Published in final edited form as:
Plant J. 2010 January ; 61(2): 364–369. doi:10.1111/j.1365-313X.2009.04052.x.

Transformation of the flax rust fungus, *Melampsora lini*: selection via silencing of an avirulence gene

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SUMMARY

Rust fungi cause devastating diseases on many important food crops, with a damaging stem rust epidemic currently affecting wheat production in Africa and the Middle East. These parasitic fungi propagate exclusively on plants, precluding the use of many biotechnological tools available for other culturable fungi. In particular the lack of a stable transformation system has been an impediment to the genetic manipulation required for molecular analysis of rust pathogenicity. We have developed an *Agrobacterium*-mediated genetic transformation procedure for the model flax rust fungus *Melampsora lini*, which infects flax (*Linum usitatissimum*). Selection of transgenic rust lines is based on silencing of *AvrL567*, which encodes a rust effector protein that is recognised by the flax *L6* immune receptor. The non-transgenic rust line is unable to infect flax plants expressing *L6*, while silenced transgenic lines are virulent on these plants, providing an effective selection system. This directly confirms that the cloned *AvrL567* gene is responsible for flax rust virulence phenotypes, and demonstrates the utility of this system to probe rust gene function.

Keywords

rust; transformation; avirulence; effector; gene silencing; *Agrobacterium*

INTRODUCTION

Rust fungi (basidiomycetes of the order Uredinales) are a diverse group, with over 7000 species known to cause disease on many different plant host species. They are particularly important pathogens of wheat, with the recent emergence of a new strain of wheat stem rust (*Puccinia graminis tritici* strain TTKS or Ug99) in East Africa posing a serious threat to global wheat production (Singh *et al.*, 2008). During infection, these pathogens produce specialised infection structures, known as haustoria, which penetrate the host cell wall and are the primary site of nutrient extraction from the host (Voegele and Mendgen, 2003). These structures also deliver effector proteins into the host cell which may facilitate infection and can be recognised by host immune receptors (Dodds *et al.*, 2004, 2006; Catanzariti *et al.*, 2006). Draft genome sequences for two rust species (wheat stem rust and poplar rust, *Melampsora larici-populina*) have been developed and contain many genes with potential pathogenicity related functions. However, analysis of the functions of these genes in disease requires the development of a reliable stable genetic transformation system to allow manipulation of gene expression in transgenic rust lines.

The interaction between the flax rust fungus, *Melampsora lini*, and its host plant flax has served as an important model for understanding the plant innate immunity system

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(Lawrence *et al.*, 2007; Dodds and Thrall, 2009). Flax immune receptors (of the nucleotide binding-leucine rich repeat class) are encoded by highly polymorphic resistance genes, and recognise rust effector proteins that are delivered into flax cells during infection. This triggers a hypersensitive response which involves localised cell death that prevents fungal development (Lawrence *et al.*, 2007). For instance the *L6* resistance gene encodes a receptor that recognises members of the *AvrL567* rust effector family and triggers host immunity to infection. Transient expression of AvrL567 proteins in *L6* plants induces a strong defence response and yeast two-hybrid assays indicate a direct interaction between these proteins (Dodds *et al.*, 2004, 2006). Rust strains that carry an unrecognised variant of *AvrL567* can cause disease on host plants containing the *L6* resistance gene. However direct evidence that *AvrL567* controls the rust avirulence phenotype is not yet available, due to the absence of genetic manipulation techniques for flax rust. We have used the strong resistance response triggered by the *AvrL567–L6* interaction as the basis of a selection mechanism to develop a transformation system for flax rust and to confirm the role of *AvrL567* in rust avirulence.

Species of fungi that can be cultivated on artificial media are usually amenable to transformation by CaCl2/polyethylene glycol or electroporation-induced DNA uptake into protoplasts or by particle bombardment or *Agrobacterium tumefaciens*-mediated DNA delivery, with antibiotic resistance or complementation of an auxotrophic mutation usually used to select transgenic cells (Weld *et al.*, 2006). However, obligate biotrophic fungal pathogens, such as rusts, either cannot, or cannot easily, be grown on artificial media, so alternative transformation protocols must be devised. Although transient transformation of rust fungi by particle bombardment has been reported (Webb *et al.*, 2006), stable transformation has remained elusive. We have developed a transformation procedure based on *A. tumefaciens* delivery of T-DNA into rust hyphae growing in stems of flax plants. The T-DNA contained a hairpin construct designed to silence *AvrL567* in the rust and allow subsequent selection of transgenic individuals by inoculation onto flax plants possessing the *L6* resistance gene.

