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ORIGINAL PAPER

GSL2 over-expression confers resistance to *Pectobacterium atrosepticum* in potato

Sara Mohan · Sathiyamoorthy Meiyalaghan · Julie M. Latimer · Michelle L. Gatehouse · Katrina S. Monaghan · Bhanupratap R. Vanga · Andrew R. Pitman · E. Eirian Jones · Anthony J. Conner · Jeanne M. E. Jacobs

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Abstract

Key message **Over-expression of the potato *Gibberellin Stimulated-Like 2* (*GSL2*) gene in transgenic potato confers resistance to blackleg disease incited by *Pectobacterium atrosepticum* and confirms a role for *GSL2* in plant defence.**

Abstract The *Gibberellin Stimulated-Like 2* (*GSL2*) gene (also known as *Snakin 2*) encodes a cysteine-rich, low-molecular weight antimicrobial peptide produced in potato plants. This protein is thought to play important roles in the innate defence against invading microbes. Over-expression of the *GSL2* gene in potato (cultivar Iwa) was achieved using *Agrobacterium*-mediated gene transfer of a plant expression vector with the potato *GSL2* gene under the regulatory control elements of the potato light-inducible *Lhca3* gene. The resulting plants were confirmed as being transgenic by PCR, and subsequently analysed for transcriptional expression of the *Lhca3-GSL2-Lhca3* chimeric potato gene. Quantitative RT-PCR analysis demonstrated that the majority of the transgenic potato lines over-expressed the *GSL2* gene at the mRNA level. Based on qRT-PCR results and evaluation of phenotypic appearance, eight lines were selected for further characterisation and evaluated in bioassays for resistance to *Pectobacterium atrosepticum* (formerly *Erwinia carotovora* subsp. *atroseptica*), the causal agent of blackleg in potato. Three independent pathogenicity bioassays showed that transgenic lines with significantly increased transcriptional expression of the *GSL2* gene exhibit resistance to blackleg disease. This establishes a functional role for GSL2 in plant defence against pathogens in potato.

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S. Mohan · S. Meiyalaghan · J. M. Latimer · M. L. Gatehouse · K. S. Monaghan · B. R. Vanga · A. R. Pitman · J. M. E. Jacobs (✉)
The New Zealand Institute for Plant & Food Research Ltd, Private Bag 4704, Christchurch 8140, New Zealand
e-mail: jeanne.jacobs@plantandfood.co.nz

S. Mohan · E. E. Jones
Faculty of Agriculture and Life Sciences, Lincoln University, P.O. Box 85084, Canterbury 7647, New Zealand

B. R. Vanga
Department of Microbiology and Immunology, University of Otago, P.O. Box 56, Dunedin 9054, New Zealand

A. R. Pitman · A. J. Conner · J. M. E. Jacobs
Bioprotection Research Centre, Lincoln University, P.O. Box 85084, Canterbury 7647, New Zealand

A. J. Conner
AgResearch Ltd, Grasslands Research Centre, Private Bag 11008, Palmerston North 4442, New Zealand

Introduction

The Gibberellin Stimulated-Like proteins, GSL1 and GSL2 (also known as Snakin-1 and Snakin-2, respectively), from potato (*Solanum tuberosum* L.) are low-molecular weight peptides with activity against a wide range of bacteria and fungi (Segura et al. 1999; Berrocal-Lobo et al. 2002; López-Solanilla et al. 2003; Kovalskaya and Hammond 2009). These peptides share common structural features with more than 500 antimicrobial peptides discovered in plants and animals, such as an N-terminal putative signal sequence, a highly divergent intermediate region and a conserved, cysteine-rich carboxyl-terminal domain (Wang and Wang 2004; Hammami et al. 2009; Pelegrini et al. 2011). GSL1 and GSL2 induce rapid aggregation of both Gram-negative and Gram-positive bacteria, and their spectrum of

antimicrobial activity is similar (Segura et al. 1999; Berrocal-Lobo et al. 2002). Although the bacterial aggregation induced by GSL1 and GSL2 does not correlate with antimicrobial activity, these proteins may play a role in controlling pathogen migration (Segura et al. 1999; Berrocal-Lobo et al. 2002; Kovalskaya and Hammond 2009).

As a consequence of their antimicrobial activity, GSL1 and GSL2 are often considered to play important roles in the innate defence against invading microorganisms (López-Solanilla et al. 1998; Segura et al. 1999; Berrocal-Lobo et al. 2002; Mao et al. 2011). An orthologue of *GSL2* from *Phaseolus vulgarus* was shown to tightly bind a 25 kD proline-rich protein and hypothesised to function as a two-component chitin receptor involved in plant pathogen interactions through antimicrobial activity and/or signalling (Bindschedler et al. 2006). Similar peptides in other species have been implicated in diverse biological processes, including cell division, cell elongation, cell growth, transition to flowering, and signalling pathways (Aubert et al. 1998; Kotilainen et al. 1999; Ben-Nissan et al. 2004; de la Fuente et al. 2006; Furukawa et al. 2006; Roxrud et al. 2007; Nahirñak et al. 2012).

Over-expression of the potato *GSL1* gene in transgenic potato plants enhances resistance to two important potato pathogens, *Pectobacterium carotovorum* subspecies *carotovorum* (formerly known as *Erwinia carotovora*) and *Rhizoctonia solani* (Almasia et al. 2008). A role for GSL1 in plant defence was also suggested from the observation of decreased virulence of GSL1-sensitive mutants of *Dickeya chrysanthemi* (formerly known as *Erwinia chrysanthemi*) to potato tubers (López-Solanilla et al. 1998). Likewise, over-expression of the tomato (*Solanum lycopersicum*) *GSL2* gene in tomato enhances tolerance to *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker and wilt disease (Balaji and Smart 2012). Viral-induced gene silencing of *GSL2* in *Nicotiana benthamiana* increases susceptibility to wilt disease development induced by *C. michiganensis* subsp. *michiganensis* (Balaji et al. 2011). Similarly, virus-induced silencing of *GSL2* in *Capsicum annuum* increases susceptibility to root-knot nematodes (*Medoidogyne* spp.) (Mao et al. 2011).

This study investigates the role of the *GSL2* gene in conferring disease resistance in potatoes. We demonstrate that over-expression of the endogenous *GSL2* gene encoding an antimicrobial peptide in potato confers resistance to blackleg disease caused by *Pectobacterium atrosepticum*.

