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Significant reduction of fungal disease symptoms in transgenic lupin (*Lupinus angustifolius*) expressing the anti-apoptotic baculovirus gene *p35*

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Summary

Narrow-leafed lupin (NLL; *Lupinus angustifolius*) is a recently domesticated but anciently propagated crop with significant value in rotation with cereals in Mediterranean climates. However, several fungal pathogens, traditionally termed necrotrophs, severely affect broad-acre production and there is limited genetic resistance in the NLL germplasm pool. Symptoms of many of these diseases appear as localized areas of dead cells exhibiting markers of programmed cell death. Based on our previous research, we hypothesized that engineered expression of the baculovirus anti-apoptotic *p35* gene might reduce symptoms of these diseases. Using *Agrobacterium tumefaciens*-mediated transformation of a cultivar highly susceptible to several pathogens, 14 independent NLL lines containing both the *p35* and *bar* genes were obtained (*p35*-NLL). Integration and expression of the transgenes were confirmed by polymerase chain reaction (PCR), progeny testing, Southern blot, Northern blot and reverse transcriptase-PCR analyses. Fecundity and nodulation were not altered in these lines. Third or fourth generation *p35*-NLL lines were challenged with necrotrophic fungal pathogens (anthracnose in stem and leaf, and *Pleiochaeta* root rot and leaf brown spot) in controlled environment conditions. Several *p35*-NLL lines had significantly reduced disease symptoms. Interestingly, as with natural resistance, no single line was improved for all three diseases which possibly reflecting spatial variation of *p35* expression *in planta*. These data support an alternative molecular definition for 'necrotrophic disease' in plants and suggest new routes for achieving resistance against a range of pathogens.

Keywords: programmed cell death, legume transformation, *Colletotrichum lupini, Pleiochaeta setosa,* disease resistance.

Introduction

Molecular research has highlighted the difficulty of classification of plant pathogens as biotrophs or necrotrophs and various explanations of these terms have arisen (Oliver and Ipcho, 2004). Clearly defined fungal biotrophs include rusts and mildews that elaborate haustorial feeding structures that invaginate but do not penetrate living cells. The host cells remain turgid and sugar transport from the host cell to the haustoria continues until feeding ceases as the pathogens initiates new feeding sites (Szabo and Bushnell, 2001). Unlike biotrophs that feed from living cells, necrotrophs trigger host cell death in the process of colonization thus creating a food base that is used for growth and reproduction of the pathogen (Stone, 2001). The process—by which necrotrophic infection leads to cell death—has been shown in several cases to involve programmed cell death with morphological features of apoptosis (Navarre and Wolpert, 1999; Richael *et al.*, 2001; Lincoln *et al.*, 2002; Wolpert *et al.*, 2002; Li *et al.*, 2008).

Fungal pathogens classified as necrotrophs have been one of the main constraints to production of narrow-leafed lupin (NLL), *Lupinus angustifolius* L. Some of the most economically important necrotrophic fungal pathogens of NLL are anthracnose stem and pod blights, caused by *Colletotrichum lupini* (Nirenberg *et al.*, 2002), and *Pleiochaeta* root rot and brown leaf spot caused by *Pleiochaeta setosa* (Kirchn.) Hughes (Sweetingham *et al.*, 1998). Limited sources of resistance to these pathogens have been identified in the lupin gene-pool although varieties with moderate resistance have been bred and commercialized (Cowling *et al.*, 1997, 2000; Cowling, 1999). Moderate resistance to these diseases results in a quantitative reduction in lesion size and severity on plant tissues and improved yield in the field (Cowling *et al.*, 1997, 2000; Thomas and Sweetingham, 2004; Thomas *et al.*, 2008).

Lupin anthracnose is a major threat to the lupin industry worldwide and in Australia, with significant yield losses (up to 80%) in susceptible lupin varieties (Thomas and Sweetingham, 2004). Anthracnose lesions can occur on all above-ground parts of the lupin plant, but characteristic symptoms are the bending and twisting of stems with a lesion (usually dark brown) in the crook of the bend. A pink spore mass is often visible within lesions (Sweetingham *et al.*, 1998).

