CHARACTERIZATION OF LATE BLIGHT (*PHYTOPHTHORA INFESTANS*) RESISTANCE OF POTATO BREEDING LINES WITH THE RB GENE FROM *SOLANUM BULBOCASTANUM*

By

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ABSTRACT

CHARACTERIZATION OF LATE BLIGHT (*PHYTOPHTHORA INFESTANS*) RESISTANCE OF POTATO BREEDING LINES WITH THE RB GENE FROM *SOLANUM BULBOCASTANUM*

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Late blight, caused by *Phytophthora infestans* (Mont.) de Bary, is the most important disease of potato (*Solanum tuberosum* L.) because it affects foliage, tuber yield and storage ability. Previous work demonstrated that the wild diploid potato species *S. bulbocastanum* is highly resistant to all known genotypes of *P. infestans*. In this study, we transformed and expressed the RB gene from *S. bulbocastanum* (*Rpi-blb1*) into conventionally bred, late blight resistant breeding lines to evaluate the effect of pyramided late blight resistance genes. All RB potato transformation events were confirmed by Polymerase Chain Reaction (PCR) and RB expression was detected by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Foliar host plant resistance was characterized using detached leaf bioassays (DLB), greenhouse whole plant bioassays (WPB) and an inoculated field trial. A set of four *P. infestans* genotypes (US-8, US-22, US-23 and US-24) were used to evaluate foliar resistance in the DLB and WPB experiments, whereas only the US-22 genotype was used in the field. The results of the greenhouse whole-plant bioassays and detached leaf bioassays were varied. However, the highest level of resistance was demonstrated in conventionally bred, late blight resistant lines, which also contained the RB gene from *S. bulbocastanum.* The results from the field trial were more explanatory and proved that pyramiding resistance genes is an effective strategy to increase the level of late blight resistance.

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1. LITERATURE REVIEW

History and importance of the potato

The potato (*Solanum tuberosum)* is native to South America and are the third largest food crop in the world (based on human consumption) following wheat and rice (CIP, 2010). In 2012, the total world production of potatoes was 364 Mt, in comparison to rice 719 Mt and wheat 670 Mt (FAO, 2012). The genetic diversity patterns of potato indicate it was grown in the vast central plateau of the Andes from the ancient city of Cuzco to Lake Titicaca 7,000 to 10,000 years ago (Zuckerman, 1998). Around 1570, potato was introduced from South America to Europe (most likely Spain). Cultivation of potatoes were slowly spread throughout Europe, and later in 1700 potatoes were brought and grown in North America (Brown, 1993). The potato was not readily adopted as a food because European was unfamiliar with how to cultivate and consume it. However, there are a number of examples where potato became an irreplaceable staple food. During the war with England in late eighteenth century in France, the potato saved millions lives, and in Ireland, potatoes led to a population increase between 1790 and 1845 (Salaman, 1970). Now, in the twenty first century the potato is grown in more than 100 countries. It is cultivated under temperate, subtropical and tropical conditions (CIP, 2010).

World potato production is increasing more rapidly than the production of other leading crops (Shekhawat et al., 1999). Production in the Asian region is expanding at approximately 6% per year. Twenty years ago, only 1/8 of the world potato production was grown in Asia, but today 1/3 is grown by Asia. India's goal is to increase production through a "Brown Revolution" (Shekhawat et al., 1999) which is realistic given the projected annual predicted increase in Indian

potato production at about 2.8% over the next two decades. In 2013, 325 million Mt of potatoes were cultivated worldwide on approximately 18.6 million hectares. The main producing countries were China (86 million Mt), India (45 million Mt), the Russian Federation (29 million Mt), and the United States (23 million Mt) (FAO, 2012). The range of potato consumption varies from a high of 180 kg per capita in Belarus to a low of 4 kg per capita in Vietnam, with average world consumption at about 33-35 kg per capita (Stanik, 2012).

Potatoes are the leading vegetable crop in the United States accounting for about 15% of vegetable sales. In 2013, 428,967 ha of potatoes were planted in the US and 424,920 ha were harvested with an average yield of 19.8 M t per ha (416 cwt per/acre) (USDA and NASS, 2012). More than 50% of potatoes are processed, 30% is sold in the fresh market, and the remainders are used for livestock and as seed. An estimated 5,500 growers produce potatoes commercially in 36 of the 50 states.

There are several seasons for expanding global potato production, including the nutritional value of the crop, the amount of food produced per land unit, and relative water-use efficiency. Potatoes contain high levels of starch and vitamin B and C, but do not contain fat, cholesterol, or sodium (Li, 1985). The nutritive value of potato per unit of land is two to three times that of cereals (Shekhawat et al., 1999). In addition, one hectare of potato can yield two to four times the food quantity of grain crops. Finally, potatoes produce more food per unit of water than any other major crop, with water-use efficiency seven time's that of cereals (Shekhawat et al., 1999).

The potato belongs to the *Solanaceae* family, which includes about 3,000 species including tomato (*Solanum lycopersicum*), pepper (*Capsicum annuum*), eggplant (*Solanum*

melongena), and tobacco (*Nicotiana tabacum*). It is a cross-pollinated, vegetatively-propagated crop with a recently sequenced a diploid genome size of 840 MB (Potato Genome Sequencing Consortium, 2011). Wild and cultivated potato species include diploids (2n=2x=24), triploids (2n=2x=36), tetraploids (2n=4x=48) and pentaploids (2x=5x=60), but hexaploids (2n-6x=72) only exist amongst wild species. Cultivated potatoes are autopolyploid and can be categorized into landraces, which include native varieties grown in the Andes of South America as well as modern varieties developed by breeders since the 19th century.

According to the 1990 morphological taxonomic system of (Hawkes, 1990) potatoes are divided into seven cultivated species: *Solanum ajanhuiri* (2x), *S. chaucha* (2x), *S. curtilobum* (5x), *S. juzepczurii* (3x), *S. phureja* (2x), *S. curtilobum* (5x), *S. stenotomum* (2x), and *S. tuberosum* (4x). Recent taxonomy work using simple sequence repeat (SSR) markers recognize only four species: *Solanum tuberosum*, *S. ajanhuiri*, *S. curtilobum*, and *S. juzepczurii*. The most commonly grown species is *S. tuberosum*, and the cultivated species are grown in the Andean region.

Potato late blight (*Phytophthora infestans* de Bary)

There are many limiting factors to potato production: fungal and bacterial diseases, viruses, nematodes, insects, and abiotic stresses. Among these constraints, potato late blight is the one of the most devastating diseases. Potato late blight, caused by the oomycete *Phytophthora infestans* (Mont. de Bary), is a fungus/like organism referred to as a water mold (Lozoya-Saldana et al., 2006). The oomycetes differ from true fungi in several ways. The cell wall composition of oomycetes includes cellulose and beta glucans, but does not include chitin. Oomycetes also produce nonseptate hyphae and are diploid, whereas true fungi are haploid or

dikaryotic (Link et al., 2002). Asexual reproduction of *P. infestans* occurs with the formation of lemon-shaped spores called sporangia born on sporangiophores. In wet and humid conditions, the sporangia germinate to produce zoospores, which are asexual spores with two flagella (one whiplash, one tinsel). Zoospores are motile in water films on the host plant surface and infect the plant by penetrating the leaf surface either directly through stomata, or through the epidermal cell wall (indirect penetration). In warmer temperatures, the sporangia produce a germ tube a single spore and also infect the plant (direct penetration) (Kirk et al., 2004). The zoospores/sporangia can also be washed down into the soil by irrigation or rain, and can directly infect tubers (Kirk et al., 2004).

Phytophthora infestans is one of the most destructive pathogens of potato worldwide and it has left its mark on history. During the Irish potato famine of the 1840s, late blight destroyed the potato crop, resulting in hundreds of thousands of deaths and triggering mass migration of people from Ireland to America and other parts of the world (Salaman, 1985). The disease was introduced to the US, specifically around New York and Philadelphia, in 1843 and then spread quickly across the continent (Ristaino, 2006).

The first symptoms of the disease are usually small black/brown lesions, first on the leaves and later on the stems. The lesions appear water-soaked and may bare white mycelium, and then quickly enlarge and become necrotic. Infected leaves fall off and infected stems become weak and eventually break down, causing the death of the plant (Henfling, 1987). A temperatures below 15ºC with humid and wet conditions, *P. infestans* produces numerous mobile zoospores on infected leaf surface, which can lead to the destruction of an entire field within an about 21 days (Fry et al., 1993).

The systemic fungicide metalaxyl was a main chemical control for late blight prior to the 1980's. As a systemic fungicide, metalaxyl was absorbed into plant leaves and stems, eliminating the need for repeated applications and rendering tubers less likely to be infected also. However, new genotypes of *P. infestans* flourished in the US during the 1980's; these were a new mating type A2 resistant to metalaxyl (Lacy and Hammerschmidt, 1995).

Until the 1980's, only the A1 mating type of *P. infestans* has reported outside of Mexico. However, in 1984 the A2 mating type of *P. infestans* was identified in Europe in Switzerland. It then spread around the world. This spread coincided with an increase in trade among Mexico, Europe, America and other countries (Fry, 2008), and it is likely that the new strains of *P. infestans* were spread by exporting infected tubers from Mexico to different countries (Fry and Goodwin, 1997). When A1 and A2 mating types grow together, they produce oospores from sexual recombination, leading to new strains of *P. infestans* (Henfling, 1987). In addition, oospores, unlike zoospores, can survive in soil from one season to the next in the absence of a host. (Singh et al., 2004) reported that oospores survived in the summer when temperature reached up to 44ºC. Oospores also overwinter in infected tubers, volunteer potatoes, and cull piles (Andrivon, 1995; Lacy and Hammerschmidt, 1995).

Phytophthora infestans causes significant annual losses around the world, and reaching epidemic proportions. Globally, the current yield loss caused by late blight is a much as 6.7 billion US dollars per year. Management practices used to control late blight, include the use of healthy, disease-free seeds produced by tissue culture, aeroponic and hydroponic seed multiplication systems, elimination of volunteer potatoes, removal of cull piles, planting resistant cultivars, and application of contact fungicides (Zwankhuizen et al., 1998). Also is very

important prior to harvesting to check if all vines are completely dead. During the harvest avoid collecting wet tubers and minimize skinning, cuts and shatter bruise. Proper storage can also reduce the risk of spreading the infected tubers. Monitoring, sorting and removing decayed tubers are good practices in the storage. To be effective contact fungicides must be applied before infection, and applications repeated at regular intervals as the plants grow. During a typical growing season in the developed countries, 10-15 fungicide applications are required which is costly and also could be harmful for the environment and humans (Jones et al., 2014). In developing countries, the control of late blights via fungicides can be financially prohibitive. Reductions in the cost of crop protection and decreased the use of fungicides can be achieved by growing resistant varieties.

