# Generation of genome wide linkage maps for a wild potato and RNA-seq analysis of transgene mediated potato defense mechanisms against late blight in the tubers and foliage

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**Abstract**

Wild potato *Solanum bulbocastanum* is a rich source of genetic resistance against a variety of pathogens. This project developed molecular tools and expanded biological knowledge useful for the improvement of cultivated potato (*S. tuberosum*) using genetic resistance from *S. bulbocastanum.* First, the genome structure of *S. bulbocastanum* relative to those of its relatives, cultivated potato and tomato (*S. lycopersicon*) was determined. Second, to facilitate efforts to improve cultivated potato through the introgression and deployment disease resistance derived from *S. bulbocastanum*, the phenotypic function and molecular mechanisms of one *S. bulbocastanum* disease resistance gene were explored in cultivated potato foliage and tubers.

For determination of genome structure for *S. bulbocastanum,* Diversity Arrays Technology (DArT) was employed to generate genome wide linkage maps for the species. Employing a pseudo-testcross mapping strategy, 631 DArT markers were integrated into a composite map comprising 12 linkage groups. Our results represent an over ten-fold increase of total marker density compared to previously available genetic maps for the species. Sequencing and alignment of corresponding DArT clones to reference physical maps from tomato and cultivated potato allowed a direct comparison of marker orders between species. Overall, the *S. bulbocastanum* genetic maps show higher collinearity with reference potato maps than tomato maps, with seven genome regions supporting a closer phylogenetic relationship between potato and *S. bulbocastanum* than between tomato and *S. bulbocastanum.*

One dominant US cultivar ‘Russet Burbank’(WT; late blight susceptible in foliage and tuber) and its *RB* (a late blight resistance gene derived from *S. bulbocastanum*) transgenic line SP2211 (*+RB;* late blight resistant in foliage and tuber) were compared in both tubers and foliage in their responses to late blight pathogen attack using an RNA-seq approach. In the tubers, a total of 483 million paired end Illumina RNA-seq reads were generated, representing the transcription of 29,319 potato genes. Differentially expressed genes, gene groups and ontology bins that exhibited differences between the WT and *+RB* lines were identified. *P. infestans* transcripts, including those of known effectors, were also identified. Faster and stronger activation of defense related genes, gene groups and ontology bins correlated with successful tuber resistance against *P. infestans*. Our results suggest that the hypersensitive response is likely a general form of resistance against the hemibiotrophic *P. infestans*—even in potato tubers, organs that develop below ground.

In the foliage, a total of 515 million paired end RNA-seq reads were generated, representing the transcription of 29,970 genes. We compared the differences and similarities of responses to *P. infestans* in potato foliage and tubers. Differentially expressed genes, gene groups and ontology bins were identified to show similarities and differences in foliage and tuber defense mechanisms. Our results suggest that disease resistance gene dosage and shared biochemical pathways contribute to *RB*-mediated incompatible potato-*P. infestans* interactions in both the foliage and tubers.

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# Chapter one: Generation of Genome-wide Linkage Maps for Wild Potato *Solanum bulbocastanum* and Comparative Genomics Studies of *Solanum* Species

Wild potato *Solanum bulbocastanum* is a rich source of genetic resistance against a variety of pathogens. However the genome architecture of the species remains largely uncharacterized. The current study employed Diversity Arrays Technology (DArT), a high throughput genotyping platform to genotype a mapping population of *S. bulbocastanum*, and generated genome-wide linkage maps for the wild species. Our results represent an over ten-fold increase in terms of total marker density compared to previously available genetic maps for the species. Sequencing and alignment of DArT clones to reference physical maps from tomato and cultivated potato allowed a direct comparison of marker orders between species. A total of eleven genomic segments informative in comparative genomic studies were identified. Overall, the *S. bulbocastanum* genetic maps show higher collinearity with the reference potato map than the tomato map, with seven genome regions supporting a closer phylogenetic relationship between potato and *S. bulbocastanum* than between tomato and *S. bulbocastanum.* We also identified four *S. bulbocastanum* genome regions that differ from cultivated potato.

## Introduction

The genus *Solanum* includes agronomically important plants such as potato, tomato and eggplant. Molecular dating suggests that potato and tomato diverged 7.3 million years ago (Wu and Tanksley 2010). Today, there are approximately 200 tuber-bearing *Solanum* species, including the cultivated potato and wild relatives comprising the secondary and tertiary gene pools. These wild species are potentially rich sources of genes for the improvement of the cultivated potato.

Different from the primary and secondary gene pool (Harlan and Wet 1971) species of potato, *Solanum bulbocastanum* (2n=2x=24) represents a taxon of wild potatoes known as "superseries *Stellata*", which encompasses approximately 20 tertiary gene pool wild potato species. The Endosperm Balance Number (EBN) model was proposed by Johnston et al. (Johnston et al. 1980) to explain intra- and interspecific crossability among potato species. A cross will be successful if the EBN ratio is 2 (maternal):1 (paternal). Within *Stellata,* most species have been assigned an EBN of 1, making them sexually incompatible with cultivated potato (4EBN). *S. bulbocastanum* is a rich source of genetic resistance against a variety of pathogens (Rodriguez and Spooner 2002). Despite not being directly crossable with cultivated potato, various important agronomic traits have been introgressed from *S. bulbocastanum* into cultivated potato through somatic hybridization and transgenic techniques (Bradeen et al. 2009; Helgeson et al. 1998).

Morphologically, *S. bulbocastanum* is one of the most distinct tuber-bearing potato species (Rodriguez and Spooner 2002). It has been suggested, based upon cytological observations, that the genome of *S. bulbocastanum* is structurally distinct (B genome) from that of cultivated potato (A genome) and many other wild potato relatives (A, C, D and P genomes) (Matsubayashi 1991; Rodriguez and Spooner 2002). However, to date, no study has explicitly compared the organization of the proposed A and B *Solanum* genomes using DNA sequence technologies. Some genetic or genomic studies have been conducted in *S. bulbocastanum* (Bradeen et al. 2003; Brown et al. 1996). However the genome architecture of *S. bulbocastanum* remains largely uncharacterized, limiting the application of comparative genomics studies between *S. bulbocastanum* and other *Solanum* species.

Diversity Array Technology (DArT, http://www.diversityarrays.com) is a community-based molecular marker technology that allows high-throughput and cost-effective genotyping of target species, without relying on prior genome sequence information. DArT involves the preparation of an array of individualized clones from a genomic representation, generated through amplified restriction fragments (Wenzl et al. 2004). Labeled genomic representations of individuals to be genotyped are hybridized to these arrays. Polymorphisms scored reflect the presence or absence of hybridization to individual array elements. The technology has been successfully utilized in various species including Arabidopsis (Wittenberg et al. 2005), wheat (Akbari et al. 2006), barley (Wenzl et al. 2004), and potatoes (Sliwka et al. 2012a; b).

Sliwka et al. (Sliwka et al. 2012b) utilized DArT technology and sequence specific PCR markers to genotype a mapping population of *Solanum* x *michoacanum* developed to map the late blight resistance gene *Rpi-mch1*. The study generated a linkage map consisting of 798 DArT markers. In a separate study, Sliwka et al. (Sliwka et al. 2012a) mapped a second late blight resistance gene *Rpi-rzc1* (derived from *Solanum ruiz-ceballosii*) to chromosome 10 using DArT markers and sequence specific PCR markers. The linkage map generated consists of 1,603 DArT markers with a map length of 1,204.8cM. Our group pioneered the development of a DArT platform for genotyping *Stellata* potato species, including *S. bulbocastanum.* The new potato array was especially enriched with elements derived from 1 EBN wild potato species. In this study, we employed this DArT array to develop a linkage map for *S. bulbocastanum.*

Importantly, as genome sequencing projects move forward, the genomes of cultivated potato and tomato have both been sequenced (Sato et al. 2012; Xu et al. 2011). Here, over 800 DArT clones, many corresponding to markers mapped in *S. bulbocastanum,* were also sequenced and aligned to reference physical maps of potato and tomato. This has allowed a direct comparison of genome structures across *Solanum* species. The current study provides insights into the genome structure of *S. bulbocastanum* and guidance for comparative genomics studies among wild potato, cultivated potato and tomato.

# Materials and methods

## Plant material, DNA isolation, and DArT genotyping

Full-sib progeny seeds from a cross between wild potato *Solanum bulbocastanum* genotypes PT29 and G15 were planted at the University of Minnesota Plant Growth Facilities green house (St. Paul, MN). Leaf tissue from seven weeks old plants was collected, frozen immediately in liquid nitrogen, and stored at -80$^{o}$C for DNA extraction using a modified CTAB method (Fulton et al. 1995).

In collaboration with Andrzej Kilian, Diversity Arrays Technology, Pty. Ltd., our group developed a DArT array for wild potatoes (http://www.diversityarrays.com/) with over 20,000 features (Sliwka et al. 2012a; b). DNA samples from 92 $F_1$ progeny of the cross PT29 X G15 together with the two parental lines (PT29 and G15) were genotyped using the DArT array and previously established protocols (Wenzl et al. 2004; Wittenberg et al. 2005).

## Linkage map construction

We employed the pseudo-testcross strategy (Grattapaglia and Sederoff 1994) to construct linkage maps. A total of 854 markers were coded into three marker classes. Markers that are heterozygous in PT29 but null homozygous in G15 were coded into the lmxll class (490 markers). Markers that are null homozygous in PT29 but heterozygous in G15 were coded into the nnxnp class (166 markers). Markers that are heterozygous in both parents were coded as hkxhk markers (198 markers).

Two parental maps were generated using lmxll (PT29 parental map) or nnxp (G15 parental map) markers respectively. The regression mapping algorithm of JoinMap 4 (http://www.kyazma.nl/index.php/mc.JoinMap/) was used in generating each parental maps. Kosambi's mapping function was used in calculating map distances. The two resulting parental maps were then merged into a composite map using anchor markers (hkxhk). Integrated map marker order is largely based on fixed marker orders from parental maps. In cases in which the two parental fixed marker orders could not be simultaneously satisfied, the marker order from PT29 was adopted.

## Comparison of marker order with potato and tomato physical maps

DArT clones polymorphic between the *S. bulbocastanum* mapping parents were subsequently sequenced and DArT sequences were aligned to both potato and tomato genome sequences using GenomeThreader (Gremme et al. 2005) with 70% minimal nucleotide coverage and sequence identity (Traini et al. submitted). Only uniquely aligned DArT clones (i.e., DArT sequences anchored to a single location in the reference genome sequence or to a cluster of identical sequences occupying a single contiguous location on the reference genome sequence) were used to compare physical and genetic maps. The comparative alignment information was summarized using a custom perl script and visualized using MapChart v2.0 (Voorrips 2002).

# Results

## Linkage map generation

A total of 458 DArT markers were integrated into the PT29 parental linkage map comprising 12 linkage groups (LGs), as expected (Figure 1.1, Table 1.1). The total genetic distance is 620.1 centimorgan (cM), averaging 0.74 markers per cM. However, many of the markers co-segregate and the number of uniquely positioned markers is only 306 (67% of all mapped markers). Overall, linkage groups corresponding to chromosomes 1 and 5 have relatively few markers.

In contrast, a total of only 138 DArT markers were integrated into the G15 parental linkage map, comprising 20 LGs—substantially more than the expected 12 LGs (Figure 1.2, Table 1.1). The total genetic distance covered in the G15 map is 506.5 cM, averaging about 0.27 markers per cM. The number of uniquely positioned markers is 135. The relatively small number of markers integrated into the G15 map (compared to the PT29 map) is likely due to the fact that PT29, but not G15, was a prominent contributor of elements to the potato DArT array.

The integrated map was generated based on marker order information from parental maps and the information provided by anchor markers (hkxhk). The integrated map comprises 12 LGs with a total of 631 markers (~64% of which are uniquely positioned) (Figure 1.3, Table 1.1). The integrated map spans a total genetic distance of 644.9 cM, averaging about 0.98 markers per cM. The linkage group corresponding to potato chromosome 4 is the largest with a total of 103 markers spanning 83.7 cM.

### Comparison to cultivated potato and tomato physical maps

Over 800 DArT clones were sequenced. Over 500 of the sequenced clones were incorporated into the newly developed *S. bulbocastanum* genetic linkage maps described above. Alignment of DArT clone sequences to reference physical maps of tomato and cultivated potato (Sato et al. 2012; Xu et al. 2011) allowed a direct comparison of the alignment information of the DArT clone sequences and the genetic position of the corresponding marker on our linkage maps. Figures 1.4 – 1.6 compare the *S. bulbocastanum* genetic maps with cultivated potato and tomato reference physical maps. In total, the *S. bulbocastanum* genetic maps covered over 86% of the total physical maps.

## Discussion

### First medium-density genome-wide linkage maps for *S. bulbocastanum*

To map a nematode resistance locus, Brown et al. (1996) developed somatic hybrids between *S. bulbocastanum* and cultivated tetraploid potato and generated a BC2 mapping population for RFLP genotyping. A *S. bulbocastanum* linkage map consisting of 48 RFLP markers (belonging to 12 linkage groups) was generated. The resistance locus was localized to the telomere of chromosome 11.

Our study utilized the DArT marker platform to generate genetic maps for *S. bulbocastanum*. The integrated map comprises over 600 DArT markers, representing a greater than 10-fold increase in marker density compared to the previously available genetic map for this species (Brown et al. 1996). The generation of medium density

genome-wide linkage maps allowed us to compare genome structures between the B genome wild potato and the A genome cultivated potato and tomato.

## High collinearity between genetic maps and physical maps

The Solanaceae comprises over 3,000 species including many important crops such as tomato, potato, eggplant, and pepper. Wu et al. (Wu and Tanksley 2010) utilized COSII markers to study the evolutionary relationships within the family, estimating that potato and tomato, sister taxa, diverged from a common ancestor 7.3 million years ago (Wu and Tanksley 2010).

Through alignment of DArT clone sequences to recently published cultivated potato and tomato genome sequences (Xu et al. 2011), we were able to compare the physical position of the clones and the genetic position of the clones in our *S. bulbocastanum* linkage maps. Overall, we found good marker collinearity between *S. bulbocastanum* and potato and tomato, as shown in Figures 1.4-1.6.

In total, we found seven genomic segments that show higher collinearity to potato chromosomes 2, 3, 5, 9, 11, 12 than to corresponding tomato chromosomes (Figures 1.4-1.6). These segments represent genome structure changes that have occurred since the initial origination of the distinct tomato and potato lineages. Nearly all of these chromosomal changes are paracentric inversions, consistent with the cytogenetic theory that predicts that paracentric inversions will have the least negative effects on fitness and thus be the most likely form of chromosomal rearrangements (Bonierbale et al. 1988). Collectively, our results provide evidence that wild potato relatives share higher

collinearity with cultivated potato than tomato, consistent with a closer phylogenetic relationship between *S. bulbocastanum* and cultivated potato than between *S. bulbocastanum* and tomato.

Our study also revealed small segments that are *S. bulbocastanum* specific (i.e., B-genome specific (Matsubayashi 1991) ). In total, we found four *S. bulbocastanum* genomic segments (located on chromosomes 2, 7, 8) that differ from cultivated potato (Figures 1.4-1.6). These segments tend to span small genetic distances (around 5-10 cM), comprise few markers, and are located near telomere positions. Further higher density mapping efforts or whole genome sequencing of *S. bulbocastanum* or other B-genome *Solanum* species may confirm the legitimacy of these regions and may reveal other B-genome specific genomic segments. Since the original A vs. B genome hypotheses are based on low resolution cytological observations (Matsubayashi 1991), we expected medium density linkage mapping in *S. bulbocastanum* to offer sufficient resolution to identify structural variations. While large scale changes in chromosome structure were not confidently established using this approach, DNA sequence level variation between the A and B genomes is still possible, but its identification will require the development of more densely populated comparative linkage maps or, ultimately, genome sequencing of B genome species.

## Conclusion

The first medium-density genome-wide linkage maps for wild potato *S. bulbocastanum* were generated, demonstrating the utility of the DArT platform for genotyping wild potato species. Over 600 markers were integrated into the linkage maps,

representing a greater than ten-fold increase in marker density compared to previously existing maps for the wild potato species. Sequencing and alignment of DArT clones to reference potato and tomato physical maps allowed a comparison of genetic and physical orders of the markers. Our results indicate that a majority of the markers shows good collinearity between genetic and physical maps. Marker orders were in higher collinearity to reference potato physical maps than to tomato physical maps. Our research will assist comparative mapping of agronomically important genes or quantitative trait loci (QTLs).

**Table 1.1 Genetic linkage maps summary for parental and integrated maps**

| | LGs | Markers | Unique loci | Map length | Density |
|---|---|---|---|---|---|
| **PT29 (P1)** | 12 | 458 | 306 | 620.1 | 0.74 |
| **G15 (P2)** | 20 | 138 | 135 | 506.5 | 0.27 |
| **Integrated** | 12 | 631 | 403 | 644.9 | 0.98 |

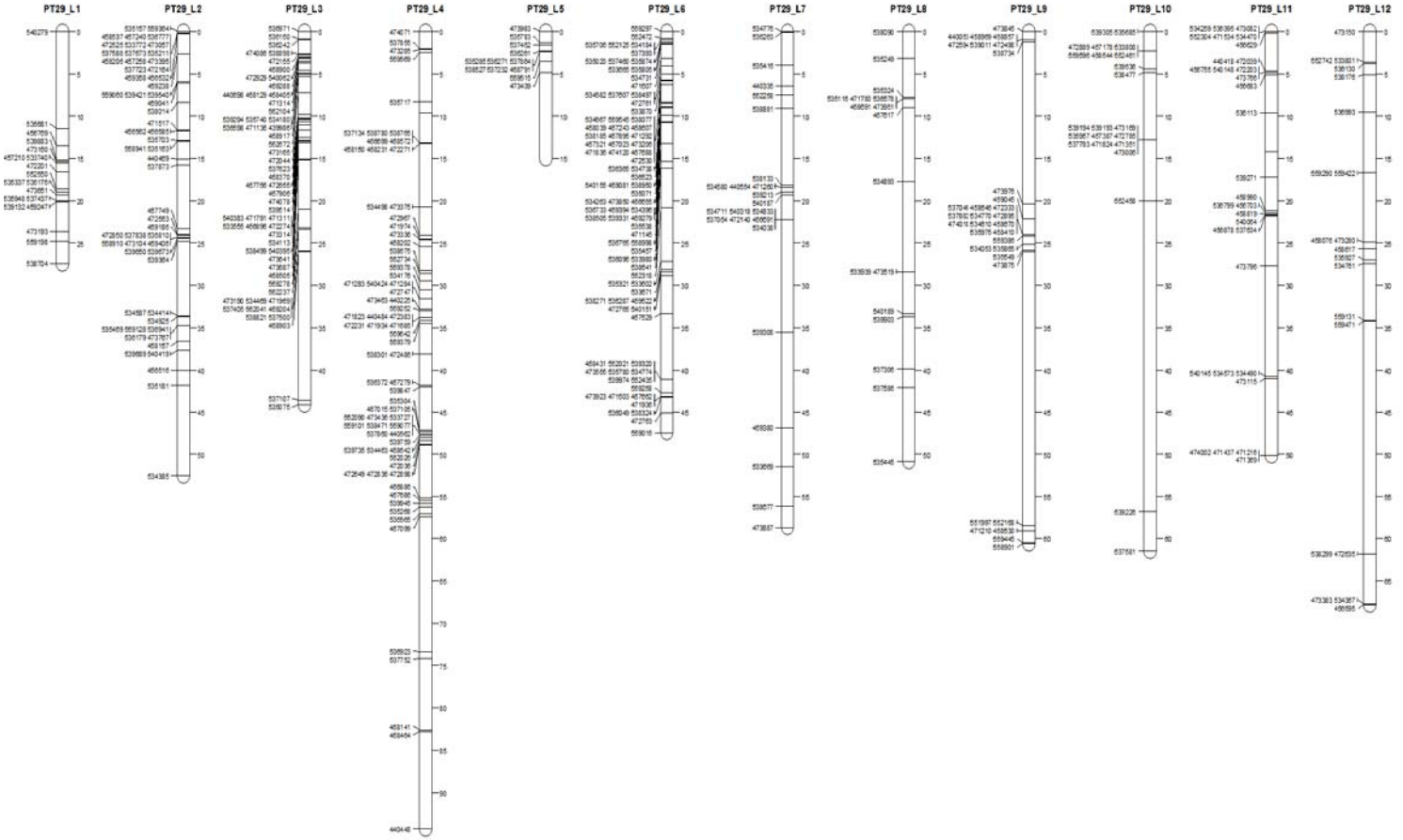


**Figure 1.1 PT29 genetic linkage map.** A total of 458 DArT markers were mapped to 12 linkage groups, corresponding to the 12 potato chromosomes. Linkage groups corresponding to chromosomes 1 and 5 are the smallest.