In brown leaf spot, caused by *P. setosa*, leaves are infected by rain splashed, soil-borne spores and develop dark brown spots, often net-like in appearance, after which leaves die and drop off within a few days. Fungal sporulation on the fallen leaf litter provides secondary inoculum for repeated infections on the same or adjacent plants. In *Pleiochaeta* root rot, seedling root infections result in dark brown lesions on the tap and lateral roots. Taproots can rot away, and severely affected plants wilt and die, while vigour is reduced in plants with a partially rotted root system (Sweetingham *et al.*, 1998).

Programmed cell death (PCD) occurs in plants during normal cell growth, development and differentiation (Hengartner and Bryant, 2000; Jones, 2001; Reape *et al.*, 2008; Williams and Dickman, 2008). There is growing evidence that a form of PCD which shows hallmarks of apoptosis, such as cell shrinkage, nuclear fragmentation into pyknotic DNA bodies, chromatin condensation and DNA laddering, is involved in many compatible plant host–pathogen interactions that have been classified as necrotrophic (Wang *et al.*, 1996; Gilchrist, 1997; Yao *et al.*, 2002; Hoat *et al.*, 2006; Kiba *et al.*, 2006; Harvey *et al.*, 2008; Li *et al.*, 2008). In animal cells, specific proteases called 'caspases' play a crucial role in regulating apoptosis (Birch *et al.*, 2000; Stennicke, 2000). Recent publications have identified caspase-like activity associated with plant development, and the cell death associated with both susceptibility and resistance in plants (Elbaz *et al.*, 2002; Bozhkov *et al.*, 2004, 2005; Chichkova *et al.*, 2004; Coffeen and Wolpert, 2004; Danon *et al.*, 2004; Hatsugai *et al.*, 2004; Boren *et al.*, 2006; Bonneau *et al.*, 2008). Introduction of highly specific mammalian caspase inhibitors has been found to block plant PCD, suppress some characteristic features of apoptosis that occur in these plant cells and limit pathogen infection (del Pozo and Lam, 1998; Dickman *et al.*, 2001; Richael *et al.*, 2001; Lincoln *et al.*, 2002; Bonneau *et al.*, 2008; Li *et al.*, 2008). Resistance to pathogens has been observed in transgenic tobacco engineered to express various anti-apoptotic proteins from animals, including chicken Bcl-Xl, nematode CED-9 and baculovirus Op-IAP (Mitsuhara *et al.*, 1999; Dickman *et al.*, 2001).

The anti-apoptosis gene, *p35,* was originally isolated from a baculovirus that infects silkworm (Clem *et al.*, 1991). The specific activity of the *p35* gene product lies in a tetrapeptide sequence, DQMD, which binds to the active site of the target caspase in the silkworm and inhibits it from further function (Zhou *et al.*, 1998; Fisher *et al.*, 1999), resulting in prevention of apoptosis in the infected host cell during viral replication. Gilchrist *et al.* (2001) and Lincoln *et al.* (2002) showed that transgenic tomatoes expressing *p35* (inherited as a single Mendelian locus) had reduced disease symptoms after inoculation with several necrotrophic pathogens of tomato. These authors further demonstrated by mutational modification of the DQMD domain to DRIL that the activity of the p35 gene was abolished in suppression of PCD and disease *in planta*. Therefore, the activity of p35 in plants is dependent on the integrity of the specific tetrapeptide sequence DQMD, as it is in animals. These results support the hypothesis that there is a conserved cross-kingdom pathway of apoptosis and that plant disease symptoms may be ameliorated by transgenic expression of anti-apoptosis genes. To further test this hypothesis and expand the breadth to other hosts and pathogens, we transferred *p35* into NLL and investigated its impact on anthracnose (*C. lupini*), root rot and brown spot (*P. setosa*) diseases of lupin.