Breeding for late blight resistance

After the historic loss of potatoes due to late blight in Ireland, many potato breeders started screening for late blight resistance. However, intensive efforts to develop late blight resistant cultivars began in the early 1990's after the dispersal of the new A2 genotypes of *P. infestans* (Colon et al., 1995). The first identification of a late blight resistance source occurred at the Edinburgh Botanical Garden, Scotland, UK in 1910. Where the entire potato collection was affected by late blight except a Mexican accession known as *Solanum demissum* (Brown, 1993). *Solanum demissum* was then introduced to cultivated potato by interspecific crosses and backcrosses with selection for late blight resistance (Haverkort et al., 2008). Eleven resistance (R) genes were characterized from *S. demissum* and mapped to a many chromosomes (El-Kharbotly et al., 1994; Leonards-Shippers et al., 1992; Li et al., 1998; Malcolmson and Black, 1966). The 11 R genes are pathogen race-specific, effective against a limited number of *P. infestans* genotypes. The resistance genes also stimulate the hypersensitive reaction (HR) instead

of preventing the pressure of the pathogen. Over time, all of these genes were overcome by *P.infestans* genotypes (Jo et al., 2011). Recently, more than 20 R-genes were found and mapped for foliage resistance to late blight in other wild species, including the following: the *Rpi-bst1* from *S. brachistotrichum*; *Rpi-edn1*.1from *S. edinense*; *Rpi-mcd1* from *S. microdontum*; *Rpi-snk1.1* and *Rpi-snk1.2* from *S. schenckii*; *Rpi-ver1* from *S. verrucosum*; *Rpi-pnt1* from *S. pinnatisectum*; *Rpi-sto1* and *Rpi-sto2* from *S. stoloniferum*; *Rpi-pta1* from *S. papita*; *Rpi-plt1* from *S. polytrichon*; *Rpi-mcq1* from *S. mochiquense*; *Rpi-phu1* from *S. phureja*, *RB/Rpi- blb1*, *Rpi-blb2*, *Rpi-blb3*, *Rpi-bt1* and *Rpi-abpt* from *S. bulbocastanum* and et al, and are promising to be useful for resistance against late blight (Ballvora et al., 2002; Lokossou et al., 2009; Huang et al., 2005; Paal et al., 2004; Bendahmane et al., 1999; Tiwari et al., 2013; Song et al., 2003; van der Vossen et al., 2003a; Park et al., 2005). The *S. bulbocastanum*-derived R gene was of most interest because this *Solanum* species was native to the central and southern part of Mexico, the center of diversity center for *P. infestans* (Lokossou et al., 2010). It is likely that *S. bulbocastanum* and *P. infestans* have a common history, which could explain why *S. bulbocastanum* has broader and stronger resistance then other species. *S. bulbocastanum* is sexually incompatible with cultivated potato due to the different endosperm balance number (EBN), therefore ploidy manipulations and somatic fusion were used to introduce late blight resistance into *S. tuberosum* from *S. bulbocastanum* (Helgeson et al., 1998). However, because these methods for introducing resistance from *S. bulbocastanum* were difficult, breeders are now using gene transfer methods to accomplish this task (Naess et al., 2000).

Three late blight resistance genes have been found from *S. bulbocastanum*: RB (also known as *Rpi-blb1*) (van der Vossen et al., 2003a), *Rpi-blb2* (van der Vossen et al., 2003a), and *Rpi-blb3* (Park et al., 2005). *Rpi-blb1* was mapped on chromosome 8 (van der Vossen et al.,

2003b). These genes confer broad resistance and differ from the *S. demissum* resistance phenotype, showing long-term durability, and delayed development and spread of the pathogen. The resistance phenotype of *S. demissum* usually produces necrotic lesions, indicating a hypersensitive response, whereas the RB phenotype does not (Halterman et al., 2008). The RB gene belongs to the largest class of R genes, which encode cytoplasmic proteins with coiled-coil, nucleotide binding sites and leucine-rich repeats (CC-NB-LRR) (Song et al. 2003; van der Vossen et al. 2003). A long-range polymerase chain reaction (PCR) product including the gene-coding region of RB, as well as approximately 5 kb of upstream regulatory sequences, was stably integrated into *S. tuberosum* using *Agrobacterium tumefaciens*-mediated transformation (Halterman et al., 2008). According to Song et al., (2003) and Kuhl et al., (2007), the insertion of the RB gene into susceptible cultivars such Katahdin and MSE149-5Y resulted in high resistance to at least three genotypes of *P. infestans.* (Halterman et al., 2008) showed that the RB gene-transformed Dark Red Norland, Katahdin, Russet Burbank and Superior were resistant to the US-8 genotype of *P. infestans.* Knowing that the RB gene enhances late blight resistance in susceptible cultivars encourages the gene stacking or pyramiding approach for the development of late blight resistant potato cultivars.

Genetically Modified Potato

Genetically modified (GM) crops were first introduced in 1994 (Kramer and Redenbaugh, 1994) and currently several GM crops are commercially grown around the world (Clive, 2013). The most widely grown GM crops worldwide are corn, canola, soybean, cotton and sugarbeet containing transgenes for disease, insect and herbicide resistance. In 2012, GM crops were planted on 175 million ha and in 28 countries (Clive, 2013). An increasing proportion of the GM crops are planted in developing countries. The advantages conferred by GM crops are

herbicide-tolerant and pest-resistant plants, improved yield and reduced environmental impacts. One study in India showed that *Bacillus thuringiensis* (Bt) cotton gave a yield boost of up to 60% and decreased use of insecticide to a minimum (Bennett et al., 2004). It is important to note that before introducing Bt cotton to India, farmers were using large amounts of pesticides to protect their crops (Qaim and Zilberman, 2003). Worldwide, GMO production since 1996 produced a reduction of 18.3% in the environmental impacts and a reduction of 8.9% in pesticide usage (Brookes and Barfoot, 2013).

During the early 1990's, a GM potato variety called NewLeaf was released by Monsanto and grown in North America (Thornton, 2003). The NewLeaf variety contained a *Btcry3A* gene conferring resistance to the Colorado potato beetle (Alyokhin et al., 2008). This variety was commercially grown to reduce insecticide use, but the popularity was short-lived. After several years of growing these GM potatoes, public concern regarding the safety of GM foods for human consumption and potentially undesirable environmental impacts lead to the removal of GM potatoes from commercial production (Zhu et al., 2012a).

The most common concern of consumers about GM crops is that the gene inserted into the crop (transgene) is from a different organism, such as bacteria, viruses, animal and non-crossable crops. To address these concerns, the plant biotechnology world developed new types of genetic modification referred to as intragenics and cisgenics. A transgenic plant is one that is transformed with a foreign gene that can come from any source such as bacteria, virus, insects etc. Intragenic plants are transformed with a gene from the same plant genus, but the promoter and/or terminator can be from a different organism. The plant, which has a modified promoter and/or terminator, could result in variability for gene expression. A cisgenic plant is one that has

been transformed with a gene containing a native promoter and terminator in a sense orientation, that has been isolated from the same plant species (Holme et al., 2013). For this definition, “same plant species” refers to any sexually compatible species that can be used in traditional breeding. However, in contrast to traditional breeding, cisgenic plants contain only the gene of interest and avoid linkage drag (Schouten et al., 2006). The RB gene falls into the cisgenic group, where gene, promoter and terminator come from the wild potato species *Solanum bulbocastanum*. This type of transformation event may improve public approval of GMO crops and bypass the need for the same safety regulations required for transgenic and intragenic crops.

Previous studies with the RB gene demonstrated that late blight-susceptible lines containing the RB gene were resistant to late blight under optimal conditions for disease. Based on this information, we transformed three late blight resistant potato cultivars with the RB gene to determine if stacking of late blight resistance mechanisms increased and broadened the resistance in the resistant lines. This thesis reports on the development of the RB-potato lines, and lab, greenhouse and field bioassays conducted to characterize the combined late blight resistance genes for foliar resistance to four *P. infe*stans genotypes.

2. FOLIAR SCREENING FOR RESISTANCE TO *PHYTOPHTHORA INFESTANS* USING FOUR GENOTYPES OF THE PATHOGEN

Introduction

Late blight of potato, one of the most economically important diseases in the US and worldwide, is caused by the oomycete *Phytophthora infestans* (Mont.) de Bary (de Bary, 1876). This devastating disease caused the Irish potato famine, hundreds of thousands of deaths, and mass migration of people out of Ireland in the 1840s (Large, 1940). The initial symptoms of the disease are black/brown lesions on leaves and stems, which might be small at first but then will rapidly grow and become water-soaked and necrotic (Henfling, 1987). Under favorable conditions, *P.infestans* produces sporangia and sporangiophores on the surfaces of infected plant leaves and stems. The sporulation results in a white growth on the underside of the leaves containing a large amount of spores. The spores are dispersed by wind or water. The pathogen can destroy extensive areas field in a few days without chemical intervention. The spores can also reach the soil and infect tubers (Cooke et al., 2011). The primary sources of inoculum are generally infected tubers (seeds), volunteer potatoes from the previous growing season, and cull piles of tubers from infected fields. The management strategies against late blight involve protectant fungicides, resistant varieties, crop rotation, disease-free seeds, and removal of infected volunteer plants and cull piles (Kirk et al., 2004; Kirk, 2003).

Prior to 1991, late blight was controlled by the systematic fungicide metalaxyl. But in 1991, after the introduction of the new metalaxyl resistant A2 mating type, the systemic fungicide failed (Goodwin et al., 1998). *Phytophthora infestans* has two mating types, A1 and A2 (Kirk et al., 2004). Before the 1990s, only the A1 mating type was present throughout the world, with the exception of Mexico, both A1 and A2 mating types were present (Lozoya-

Saldana et al., 2006). In 1991, the A2 mating type was reported in North America, Europe and the Middle East (Deahl et al., 1991). The presence of both mating types allows for sexual reproduction resulting in oospores and the production of new genotypes of the pathogen and increased genetic diversity (Kuhl et al., 2001). Oospores can also survive in extreme temperatures and can overwinter in infected tubers (Kirk et al., 2004). According to Hu et al. (2012) and Fry et al. (2012) there were seven genotypes of *P. infestans* in the US: US-8, US-11, US-20, US- 21, US-22, US-23 and US-24. US-8, US-20, US-21 and US-22 are A2 mating type and US-8, US-20 and US-21 are resistant to metalaxyl, whereas US-22 is sensitive to metalaxyl. US-11, US-23 and US-24 are A1 mating type and US-11 are resistant and US-23 and US-24 are sensitive to metalaxyl, respectively (Hu et al., 2012; Gevens and Seidl, 2012b, a). Although contact fungicides control late blight if applied correctly, the most cost-effective way to control the disease is to grow potato cultivars with broad-spectrum resistance conferred by a multiple R genes.

Breeding for late blight resistance and other diseases and insect resistance are major goals of many potato breeding programs. Wild *Solanum* germplasm has a diverse R-gene pool that can be accessed by breeding programs to develop lines that have late blight resistance. The initial R-genes from wild species were from *S. demissum*. These R-genes have been introduced to different potato cultivars to confer late blight resistance. However, R-genes from *S. demissum* failed to provide durable resistance because the new genotypes of *P. infestans* quickly overcame lines/cultivars that contained one or more R-genes (van der Vossen et al., 2003a). Since then, several novel resistance genes were discovered and have been mapped in different chromosomes of potato from other wild potato species (Ballvora et al., 2002; Lokossou et al., 2009; Huang et al., 2005; Bendahmane et al., 1999; Paal et al., 2004; van der Vossen et al., 2003a; van der

Vossen et al., 2005; Song et al., 2003). Among the resistance sources from wild species, *S.bulbocastanum* was considered to be a highly and effective resistant source against all known genotypes of *P. infestans*.

