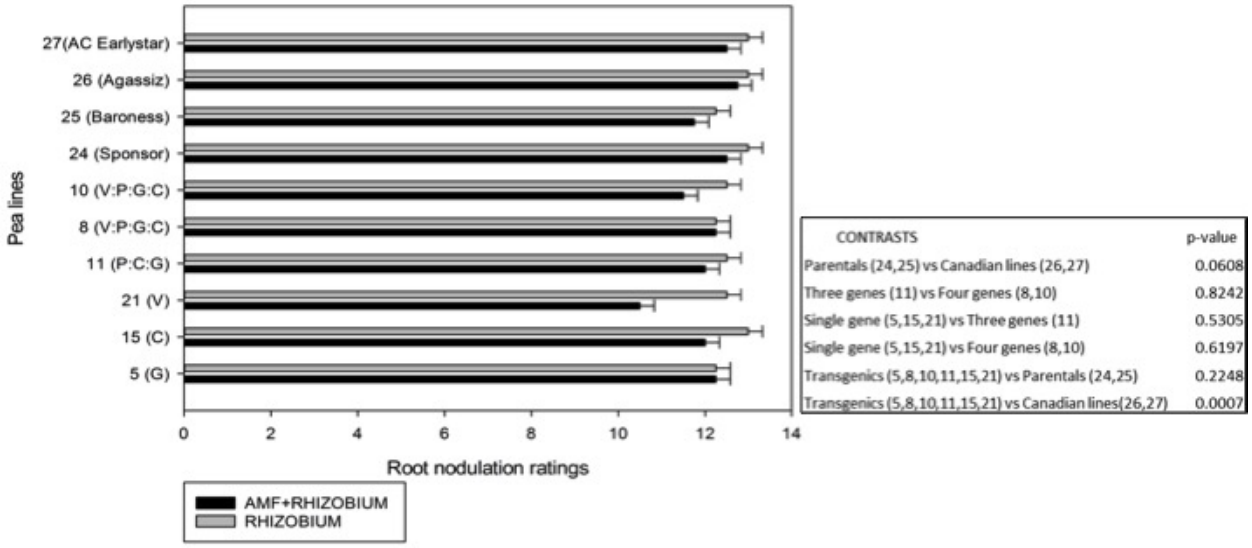

Figure 6-3. Root nodulation ratings in presence of inoculants AMF+rhizobium (MYKE® PRO PS3+R) or rhizobium only; in transgenic and conventional lines (transgenic lines: 5 (G), 15(C), 21(V), 11(P:C:G), 8(V:P:G:C), 10(V:P:G:C); German parental lines 24(Sponsor), 25(Baroness) and Canadian lines 26(Agassiz), 27(AC Earlystar). Values are presented as LSmeans ± SE and p values are listed for the contrasts