Materials and methods

Vector construction

Genomic DNA was isolated from the leaves of potato plants (cultivar Iwa) based on a previously described method (Bernatzky and Tanksley 1986). The *StGSL2* gene was amplified by PCR using the primers GSL2-F and GSL2-R (Table 1). The 50 μL PCR amplification contained 1× Expand High Fidelity^{PLUS} Reaction Buffer with 1.5 mM $MgCl_2$ (Roche Applied Science, Mannheim, Germany), 0.2 mM of each dNTP, 0.4 μM of each primer, 1 μL of DNA (~100 ng) and 2.5 U of Expand High Fidelity *Taq* polymerase (Roche Applied Science). The PCR involved 1 min at 93 °C, then 34 cycles of 30 s at 92 °C, 30 s at 58 °C, and 90 s at 72 °C followed by a 6 min extension at 72 °C. The expected 955 bp fragment was cloned into pGEM®-T Easy (Promega, Mannheim, Germany) and

Table 1 Primers used in this study

Target gene	Primer pairs	Primer sequence (5′–3′)	Product size (bp)
GSL2	GSL2-F	AAATATTTCAAATTCCAATGGC	955
	GSL2-R	CAATACAATGCAAACCAGAACAA	
Lhca3-GSL2	GSL2-bF4	TCATCAAGGCCAAGATTGTG	461
	Cab-Ra	TGTTACATTACACATAAGAGAAGG	
Actin	Actin-F	GATGGCAGAAGGCGAAGATA	1,069
	Actin-R	GAGCTGGTCTTTGAAGTCTCG	
nptII	NptII-F	ATGACTGGGCACAACAGACAATTCGGCTGCT	612
	NptII-R	CGGGTAGCCAACGCTATGTCCTGATAGCGG	
virG	GMT25virGF	GCGGTAGCCGACAG	692
	GMT25virGR	GCGTCAAAGAAATA	
GSL2	GSL2-exF2	CAAGTGACCAGCAATGCTATTTCT	231
	GSL2-exR2b	AGGGCATTTACGTTTGTTGC	
Eflα	Eflα-F	ATTGGAAACGGATATGCTCCA	101
	Eflα-R	TCCTTACCTGAACGCCTGTCA	
ECA0515	ECA0515-F	ATGTCAGAACTGACCGCCTT	215
	ECA0515-R	ATTAAGAACACGGCCACCTG	

sequenced to confirm the authenticity of the sequence as the b1 allele (EU848497) of *StGSL2*. The b1 allele of *StGSL2* was then isolated from plasmid DNA by PCR using the GSL2-F and GSL2-R primers (Table 1). The 50 μL PCR mix contained 1× ThermoPol Reaction Buffer [20 mM Tris–HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1 % Triton X-100, pH 8.8 at 25 °C], ~1 ng plasmid DNA, 0.2 mM of each dNTP, 0.4 μM of each primer and 0.6 U of Vent$^{®}_{R}$ DNA polymerase (New England BioLabs, MA, USA), to amplify a blunt-end fragment. The PCR involved 1 min at 93 °C, then 34 cycles of 30 s at 92 °C, 15 s at 58 °C, and 90 s at 72 °C, followed by 6 min extension at 72 °C. The 955 bp PCR product was gel-purified using QIAquick Gel Extraction Kit (QIAGEN, Venlo, The Netherlands) and 5′-phosphorylated for efficient blunt-end ligation using Quick Blunting Kit (New England BioLabs) according to the manufacturer's instructions. The resulting fragment was then ligated into the *Psi*I site of the potato *Lhca3* expression cassette (Meiyalaghan et al. 2009) using T4 DNA Ligase (New England BioLabs) according to manufacturer's recommendation. Clones with the inserted PCR product in the desired orientation were identified by sequencing using Applied Biosystems BigDye® Terminator kit. The primers Cab-Fa (5′TTCTAGTGGAGCTAAGTGTTCA3′; in the *Lhca3* promoter region) and Cab-Ra (5′TGTTACATTACACATAAGAGAAGG3′; in the *Lhca3* 3′ terminator region) were used individually as sequencing primers in each sequencing reaction. Sequencing reactions were analysed using an ABI 3130xl automated sequencer (Applied Biosystems, USA). A plasmid verified to have the *Lhca3-GSL2-Lhca3* chimeric potato gene in the sense orientation was digested with *Hin*dIII and the 1,770 bp *Lhca3-GSL2-Lhca3* fragment was blunt-ended using Quick Blunting Kit (New England BioLabs) prior to ligation into the blunt-ended *Not*I site of the binary vector pMOA33 (Barrell and Conner 2006) using T4 DNA Ligase (New England Biolabs) to produce pMOA33-Lhca3-GSL2. The orientation of the chimeric *Lhca3-GSL2-Lhca3* gene within the T-DNA in binary vector pMOA33-Lhca3-GSL2 was tested by restriction analysis using *Eco*RV and *Xho*I to select the binary vector that contains the *Lhca3* promoter adjacent to the right border within the T-DNA.

Potato transformation

The pMOA33-Lhca3-GSL2 binary vector was transferred to *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) using the freeze–thaw method (Höfgen and Willmitzer 1988). *Agrobacterium* cultures harbouring the binary vector were cultured overnight on a shaking table at 28 °C in LB broth supplemented with 300 mg/L spectinomycin. Leaf segments from virus-free plants of potato (cultivar Iwa) were transformed using 100 mg/L kanamycin as a selection agent as previously described (Meiyalaghan et al. 2006).

Molecular confirmation of transformation

Genomic DNA was extracted from 0.2 g of leaf material from putative independent transgenic plants and control plants using a modified CTAB method (Doyle and Doyle 1990). To verify the quality of the genomic DNA template, PCRs were conducted to amplify the endogenous actin gene using Actin-F and Actin-R primers (Table 1). The PCR involved 1 min at 93 °C, then 40 cycles of 30 s at 92 °C, 30 s at 58 °C, and 90 s at 72 °C, followed by a 6 min extension at 72 °C. The NptII-F and NptII-R primers (Table 1) were used to amplify the *npt*II gene from the samples. Each 10 μL reaction mix and the thermal cycling profile were as described above, except for an annealing step of 20 s at 60 °C and extension step of 45 s at 72 °C. To amplify the *Lhca3-GSL2-Lhca3* chimeric potato gene, a primer specific to *GSL2* (GSL2-bF4) and a primer specific to the *Lhca3* 3′ terminator (Cab-Ra) (Table 1) were used to avoid endogenous gene amplification. The conditions for PCR involved 1 min at 94 °C, then 34 cycles of 20 s 93 °C, 20 s 55 °C, 80 s 72 °C, followed by a 6 min extension at 72 °C. Finally, primers specific to the *Agrobacterium virG* gene (GMT24virGF and GMT25virGR) (Table 1) were used to investigate the possible presence of *Agrobacterium* contamination remaining in the plant tissue. The PCR conditions involved 2 min at 94 °C, then 34 cycles of 30 s 94 °C, 30 s 45 °C, 30 s 72 °C, followed by a 5 min extension at 72 °C. Each 10 μL PCR mix contained 1× ThermoPol Reaction Buffer [20 mM Tris–HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1 % Triton X-100, pH 8.8 at 25 °C], 0.2 mM of each dNTP, 0.2 μM of each primer and 0.4U of *Taq* DNA Polymerase (New England BioLabs). All PCRs were carried out in a Mastercycler (Eppendorf, Hamburg, Germany).