The late blight resistance gene, RB, was isolated from the wild potato species *Solanum bulbocastanum* and belongs to the largest class of R-genes that encode proteins with a nucleotide binding site and leucine-rich repeats (van der Vossen et al., 2003a; Song et al., 2003). A long-range PCR product including the promoter gene coding region of RB, as well as approximately 5 kb of upstream regulatory sequences, was stably integrated into *S. tuberosum* using *Agrobacterium*-mediated transformation (Kuhl et al., 2007; Halterman et al., 2008).

There are three main research objectives of this study. First, is to create transgenic lines using three late blight resistant advanced breeding lines from the MSU Potato Breeding and Genetics Program. Then, characterize RB presence and expression in the generated lines. Lastly, using three different methods to screen the RB-lines and their parent lines using four major (US-8, US-22, US-23 and US-24) North American genotypes of *P. infestans*.

Materials and Methods

Plant transformation and Plant Materials

Four advanced breeding lines from the MSU Potato Breeding and Genetics Program were transformed with the RB gene for this experiment. These breeding lines included, MSE149-5Y, a susceptible control with no known R genes (Kuhl et al., 2007), and MSM171-A, MSI152-A and MSR061-01 which have different sources of foliar late blight resistance as described in Table 1.

Putative RB-transformants were named using the MSU protocols, i.e., the name of the parental line (M171, E149, R061 etc.) followed by the construct number (.69 or .82) and a consecutive line number (ex. M171.69.01; R061.69.01 etc.).

Table 1. R genes presenting three late blight resistant parental lines (MSI152-A, MSM171-A and MSR061-01) that were transformed with the RB gene from *Solanum bulbocastanum.* These three parental lines have different sources of late blight resistance. *R1, R2, Rphi-blb3, 2, R3a, R3b/R10, R8, R9* and *chc1* are different late blight resistant genes. MSR061-01 did not have any R genes in this category of genes, but there are additional R genes that were not characterized here.

		Late Blight resistance genes							
Line	**RB Source**	***R1***	***R2***	***Rpi-blb3 2***	***R3a***	***R3b/R10***	***R8***	***R9***	***chc1***
MSI152-A	B0718	1	1		0	0	0		
MSM171-A	Stirling	0	0	0	0	1	0	0	0
MSR061-01	NY121	0	0		0	0	0		

Agrobacterium tumefaciens-mediated transformation was used to generate transgenic RB-potato lines as described by (Douches et al., 1998). For transformation experiments, the internodes of virus-free, tissue culture plantlets were cut into small pieces and transformed with the pSPUD69 construct (Figure 1), which contains the full length (8.6 bp) RB gene, *nptII* (neomycin phosphotranferase) gene for kanamycin resistance, and the native promoter (Kuhl et al., 2007) and with the pSPUD82 construct, which is a shortened version (5.3 bp) of the pSPUD69 (Figure 2). When shoots from callus were 5-7mm, they were cut and put into selection medium (Murashige and Skoog with 50 mg L^{-1} of kanamycin). Any shoots that rooted in the selection medium were tested via PCR and RT-PCR analysis to confirm the presence of the RB gene and its transcription according to (Kuhl et al., 2007). A total of 18 RB-lines and four parental lines were evaluated. Parental and RB-lines were grown in Magenta boxes with general

Murashige and Skoog media for 2-3 wk with five plants per box. Plants were then transferred to the greenhouse into the 10.6 cm pots for late blight assays.

Figure 1. T-DNA region of pSPUD69. This construct includes the *nptII* gene for kanamycin resistance (Kan r) controlled by the ubiquitin (*Ubi3*) promoter (Pro) and terminator (Term), late blight resistant RB gene containing native promoter and terminator (8.6 kb) and left (LB) and right (RB) borders.

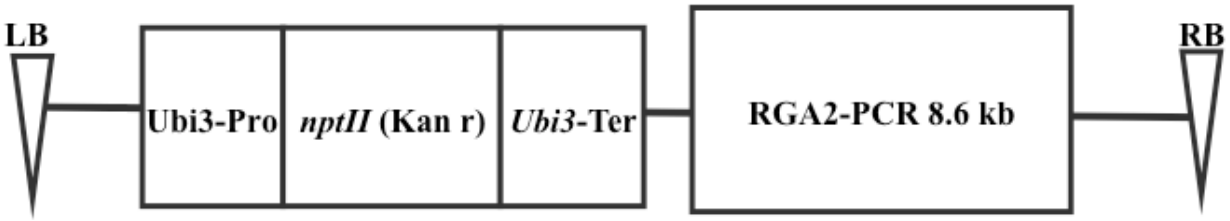
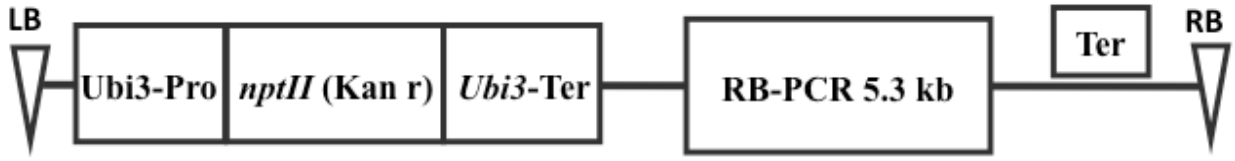


Figure 2. T-DNA region of the pSPUD82 construct. The pSPUD 82 construct is a shortened version of the pSPUD69 construct. This construct includes the *nptII* gene for kanamycin resistance (Kan r) controlled by the ubiquitin (*Ubi3*) promoter (Pro) and terminator (Term), late blight resistant RB gene (5.3 kb), left (LB) and right (RB) borders, and non-native terminator on the 3' end.



Molecular characterization

Twenty-seven putative transgenic events were tested for the presence of RB+*nptII* by PCR and for the expression of the RB gene by RT-PCR. DNA was extracted from 100 mg leaf tissue sampled from 3-4 wk old plants. The DNA extractions were conducted using the Qiagen

Plant Dneasy Kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). The final volume of DNA was 60 µL. The PCR reaction used 10 µL of Go Taq® Master Mix 2x (Promega, Madison, WI, USA), 1 µL of RB reverse and RB forward primers (forward, 5-'CACGAGTGCCCTTTTCTGAC-3'and reverse,

5'-ACAATTGAATTTTTAGACTT-3') (Integrated DNA Technologies, Coralville, IA, USA) specific for the resistance allele (Colton et al., 2006), 6 µL of ddH_2O and 2 µL of genomic DNA. The amplified RB gene product was 213 bp in length. The PCR reaction for the selectable marker *nptII* was the same as above, except *nptII* primers (forward, 5'-CGCAGGTTCTCCGGCCGCTTGGGTGG- 3'and reverse,

5'-AGGAGCCAGTCCCTTCCCGCTTCA-3') were substituted for RB primers, and the resulting *nptII* gene product was a 267-bp fragment. A thermal cycler (PerkinElmer 9600,Wellesley, MA) was programmed for the RB primers for a hot start (95℃, 5 min) and 35 cycles of 95℃ for 45 s denaturing, 50℃ for 45 s annealing, and 72℃ for 45 s extension, with a final extension of 72℃, 5 min. For *nptII*, the thermal cycler was programmed for a hot start (95℃ for 5 min) and 35 cycles of 95℃ for 30 s denaturing, 60℃ for 30 s annealing, and 72℃ for 30 s extension, with a final extension of 72℃ for 5 min.

The RNA was extracted from 100 mg of leaf tissue with the RNeasy Plant Mini Kit according to the manufacturer's guidelines (Qiagen, Valencia, CA, USA). The final volume of RNA was 30 µL. RT-PCR was done using Super Script One-Step RT-PCR with Platinum Taq System according to the manufacturer's directions (Invitrogen, Carlsbad, CA, USA). The RT-PCR reaction was set up using 10µL of 2X Reaction, 1 µL of each primer (RB primers were the same as above), 1 µL of Platinum Taq and 4 µL of ddH_2O. The thermal cycle was programmed

for a 50℃ for 30 m then, 94℃ for 2 m, and 35 cycles of 94℃ for 15 s denaturing, 45℃ for 30 s annealing, and 72℃ for 45 s elongation, with a final extension of 72℃ for 5 min. Fragments from PCR and RT-PCR were separated on 1.0% agarose gels in 1 x Tris-borate EDTA, stained with 10mg/µL ethidium bromide, then visualized and photographed with the Gel Doc-It TS Imaging System (UVP- Upland, CA, USA) under UV light.

Culturing of Phytophthora infestans

Four genotypes of *P. infestans* (US-8, US-22, US-23, and US-24) were grown on rye B medium (components per one L of media: Rye-60g, sucrose-20g, B-sitosterol-0.05g and agar-15g) for 14 d in the dark at 18℃, and exposed to light from 24 h to 2 d to encourage sporulation (Kirk et al., 1999). All *P. infestans* genotypes were obtained from Dr. Kirk (Plant, Soil and Microbial Science Department, Michigan State University). Plates with sporangia and mycelia were harvested by flooding with sterile, distilled water and gently scraping the surface of the culture using a scalpel (Rojas et al., 2014). The solution containing sporangia and mycelia was poured into a beaker, and plates were rinsed into the same beaker to obtain as much mycelium as possible. The suspension was strained through four layers of cheesecloth and the sporangia concentration was measured with a hemacytometer and adjusted to 10^6 total sporangia mL^{-1} with sterile distilled water (Kirk et al., 1999). The sporangial suspension was stored for 4 h at 4℃ to encourage zoospore release. And then was used for inoculation of detached leaves and whole plants in the greenhouse experiments

Detached Leaf Bioassays (DLBs)

Detached-leaf bioassays (DLB) were conducted in the fall of 2012 and 2013 years using four MSE149-5Y-derived RB lines, six MSM171-A-derived RB lines, one MSI152-A-derived RB line, one MSR06-01-derived RB line, one MSG227-2-derived RB line, and one Spunta-derived RB line. Lines were challenged separately with each of the 4 *P. infestans* genotypes (US-8, US-22, US-23 and US-24). Assays were repeated over a 2-year period with three replications in the growth chamber.