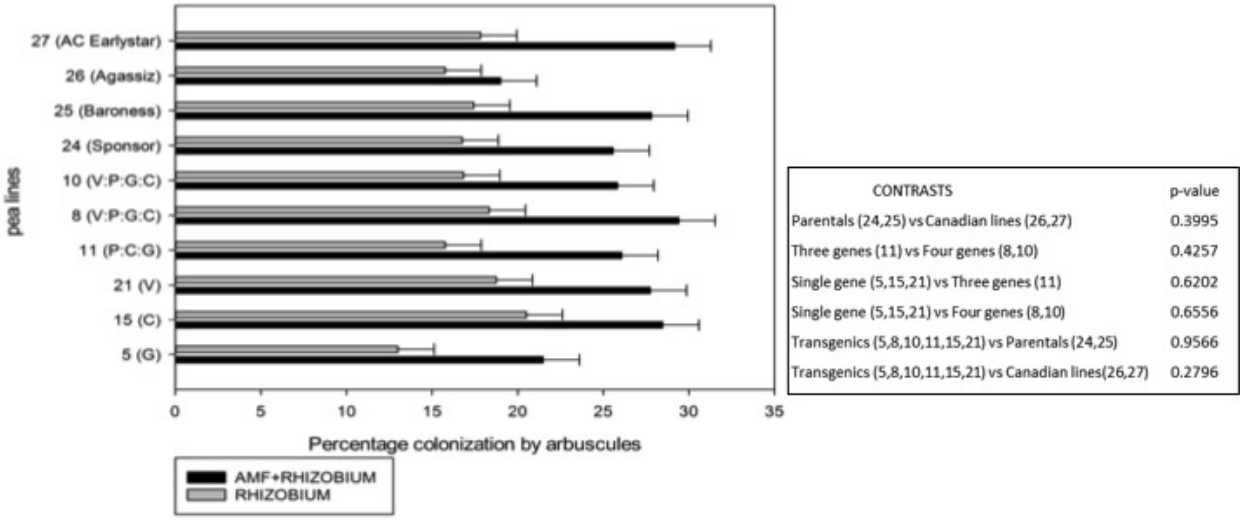

Figure 6-4. Arbuscular colonization (%) root length in presence of inoculants AMF+rhizobium (MYKE® PRO PS3+R) and rhizobium only in transgenic lines and conventional lines. Transgenic lines 5 (G), 15(C), 21(V), 11(P:C:G), 8(V:P:G:C), 10(V:P:G:C); non transgenic German parental lines 24(Sponsor), 25(Baroness) and Canadian lines 26(Agassiz), 27(AC Earlystar). Values are presented as LSmeans ± SE and p values for contrasts are presented

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Chapter Seven: General discussion and conclusions

7.1. Significance of research

Pea yields are significantly affected by biotic and abiotic stress, but there is limited genetic resistance in the germplasm. Genetic modification suggests a potential solution; however, the trait(s) should demonstrate commercially translatable efficacy. A plethora of research documenting incorporation of PR genes into various crops against diseases has been published in the past 15 years (Moosa et al. 2017). Although such research builds our knowledge of gene modification techniques and is an important tool for understanding the role of PR genes in combating stresses and their interactions, the lack of commercialized products purports the idea of successful disease resistance through expression of PR proteins.

There is a paucity of publications showing negative results (Knight 2003), such as lack of efficacy of the inserted transgenes in a field environment and in the presence of the stress. As such, the deficiency of publications substantiating the efficacy of PR genes against biotic stresses in field environment leaves a gap in our knowledge the consequences of laboratory based experiments. For a commercialized end product, the transgenes must demonstrate consistent and sustained resistance against stresses in a field environment. Research such as ours, i.e. reporting lack of efficacy of antifungal genes against two fungal pathogens in the field, contribute towards the importance of reporting negative results, which can aid in targeting the focus of research and allocation of monetary resources.

7.2 Results by each research objective

7.2.1. Genetic transformation of Canadian pea cultivars using drought tolerant genes

Agrobacterium mediated genetic transformation of pea was conducted in the Plant Biotechnology Laboratory, University of Hannover, Germany. With the successful integration of T-DNA, transgenic progeny were maintained after confirmation of successful gene integration in two generations, quantification of PR10a expression using qPCR and import of plant material back to Canada. Briefly, Canadian pea cultivar AC Earlystar was used to co-express PR10A from potato and transcription factor DREB2A from rice using dicistronic vector through *Agrobacterium* mediated gene transfer method practiced at University of Hannover, Germany. *Agrobacterium* strain EHA105 harboring a helper plasmid pSoup and transformation vector pGIIPR10acp148DREB2A was employed for the transformation along with herbicide resistant bar gene (selectable marker). Co-expression was enabled by single promoter mannopine synthase and terminator 35S CaMV. Embryo slices were subjected to inoculation and co-cultivation with *Agrobacterium*, selected by selectable marker, recovered by micro grafting onto seedling rootstock and leaf tissue subjected to PCR confirmations for T-DNA integration. Gene expression using RT-PCR was conducted on PCR positive plants. Subsequent generations of transgenic pea maintained and confirmed for transgenic inheritance and later imported to Canada. Transformation efficiency of 3.65% and a cultivar-independent method to transform pea were demonstrated. A greenhouse water stress experiment showed greater drought tolerance of transgenic lines compared to non-transgenic lines. Although, southern blot on transgenic plants and gene expression of both the PR10A and DREB2A would have provided us elaborate information however, due to resource

limitations, this could not be accomplished. This research serves as a technology transfer opportunity for pulse researchers in Canada.