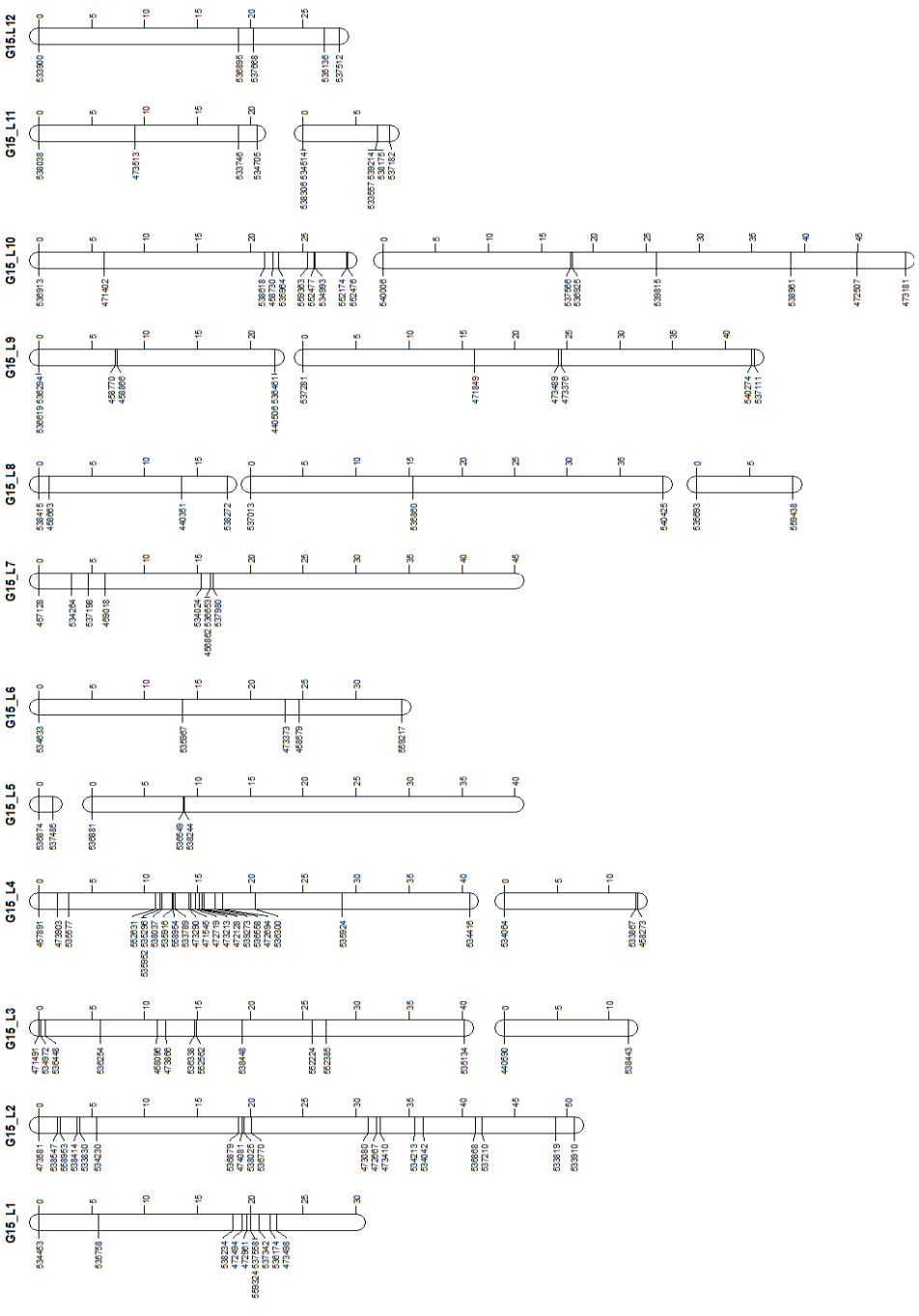

**Figure 1.2 G15 genetic linkage map.** A total of 138 DArT markers were mapped to 20 linkage groups. Linkage groups are arranged based on clone sequence alignment to the 12 cultivated potato chromosomes.

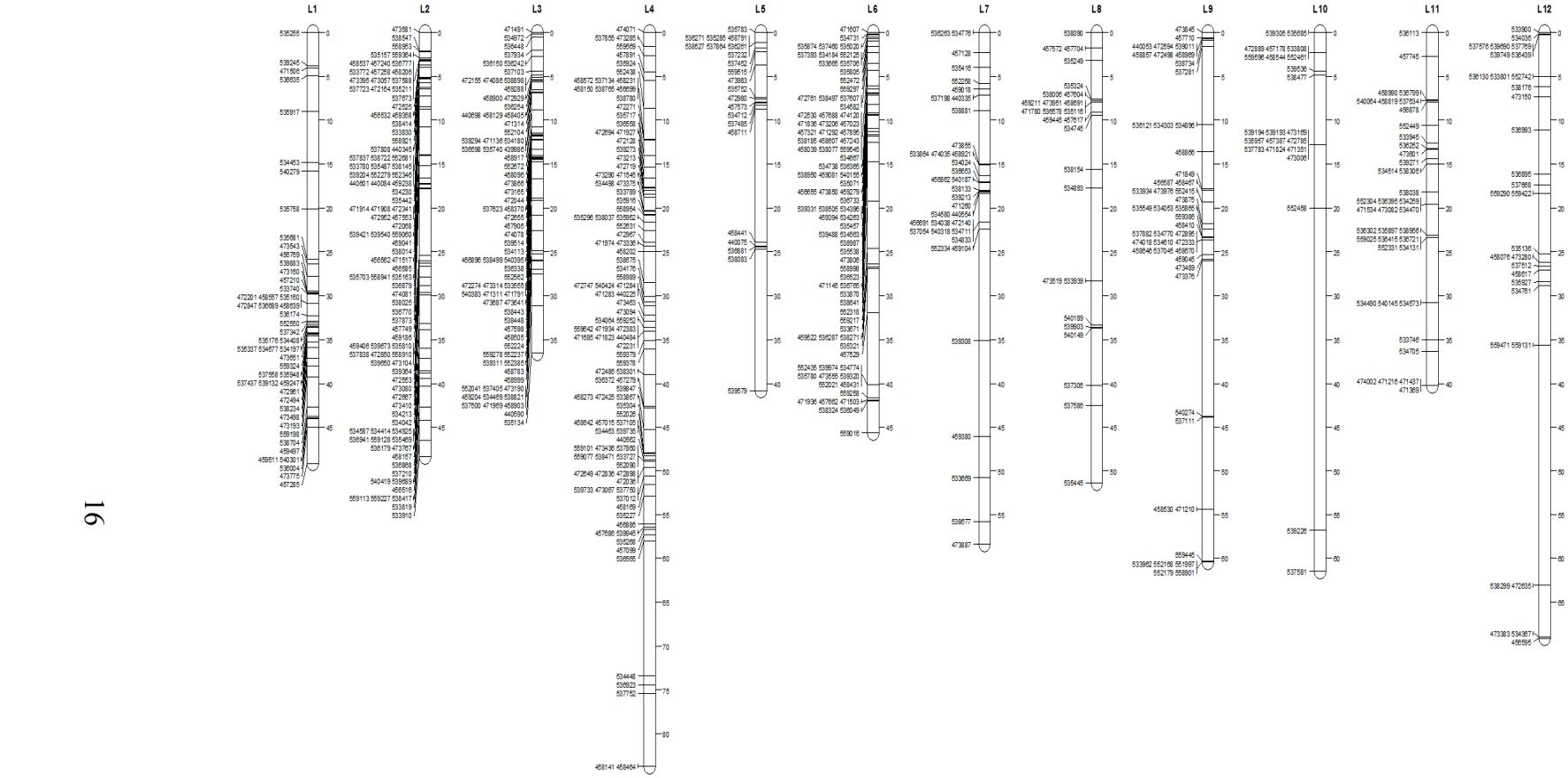


**Figure 1.3 Integrated genetic linkage map.** A total of 631 DArT markers were mapped to 12 linkage groups representing 12 chromosomes.

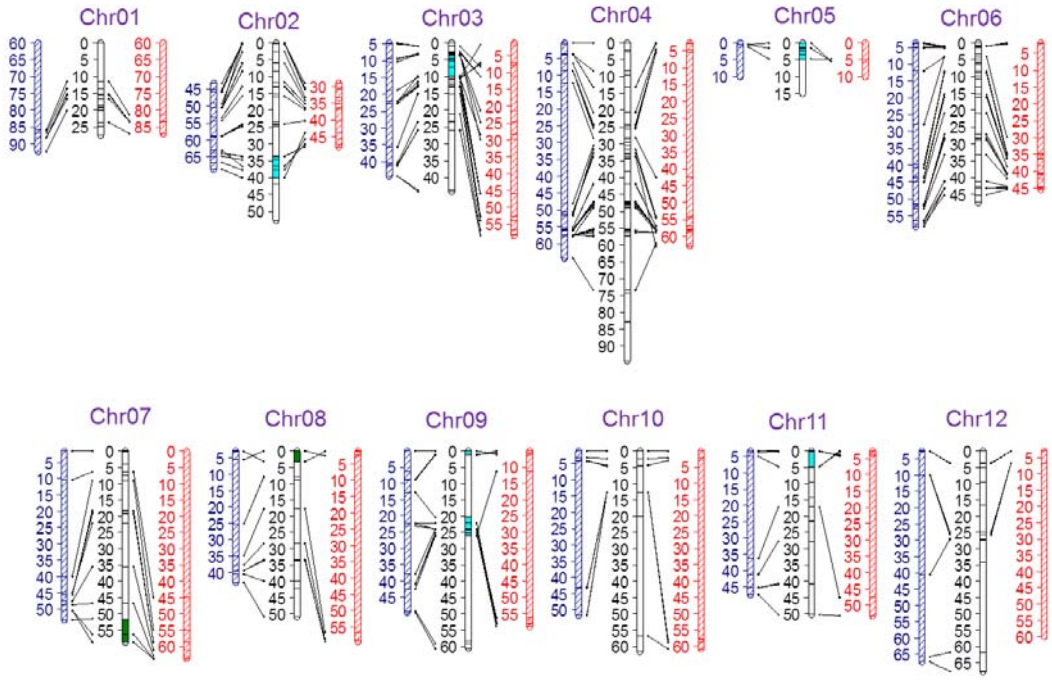


**Figure 1.4 Comparison of the *S. bulbocastanum* PT29 genetic map with tomato and cultivated potato physical maps.** Dark blue: potato physical map (genome sequence); Red: tomato physical map (genome sequence); black: genetic map (PT29 DArT marker map). On the *S. bulbocastanum* map, regions highlighted in blue show higher collinearity to cultivated potato than to tomato. Regions of the *S. bulbocastanum* map highlighted in green are segments with an arrangement distinct from that found in cultivated potato or tomato. These segments may be specific to *S. bulbocastanum* and other B genome *Solanum* species.

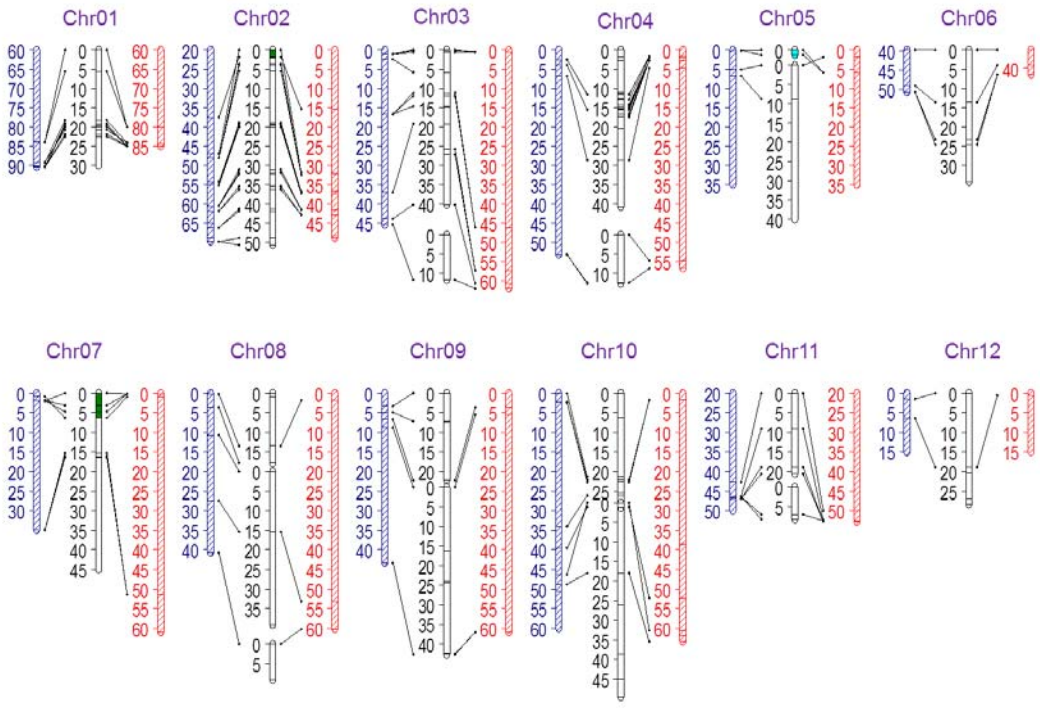


**Figure 1.5 Comparison of the *S. bulbocastanum* G15 genetic map with tomato and cultivated potato physical maps.** Dark blue: potato physical map (genome sequence); Red: tomato physical map (genome sequence); black: genetic map (G15 DArT marker map). On the *S. bulbocastanum* map, regions highlighted in blue show higher collinearity to cultivated potato than to tomato. Regions of the *S. bulbocastanum* map highlighted in green are segments with an arrangement distinct from that found in cultivated potato or tomato. These segments may be specific to *S. bulbocastanum* and other B genome *Solanum* species.

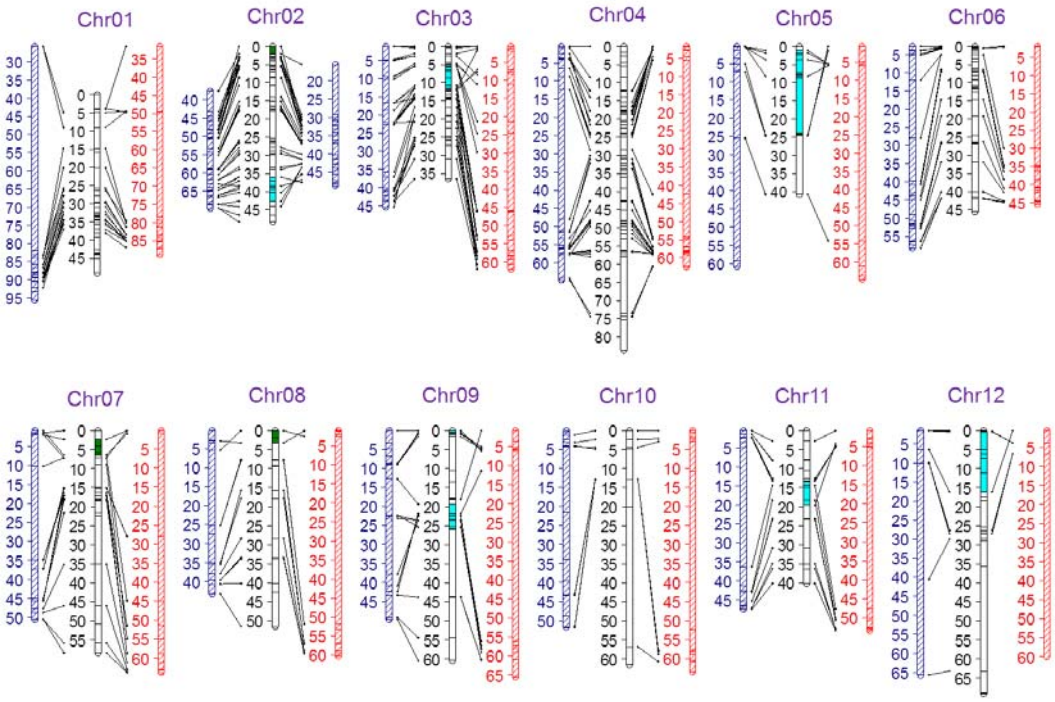


**Figure 1.6 Comparison of the *S. bulbocastanum* integrated genetic map with tomato and cultivated potato physical maps.** Dark blue: potato physical map (genome sequence); Red: tomato physical map (genome sequence); black: genetic map (integrated DArT marker map). On the *S. bulbocastanum* map, regions highlighted in blue show higher collinearity to cultivated potato than to tomato. Regions of the *S. bulbocastanum* map highlighted in green are segments with an arrangement distinct from that found in cultivated potato or tomato. These segments may be specific to *S. bulbocastanum* and other B genome *Solanum* species.

# Chapter Two: Insights into organ-specific pathogen defense responses in plants: RNA-seq analysis of potato tuber-*Phytophthora infestans* interactions

The late blight pathogen *Phytophthora infestans* can attack both potato foliage and tubers. Although interaction transcriptome dynamics between potato foliage and various pathogens have been reported, no transcriptome study has focused specifically upon how potato tubers respond to pathogen infection. When inoculated with *P. infestans*, tubers of nontransformed 'Russet Burbank' (WT) potato develop late blight disease while those of transgenic 'Russet Burbank' line SP2211 (+*RB*), which expresses the potato late blight resistance gene *RB* (*Rpi-blb1*), do not. We compared transcriptome responses to *P. infestans* inoculation in tubers of these two lines. We demonstrated the practicality of RNA-seq to study tetraploid potato and present the first RNA-seq study of potato tuber diseases. A total of 483 million paired end Illumina RNA-seq reads were generated, representing the transcription of around 30,000 potato genes. Differentially expressed genes, gene groups and ontology bins that exhibited differences between the WT and +*RB* lines were identified. *P. infestans* transcripts, including those of known effectors, were also identified. Faster and stronger activation of defense related genes, gene groups and ontology bins correlate with successful tuber resistance against *P. infestans*. Our results suggest that the hypersensitive response is likely a general form of resistance against the hemibiotrophic *P. infestans*—even in potato tubers, organs that develop below ground.

## Introduction

Cultivated potato is the world's third most important human food crop and the number one non-grain food commodity (FAOSTAT 2010). Unfortunately, potato is also host of a broad range of pathogens (Stevenson et al. 2001). Late blight disease is caused by *Phytophthora infestans*, resulted in the Irish Potato Famine in the 1840s, and still today results in multi-billion dollar losses worldwide annually (Fry 2008). *P. infestans* is a notorious plant destroyer with the capacity to attack both potato foliage and tubers. Foliage resistance against late blight does not guarantee tuber resistance—contrasting disease resistance phenotypes can be evident in comparing foliage and tubers from a single genotype (Kirk et al. 2001).

Gene *RB* (*Rpi-blb1*) (Song et al. 2003; van der Vossen et al. 2003), a disease resistance gene cloned from a wild potato species *S. bulbocastanum*, confers broad-spectrum foliar resistance against all major late blight pathogen isolates. The gene has been introduced into several potato cultivars using transgenic approaches (Bradeen et al. 2009). Transgenic lines have been or are currently being tested for eventual commercial release in Europe, India, Bangladesh, US and other places. Despite being foliar late blight resistant, most +*RB* transgenic potato lines lack statistically significant tuber blight resistance (Halterman et al. 2008; Millett 2008). However, these conclusions are all based on ANOVA models, and the phenotypic data are often associated with very large variances, resulting in low statistical powers. We are re-analyzing the data using alternative statistical models (Appendix one). Our results indicate that enhanced *RB*

transcript levels correlate with enhanced disease resistance (Appendix one). We identified two transgenic lines with unusually high *RB* transcript levels in the tuber that are also tuber blight resistant (Millett 2008). Thus, *RB* has the potential to function in both foliage and tuber and the *RB*-potato tuber-*P. infestans* interaction provides a tractable system to study how potato tubers defend against plant pathogens.

Next-generation sequencing (NGS) technologies are fast evolving and are transforming biology research (Metzker 2010; Morozova and Marra 2008). Genome sequences of potato and *P. infestans* have been published (Haas et al. 2009; Xu et al. 2011), making sequencing-based transcriptome studies (RNA-seq) more accessible to potato late blight researchers. RNA-seq is a relatively new approach towards study of the transcriptome (Marioni et al. 2008; Mortazavi et al. 2008). To our knowledge, there has been no RNA-seq study focused on potato-microbe interactions. Indeed, one NGS-based transcriptome study focused on the interaction between potato and *P. infestans* has been published (Gyetvai et al. 2012). However, that study utilized the DeepSAGE method, not RNA-seq. DeepSAGE differs substantially from RNA-seq, and researchers in that study relied heavily on assembled tags; the newly available genome sequence data were mostly not utilized. Furthermore, that and all other published studies focused on potato foliage transcriptome dynamics (Birch et al. 1999; Gyetvai et al. 2012; Restrepo et al. 2005); no previous study has reported transcriptome dynamics of potato tubers in response to pathogen attack.

In this study, we employed RNA-seq to study the transcriptome dynamics of potato tuber- *P. infestans* interactions in compatible and incompatible potato genotypes.

We employed genome-wide sequence data from both potato and *P. infestans* in our analyses. Differentially expressed genes and ontology bins were identified that distinguish compatible and incompatible interactions. Transcripts of *P. infestans*, including those from candidate effectors were also identified. Our study has important implications for potato *R* gene deployment and contributes to scientific understanding of organ-specific defense regulation in plants.