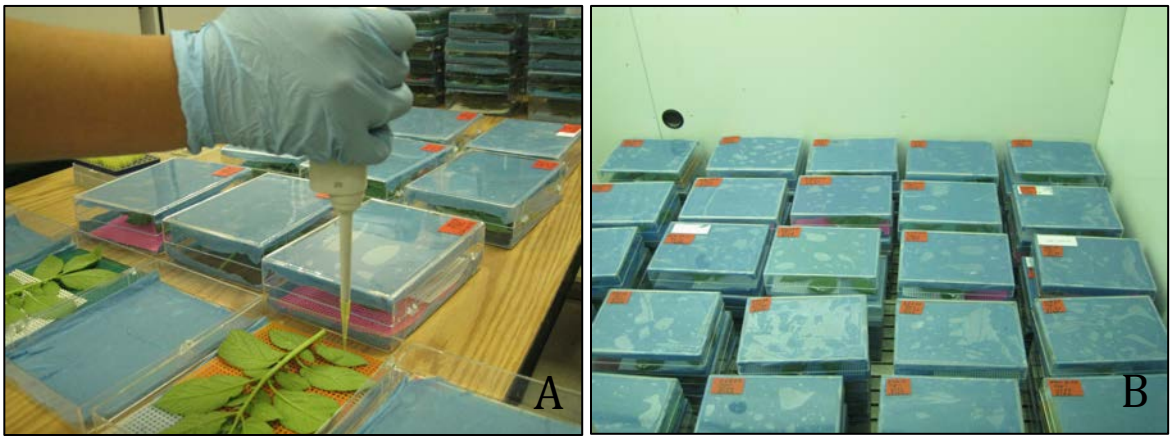
When the tissue culture plantlets were 6.0 to 7.5 cm tall they were transferred to pots (3.8 L) containing potting mixes (Suremix perlite, Michigan Growers Products, Inc. Galesburg, MI, US) in the greenhouse maintained at 16 hr day length. Plants were watered every three days and were fertilized (20-20-20, Proturf and Peters Professional®, Brantford, ON, Canada) weekly. After 5 weeks, then, fourth leaf from the growing point (fully expanded) were cut with scissors, washed with distilled H_20, then dried with Kimwipes®. Six leaves per line were placed in plastic boxes (17.8 cm x 12.7 cm x 4.5 cm, Uline, Pleasant Prairie, WI, USA) lined with a moist paper towel to maintain humidity, and with plastic mesh on the bottom to keep leaves from directly contacting the paper towel. Detached leaves were placed abaxial side up individual boxes and sprayed with 20 µL of a zoospore suspension, while the control leaves were sprayed with 20 µL of ddH_2O. After inoculation all boxes were placed in a growth chamber set at 15°C using a completely randomized design (Figure 3). Samples were kept in the dark for the first 24 h, then switched to a light period of 14 h for 14 days. Once every day the leaves were sprayed with distilled water to maintain humidity. Disease ratings were taken at 7, 9, 11 and 14 days after inoculation (DAI) by estimating the percentage of leaf tissue infected according to the method of

(Forbes and Korva, 1994). The severity of the foliar tissue late blight was expressed as the Relative Area Under the Disease Progression Curve (RAUDPC). The RAUDPC was calculated as:

$$RAUDPC = \frac{\Sigma(T_{i+1} - T_i) * (\frac{D_{i+1} + D_{i)}}{2}}{T_{Total} * 100}$$

where T_i was the i^{th} day after emergence when an estimation of percent foliar late blight was made, D_i was the estimated percentage of area with blighted foliage at T_i and T_{Total} was the number of days after emergence at which the final foliar assessment was recorded (Muhinyuza et al., 2008).

Figure 3. Detached leaf bioassay experiment in the growth chamber: A) inoculating the leaves with zoospore suspension of *Phytophthora infestans* B) inoculated leaves inside of the plastic boxes in the control growth chamber using completely randomized design.



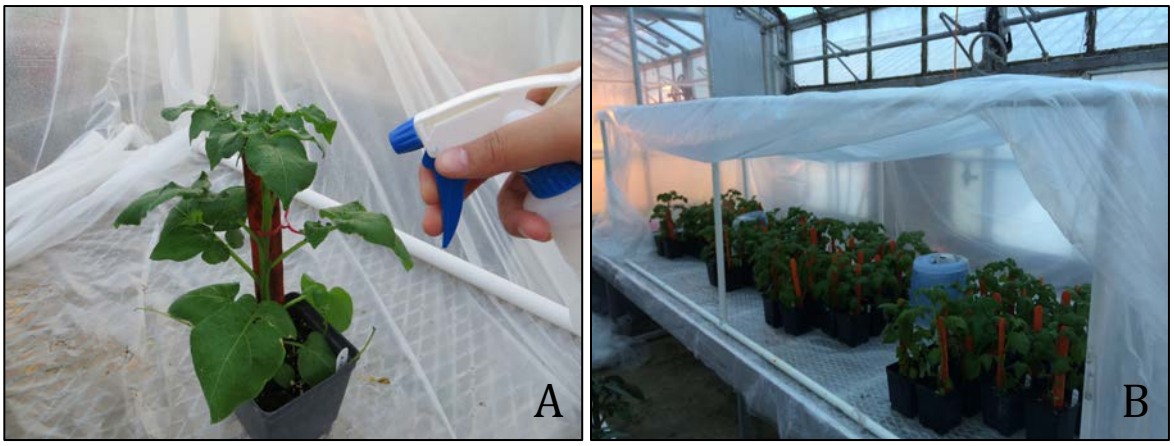
Whole-Plant Bioassays (WPBs)

Whole-plant bioassays (WPB) were conducted using the same lines and *P. infestans* genotypes tested in the DLBs. Similarly as in the DLB experiment, potato lines were challenged separately with each of the 4 *P. infestans* genotypes (US-8, US-22, US-23 and US-24).

WPBs were conducted in a tent (3 x 1 x 0.9 m) set on greenhouse bench. Six m clear plastic sheets enclosed the tent from the top to the sides and 3m to enclose the bottom.

Plants for WPBs were grown in the similar standard conditions as DLBs in the greenhouse. After reaching 5 weeks growth, four plants per line were misted with distilled water, inoculated with the zoospore suspension using a hand-held sprayer, and then placed in the tent (Figure 4). Plants in the pots were placed in a randomized complete block design (RCBD) with four replications. The first 24 h plants were kept in the dark then switched to a light period of 16 h for 18 days. Humidifiers (Holmes HM2610 and Sunbeam SUL2512, Boca Raton, FL, USA) were used to maintain high humidity inside of the tent continuously until the end of the experiment. Twice a day for the duration of the experiment, plants were hand-misted with distilled water and humidifiers were refilled with water. Data were taken on 7, 9, 11, 14, 16 and 18 DAI days after inoculation (DAI) by estimating the percentage of leaf tissue infected according to the method of (Forbes and Korva, 1994). The severity of the foliar tissue late blight was expressed as the Relative Area Under the Disease Progression Curve (RAUDPC), and RAUDPC was calculated, as described above for the DLB study.

Figure 4.Whole plant bioassay in the greenhouse: A) inoculating the plants with a hand-held sprayer with zoospore suspension of *Phytopthtora infestans*, B) whole plants in the greenhouse inoculation tent. The front plastic sheet has been lifted to reveal the plants and the humidifiers.



Field trial

A field trial was conducted in 2013 at the Michigan State University Clarksville Research Center (CRC), Clarksville, MI to determine the susceptibility of cultivars and lines under more natural field conditions. Minitubers of the eighteen lines were produced from tissue culture plants in the greenhouse from October to March 2012-2013 and were used as seed tubers for the field disease assessment. Five minitubers per plot were planted by hand June 4, 2013 into 1.5 m long plots in two-row blocks as 86.3 cm row spacing separated by two fallow rows. The experimental design for this experiment was a RCBD with three replications. A late blight susceptible line (Atlantic) planted around the perimeter and between blocks to separate treatments and enhance the spread of *P. infestans* inoculum. The trial was inoculated with a

zoospore suspension (prepared as described above) of the US-22 genotype of *P. infestans* on July 31, 2013. No fungicides were applied to the field during the season. The field was sprinkler irrigated as needed. Disease was rated visually based on percentage of foliar area infected by late blight. Ratings were done from August 8, 2013 (8 DAI) to September 9, 2013 (40 DAI) at intervals between 3 and 7 days, based on the rate of disease progression. The data was used to calculate RAUDPC as noted in the DLB and WPB assays.

Statistical analyses

Data were analyzed using analysis of variance (ANOVA). For the DLB and WPB assays, there were significant differences between years, so data from each year were analyzed separately. Pairwise correlation tests were done for each *P. infestans* genotype between years for DLBs and WPBs (DLB 2012 vs DLB 2013; WBP 2012/13 vs WPB 2013/14) and between the different greenhouse methods in all possible combinations. For the pairwise comparison of the field, DLB and WPB experiments, only data from the US-22 genotype was used. All analyses were done using the JMP v. 9 statistical software (SAS Institute, Cary, NC, USA).

Results

Molecular evaluations

After the transformation experiments, 29 lines were regenerated with nine from the susceptible MSE149-5Y line, seven from the late blight resistant (LBR) MSM171-A line, five from the LBR line MSI152-A, two from a third LBR line MSR061-01 and two from the late blight susceptible lines MSG227-2 and Spunta. Of the 29 regenerated lines, 14 were kanamycin

resistant, and 13 of those were PCR positive for both the RB and *nptII* genes. However, the RB transcript was detected in only 10 lines (Table 2).

Table 2. Summary of the four parental lines and 29 regenerated RB-lines tested for rooting in kanamycin selection media, presence/absence of the RB[a] and *npt*II[b] genes via Polymerase Chain Reaction (PCR) and presence/absence of RB transcripts via Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). The order of the lines listed below is the first the parent line followed by its RB-transformed lines.

Lines	Kanamycin (25ppm) rooting	RB (PCR)	*nptII* (PCR)	RB (RT-PCR)
MSE149-5Y[c]	-	-	-	-
E149.69.03[d]	+	+	+	+
E149.69.06[d]	+	+	+	+
E149.69.11[d]	+	+	+	-
E149.82.01[e]	+	+	+	-
E149.89.02[e]	-	-	-	-
E149.89.03[e]	-	-	-	-
E149.89.04[e]	-	-	-	-
E149.82.05[e]	-	-	-	-
E149.82.06[e]	-	-	-	-
MSM171-A[c]	-	-	-	-
M171.A-11[f]	-	-	-	-
M171.69.06[d]	+	+	+	-
M171.69.14[d]	+	+	+	+
M171.69.19[d]	+	+	+	+
M171.69.21[d]	-	-	-	-
M171.82.01[e]	+	+	+	+
M171.82.02[e]	+	+	+	+
MSI152-A[c]	-	-	-	-
I152.69.01[d]	+	+	+	+
I152-A.04 [d]	-	-	-	-
I152.69.02[d]	+	-	-	-
I152.69.03[d]	-	-	-	-
I152.69.04[d]	-	-	-	-
MSR061-01[c]	-	-	-	-
R061.69.01[d]	+	+	+	+
R061-01.02 [f]	-	-	-	-
CG227C4.5[d]	+	+	+	+
CSPAG.13[d]	+	+	+	+

[a] RB gene is confer resistance to late blight
[b] nptII gene neomycin phosphotranferase gene is used in selection of transformed organisms
[c] Parental lines
[d] pSUD69 construct lines
[e] pSUD82 construct lines
[f] Plasmid check lines (no RB gene)

Foliar late blight development in detached leaf bioassays

Detached leaf bioassay using four different genotypes of *Phytophthora infestans* showed significant differences between two years, two main factors (line and genotype) and the two-way interactions (Table 3).

2012 Detached leaf bioassay

In 2012, four parental and 10 RB-lines were used for detached leaf bioassays to measure the foliar response to inoculation with different genotypes of *P. infestans*. Four RB-lines were missing in this study because they did not grow at the same rate as the other RB lines in the greenhouse.

