Functional genomics of *Solanum sisymbriifolium* immunity to *Globodera pallida*

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Summary

Stable resistance or immunity against *G. pallida* does not exist in Russet-type potatoes. Our project will first establish the nature of immunity of the trap crop *Solanum sisymbriifolium* to *G. pallida*, and then use this information to identify genes associated with these responses. Roots of potato become infested with *G. pallida* as early as 2 weeks after infestation, whereas infestation in *S. sisymbriifolium* may be delayed by as much as 6 weeks. In these studies, eggs were used as the inoculum as opposed to using hatched second-stage juveniles (J2) which means that infestation may not have been synchronous. While the genome sequence for the cultivated potato species, *Solanum tuberosum* Group Tuberosum, has been assembled and studied for many years, genomic resources for *S. sisymbriifolium* are lacking. Total RNA from a single genotype of *S. sisymbriifolium* was used to generate two RNA pools, one uninfested and the other from roots infested with *G. pallida*.

Key words: *Solanum sisymbriifolium*, *Globodera pallida*, *Solanum tuberosum*, immunity, cyst nematodes

Introduction

Potato is the world’s most important non-grain crop and, overall, the third most important food crop. The United States of America (USA) ranks fifth in global production. In the USA, potatoes are grown commercially on more than 1 million acres throughout 30 states, with Idaho leading production, followed by Washington, Wisconsin, Colorado, and North Dakota. The estimated farm gate value of this crop is more than US$4 billion, and the total value of production exceeds US$4 trillion. Potato production in the USA, and the ability to market USA potatoes abroad, is threatened by the presence and potential geographic spread of invasive cyst nematodes in the genus *Globodera*.

*Globodera* is a worldwide regulatory concern, and one of the most economically important pests of potato, causing as much as 80% yield loss in infested fields. At the present time, *Globodera* infestations have only been become established in a few, widely scattered places in the USA. *Globodera rostochiensis* was first reported in New York in 1941 (http://plpnemweb.ucdavis.edu/nemaplex/Taxadata/G053s2.htm). However, the unexpected appearance of *G. pallida* in Idaho in
2006, the recent discovery of a new species (G. elliingtonae) in Oregon and Idaho in 2012, and the emergence of a new pathotype of G. rostochiensis in New York, all highlight the threat that this group of nematodes poses to the multi-billion-dollar USA potato industry.

The introduction and potential spread of potato cyst nematodes has serious implications for USA potato exports and the presence of G. pallida in Idaho has been viewed with alarm by other states and countries that import Idaho potatoes and other farm products. At one point prior to implementation of the current quarantine measures, import of Idaho fresh potato products and nursery stock was banned in Canada and Mexico, while, for some time, Japan entirely cut off imports of USA potatoes.

Surveys in 2006 to determine the possible origin and distribution of G. pallida in Idaho confirmed nine infested fields totaling 911 acres, all within a five mile radius in Bingham and Bonneville Counties, Idaho. The fields associated with infested acreage through shared tenancy, farming practices, equipment, and/or shared borders have been extensively surveyed and regulated. As of January 2015, 7,773 acres of farmland are regulated, of which 26 fields comprising 2,897 acres are infested; all are within a 7.5 mile radius in Bonneville and Bingham counties. In New York, because of the presence of G. rostochiensis, 312,708 acres are regulated, and, of these, 5,785 are infested.

The total economic impact from Globodera spp. for the USA has not been calculated, but for Idaho the economic impact was estimated to be US$4.4 million in direct losses and US$8.6 million in lost income, producing a loss of US$33.2 million in state GDP, and US$1.2 million in state tax revenues (REF). The impact of this loss on the Idaho economy has been particularly harsh since potatoes make up an estimated US$950 million or 28% of Idaho’s crop cash receipts. Consequently, containment and eradication of G. pallida is a top priority for the Idaho potato industry, including the Idaho Potato Commission, the Idaho State Department of Agriculture, and USDA-APHIS. Millions of dollars have been spent in Idaho in eradication efforts. A critical component of this work has been fumigation of infested fields with methyl bromide with bioassays for egg viability conducted at the University of Idaho in Moscow. However, use of soil fumigation is becoming increasingly challenging due to regulatory pressures that make strategies relying primarily on soil fumigation both difficult and costly.

To maintain Globodera-free deregulated potato production acreage, growers will need access to potato cultivars with resistance to the spectrum of species and pathotypes of Globodera that are currently present and/or which could potentially be introduced. However, presently there is very little potato germplasm available with resistance to G. pallida. Potential sources of resistance are limited. Thus, fundamental to the success of the Globodera program in the USA is development of effective and durable resistance in commercially viable potato varieties.