## Materials and methods

### Plant material and RNA preparation

The tuber late blight susceptible nontransformed 'Russet Burbank' (WT, provided by Dr. Carl Rosen, UMN) and SP2211, a tuber late blight resistant transformed 'Russet Burbank' line carrying the *RB* transgene (+*RB* (Bradeen et al. 2009)) were examined in this study. Tubers were produced under standard cultural practices at the University of Minnesota Sand Plain Research Farm (Becker, MN). Tubers of each line were harvested and held for three days at room temperature before storage for six weeks at 11-13$^{o}$C. *P. infestans* US8 isolate US940480 (Song et al. 2003) was maintained on Rye A medium (Tumwine et al. 2000). Prior to inoculation, sporangia were harvested from plates by physical scraping into distilled water. Inoculum was adjusted to $6.75*10^4$ sporangia/ml. Prepared inoculum was incubated for 1 hour at 4$^{o}$C and then at room temperature for 30 minutes. Six week old WT and +*RB* tubers were inoculated using a modified whole tuber assay (Millett 2008), as detailed below.

For RNA extractions and RNA-seq, three tubers of each genotype were randomly selected, washed with deionized water, and allowed to air dry at room temperature for 24 hours. Tubers were wounded [0.2cm*0.3cm (depth*diameter)] at six sites spaced uniformly (~3cm apart) across the tuber equator. Each wound was inoculated with 10 µl sporangial suspension or water (mock treatment). Inoculated tubers were stored in tightly sealed dark boxes under high (~95%) humidity and at room temperature for 72 hours (during which time all RNA-seq samples were collected as specified below) and then moved to 11-13$^{o}$C for disease development and phenotyping at 11 days post inoculation. From each of three replicate tubers per genotype, tuber tissue (0.7cm*(0.5-0.8)cm) was collected using a cork borer from an inoculation site at time point 0 (pre-inoculation) and at 6, 12, 24, and 48 hours post inoculation (hpi). The sampled tissue includes cells from the periderm, cortex and medulla layers. The sixth inoculation site on each tuber was left intact and the tubers were phenotyped for late blight disease development 11 days later by multiplying measured length, width, and depth of disease lesions. Collected tissue samples (one tuber core per replicate tuber for each time point) were immediately frozen in liquid nitrogen and stored at -80$^{o}$C. In total, 36 tissue samples from the two plant genotypes (WT and +*RB*) x three time points (0, 24, and 48 hpi) x two inocula (*P. infestans* or water) x three replicates were employed for RNA extraction. [Note: We collected tissue from 0, 6, 12, 24, 48 hpi, but only 0, 24, 48 hpi samples were subjected to RNA-seq]. Total RNA was extracted from frozen tissue using the SV Total RNA Isolation System (Promega Corporation, Madison, WI) according to manufacturer's instructions. The quantity and quality of RNA samples were assessed using a Nanodrop

1000 machine (Thermo Fisher Scientific Inc., Wilmington, DE). High quality total RNA (5ug, 100ng/µl) samples were sent to the University of Minnesota BioMedical Genomics Center (BMGC) for RNA-seq library preparation using the TruSeq SBS Kit (50 Cycles) (Illumina Inc., San Diego, CA) and paired end sequencing using an Illumina Hi-Seq 2000 machine (Illumina).

A separate, additional set of tubers (nine for WT and eighteen for +*RB*) were simultaneously subjected to the whole tuber assay (using the same inoculum and protocol described above), but without tissue collection, for the purposes of disease phenotyping.

## RNA-seq reads mapping and DE genes clustering

RNA-seq reads were quality filtered using SolexaQA packages (Cox et al. 2010) with default parameters and a length filter of greater than 27bp for both ends of each read pair. Quality filtered RNA-seq reads were analyzed using the "Tuxedo Suite" software packages (Trapnell et al. 2012): Bowtie (Langmead et al. 2009) v0.12.7, Tophat (Trapnell et al. 2009) v1.3.2, Cufflinks (Trapnell et al. 2010) v1.1.0 and a reference genome of potato (Xu et al. 2011). We suppressed Tophat (Trapnell et al. 2009) from identifying novel junctions. A total of eleven pair-wise comparisons [between time (4), between line (3), between treatment (4)] were made using Cuffdiff of the Cufflinks software packages. Differentially expressed (DE) genes are those genes that showed significantly (FDR adjusted p-value <0.001) different transcript levels among comparisons. False discovery rate correction (FDR) was done using the Benjamini Hochberg method (Benjamini and Hochberg 1995).

Quality filtered RNA-seq reads were also mapped to reference transcript sequences of *P. infestans* (Haas et al. 2009) and different classes (see Champouret et al. (Champouret et al. 2009) for details of classification) of *ipiO* (potential cognate avr effector of *RB*) gene sequences. The mapping was done using Bowtie (Langmead et al. 2009) v0.12.7.

Potato gene expression fold change values were used to cluster and partition genes into groups using hierarchical clustering and the complete linkage method in Cluster 3.0 (Eisen et al. 1998) and visualized in Treeview software (Saldanha 2004) using adjusted pixel settings (threshold 1.0).

The UNIX command line options for Tophat and Cuffdiff were as follows: "tophat -G genes.gff -o sample.out --no-novel-juncs -r 100 genome.fasta read.left.fq read.right.fq; cuffdiff -N -u -O cuffdiff.out genes.gff 1.bam,2.bam,3.bam 4.bam,5.bam,6.bam". The UNIX command line options for Bowtie were as follows: "Bowtie -a -v 2 --best -M 1--fr ref_bowtie_build -1 read1.fq -2 read2.fq". Custom perl scripts were used to parse the mapping/alignment results into tabular formats. R statistical software (R Development Core Team 2011) was used to generate various plots.

## Functional assignment and MapMan analysis of potato genes

To assign potato genes into functional categories (bins), we adopted MapMan ontology (Thimm et al. 2004). The Mercator annotation pipeline (http://mapman.gabipd.org/web/guest/app/mercator) was used to assign potato genes into functional bins by searching a variety of reference databases. The ratios of the numbers

of the genes in to not in an ontology bin were compared between the whole gene set and selected gene set using Fisher's exact test. If the ratio is significantly higher for the selected gene set, the set is called enriched in the ontology category. The log2 transformed fold change values for genes within an ontology bin were compared to the log2 transformed fold change values for all genes outside of the ontology bin using Wilcoxon rank-sum test to identify functional bins that show higher or lower induction. Resulting p-values from Fisher's exact tests or Wilcoxon rank-sum tests were subjected to multiple test correction using the Benjamini Hochberg method (Benjamini and Hochberg 1995).

## Real-time quantitative RT-PCR

For determining the transcript levels of the transgene *RB*, we used a previously established qRT-PCR protocol (Bradeen et al. 2009; Millett and Bradeen 2007). For RNA-seq validation, Primer Express 3.0 (Applied Biosystems, Foster City, CA, USA) was used with default parameters to generate primer pairs for selected transcripts (Table 2.1). Total RNA (150ng, 30ng/ul) from diluted stocks of the same RNA that was subjected to RNA-seq was used in each reverse transcription reaction using the SuperScript III First Strand Synthesis Kit (Life Technologies Inc., Carlsband CA) according to manufacturer's instructions. All qPCRs were performed using the Power SYBR Green Master Mix (Life Technologies) and an ABI 7500 Real Time System (Applied Biosystems). Target gene transcript levels were normalized to *EF1α* (Nicot et al. 2005) using $2^{-\Delta CT}$ values. Normalized gene expression levels were compared with RNA-seq FPKM values derived from Cufflinks.

## Results

### The +*RB* transgenic line exhibits enhanced tuber late blight resistance

In previous field-based evaluations, the +*RB* line examined in this study, SP2211, was ranked as the third most foliar late blight resistant of 57 transgenic lines tested and was rated as "Resistant" to foliar late blight (Bradeen et al. 2009). In contrast, the WT line (nontransformed 'Russet Burbank') was rated as "Susceptible" to foliar late blight (Bradeen et al. 2009). Here, in replicated whole tuber assays performed six weeks after harvest, the +*RB* line showed no tuber late blight disease after normalization to the water-inoculated controls, whereas the WT line showed clear tuber late blight disease development (Figure 2.1). Results from two sets of whole tuber experiments were in agreement (32 of 33 *P. infestans* inoculations of WT tubers resulted in disease, none of the 60 inoculation sites in +*RB* tubers developed late blight disease). Thus, while WT is susceptible to *P. infestans* infection in both foliage and tuber, the *RB* transgene renders the +*RB* line resistant to *P. infestans* infection in both foliage and tuber. *RB* gene transcription in the tubers of the +*RB* line was unaltered by inoculation with *P. infestans* at 0, 24, 48 hpi ($p>0.05$).

### RNA-seq reads aligned well with the potato reference genome sequence

A total of 36 RNA samples, collected from three bio-reps of *P. infestans*- or water-inoculated tuber tissues of two potato genotypes (WT and +*RB*) at three time points post inoculation, were subjected to RNA-seq. Approximately 483 million paired end reads were generated, yielding an average of 13.4 million reads per sample. A total of

436.3 million Illumina reads (90.3%) passed quality filtering. The majority of reads (380.2 million or 78.7% of all reads) could be mapped uniquely to one location within the doubled monoploid (DM) potato reference genome sequence (Xu et al. 2011) (Figure 2.2). An additional 16.6 million reads (3.5%) were mapped to multiple locations within the reference genome sequence (Figure 2.2).

We detected transcription of 29,319 potato genes based on cufflinks FPKM information and gene models reported by the Potato Genome Sequencing Consortium (PGSC) (Xu et al. 2011). Around 20% of RNA-seq reads that passed quality filters were mapped to regions outside of the current potato reference genome gene models (or predicted exons). qRT-PCR results correlated well with RNA-seq data with an average Pearson correlation coefficient of r=0.86 (Table 2.2). These results suggest that RNA-seq and qPCR approaches can be cross-validated, confirming that the potato gene models (Xu et al. 2011) are appropriate for functional genomics studies of potato tubers.

## Time rather than genotype has a greater influence on overall transcriptome dynamics

A principal component analysis (PCA) of log2 transformed FPKM values for 29,319 genes from the 36 RNA-seq samples is shown in Figure 2.3. Together, PC1 and PC2 explained >40% of the total variance of this dataset. Samples collected at 0 hpi were distinct from all other samples, with PC1 providing clear separation of 0 hpi samples from 24 hpi and 48 hpi samples. PC1 also differentiates water- and *P. infestans*-inoculated samples at 48 hpi. Overall, the distribution of +*RB* samples is similar to that of

WT samples. These results indicate that time and treatments (*P. infestans*- vs. water-inoculation) play a greater role than genotype in defining overall transcriptome dynamics.

We identified 2,531 genes that are differentially expressed (DE) during at least one of the between time transitions in *P. infestans*-inoculated samples. Transcriptome dynamics of both genotypes during transitions from 0 hpi to 24 hpi and from 24 hpi to 48 hpi were visualized using MA plots (Figure 2.4). In general, the 0 hpi to 24 hpi transition revealed a greater number of DE genes. At later stages of infection (24 hpi – 48 hpi), WT tubers exhibited mostly up-regulation of genes, whereas the +*RB* line displayed approximately equal numbers of up- and down-regulated genes (Figure 2.4), potentially reflecting contrasting rates of pathogen proliferation (as indicated by total pathogen reads counts, see results sections below) and corresponding host response patterns. Consistent with PCA analysis which shows that genotype has a lesser role (compared to time) in defining overall transcriptome dynamics, fewer genes were determined to be DE during between line comparisons, despite a trend of increasing numbers of DE genes over time.

## The +*RB* transgenic line has faster and stronger induction of DE genes at 48 hpi

A total of 1,102 DE genes were identified when water- and *P. infestans*-inoculated samples were compared (between treatment comparisons) at the same time points (24 hpi or 48 hpi). We identified two representative hierarchical clusters of genes showing interesting regulation patterns at 48 hpi (Figure 2.5). In WT, these genes were suppressed (compared to water-inoculated); in +*RB*, these genes were induced (compared to water-inoculated). These genes include ethylene response factors (*ERF2*), cellulose

synthase, chitinase, and elicitor inducible cytochrome P450. Two genes specifically associated with the hypersensitive response, PGSC0003DMG400025335 [*Hypersensitive-induced reaction protein* (*HIR*); Figure 2.5] and PGSC0003DMG400027473 (*Hypersensitive response assisting protein*) were significantly induced in the +*RB* line but not in the WT line at 48 hpi. The +*RB* line displayed 903 DE genes at 48 hpi, accounting for about 82% of the total DE genes identified during between treatment comparisons (Figure 2.6). These results suggest that the +*RB* line responds to *P. infestans* attack by rapid differential regulation of large sets of genes, whereas the WT line is slower to respond.

## Ontology bins and gene groups distinguishing compatible and incompatible interactions

MapMan ontology enrichment analysis of DE genes (Figure 2.6) revealed that WRKY transcription factors are up-regulated in +*RB* but not in WT at 48 hpi. Strikingly, 13 of the 14 (92.8%) DE (during water- vs. *P. infestans*-inoculated comparisons) WRKY transcription factor genes show significant induction in the +*RB* line at 48 hpi (Table 2.3). In contrast, none of the 14 WRKY transcription factor genes showed statistically significant (FDR=0.001) induction in the WT line at 48 hpi. Homologs of several of these WRKY transcription factors have been previously reported to be involved in defense against *Phytophthora* spp. and other pathogens (Katou et al. 2005; Nakazawa-Ueji et al. 2010). We hypothesize that DE WRKY transcription factors identified in this study are downstream regulatory components of *RB*-mediated defenses in potato tubers.

Very importantly, we discovered that when WT and +*RB* were compared directly, the +*RB* line displays faster and stronger transcription of genes within receptor kinase and defense related bins, even at 0 hpi (Figure 2.7). It is especially relevant to note that many of the ontology bins commonly associated with plant defense, including receptor kinases and *PR* (pathogenesis-related) protein genes are more highly expressed in the +*RB* line. Interestingly, WT actually has much higher transcription for genes within those similar defense-related bins at 24 hpi (but not 48 hpi) if an indirect comparison (adjusting FPKM values of *P. infestans*-inoculated samples by FPKM values of water-inoculated samples of the same genotype) was made, indicating that WT and +*RB* probably share large sets of defense components. These findings suggest that faster and stronger expression of defense related genes plays a role in enhanced disease resistance in the +*RB* line. Faster and stronger activation of defense related bins was also observed in an independent pilot RNA-seq study (Illumina GAIIx single end reads) using field grown tubers of the same genotypes from a different year.

## RNA-seq reads mapping to *Phytophthora infestans* reference transcripts

A total of 17,353 paired end sequence reads were mapped to the *P. infestans* reference transcript set (Haas et al. 2009), representing the transcription of over 4,600 genes (out of 17,797 genes total). Ninety-six *P. infestans* transcripts are predicted to encode RxLR effectors (Haas et al. 2009). Throughout the infection time course, a trend of increasing mappable reads is evident for WT samples. This increase of mappable reads was not seen in the +*RB* line from 24 hpi to 48 hpi (Figure 2.8) and a majority (>82%) of all mapped reads originated from infected WT potato tuber samples collected at 48 hpi.

This is in agreement with tuber late blight disease development observed in WT but not +*RB* tubers. In total, a majority of all *P. infestans* reads (>95%) were from infected WT potato tuber samples; less than 5% of the reads were from infected +*RB* potato tuber samples.

Class I and II ipiO proteins, members of the RxLR family, induce avirulence responses in potato lines containing *RB* (Champouret et al. 2009; Pieterse et al. 1994; Vleeshouwers et al. 2008). After inoculation (24, 48 hpi), class I *ipiO* transcripts were detected (though in low abundance) in five of six tuber samples collected from the *P. infestans*-inoculated WT line, but from no samples derived from the +*RB* line. This is probably due to suppression of *P. infestans* proliferation in the +*RB* line.

## Discussion

### Faster and stronger activation of defense related genes, gene groups and ontology bins correlate with successful tuber defense

Direct comparison between WT and +*RB* lines at 0 hpi revealed a total of 11 DE genes. Seven of the eleven DE genes were more highly transcribed in +*RB*. These include Carbonic anhydrase (PGSC0003DMG400006956), Aquaglyceroporin (PGSC0003DMG400009604), Cytochrome P450 (PGSC0003DMG400015185), Malic enzyme (PGSC0003DMG401026923), and 1-aminocyclopropane-1-carboxylate oxidase (ACO) (PGSC0003DMG401026923). Interestingly, these genes or homologs of these genes were previously reported to be involved in basal or pathogen-induced defense

responses in plants (Ishihama et al. 2011; Restrepo et al. 2005; Yu et al. 2011). These results demonstrate constitutive transcription of defense related genes in the +*RB* line.

GO enrichment analysis of DE genes identified during between treatment comparisons (Figure 2.6) suggests that +*RB* has faster response to pathogen attack, as evidenced by higher and statistically significant induction of ontology bins commonly associated with plant defense (e.g., ET, WRKY, Signaling receptor kinases) at 48 hpi. One notable example is WRKY transcription factors (Table 2.3), with 13 out of 14 WRKY transcription factors significantly induced in +*RB*. Many of the same transcription factors are also induced, but at less significant levels, in the WT line, documenting that more potent and rapid activation of defense related genes, rather than novel resistance responses *per se,* might play a role in enhanced resistance in the +*RB* line.

Furthermore, when a more holistic approach (using all available data points: 39,031 gene transcript values per sample) was employed to identify differentially regulated ontology bins, various defense related ontology bins were discovered (Figure 2.7). +*RB* potato tubers displayed faster and stronger transcription of genes within bins commonly associated with plant defense such as receptor kinases and pathogenesis-related (*PR*) genes (Figure 2.7). Together, these results suggest that the +*RB* line is tuber late blight resistant due to faster and stronger activation of defense related components.

Cao et al. (Cao et al. 2007) found that increased transcription of the rice *Xa3* resistance gene correlated with enhanced expression of defense-responsive genes and an enlarged resistance spectrum to *Xanthomonas oryzae* pv. *oryzae* (Xoo). Previously, we

demonstrated that higher transgene *RB* copy numbers correlated with both higher *RB* transcript levels and enhanced late blight resistance in the foliage (Bradeen et al. 2009). The *+RB* line employed in this study, SP2211, displayed the highest tuber transgene transcription levels among 11 transgenic lines examined (Millett 2008). Thus, the unusually high levels of *RB* gene transcription in the tubers of SP2211 correlates with faster and stronger activation of defense related components (Figure 2.6-7). The faster and stronger up-regulation of these defense-related components very likely contributed to the observed successful defense against *P. infestans* (Figure 2.1) in this line. One interesting observation is that WT actually has much higher transcription of similar defense-related bins at 24 hpi (but not 48 hpi) in indirect (adjusting FPKM values of *P. infestans*-inoculated samples by FPKM values of water-inoculated samples of the same genotype) comparisons. Collectively, our data support a model of tuber blight resistance resulting as a function of faster and stronger expression of defense related genes due to high *RB* transcript levels.

## HR is likely a general form of resistance to *P. infestans*, even in potato tubers

Prevailing scientific thought suggests that the phyllosphere frequently encounters biotrophic or hemibiotrophic pathogens, against which the HR is effective. In contrast, roots are more likely to encounter necrotrophic microbes that would theoretically benefit from the HR (Glazebrook 2005). Consistently, working in Arabidopsis, Hermanns et al. (Hermanns et al. 2003) showed that HR is present in above ground incompatible host-pathogen interactions, but absent during the same host-pathogen interaction in the roots—

despite R gene transcription in both leaves and roots. These authors concluded that R gene function is modulated in an organ-specific, but mechanistically poorly defined manner for the purpose of suppressing HR in the roots. Observations of differential late blight resistance levels in potato foliage and tubers within a single genotype (Kirk et al. 2001) suggest that R gene function in potato may also be modulated in an organ-specific manner. But the potato tuber, although it develops below ground, is a modified stem, not a root. The identification of SP2211, a +*RB* transgenic line with both foliar and tuber late blight resistance provided opportunity to examine the role of HR in defending non-root organs against pathogen attack.

*P. infestans* is a hemibiotrophic pathogen and Kamoun et al. (Kamoun et al. 1999) argued that HR is likely a general form of resistance against *Phytophthora* pathogens. In agreement, Chen and Halterman (Chen and Halterman 2011) demonstrated that *RB* triggers an HR in potato foliage challenged with *P. infestans.* In examining *RB*-mediated tuber responses to *P. infestans*, we identified DE genes associated with the HR (potato *HIR* and hypersensitive response assisting genes) that are highly up-regulated in pathogen-inoculated tuber samples of SP2211 compared to water-inoculated tuber samples at 48 hpi. Previous studies have shown that the pepper homolog of *HIR* is a positive regulator of hypersensitive cell death (Jung and Hwang 2007; Jung et al. 2008). Qi et al. (Qi et al. 2011) also demonstrated that the NBS-LRR protein RPS2 forms complexes with AtHIR proteins in Arabidopsis and tobacco plants, providing mechanistic insight into R protein function and the HR. We hypothesize that RB and HIR might form similar complexes in potato, a topic that warrants further experimentation. Rapid

production of reactive oxygen species by plants is a hallmark of pathogen recognition and correlates with the HR. Working with disks cut from potato tubers that carry the *R1* late blight resistance gene, Doke (Doke 1983) demonstrated that incompatible but not compatible races of *P. infestans* triggered production of reactive oxygen species. In our study, a gene (PGSC0003DMG400024754) encoding respiratory burst oxidase homolog protein B (NADPH oxidase RBOHB) was induced (based on between treatment comparisons) 6.6 fold in the *+RB* line but not in the WT line 48 hours after *P. infestans*-inoculation. This gene is known to be involved in the massive phase II oxidative burst induced in potato by pathogen infection (Yoshioka et al. 2001) and offers further support that *RB*-mediated resistance to tuber late blight likely entails an HR or HR-like phenomenon.