Results

A p35 transformation construct and production of transgenic lupin plants

An existing anti-apoptotic baculovirus *p35* gene cassette (CaMV 35S promoter-*p35* ORF-Nos terminator; Lincoln

et al., 2002) was transferred to a twin T-DNA transformation vector that contained the *bar* gene as a selectable marker in the second T-DNA (pRM66, courtesy R. Morton and T.J. Higgins, CSIRO, Australia) and that had been modified by addition of a multiple cloning site. The resultant construct was designated pTW35 (Figure 1). *Agrobacterium*-mediated transformation of NLL cultivar Unicrop (Gladstones, 1972) yielded 74 independent T_0 transformation events (an average of about 3.3% transformation efficiency) after several cycles of selection, from a total of 2220 inoculated meristems. Putative transformed shoots from 47 transformation events (63.5% of the total 74 events) were successfully rooted and grew to maturity. All T_0 putative transformed plants appeared normal, were fertile, and produced T_1 seeds of similar size and appearance to the untransformed cultivar (data not shown).

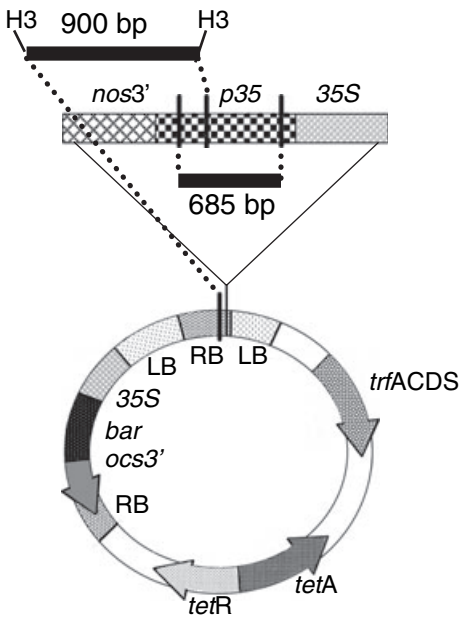

Figure 1 Structure of pTW35. Cartoon map (not to scale) of the plasmid pTW35 that was constructed for NLL transformation by *Agrobacterium tumefaciens*. The *p35* gene cassette is shown as a bar above the circular vector pRM66mcs, in the orientation of the insertion event that is in the *Pac*I restriction enzyme site of a mcs positioned between the second T-DNA left and right borders. The second left border and *trf*A CDS flank the *oriT* sequence. The arrowheads on the vector indicate the direction of gene transcription. The two black horizontal bars connected with heavy dots to vertical bars on the plasmid map indicate the probes used for Southern blot analysis of *p35*-NLL lines. The 900-bp probe is a *Hin*dIII (H3) digest product. The 685-bp probe is a PCR amplification product. *tet*A and *tet*R encode tetracycline resistance; RB is right border; *ocs3'* is octopine synthase gene transcript terminating sequence; *bar* is the phosphinothricin resistance gene from *Streptomyces hygroscopicus*; 35S5′ is the cauliflower mosaic virus 35S promoter; LB is left border; *nos3'* is nopaline synthase gene transcript terminating sequence; *p35* is the p35 gene open reading frame.

Determining insert identity, integrity and copy number in p35 transgenic lines

To confirm the genetic composition of the putative *p35* transgenic lines obtained, we first examined the presence of the two T-DNA inserts in T_1 progeny by PCR. Of 45 independent T_0 events, 14 T_1 lines (31.3%) had inherited both the *p35* and *bar* genes and three T_1 lines (6.7%) inherited the *bar* gene only. Several T_1 plants from each line that contained *p35* were grown to maturity and selfed progeny was tested for the presence of the transgenes by PCR. Table 1 shows that some lines were apparently fixed for the presence of *p35* while others were still segregating. However, data from 'fixed lines' also were consistent with multiple independent insert events (Sedecole, 1977). Therefore, genomic DNA samples from T_1 or T_2 progeny were analysed by Southern blot hybridization in a reconstruction experiment, to confirm presence or absence of *p35*, its approximate copy number and number of insertion events.