Solanum sisymbriifolium (sticky nightshade, SN) is a solanaceous plant that has been investigated as a trap crop for both G. rostochiensis and G. pallida (Scholte, 2000; Timmermans et al., 2007; Dias et al., 2012). It was shown to be nearly as effective as potato at inducing potato cyst nematode hatching, yet thereafter is unable to support either G. pallida or G. rostochiensis parasitism (Scholte, 2000). Our project has been established to identify the nature of the resistance shown by S. sisymbriifolium and then use this information to identify genes associated with these responses. Our future goal will be to determine whether this resistance can be moved from S. sisymbriifolium into potato lines suitable for production in Idaho and the Northwest. These research efforts will have a positive impact on the potato industry by providing alternatives to current control efforts and will help regulators deal with current infestations and future risks.

Materials and Methods

Organisms used

A population of G. pallida, obtained from an infested field in Shelley, ID, was propagated on the susceptible potato cultivar ‘Desirée’ in clay pots filled with sterilized sandy loam soil and sand (2:1). After 16 weeks, cysts for experimental use were recovered by extracting from soil and roots
using the Fenwick Can method (Turner, 1998). Cysts were placed in sealed pouches (2.54 cm²) made of nylon mesh, and were hydrated for 3 days prior to experimental use.

For non-host assays and hatching assays, Solanum sisymbriifolium was grown from seed in plastic pots for 4 weeks at 18°C and a 16:8 h (day:night) photoperiod before being transplanted into pots for experiments. For all experiments, potato plants (S. tuberosum var. 'Russet Burbank' or 'Desirée') were grown from tissue culture plantlets before being transplanted into pots for experiments. A single genotype of S. sisymbriifolium has been identified for use in sequencing and for asexual propagation. This genotype has been transferred to tissue culture for long-term storage and propagation.

**Host assay**

Experiments were conducted using Prosser fine sandy loam soil, which was air-dried and sieved through a 5 mm mesh. A 2:1 sand:soil mixture was autoclaved twice for 90 mins at 121°C prior to use. The host assay consisted of a 16 wk incubation of PCN cysts (five eggs g soil) planted to potato (Russet Burbank) or S. sisymbriifolium in clay pots (16 cm diameter) with six replicates/treatment. Pots were arranged in the greenhouse in a randomized block design. After 16-weeks, plants were destructively sampled by extracting and counting PCN cysts from soil using the Fenwick Can method (Turner, 1998).

**Hatching assay**

Potato (cv 'Desirée') and S. sisymbriifolium plants were grown for 4 weeks under greenhouse conditions (18 hr light, 20°C). Diffusate was collected from six reps of each host by running 200 mL of water through the pot and collecting the flow-through. The diffusate was then filter sterilized using a 0.22 µm filter. Diffusate was stored at -20°C until use. For hatching assays cysts were crushed then mixed with a 1:1 diffusate:water and 200 µL was dispensed in 96 well plates (100 eggs well). Initial counts of eggs and juveniles were performed the day the hatching assay was set up. Counts were repeated after 2 weeks.

**Infestation assays**

Potato (cv 'Russet Burbank') and S. sisymbriifolium plants were grown under greenhouse conditions (18 h light, 20°C) as described above. For infestation assays, either potato or S. sisymbriifolium were exposed to G. pallida (five eggs g soil, inoculated as cysts contained in cyst bags). Plants were destructively sampled every 2 weeks post inoculation and roots were stained with acid fuchsin (Byrd et al., 1983). Counts of all G. pallida life stages were recorded over the course of 8 weeks for each plant species.

**Characterization and analysis of S. sisymbriifolium genome**

While the genome sequence for the cultivated potato species, Solanum tuberosum Group Tuberosum, has been assembled and studied for many years, genomic resources for S. sisymbriifolium are lacking. As of July 2015, the national sequence database, GenBank, listed 14 different S. sisymbriifolium protein-coding sequences. Because so little effort has been put into studying this species, researchers do not have the genomic resources needed to identify the genes and pathways that confer immunity to Globodera. Total RNA from a single genotype of S. sisymbriifolium was used to generate two RNA pools, one from four tissues (leaf, root, bud and stem) and the other from roots infested with G. pallida. Tissue collected for RNA extraction was immediately flash frozen with liquid nitrogen and extracted using a modified RNAzol method. All RNA samples were evaluated at the time of extraction using a NanoDrop spectrophotometer and Agilent TapeStation and frozen at -80°C until processed for library generation. Primary libraries were generated when first strand cDNA was made from mRNA using an oligo dT primer. After second strand synthesis occurred, the double-stranded cDNA was size fractionated and directionally cloned into the pExpress I vector. Normalized libraries were generated from the
respective primary cDNA library when cRNA:cDNA hybrids hybridized at a low C_{t} value were removed by phenol extraction and the remaining single-stranded target cDNA was converted to double-stranded DNA with a repair oligo and Taq DNA polymerase.