Interestingly, HR is commonly associated with SA (salicylic acid) mediated responses. But our ontology bin analysis did not reveal SA metabolism as a differentiating factor for the tuber blight resistant and susceptible lines. Instead, our study reveals that the ET (ethylene) bin is predominantly associated with the incompatible interaction. Leon-Reyes et al. (Leon-Reyes et al. 2009) reported that the antagonistic relationship between SA and JA (Niki et al. 1998) only exists when there is no strong production of ET. Nunez-Pastrana et al. (Nunez-Pastrana et al. 2011) reported that ethylene but neither SA nor Methyl JA (jasmonic acid) induces a resistance response against *Phytophthora capsici* in pepper. Extensive hormonal crosstalk (Robert-Seilaniantz et al. 2011) during plant responses to pathogens is a topic of extensive ongoing research and future results will likely yield new insights that will allow fine-

tuning of our understanding of potato tuber-pathogen interactions, and organ-specific defense responses in plants.

## Conclusion

We presented the first RNA-seq (or transcriptome dynamics) study focused on potato tuber responses to pathogen attack. We mapped approximately 400 million RNA-seq reads onto the recently published potato reference genome sequence, documenting the utility of RNA-seq for biological study of tetraploid cultivated potato. Our results showed that time and treatment rather than genotype has a larger influence on overall transcriptome dynamics, although sets of DE genes that distinguish resistant and susceptible lines were identified. Our data suggest that potent regulation of defense genes and gene groups or ontology bins (e.g., *HIR* and WRKY transcription factors) plays a role in *RB*-mediated tuber defense. In particular, faster and stronger expression of defense related genes correlates with enhanced tuber blight resistance in the +*RB* transgenic line. In agreement with Kamoun et al. (Kamoun et al. 1999) and Doke (Doke 1983), our data suggest that HR is likely a general form of resistance against late blight even in the potato tuber, an organ that develops below ground.

**Table 2.1 qRT-PCR primer sequences for 20 selected potato genes**

| gene | transcripts | Forward | Reverse |
|---|---|---|---|
| PGSC0003DMG400001227 | PGSC0003DMT400003089 | GCCAAGACTGTACGAGGAGAAAA | CACAATGACGGATAGCGTACAAG |
| PGSC0003DMG400005455 | PGSC0003DMT400013939 | TCTTGATGTTGGTGGGTTGACT | TCATTTGCAGTGTTCTTGATATCATTC |
| PGSC0003DMG400006313 | PGSC0003DMT400016152 | GCCTTTCGGGTTGGTTGTG | CATGGTCATGGGTGCTTCAA |
| PGSC0003DMG400007996 | PGSC0003DMT400020638 | GCACCATGGACAGAGACTTTTACC | TGATAGCCTTCAAATGATCCATAGG |
| PGSC0003DMG400008021 | PGSC0003DMT400020698 | GCTGGCTCCTCGAATACAAGA | TCTCCGTCCTTCCATAGCTTCT |
| PGSC0003DMG400009671 | PGSC0003DMT400025041 | AGTAGCCAATGTTCAGGTTCAGAGT | TCCTCCACAACAGCTCAGTACAA |
| PGSC0003DMG400010713 | PGSC0003DMT400027828 | GCTTCGTCAAGCGATTCCA | CACGAAGTGGCTCCCCTGTA |
| PGSC0003DMG400011627 | PGSC0003DMT400030381 | GCTTCTTCGGTGGTCGTAAGA | GGGAAGCCCTCGAATGGAT |
| PGSC0003DMG400012797 | PGSC0003DMT400033319 | CGGGCACGAGCACACTTC | GTTCACGGTCCTCTTTGCAAA |
| PGSC0003DMG400013901 | PGSC0003DMT400036094 | GCAGAAGATCTTTGTGTCACTTCAG | AGGATGCCACAGGAGAAGAGAA |
| PGSC0003DMG400014207 | PGSC0003DMT400036845 | GCTTCTTCGATGTTGACTCAATATG | CAAAACCTCTGATACAGTGACACAATT |
| PGSC0003DMG400015489 | PGSC0003DMT400040041 | GAAACGGGTCAAGCCATTGA | GCCTTGACAGTTGTTCATGGAA |
| PGSC0003DMG400017230 | PGSC0003DMT400044378 | ACCGTTATCCGCCCTAACACT | TTCATCAGATGGACCGGTGAT |
| PGSC0003DMG400017609 | PGSC0003DMT400045386 | GGCTTCCATAATCCATAACAACTCTACT | GGGATGTAGATGGCGAAGAGAT |
| PGSC0003DMG400019257 | PGSC0003DMT400049574 | GGAGCTATGATGATATCAGGACAGAA | GGGCGTTGGGCAATCC |
| PGSC0003DMG400020334 | PGSC0003DMT400052381 | TCCATTAATTCACTCGAAAAAGTCAT | TTGGGAATTCTTTGACTGATAATACG |
| PGSC0003DMG400027391 | PGSC0003DMT400070451 | TCAGATTGGTGATGGCAAAAAG | TGAGAGTTGAAGGATGGGTGATT |
| PGSC0003DMG400027682 | PGSC0003DMT400071177 | CCGGAATACCACCAAATGCT | CGTCATTGTAATCAGCCCAAAG |
| PGSC0003DMG400032159 | PGSC0003DMT400081918 | ACGACTTGCTCTGGTTCTTAAAAAG | CCTCCTATGGAATGTGTAACATATGAA |
| PGSC0003DMG402021263 | PGSC0003DMT400054790 | GGTGAAGGCTGCAAGAAGAGA | GCAAGCTTTCTGTAAACAGCCATA |

| | G400007996 | G400010713 | G400013901 | G400014207 | G400001227 | G400011627 | G400032159 | G400005455 | G400006313 | G400008021 | G400009671 | G400012797 | G400015489 | G400017230 | G400017609 | G400019257 | G400020334 | G402021263 | G400027391 | G400027682 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| samples | qPCR | qPCR | qPCR | qPCR | qPCR | qPCR | qPCR | qPCR | qPCR | qPCR | qPCR | qPCR | qPCR | qPCR | qPCR | qPCR | qPCR | qPCR | qPCR | qPCR | |
| RB_0h | 0.022017 | 0.688369 | 0.052932 | 0.036012 | 0.001176 | 0.065771 | 0.000484 | 0.013791 | 0.011197 | 0.015466 | 0.02948 | 0.000163 | 0.012257 | 0.043959 | 0.012057 | 0.000196 | 0.003851 | 0.00523 | 0.007414 | 0.004346 | |
| RB_24h | 0.023645 | 0.002428 | 0.016707 | 0.026639 | 0.011111 | 0.007547 | 0.001159 | 0.006743 | 0.006114 | 0.017349 | 0.016336 | 0.000118 | 0.052302 | 0.023706 | 0.009641 | 7.79E-05 | 0.037953 | 0.008175 | 0.004001 | 0.008709 | |
| RB_48h | 0.014662 | 0.003649 | 0.005823 | 0.035312 | 0.008251 | 0.005491 | 0.001023 | 0.009018 | 0.002651 | 0.023879 | 0.01399 | 0.000157 | 0.046322 | 0.027777 | 0.007302 | 6.28E-05 | 0.040312 | 0.005993 | 0.002937 | 0.006186 | |
| RB_mock_0h | 0.026259 | 0.461358 | 0.022498 | 0.027333 | 0.001331 | 0.03561 | 0.000328 | 0.025079 | 0.012207 | 0.041655 | 0.031837 | 0.000123 | 0.013668 | 0.047903 | 0.01083 | 0.000153 | 0.005507 | 0.003963 | 0.002706 | 0.004763 | |
| RB_mock_24h | 0.017625 | 0.003605 | 0.015404 | 0.040249 | 0.008197 | 0.010267 | 0.001771 | 0.006554 | 0.005085 | 0.014848 | 0.013643 | 0.000156 | 0.049136 | 0.027537 | 0.011924 | 9.07E-05 | 0.012145 | 0.01099 | 0.002264 | 0.007399 | |
| RB_mock_48h | 0.01094 | 0.002099 | 0.008603 | 0.027107 | 0.009324 | 0.005824 | 0.00082 | 0.006539 | 0.005637 | 0.008426 | 0.011319 | 0.000244 | 0.023303 | 0.022232 | 0.010935 | 0.000101 | 0.007873 | 0.006536 | 0.001695 | 0.003889 | |
| WT_mock_0h | 0.034832 | 0.131898 | 0.043333 | 0.035879 | 0.000711 | 0.027414 | 0.00047 | 0.010473 | 0.011095 | 0.028577 | 0.027781 | 8.79E-05 | 0.017085 | 0.046555 | 0.011717 | 0.000273 | 0.009229 | 0.004891 | 0.011327 | 0.005519 | |
| WT_mock_24h | 0.020752 | 0.004625 | 0.018331 | 0.039848 | 0.008072 | 0.010358 | 0.001508 | 0.00642 | 0.003903 | 0.014725 | 0.013859 | 0.000136 | 0.056265 | 0.030383 | 0.011083 | 8.43E-05 | 0.023175 | 0.009935 | 0.004233 | 0.009102 | |
| WT_mock_48h | 0.011627 | 0.003722 | 0.011189 | 0.030517 | 0.003611 | 0.00603 | 0.001264 | 0.005216 | 0.004476 | 0.009334 | 0.016227 | 0.000207 | 0.028057 | 0.023813 | 0.009478 | 0.000117 | 0.014755 | 0.006769 | 0.003542 | 0.004641 | |
| WT_0h | 0.014758 | 0.811712 | 0.041763 | 0.034218 | 0.001395 | 0.062894 | 0.000661 | 0.012871 | 0.020742 | 0.004618 | 0.031037 | 0.000178 | 0.012656 | 0.026651 | 0.012634 | 0.000259 | 0.007977 | 0.00695 | 0.015012 | 0.004862 | |
| WT_24h | 0.023056 | 0.002091 | 0.016173 | 0.044357 | 0.007448 | 0.00695 | 0.00119 | 0.008232 | 0.006851 | 0.012608 | 0.012257 | 0.000125 | 0.06063 | 0.024623 | 0.011368 | 7.81E-05 | 0.03225 | 0.009245 | 0.004366 | 0.010035 | |
| WT_48h | 0.017259 | 0.005721 | 0.007627 | 0.034317 | 0.008395 | 0.004399 | 0.00147 | 0.006929 | 0.003509 | 0.017349 | 0.020998 | 0.000172 | 0.036542 | 0.032627 | 0.007623 | 7.67E-05 | 0.063788 | 0.006415 | 0.003121 | 0.003956 | |
| | | | | | | | | | | | | | | | | | | | | | |
| samples | RNA-seq | RNA-seq | RNA-seq | RNA-seq | RNA-seq | RNA-seq | RNA-seq | RNA-seq | RNA-seq | RNA-seq | RNA-seq | RNA-seq | RNA-seq | RNA-seq | RNA-seq | RNA-seq | RNA-seq | RNA-seq | RNA-seq | RNA-seq | |
| RB_0h | 9.370953 | 466.345 | 67.99487 | 18.4105 | 1.531679 | 90.5075 | 0.969719 | 17.21627 | 3.57804 | 11.17962 | 20.25573 | 11.1449 | 12.83476 | 43.23313 | 10.13824 | 89.2666 | 6.81094 | 8.31256 | 12.23591 | 10.33177 | |
| RB_24h | 9.106957 | 1.098295 | 29.96483 | 28.06367 | 28.42513 | 8.144493 | 1.864873 | 9.17313 | 1.018827 | 8.034093 | 10.25739 | 13.31227 | 64.6428 | 25.26153 | 6.049603 | 38.3778 | 26.7947 | 10.51053 | 4.795813 | 14.8926 | |
| RB_48h | 9.237457 | 2.910833 | 24.17373 | 32.50013 | 16.8261 | 6.464883 | 2.595493 | 13.8756 | 0.672317 | 11.5853 | 12.3889 | 12.25157 | 74.35527 | 32.18587 | 5.81777 | 34.76223 | 35.33063 | 9.057663 | 3.774863 | 11.11017 | |
| RB_mock_0h | 15.48009 | 341.1127 | 59.95117 | 21.95183 | 1.482992 | 49.88643 | 0.663221 | 24.51897 | 2.56671 | 34.15793 | 19.60253 | 9.761403 | 18.57335 | 59.16877 | 6.694493 | 65.40103 | 10.03822 | 6.101823 | 6.620697 | 11.27248 | |
| RB_mock_24h | 7.189817 | 2.02819 | 30.632 | 24.0745 | 16.46503 | 9.38093 | 2.601 | 11.20882 | 1.2478 | 5.41523 | 6.96429 | 13.93897 | 64.80917 | 29.17917 | 7.653793 | 48.4998 | 13.24278 | 13.29023 | 5.15643 | 16.54987 | |
| RB_mock_48h | 5.04011 | 0.922497 | 30.3524 | 25.30733 | 18.28263 | 7.417783 | 2.48271 | 9.463 | 1.477841 | 4.846553 | 6.73655 | 22.55877 | 39.06063 | 24.77037 | 7.283403 | 72.26413 | 10.8277 | 9.260777 | 5.541937 | 10.35963 | |
| WT_mock_0h | 14.13228 | 156.2986 | 54.53613 | 22.7754 | 3.07192 | 32.4936 | 0.6881 | 24.71393 | 3.028138 | 36.57142 | 21.5127 | 9.434177 | 21.1456 | 58.44257 | 12.40329 | 92.0168 | 12.26568 | 6.445303 | 18.54242 | 12.77227 | |
| WT_mock_24h | 9.325093 | 2.298177 | 33.55087 | 24.0397 | 20.58813 | 11.82181 | 2.78213 | 10.48376 | 0.696432 | 5.493563 | 7.573707 | 14.94213 | 64.1722 | 31.073 | 7.846567 | 36.0072 | 16.50282 | 11.71537 | 4.434323 | 15.28363 | |
| WT_mock_48h | 6.433903 | 3.490754 | 38.55577 | 22.57147 | 15.48548 | 7.19032 | 2.899733 | 10.43903 | 1.798227 | 4.10078 | 10.53809 | 20.08463 | 32.48897 | 24.30537 | 8.57638 | 58.65157 | 12.13471 | 9.489643 | 5.357267 | 10.45744 | |
| WT_0h | 6.273053 | 528.038 | 59.4621 | 14.03893 | 0.94209 | 110.2415 | 1.365359 | 15.2384 | 6.37627 | 0.877012 | 24.9362 | 14.05413 | 14.49103 | 27.54817 | 13.12427 | 118.1887 | 8.547827 | 10.68265 | 19.79367 | 9.613573 | |
| WT_24h | 8.582747 | 1.067026 | 28.72257 | 28.99927 | 17.5523 | 10.38663 | 3.567663 | 10.64108 | 1.527503 | 5.248167 | 7.588367 | 14.87003 | 69.30943 | 26.116 | 6.03984 | 27.9304 | 22.48343 | 11.08992 | 4.43141 | 17.57173 | |
| WT_48h | 10.2695 | 3.44658 | 25.49863 | 26.7658 | 19.63607 | 6.801893 | 3.049347 | 13.6721 | 1.656323 | 10.14416 | 19.65077 | 14.43133 | 50.72297 | 39.71873 | 4.946103 | 44.08997 | 47.88247 | 8.404817 | 4.63022 | 11.87807 | |
| | | | | | | | | | | | | | | | | | | | | | average |
| **Correlation_rm** | **0.825176** | **0.994088** | **0.888481** | **0.093046** | **0.9472** | **0.985698** | **0.82015** | **0.782713** | **0.950391** | **0.897841** | **0.933212** | **0.830691** | **0.959527** | **0.96681** | **0.682471** | **0.918851** | **0.981484** | **0.953502** | **0.950319** | **0.893122** | **0.862739** |

Table 2.2 RNA-seq FPKM and qPCR correlation. First column is potato tuber sample IDs. Remaining columns indicate qPCR results and RNA-seq FPKM values for each potato gene, as indicated. The per gene and average correlations between RNA-seq and qPCR are listed at the bottom.

**Table 2.3 Transcription of WRKY genes is highly induced in *+RB* but not WT**. Column one and two are gene ID and PGSC annotation descriptions. Columns three to six are log2 fold change values derived from between treatment comparisons (*P. infestans*- vs. water-inoculated). Values highlighted in red indicate statistically significant up-regulation (FDR=0.001).

| Gene | PGSC.annot | WT_24h | +RB_24h | WT_48h | +RB_48h |
|---|---|---|---|---|---|
| PGSC0003DMG400000211 | WRKY transcription factor | 0.361932 | -0.10115 | 1.3649 | 2.76934 |
| PGSC0003DMG400005835 | WRKY transcription factor-30 | -0.24461 | 0.227315 | 2.54673 | 3.62647 |
| PGSC0003DMG400008188 | WRKY transcription factor | -0.60055 | -0.4471 | 2.92134 | 5.6014 |
| PGSC0003DMG400011633 | WRKY-type transcription factor | 0.592792 | 0.604168 | 0.692155 | 2.70031 |
| PGSC0003DMG400016441 | WRKY protein | 0.763239 | 0.177539 | 0.007094 | 1.09009 |
| PGSC0003DMG400016769 | Double WRKY type transfactor | 0.098889 | -0.23761 | 0.283502 | 1.98727 |
| PGSC0003DMG400019824 | JA-induced WRKY protein | -0.63359 | -1.14316 | 0.855998 | 3.55835 |
| PGSC0003DMG400020206 | WRKY transcription factor-b | 1.52349 | 0.511368 | 2.55535 | 4.27083 |
| PGSC0003DMG400020608 | DNA-binding protein 3 | 0.908722 | -0.45846 | 1.61096 | 4.33967 |
| PGSC0003DMG400021895 | WRKY-type DNA binding protein | 0.182197 | -1.13779 | 0.576008 | 1.97609 |
| PGSC0003DMG400028520 | WRKY transcription factor 1 | -0.26909 | -0.6326 | 0.270862 | 1.58399 |
| PGSC0003DMG400031140 | WRKY transcription factor | 0.383975 | -3.23026 | 2.4424 | 6.9517 |
| PGSC0003DMG401010558 | WRKY-A1244 | 0.056465 | -0.15241 | -0.95852 | -2.05383 |
| PGSC0003DMG402007388 | MRNA, 1346 bp sequence | 2.87749 | -10 | 3.13664 | 5.30573 |

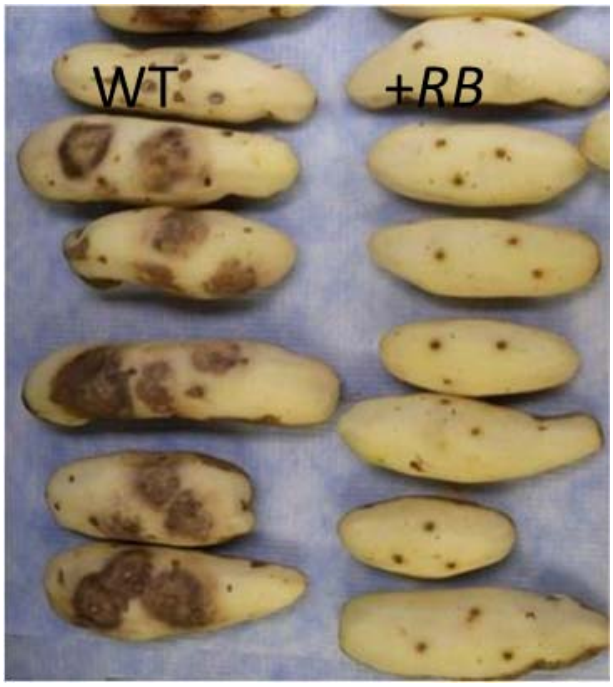


**Figure 2.1. Gene *RB* confers enhanced tuber late blight disease resistance**. Field grown tubers of nontransformed 'Russet Burbank' (WT) and SP2211 (+*RB*), a transformed 'Russet Burbank' line carrying the *RB* transgene, were mechanically wounded, inoculated with *Phytophthora infestans,* and incubated for 11 days under conditions that favor disease development. Tubers were peeled to allow assessment of diseased tissues. WT tubers consistently show robust tuber late blight disease development, revealed as darkened tissue radiating from inoculation sites. In contrast, +*RB* tubers displayed no tuber late blight disease. Note that brown spots present on +*RB* tubers are in response to mechanical wounding, not late blight disease; similar wound response was observed in water-inoculated tubers (not shown).