Analysis across P. infestans genotypes

The analysis across four *P. infestans* genotypes (Table 4) showed that the three late blight resistant (LBR) parental lines had lower RAUDPC values than the late blight susceptible (LBS) line (MSE149-5Y), but only MSR061-01 (RAUDPC=22.9) was significantly different from MSE149-5Y (RAUDPC=36.6). The highest mean RAUDPC was for E149.69.11 (38.9) and none of the MSE149-5Y derived RB-lines were statistically different from their parental line. Only one of the MSM171-A derived RB lines (M171.69.19, RAUDPC=12.8) had an RAUDPC that was significantly different from the parental line (MSM171-A, RAUDPC=30.0) The other four RB-lines derived from MSM171-A (M171.69.06, M171.69.14, M171.A-11 and M171.82.01) had RAUDPC values ranging from 19.9 to 29.1. RB-derived lines from LBR parental lines MSI152-A and MSR061-1 were similar to the parental lines.

Table 3. Summary of the analysis of variance of the main effects of parental and RB-transformed potato lines inoculated with US-8, US-22, US-23 and US-24 genotypes of *Phytophthora infestans* in a detached leaf bioassay conducted in 2012 and 2013.

Source	P-Value					
Year	<.0002					
		2012			**2013**	
	F [a]	df	P-Value	F	df	P-Value
Line	7.7	13	<.0001	19.3	16	<.0001
P.i [b] Genotype	174.8	3	<.0001	40.9	3	<.0001
Line * *P.i* Genotype	3.7	39	<.0001	5.2	48	<.0001

[a] F ratio=the model mean square divided by the error mean square and Prob < F lists the p-value for the test. P-values of 0.05 or less were considered evidence that there was at least one significant effect in the model.
[b] *P.i -Phytophthora infestans*

Table 4. Foliar late blight progression [measured as the Relative Area Under Disease Progress Curve (RAUDPC)] and *Phytophthora infestans* aggressiveness in two years of detached leaf bioassays. Parental potato lines and RB- transformed potato lines were inoculated with the US-8, US-22, US-23 and US-24 genotypes of *P. infestans.*

Year	Potato lines	Mean RAUDPC (%) [a]		Aggressiveness of *Phytophthora infestans*	
				Genotypes	Mean RAUDPC (%) [a]
2012	MSE149-5Y (SP) [b]	36.6	ab [c]	US-8	6.9 c
	E149.69.03 (T) [d]	30.8	abc	US-22	43.3 a
	E149.69.06 (T) [d]	30.1	abc	US-23	16.6 b
	E149.69.11 (T) [d]	38.9	a	US-24	39.1 a
	MSM171-A (RP) [g]	30.0	abc		
	M171.A-11 (C) [h]	20.1	cd		
	M171.69.06 (T) [d]	22.7	cd		
	M171.69.14 (T) [d]	19.9	cd		
	M171.69.19 (T) [d]	12.8	d		
	M171.82.01 (T) [d]	29.1	abc		
	MSI152-A (RP) [g]	27.0	abc		
	I152.69.01 (T) [d]	23.5	cd		
	MSR061-01 (RP) [g]	22.9	cd		
	R061.69.01 (T) [d]	26.5	bc		
2013	MSE149-5Y (SP) [b]	35.0	a [c]	US-8	24.4 ab
	E149.69.03 (T) [d]	31.2	ab	US-22	9.0 c
	E149.69.06 (T) [d]	21.1	bcd	US-23	25.7 a
	E149.69.11 (T) [d]	37.4	a	US-24	21.2 b
	E149.82.01 (T) [d]	38.1	a		
	CG227C4.5 (T) [e]	4.8	f		
	CSPAG.13 (T) [f]	14.7	def		
	MSM171-A (RP) [g]	6.0	ef		
	M171.A-11 (C) [h]	13.8	def		
	M171.69.06 (T) [d]	15.0	def		
	M171.69.14 (T) [d]	14.9	def		
	M171.69.19 (T) [d]	4.9	f		
	M171.82.01 (T) [d]	18.2	cde		
	MSI152-A (RP) [d]	21.3	bcd		
	I152.69.01 (T) [g]	16.0	def		
	MSR061-01 (RP) [g]	29.3	abc		
	R061.69.01 (T) [d]	19.8	bcd		

Table 4 (cont'd)

[a] Resistance/susceptibility score was expressed as RAUDPC (%) = [AUDPC/ last day reading-First day reading)]*100 ; RAUDPC has a minimum value of zero (no infection) and maximum value of 100 (completely infected tissue)
[b] (SP) Susceptible parental line
[c] Values followed by the same letter are not significantly different at $p = 0.05$ for comparisons of mean RAUDPC values within a) potato lines and b) different *Phytophthora infestans* genotypes using Tukey honestly significant different (HSD) test
[d] (T) Transformed with RB gene lines
[e] RB line from MSG227-2
[f] RB line from Spunta
[g] (RP) Resistant parental line
[h] Plasmid control check line (no RB gene)

Based on mean RAUDPC across all potato genotypes, the US-22 and US-24 *P. infestans* genotypes were more aggressive (RAUDPC=43.3, 39.1, respectively) than US-23 (16.6) and US-8 (6.9) (Table 4). US-23 and US-8 were significantly different from each other and from US-22 and US-24.

Analysis for individual P. infestans genotypes

Analysis of each genotype of *P. infestans* against each potato lines revealed differences in the response based on *P. infestans* genotypes (Table 5). The US-8 genotype was the least aggressive and the RAUDPCs ranged from 0.0 to 39.0. None of the LBR parental lines were significantly different from the LBS line (MSE149-5Y) and none of the RB lines were significantly different from their parental line. The US-22 and US-24 *P. infestans* genotypes were more aggressive and all lines had high RAUDPC values except for M171.69.19 and M171.69.19 (RAUDPC=19.3 and 2.1 for US-22 and US-24, respectively) was significantly different from MSM171-A (RAUDPC=49.3 and 39.7, for US-22 and US-24, respectively) for both *P. infestans* genotypes. The highest RAUDPC value for US-22 was on MSE149-5Y (50.7) and in US-24 RB-line I152.69.01 (52.4). As with US-8, none of the RB lines were significantly different from their parental line with the exception of M171.69.19 noted above. For US-23, none of the lines were significantly different in comparison to their parental lines.

Table 5. Foliar late blight progression in detached leaf bioassays [measured as the Relative Area Under Disease Progress Curve (RAUDPC)] on parental potato lines and lines transformed with a RB gene. Leaves were inoculated with US-8, US-22, US-23 and US-24 genotypes of *Phytophthora infestans* in 2012 and 2013

		Severity of *Phytophthora infestans* (RAUDPC [a])							
Year	Potato lines	US-8		US-22		US-23		US-24	
2012	MSE149-5Y (SP) [b]	18.9	d-l [c]	50.7	ab	26.1	a-l	50.5	ab
	E149.69.03 (T) [d]	8.2	i-l	48.3	a-c	19.1	d-l	47.6	a-d
	E149.69.06 (T) [d]	17.0	e-l	43.5	a-f	13.4	g-l	46.4	a-d
	E149.69.11 (T) [d]	24.4	a-l	48.6	ab	31.1	a-j	51.5	ab
	MSM171-A (RP) [g]	19.4	c-l	49.3	ab	11.6	h-l	39.7	a-h
	M171.A-11(C) [h]	0.1	l	39.3	a-h	14.7	f-l	26.5	a-l
	M171.69.06 (T) [d]	0.0	l	40.1	a-h	22.9	a-l	27.8	a-l
	M171.69.14 (T) [d]	0.3	l	38.9	a-h	8.3	i-l	31.9	a-i
	M171.69.19 (T) [d]	0.1	l	19.3	c-l	29.9	a-k	2.1	j-l
	M171.82.01 (T) [d]	1.0	kl	45.0	a-e	28.0	a-l	42.5	a-g
	MSI152-A (RP) [g]	4.1	i-l	49.1	ab	3.4	i-l	51.4	ab
	I152.69.01 (T) [d]	1.1	kl	39.8	a-h	0.6	l	52.4	ab
	MSR061-01 (RP) [g]	1.8	kl	49.6	ab	16.2	e-l	23.8	b-l
	R061.69.01 (T) [d]	0.7	kl	44.7	a-e	7.0	i-l	53.6	a
2013	MSE149-5Y (SP) [b]	42.0	a-g [c]	11.2	i-o	43.0	a-g	43.6	a-f
	E149.69.03 (T) [d]	45.7	a-c	22.1	a-o	21.9	a-o	35.0	a-k
	E149.69.06 (T) [d]	3.2	no	4.0	no	40.5	a-i	36.7	a-j
	E149.69.11 (T) [d]	45.3	a-d	10.6	j-o	49.7	a	44.0	a-e
	E149.82.01 (T) [d]	47.5	ab	16.9	c-o	45.6	a-d	42.4	a-g
	CG227C4.5 (T) [e]	5.9	k-o	3.5	o	2.8	n-o	10.7	j-o
	CSPAG.13 (T) [f]	13.8	g-o	0.9	o	18.7	b-o	25.2	a-o
	MSM171-A (RP) [g]	0.8	k-o	1.1	no	11.1	j-o	5.0	m-o
	M171.A-11 (C) [h]	21.9	a-o	7.0	k-o	16.3	d-o	9.9	j-o
	M171.69.06 (T) [d]	30.4	a-n	1.0	o	28.7	a-o	1.1	o
	M171.69.14 (T) [d]	37.9	a-j	12.1	h-o	1.2	n-o	8.6	j-o
	M171.69.19 (T) [d]	15.9	e-o	1.7	n-o	1.5	n-o	0.5	o
	M171.82.01 (T) [d]	17.5	c-o	25.4	a-o	25.0	a-o	4.9	m-o
	MSI152-A (RP) [g]	5.5	l-o	10.6	j-o	41.0	a-h	28.0	a-o
	I152.69.01 (T) [d]	1.8	no	14.5	f-o	28.0	a-o	19.8	b-o
	MSR061-01 (RP) [g]	45.6	a-d	11.2	i-o	26.5	a-o	33.9	a-m
	R061.69.01 (T) [d]	28.9	a-o	3.3	no	34.5	a-l	12.3	h-o

Table 5 (cont'd)

[a] Resistance/susceptibility score was expressed as RAUDPC (%) = [AUDPC/ last day reading-First day reading)]*100 ; RAUDPC has a minimum value of zero (no infection) and maximum value of 100 (completely infected tissue)
[c] (SP) Susceptible parental line
[b] Values followed by the same letter are not significantly different at $p = 0.05$ for comparisons of mean RAUDPC values within a) potato lines and b) different *Phytophthora infestans* genotypes using Tukey honestly significant different (HSD) test
[d] (T) Transformed with RB gene lines
[e] RB line from MSG227-2
[f] RB line from Spunta
[g] (RP) Resistant parental line
[h] Plasmid control check line (no RB gene)

2013 Detached leaf bioassays results

In 2013, four parents and 13 RB-lines were used for detached leaf bioassays to measure the foliar response to inoculation with different genotypes of *P. infestans*. M171.82.02 did not emerge well in tissue culture and was not included in the study.

Analysis across all P. infestans genotypes

For the overall analysis in 2013, the three LBR parental lines had significantly lower RAUDPC values than the LBS line with the exception of MSR061-1 (Table 4). The RAUDPC values ranged from 4.8 (CG227C4.5) to 38.1 (E149.82.01). There were no significant differences between parental lines and their corresponding RB lines with the exception of E149.69.06 (RAUDPC=21.1), which was significantly lower than MSE149-5Y (RAUDPC=35.0). Two RB-lines from susceptible parents (CG227C4.5 and CSPAG.13) also were significantly lower than the LBS line (MSE149-5Y) with RAUDPC values of 4.8 and 14.7, respectively.