qRT-PCR for *GSL2* transcript abundance

RNA was extracted from leaves, stems, tubers and roots of 12-week-old greenhouse-grown plants using Illustra RNAspin Mini Isolation kit (GE Healthcare, Buckinghamshire, UK), including DNase treatment, according to the manufacturer's instructions. First-strand cDNA synthesis was performed with 1 μg of total RNA per sample using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. The cDNA was diluted tenfold, then used as template in the qRT-PCR experiments. Constitutively expressed *Ef*1α was used as an internal standard using the primers Ef1α-F and Ef1α-R (Table 1) (Nicot et al. 2005) to normalise for small differences in template amounts. Primers

were designed to amplify the *GSL2* gene (GSL2-exF2 and GSL2-exR2b) using Primer express 3.0 software (Applied Biosystems, USA).

qRT-PCRs were carried out in the StepOne™ Real-Time PCR System (Applied Biosystems, USA). Initially, the 32 Lhca3-GSL2 lines were analysed for *GSL2* expression in leaf samples from plants grown during late summer in a containment greenhouse in Lincoln (latitude 43°38′ S, New Zealand). Subsequently, qRT-PCR experiments were carried out in leaf, stem, tuber and root samples using three biological replicates for each of the 12 selected transgenic lines from plants grown during late spring in a containment greenhouse in Palmerston North (latitude 40°21′ S, New Zealand). Reactions were performed in triplicate for each sample. PCRs were in a total volume of 20 μL containing 0.2 μM of each primer (forward and reverse), 1X EXPRESS SYBR® GreenER™ qPCR Supermix with premixed ROX reference dye (500 nM) (Invitrogen) and 1 μL cDNA template. PCR conditions were as follows: 2 min at 50 °C, 2 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 60 s at 60 °C, finally dissociation curve analysis was carried out by incubation for 15 s at 95 °C, 60 s at 60 °C, 15 min at 95 °C. The relative expression levels were determined using the StepOne™ software v2.1. Quantification was based on the comparative C_T method ($\Delta\Delta C_T$).

Assays for resistance to *Pectobacterium atrosepticum* SCRI1043

Eight independently derived Lhca3-GSL2 lines were assayed for their resistance to blackleg disease caused by *P. atrosepticum* SCRI1043 in three independent experiments:

Experiment 1 was conducted in a containment greenhouse facility at Lincoln in New Zealand, using in vitro micro-propagated plants.

Experiment 2 was conducted in the Biotron containment facility at Lincoln University in New Zealand (http://bioprotection.org.nz/the-new-zealand-biotron) using in vitro micro-propagated plants.

Experiment 3 was performed in a different containment greenhouse facility at Palmerston North in New Zealand using plants established from tubers.

P. atrosepticum SCRI1043 was stored in 15 % glycerol at −80 °C and cultured onto Luria–Bertani (LB) agar at 28 °C as required. To produce inoculum for the resistance assays, 20 mL of LB broth was inoculated with one colony picked off a newly cultured LB agar plate and incubated overnight in a shaking incubator at 28 °C and 200 rpm. The cells were harvested by centrifugation at 4,000x*g* for 10 min. The supernatant was discarded and the cells were washed in 10 mM $MgCl_2$, and resuspended (OD 1.2 at 600 nm) in 20 mL of 10 mM $MgCl_2$.

In all experiments, individual plants were established in pots (6 × 6 × 7 cm deep) containing potting mix (Conner et al. 1994) and after 4 weeks growth transferred to an inoculation chamber (a tent made of plastic sheets over a metal frame) for 1 week acclimatisation before bacterial inoculation. For each transgenic line and the Iwa control, 14 replicate plants were inoculated with *P. atrosepticum* SCRI1043. The stem under the second fully expanded leaf was wounded using a micropipette tip and inoculated with 10 μL of the bacterial suspension (equivalent to about 10^8 CFU). The inoculation site was then sealed with vaseline to avoid desiccation. The controls consisted of an additional 14 plants inoculated with 10 mM $MgCl_2$ and 14 plants that remained uninoculated. All plants, including controls, were randomly positioned in the inoculation chamber.

Blackleg disease assessment

The severity of blackleg disease on each replicate plant was scored by observing symptoms, such as chlorosis, necrosis, wilted/rolled leaves, and stem collapse. Lesion length at each inoculation site was measured daily for 14 days in experiment 1 and for 11 days in experiment 2. In experiment 3, disease assessment was made only at 10 dpi. In experiment 3, PCR analysis, qPCR and dilution plating were also conducted to determine the persistence and invasion of *P. atrosepticum* SCRI1043 from the inoculation sites. For these assays, a 3 cm long stem segment was excised just below the visible symptoms of the lesion at 10 dpi.

To establish the number of viable cells, stem segments were washed in running tap water, surface-sterilised in 1 % sodium hypochlorite (commercial bleach) for 5 min, washed once with sterile water and subsequently sterilised with 70 % ethanol for 5 min. After sterilisation, the stem samples were washed twice with sterile water and the surface blotted dry with sterile tissue paper. The numbers of viable *P. atrosepticum* SCRI1043 were estimated by grinding 40 mg of the post-inoculated stem tissue in 1 mL of 10 mM $MgCl_2$ and plating 100 μL of serially diluted samples (10^0–10^{-6}) onto three crystal violet pectate agar (CVP) plates (Hyman et al. 2001). After incubation for 72 h at 28 °C, the number of colonies causing characteristic pitting was counted.

Genomic DNA was also isolated from 40 mg of the post-inoculated stem tissue of three randomly selected plants for each treatment using a modified CTAB method (Doyle and Doyle 1990). The presence of the pathogen was confirmed by PCR using the primers ECA0515F and ECA0515R (Table 1) to amplify a 215 bp fragment from ECA0515, a gene which is located on the core genome of *P. atrosepticum* SCRI1043. Each 25 μL PCR mix contained 10× PCR buffer, 0.2 mM of each dNTP, 0.5 μM of

forward primer, 3 μM of reverse primer and 0.4 U of Taq DNA Polymerase (New England Biolabs). The conditions for PCR were: 5 min at 94 °C, 40 cycles of 30 s 94 °C, 30 s 60 °C, 40 s 72 °C, followed by 7 min extension at 72 °C. Amplified products were separated by electrophoresis in a 1 % agarose gel in 1×TAE buffer at 5.5 V/cm for 80 min and visualised under UV light after staining with ethidium bromide (1 mg/L) for 15 min.