RESULTS AND DISCUSSION

The main infection stage of the flax rust life cycle involves dikaryotic (binucleate) urediospores. As flax rust strains heterozygous for avirulence genes can give rise to spontaneous virulent mutants due to mutation or deletion of their single avirulence gene at significant frequencies (Lawrence, 1977), we chose as the T-DNA recipient rust strain CH5–89. This strain is homozygous for a haplotype of the *AvrL567* locus with two adjacent genes, *AvrL567-A* and *-B*, whose products are both recognised by *L6* (Dodds *et al.*, 2004). A binary T-DNA vector (siAvrL567) was designed that contained a silencing construct encoding a 480-bp hairpin RNA that included most of the *AvrL567-A* coding region, expressed under its own promoter (Figure 1a). An *A. tumefaciens* strain carrying this binary T-DNA vector was introduced into the stems of Hoshangabad plants (no resistance genes) that had been inoculated 5 days previously with strain CH5–89. Access of the bacteria into the infected stems was achieved by extensively puncturing the stems while immersed in a suspension of the *Agrobacterium* (Figure S1a). Urediospores were collected from the stems 9 days after *Agrobacterium* infiltration with two subsequent collections made at 4- or 5-day intervals (Figure S1b). In two separate experiments, a total of 15 pots of rust-infected flax stems were infiltrated with *Agrobacterium* cultures containing the siAvrL567 construct. Urediospore production from the stems in each pot varied due to differences in initial infection rates and because stem puncture sometimes resulted in the death of the stem or the formation of regions of scar tissue from which no spores were produced. Only spores collected from the six highest yielding pots (three from each experiment) were screened for putative transgenics. Of the three spore collections made from each pot, the first yielded the least

amount of spores. Therefore spores from either the second or third collections were used in the screening experiment.

Urediospores collected from the six high-yielding pots were inoculated separately onto flax plants of the line H3 × Birio, which possesses the *L6* resistance gene. Each of these inoculations gave rise to between 18 and 69 virulent pustules (Table 1, Figure S1c) which were considered as putative transgenic lines. Ten pustules originating from the pot 1 collection and two from each of the other five pot collections were multiplied by inoculation onto the susceptible flax line Hoshangabad for two infection cycles. PCR amplification of six isolates, one from each pot and therefore representing independent events, showed that all six harboured the T-DNA construct (Figure 1b). Subsequently, DNA gel blot analysis was used to confirm the presence of an integrated T-DNA. Genomic DNA was digested with *Xba*I and DNA gel blots were hybridised with an *AvrL567*-derived probe (Figures 1c and S2a and data not shown). This probe detects two fragments in the untransformed CH5–89 strain, which correspond to the endogenous *AvrL567-A* and *-B* genes. All 20 of the virulent isolates contained these two bands, indicating that they still contained undisrupted copies of the endogenous *AvrL567-A* and *-B* genes, but they also contained additional fragments corresponding to the introduced T-DNA. These included a single internal T-DNA fragment of 2.6 kb and an external T-DNA-fungal DNA junction fragment whose size varies depending on the distance between the right hand border of the T-DNA and the first *Xba*I site in the adjacent fungal DNA (Figure 1a). A probe derived from the 35S promoter of the spectinomycin resistance gene cassette hybridised only to the 2.6 kb internal fragment in the transgenic rust strains and did not detect any bands in the untransformed parent line (Figure S2b). Nineteen isolates contained a single junction fragment, indicating the presence of one copy of the T-DNA inserted in the fungal genome, while one isolate contained two copies of the T-DNA (Figure S2, lane pot 1-1). Based on the junction fragment lengths the ten virulent isolates from pot 1 were derived from eight different transgenic events (there were two pairs in which each member of the pair had identical band patterns) while the two isolates tested from each of the other five pots were, in all cases, derived from different transgenic events (Table 1). The isolates with identical fragments are likely to have originated from urediospores that came from the same transgenic sporogenous cell. In the process of urediospore formation, hyphae orientate towards the surface and, after the formation and release of peridial and intercalary cells, produce terminal sporogenous cells which bud off twin cells, one of which develops into a pedicel and the other into an urediospore (Lawrence *et al*., 2007). As sporogenous cells can bud off a succession of urediospores a transformed sporogenous cell could produce a number of urediospores possessing the same transgenic event. The maintenance of the T-DNA insertions after two asexual generations on a non-selective (no *L6* gene) host line indicates stable incorporation of the T-DNA in the original urediospore from which each virulent isolate was derived. The relatively high numbers of putative transformants isolated, and the lack of any apparent untransformed escapes (contaminating spores or spontaneous mutants), suggests that ample transgenic isolates for most investigations could be obtained from transformations performed on ten to fifteen infected stems (two or three pots).