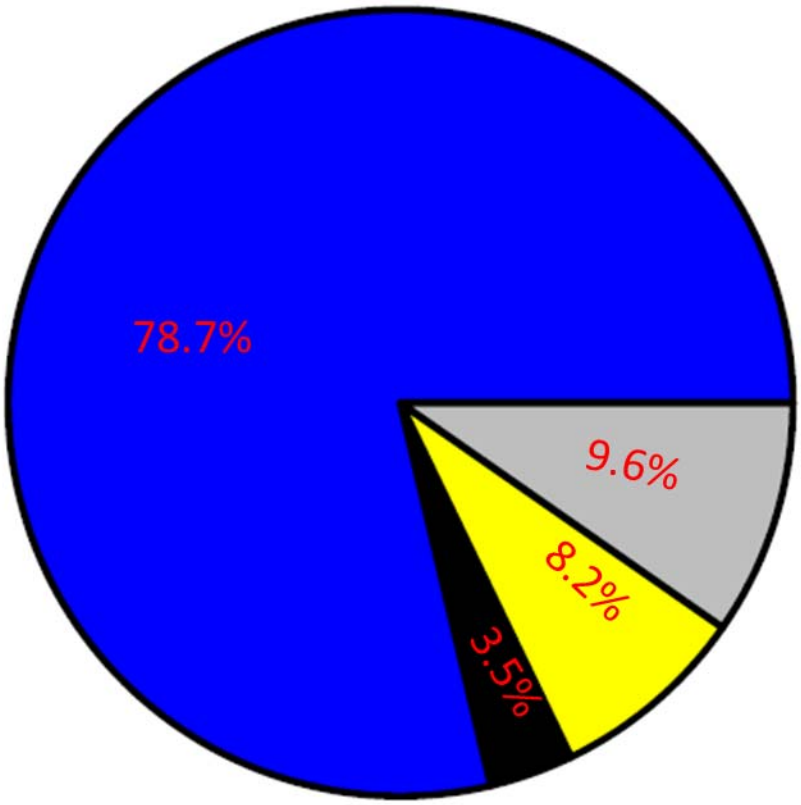


**Figure 2.2**. **A majority of RNA-seq reads map uniquely to the potato reference genome sequence**. A pie chart summarizing results of alignment of Illumina RNA-seq reads from 36 water- or *P. infestans*-inoculated WT and *+RB* tuber samples to the potato reference genome sequence. The grey portion of the chart represents RNA-seq reads that failed quality checks and were filtered out of our data set (9.6% of all RNA-seq reads). The remaining 90.4% of RNA-seq reads passed quality checks. The blue portion of the chart represents reads that mapped uniquely to the potato genome sequence (78.7% of all RNA-seq reads). The black portion of the chart represents reads that mapped to multiple locations of the potato genome sequence (3.5%). The yellow portion of the chart represents reads that failed to map to the potato genome sequence (8.2%), including <0.01% of RNA-seq reads that mapped to *P. infestans* transcripts.

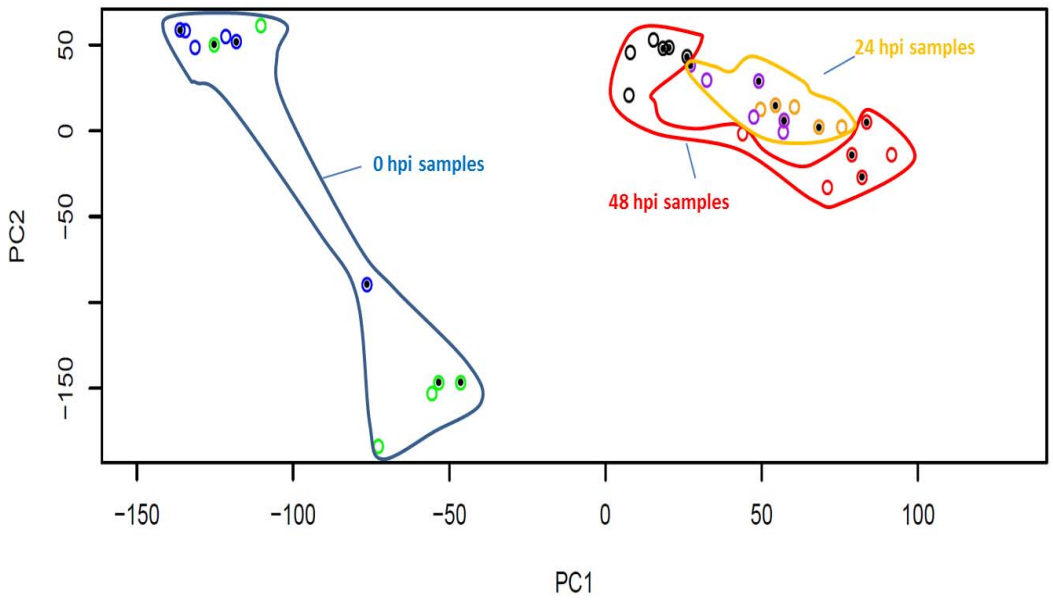


**Figure 2.3. Time rather than genotype has a larger influence on overall transcriptome differences**. FPKM (Fragment per kilobase of exon per million mapped reads) values for transcriptome sets from 36 potato tuber samples were subjected to PCA using the R statistical package (R Development Core Team 2011). Blue circles represent *P. infestans*-inoculated tuber samples collected at 0 hpi; green circles represent water-inoculated samples at 0 hpi; orange circles represent *P. infestans*-inoculated samples collected at 24 hpi; purple circles represent water-inoculated samples collected at 24 hpi; red circles represent *P. infestans*-inoculated samples collected at 48 hpi; black circles represent water-inoculated samples collected at 48 hpi. Circles containing dots were collected from the transgenic line SP2211 (+*RB*); circles without dots were collected from nontransformed 'Russet Burbank' (WT). Note that tuber samples collected at a similar time tend to cluster, regardless of genotype of origin.

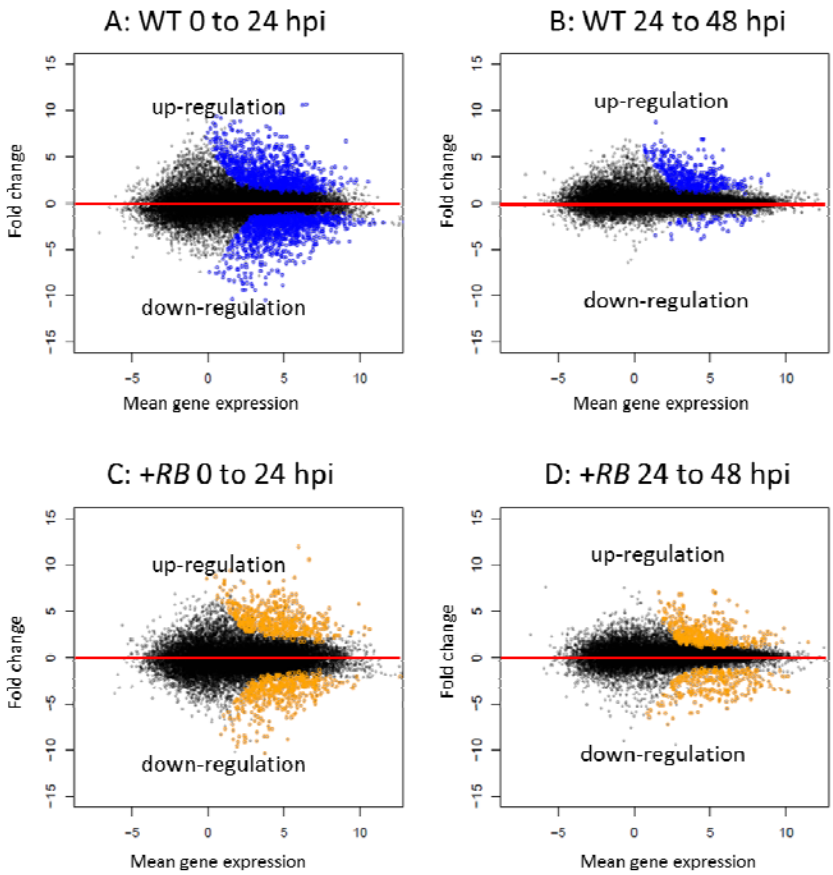


**Figure 2.4. Between time point transcriptome dynamics reveal different patterns of gene regulation in compatible and incompatible potato tuber – *Phytophthora infestans* interactions**. MA plots displaying between time point transcriptome dynamics in nontransformed 'Russet Burbank' (WT) and transgenic SP2211 (+*RB*) lines in response to *P. infestans*. X-axes indicate mean gene expression levels [0.5*(log2(FPKM1)+log2(FPKM2))] across the two selected time points for each comparison. Y-axes indicate fold change values [log2(FPKM1/FPKM2), where FPKM1 represents the later time point and FPKM2 represents the earlier time point] across the selected time points. A: changes in WT transcriptome dynamics from 0 to 24 hpi; B: changes in WT transcriptome dynamics from 24 hpi to 48 hpi; C: changes in +*RB* transcriptome dynamics from 0 hpi to 24 hpi; D: changes in +*RB* transcriptome dynamics from 24 hpi to 48 hpi. Within each panel, colored dots represent genes that are significantly (FDR=0.001) differentially regulated among comparisons. Black dots represent genes that are not significantly differentially regulated. Note that the WT line shows mostly up-regulation of genes during later stages of infection (panel B) while the +*RB* line displays approximately equal up- and down-regulation of genes (panel D).

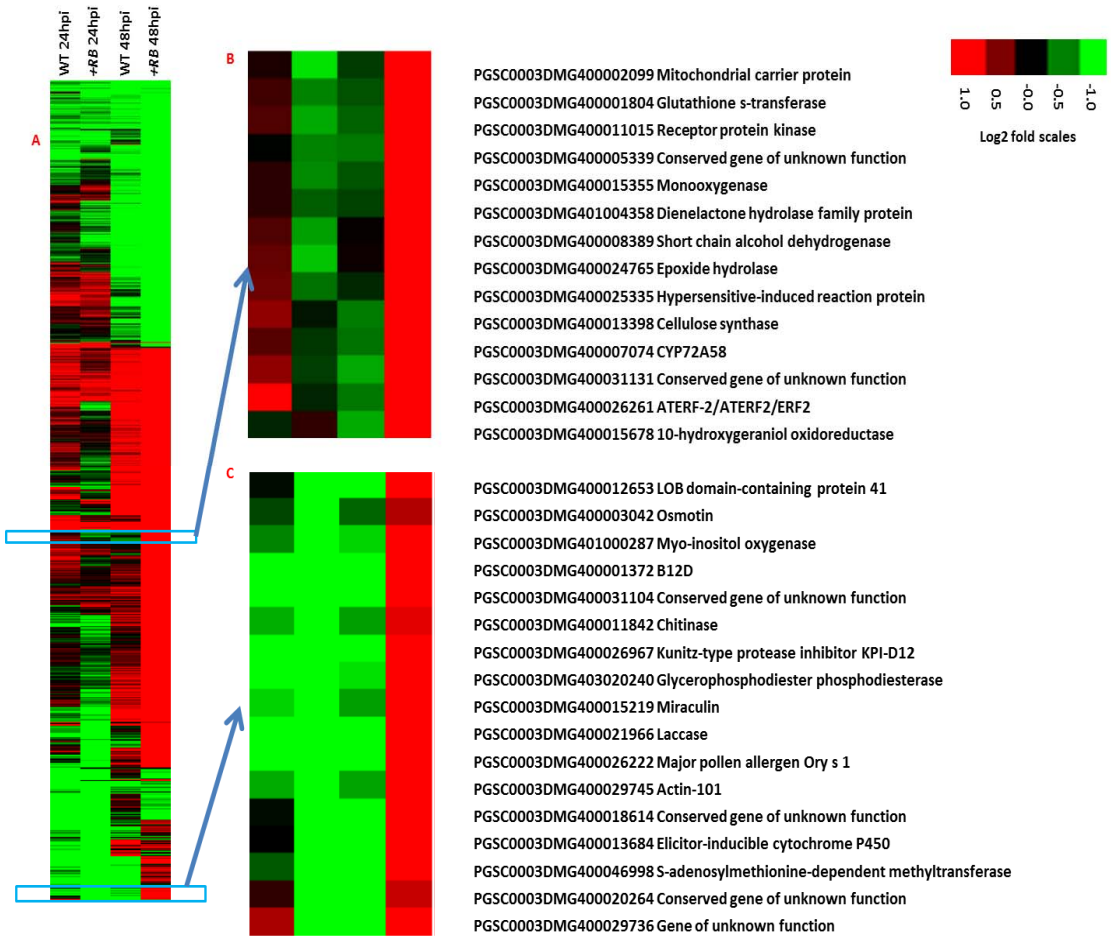


**Figure 2.5. Hierarchical clustering of differentially expressed (DE) genes in potato tubers following inoculation with *Phytophthora infestans*.** Tubers of nontransformed 'Russet Burbank' (WT) and transgenic SP2211 (+*RB*) were inoculated with *P. infestans* or water. Tuber samples collected 0, 24, and 48 hpi were subjected to RNA-seq, revealing a total of 1,102 DE genes between water- and *P. infestans*-inoculated comparisons within the same genotype and the same time. Log2(FPKM_p.inf/FPKM_mock) values were used to cluster 1,102 DE genes (FDR=0.001) in Cluster 3.0 (Eisen et al. 1998) using uncentered correlation and the complete linkage method. Results were visualized using Treeview (Eisen et al. 1998). (A) Global visualization of the 1,102 DE genes; (B) A small gene cluster differentially regulated in +*RB* and WT at 24 and 48 hpi; (C) A small gene cluster differentially regulated in +*RB* and WT only at 48 hpi. Red indicates genes that are up-regulated, green indicates genes that are down-regulated.

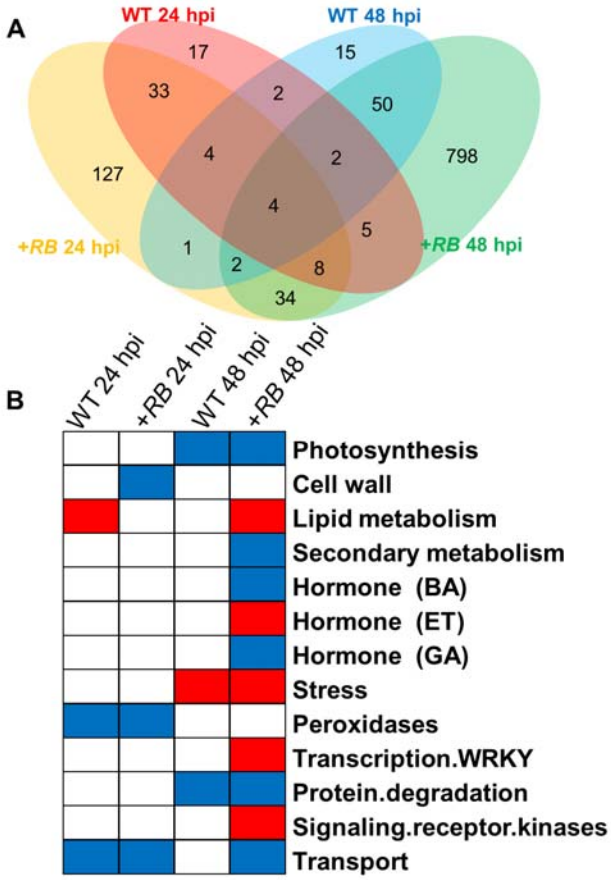


**Figure 2.6. Tubers of the +*RB* line have a higher frequency of differentially expressed (DE) genes 48 hours post inoculation (hpi) with *Phytophthora infestans*.** Tuber samples collected 0, 24, and 48 hpi were subjected to RNA-seq, revealing a total of 1,102 DE genes between water- and *P. infestans*-inoculated comparisons within the same genotype and the same time. (A) All 1,102 DE genes were analyzed using the "Venn count" function in the limma packages of R (R Development Core Team 2011) and results were summarized as a Venn diagram. Red: WT 24 hpi; orange: +*RB* 24 hpi; blue: WT 48 hpi; green: +*RB* 48 hpi. The results show that the +*RB* line is the main contributor of DE genes during water- vs. *P. infestans*-inoculated comparisons. (B) All 1,102 DE genes were also assigned to a MapMan ontology based on the Mercator mapping file (see methods), and subjected to Fisher's exact test. Bins in red were significantly up-regulated; bins in blue were significantly down-regulated; transcription of bins in white did not change significantly. The results indicate that ontology bins encompassing ET metabolism and signaling are enriched for DE genes in +*RB* but not in WT at 48 hpi.

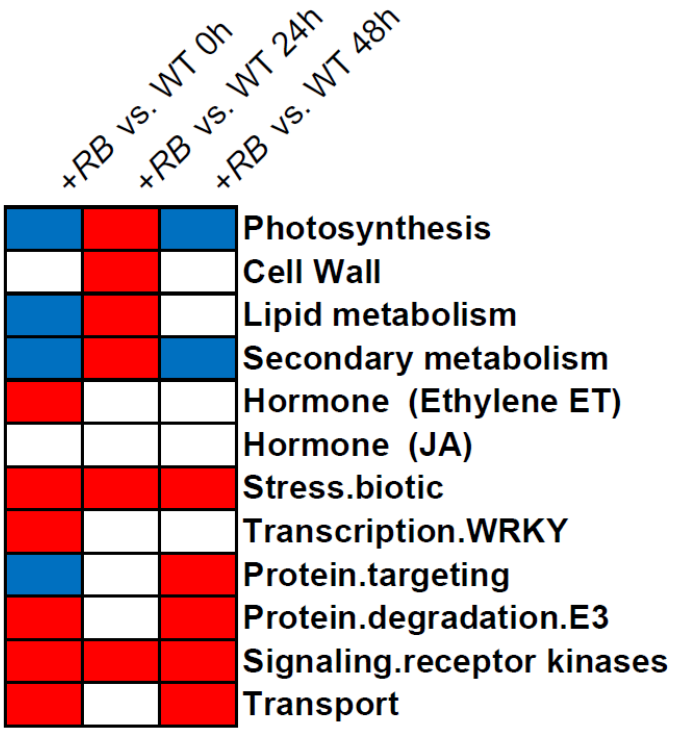


**Figure 2.7. Faster and stronger activation of defense related genes or gene groups correlates with successful tuber resistance against *P. infestans***. Tubers of 'Russet Burbank' (WT) and SP2211 (+*RB*) were inoculated with *P. infestans* and water. We compared the RNA-seq FPKM counts for WT and +*RB* using all 39,031 gene models included in the Potato Genome Sequencing Consortium (PGSC) v3 dataset (i.e., all genes were included, regardless of whether or not a given gene was DE). Genes were grouped into ontology bins using a MapMan mapping file. Each column represents a comparison between the two genotypes at a defined time point post inoculation, as indicated. Bins in blue are transcribed at higher levels in WT than in +*RB;* bins in red are transcribed at higher levels in +*RB* than in WT; bins in white did not significantly differ in transcript levels between WT and +*RB*. Results indicate that faster and stronger activation of defense bins, most notably biotic stress response and receptor kinase bins, occurred in tubers of the tuber late blight resistant +*RB* line.

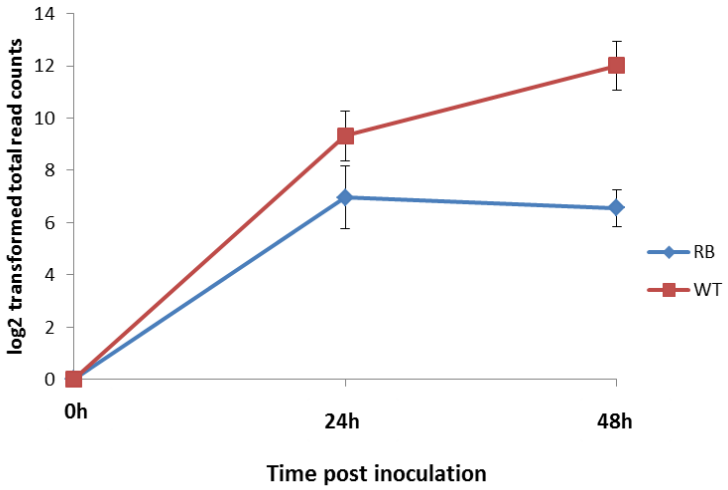


**Figure 2.8 Log2 transformed total read counts that mapped to *Phytophthora infestans* transcripts**. The X-axis indicates different time points (0, 24, 48 hpi) post *P. infestans* inoculation. The Y-axis indicates log2 transformed total mapped reads count. Results indicate an increase in *P. infestans* RNA-seq reads in the WT but not the *+RB* line over time.

# Chapter Three: Contrasting potato foliage and tuber defense mechanisms against the late blight pathogen *Phytophthora infestans*

The late blight pathogen *Phytophthora infestans* can attack both potato foliage and tubers. When inoculated with *P. infestans*, foliage of nontransformed 'Russet Burbank' (WT) develops late blight disease, while that of transgenic 'Russet Burbank' line SP2211 (+*RB*) does not. We compared transcriptome responses of these two lines to *P. infestans* inoculation using an RNA-seq approach. A total of 515 million paired end RNA-seq reads were generated, representing the transcription of 29,970 genes. We compared the differences and similarities of defense mechanisms against *P. infestans* in potato foliage and tubers. Differentially expressed genes, gene groups and ontology bins were identified to show similarities and differences in foliage and tuber defense mechanisms. Our results suggest that *R* gene dosage and shared biochemical pathways contribute to *RB*-mediated incompatible potato-*P. infestans* interactions in both the foliage and tubers.

## Introduction

*Phytophthora infestans* is a notorious plant destroyer with the capacity to attack both potato foliage and tubers. Importantly, foliage resistance against *P. infestans* does not guarantee tuber resistance (Kirk et al. 2001), although some correlations between tuber and foliage resistance have been reported (Bradshaw et al. 2006; Park et al. 2005).