A count of *P. atrosepticum* SCRI1043 was also estimated by qPCR using the genomic DNA from triplicate samples of 40 mg of stem tissue excised below visible symptoms. PCRs were in a total volume of 20 μL containing 0.5 μM of forward primer (ECA0515F) and 3 μM of reverse primer (ECA0515R), 1X EXPRESS SYBR® GreenER™ qPCR Supermix with premixed ROX reference dye (500 nM) (Invitrogen) and DNA template. The PCR conditions were 2 min at 50 °C, 2 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 60 s at 60 °C. A dissociation curve analysis was then carried out by incubation for 15 s at 95 °C, 60 s at 60 °C, 15 min at 95 °C to confirm specific amplification of the ECA0515 fragment. Calibration standards were developed using a series of tenfold serial dilutions (10^{-2}–10^{-7}) of *P. atrosepticum* DNA. Calibration equations were derived by linear regression of the C_T values on $\log_{10}$ dilutions. R^2 was 97.2 %, with an amplification efficiency (E) calculated to be 1.61 from the estimated slopes of the regressions using the equation $E = 10^{(-1/\text{slope})}$. For converting the estimated dilutions into DNA/CFU, the known $\log_{10}$ dilutions were converted into $\log_{10}$ CFU or $\log_{10}$ DNA concentrations on the basis of the known CFU for the 10^0 dilution.

Statistical analysis

The data from the qRT-PCR estimates of *GSL2* transcript accumulation and lesion length from blackleg pathogenicity experiments were subjected to analysis of variance. The viable pathogen CFU data and the total pathogen cell count estimated by qPCR were $\log_{10}$ transformed prior to analysis.

Results

Potato transformation

Following co-cultivation of potato leaf discs with *Agrobacterium* possessing the pMOA33-Lhca3-GSL2 binary vector (Fig. 1), a total of 45 putative transgenic lines were recovered. Independent PCR tests confirmed the presence of an endogenous *Actin* gene, the *npt*II gene and the *Lhca3-GSL2-Lhca3* chimeric gene in the regenerated lines (Fig. 2). The same DNA samples failed to amplify PCR products using primers specific to the *Agrobacterium virG* gene. This confirms the absence of any *Agrobacterium* cells remaining in the plant tissue that would otherwise compromise the PCR testing of the transgenic potato lines. Thirty-two independently derived Lhca3-GSL2 lines originating from different co-cultivated explants were selected for further analysis based on their vigour and appearance resembling the non-transgenic controls during in vitro growth. These Lhca3-GSL2 transgenic lines were labelled from 201 to 232. All selected transgenic plants were multiplied in vitro and transferred to the greenhouse. Of the 32 Lhca3-GSL2 lines, 30 developed normally and exhibited a similar phenotypic appearance to the untransformed control plants in the greenhouse. These continued to exhibit a normal phenotypic appearance during subsequent cycles of vegetative propagation. Two lines (#204 and #215) showed abnormal characteristics such as marginal leaf curl and leaf wrinkling and small misshapen tubers.

Quantitative RT-PCR of *GSL2* transcript abundance in leaves

The relative transcript levels of the *GSL2* genes (the endogenous *GSL2* gene and the *Lhca3-GSL2-Lhca3* chimeric potato gene) in all 32 transgenic lines were determined by qRT-PCR analysis relative to the endogenous *GSL2* levels in leaves of non-transgenic potato (cultivar Iwa). Some transgenic lines had similar expression levels to the wild type, whilst in others expression was increased up to 15-fold (Fig. 3). Twelve Lhca3-GSL2 lines with varying levels of *GSL2* expression in leaves were selected for more detailed qRT-PCR assessment in a range of organs. These included 11 lines with medium–high transcriptional over-expression (202, 203, 205, 206, 211, 214, 218, 219, 223, 224 and 232) and a line with no over-expression (228).

Quantitative RT-PCR of *GSL2* transcript abundance in various organs

The expression of the *GSL2* genes was further evaluated by qRT-PCR analysis in the leaves, stems, tubers and roots of the 12 selected transgenic lines (Fig. 4). The *GSL2* transcript level in the leaf tissues exhibited similar trends among lines to that observed in the earlier experiment (Fig. 3); 11 lines retained a 2.5- to 12.5-fold increase in transcription of *GSL2* over the control, while the line with no over-expression (228) retained similar expression to the non-transgenic Iwa control (Fig. 4). Stems of the selected Lhca3-GSL2 transgenic lines showed considerably higher over-expression of *GSL2* transcripts in most transgenic lines, except for the line 228 which also showed no over-expression in the leaves. The transgenic lines 202, 203, 205, 206, and 218 showed a 40- to 110-fold increase in *GSL2* transcripts over the Iwa control, whereas lines 211, 214, 219, 223 and 232 showed a 15- to 30-fold increase

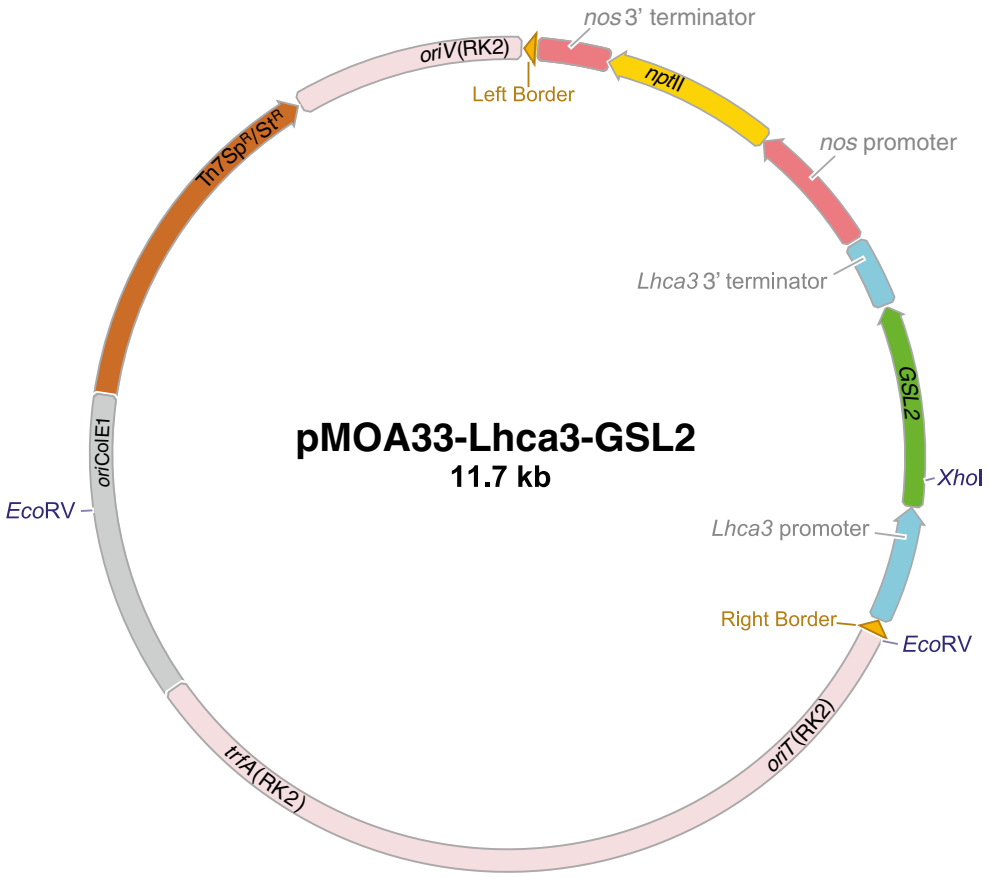

Fig. 1 The pMOA33-Lhca3-GSL2 binary vector. The minimal T-DNA region has two chimeric genes designed for plant expression between the right and left borders: the *Lhca3-GSL2-Lhca3* chimeric gene for overexpression of *GSL2* and the *nos-nptII-nos* chimeric gene conferring kanamycin resistance as a selectable marker for plant transformation. The *Eco*RV and *Xho*I restriction sites were used to determine orientation of the *GSL2* chimeric gene on the T-DNA