The two probes used for Southern blot analysis are shown in Figure 1. The 900-bp probe was a control that should hybridise only to the 900-bp *Hin*dIII fragment containing the *p35* insert. Predicted results for hybridization of the 685-bp probe to *Hin*dIII-digested genomic DNA from transgenic lines containing some of the simpler possible insert structures and an example of a Southern blot using this probe are illustrated in Figure S1. Data from hybridization of both probes for all lines are summarized in Table 1. The 685-bp probe (Figure 1) hybridized to the expected 900-bp T-DNA fragment (for estimation of copy number) and to some larger size bands as expected for *p35* T-DNA integration site 'end fragments' in the lupin genome (Figure S1). However, the control 900-bp probe also detected some extra larger bands in some *p35*-NLL lines (Table 1). As the same sized bands also were detected on the blot hybridized with the 685-bp probe in every case (Figure S1 and data not shown), these extra bands were considered artefacts. The remaining larger bands (Figure S1, Table 1) were used to estimate the number of *p35* integration sites (Table 1). Genomic DNA from the line T764, which did not contain an amplifiable *p35* sequence, also did not detectably hybridise to the *p35* probes from Southern blot analysis (Table 1), indicating that the negative PCR results were due to lack of the *p35* T-DNA insert, not a minor alteration at a primer binding site. This line was used as an empty-vector negative control in the gene expression and plant–microbe interaction analyses.

Table 1 Analysis of *p35* copy and insert number in the genome of some *p35*-transformed lupin lines

Transformed lupin line	Observed p35 present: p35 absent in T1	Observed p35 present: p35 absent in T2 (-x is T2 line no.)	χ^2 *P* value testing 3:1 ratio*	Observed hybridized bands (bp)†	Observed independent inserts	Confirmed fixed T4 line
T363	4 : 0	13 : 5 (-1)	0.79	900 (1×), 2800	1	Yes
		16 : 0 (-2)	0.02			
T401	7 : 1	14 : 0 (-1)	0.04	900 (5–6×), ++	≥5	No
		22 : 0 (-2)	0.007			
		18 : 0 (-11)	0.01			
T563	5 : 1	20 : 0 (-5)	0.01	900 (∼10×), 1300, 2600, ++	≥5	No
		19 : 0 (-6)	0.01	900 (1×), 2100	1	Yes
T763	2 : 1	15 : 0 (-1)	0.03	900 (1×), 2600, 5000‡	1	Yes
		14 : 2 (-2)	0.19			
T821	9 : 9	15 : 2 (-1)	0.16	900 (2×), ∼1600, ∼2400, ∼3500‡, ∼5000‡, ∼7000‡	2	No
		12 : 5 (-2)	0.71			
		11 : 7 (-3)	0.21			
		11 : 5 (-8)	0.62			
		11 : 3 (-16)	0.59			
		12 : 4 (-18)	0.78			
T824	29 : 4	6 : 2 (-1A1)	0.68	900 (∼10×), 1100, 1300, 1500, ++	>5	No
		20 : 0 (-1C1)	0.01			
		11 : 5 (-1C2)	0.62			
		20 : 0 (-1C3)	0.01			
		20 : 0 (-IIB1)	0.01			
T961	11 : 0			900 (4–5×), ∼1500, ∼2300, ∼2700, ∼3500, ∼4500	5	No
T1562	12 : 0			900 (1×), ∼3000, ∼5000	2	No
T1563	11 : 1			900, ∼5000	1	Yes
T1661	7 : 5			900 (2×), ∼1500, ∼2000‡, ∼3000‡, ∼4000‡, 7500	2	No
T1765	26 : 12			900 (1×), ∼5000	1	Yes
Unicrop (cv.)§					0	
Tanjil/Kalya (cv.)§					0	
T764¶	9 : 3	16 : 0 (-2)	0.02		0	Yes*

*A *P* value of >0.05 supported a 3 : 1 ratio, indicative of a single *p35* insert locus or two (or more) very closely linked loci.