**Agroinfection of Solanum sisymbriifolium: Transient expression assays**

All *S. sisymbriifolium* plants used were clones of a single founder to reduce the confounding effects of genotype on reproducibility. Agroinfection studies were done using GV3101 pTi2260 (Anand et al., 2007) harboring pCAMBIA1301 (http://www.cambia.org/). Bacteria were cultured and induced following Van der Ackenveken et al. (1996) and then either pressure infiltrated (Kapila et al., 1997) or gently painted onto unwounded LT leaves using a nylon paint brush sterilized in 70% EtOH. â-glucuronidase assays were carried out (Jefferson et al., 1987) after culturing plants 6 days at 22°C, 16 h light. The stained areas of each leaf was determined using CompuEye (http://www.ehabsoft.com/CompuEye/LeafSArea/).

**Results and Discussion**

**Host and hatching assays**

*Solanum sisymbriifolium* was found to be a non-host to the Idaho *G. pallida* population (Table 1). Furthermore, hatching of *G. pallida* did not significantly differ from root diffusates of *S. sisymbriifolium* or potato (Table 1).

Table 1. Solanum sisymbriifolium host assay and % hatch for Globodera pallida. Number of *G. pallida* eggs g soil from either potato or *S. sisymbriifolium* after 16 weeks. Percent hatch after 2 week exposure to either *S. sisymbriifolium* or potato root diffusate

<table>
<thead>
<tr>
<th>Crop</th>
<th>Eggs g^{-1} soil</th>
<th>Hatch %*</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. sisymbriifolium</em></td>
<td>0a</td>
<td>58a</td>
</tr>
<tr>
<td>Potato</td>
<td>96b</td>
<td>66a</td>
</tr>
</tbody>
</table>

Values are means of six replicates. *Means followed by the same letter do not differ significantly (*P<0.05*) according to protected LS means.

**Infestation assays**

Development of *G. pallida* in potato followed a normal course of development with highest number of second stage juveniles found at 2 weeks (Fig. 1), followed by an increase in third and fourth stage juveniles and development of females by 8 weeks. In *S. sisymbriifolium*, second stage juveniles were not observed until 6 weeks post inoculation; thereafter no further second stage or other stages of the nematode were observed for the duration of the experiment (Fig. 1). Fewer nematodes were observed in *S. sisymbriifolium* than in potato, and infestation of *S. sisymbriifolium* roots by *G. pallida* occurred much later than for potato. As cysts were used in these experiments, the decrease observed may be caused by less diffusate released from *S. sisymbriifolium* than from potato. Further studies are being conducted to determine if infestation by hatched juveniles is similar for both potato and *S. sisymbriifolium*.

**Characterization and analysis of S. sisymbriifolium genome**

RINe values for the four tissue RNAs ranged from 8.3 to 9.8 while 260/280 ratio ranged from 2.07–2.14 and 260/230 ratio ranged 2.28–2.47. Several RNA samples contributed to the infected root library with an average RINe value of 9.0, and averaged 2.08 and 2.16 for the 260/280 and 260/230 ratios, respectively. Titers for the four libraries ranged from 1.6 × 106 to 1.5 × 107 (Table 2). Average insert size ranged from 1.0 kb to 1.2 kb. The normalized libraries had a lower
percent recombinants compared to the primary libraries from which they were derived (Table 2). Normalization successfully reduced actin by 25 fold for the four-tissue library while the infected root library actin was reduced 30 fold.

RNA used to generated the four cloned libraries of *S. sisymbriifolium* was high quality with average RINe values of 8.3 and 9.0 for the four tissue and infected roots libraries, respectively. This contributed to a high rate of recombinant clones (87–97%) and an average insert size of 1.1 kb. Normalization reduced library redundancy as indicated by a 25–30 fold reduction in actin levels.

<table>
<thead>
<tr>
<th>Library</th>
<th>Titer (cfu mL)</th>
<th>Insert size (average kb)</th>
<th>Recombinants (%)</th>
<th>Actin reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary 4 tissue</td>
<td>$1.6 \times 10^6$</td>
<td>1.0</td>
<td>97</td>
<td>n/a</td>
</tr>
<tr>
<td>Normalized 4 tissue</td>
<td>$7.0 \times 10^6$</td>
<td>1.0</td>
<td>&gt;87</td>
<td>25 fold</td>
</tr>
<tr>
<td>Primary infected roots</td>
<td>$3.0 \times 10^6$</td>
<td>1.2</td>
<td>92</td>
<td>n/a</td>
</tr>
<tr>
<td>Normalized infected roots</td>
<td>$1.5 \times 10^7$</td>
<td>1.1</td>
<td>&gt;90</td>
<td>30 fold</td>
</tr>
</tbody>
</table>