Gene *RB* (*Rpi-blb1*) (Song et al. 2003; van der Vossen et al. 2003) is a disease resistance (*R*) gene conferring broad spectrum resistance against complex *P. infestans* races in potato foliage. Previously, we reported that higher *RB* gene copy numbers correspond to higher transcript levels and enhanced late blight resistance in the foliage (Bradeen et al. 2009). Recently our research group discovered two transgenic (+*RB*) potato lines (SP2211 and SP2213) with extraordinary *RB* transcript levels that are resistant to the late blight pathogen not only in the foliage but also in the tubers (Millett 2008). Thus, the *RB*-potato-*P. infestans* pathosystem provides a tractable system to study how different plant organs respond to a common pathogen.

Previous transcriptome studies have documented potato foliar defense strategies against the late blight pathogen. Restrepo et. al. (Restrepo et al. 2005) utilized a microarray technique to examine potato leaf – *P. infestans* interactions, highlighting a possible role for carbonic anhydrase (CA) in defining the interaction outcome. Gyetvai et al. (Gyetvai et al. 2012) utilized the DeepSAGE method to analyze potato leaf – *P. infestans* interactions. That study relied mostly on assembled tags for functional analysis. Both of these studies focused on how potato foliage defends against the late blight pathogen; research goals of these studies did not include comparing potato foliage and

tuber responses to pathogen attack. In addition, neither of these studies made extensive use of the potato genome reference sequence (Xu et al. 2011).

In the current study, we employed RNA-seq to study transcriptome dynamics of potato foliage-*P. infestans* interactions. We employed whole genome sequence data from potato for our analysis. We also compared potato foliage-*P. infestans* interactions with those of tuber-*P. infestans* interactions (Chapter two). We identified differentially expressed (DE) genes and ontology bins that are shared components of foliage and tuber response to *P. infestans* and others that are organ-specific components of potato response to pathogen attack. Our study contributes to scientific understanding of organ-specific defense responses in plants.

## Materials and methods

### Plant materials and RNA preparation

Untransformed 'Russet Burbank' (WT) and transgenic line SP2211 (+*RB*) were examined in this study (Bradeen et al. 2009). RNA from tuber samples was prepared and subjected to RNA-seq analysis as described in Chapter Two.

Foliage samples were generated and collected from six week old, greenhouse-grown WT and +*RB* plants. Three WT and three +*RB* plants were each inoculated with either *P. infestans* or water, providing three bioreps for each genotype x treatment combination. *P. infestans* US8 isolate US940480 (Song et al. 2003) was maintained on Rye A medium (Tumwine et al. 2000) and sporangia were harvested from plates by physical scraping into distilled water. The resulting inoculum was adjusted to 1,200

sporangia/ml and incubated for 1 hour at 4$^{o}$C and then at room temperature for 30 minutes. The prepared inoculum or water (mock treatments) was sprayed onto the leaves until runoff. The greenhouse chamber was maintained at >95% humidity by frequent overhead misting. Three leaflets from each of the bio-rep plants were collected at 0 (pre-inoculation), 6, 12, 24, and 48 hours post inoculation. Collected tissue samples were immediately frozen in liquid nitrogen and stored at -80$^{o}$C. Plants were allowed to develop disease symptoms and were visually rated on a 0-9 scale (Bradeen et al. 2009) 21 days after inoculation.

In total, 36 foliage samples from the two plant genotypes (WT and +*RB*) x three time points (0, 6, and 24 hpi) x two inocula (*P. infestans* or water) x three bio-replicates were employed for RNA extraction and RNA-seq. [Note: We collected tissue from 0, 6, 12, 24, 48 hpi, but only 0, 6, 24 hpi samples were subjected to RNA-seq]. Total RNA was extracted from the samples using the SV Total RNA Isolation System (Promega Corporation, Madison, WI) according to manufacturer's instructions. The quantity and quality of RNA samples were assessed using a Nanodrop 1000 (Thermo Fisher Scientific Inc., Wilmington, DE). High quality total RNA (3ug, 50ng/µl) samples were sent to the University of Minnesota BioMedical Genomics Center (BMGC) for RNA-seq library prep using the TruSeq SBS Kit (50 Cycles) (Illumina Inc., San Diego, CA) and sequencing using an Illumina Hi-Seq 2000 machine (Illumina).

## RNA-seq reads mapping and DE genes clustering

RNA-seq reads were quality filtered using SolexaQA packages (Cox et al. 2010) with default parameters and a length filter of greater than 27bp for both ends of each read

pair. Quality filtered RNA-seq reads were analyzed using the "Tuxedo Suite" software packages (Trapnell et al. 2012): Bowtie (Langmead et al. 2009) v0.12.7, Tophat (Trapnell et al. 2009) v1.3.2, Cufflinks (Trapnell et al. 2010) v1.1.0 and a reference genome of potato (Xu et al. 2011). We suppressed Tophat (Trapnell et al. 2009) from identifying novel junctions. A total of eleven pair-wise comparisons [between time (4), between lines (3), and between treatments (4)] were made using Cuffdiff of the Cufflinks software packages. Differentially expressed (DE) genes are those genes that showed significantly (FDR adjusted p-value <0.001) different transcript levels among comparisons. False discovery rate correction (FDR) was done using the Benjamini Hochberg method (Benjamini and Hochberg 1995).

Quality filtered RNA-seq reads were also mapped to reference transcript sequences of *P. infestans* (Haas et al. 2009) and different classes (see Champouret et al. (Champouret et al. 2009) for details of classification) of *ipiO* (potential cognate avr effector of *RB*) gene sequences. The mapping was done using Bowtie (Langmead et al. 2009) v0.12.7. Cufflinks software suites were not used in this analysis as underlying normalization assumptions were not met. Potato gene expression fold change values were used to cluster and partition genes into groups using hierarchical clustering and the complete linkage method in Cluster 3.0 (Eisen et al. 1998) and visualized in Treeview software (Saldanha 2004) using adjusted pixel settings (threshold 1.0).

The UNIX command line options for Tophat and Cuffdiff were as follows: "tophat -G genes.gff -o sample.out --no-novel-juncs -r 100 genome.fasta read.left.fq read.right.fq; cuffdiff -N -u -O cuffdiff.out genes.gff 1.bam,2.bam,3.bam

4.bam,5.bam,6.bam". The UNIX command line options for Bowtie were as follows: "Bowtie -a -v 2 --best -M 1--fr ref_bowtie_build -1 read1.fq -2 read2.fq". Custom perl scripts were used to parse the mapping/alignment results into tabular formats. R statistical software (R Development Core Team 2011) was used to generate various plots

## Functional assignment and MapMan analysis of potato genes

To assign potato genes into functional categories (bins), we adopted MapMan ontology (Thimm et al. 2004). The Mercator annotation pipeline (http://mapman.gabipd.org/web/guest/app/mercator) was used to assign potato genes into functional bins by searching a variety of reference databases. The ratios of the numbers of the genes in to not in an ontology bin were compared between the whole gene set and selected gene set using Fisher's exact test. If the ratio is significantly higher for the selected gene set, the set is called enriched in the ontology category. The log2 transformed fold change values for genes within an ontology bin were compared to the log2 transformed fold change values for all genes outside of the ontology bin using Wilcoxon rank-sum test to identify functional bins that show higher or lower induction. Resulting p-values from Fisher's exact tests or Wilcoxon rank-sum tests were subjected to multiple test correction using the Benjamini Hochberg method (Benjamini and Hochberg 1995).

## Real-time quantitative RT-PCR

For determining the transcript levels of the transgene *RB*, we used a previously established qRT-PCR protocol (Bradeen et al. 2009; Millett and Bradeen 2007). For

RNA-seq validation, Primer Express 3.0 (Applied Biosystems, Foster City, CA, USA) was used with default parameters to generate primer pairs for selected transcripts (Table 3.1). Total RNA (150ng, 30ng/ul) from diluted stocks of the same RNA that was subjected to RNA-seq was used in each reverse transcription reaction using the SuperScript III First Strand Synthesis Kit (Life Technologies Inc., Carlsband CA) according to manufacturer's instructions. All qPCRs were performed using the Power SYBR Green Master Mix (Life Technologies) and an ABI 7500 Real Time System (Applied Biosystems). Target gene transcript levels were normalized to *EF1α* (Nicot et al. 2005) using $2^{-\Delta CT}$ values. Normalized gene expression levels were compared with RNA-seq FPKM values derived from Cufflinks.

## Results

### Gene *RB* confers resistance against late blight in the foliage; *RB* transcript levels differ between foliage and tubers

In previous field-based evaluations, the +*RB* line examined in this study, SP2211, was ranked as the third most foliage late blight resistant of 57 transgenic lines tested and was rated as "Resistant" to foliage late blight (Bradeen et al. 2009). In contrast, the WT line (nontransformed 'Russet Burbank') was rated as "Susceptible" to foliage late blight (Bradeen et al. 2009). Here, in greenhouse late blight inoculation experiments, the WT line developed symptoms of late blight disease while the +*RB* line did not (Figure 3.1). Similar to previous research documenting that the *RB* transgene is transcriptionally up-regulated following pathogen inoculation (Kramer et al. 2009), in this study, the *RB*

transgene transcript levels increased to 234% at 6 hpi and then decreased to 165% at 24 hpi in the foliage. In contrast, *RB* transgene transcript levels were statistically identical in the tubers at 0, 24, and 48 hpi (Chapter two).

Interestingly, foliar +*RB* samples consistently displayed higher *RB* transgene transcript levels than tuber +*RB* samples ($p<1E-4$). Before pathogen inoculation, *RB* transgene transcript levels in the foliage are 2.9 fold higher than those in the tubers. After pathogen inoculation, averaged across selected time points (0, 6, and 24 hpi for foliage, and 0, 24, and 48 hpi for tubers), *RB* transgene transcript levels in the foliage are 6.7 fold higher than in the tubers. These results suggest that potato plants modulate *RB* gene transcript levels in different organs, with below ground organs displaying less *R* gene transcription compared to above ground foliage.

## Foliar RNA-seq reads alignment to the potato reference genome sequence

From a total of 36 leaf RNA samples, approximately 515 million paired end reads were generated, yielding an average of 14.3 million reads per sample. Around half of these reads (258.4 million or 50.1%) could be mapped uniquely to one location on the doubled monoploid (DM) potato reference genome sequence (Xu et al. 2011). An additional 7.6 million (1.5%) reads were mapped to multiple locations within the reference genome sequence (Figure 3.2).

We detected transcription of 29,970 potato genes based on cufflinks FPKM information and gene models reported by the Potato Genome Sequencing Consortium

(PGSC) (Xu et al. 2011). The detected foliar transcripts largely overlap those detected in the tubers with 28,050 out of the 29,970 (93.6%) genes transcribed in the foliage also detected in the tubers. Around 20% of RNA-seq reads that passed quality filters were mapped to regions outside of the current potato reference genome gene models. Nonetheless, qRT-PCR results correlated well with RNA-seq data with an average Pearson correlation coefficient of r=0.87 (Table 3.2). These results suggest that RNA-seq and qPCR approaches can be cross-validated, confirming that the PGSC gene models are appropriate for functional genomics studies on potato foliage.

## Foliar and tuber transcriptomes differ dramatically; treatment (*P. infestans* vs. water) has a greater influence than genotype (WT vs. +*RB*) on overall transcriptome dynamics in the foliage

A principal component analysis (based on log2 transformed FPKM values of 31,239 genes) for both the foliar and tuber samples is shown in Figure 3.3A. PC1 explained >25.8% of the total variance in this dataset and clearly separates foliage and tuber sample transcriptomes. A principal component analysis of log2 transformed FPKM values for 29,970 genes represented in the 36 foliage RNA-seq samples is shown in Figure 3.3B. Together PC1 and PC2 explained >28% of the total variance of this dataset. Overall, neither time nor genotype has a clear impact on overall sample distributions. Treatment (water vs. *P. infestans*) effects seem to be captured by PC2 but not PC1, with *P. infestans*-inoculated samples tending to be associated with negative PC2 values, and water-inoculated samples tending to be associated with positive PC2 values.

In foliage, at an FDR threshold of 0.01, a total of 475 genes can be identified as DE for between treatment comparisons (water- vs. *P. infestans*-inoculated). A majority of these (90%) was identified by comparison of water and *P. infestans*-inoculated +*RB* samples at 24 hpi. Hierarchical clustering of the 475 DE genes revealed representative gene clusters that are known to be correlated with plant defense (e.g., *PR1* genes and cysteine protease inhibitors) (Figure 3.4).

## Foliar and tuber responses *to P. infestans* share some ontology bins, while other response components are organ-specific

GO enrichment analysis of the 475 foliar DE genes (derived from between treatment comparisons of water and *P. infestans* +*RB* samples at 24 hpi) reveals that ontology bins such as lipid metabolism, amino acid metabolism, secondary metabolism, ethylene (ET), jasmonic acid (JA), stress, proteinase inhibitors, and peroxidases are overrepresented by up-regulated genes in *P. infestans* treated +*RB* samples (Figure 3.5). In contrast, ontology bins including photosynthesis and major CHO (carbohydrate metabolism) are overrepresented by down-regulated genes in *P. infestans* treated +*RB* samples (Figure 3.5). Overall, our results suggest that the +*RB* potato line shifted its metabolic prioritization from photosynthesis to pathogen defense upon *P. infestans* infection, similar metabolic transition has also been documented in Arabidopsis plants under pathogen attack (Depuydt et al. 2009). Interestingly, the WT samples do not show a similar transition, with few genes determined to be DE genes. This suggests that the WT line has failed to mount active defense components within the first 24 hours

following *P. infestans* attack, consistent with its susceptibility to foliar late blight (Figure 3.1).

Similar analysis in the tubers (Figure 2.6) revealed another set of MapMan bins that likely contribute to successful tuber defense against *P. infestans*. The regulation patterns of these two sets of MapMan bins (derived from foliage and tuber studies) share certain components. For example, ethylene (ET) metabolism and stress bins are highly induced both in the foliage and in the tubers of *P. infestans* challenged +*RB* samples. (Figure 3.5, Figure 2.6). At 0hpi, when WT and +*RB* lines were compared directly using Wilcoxon rank sum test, stress.biotic, signaling.receptor.kinases bins were transcribed at higher levels in the +*RB* line in both of the foliage and tubers (Figure 3.6). These ontology bins highlight potentially shared biochemical and signaling pathways in tuber and foliage defense responses.

Besides shared components, we also identified apparent organ-specific pathogen response components. For between treatment comparisons, secondary metabolism was found to be down-regulated in tubers of *P. infestans* challenged +*RB* samples (compared to water challenged samples) (Figure 2.6), but up-regulated in the foliage of *P. infestans* challenged +*RB* samples (compared to water challenged samples) (Figure 3.5). For between genotype comparisons (Figure 3.6), lipid metabolism and secondary metabolism were transcribed at higher levels in WT (compared to +*RB*) in the tuber (at 0 hpi), but higher in the +*RB* (compared to WT) line in the foliage (at 0 hpi) (Figure 3.6).

### RNA-seq reads mapping to *Phytophthora infestans* reference transcripts

A total of only 89 paired end Illumina reads mapped to the *P. infestans* reference transcript set (matching 86 genes total), none of these belongs to the RXLR effector family (Haas et al. 2009). Different from the tuber inoculations (Chapter two, additional file 8), a drop in total reads mapping to *P. infestans* was identified in both WT and +*RB* samples during 6 hpi to 24 hpi transitions, possibly reflecting different pathogen colonization strategies in the foliage compared to that in the tubers.

## Discussion

### Foliar and tuber transcriptomes differ dramatically but certain defense components are shared (or conserved) in both organs

Principal component analysis (Figure 3.3) reveals that tuber and foliage transcriptomes differ dramatically, with PC1 clearly separating the transcriptome samples into foliage and tuber clusters. A majority of DE genes is not shared between the two organs. Of the 475 DE genes (between treatment comparisons) identified in the foliage, only 127 of them (26.7%) are also DE genes in the tubers.

Hierarchical visualization of the regulation patterns of the 127 shared DE genes reveal groups of interesting genes (Figure 3.7). Both foliage and the tubers show down regulation of photosynthesis related gene groups (Figure 3.7B). This is consistent with GO enrichment analysis (Figure 2.6, Figure 3.5). *P. infestans* challenged foliage of both WT and +RB displays up-regulation (compared to water-inoculated samples) of carbonic anhydrase at 24 hpi (Figure 3.7C). Other groups of genes (Figure 3.7D-F) including

WRKY transcription factors, PAR-1c protein and polyphenoel oxidases are more highly transcribed in *P. infestans* challenged tuber and foliage samples (at 24 hpi for foliage, at 48 hpi for tubers).

Consistently, in both organs, faster and stronger activation of defense related ontology bins such as stress and ethylene (ET) metabolism correlates with successful defense (Figure 2.6, Figure 3.5). These results suggest that despite being different organs (below vs. above ground), the defense mechanisms in tubers and foliage could overlap.

## *RB* gene transcription levels and shared ontology bins likely contribute to incompatible potato-*P. infestans* interactions in both the foliage and tubers

Certain previous studies documented correlation between tuber and foliage late blight resistance for particular *R* genes (Bradshaw et al. 2006; Park et al. 2005). But, in general, whether or not foliar and tuber late blight resistances are conditioned by the same gene is considered to be a function of the *R* gene itself (Bradshaw et al. 2006; Park et al. 2005). Gene *RB* has been previously described only as a foliage *R* gene (Halterman et al. 2008). But our study reveals that gene *RB* can function even in the tubers. In particular, enhanced *RB* gene transcription correlates with enhanced tuber disease resistance (Appendix one). Thus transgenic (+*RB*) genotypes with low tuber transcription of *RB* may be foliar late blight resistant but tuber blight susceptible. In contrast, transgenic lines SP2211 (examined in this study) and 2213 have comparatively high levels of *RB* transcripts even in the tuber and display late blight resistance in both foliage

and tuber (Appendix one). Importantly, we document for SP2211 that *RB* transcription is much higher in foliage than in tubers. Previously, we concluded that *RB* transgene dosage correlated with both *RB* transcript levels and foliar late blight resistance (Bradeen et al. 2009). Results in the current study suggest a similar correlation in potato tubers and thus we conclude that *R* gene dosage and the resulting variation in *R* gene transcript levels may be determining factors in whether disease resistance is manifested in an organ-specific manner.

We identified ontology bins that show conserved regulation patterns across different potato organs. For example, photosynthesis is down-regulated upon pathogen infection in both foliage and tubers. Stress and ethylene (ET) metabolisms were up-regulated following pathogen inoculation in both foliage and tubers. The +*RB* transgenic line appears to have pre-primed defense bins, transcribed even in the absence of the pathogen, including signaling receptor kinases and stress. It is possible that these shared defense components are downstream elements of *R* gene signaling. We hypothesize that *RB*-mediated tuber and foliage resistance to late blight pathogen infection reflects both an *R* gene dosage effect and shared biochemical pathway effects.

## Conclusion

The current study employed RNA-seq to study potato foliage-*P.infestans* interactions and compared the similarities and differences of potato foliage-*P. infestans* interactions with those of potato tuber-*P. infestans* interactions. DE genes, gene groups and ontology bins were identified to show potato foliage transcriptome dynamics in response to *P. infestans* inoculation. Shared DE genes and ontology groups between

potato foliage-*P. infestans* and tuber-*P. infestans* interactions were identified (e.g. down regulation of photosynthesis, up-regulation of ethylene (ET) and stress bins). *RB* gene transcription levels and shared ontology bins likely contribute to incompatible potato-*P. infestans* interactions in different plant organs.