†Hybridized bands on Southern blots. Numbers in brackets are estimated copy number of the 900-bp *p35* insert band, based on the intensity of 685-bp probe hybridization (Figure 1), compared to the 900-bp pTW35 plasmid DNA fragments used for copy number reconstruction (++ means multiple additional bands).

‡Bands that also appeared on control blots hybridized with the 900-bp probe (Figure 1) and are considered artefactual.

§Untransformed wild-type Unicrop (susceptible cultivar), and untransformed wild-type Tanjil and Kalya (fungal resistant cultivars).

¶An 'empty-vector' line. Segregation data are for the *bar* gene, assessed by PCR and Pearson's Chi squared test.

Assessment of p35 transcript accumulation in propagated p35-NLL lines

To test expression of *p35*, we performed Northern gel blot hybridization for total leaf RNA of several transgenic lines, using *p35*-tomato leaf RNA (Lincoln *et al.*, 2002) as a positive control. A transcript of the expected size close to 1 kb was detected in the *p35*-NLL line T363, but compared to *p35*-tomato leaf transcripts was present at very low abundance (data not shown). Therefore, we used the more sensitive detection method of RT-PCR to detect *p35* transcripts in leaf, stem and root RNAs of *p35*-NLL lines (Figure 2 and data not shown). These analyses detected *p35* transcripts in at least one organ of most *p35*-NLL lines. No signal was obtained for negative control plants (T764 and untransformed NLL plants; Figure 2b). RT-PCR results are not a precise measure of transcript abundance. However, as equivalent amplification conditions were used for all samples and amplification product abundances were consistent between repeated reactions, we derived a semi-quantitative measure of abundance (Table 2). These data suggest that there were differences in the abundance

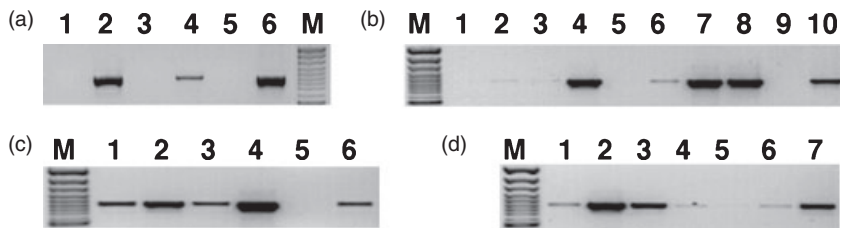

Figure 2 RT-PCR analysis of *p35* gene expression. Agarose gel electrophoresis of reverse transcriptase (RT) PCR products of total RNA. (a) Control and test reactions of T363 leaf RNA. Lane 1 DNAsed RNA, Lane 2 reverse transcribed T363 leaf cDNA, Lane 3 Blank, Lane 4 T363 Leaf DNA (positive control), Lane 5 Unicrop leaf DNA (negative control), Lane 6 p35 plasmid DNA (positive control). (b) RT-PCR products of leaf cDNAs from the designated p35 and control lines. Lane 1 T563-6; Lane 2 T763; Lane 3 T563-5; Lane 4 T1562; Lane 5 T961; Lane 6 T1661; Lane 7 T1765, Lane 8 T1563, Lane 9 Unicrop, Lane 10 T363 Leaf DNA (positive control). (c) RT-PCR products of stem cDNA from the designated genotypes. Lane 1 T363; Lane 2 T821; Lane 3 T824; Lane 4 T1765; Lane 5 Blank; Lane 6 T363 Leaf DNA (positive control). (d) RT-PCR products of root cDNA from the designated genotypes. Lane 1 T821; Lane 2 T1765; Lane 3 T363 (leaf control); Lane 4 T363; Lane 5 T563-5; Lane 6 T401; Lane 7 T363 Leaf DNA (positive control). In all panels M is GeneRuler^{TM} 100-bp DNA ladder plus (MBI Fermentas, Maryland, USA).