1 Pooled RNA from leaf, root, bud and stem tissue.
2 Roots infected with *G. pallida*.

*Fig. 1. Invasion and development of second and third stage *Globodera pallida* juveniles in potato or *Solanum sisymbriifolium* over 16 weeks.*

*Agroinfection of Solanum sisymbriifolium*

The defense mechanism that *S. sisymbriifolium* uses against PCN is unknown. We are characterizing the symptoms associated with nematode infestation in *S. sisymbriifolium*, and
we will investigate several possible mechanisms that could account for how SN acts as a trap crop. Gene identification in *S. sisymbriifoium* genes will in the end hinge upon our ability to transform it. We are in the process of carrying out transcriptome analysis with the help of annotation programs such as Blast2Go (Conesa et al., 2005) that assign potential functions to each identified translation product. However, verification of each assignment will depend upon showing that reducing its expression in its native host through some form of silencing or targeted mutagenesis and determining whether this reduces nematode resistance. To date, gene transfer protocols have not been developed for SN. Our own attempts to use standard pressure-infiltration protocols (Kapila et al., 1997) with *Agrobacterium* GV3101 pTi2260 containing various eGFP constructs have failed so far, although the same conditions and vectors worked well on *Nicotiana benthamiana* (data not shown).

Because of the difficulties in developing a protocol for agroinfection, we investigated whether we could get better agroinfection by gently painting induced bacteria onto leaves. While we still did not detect eGFP expression, we could detect *uidA* reporter activity on minimally-wounded leaves. Unexpectedly, preliminary studies showed that the percentage of the leaf area expressing GUS was not influenced by which leaf was infected, but rather increased as leaf number decreased (Fig. 2). One interpretation of this result is that some aspect of an antibacterial defense either controlling the penetration of the leaf surface, or the survival of the bacteria attempting infection, improved as the plant aged. Additional studies are underway using plants with as few as 10 to as many as 27 leaves to determine if this trend persists.

Fig. 2. *Solanum sisymbriifoium* shows more severe darkening and necrosis than *Nicotiana benthamiana* to Agrobacteria. Leaves of *S. sisymbriifoium* and *N. benthamiana* were uniformly wounded with a wire dog brush, then dipped into induced *Agrobacterium tumefaciens*. Photographs of leaf discs were taken after 6 days.

One of the major differences between the way that *N. benthamiana* and *S. sisymbriifoium* reacted to agroinfection was that *N. benthamiana* remained healthy up to the very edge of the wound whereas *S. sisymbriifoium* developed a melanic ring and extensive cell death around each puncture (Fig. 3). This disfiguration was not seen when bacteria were not applied to leaves indicating the reaction might be defensive. Based on this observation, we investigated whether we could get better agroinfection by gently painting induced bacteria onto leaves. While we still did not detect eGFP expression, we could detect *uidA* reporter activity on minimally-wounded leaves. Unexpectedly, preliminary studies showed that the percent of the leaf area expressing GUS was not influenced by which leaf was infected, but rather increased as leaf number decreased (Fig. 3). One interpretation of this result is that some aspect of an antibacterial defense either controlling the penetration of the leaf surface, or the survival of the bacteria attempting infection, improved as the plant aged. Additional studies are underway using plants with as few as 10 to as many as 27 leaves to determine this trend persists.
Current goals

Several decades of study (Niebel et al., 1993; Gheysen & Fenoll, 2002) have shown that many plant responses to infestation by Globodera can only be detected at the site of infestation. Localised gene expression at the feeding site during its establishment can lead either to the formation of a syncytium as observed in a susceptible reaction, or to localised root cell death resulting in death of the nematode as observed in resistant or immune responses. Although our studies are not yet complete, we have found evidence that nematode infestation triggers localised cell death or a hypersensitive response at or near putative feeding sites in SN. Transcript analysis of expression changes in these responding cells could lead to the discovery of novel defence genes with potential uses in many different crops. However, these cells are a small fraction of the entire root so that when mRNA is isolated and analysed, transcript changes occurring in the few infested cells are overshadowed by the expression of housekeeping functions and secondary responses in uninfested ones. Comparisons and analyses of transcriptional events occurring in cells from the feeding site of a susceptible potato or from cells from the feeding site of an immune SN reaction will help us identify genes and pathways induced during the course of an immune reaction. We have been perfecting protocols to isolate messenger RNA specifically from the infestation centres of susceptible potato and resistant SN roots for sequencing and analysis. The comparison of these pools will reveal which genes are up- or down-regulated during infestation of each plant species. The SN sequences will be used to identify, and if possible, isolate close homologues from the potato genome. Finally, the pairs of SN and potato genes will be hybridized to RNA from infested and uninfested plants of the clones' original host species to compare their expression profiles. Genes that are significantly induced or repressed will be further characterized for their role in PCN immunity in SN and potential candidate genes will be used for development of immune potato.

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References