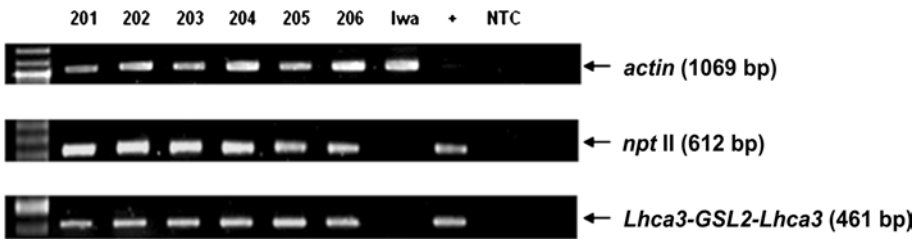

Fig. 2 PCR analysis of representative Lhca3-GSL2 regenerated lines. Amplicons produced following PCR with primers specific to the endogenous *Actin* gene, the neomycin phosphotransferase (*npt*II) gene, and the *Lhca3-GSL2-Lhca3* chimeric potato gene. Lane 1, GeneRuler™ DNA ladder (Fermentas); lanes 2–7, putative transformed lines 201–206; lane 8, non-transgenic Iwa (negative control); lane 9, pMOA33-Lhca3-GSL2 plasmid (positive control), lane 10, no template control. The standard markers in lane 1 are, from top to bottom; 1,500, 1,200 and 1,000 bp (*upper panel*); 800, 700 and 600 bp (*middle panel*); 500 and 400 bp (*bottom panel*)

in expression (Fig. 4). No statistically significant change in *GSL2* expression was observed in root and tuber tissue of the transgenic lines relative to the Iwa control plants (Fig. 4). Although the analysis of variance established marginally significant differences in *GSL2* transcript levels between the transgenic lines, none of the lines was significantly different from the non-transgenic control plants.

Resistance of transgenic lines to *Pectobacterium atrosepticum* SCRI1043

Eight Lhca3-GSL2 lines with varying levels of *GSL2* overexpression were selected based on availability of plant material. Each line was challenged with *P. atrosepticum* SCRI1043 in three independent experiments under varying

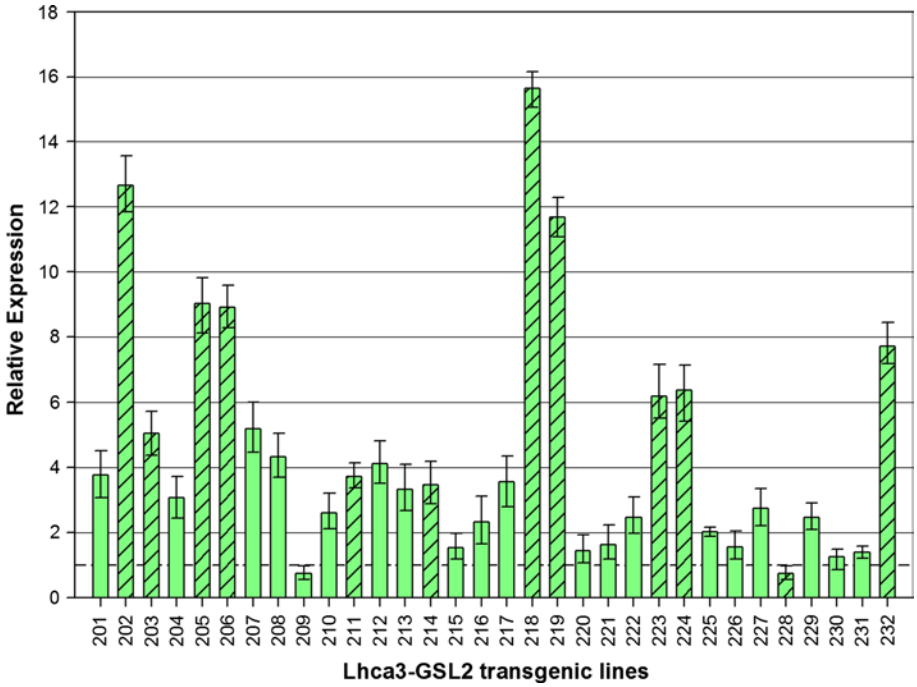

Fig. 3 Quantitative PCR amplification of the *GSL2* transcripts in leaves. Plants were grown in the same containment greenhouse facility as in experiment 1 of the pathogenicity assays. The qRT-PCR data for each sample were normalised to the amount of *Ef1α* transcript using the same amplification conditions. The relative abundance of the *GSL2* gene transcript in transgenic plants was determined using the $\Delta\Delta C_T$ method relative to the non-transgenic Iwa control. The relative expression value of the non-transgenic control is set at 1 and is represented by the *dashed horizontal line*. The 95 % confidence limits for each mean are represented by the *vertical line* ($n = 3$ technical replicates of each sample). The lines selected for further analysis are shown with *hatched bars*

environments. In the first experiment in a greenhouse, disease symptoms were observed on the Iwa control plants 2-day post-inoculation (dpi) with the pathogen. Rapid disease progression was observed and resulted in necrotic stems with folded and curled leaves after 5 days, followed by stem collapse and death of virtually all plants by 12 dpi. All lines showed a small swelling around the lesion (wound response) within 4 days, which developed into necrotic lesions in susceptible lines. For transgenic lines 202, 203, 211 and 219, some necrosis developed in a few plants, from which most recovered by 9 dpi. At 14 dpi, these four Lhca3-GSL2 lines (202, 203, 211 and 219) showed no symptoms of blackleg and were considered to be resistant to blackleg disease (Table 2; Fig. 5). Two lines (205 and 223) showed significantly reduced lesion length compared to the non-transgenic control and were given the status of partially resistant (Table 2). Line 228, with no measurable transcriptional over-expression of *GSL2*, was susceptible to *P. atrosepticum* SCRI1043 (Table 2; Fig. 5). Surprisingly, line 218 also proved to be susceptible in this bioassay. The $MgCl_2$-inoculated control and non-inoculated plants did not develop any disease symptoms or necrotic lesions (Table 2).