Three independent transgenic isolates were inoculated onto a set of 32 flax differential lines containing all known rust resistance genes, to test their virulence phenotypes. In each case, the transgenic lines showed an identical phenotype to the non-transgenic CH5–89, except on lines containing the *L5* (Wilden), *L6* (Birio) or *L7* (Barnes) resistance genes which recognise *AvrL567* (Table 2). Whereas the non-transgenic strain is completely avirulent on Wilden and Birio and slightly less than fully virulent on Barnes, the three transgenics were fully virulent on all three of these differential lines. The non-transgenic CH5–89 is not avirulent on Barnes (*L7*) because CH5–89 contains an inhibitor gene (*I*) that suppresses the *L7–AvrL567* interaction resulting in a slightly less than fully virulent reaction (Lawrence *et al*., 1981).

These results confirm that the transgenic lines are derived from CH5–89, as no other rust strain in our collection displays this pattern of virulence phenotypes. The recovery of transgenic isolates virulent on *L5*, *L6* and *L7* suggests that the transgene is triggering an RNAi mechanism leading to a reduction in RNA transcript levels of *AvrL567-A* and *-B*. To test whether these genes were indeed silenced, RNA was extracted from Hoshangabad plants infected with non-transgenic CH5–89 and the three transgenic isolates. RNA gel blot analysis detected expression of the rust tubulin gene in all samples, while *AvrL567* transcripts were detected in CH5–89, but not in the transgenic lines (Figure 2). The observed high level of silencing in all three transgenic lines probably reflects the strong selection applied by the requirement for growth on plants with the *L6* resistance gene – i.e. transgenics with only partial silencing of *AvrL567* may not come through the selection screen.

These results confirm that the *AvrL567* genes in flax rust are indeed responsible for the conferring the avirulence phenotype on plants containing *L5*, *L6* or *L7* resistance genes. Previously this was inferred from the genetic mapping, in-planta expression and yeast two-hybrid protein interaction studies (Dodds *et al.*, 2004, 2006). The AvrL567 protein is presumed to have some effector function in promoting infection in the absence of host resistance genes. However, the *AvrL567*-silenced transgenic rust lines appear to be just as vigorous with regard to growth rate and urediospore production as the non-transgenic CH5–89 strain when grown on the susceptible flax variety Hoshangabad. Thus, any effector role of AvrL567 does not appear to be essential for flax rust pathogenicity, at least under the environmental conditions employed. This may be due to redundancy in the flax rust effector repertoire, with another protein(s) substituting for the missing AvrL567 function, or may indicate that its function is of only minor consequence for the rust with any subtle effects on urediospore production difficult to quantify. It is also possible that AvrL567 proteins may have an important role in the sexual stage of the rust life cycle.