**Table 3.1 qRT-PCR primer sequences for 12 selected potato genes**

| | | |
|---|---|---|
| **PGSC0003DMG400010713** | GCTTCGTCAAGCGATTCCA | CACGAAGTGGCTCCCCTGTA |
| **PGSC0003DMG400017350** | TGGGAAGCCCGTGTGAAG | CGGTGGATTACTTTAGGTTCTATGG |
| **PGSC0003DMG400009671** | AGTAGCCAATGTTCAGGTTCAGAGT | TCCTCCACAACAGCTCAGTACAA |
| **PGSC0003DMG400019257** | GGAGCTATGATGATATCAGGACAGAA | GGGCGTTGGGCAATCC |
| **PGSC0003DMG400020334** | TCCATTAATTCACTCGAAAAAGTCAT | TTGGGAATTCTTTGACTGATAATACG |
| **PGSC0003DMG402021263** | GGTGAAGGCTGCAAGAAGAGA | GCAAGCTTTCTGTAAACAGCCATA |
| **PGSC0003DMG400004711** | CGGGCAGGGAGGCTAAA | AGCTTTGCAGCACGGATATTC |
| **PGSC0003DMG400006956** | CAGCCCGGTGAGGCTTT | CCCGAGTACCTAAGCTTGTCATAAG |
| **PGSC0003DMG400010663** | AGTACACAAGACTCAGAAGCTGATCTG | ATGCACAGTGTGAGATGGTGTTG |
| **PGSC0003DMG400011169** | GCCCAACAAACTGGACAAAAC | CGGGTCGTTGTTCCAGCTAT |
| **PGSC0003DMG400012234** | AAACCATTCACAAGAAGCTGAAAA | TGACAACCTTTGGGAATTCGA |
| **PGSC0003DMG400014823** | TCCATCACTTGGAATCCACAAC | AAACTCCCATTGATTCACTATTCTTGTA |

**Table 3.2 Correlation between qPCR and RNA-seq**

RNA-seq FPKM and qPCR correlation. The first column is potato foliage sample IDs. The remaining columns indicate qPCR results and RNA-seq FPKM values for each potato gene, as indicated. The per gene and average correlations between RNA-seq and qPCR are listed at the bottom of the page.

| norm_EF1 | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sample | G400010713 | G400009671 | G400019257 | G400004711 | G400010663 | G400011169 | G400006956 | G400012234 | G400014823 | G400020334 | G402021263 | G400017350 | |
| +RB_0h | 0.0830 | 0.0185 | 0.0069 | 0.0009 | 0.0118 | 0.7471 | 0.002532 | 0.00889 | 0.008142 | 0.01304 | 0.024883 | 0.008031 | |
| +RB_6h | 0.0084 | 0.0209 | 0.0003 | 0.0007 | 0.0076 | 0.3891 | 0.025587 | 0.010868 | 0.008522 | 0.161681 | 0.020072 | 0.012239 | |
| +RB_24h | 0.0104 | 0.0230 | 0.0008 | 0.0008 | 0.0113 | 0.5195 | 0.06247 | 0.002185 | 0.003242 | 0.187015 | 0.008535 | 0.004562 | |
| mock +RB_0h | 0.1026 | 0.0145 | 0.0023 | 0.0014 | 0.0095 | 0.6417 | 0.01517 | 0.012144 | 0.047077 | 0.01362 | 0.039445 | 0.009484 | |
| mock +RB_6h | 0.0057 | 0.0120 | 0.0003 | 0.0006 | 0.0083 | 0.3977 | 0.020897 | 0.012883 | 0.004785 | 0.043427 | 0.011794 | 0.012168 | |
| mock +RB_24h | 0.0036 | 0.0223 | 0.0081 | 0.0016 | 0.0092 | 0.6379 | 0.001259 | 0.004632 | 0.00385 | 0.030491 | 0.024031 | 0.00671 | |
| mock WT_0h | 0.1129 | 0.0187 | 0.0039 | 0.0008 | 0.0098 | 0.8671 | 0.007973 | 0.004218 | 0.012779 | 0.016589 | 0.014152 | 0.004534 | |
| mock WT_6h | 0.0086 | 0.0076 | 0.0002 | 0.0006 | 0.0091 | 0.3155 | 0.113682 | 0.008001 | 0.005422 | 0.033072 | 0.0151 | 0.007216 | |
| mock WT_24h | 0.0055 | 0.0194 | 0.0079 | 0.0019 | 0.0078 | 0.8440 | 0.017428 | 0.005107 | 0.006032 | 0.02834 | 0.028863 | 0.006519 | |
| WT_0h | 0.0845 | 0.0152 | 0.0061 | 0.0010 | 0.0122 | 0.8694 | 0.005412 | 0.005779 | 0.008012 | 0.011193 | 0.023675 | 0.005587 | |
| WT_6h | 0.0061 | 0.0158 | 0.0002 | 0.0007 | 0.0098 | 0.4024 | 0.03342 | 0.004425 | 0.004486 | 0.105108 | 0.010266 | 0.005812 | |
| WT_24h | 0.0051 | 0.0214 | 0.0008 | 0.0017 | 0.0095 | 0.6197 | 0.158172 | 0.003063 | 0.003808 | 0.136341 | 0.019466 | 0.003729 | |
| | | | | | | | | | | | | | |
| fpkm_avg | | | | | | | | | | | | | |
| sample | G400010713 | G400009671 | G400019257 | G400004711 | G400010663 | G400011169 | G400006956 | G400012234 | G400014823 | G400020334 | G402021263 | G400017350 | |
| +RB_0h | 96.3360 | 8.9396 | 880.5837 | 0.6847 | 37.7254 | 314.9260 | 5.532967 | 23.01133 | 12.53942 | 6.98089 | 47.0977 | 8.8165 | |
| +RB_6h | 7.2536 | 10.6836 | 66.3062 | 0.3540 | 25.4546 | 185.2053 | 41.6899 | 23.87687 | 9.896915 | 66.0878 | 39.71197 | 11.26879 | |
| +RB_24h | 7.9166 | 13.2167 | 157.0622 | 0.6496 | 37.9536 | 270.6280 | 164.3303 | 4.921818 | 2.816575 | 101.062 | 19.25187 | 4.68394 | |
| mock +RB_0h | 102.9213 | 6.1841 | 494.9623 | 0.8009 | 29.5605 | 267.1280 | 12.72187 | 27.8403 | 50.52625 | 8.14047 | 74.58 | 16.66804 | |
| mock +RB_6h | 5.8663 | 6.8832 | 109.4383 | 0.3742 | 32.1771 | 174.5774 | 191.2957 | 34.1345 | 3.877953 | 46.83463 | 30.35037 | 12.68277 | |
| mock +RB_24h | 2.2819 | 8.7270 | 837.2437 | 1.0357 | 23.5773 | 255.5403 | 2.022495 | 11.58889 | 5.755587 | 13.21691 | 50.50307 | 7.126113 | |
| mock WT_0h | 152.3726 | 10.0822 | 685.1157 | 0.4642 | 36.4736 | 411.0647 | 14.47901 | 12.12762 | 20.83262 | 11.16429 | 35.6889 | 5.05161 | |
| mock WT_6h | 6.5662 | 3.2048 | 105.4016 | 0.3029 | 28.7119 | 165.2453 | 160.5415 | 26.29147 | 6.884778 | 36.55929 | 34.84 | 12.52031 | |
| mock WT_24h | 6.2466 | 6.2114 | 772.5727 | 0.8953 | 25.3898 | 305.0177 | 24.6235 | 10.45872 | 7.015816 | 20.26968 | 40.98497 | 6.774867 | |
| WT_0h | 141.8254 | 7.3400 | 824.5383 | 0.6496 | 35.5051 | 368.9363 | 9.261267 | 17.3185 | 14.5769 | 10.66252 | 43.707 | 6.429003 | |
| WT_6h | 83.2863 | 7.8530 | 180.2029 | 0.3899 | 38.5194 | 255.4243 | 113.2822 | 11.32067 | 11.43665 | 63.14367 | 29.67813 | 8.47546 | |
| WT_24h | 6.1335 | 7.3666 | 235.0323 | 0.7571 | 30.2000 | 212.8527 | 189.7928 | 8.279417 | 1.450694 | 101.2751 | 29.99147 | 7.019443 | |
| | | | | | | | | | | | | | |
| mean | 51.5839 | 8.0577 | 445.7050 | 0.6132 | 31.7707 | 265.5455 | 77.4645 | 17.5975 | 12.3008 | 40.4498 | 39.6988 | 8.9597 | |
| **Correlation** | **0.90** | **0.78** | **0.96** | **0.88** | **0.73** | **0.88** | **0.750357** | **0.958936** | **0.969877** | **0.930662** | **0.915434** | **0.743893** | **0.8658** |

**Figure 3.1 Gene *RB* confers foliar resistance against the late blight pathogen *P. infestans.*** *Phytophthora infestans* isolate US940480 was used to inoculate six weeks old WT and +*RB* plants in greenhouse trials. Visual phenotype assessment was done 21 days after inoculation. The left panel is a representative WT plant; the right panel is representative +*RB* plant. The susceptible WT plants were mostly killed (scored as 9) by the pathogen, while those of the resistant +*RB* line were not (scored as 1 or 2).

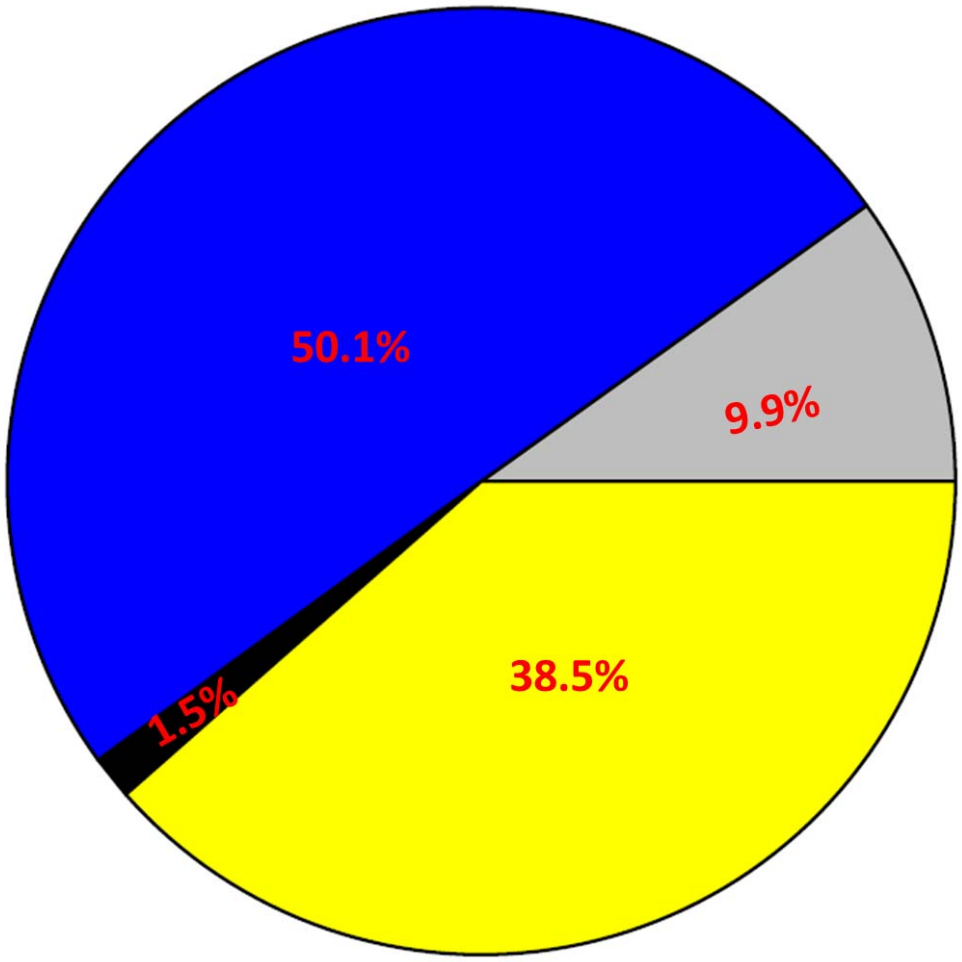


**Figure 3.2 RNA-seq reads mapping results for foliage data.** A pie chart summarizing results of alignment of Illumina RNA-seq reads from 36 water- or *P. infestans*-inoculated WT and *+RB* foliage samples to the potato reference genome sequence. The grey portion of the chart represents RNA-seq reads that failed quality checks and were filtered out of our data set (9.9% of all RNA-seq reads). The remaining 90.1% of RNA-seq reads passed quality checks. The blue portion of the chart represents reads that mapped uniquely to the potato genome sequence (50.1% of all RNA-seq reads). The black portion of the chart represents reads that mapped to multiple locations of the potato genome sequence (1.5%). The yellow portion of the chart represents reads that failed to map to the potato genome sequence (38.5%).

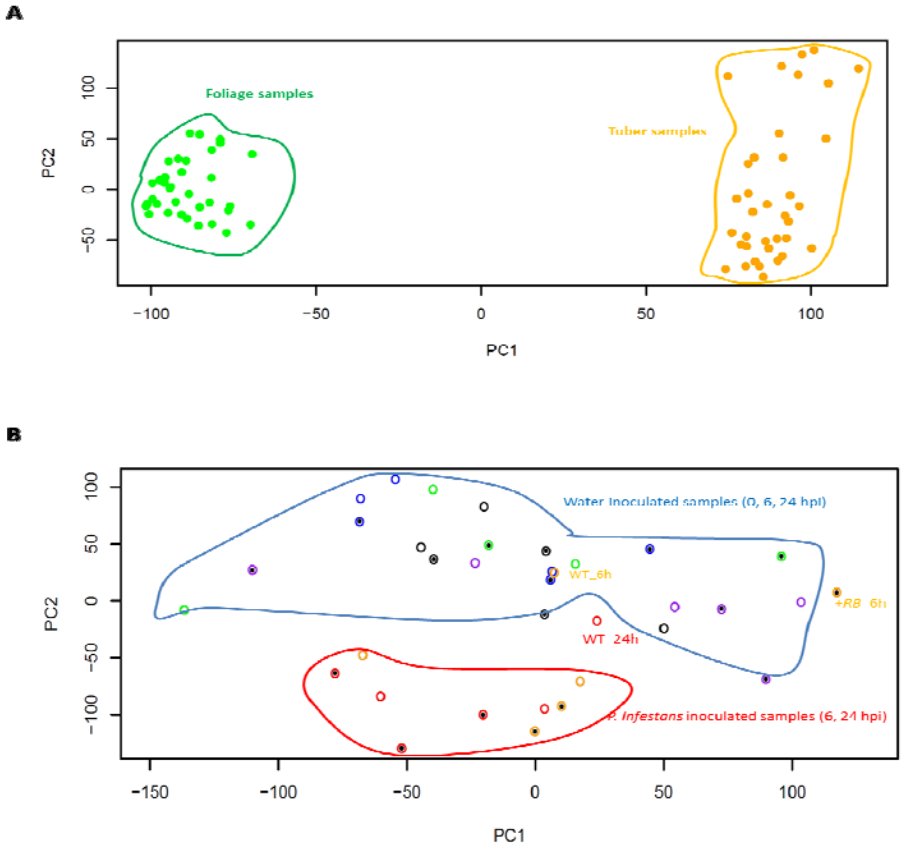


**Figure 3.3 Principal component analyses of tuber and foliage transcriptome sets.** (A) FPKM (Fragment per kilobase of exon per million mapped reads) values for transcriptome sets from 72 (36 tuber +36 foliage) potato samples were subjected to PCA using the R statistical package (R Development Core Team 2011). Yellow circles represent tuber samples, green circles represent foliage samples. PC1 clearly separates foliage and tuber samples into two clusters. (B) FPKM values for transcriptome sets from 36 potato foliage samples were subjected to PCA using the R statistical package (R Development Core Team 2011). Blue circles represent *P. infestans*-inoculated samples collected at 0 hpi; green circles represent water-inoculated samples at 0 hpi; orange circles represent *P. infestans*-inoculated samples collected at 24 hpi; purple circles represent water-inoculated samples collected at 24 hpi; red circles represent *P. infestans*-inoculated samples collected at 48 hpi; black circles represent water-inoculated samples collected at 48 hpi. Circles containing dots were collected from the transgenic line SP2211 (+*RB*); circles without dots were collected from nontransformed 'Russet Burbank' (WT). PC2 separates a majority of the water and *P. infestans* inoculated samples, suggesting that treatment has an effect on overall transcriptome dynamics.

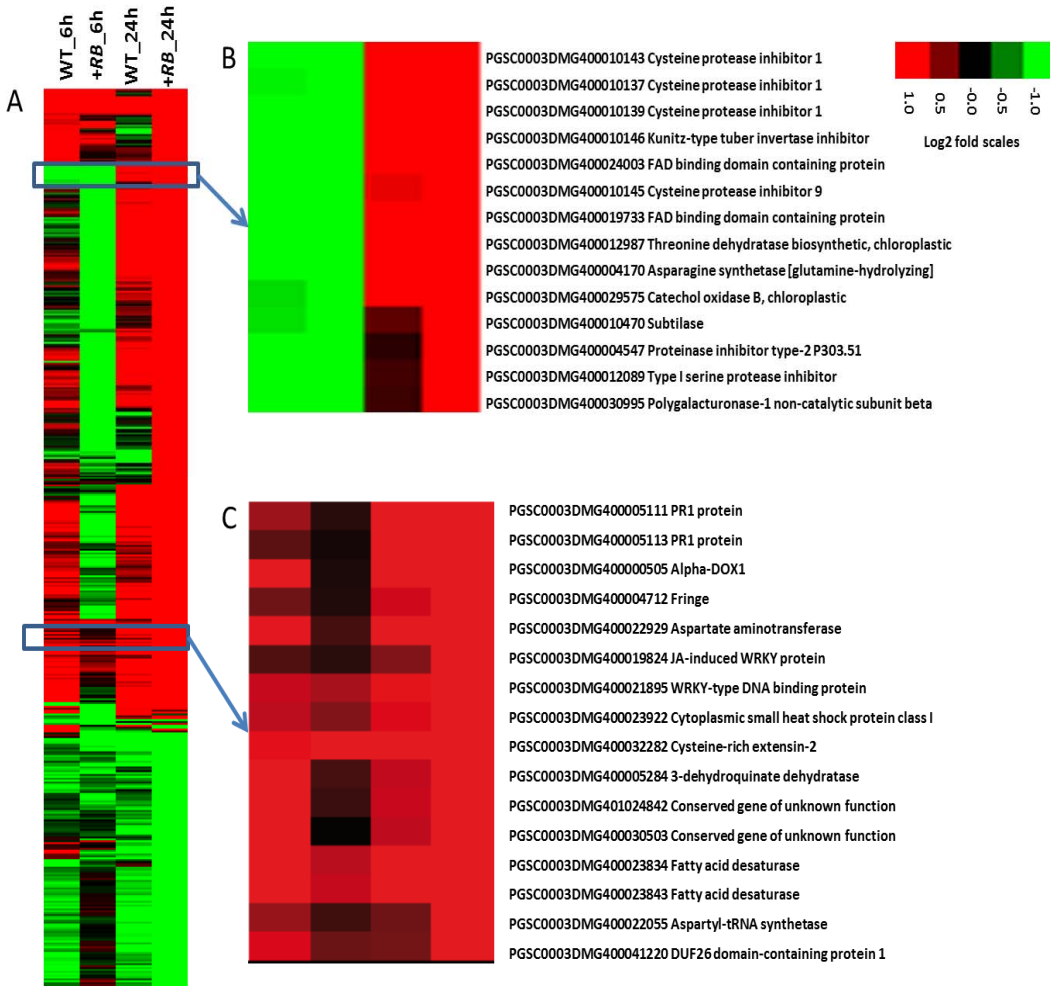


**Figure 3.4 Hierarchical clustering of foliage DE genes (between treatment comparisons).** Foliage of nontransformed 'Russet Burbank' (WT) and transgenic SP2211 (+*RB*) was inoculated with *P. infestans* or water. Foliage samples collected 0, 6, and 24 hpi were subjected to RNA-seq, revealing a total of 475 DE genes between water- and *P. infestans*-inoculated comparisons within the same genotype and the same time point. Log2(FPKM_p.inf/FPKM_mock) values were used to cluster these 475 DE genes (FDR=0.01) in Cluster 3.0 (Eisen et al. 1998) using uncentered correlation and the complete linkage method. Results were visualized using Treeview (Eisen et al. 1998). Red indicates genes that are up-regulated, green indicates genes that are down-regulated. (A) Global visualization of the 475 DE genes; (B) A small gene cluster differentially regulated in +*RB* and WT at 24 hpi; (C) A small gene cluster generally up-regulated in +*RB* and WT. These clusters highlight the role of cysteine protease inhibitors and other pathogenesis related proteins (e.g., PR1) in foliar defense response to *P. infestans*.

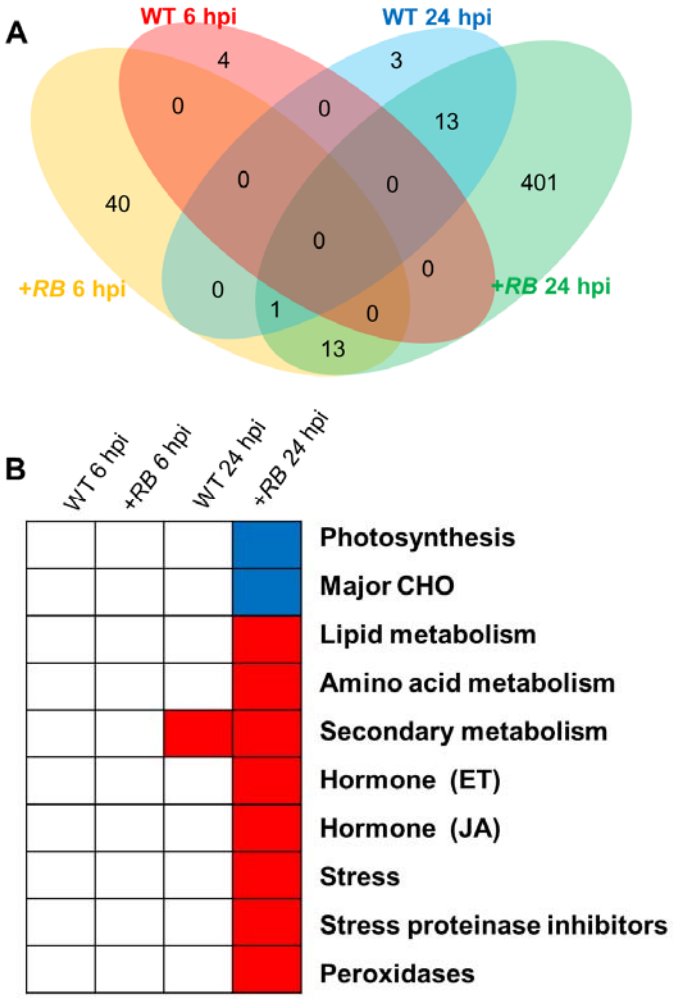


**Figure 3.5 Foliage of the +*RB* line has a higher frequency of differentially expressed (DE) genes 24 hours post inoculation (hpi) with *Phytophthora infestans*.** Foliage samples collected 0, 6, and 24 hpi were subjected to RNA-seq, revealing a total of 475 DE genes between water- and *P. infestans*-inoculated comparisons within the same genotype and the same time point. (A) All 475 DE genes were analyzed using the "Venn count" function in the limma packages of R (R Development Core Team 2011) and results were summarized as a Venn diagram. Red: WT 6 hpi; orange: +*RB* 6 hpi; blue: WT 24 hpi; green: +*RB* 24 hpi. The results show that the +*RB* line is the main contributor of DE genes during water- vs. *P. infestans*-inoculated comparisons. (B) All 475 DE genes were also assigned to a MapMan ontology based on the Mercator mapping file (see methods), and subjected to Fisher's exact test. Bins in red were significantly up-regulated; bins in blue were significantly down-regulated; transcription of bins in white did not change significantly. The results indicate that ontology bins encompassing ET metabolism and stress are enriched for DE genes in +*RB* but not in WT at 24 hpi.