Table 2 Summary of *p35* gene expression analysis in leaf, stem and root

	p35 Transcript abundance* in		
Genotype	Leaf	Stem	Root
T363	High (2)	Mod-High (2)	Low-Mod (2)
T401	Moderate	Low-Mod (2)	Low-Mod (2)
T563–5	Low	ND	Low (2)
T563–6	No-V Low (2)	ND	No
T763	Low	Low (2)	Low
T821	Moderate (2)	High (2)	Low-Mod (2)
T824	Low (2)	High	Low (2)
T961	Low	ND	ND
T1562	High	ND	ND
T1563	High	High	ND
T1661	Moderate	Low	ND
T1765	High (2)	High (2)	High (2)
Unicrop‡	No	ND	No
Tanjil/Kalya‡	No	ND	No
T764†	No (2)	No	No

*Designated product abundance is based on the intensity of amplified band after RT-PCR compared to a loading standard. Repeated RT-PCR experiments (number of experiments indicated in brackets) were conducted with a different RNA preparation.

ND means experiment was not performed.

†An empty-vector line (contains no *p35* transcript but is homozygous for the *bar* gene).

‡Untransformed wild-type Unicrop (susceptible cultivar) and untransformed wild-type Tanjil and Kalya (fungal resistant cultivars).

of *p35* gene expression between *p35*-NLL lines and also within each line in the different organs tested. Total RNA extracts from a few transgenic lines (i.e. T1765 and T363) consistently gave high abundance *p35* RT-PCR product from leaf, stem and root while other transgenic lines gave moderate, low or even no *p35* RT-PCR product. In general, less *p35* RT-PCR product was derived from root RNA than from leaf and stem (Table 2).

Fungal pathogenicity tests

The molecular analyses described above established that we had derived a suitable number and quality of *p35*-NLL lines to assess the impact of *p35* on necrotrophic fungal pathogen diseases of NLL. Pooled T_3 or T_4 progeny were tested and, except for lines with multiple inserts, these were fixed for the transgene at $P \geq 0.95$. We performed whole plant inoculation tests using locally derived fungal isolates and standard methodology and data analysis employed in the national NLL breeding program at the Department of Agriculture and Food Western Australia (Yang and Sweetingham, 2002; Thomas and Sweetingham, 2003).

Two separate anthracnose disease tests were performed. Anthracnose symptoms (i.e. bending of upper stem internode) started to occur within 3–4 days after inoculation (DAI) of susceptible control plants. Within five to six DAI, small lesions became visible on stems of some NLL plants. Almost all upper stem parts and growing points of control plants died within a month, and the fungus sporulated abundantly on the dead parts. Disease symptoms were scored at 13 DAI in both experiments. Results for Unicrop and T764, compared to the most tolerant *p35*-NLL line T821, are shown in Figure S2, and results for all lines are presented in Table 3. Resistant cultivar Tanjil had the lowest anthracnose disease rank. Line T821 consistently had significantly lower disease scores than all other *p35*-NLL lines, which were not significantly different from the susceptible parent cultivar Unicrop and the empty-vector control line. The only symptoms noted on T821 were slight bending of the growing points of some plants, and some spores occurring on lesions. In the second disease trial, ranks of anthracnose symptoms on the resistant cultivar Tanjil and line T821 were not significantly different (Table 3).