From the work reported above it is evident that *A. tumefaciens* can transform cells in flax rust hyphae growing in the stems of flax plants, with the transferred T-DNA being stably integrated into the rust genome and inherited through successive asexual generations. Furthermore, expression of a hairpin RNA triggers a robust gene silencing mechanism that can suppress expression of a rust gene target. These are critical tools for the analysis of rust gene function because they now allow the delivery of novel gene variants, including for instance epitope or fluorescently tagged proteins for localisation or protein interaction analyses. This may require the development of gene expression constructs using homologous promoter and terminator sequences. Robust gene silencing will also allow the targeted ablation of specific gene functions, which would be difficult to achieve by targeted gene knockouts or insertional mutation, as the dikaryotic nature of the rust would require passage through a sexual phase to obtain homozygous strains. Two key factors in this selection system were firstly the use of a rust strain homozygous for the *Avr* gene silencing target to minimise the isolation of rust pustules that had become virulent due to mutation of the target gene, and therefore reduce the need to screen out these potential contaminants, and secondly the very robust resistance selection afforded by the *L6–AvrL567* interaction, which completely prevents any pustule development by the parent rust strain. While the current selection system based on *AvrL567* silencing will allow delivery of other genes of interest into flax rust, it does not permit delivery of modified *AvrL567* genes. However this could be overcome by developing a selection screen based on the silencing of other cloned avirulence genes in flax rust, such as *AvrM*, *AvrP4* and *AvrP123* (Catanzariti *et al*., 2006), provided that, like *AvrL567*, they are not essential for rust viability. Our results demonstrate the feasibility of *Agrobacterium*- mediated rust transformation coupled with selection for a silencing-induced virulence phenotype, which should be adaptable to other rust species including the agronomically important cereal rusts. Many avirulence genes have been

genetically defined in wheat stem rust for instance (Zambino *et al.*, 2000; Leonard and Szabo, 2005) and if cloned would serve as gene silencing targets allowing efficient selection for transgenic urediospores by screening on a wheat line carrying the corresponding resistance gene. The demonstration that *A. tumefaciens* can deliver T-DNA to rust hyphae in infected plants, should also allow the development of alternative selection strategies for rust transformation based on fungicide or herbicide resistance (Anderson and Kolmer, 2005; Feng *et al.*, 2005).

EXPERIMENTAL PROCEDURES

Plant and rust materials

The flax line Birio possesses the *L6* resistance gene (Flor, 1956) while the Hoshangabad line contains no known rust resistance genes (Mayo and Shepherd, 1980). The line H3 × Birio, homozygous for *L6*, was selected amongst the selfed progeny of a second backcross line from the cross [(Birio × Hoshangabad) × Hoshangabad] × Hoshangabad. A set of 32 differential flax lines containing different R genes includes a standard set described by Islam and Mayo (1990) with the addition of two lines containing the recombinant *L* alleles *Lx* and *RL10* (Luck *et al.*, 2000). Rust strain CH5–89 is an F2 individual from a cross between rust strains C and H (Lawrence *et al.*, 1981), and is homozygous for the *AvrL567* haplotype conferring avirulence to *L5*, *L6* and *L7* (Dodds *et al.*, 2004). A stock of CH5–89, free of any contaminant spores that might be virulent to *L6*, was obtained by successively growing the strain on host lines possessing the *N*, *P4*, *L2* and *P* resistance genes, while growing no other rust strain. Strain CH5– 89 is the only strain held at this laboratory that is virulent to all four of these resistance genes.

Silencing construct

A silencing construct (siAvrL567) encoding a hairpin RNA was generated in the pTNotTReg T-DNA binary vector (Dodds *et al.*, 2001) (Figure 1a). It consists of a 2.25-kb fragment amplified from flax rust genomic DNA with primers 10–1.28 (GGATGTTCGAGGTAGTGTC) and 10–1.17 (CTTCAATTGTACGGCGAGTC) which contains the *AvrL567* promoter (1.5 kbp) and most (750 bp) of the transcribed gene (120 bp at the 3′ end was deleted), including two small introns near its 5′ end. This fragment was fused to an inverted cDNA copy (600 bp) of the same gene amplified with 10–1.15 (AAGCTTGAGAGCTCCGCTC) and 10–1.16 (TAATCCTCGTTGACATCAGTC) primers. Thus the predicted transcript contains a 480-bp inverted repeat separated by a 120-bp linker derived from the 3′ end of the gene. The T-DNA also contained a gene conferring resistance to spectinomycin driven by the 35S promoter.