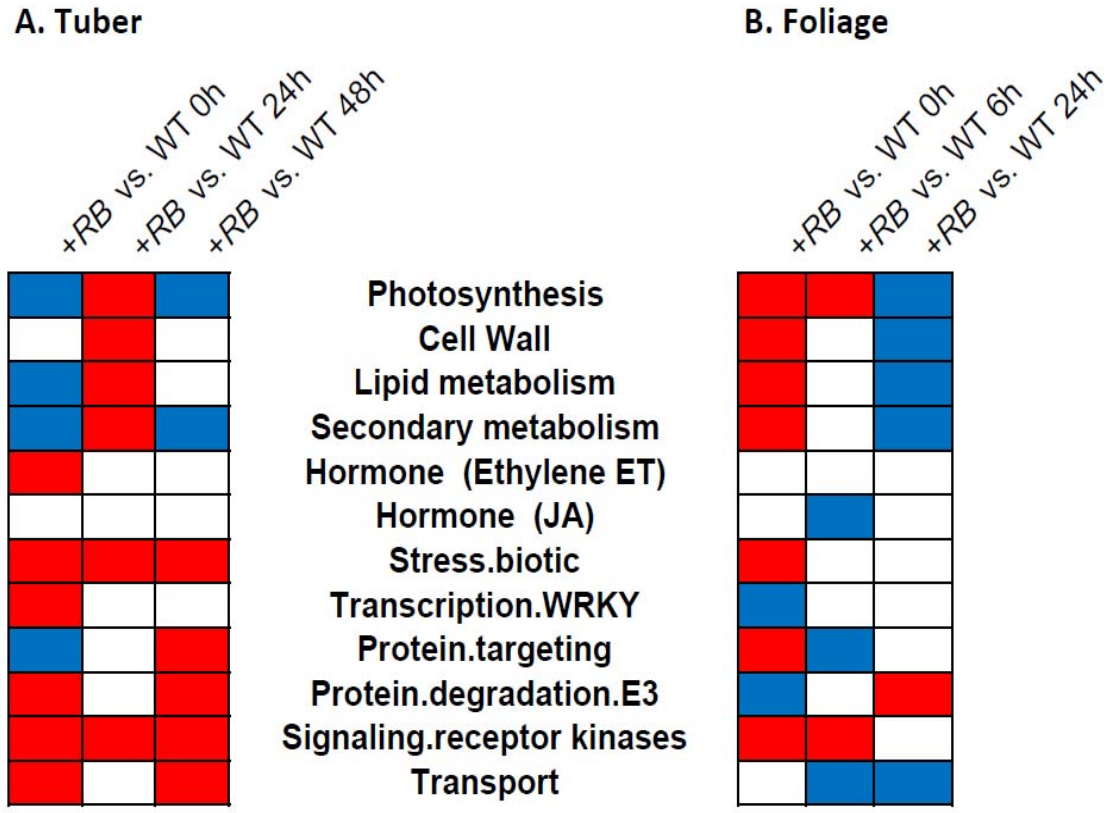


**Figure 3.6 Faster and stronger activation of defense related genes or gene groups correlates with successful foliage and tubers resistance against *P. infestans*.** Foliage and tubers of 'Russet Burbank' (WT) and SP2211 (+*RB*) were inoculated with *P. infestans* and water. We compared the RNA-seq FPKM counts for WT and +*RB* using all 39,031 gene models included in the Potato Genome Sequencing Consortium (PGSC) v3 dataset (i.e., all genes were included, regardless of whether or not a given gene was DE). Genes were grouped into ontology bins using a MapMan mapping file. (A) Each column represents a tuber comparison between the two genotypes at a defined time point post inoculation, as indicated. (B) Each column represents a foliar comparison between the two genotypes at a defined time point post inoculation, as indicated. Bins in blue are transcribed at higher levels in WT than in +*RB;* bins in red are transcribed at higher levels in +*RB* than in WT; bins in white did not significantly differ in transcript levels between WT and +*RB*. Results indicate that faster and stronger activation of defense bins, most notably biotic stress response and receptor kinase bins, occurred in tubers and foliage of the tuber late blight resistant +*RB* line.

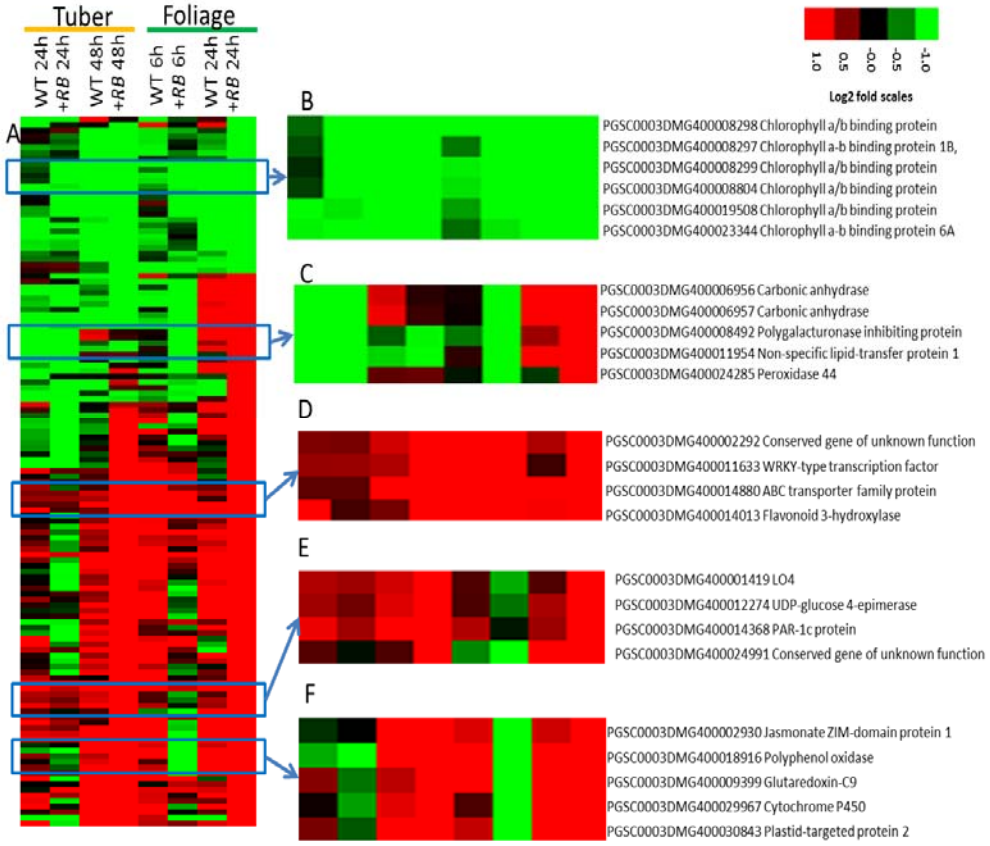


**Figure 3.7 Hierarchical clustering of shared DE genes between foliage and tubers.** Foliage and tubers of nontransformed 'Russet Burbank' (WT) and transgenic SP2211 (+*RB*) were inoculated with *P. infestans* or water. Tuber samples collected at 0, 24, 48 hpi and foliage samples collected 0, 6, and 24 hpi were subjected to RNA-seq, revealing a total of 1,102 (for tubers) and 475 (for foliage) DE genes between water- and *P. infestans*-inoculated comparisons within the same genotype and the same time point. A total of 127 DE genes are shared between both foliage (FDR=0.01) and tubers (FDR=0.001). Log2(FPKM_p.inf/FPKM_mock) values were used to cluster these 127 DE genes in Cluster 3.0 (Eisen et al. 1998) using uncentered correlation and the complete linkage method. Results were visualized using Treeview (Eisen et al. 1998). Red indicates genes that are up-regulated, green indicates genes that are down-regulated. (A) Global visualization of the 127 DE genes; (B) a small gene cluster representing down-regulated photosynthesis genes in all tissues; (C) A small gene cluster harboring carbonic anhydrase (CA) (D) A small cluster representing genes that are generally up-regulated in all conditions. (E-F) Small clusters of DE genes showing higher induction in +*RB* in both foliage (at 24 hpi) and tubers (at 48 hpi). The highlighted clusters indicate shared responses such as down regulation of photosynthesis genes and up-regulation of defense related genes in both tubers and foliage.

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# Appendix One: Enhanced *RB* transgene transcription correlates with enhanced tuber resistance against the late blight pathogen *Phytophthora infestans*

Gene *RB* (Song et al. 2003) is generally viewed as a foliar R gene. Our group discovered that enhance *RB* copy numbers and transcript levels correlate with enhanced foliar resistance against the late blight pathogen (Bradeen et al. 2009). In a survey of 11 transgenic (+*RB*) genotypes, our group discovered two transgenic lines (SP2211, SP2213) that are not only foliage resistant but also tuber resistant to the late blight pathogen. Importantly, these two transgenic lines also happen to have the highest *RB* transgene transcript levels in the tuber. Due to the highly variable tuber late blight disease phenotypes, the assumptions of ANOVA models to identify resistant/susceptible lines are often not met. Through transforming tuber blight disease phenotype data into "resistant" and "susceptible" categories, we were able to analyze the correlation of disease incidence with *RB* transgene transcript levels using a generalized linear model (logistic regression). Our results indicate that there is a significant correlation between *RB* gene transcript levels and enhanced tuber resistance.

## Materials and methods

### Plant materials

Cultivated potato carrying the *RB* transgene [*Solanum tuberosum* L. cvs. Katahdin (lines SP922, SP951, SP966), Russet Burbank (lines SP2105, SP2182, SP2211, SP2213), and Dark Red Norland (lines SP2564, SP2572, SP2577, SP2585)], and nontransformed cultivated potato (*S. tuberosum* cultivars Katahdin, Russet Burbank and Dark Red Norland) were kind gifts from John Helgeson (USDA-ARS and University of Wisconsin-Madison) and Sandra Austin-Phillips (University of Wisconsin-Madison) and have been previously described (Bradeen et al. 2009). All plant materials were produced in greenhouses on the University of Minnesota (St. Paul, MN) campus or in field plots at the University of Minnesota Sand Plain Research Farm (Becker, MN) using standard cultural practices. For molecular assays, tuber tissue (a 1x1x2.5 centimeter tuber core taken halfway between the stolon and apical ends) was collected prior to inoculation, frozen in liquid nitrogen and stored at -80°C.

#### Whole tuber assay

Tuber blight resistance was tested in two years (2006 and 2008) as previously described (James et al. 2001), with slight modifications. Inoculum (7,000 sporangia/mL H2O) was prepared from *P. infestans* US-8 [isolate 940480 (A2)] sporangia (Millett et al. 2009). Whole, unwounded, field grown tubers were stored at 4°C after harvest, until one,

four, six, fifteen, or nineteen weeks post-harvest, when five tubers per line, per tuber age, were hand-washed and air-dried at room temperature for 24 hours prior to inoculation. Three of the five tubers were inoculated with *P. infestans* as described below, one was water-inoculated, and one was reserved for RNA extraction. For *P. infestans*- and water-inoculated tubers, a wound was made halfway between the stolon and apical ends using a flat end screw (of 2 millimeter diameter) that extended 2 millimeters through a wooden handle, yielding a wound approximately 6.3 cubic millimeters in size. Ten µL of prepared inoculum or sterile water (for the control tuber) were pipetted into each wound. Inoculated tubers were then maintained in the dark, first at 22°C, ~95% RH for 72 hrs, and then at 13°C, ~90% RH for 11 days. Inoculated tubers were peeled around the inoculation site at the termination of the incubation period and cut in half across the longitudinal axis through the inoculation site to measure radii and depth of diseased tissue in order to calculate volume of diseased tissue. Blight data were not collected for week four in 2006, or for week one in 2008. Additionally, transgenic lines SP966 and SP2213 ('Russet Burbank') were not evaluated in 2008.

## qRT-PCR methods

Primer design, RNA isolation, and qRT-PCR methodologies have been previously described(Bradeen et al. 2009; Millett and Bradeen 2007; Millett et al. 2009). Briefly, *RB* transgene specific primers 2MAMA5'3 and 2MAMA3'1 and *EF1α* primers developed by Nicot et al. (2005) (EF1-f and EF1-r) were utilized in 25 µL total volume [5 µL (7-50 µM) template] qRT-PCRs using SuperScript™ III Platinum® SYBR® Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA). *RB* and *EF1α* fragments were

amplified in independent reaction tubes and each sample was tested in triplicate. Results were evaluated using Sequence Detection Software v. 1.4.0.25 (Applied Biosystems). The threshold value was adjusted against control reactions for consistent Ct values across multiple experimental plates. Ct values of *RB* transgene and *EF1α* were then processed with the Visual Basic software Q-Gene (Muller et al. 2002) in Microsoft® Excel® and subsequently analyzed using R. In these analyses, *RB* transcript levels in 2006 and 2008 were scaled using upper quartile values (Bullard et al. 2010).

### Logistic regression method

For each week within a given year, a tuber was categorized as resistant if the tuber disease volume was ranked below the 1$^{st}$ quartile, which usually means absence of late blight disease lesions. Categorized disease phenotype data (resistant or susceptible) and *RB* gene transcript data were correlated using a logistic regression model: logit[probability of resistance]=α+βx; whereas x is *RB* gene transcription levels. The analysis was done using R (R Development Core Team 2011) statistical software.

## Results and discussion

Across all weeks examined (week 1, 6, 19 for 2006 and week 4, 19 for 2008), 'Russet Burbank' transgenic line SP2211 and SP2213 showed visually the least disease development (Figure A1). Interestingly, the same lines possess the highest *RB* gene transcript levels (Millett 2008). Disease development for three of the four 'Dark Red Norland' transgenic lines also showed visually less disease development than the

corresponding nontransgenic control, despite a lack of significance using ANOVA models and Tukey HSD mean separation methods (Millett 2008).

Logistic regression using two years' data suggests that overall there is a correlation (p=0.01) of *RB* transcript levels and disease resistance (measured as 1-disease incidence probability) (Figure A2). It was previously reported that high *RB* transcript levels correlated with enhanced foliar resistance (Kramer et al. 2009) (Bradeen et al. 2009). The current study suggests that the enhanced *RB* gene transcript and enhanced tuber resistance are also correlated. Thus, *RB* can confer both foliage and tuber blight resistance, similar to those observed for other R genes (Bradshaw et al. 2006; Park et al. 2005).

## Conclusion

In the foliage, enhanced *RB* transcript levels correlate with enhanced late blight resistance (Bradeen et al. 2009). Interestingly in the tubers, enhanced *RB* transcript levels also correlated with enhanced late blight resistance (Figure A2). Through logistic regression analysis, we were able to establish a significant correlation between transgene transcript levels and disease resistance in transgenic tubers. Our results have important implications in *R* gene deployment, documenting that gene *RB* can impart enhanced late blight resistance to both potato foliage and tubers.

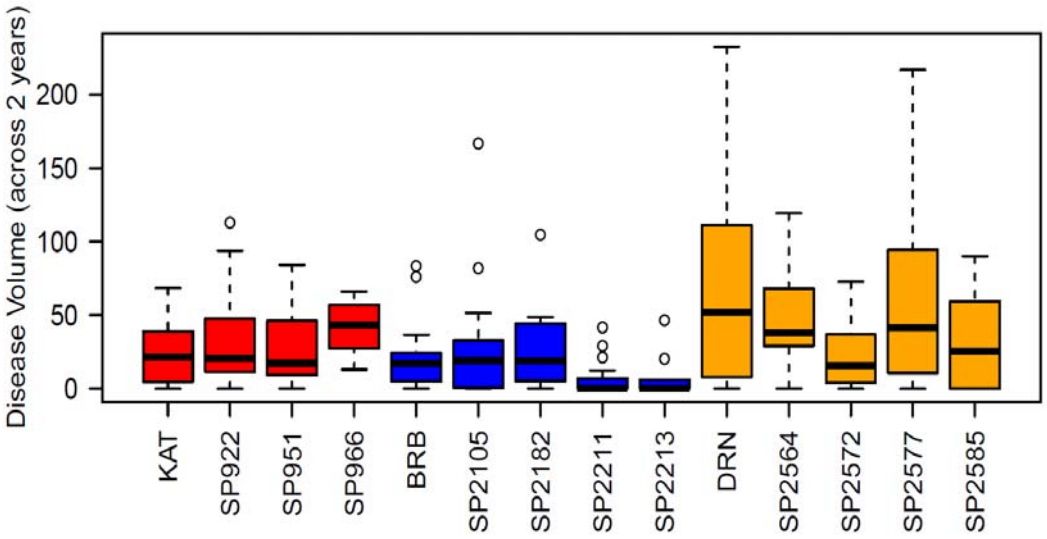


**Figure A1. Boxplot of tuber late blight disease volumes.** Observed disease volume in $cm^3$ represented as a box and whisker plot for each nontransformed (control cultivars KAT: 'Katahdin', BRB: 'Russet Burbank' and DRN: 'Dark Red Norland') and transgenic (+*RB;* "SP") potato lines across all replicates, all tuber ages, and two years. 'Russet Burbank' lines SP2211 and SP2213 showed the least disease development among all lines examined. Importantly, these two lines also have the highest *RB* transcript levels (Millett 2008).

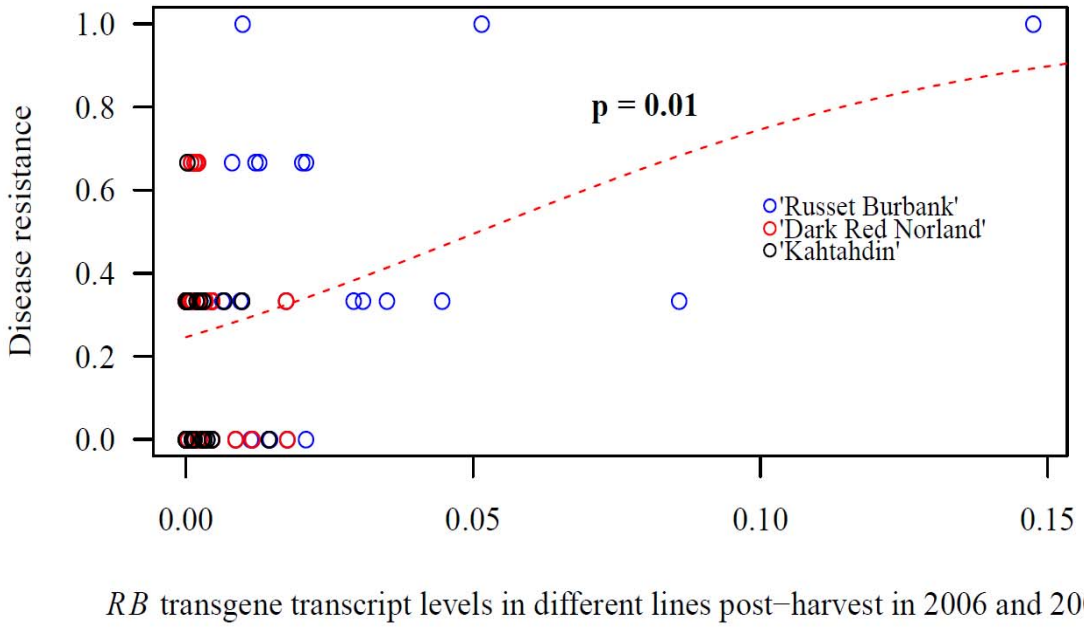


**Figure A2. Tuber blight resistance correlates with *RB* transcript levels in the tubers.** X-axis indicates normalized *RB* transcript levels measured in uninoculated potato tubers. Y-axis indicated tuber late blight disease resistance measured as 1-tuber disease incidence. 'Katahdin' transgenic lines are represented as black open circles; 'Dark Red Norland' transgenic lines are represented as red open circles. 'Russet Burbank' transgenic lines are represented as open blue circles. The dashed line indicates the fitted logistic curve based on a generalized linear model. The correlation of *RB* transcript levels and observed tuber blight resistance is significant (p-value=0.01).