7.2.2. Quantify disease resistance of transgenic pea lines to Fusarium root rot (*Fusarium* spp.) under field conditions

GM technology remains unexplored in transgenic disease tolerant pea development. Previously transformed pea with four antifungal genes i.e. *β-1,3 glucanase* (G), *endochitinase* (C), *polygalacturonase inhibiting proteins* (PGIPs) (P) and anti-microbial small molecules (*stilbene synthase*) (V) with the genes inserted either individually or stacked through crossing were tested for their efficacy against Fusarium root rot in confined trials over three years (2013 to 2015) in comparison with two parental German lines and three Canadian lines. Confined field trials from 2013-15, under auspices of CFIA were established. Seventeen treatments, comprised of nine transgenic lines (five lines with single gene insertions {5(G), 18(C), 20(C), 21(V) and 23(P)} one line with double gene insertion {4(V:P)}, one line with triple gene insertion {11(P:C:G)}, two lines with four gene insertions {8(V:P:G:C), 10(V:P:G:C)} and as comparator, four lines including two German parental lines, Sponsor and Baroness and three Canadian lines with/without pathogen inoculum : Agassiz, AC Earlystar and AAC Royce (each with some disease resistance against various fungi). Seeds were individually planted by hand in randomized complete block design with pea lines as treatment (randomly arranged in blocks), six replicates per treatment. All transgenic lines, German parental controls and three Canadian lines were also treated with *Fusarium avenaceum* inoculum, ground into fine power from previous year's infected wheat plants and was applied in contact with the seed at the time of seeding to promote disease establishment. Data was acquired for verification of pathogen (by plating soil, root tissue and inoculum from the trials and

PCR confirmations), seedling emergence, plant heights, disease severity ratings and fresh biomass. Plant tissue was also used to test for gene expression in each transgenic line.

Consistent patterns of superior emergence, higher fresh weight or lower disease ratings above and below ground, of transgenic lines in presence of pathogen inoculum were not observed in the three years of field experiments when compared to the parental and Canadian lines in the presence of pathogen inoculum. No indication of an advantage of stacked genes over single genes was observed. Most transgenic lines had lower relative gene expression in the roots than in the leaves in greenhouse, suggesting a possible explanation for poor tolerance to Fusarium root rot. There were several pathogens other than just *Fusarium avenaceum* (the inoculum), which were detected in the soil and roots of infected transgenic seedlings, suggesting multiple pathogens were acting at the same time. The results obtained were against our initial hypothesis that transgenic lines will have superior disease tolerance than control plants and that stacked genes will have better disease tolerance. Although multiple years and multiple location data over many years would perhaps yield different results, but the restrictions in working with GM material especially regulatory and monetary limits the input resources.

7.2.3. Quantify disease resistance of transgenic pea lines to Mycosphaerella blight (*P. pinodes*) under field conditions

Mycosphaerella blight is a commercially important pea disease in Canada and germplasm has partial resistance (Conner et al. 2012). Disease tolerance traits introduced in European pea (*β-1,3 glucanase (G)*, *endochitinase* (C), *polygalacturonase inhibiting proteins* (PGIPs) (P) and anti-microbial small molecules (*stilbene synthase*) (V)) were tested in comparison to partially resistant Canadian lines and parental lines were evaluated for to Mycosphaerella blight severity in three years (2013-15) of confined field

trails. 30 seeds plot^{-1} were arranged in randomized complete block design with six replications. Data were collected for plant growth assessment (seedling emergence, heights, seed numbers, yield) and disease severity ratings were obtained. No consistent superior emergence, higher yield, seed numbers and lower disease ratings was observed among the lines tested over three subsequent years of field trials. No benefit of stacking antifungal genes over individual genes was measured. Most transgenic lines had lower relative gene expression in roots than leaves suggesting the effect of promoters chosen or silencing of genes. The findings from the experiment refuted the initial hypothesis that transgenic lines will have superiority for disease tolerance than relative to the control lines and that stacked lines will have an enhanced disease tolerance to Mycosphaerella blight in field. Field trials where multiple stresses are present are crucial in testing the agronomic and ecological relevance of trait of interest such as disease tolerance.