The growth environment in the Biotron facility during Experiment 2 had a major influence on the physiology of the plants, resulting in the plants having 'woody' stems. As a consequence, external disease symptoms were not visible. Nevertheless, an association between disease resistance and the over-expression of the *GSL2* gene was visible in longitudinal sections through the inoculated stems (Fig. 6). The internal lesion length and the percentage of plants with internal symptoms established lines 202, 203, 211, and 219 as resistant to *P. atrosepticum* SCRI1043, whereas lines 205, 218 and 223 were partially resistant, and line 228 was susceptible (Table 3).

The third experiment was conducted in a different greenhouse, where rapid disease progression in non-transgenic control plants was also observed with stem collapse and death of plants at 10 dpi (Table 4). Similar to experiment 1, lines 202, 203, 211 and 219 exhibited resistance to *P. atrosepticum* SCRI1043 with no (or minimal) plant collapse and significantly reduced lesion length observed when compared with the Iwa control (Table 4). Lines 205 and 223 both exhibited partial resistance with a low frequency of plant collapse and significantly reduced lesion length. As expected, line 228 with no *GSL2* over-expression was susceptible and exhibited a similar response to the non-transgenic control. Consistent with experiment 1, line 218 also proved to be susceptible in this bioassay. The $MgCl_2$-inoculated control and non-inoculated controls plants failed to develop any disease symptoms or necrotic lesions (Table 4).

Detection of *Pectobacterium atrosepticum* SCRI1043 in potato stems

The viable bacterial colony counts of *P. atrosepticum* SCRI1043 taken from below the visible symptoms of stem samples closely reflected the magnitude of disease symptoms (Table 4). The colony forming unit (CFU) counts for the over-expressing *GSL2* lines with blackleg resistance (202, 203, 211 and 219) were significantly lower

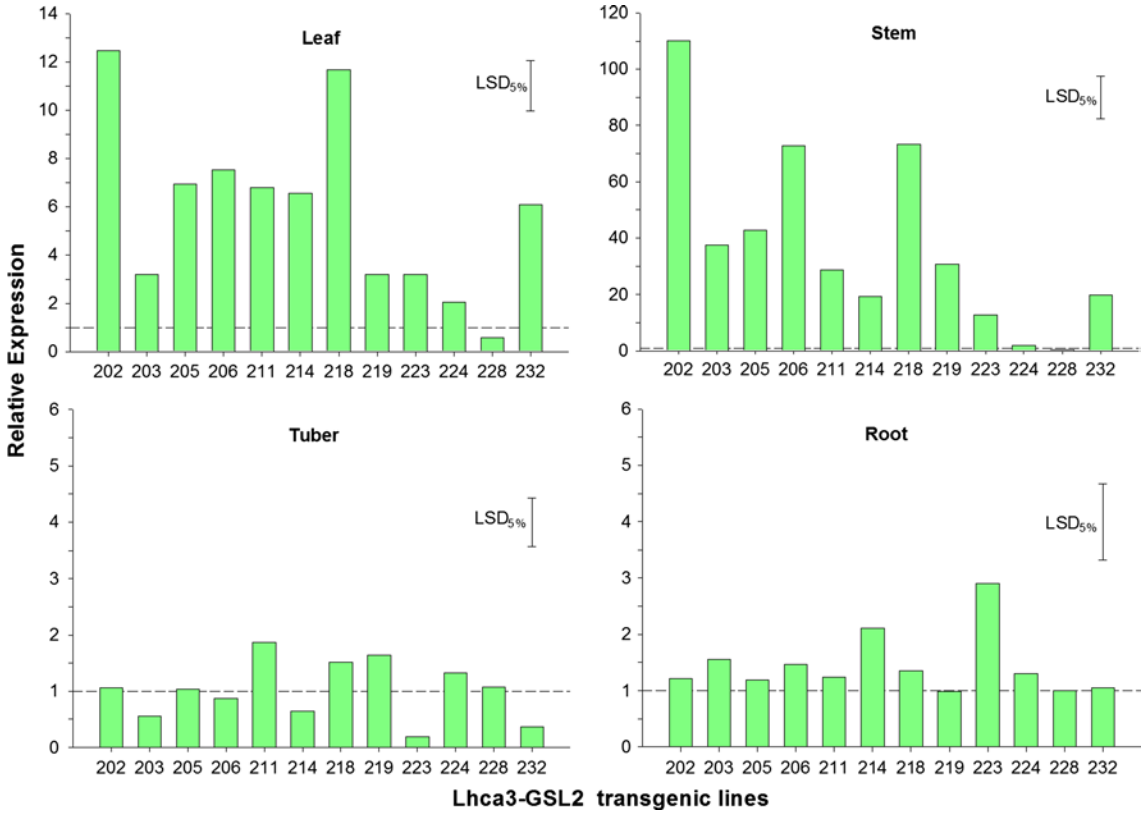

Fig. 4 *GSL2* transcript levels in the different organs of selected lines. Plants were grown in the same containment greenhouse facility as in experiment 3 of the pathogenicity assays. The qRT-PCR data for each sample were normalised to the amount of *Ef1α* transcript using the same amplification conditions. The relative abundance of *GSL2* gene transcript in transgenic plants was determined for three biological replicates, each with three technical replicates, using the $\Delta\Delta C_T$ method relative to the non-transgenic Iwa control. The relative expression value of the non-transgenic control is set at 1 and is represented by the dashed horizontal line. Analysis of variance established highly significant differences between the transgenic lines for *GSL2* expression in leaves ($F_{s\,[11,23]} = 25.308$, $P < 0.001$) and stems ($F_{s\,[11,\,22]} = 43.915$, $P < 0.001$), marginally significant differences in tubers ($F_{s\,[11,19]} = 2.607$, $P = 0.032$) and no significance in roots ($F_{s\,[11,24]} = 1.394$, $P = 0.239$). *Vertical bars* represent the least significant difference (LSD) at the 0.05 probability level

($P < 0.001$) than the Iwa control and the low-expressing line (228). No colonies of *P. atrosepticum* SCRI1043 were recovered from the negative controls.

The presence of *P. atrosepticum* SCRI1043 in the same tissue samples was confirmed by PCR. A 215 bp amplicon specific to the ECA0515 gene of *P. atrosepticum* was detected in all replicates of the inoculated lines using primers ECA0515F and ECA0515R, but absent from the $MgCl_2$-inoculated plants and the non-inoculated plants. The presence of *P. atrosepticum* SCRI1043 cells was also estimated by qPCR with DNA isolated from stem samples taken from below the visible symptoms (Table 4). The estimated densities of bacterial cells were higher than those observed from viable plate counts. Nevertheless, *P. atrosepticum* cell densities from the high *GSL2* expressing lines with blackleg resistance (202, 203, 211 and 219) were again significantly lower by several orders of magnitude ($P < 0.001$) than the Iwa control and the low expressing line (228). Absence of *P. atrosepticum* SCRI1043 was confirmed in the $MgCl_2$-inoculated and non-inoculated negative controls.