Based on mean RAUDPC values across all potato genotypes, the US-8, and US-23 *P. infestans* genotypes were more aggressive and were not statistically different from each other. Although the US-24 genotype was also aggressive, it was significantly less so than the US-8 genotype. The US-22 genotype was the least aggressive (Table 4).

Analysis for individual P. infestans genotypes

For the US-8 genotype, the RAUDPC values ranged from 0.8 (MSM171-A) to 47.5 (E149.82.01) (Table 5). Among the MSE149-5Y-derived RB lines, only one (E149.69.06, RAUDPC=3.2) was significantly lower than the parental line (RAUDPC=42.0). None of the MSM171-A-derived lines were better than the parental line and M171.69.14 had a significantly higher RAUDPC value (37.9) than MSM171-A (RAUDPC=0.8). For MSI152-A and MSR061-

01 the derived lines were not significantly different from the parental line. CG227C4.5, (derived from susceptible parent MSG227-2) had a significantly lower RAUDPC (5.9) than the susceptible parental line (RAUDPC=42.0). For the US-22 genotype the RAUDPC values ranged from 0.9 (CSPAG.13) to 25.4 (M171.82.01) and none of the RB-lines were significantly different from their parents (Table 5). However, E149.69.06, CG227C4.5, CSPAG.13, M171.69.06, M171.69.19 and R061.69.01 lines had low mean RAUDPCs (4.0, 3.5, 0.9, 1.0, 1.7 and 3.3, respectively). For the US-23 genotype the RAUDPC values ranged from 1.2 (M171.69.14) to 49.7 (E149.69.11) and only one of the LBR parents (MSM171-A, RAUDPC=11.1) was significantly different than the LBS parent (Table 5). None of the RB lines were significantly different from their parental lines but two RB lines derived from susceptible parents (CG227C4.5 and CSPAG.13) were significantly better than the LBS parent. The range in RAUDPC values for the US-24 genotype was 0.5 (M171.69.19) to 43.6 (MSE149-5Y) (Table 5). Only one LBR parent (MSM171-A, RAUDPC=5.0) was significantly different from the LBS parent (MSE149-5Y, RAUDPC=43.6) as was the case for the US-23 genotype as well. The RB-line (CG227C4.5) was significantly different compared to MSE149-5Ywith RAUDPCs of 10.7 and 43.6, respectively. The rest of the RB-lines were not significantly different from their parent lines.

Foliar late blight development in whole plant bioassays

Whole plant bioassay using four different genotypes of *Phytophthora infestans* showed significant differences between two years, two main factors (line and genotype) and the two-way interactions (Table 6).

2013 Whole plant bioassays results

In 2013, one parent line (MSR061-01) and seven RB-lines (E149.82.01, CG227C4.5, CSPAG.13, M171.69.14, M171.69.19, M171.A-11, and I152.69.01) were not tested due to poor growth in the greenhouse limiting the experiment to three parental and seven RB-lines.

Analysis across all P. infestans genotypes

The RAUDPC values ranged from 5.2 (R061.69.01) to 17.2 (E149.69.11) with all LBR parental lines significantly different from the LBS parent (MSE149-5Y) (Table 7). Amongst the RB lines derived from MSE149-5Y only one (E149.69.03, RAUDPC=9.7) was significantly different from its parental line. MSM171-A and its RB-lines were not statistically significant from each other, with RAUDPC values ranging from 6.3 to 8.6. MSI152-A had a RAUDPC value of 7.9, but no RB-lines from this parent were tested in 2013. R061.69.01 had RAUDPC of 5.2, but could not be compared to its parental line (MSR061-01), which was not included in the experiment as noted above.

Based on mean RAUDPCs across all potato genotypes, the most aggressive *P. infestans* genotypes were US-8 (14.1), US-24 (12.8), and they were not significantly different from each other (Table 7). The least aggressive genotypes were US-22 (6.5), and US-23 (7.0) and they were not significantly different from each other. However, the US-22 and US-23 were significantly different from US-8 and US-24 (Table 7).

Table 6. Summary of the analysis of variance of the main effects of parental and RB-transformed potato lines inoculated with US-8, US-22, US-23 and US-24 genotypes of *Phytophthora infestans* in a whole plant bioassay conducted in 2013 and 2014.

Source	P-Value					
Year	<.0001					
	2013			**2014**		
	F [a]	df	P-Value	F	df	P-Value
Line	18.7	9	<. 0001	20.3	17	<.0001
P.i [b] Genotype	39.8	3	<. 0001	322.9	3	<.0001
Line * *P.i* Genotype	2.9	27	<. 0001	3.9	51	<.0001

[a] F ratio=the model mean square divided by the error mean square and Prob < F lists the P-value for the test. P-values of 0.05 or less were considered evidence that there was at least one significant effect in the model.

[b] *P.i – Phytophthora infestans*

Table 7. Foliar late blight progression [measured as the Relative Area Under Disease Progress Curve (RAUDPC)] and *Phytophthora infestans* aggressiveness in two years of whole plant bioassays. Parental potato lines and RB- transformed potato lines were inoculated with the US-8, US-22, US-23 and US-24 genotypes of *P. infestans.*

Year	Potato lines	Mean RAUDPC (%) [a]		Aggressiveness of *Phytophthora infestans* Genotypes	Mean of RAUDPC (%) [a]	
2013	MSE149-5Y (SP) [b]	16.7	a [c]	US-8	14.1	a
	E149.69.03 (T) [d]	9.7	bc	US-22	6.5	b
	E149.69.06 (T) [d]	13.3	ab	US-23	7.0	b
	E149.69.11 (T) [d]	17.2	a	US-24	12.8	a
	MSM171-A (RP) [g]	6.7	cd			
	M171.69.06 (T) [d]	6.3	cd			
	M171.82.01 (T) [d]	9.6	bcd			
	M171.82.02 (T) [d]	8.7	cd			
	MSI152-A (RP) [g]	7.9	cd			
	R061.69.01 (T) [d]	5.2	d			
2014	MSE149-5Y (SP) [b]	28.7	a [c]	US-8	4.0	c
	E149.69.03 (T) [d]	14.7	cdef	US-22	32.5	a
	E149.69.06 (T) [d]	22.1	abc	US-23	22.2	b
	E149.69.11 (T) [d]	27.5	ab	US-24	4.0	c
	E149.82.01 (T) [d]	27.7	ab			
	CG227C4.5 (T) [e]	2.7	g			
	CSPAG.13 (T) [f]	19.7	bcd			
	MSM171-A (RP) [g]	13.4	def			
	M171.A-11 (C) [h]	14.9	cdef			
	M171.69.06 (T) [d]	12.8	def			
	M171.69.14 (T) [d]	9.7	fg			
	M171.69.19 (T) [d]	2.3	g			
	M171.82.01 (T) [d]	15.6	cdef			
	M171.82.02 (T) [d]	14.2	cdef			
	MSI152-A (RP) [g]	18.4	cde			
	I152.69.01 (T) [d]	10.2	efg			
	MSR061-01 (RP) [g]	14.8	cdef			
	R061.69.01 (T) [d]	13.3	def			

[a] Resistance/susceptibility score was expressed as RAUDPC (%) = [AUDPC/ last day reading-First day reading)]*100 ; RAUDPC has a minimum value of zero (no infection) and maximum value of 100 (completely infected tissue)
[b] (SP) Susceptible parental line

Table 7 (cont'd)

[c] Values followed by the same letter are not significantly different at $p = 0.05$ for comparisons of mean RAUDPC values within a) potato lines and b) different *Phytophthora infestans* genotypes using Tukey honestly significant different (HSD) test
[d] (T) Transformed with RB gene lines
[e] RB line from MSG227-2
[f] RB line from Spunta
[g] (RP) Resistant parental line
[h] Plasmid control check line (no RB gene)

Analysis for individual P. infestans genotypes

For the US-8 genotype the RAUDPC values ranged from 5.1 (R061.69.01) to 25.0 (MSE149-5Y) (Table 8). Of the two LBR parents in the experiment only MSM171-A (RAUDPC=12.7) was significantly different from the LBS parent (MSE149-5Y). Of the RB lines derived from MSE149-5Y only E149.69.03 (RAUDPC=11.1) was significantly different from the parental line (RAUDPC=25.0). There were no MSM171-A derived lines that were significantly different from the parental line. The RB lines R061.69.01 (5.1) could not be compared with its parental line but it was significantly different from the LBS line MSE149-5Y (25.0). For the US-22 genotype, the RAUDPC ranged from 0.0 (MSI152-A) to 12.0 (E149.69.11) and only one of the two LBR parents (MSI152-A, RAUDPC=0.0) was significantly different from the LBS parent (MSE149-5Y, RAUDPC=11.2) (Table 8). There were no significant differences among any of the RB lines and their respective parental lines and R061.69.01 was not significantly different from the LBS line. For the US-23 genotype, the RAUDPC values ranged from 2.9 (MSI152-A) to 13.2 (E149.69.11) (Table 8). Neither of the LBR parental lines were significantly different from the LBS parental line nor none of the RB lines were significantly different from their respective parental lines. RB line R061.69.01 was not significantly different from the LBS parental line. For the US-24 genotype, RAUDPC values ranged from 4.0 (MSM171-A) to 25.2 (E149.69.11) and both LBR parental lines and R061.69.01 were significantly different from the LBS parental line. However, there were no significant differences among any of the RB lines and their respective parental lines (Table 8).

Table 8. Foliar late blight progression in two years of whole plant bioassays [measured as the Relative Area Under Disease Progress Curve (RAUDPC)] on parental potato lines and lines transformed with the RB gene. Plants were inoculated with US-8, US-22, US-23 and US-24 genotypes of *Phytophthora infestans*.