7.2.4. Quantify potential non target effects of the anti-fungal genes to beneficial soil associates

This experiment addresses the concern of potential non-target consequences of antifungal genes in pea on non-target organisms including beneficial soil associates. Pea transformed with four antifungal genes (*β-1,3 glucanase*, *endochitinase*, *polygalacturonase inhibiting proteins* and *stilbene synthase*) were tested in a confined field trial in 2013 for their effects on root nodulation and colonization by two commonly used inoculants, Rhizobium and arbuscular mycorrhizae fungus (AMF) respectively. Transgenes were tested for gene expression from greenhouse grown material and lower expression in the roots than leaves was found. To determine the impact of disease tolerant pea or gene products on colonization by non-target AMF and nodulation by Rhizobium, field trial with a split plot design (5 plants plot^{-1}, cultivars as main plot and inoculation as

subplot, replicated four times per treatment) was established. Data was acquired for seedling emergence, Rhizobium nodulation ratings and root colonization potential by arbuscular mycorrhizae. Transgene insertion, as single gene or stacked genes, did not alter root colonization by AMF or root nodulation by Rhizobium inoculation in the field. No effect of transgenes on the plant growth and performance were observed, although having a dual inoculant with both AMF and Rhizobium yielded higher fresh weight shoot-to-root ratio in all the lines tested. This initial risk assessment of transgenic peas expressing antifungal genes showed no deleterious effect on non-target organisms. The results from this part of project provide the crucial information, which will be required by CFIA, should transgenic pea were to move towards commercialization. However, non-target effects on other beneficial soil associates like *Pseudomonas* spp., which have synergistic relationship with pea need further exploration and testing. The lower gene expression in chapters 4,5 and 6 seems to be consistent and perhaps a contributor towards lack of disease resistance.

7.3 Conclusion

The scope of our research has allowed us to assess the efficacy of four antifungal PR genes in field in conferring disease resistance, in presence of hemibiotrophic and necrotrophic fungal pathogens. The PR genes were inserted as both single and stacked genes with inducible and constitutive promotors and were being expressed differentially in root and leaf tissue. Our results reinforce that disease resistance, using transgenes from single or multiple PR proteins, provides insufficient protection against biotic stresses tested to confer consistent efficacy in the field.

Hemibiotrophic fungi (such as *Fusarium* spp.) can have differential gene expression in response to plant defenses during the two phases (i.e. biotrophic and necrotrophic) it undergoes during its life cycle and requires co-ordinated and ordered expression of diverse defense signaling pathways (Ding et al. 2011). In biotrophic phase, specific proteins target cell wall degradation and mask fungal cell surfaces to avoid plant defenses however, during the nectrotrophic phase, the fungus undergoes life style change of not only protecting itself from the plant defenses but also actively releasing enymes and toxins to attack the plant cell walls and utlize plant nutrients released during cell degradation (Meinhardt et al. 2014; Yang et al. 2013). Hence, the low gene expressions of antifungal genes under investigation in our field trials, were perhaps, not able to terminate the activity of the hemibiotrophic *Fusarium* spp. because of its specialized way of combating plant defenses.

Often, disease resistance conferred due to chitinolytic activity of transgenic plants expressing chitinase is attributed not only to chitinase itself but the triggering of other defense-related mechanisms due to presence of chitinase in the cell (Jayraj and Punja 2007). Lower gene expression levels reported in our field experiments may have been insufficient to trigger the response. The amount of chitinase enzyme produced and the proportion of chitin present in phytopathogenic fungal cell walls may also contribute to the lower disease reduction in our field trials. Acidic chitinases such as Chit30 (used in transformation for chitinase gene), that aim the cell wall are less effective at reducing disease caused by necrotrophic pathogens such as *P. pinodes*, than intracellular basic chitinase isoforms (Ferreira et al. 2007; Prasad et al. 2013; Punja and Raharjo 1996; Wally et al 2009). Recently, chitosan has been postulated to be present on the surface of