Discussion

Over-expression of the potato *GSL2* gene (also known as *Snakin 2*) which encodes a cysteine-rich, low-molecular weight antimicrobial peptide, is anticipated to confer disease resistance. To test this hypothesis, we developed and validated a series of transgenic potato lines obtained by *Agrobacterium*-mediated gene transfer of the *GSL2* gene under the regulatory controls of the potato light-inducible *Lhca3* gene to limit over-expression to the foliage. This was imperative to ensure that over-expression did not occur in the edible tubers to minimise food safety issues associated with the resulting transgenic plants (Conner and Jacobs 2000). To test disease resistance in potato plants over-expressing *GSL2*, *P. atrosepticum* was used as a model

Table 2 Resistance of Lhca3-GSL2 transgenic lines to *Pectobacterium atrosepticum* SCRI1043 under greenhouse conditions (Experiment 1)

Plant line	Incidence of blackleg (% at 14 dpi)	Frequency of collapsed plants (% at 14 dpi)	Mean blackleg lesion length (cm at 9 dpi)	Status[a]
Iwa control	93	71	3.5	Susceptible
202	0	0	0	Resistant
203	0	0	0	Resistant
205	71	14	0.8	Partially resistant
211	0	0	0.1	Resistant
218	100	100	3.6	Susceptible
219	0	0	0.2	Resistant
223	93	0	0.8	Partially resistant
228	93	86	3.5	Susceptible
$MgCl_2$-inoculated plants	0	0	–	–
Non-inoculated plants	0	0	–	–
F_s^b (*df*)			33.45*** (8.117)	
LSD (5 %)			0.77	

Summary of disease parameters from 14 replicates of each transgenic line, non-transgenic control Iwa and $MgCl_2$-inoculated plant

LSD least significant differences between two means at the 0.05 probability level

*** Represents significant difference between means at the 0.001 probability level

[a] Status was calculated on the basis of an arbitrary scale: resistant (mean lesion length <0.5 cm), partially resistant (0.5–2 cm) and susceptible (>2 cm)

[b] *F* value from analysis of variance

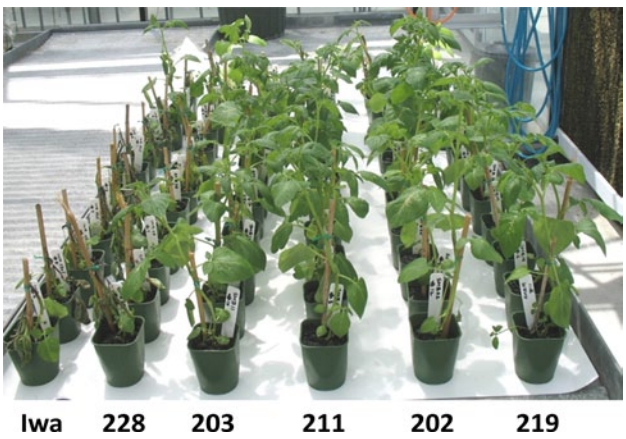

Fig. 5 Disease symptoms in potato stems in response to *Pectobacterium atrosepticum* SCRI1043 (Experiment 1). Variation in the disease response following pathogen challenge among the Lhca3-GSL2 transgenic lines compared with the non-transgenic control (Iwa). From *left to right*, each row represents a line that has 14 replicate plants. The photograph was taken at 14 dpi

Fig. 6 Internal necrotic lesions in potato stems in response to *Pectobacterium atrosepticum* SCRI1043 (Experiment 2). Variation in the internal necrotic lesion following pathogen challenge among Lhca3-GSL2 transgenic lines compared with the non-transgenic control (Iwa) and the $MgCl_2$-inoculated negative control. The photograph was taken at 11 dpi

pathogen since it incites blackleg, an important disease of potato foliage (Czajkowski et al. 2011). Blackleg disease in the field usually begins from contaminated seed tubers or plant entry from soil via wounds in roots or tubers. *P. atrosepticum* then invades the stem and multiplies in the xylem, resulting in typical inky black symptoms during stem decay and foliage collapse (Czajkowski et al. 2011). Elimination of the field multiplication of the pathogen is anticipated to provide a break in the disease cycle and provide disease control by minimising the tuber-borne and soil presence of the pathogen.

A wide spectrum of *GSL2* over-expression at the transcriptional level was observed among 32 independently transformed Lhca3-GSL2 lines (Fig. 3), a phenomenon typical for populations of independently derived transgenic events and usually attributed to position effects and/or transgene copy number (Conner and Christey 1994; Barrell et al. 2013). Despite the varying levels of *GSL2* over-expression, the resulting plants exhibit a phenotypically

Table 3 Resistance of Lhca3-GSL2 transgenic lines to *Pectobacterium atrosepticum* SCRI1043 in a Biotron (Experiment 2)

Plant line	Incidence of blackleg (% at 11 dpi)	Mean internal blackleg lesion length (cm at 11 dpi)	Status[a]
Iwa control	100	2.8	Susceptible
202	7	0.2	Resistant
203	0	0.0	Resistant
205	79	1.4	Partially resistant
211	14	0.3	Resistant
218	86	1.3	Partially resistant
219	14	0.6	Resistant
223	86	1.3	Partially resistant
228	93	2.2	Susceptible
$MgCl_2$-inoculated plants	0	–	–
Non-inoculated plants	0	–	–
F_s^b (*df*)	–	44.28*** (8.117)	
LSD (5 %)		0.46	

Summary of disease parameters from 14 replicates of each transgenic line, non-transgenic control Iwa and $MgCl_2$-inoculated plant

LSD least significant difference between two means at the 0.05 probability level

*** Represents significant differences between means at the 0.001 probability level

[a] Status was calculated on the basis of an arbitrary scale: resistant (mean internal lesion length <1 cm), partially resistant (1–2 cm) and susceptible (>2 cm)

[b] *F* value from analysis of variance

normal appearance when grown under greenhouse conditions. This is similar to previous observations from over-expression of *GSL1* in potato (Almasia et al. 2008) and *GSL2* in tomato (Balaji and Smart 2012).