		Severity of *Phytophthora infestans* (RAUDPC[a])							
Year	Potato Lines	US-8		US-22		US-23		US-24	
2013	MSE149-5Y (SP)[b]	25.0	a[c]	11.2	c-h	8.2	c-i	22.4	ab
	E149.69.03 (T)[d]	11.1	c-i	7.5	d-i	8.7	c-i	11.4	b-h
	E149.69.06 (T) [d]	18.2	a-d	7.0	e-i	11.6	b-h	16.6	a-f
	E149.69.11 (T) [d]	18.6	a-c	12.0	b-g	13.1	b-g	25.2	a
	MSM171-A (RP) [g]	12.7	b-g	5.5	f-i	4.5	g-i	4.0	g-i
	M171.69.06 (T) [d]	10.6	c-i	4.6	g-i	3.8	g-i	6.3	f-i
	M171.82.01 (T) [d]	11.4	b-h	10.8	c-i	5.9	f-i	10.2	c-i
	M171.82.02 (T) [d]	11.3	c-h	6.0	f-i	5.6	f-i	11.8	b-h
	MSI152-A (RP) [g]	17.6	a-e	0.0	i	2.9	g-i	11.0	c-i
	R061.69.01 (T) [d]	5.1	g-i	0.8	hi	5.9	f-i	9.0	c-i
2014	MSE149-5Y (SP)[c]	8.5	n-t	52.3	ab	42.5	a-d	11.5	l-t
	E149.69.03 (T)[d]	6.0	o-t	29.3	c-m	16.0	i-t	7.3	n-t
	E149.69.06 (T) [d]	10.8	m-t	41.0	a-e	28.8	c-m	8.0	n-t
	E149.69.11 (T) [d]	1.3	st	54.0	a	37.8	a-f	17.0	h-t
	E149.82.01 (T) [d]	10.3	m-t	45.3	a-c	41.0	a-e	14.3	j-t
	CG227C4.5 (T) [e]	0.8	st	3.8	p-t	5.8	o-t	0.5	t
	CSPAG.13 (T) [f]	4.0	p-t	45.3	a-c	27.0	c-n	2.5	r-t
	MSM171-A (RP) [g]	2.0	r-t	36.5	a-h	14.3	j-t	1.0	st
	M171.69.06 (T) [d]	2.8	q-t	25.5	c-o	22.5	e-q	0.5	t
	M171.69.14 (T) [d]	6.0	o-t	21.3	e-r	9.8	m-t	1.3	st
	M171.69.19 (T) [d]	0.3	t	5.0	p-t	4.0	p-t	0.0	t
	M171.82.01 (T) [d]	3.3	q-t	33.8	b-j	23.5	d-p	1.8	r-t
	M171.82.02 (T) [d]	1.8	r-t	33.0	b-k	18.5	f-t	3.5	q-t

Table 8 (cont'd)

M171.A-11 (C) [h]	5.0	p-t	33.3	b-k	20.5	f-s	0.8	st
MSI152-A (RP) [g]	1.3	st	34.5	a-i	37.0	a-g	1.0	st
I152.69.01 (T) [d]	0.3	t	27.0	c-n	13.5	k-t	0.0	t
MSR061-01 (RP) [g]	7.0	o-t	33.5	b-j	17.8	g-t	1.0	st
R061.69.01 (T) [d]	0.8	st	31.3	c-l	19.5	f-t	1.5	r-t

[a] Resistance/susceptibility score was expressed as RAUDPC (%) = [AUDPC/ last day reading-First day reading)]*100 ; RAUDPC has a minimum value of zero (no infection) and maximum value of 100 (completely infected tissue)
[b] (SP) Susceptible parental line
[c] Values followed by the same letter are not significantly different at p = 0.05 for comparisons of mean RAUDPC values within a) potato lines, b) different Phytophthora infestans genotypes using Tukey honestly significant different (HSD) test
[d] (T) Transformed with RB gene lines
[e] RB line from MSG227-2
[f] RB line from Spunta
[g] (RP) Resistant parental line
[h] Plasmid control check line (no RB gene)

2014 Whole plant bioassays results

Analysis across all P. infestans genotypes

In 2014 all parents and RB-lines were tested and RAUDPC values ranged from 2.3 (M171.69.19) to 28.7 (MSE149-5Y) (Table 7). Both LBR parents were significantly different from the LBS parent as were the two RB lines CG227C4.5 and CSPAG.13. Amongst the RB-lines derived from MSM171-A (RAUDPC=13.4), only one (M171.69.19, RAUDPC 2.3) was significantly different from the parental line. There were no significant differences among the parental lines and the RB-lines for either MSI152-A or MSR061-1.

The most aggressive *P. infestans* genotype was US-22 (32.5), and followed by US-23 (22.2), and this difference was significant (Table 7). The US-8 and US-24 genotypes were less aggressive and had the same RAUDPC value (4.0), which was significantly different from both US-22 and US-23.

Analysis for individual P. infestans genotypes

The infection level was very low for the US-8 genotype with a range of RAUDPC values from 0.3 (I152.69.01) to 10.8 (E149.69.06) and no significant difference between the LBR parental lines and the LBS line (Table 8). In addition, the RB-lines CG227C4.5 and CSPAG.13 were not significantly different from the LBS parental line. For the US-22 genotype, the range of RAUDPC values was 3.8 (CG227C4.5) to 52.3 (MSE149-5Y) with no significant difference between the LBR and LBS parental genotypes (Table 8). The RB-line CG227C4.5 (RAUDPC =3.8) was significantly different from the LBS parental line (MSE149-5Y, RAUDPC=43.3). Amongst the RB-lines derived from MSE149-5Y, E149.69.03 (RAUDPC=29.3) was significantly different from the parental line. One line (M171.69.19, RAUDPC=5.0) derived

from MSM171-A (RAUDPC=36.5) was significantly different from the parental line, but none of the RB-lines derived from MSI152-A or MSR061-01 were significantly different from their non-transgenic parental lines. For the US-23 genotype, RAUDPC values ranged from 4.0 (M171.69.19) to 42.5 (MSE149-5Y) and two of the LBR parental lines, MSM171-A and MSR061-01 were significantly different from the LBS parent with RAUDPC values of 14.3 and 17.8, respectively (Table 8). RB-line E149.69.03 (RAUDPC=16.0) was the MSE149-5Y-derived line that was significantly different from the parental line. None of the RB-lines derived from MSM171-A or MSR061-01 were significantly different from their respective parental lines. However, I152.69.01 (RAUDPC=13.5) was significantly different from its parental line MSI152-A (RAUDPC=37.0). For the US-24 genotype, RAUDPC values ranged from 0.0 (I152.69.01 and M171.69.01) to 17.0 (E149.69.11) and none of the LBR parental lines were significantly different from the LBS parental line (Table 8). None of the RB-lines were significantly different from their respective parental lines and neither CG227C4.5 nor CSPAG.13 were significantly different from the LBS parental line. In general, the ability to differentiate between lines varied with the isolate infection levels. When the disease pressure was high the RB-lines showed lower RAUDPCs and were significantly different from their respective parental lines, and when infection was low the parental lines and RB-lines had similar RAUDPCs that could not be separated.

Field experiment with US-22

The results of the field experiment to evaluate the foliar late blight resistance are shown in Table 9. In the field, the three LBR parent lines MSM171-A, MSI152-A and MSR061-1 all had lower RAUDPCs (14.5, 3.1 and 4.1, respectively) than the LBS parent lines (MSE149-5Y,

RAUDPC=20.5) but the difference between MSM171-A and MSE149-5Y was not significant. Amongst the RB lines derived from MSE149-5Y all but one (E149.69.11) had lower RAUDPC values than the parental line and the differences were significant. RAUDPC differences for the MSM171-A-derived RB-lines were not significant with the exception of M171.69.19 (RAUDPC=1.1). RB-lines derived from MSI152-A and MSR061-01 was not significantly different from their respective parental lines. CG227C4.5 and CSPAG.13 (from late blight susceptible parents MSG227-2 and Spunta) had very low RAUDPCs (5.0 and 1.1, respectively) that were significantly different from the LBS parental line.

Table 9. Foliar late blight progression in an inoculated field trial [measured as the Relative Area Under Disease Progress Curve (RAUDPC)] on parental potato lines and lines transformed with the RB gene. Plants were inoculated with US-22 genotype of *Phytophthora infestans* at the Clarksville Research Center at Michigan State University, 2013.

Severity of *Phytophthora infestans* (RAUDPC[a]), US-22		
Potato line	RAUDPC [a]	
MSE149-5Y (SP) [b]	20.5	ab [c]
E149.69.03 (T) [d]	8.2	cd
E149.69.06 (T) [d]	7.8	cd
E149.69.11 (T) [d]	26.7	a
E149.82.01 (T) [d]	5.9	cd
CG227C4.5 (T) [e]	5.0	cd
CSPAG.13 (T) [f]	1.1	d
MSM171-A (RP) [g]	14.5	bc
M171.A-11 (C) [h]	7.5	bcd
M171.69.06 (T) [d]	9.5	bcd
M171.69.14 (T) [d]	8.0	cd
M171.69.19 (T) [d]	1.1	d
M171.82.01 (T) [d]	6.7	cd
M171.82.02 (T) [d]	8.4	bcd
MSI152-A (RP) [g]	3.1	d
I152.69.01 (T) [d]	0.3	d
MSR061-1 (RP) [g]	4.1	cd
R061.69.01 (T) [d]	0.3	d

[a] Resistance/susceptibility score was expressed as RAUDPC (%) = [AUDPC/ last day reading-First day reading)]*100 ; RAUDPC has a minimum value of zero (no infection) and maximum value of 100 (completely infected tissue)

[b] (SP) Susceptible parental line

[c] Values followed by the same letter are not significantly different at $p = 0.05$ for comparisons of mean RAUDPC values within a) potato lines, b) different *Phytophthora infestans* genotypes using Tukey honestly significant different (HSD) test

[d] (T) Transformed with RB gene lines

[e] RB line from MSG227-2

[f] RB line from Spunta

[g] (RP)Resistant parental line

[h] Plasmid control check line (no RB gene)

Correlation tests among DLBs, WPBs and field assays

The correlation between the two years of DLBs was generally low and non-significant for each of the *P. infestans* genotypes with the exception of US-24 (r=0.53, p=0.05) (Table 10). For WPBs the correlations between years for each of the *P. infestans* genotypes were higher than for the DLBs, ranging from r=0.43 to 0.91, but only the US-24 correlation was significant (r=0.91, p<0.01) (Table 10). The correlations between the two greenhouse methods (DLB and WPB) were variable between years and *P. infestans* genotypes. Correlations coefficients ranged from r=0.23 to 0.67 with only three comparisons being significant: DLB2012 vs. WPB 2013 for the US-8 *P. infestans* genotype (r=0.67, p=0.05), DLB2013 vs. WPB2013 for the US-23 *P. infestans* genotype (r=0.75, p=0.0005) and DLB2013 vs. WPB2013 (r=0.77, p <0.001). When comparing greenhouse methods (DLB and WPB) with the field trial, the correlations ranged from r=0.08 to 0.44 for DLB/field comparisons and from r=0.42 to 0.75 for WPB/field comparisons (Table 11). The only significant correlations were between the 2012 WPB and field trial r=0.75, p=0.01.

Table 10. Correlation coefficient for the relationship between detached leaf bioassays (DLB) and whole plant bioassays (WPB) for the four (US-8, US-22, US-23 and US-24) *P. infestans* genotypes in 2012 and 2013.

DLB 2012	DLB 2013	R	P-value
US-8	US-8	0.06	0.83
US-22	US-22	0.33	0.25
US-23	US-23	0.05	0.83
US-24	US-24	0.53	0.05
WPB 2012	WPB 2013		
US-8	US-8	0.43	0.21
US-22	US-22	0.6	0.06
US-23	US-23	0.43	0.21
US-24	US-24	0.91	<0.001
DLB 2012	WPB 2012/13		
US-8	US-8	0.67	0.05
US-22	US-22	0.23	0.56
US-23	US-23	0.48	0.19
US-24	US-24	0.53	0.12
DLB2013	WPB 2013/14		
US-8	US-8	0.35	0.17
US-22	US-22	0.07	0.79
US-23	US-23	0.75	0.0005
US-24	US-24	0.77	<0.001

[a] Correlation significant at $p<0.05$

Table 11. Correlation coefficients for the relationship between detached leaf bioassays, whole plant bioassays and field test for US-22 genotype in 2012 and 2013.