the cell walls of fungal infection structures, suggesting that chitinases, although effectively degrading cell walls of vegetative fungal hyphae, may not be sufficient for the digestion of cell walls of infection hyphae alone (El Gueddari et al. 2003). This may have contributed to lack of disease resistance. Necrotrophs such as *P. pinodes* usually secrete a large amount of cell wall-degrading enzymes to degrade plant cell wall polymers causing significant cell damage (Horbach et al. 2011). It is conceivable that expressing two cell degrading genes such as chitinase and glucanase may not pose a solution suggesting that with higher gene expression in transgenic leaves, the foliar necrotroph *P. pinodes* was perhaps well equipped with plethora of plant cell wall degrading enzymes to deal with our antifungal genes.

Mutualistic fungi such as AMF, is suggested to have capacity to adapt to presence of antifungal properties of transgenes (Vierheilig et al. 1995). The differential targeting of pathogenic fungus (*Fusarium* spp. and *P. pinodes*; chapter 4 and 5) and beneficial soil organisms (AMF and Rhizobium; chapter 6) by the antifungal genes in pea lines may also be ascribed to recognition specificity (Khan et al. 2010; Stefani and Hamelin 2010), genotype related functional specificity between beneficial soil organisms (Djordjevic et al. 1987; Gianinazzi-Pearson 1996; Saxena et al. 2006) and/or type of genetic modification and gene being expressed (Giovannetti et al. 2010). However, in wake of these results from chapter 4 and 5 (inability of our transgenes to confer disease resistance), our results for absence of deleterious effects of antifungal pathogens on arbuscular mycorrhizal colonization and root nodulation by Rhizobium (chapter 6) should be viewed with caution.

Chapter 3 describes a cultivar independent methodology for Agrobacterium mediated gene transfer to pea ultilizing drought tolerance traits. This serves as a technology transfer opportunity to researchers engaged with pulse research in Canada. Especially in case of disease resistance where many years of breeding efforts have only resulted in partial disease tolerant pea varieties transgenetic technologies may pose a solution or be used as a research tool to identify putative genes of interest. No fungicides or cultural control methods have been able to combat the disease spread in pea. Although research is underway for identification of natural resistance resources through breeding and chemical options are being sought, this was a proactive step to test the efficacy of genetically modified disease resistant peas in field against two commercially relevant crop pathogens in their target environment in comparison to best Canadian lines in market. Acknowledging that genetic modification can be a resource and time consuming venture, a company can only commercialize a trait if return surpasses the time and resources required to create, test and validate genes of interest in superior cultivars. This research evaluates the utility of GM technology using *Agrobacterium* mediated gene transfer for pulse crops in Canada. Stacked genes are of a great interest and believed to stay in commercial interest in near future as well (Parisi et al. 2016). Also, with the advent of incorporating different antifungal genes transgenically, especially PR proteins in various crops (Moosa et al. 2017), this research can serve to identify barrier to success that may aid in appropriate resource allocation by academic and industrial stakeholders. Especially with an example of French pea industry whose root rot epidemic is seen as the biggest challenge to the industry since 1993 (Wicker et al. 2001) with no apparent

solution in sight, this research was a proactive approach for safety of Canadian pulse industry.

7.4 Future research

7.4.1. Transgenic stress tolerant pea

PR proteins have been incorporated in various crops as a tool against pathogenic fungi for over 15 years (Moosa et al. 2017), yet no registered transgenic product is commercially available for any crop, although, disease in crops is a significant constraint for crop production. CFIA has not received a submission for approval of a disease resistance PNT crop in past 13 years (CFIA 2017b). Worldwide, the International Service for the Acquisition of Agri-biotech Applications (ISAAA) reports only three similar fungal disease events (in potato (*Solanum tuberosum* L.) transgenically expressing late blight resistant "Rpi-vnt1"protein (encoding for a coiled coil nucleotide-binding leucine-rich repeat protein from *Solanum venturii)* registered for fungal disease resistance, a few for viral resistance (in potato, bean and some horticultural crops), yet none utilizes PR proteins (ISAAA 2017). Even in the case of the most commercially utilized legume crop, soybean, no disease resistant cultivar has been registered to date in Canada or USA (CFIA 2017b; USDA/APHIS 2017). Lack of field trials or agri-biotech applications in case of PR proteins, suggests lack of the evidence of efficacy, other than laboratory or controlled environment experiments. Future research is warranted only if there is an evidence of proof of concept for the gene(s) under consideration.