Twelve transgenic lines were selected for more detailed assessment based on their elevated transcription of *GSL2* in leaves. A series of 11 medium–high expressing lines (202, 203, 205, 206, 211, 214, 218, 219, 223, 224 and 232) and a low expressing line (228) were subjected to further analysis of transcript levels in leaves, stems, tubers and roots (Fig. 4). The magnitude of *GSL2* over-expression in leaves was similar in the two experiments, despite the plants being grown in different environments at different locations in different seasons. The exception was line 219 which exhibited an almost 12-fold increase in *GSL2* expression in the first experiment (Fig. 3), but only a threefold increase in the second experiment (Fig. 4). Given the influence of position effect on transgene expression, the inconsistent performance of occasional transgenic lines in different environments is not unexpected (Conner and Christey 1994; Barrell et al. 2013). The stem tissue exhibited very high over-expression of the *Lhca3-GSL2-Lhca3* chimeric potato gene, with minimal change observed in roots and tubers. The absence of over-expression in roots and tubers is expected given the foliage-specific nature of the light-inducible *Lhca3* promoter controlling the *GSL2* over-expression (Nap et al. 1993; Meiyalaghan et al. 2006).

Disease screening in three different environments established that elevated transcription of *GSL2* confers resistance to blackleg disease in potato. The *P. atrosepticum* isolate used (SCRI1043; American Type Culture Collection BAA-672) is often used in epidemiological and molecular studies (e.g. Hinton et al. 1985; Toth et al. 1997) and is a well-characterised strain with a full genome sequence (GenBank accession NC-004547) (Bell et al. 2004). All *GSL2* over-expressing lines generally showed consistent responses across all three pathogen bioassays and exhibited significant reduction in disease symptoms compared to low-expressing lines and the non-transformed control. This is consistent with the reports of bacterial resistance from the over-expression of *GSL1* in potato (Almasia et al. 2008) and *GSL2* in tomato (Balaji and Smart 2012). Most importantly, this study in potato validates *GSL2* over-expression by quantifying transcript accumulation and relating this to the phenotypic expression of disease resistance.

Of the eight transgenic lines inoculated with *P. atrosepticum*, four (202, 203, 211 and 219) were classified as resistant, two (205 and 223) as partially resistant, and two as susceptible (218 and 228). This response generally reflected the magnitude of transcriptional over-expression. An exception was line 218 which had high transcriptional over-expression in leaves and stems (Fig. 4), but was susceptible to *P. atrosepticum*. Despite a high *GSL2* transcript level, line 218 may have compromised protein accumulation due to either poor translation of transcripts to protein

Table 4 Resistance of Lhca3-GSL2 transgenic lines to *Pectobacterium atrosepticum* SCRI1043 under greenhouse conditions (Experiment 3)

Plant line	Incidence of blackleg (% at 10 dpi)	Frequency of collapsed plants (% at 10 dpi)	Mean blackleg lesion length (cm at 10 dpi)	*P. atrosepticum* count$\log_{10}$ CFU/g plant stem (back transformed mean)	*P. atrosepticum* qPCR$\log_{10}$ cells/g plant stem (back transformed mean)	Status[a]
Iwa control	86	83	9.4	6.80 (6.3×10^6)	6.99 (9.9×10^6)	Susceptible
202	29	0	0.6	0.56 (3.7×10^0)	2.93 (8.5×10^2)	Resistant
203	29	0	0.8	0.30 (2×10^0)	2.15 (1.4×10^2)	Resistant
205	86	14	5.2	ND[b]	ND	Partially resistant
211	43	7	1.6	1.13 (1.7×10^1)	3.18 (1.5×10^3)	Resistant
218	86	29	8.4	ND	ND	Susceptible
219	50	0	1.5	1.01 (1.1×10^1)	4.23 (1.7×10^4)	Resistant
223	86	7	6.0	ND	ND	Partially resistant
228	79	50	10.1	4.56 (3.6×10^4)	6.34 (2.2×10^6)	Susceptible
$MgCl_2$-inoculated plants	0	0	–	0	0	
F_s[c] (*df*)			15.81*** (8.117)	14*** (6.12)	13*** (6.48)	
LSD			2.6	0.16	0.23	

Summary of disease parameters and pathogen density from 14 replicates of each transgenic line, non-transgenic control Iwa and $MgCl_2$-inoculated plant

LSD least significant difference between two means at the 0.5 probability level

*** Represents significant differences between means at the 0.001 probability level

[a] Status was calculated on the basis of an arbitrary scale: resistant (mean lesion length <2 cm), partially resistant (2–6 cm) and susceptible (>6 cm)

[b] *ND* not determined

[c] *F* value from analysis of variance

or failure of appropriate protein processing, both of which have been observed in some transgenic lines over-expressing genes encoding other small antimicrobial peptides (Barrell and Conner 2009).

In this study, the results of phenotypic assessments of disease symptoms were validated by determining the persistence and invasion of *P. atrosepticum* from the inoculation sites in the stem. Re-isolation of *P. atrosepticum* is crucial for confirming its role as the causal agent of the observed disease and to establish the mode of action for GSL2. The estimated densities of *P. atrosepticum* cells from the qPCR assays were higher than those observed from viable plate counts of CFU (Table 4). This difference was about 100-fold for all the *GSL2* over-expressing lines, but not different for the non-transgenic control (Table 4). The qPCR estimates of cell density are based on DNA and detect the presence of both viable and non-viable cells. Since these estimates are higher than the viable plate counts, the vast majority of *P. atrosepticum* detected by qPCR in apparently healthy tissue below the visible symptoms are probably non-viable. This is expected given the antimicrobial activity of the *GSL2* peptide against a wide range of bacteria and fungi (Berrocal-Lobo et al. 2002; López-Solanilla et al. 2003).

The blackleg bioassay used in this study applied severe disease pressure to potato plants. It is very rare that plants grown under normal field conditions would encounter the infection intensity (equivalent to 10^8 cells per inoculation site) of *P. atrosepticum* as used in these experiments at any one time. Nevertheless, significant delays in infection and substantial reductions in disease symptoms were found in the Lhca3-GSL2 transgenic lines. A similar type of delay in disease was observed in other over-expression studies with *GSL* genes (Almasia et al. 2008; Balaji and Smart 2012), suggesting that these transgenic plants overcome the initial intense pathogen attack and prevent further disease development.

In conclusion, resistance to *P. atrosepticum* infection can be successfully engineered into potato through the over-expression of an endogenous gene encoding the antimicrobial protein GSL2. Bacteria related to *P. atrosepticum* (such as *P. carotovorum* subsp. *carotovorum* and *Dickeya* spp.) can cause disease in a wide range of hosts such as tomato, tobacco, and eggplant (Czajkowski et al. 2011). Identifying and over-expressing *GSL* genes in these crops may also provide valued resistance to these bacteria. Furthermore, given the broad spectrum of activity of the GSL2 protein against a wide range of bacteria, fungi and nematodes (Segura et al. 1999; Berrocal-Lobo et al. 2002; López-Solanilla et al. 2003; Kovalskaya and Hammond 2009; Mao et al. 2011), over-expression of *GSL2* may confer improved resistance to other important diseases in potato such as late blight and zebra chip, as well as resistance to pathogens in other crops.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards The authors declare that the experiments comply with the laws of New Zealand.

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