Transformation protocol

Hoshangabad plants (four or five per pot) were grown in a line in 15 cm pots and kept upright by dowelling and wire supports to ensure straight stems. When approximately 45 cm tall, side stems were removed from each plant and the main stems, stripped of leaves except for the top 5 cm, were inoculated with rust strain CH5– 89 using a small camel's hair brush dipped in urediospores dispersed on the surface of a few ml of water in a small petri dish. After inoculation plants were sprayed with a fine mist of water and kept at high humidity and below 24°C overnight in large plastic bins before removal to a glasshouse. *A. tumefaciens* strain GV3101 pMP90 containing the T-DNA silencing construct was grown overnight at 28°C in LB containing 50 μg ml^{-1} gentamycin and 10 μg ml^{-1} tetracycline. Acetosyringone (200 μM) was added and cultures grown for a further 5–6 h before harvesting cells by centrifugation (3500 *g*, Jouan C412 benchtop centrifuge). *Agrobacterium* cells were resuspended in an equal volume of 5% sucrose containing 200 μM acetosyringone and were introduced into the infected stems 5 days after rust inoculation. To

introduce the *Agrobacterium* into the rust-infected stems, a pot with the infected stems was laid horizontally with one stem lying across the top of a file brush (110 × 50 mm; Figure S1a) immersed in the *Agrobacterium* suspension in a plastic dish (120 × 80 × 15 mm high). The stem was then punctured by pressing down with both thumbs and rolling the stem across the wire bristles of the brush to puncture all sides of the stem. This procedure was then repeated until the full length of each stem in the pot had been punctured. Plants were kept under high humidity at approximately 23°C for 2 days before removal to a glasshouse. Urediospores were collected from the stems in each pot 9 days after *Agrobacterium* treatment and stored at 4°C in sealed glass tubes under vacuum. Second and third collections were made at 4- or 5-day intervals.

To screen for transgenic rust spores virulent to *L6*, H3 × Birio plants were planted in 10 × 10 cm pots (nine plants per pot) and the primary shoot was cut off when a few cm high to promote the side shoot growth to increase leaf area. Spores from one collection (approximately 20–30 mg) were mixed with 1.5 g talc and dusted over 24 pots of H3 × Birio plants (approximately 12 cm tall) and kept at high humidity overnight. Plants were examined for virulent pustules 12–14 days after inoculation. Stems without pustules were removed and pots with pustules separated from each other. Spores collected from single pustules were put through two rounds of increase on Hoshangabad plants to obtain sufficient urediospores for DNA extraction.

DNA extraction, southern analysis, RNA extraction and northern analysis

Rust spores (800–1000 mg) were dispersed over water in two trays (52 × 36 cm) and incubated overnight below 23°C and DNA was extracted from the mat of germinated spores as described (Lawrence *et al.*, 2010). For PCR analysis, genomic DNA was amplified with combinations of primers 10-1.15, 10-1.16, 10-1.19 (CCAGACTACATCAAAATCAAG) and 35Srev (ATTGGCCGTCCGCTCTAC). For gel blot analysis, genomic DNA was digested with restriction enzyme *Xba*1, separated on 1.0% agarose gels, transferred to Hybond N+ nylon membranes and hybridised with a 32P-dCTP- labelled 10–1.15/16 PCR fragment of *AvrL567* as described (Lawrence *et al.*, 2010). RNA was extracted from 1 g of infected flax leaves as described (Jones *et al.*, 1985), separated on 1.5% agarose gels and transferred to Hybond N+ nylon membranes. RNA blots were hybridized with either the 10–1.15/16 *AvrL567* fragment or a fragment from the flax rust tubulin gene (Ayliffe *et al.*, 2001) as described (Catanzariti *et al.*, 2006).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Excellent technical assistance was provided by Patricia Moore and Kim Newell. This work was funded by CSIRO and the Australian Grains Research and Development Corporation grant CSP00017.