Since the ultimate goal of research programs with potential crop improvement is to enhance yield and productivity (Godfray et al. 2010), strategic field trial experiment allowing realistic evaluation of genotype x environment interaction like ours, become

crucial. Field testing is important to test the agronomic performance and ecological relevance of trait in the in the local environment (Wozniak and McHughen 2012). Transgenes must be evaluated for yield trade offs, their interactions and interplay with various biotic and abiotic stresses in their natural form (Bostock et al. 2014), to identify issues with stability and resultant pleotropic effects (Pons et al. 2012) and to satisfy regulatory agencies (Romeis et al. 2008). The antifungal traits examined in peas did not consistently impart disease tolerance in transgenic pea in our experiments when scaled to field environment and, hence the results do not justify of the investment of time and labor further testing these events. However, we had only one trial site and limited seed for the experiment. Multi-year and multi-location trials are highly desirable, however, the regulatory conditions such as confinement, isolation and monitoring, make them unique, to be handled by highly specialized personnel and under the auspices of CFIA, in Canada. In the case of pea, with these antifungal gene/gene combinations, and in Canadian conditions, we have lack of evidence to support further investigation for commercialization of the trait. With other pathogens and other crop species, these genes may be useful and will need further testing. Nevertheless, such trials are capital and labor intensive, yet crucial for GM crop commercialization and hence should be encouraged.

Other transgenic approaches should be explored beyond classical antifungal PR genes (such as glucanases and chitinases) including exploration of other antifungal strategies such as targeted use of basal resistance against specific pathogens i.e. the possibility to enhance the perception and regulation of induced resistance without metabolic costs (Collinge et al. 2016) or the use of transcription factors (Dobón et al. 2015; Wang et al. 2016) and effectors (Vleeshouwers and Oliver 2014) could be the

future of biotic and abiotic stress tolerance in crops. Even within the realm of PR proteins, other PR proteins which have received recent interest against abiotic and biotic stresses such as thaumatin like proteins (TLPs) (He et al. 2017), osmotin (Sripriya et al. 2017) and/or their combinations with broad acting PR proteins such as chitinase and glucanase also need to be explored as tools against the fungal pathogens of pea.

Disease resistance is never complete and the level of disease reduction depends on the strategy deployed and the characteristic(s) of the pathogen(s) (Punja 2004). For example, life strategies of the pathogen, i.e., whether they are hemibiotrophic (such as *Fusarium* spp.), necrotrophic pathogens (such as *Mycosphaerella* spp.) or biotrophs (e.g., *Blumeria* spp.) may affect the choice of the genes to be introduced. Often when pathogens are genetically variable, as has been reported in cases of *F. graminearum* (O'Donnell et al. 2004) and *F. avenaceum* (Holtz et al. 2011) and other *Fusarium* species, disease resistance becomes extremely difficult due to facilitation of rapid evolution of resistance (Feng et al. 2010). Also, the gene being expressed could have specific properties under certain circumstances, which can affect the disease reduction. For example, chitinase expression based on the source of gene can vary in specificity to substrate binding, optimal pH, and localization of cell and hence have a varied antifungal activity (Punja 2004; Sandhu et al. 2017). Hence, if compared to the demonstrable scientific and economic success in genetically modified crop plants for resistance to herbicides, insect pests, and virus diseases, enhanced disease resistance has lagged behind (Punja 2004).

Since multiple pathogens seemed to contribute towards disease pressure development in Chapter 4, it is possible a step back towards more controlled environment

experiments (in greenhouse etc.,) in presence of single and/or multiple disease causal organisms at the same time, should be able to give us more information on the pathogen-pathogen interactions and hence contribute towards strategies required to combat these pathogens more effectively.