Methods	By Methods	R	P-value
Field	DLB 2012 US-22	0.44	0.11
Field	DLB 2013 US-22	0.08	0.75
Field	WPB 2012 US-22	0.75	0.01
Field	WPB 2013 US-22	0.42	0.08

[a] Correlation significant at $P<0.05$

Discussion

The purpose of this study was to determine if the combination of the RB gene and host-plant resistance could enhance and broaden resistance to *P. infestans*.

The *Agrobacterium*-mediated transformation method was used to generate transgenic lines containing and expressing the RB gene. In general, the transformation efficiencies were very low for all four lines with only a total of 25 regenerated lines, of which 14 were rooted transformation events, and of those, only 13 were PCR positive for the RB gene. Kuhl et al. (2007) also noted low efficiency in experiments to transform MSE149-5Y with the RB gene. MSE149-5Y had a high efficiency (75% regeneration rate) when transformed with other genes (Kuhl et al., 2007) and was used effectively in the Douches lab for transformation experiments with various genes (Dr. Kim Felcher, personal communication). One explanation for this discrepancy may be the large size of the RB construct T-DNA region (8.6 kb). Another factor influencing transformation efficiency can be the protocol. For this study, transformations were accomplished using a Douches et al. (1998) protocol. Additional attempts to transform MSM171-A with the RB gene using modified protocols yielded higher transformation efficiencies (150 rooted events, 60 PCR positive for the RB gene). However, the same modified protocol used with other LBR lines (MSI152-A, MSR061-01, Jacqueline Lee, MSL268-A and Missaukee) resulted in low transformation frequencies (data not shown), indicating that transformation efficiency depends not only on the size of the insert and the transformation protocol, but on the genotype of the potato variety as well. (Heeres et al., 2002) found that transformation of potato is a genotype-dependent process and also different procedures and protocols needs to be adjusted for individual genotype. (Chakravarty et al., 2007) reviewed that

there are several problems affects the transformation efficiency in the potato and one of them is a genotype dependently.

Three different screening methods were used to test four genotypes of *P. infestans:* detached leaf bioassay (DLB), whole plant bioassay (WPB) and inoculated field trials. The DLB and WPB methods are very quick, inexpensive and labor/space saving, where the environment can be controlled to improve the disease pressure and to reduce the effects of biotic and abiotic factors. Another advantage of both screening methods is that they allow for testing of genotypes of the pathogen that cannot be tested in the field. For example, the US-24 genotype of *P. infestans* does not exist in Michigan, and no studies can be done with this genotype in the field. However, we were able to test the RB-transgenic lines against the US-24 genotype using both DLB and WPB. Field evaluations for late blight response are very valuable as they allow breeders to the assess plants under the conditions in which they will eventually be grown and allow for longer interactions between the host and pathogen. As a result, the classification of resistant and susceptible lines should be more accurate.

Although all three screening methods are important and have their advantages, many studies found correlations and similarities between laboratory, greenhouse and field assays (Dorrance and Inglis, 1997; Sharma et al., 2013; Vleeshouwers et al., 1999). (Vleeshouwers et al., 1999) found that assays between field and laboratory were significantly correlated; however the field lesion growth rate (LGR) was lower compared to the detached leaf and intact plants in the laboratory. Another study by (Douches et al., 2002) reported a moderate correlation between field and greenhouse studies and in another year no correlation between the two methods. The different results was obtained might be due to the high disease severity in the greenhouse in one

year and not in another. However, no close relationship between laboratory and field assays was documented by (Lal et al., 2013). The reason may be that the ideal temperature, fixed amount of inoculum and humidity was maintained in the laboratory, while in the field, the weather and amount of inoculum could be variable. A study by (Ali et al., 2012) found that the stem, meristem and roots play an important role in strengthening the physical barriers to pathogen invasion. Their study showed that leaves with meristem vs. no meristem and stem vs. no stem had different responses to the disease. Leaves with meristems and stems, displayed a hypersensitive response (HR), which contributed to resistance against *P. infestans*, whereas, leaves lacking meristems and stems did not exhibit HR. (Haverkort et al., 2008) reported that the agro-infiltration method using avirulence proteins could be used additionally for detached leaf assay in the laboratory to detect the R-gene based resistance.

In the current study, results from DLB and WPB experiments were variable between years and among *P. infestans* genotypes. Correlations between years for each method and with the other screening methods were generally low to moderate (with some exceptions) and often non-significant (Tables 10 and 11). In general, WPB results were more strongly correlated with the field results than DLB results.

The low correlation between years for the DLB experiments may be attributed to the isolates used, environmental conditions and protocol used. Overall, in our experiment we noticed that using different genotypes of *Phytophthora infestans* resulted in a different level of virulence of the pathogen. Studies by (Danies et al., 2013) and Johnson et al. (2014) confirmed that the US-8 and US-24 were mainly pathogenic on potatoes and were not very aggressive on tomatoes, whereas the US-22 and US-23 primarily showed a preference on tomatoes and they also showed

pathogenicity on potatoes. Finding by (Rojas et al., 2014) in potato late blight tuber assays was that the US-8 genotype was more aggressive than US-22 genotype, but the US-22 genotype collected on potatoes tissue was more aggressive on potatoes rather than on tomatoes, which could explain the host specificity. This might also explain in our study the less virulence of the US-23 genotype in some years, because this genotype was collected from tomato plants (W. Kirk, personal communication). According to the disease triangle concept, three factors (environment, pathogen and temperature) need to be optimized for disease progression. In the DLB experiments, steps were taken to ensure the optimization of the pathogen (i.e. healthy cultures and high inoculum concentration) and temperature, but it was not possible to optimize the humidity of the growth room. This may have had an impact on the aggressiveness of the pathogen. Other protocols have been used for DLB assays in an attempt to more closely mimic whole plants. Physiological changes in detached leaves may also account for the poor correlation between DLB and WPB as well as DLB and field results (Ali et al., 2012).

The WPB experiments were set up in the greenhouse in the late fall of 2012 and continued through the early spring of 2014. In order to achieve good disease pressure the temperature and humidity of the growth chamber must be optimized and maintained (22-25°C; humidity 70-80 %). The growth chambers were inside the greenhouse and the temperature inside the chamber was affected by the temperature of the greenhouse and ultimately by the outside temperature. For example during the warmer weather of winter 2013 (http://www.agweather.geo.msu.edu(Michigan State Univeristy and Agriculture Weather Office, 2014), the chamber temperature was as high as 32°C. These higher temperatures could have inhibited pathogen aggressiveness and disease progress, whereas we found during the 2014 winter the high aggressiveness in two genotypes (Table 8). Despite successful bioassays, we

were not able to use mist and humidity control in the growth chamber for our experiments. According to (Ali et al., 2012) they found that prior inoculation the plants needs to be kept in the humidity and mist chamber for 2 h and after inoculation they need to kept in the controlled humidity growth chamber to obtain a good infection. This would contribute to lower year-to-year correlations as well as poor correlation with DLB and field results. In addition, poor disease progress added to high variability between replications making it difficult to detect differences between potato genotypes for response to *P. infestans*. This is particularly notable in 2013/14 within with *P. infestans* genotypes US-8 and US-24 (Table 7,8).

Although the DLB and WPB have advantages in comparing *P. infestans* genotypes vs. plant genotypes, field assessment can be the most valuable method for screening late blight resistance. The significant correlation found among field and WPB 2012 with US-22 genotypes. All other interactions with the US-22 genotype between field, DLB and WPB were not significantly correlated. In 2012 the field study did not occur because non-dormant tubers were not available for planting. Due to the fact that there were no data for 2012 field season it was impossible to compare to result of 2013. Nevertheless, the field results from 2013 reveals that RB-lines had equal or less RAUDPCs than the parental lines. Similar research was done by (Jones et al., 2014), but using Desiree variety, a late blight susceptible line with an R-gene from *S. venturii* (*Phi-vnt1.1*). They have noted due to the weather conditions in three field seasons the *P. infestans* infection was variable, but lines with *Rpi-vnt1.1* genes showed consistent high resistance in the less infection years as well as under high pressure of the disease.

The following conclusions can be drawn from this research. First, the important factors for a good infection for late blight are moderate temperature, high humidity, moist conditions

and good concentration of the inoculum. The DLB experiments were not consistent and similar infection did not occur among all four genotypes and among the years because the combination of factors for good infection was difficult to control simultaneously. Secondly, the WPB result was more correlated to the field results. Also, screening the whole plant it gives more comparison to the field because the intact plant tested instead of the detached leaf. This statement could supported by (Orlowska et al., 2012), where they reported that the roots played significant role for the Sarpo Mira late blight resistant potato variety for defense from the foliar late blight. Last, the field test is a crucial and since the potato lines eventually will grow in the field it is very important test them in the same environment.

Potato late blight has been, and remains to this day, the most destructive disease impacting potato production leading to economic losses upwards of 6 US billion dollars worldwide (Haverkort et al., 2008; Fry, 2008). Breeders have been breeding for resistance to this disease for more than 120 years, and still there are few, if any commercial potato varieties with resistance to many different genotypes of *P. infestans* (Li et al., 2011). Breeding programs throughout the world have developed potato varieties with resistance to a sub-set of *P. infestans* genotypes, but traditional breeding approaches have not yielded broadly resistant varieties (Douches et al., 2001; Douches et al., 2010; Novy et al., 2006; Thurston et al., 1985). The naturally resistant lines could or could not be a solution for a good resistance, and it also can be time-consuming process. The conventional breeding for a late blight resistance using accessions from the wild potato *S. bulbocastanum,* took 46 years to finally release two resistant cultivars (Toluca and Bionica) (Haverkort et al., 2009). Because late blight control is economically important and host-plant resistance could save millions of dollars and reduce environment

impacts, it a crucial to pursue alternative breeding strategies while continuing traditional breeding approaches.

In this study we pyramided RB with the resistance genes conventionally bred into the breeding lines, to increase the resistant level. However, the researchers have been studying stacking two or three genes in one vector, and insert into the potato line (Storck et al., 2011; Zhu et al., 2012b; Kim et al., 2012). (Storck et al., 2011) reported that over the five years of testing against to late blight the resistant variety "Fortuna" that is expressing the combination of the *Rpi-blb1* and *Rpi-blb2* genes from *S. bulbocastanum* was never infected with late blight. Pyramiding and stacking genes approaches to enhance the resistance it seems promising strategy to apply against late blight (Jo et al., 2014; Zhu et al., 2012a).

The other examples of gene stacking to enhance and broadened the resistance for one or multiple traits were used for other crops such as: pea (*Pisum sativum*), walnut (*Juglans regia*), canola (*Brassica napus*), corn (*Zea mays*) and cotton (*Gossypium hirsutum*) (Chan et al., 2005; Que et al., 2010; Walawage et al., 2013). The knowledge about genomics is continuing to expand and more R-genes from the wild species or relatives will be revealed and the stacking and multiple gene approaches will be more commonly used.

The future work with these lines can be constructing RB and other resistant genes from wild potatoes in one vector and introducing the marker free transformation method into LBR lines. The additional value of the using the RB gene that it creates a cisgenic event using the indigenous promoter and terminator and it makes for a greater possibility of public approval. Cisgenesis strategy provides us to keep the old and popular varieties in the market but introducing to them a new and important trait such as a late blight resistance (Haverkort et al.,

2009). The other future work can be done by planting LBR+RB lines and combining with the fungicide studies to achieve good late blight control for commercial growers (Kirk et al., 2005).

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