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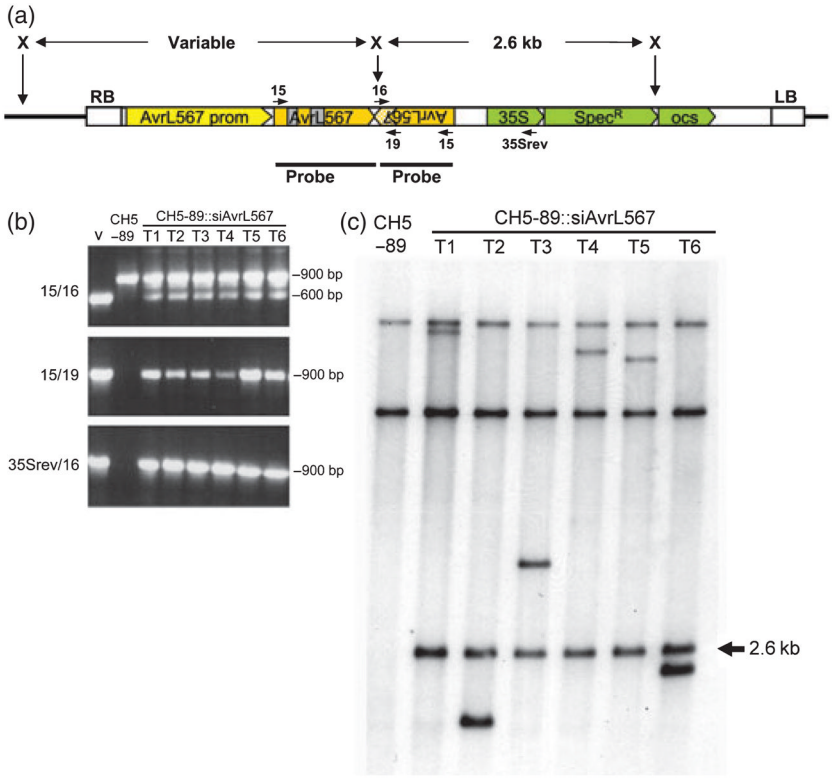

Figure 1. Analysis of transgenic flax rust isolates

(a) Schematic representation (not to scale) of the siAvrL567 T-DNA construct used to transform flax rust strain CH5–89. The *AvrL567* promoter region (yellow) drives expression of the inverted repeat *AvrL567-A* transcript (orange). Two intron sequences are shown in grey, while a 120-bp non-repeated region is hatched orange. The T-DNA also contains a spectinomycin resistance gene (SpecR) under the control of the 35S promoter and OCS terminator sequences (green). The left (LB) and right (RB) borders of the T-DNA and internal *Xba*I (X) restriction sites are indicated, as is the location of the *AvrL567* probe. The location on the T-DNA of oligonucleotide primers 10-1.15, 10-1.16, 10-1.19 and 35Srev are indicated by small arrows.

(b) Genomic DNA of non-transgenic flax rust strain CH5–89 and six transgenic (T1–T6) isolates, each from a separate pot, was amplified by PCR with primers directed to the siAvrL567 T-DNA construct (v). The primer pair 10–1.15/10–1.16 (15/16) amplifies a 600-bp fragment from the T-DNA and a 900-bp fragment from genomic DNA, while the 10-1.15/10-1.19 (15/19) and 35Srev/10-1.16 (35Srev/16) primer pairs amplify 900-bp fragments from the T-DNA only.

(c) Genomic DNA of non-transgenic flax rust strain CH5–89 and six transgenic (T1–T6) isolates, each from a separate pot, was digested with the restriction enzyme *Xba*I, separated by agarose gel electrophoresis, transferred to nylon membranes and hybridised with a probe derived from the coding region of *AvrL567-A*. This probe detects two fragments in CH5–89 derived from the endogenous *AvrL567* locus. In the transgenic lines the probe hybridises additionally to a 2.6-kb fragment originating from within the T-DNA and to a single T-DNA–fungal DNA junction fragment of variable size, indicating that each of the transgenic isolates contains a single T-DNA insert.