Successful *Agrobacterium*-mediated gene transfer methodology in the rather recalcitrant pea, has been described in chapter 3. However, Southern blotting will further elaborate the gene expression and subsequent protein formation of *PR10A* and *DREB2A* genes. In the future, field trials with confirmed homozygous progeny of transgenic drought tolerant pea should be helpful in determining the efficacy of the trait for pea crops in drought conditions, where the environment x genotype interaction prevails. Since the methodology described is cultivar independent, future research could utilize many other gene combinations (for example, other transcription factors such as the recent success with MYB and bHLH family transcription factor in sugarcane (Guo et al. 2017) and *Arabidopsis* (Le Hir et al. 2017) for creating drought tolerant pea.

As discussed in Chapter 6, no deleterious effects of antifungal genes on beneficial associations of arbuscular mycorrhizae and Rhizobium with transgenic pea were recorded. However, as every risk assessment should be carried out on case-by-case basis (CFIA 2016; Conner et al. 2003), this information cannot be interpreted for other transgenic pea with new or similar genes to those used in this research. For example, the drought tolerant peas described in chapter 6 will need to be evaluated for their non-target effects on such soil beneficial associations and previous data cannot be extrapolated. Similarly, the findings in the case of arbuscular mycorrhiza and Rhizobium cannot be extrapolated to other pea-non target organism associations such as *Burkholderia* spp.,

Pseudomonas spp. or endophytic root colonizing fungi such as *Fusarium equiseti*. With the advent of next generation sequencing in biomonitoring, which gives us insight into the ecosystem-wide biotic responses, the quantification of non-target effects of various genes should become easier.

Issues with the expression of multiple genes and possibly gene silencing over the generations of transgenic plants are described in Chapters 4, 5 and 6. With the advent of newer technologies for co-expression of multiple plant genes for example, plastid genome engineering (Ferrer et al. 2016), the future of GM crop development could be expected to have a improved system to incorporate several genes at once and could be utilized for abiotic or biotic stress tolerant pea development.

7.4.2. Novel technologies for improving stress tolerance

Pulse crops, especially pea, are yet to benefit from newer technologies in PNT crop development, such as site directed nuclease (SDN), transgenesis, RNAi, zinc finger nucleases (ZFN), transcription activator like effector nucleases (TALENs) or clustered regularly interspaced short palindromic repeats (CRISPR/Cas) (Altpeter et al. 2016; van de Wiel et al. 2017). With these in use, production of high quality transgenic events (as they are highly targeted due to their integration specificity and single-copy insertions) is expected. These techniques could overcome the currently used iterative approach to generate several events with random transgene insertions, and the events need to be selected, screened, and evaluated over many subsequent generations to ensure stability and effiacy (Altpeter et al. 2016). However, many of these are genome-editing techniques that may have limited applicability due to tissue culture and regeneration issues and resultant poor plant performance (van de Wiel et al. 2017). While there is worldwide a

debate if genome-edited crops will fall under existing regulatory systems that have been designed for transgenic plants (for an overview, see Sprink et al. (2016), Canada's current trait based regulatory system will evaluate genome edited crops just as another PNT (CFIA 2016).

We examined two different type of pathogens i.e. hemibiotrophic *Fusarium spp*. and nectrotrophic *P. pinodes* and mechanisms underlying resistance to these in plants, is complex. Multiple layers of active and passive defense are activated in a plant during a pathogen attack as responses at cellular level begin. The timing and strength of these defense reaction activations determine the resistance level (Ding et al. 2011). The first defense system is innate immunity (also known as pathogen-associated molecular patterns (PAMP), or PAMP-triggered immunity (PTI)), which triggers microbe-associated molecular patterns (MAMPs) including activation of cascades such as mitogen-activated protein kinase (MAPK), production of reactive oxygen species (ROS) and activation of transcription factors (Ausubel 2005; Boller and He 2009). Second defense system is the effector-triggered immunity (ETI), which occurs after recognition of the pathogen effectors by host resistance proteins including defense responses such as local programmed cell death, known as the hypersensitive response (HR) (Nimchuk et al. 2003). Both these defense systems trigger similar processes such as accumulation of ROS, generation of antimicrobial secondary metabolites and cell-wall reinforcement via the oxidative cross-linking of cell-wall components (Kishi- Kaboshi et al. 2010; Nurnberger et al. 2004). Cellular responses during activation of these defense systems are proposed to be regulated by concentration gradient of salicyclic acid (SA) and jasmonic acid (JA) (Betsuyaku et al. 2017). A number of studies have demonstrated that JA and