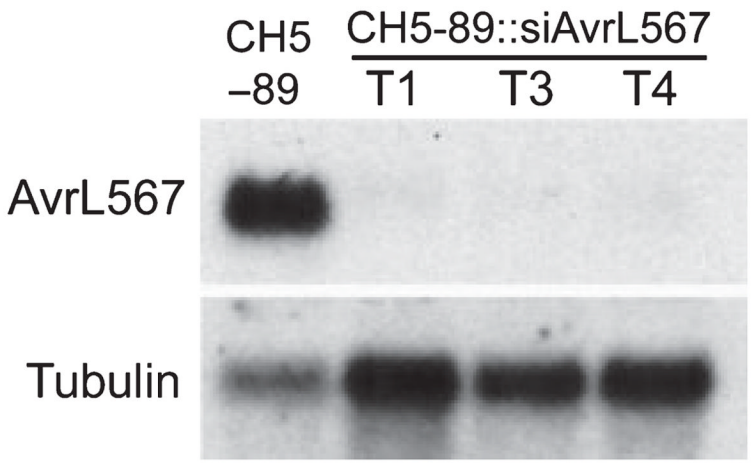

Figure 2. RNA gel blot analysis of transgenic rust lines

RNA extracted from flax leaves heavily infected with non-transgenic flax rust strain CH5–89 and three transgenic (T1, T3, T4) isolates was separated by gel electrophoresis, transferred to nylon membranes and hybridised with probes derived from *AvrL567-A* or from the flax rust tubulin gene.

Table 1

Characterisation of virulent pustules (putative transgenics) recovered from screening flax rust urediospores of strain CH5–89 collected from *Agrobacterium*-infiltrated flax stems in each of six pots on H3 × Birio (*L6*) plants

Spore collection	Number of pustules virulent on H3 × Birio	Number tested by Southern analysis	Number transformed	Number of transgenic events
Pot 1	23	10	10	8
Pot 2	45	2	2	2
Pot 3	18	2	2	2
Pot 4	48	2	2	2
Pot 5	20	2	2	2
Pot 6	69	2	2	2

Table 2

Infection reaction types of 32 flax differential lines to CH5–89 and transgenic rust isolates[a]

			Rust strains		
			CH5–89::siAvrL567		
Flax lines	***R* gene**	***CH5–89***	**T1**	**T3**	**T4**
Clay	K	+	+	+	+
Raja	K1	+(+/−)	+	+	+
Ottawa	L	−−	−−	−−	−−
B14 × Burke	L1	+	+	+	+
Stewart	L2	+	+	+	+
P.B.C.	L3	+	+	+	+
Kenya	L4	−	−(−−)	−(+/−)	−(+/−)
Wilden	L5	−−	+	+	+
Birio	L6	−−	+	+	+
Barnes	L7	+(+/−)	+	+	+
B13 × Towner	L8	+	+	+	+
Bison	L9	+	+	+	+
Bolley Golden selection	L10	+	+	+	+
Linore	L11	+	+	+	+
B6 × Kugler C	L12	−−	−−	−−	−−
Lx	Lx	+	+	+	+
RL10–1	RL10	+	+	+	+
Dakota	M	−−	−−	−−	−−
Williston Brown	M1	+	+	+	+
Ward	M2	+	+	+	+
Cass	M3	−−	−−	−−	−−
Victory ‘A’	M4	−−	−(−−)	−−	−
Cortland	M5	+/−	+/−	+/−	+/−
C.I. 2008	M6	−−	−	−	−
Bombay	N	+	+	+	+
Polk	N1	−−	−−	−−	−−

			Rust strains		
			CH5–89::siAvrL567		
Flax lines	***R* gene**	***CH5–89***	**T1**	**T3**	**T4**
Marshall	N2	−	−−	−−	−−
Koto	P	+	+	+	+
Akmolinsk	P1	−−	−−	−−	−−
Abyssinian	P2	−−	−−	−−	−−
Leona	P3	−−	−−	−−	−−
C.I. 1911	P4	+	+	+	+

[a]Reaction types were scored on a four point scale according to Lawrence *et al*. (1981), ranging from a fully virulent reaction with many large pustules (+) through restricted growth (+/−) and (−) to no growth (−−). Reaction types intermediate between two scores were scored as −(+/−) or +(+/−) etc.