ethylene (ET) signaling pathways play pivotal roles in resistance against necrotrophic pathogens and hemi-biotrophic pathogens such as, *COI1* gene (mutuation of JA receptor protein) alters resistance to necrotrophic pathogens such as *Alternaria brassicicola* and *Botrytis cinerea* (Thomma et al. 1998) or in case of Arabisposis, the ERF1 and ORA59 transcription factors integrate JA/ET pathways and activate expression of defense-related genes such as *PDF1.2* against nectrotrophic pathogens (Pré et al. 2008). Similarly, in *Arabidopsis,* DELLA stabilization contributes to flg22 (flagellin-derived peptide)-induced growth inhibition and promotes susceptibility to virulent biotrophs and resistance to necrotrophs, partly by altering the relative strength of SA and JA signaling (Navarro et al. 2008). Perhaps a similar approach can be used for targeting *Fusarium spp* and *P. pinodes* by upregulating genes that contribute to the SA/JA/ET signaling rather than relying on end products of defense response cascades such as chitinase and glucanase as attempted in our research.

Among other strategies of utilizing plant's innate immune system, interspecies transfer of pattern recognition receptors (PRRs) are being used to confer responsiveness to previously unrecognized elicitors (Boutrot and Zipfel 2017). Progress of enhancing the innate immune system by transfer of dicotyledonous elongation factor (Lu et al. 2015) and *Arabidopsis* elongation factor inserted in wheat (Schoonbeek et al. 2015) are encouraging and hopefully more progress will be seen in pea as well. Recent interest in utilizing ergosterol (a common component of many plant fungi essential for fungal growth) as an antifungal tool alone or in combination with PR-1 proteins (Breen et al. 2017; Kazan and Gardiner 2017) could be another interesting tool in the quest for genetic modification of pea for disease tolerance, but it has not been yet utilized in pea yet.

One of the biggest constraints in engineering novel cultivars for disease resistance is the choice of promoters. As suggested by Collinge et al. (2016), promoters with organ- and response- specificity can avoid expression in the tissues where they are consumed hence avoiding any allergenicity risks associated with the resultant proteins. Transgenic peas expressed antifungal genes under D35S from CAMV or *Stilbene synthase* promoters in the current experiment. However repetitive use of, D35S could increase probability of gene silencing (Matzke and Matzke 1995). Perhaps transformation with a stronger, constitutive and ubiquitously expressed promoter for example, ubiquitin (Christensen and Quail 1996) (most commonly derived from maize (Ma and Xiu 2016), sugarcane (Wei et al. 2003) and several other plants for example soybean (Hernandez-Garcia et al. 2009)) can provide pea with more stable gene expression. The rice ubiquitin promoters (RUBQ1 or RUB2) drove higher (8- to 35-fold higher) constitutive levels of GUS expression in all of the rice tissues studied as compared to the 35S promoter (Wang and Oard 2003). *JcUEP* – a ubiquitin promoter derived from *Jatropha curcas* retained its activity under stress conditions in low temperature, high salt, dehydration and exogenous ABA treatments suggesting constitutive as well as inducible activity of ubiquitin promoters (Tao et al. 2015). However, with newer genes being discovered and engineered into plant tissues, some shortcomings such as a lack of consensus of biosafety around the world, the allergenicity of some common promoters and their lack of specificity of expression sites remain. The ongoing focus on the need for "effective" biosafety regulations to allow evidence based considerations of benefits and risks of disease tolerant GM crops under experimental conditions could provide more avenues for creation of the GM disease tolerant peas in future. Very few examples of registered disease resistant crops are

available worldwide. No minor crop such as pea has been effectively released with disease tolerant traits (Scott et al. 2016). Regulatory systems worldwide need to work collectively so as to have a balanced regulatory system in terms of its workability and costs, so that they do not unduly restrain the benefits of innovative plant products and technologies with potential advantages to agricultural sustainability.

7.5 Literature cited

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