Table 3 Assessment of necrotrophic fungal pathogen disease and nodulation of *p35* transgenic lines

	Anthracnose*		Root rot*		Brown spot*		Nodulation†	
Lupin genotype	Exp 1 (13 DAI)	Exp 2 (13 DAI)	Exp 1 (21 DAI)	Exp 2 (30 DAI)	Exp 1 (8 DAI)	Exp 1 (12 DAI)	No. per plant	DW (mg) per plant
T363	4.78^{c}	3.77^{b}	2.27^{b}	1.60^{a}	1.99^{abc}	3.44^{bcd}	13.0	38.9
T401	4.57^{c}	4.20^{b}	2.61^{bc}	2.03^{abc}	2.53^{efg}	3.67^{cd}	14.0	37.5
T563–5	4.89^{c}	4.34^{b}	2.74^{bc}	1.74^{ab}	2.35^{cdef}	3.59^{bcd}	13.7	35.0
T563–6	4.86^{c}	4.07^{b}	3.04^{cd}	1.88^{ab}	2.04^{abcd}	3.53^{bcd}	11.6	40.0
T763	4.67^{c}	4.08^{b}	2.97^{cd}	2.37^{bc}	2.20^{bcde}	3.35^{bc}	10.6	37.5
T821	3.81^{b}	2.11^{a}	2.39^{b}	1.39^{a}	1.89^{ab}	3.37^{bcd}	10.6	35.6
T824	4.91^{c}	3.83^{b}	1.70^{a}	1.67^{ab}	2.35^{cdef}	3.49^{bcd}	11.1	31.6
T961	4.50^{c}	4.23^{b}	2.94^{cd}	2.31^{bc}	2.79^{g}	3.41^{bcd}	9.9	32.0
T1562	4.88^{c}	4.46^{b}	3.28^{d}	2.76^{c}	2.62^{fg}	3.33^{bc}	9.5	37.6
T1563	4.72^{c}	3.79^{b}	2.98^{cd}	2.03^{abc}	2.78^{g}	3.45^{bcd}	11.4	33.1
T1661	4.89^{c}	4.40^{b}	3.00^{cd}	2.35^{bc}	2.13^{bcde}	3.74^{d}	12.0	32.1
T1765	4.63^{c}	3.80^{b}	1.76^{a}	1.37^{a}	1.68^{a}	2.42^{a}	6.8	36.5
Unicrop‡	4.92^{c}	4.03^{b}	2.94^{cd}	2.66^{c}	2.46^{defg}	3.46^{bcd}	12.8	37.8
Tanjil/Kalya§	2.86^{a}	1.42^{a}	2.31^{b}	1.37^{a}	2.23^{bcdef}	3.25^{b}	11.6	49.0
T764¶	4.83^{c}	4.20^{b}	3.02^{cd}	2.34^{bc}	2.83^{g}	3.44^{bcd}	11.9	32.6
$MS_{genotype}$	1.841***	2.214***	1.377***	0.624***	0.727***	0.538***	9.68 (NS)	58.4 (*)
CV	8.4%	11.3%	14.8%	18.4%	13.6%	8.2%	ND	ND

*Based on 0–5 scale; DAI = days after inoculation; Means in each column followed by a common letter are not different at the 5% probability level according to the Duncan test. ***Significant, with $P < 0.001$.

†Mean values; DW, dry weight; (NS), not significant; (*), LSD ($P = 0.05$) = 14.6; ND, not determined.

‡Untransformed wild-type Unicrop cultivar.

§Resistant cultivars: Tanjil was used in the anthracnose experiments while Kalya was used in the root-rot and brown spot experiments.

¶Contains the *bar* gene but has no *p35* gene.

The results for two independent experiments testing *Pleiochaeta* root rot severity in *p35*-NLL are summarized in Table 3. There was slight variation in disease progression between the two experiments, with less severe disease symptoms and a longer experimental period required in the second experiment. Despite the variation in *Pleiochaeta* root rot severity scores between the two experiments, lines T1765 and T824 performed significantly better than the susceptible parent cultivar Unicrop, and were comparable to the moderately resistant Kalya variety. Lines T821 and T363 had an intermediate disease severity. Severely affected plants, including Unicrop and the empty-vector control, were wilted and died when symptoms were scored (21 DAI in Experiment 1; 30 DAI in Experiment 2). Examples of disease symptoms in the three classes of response are shown in Figure S3.

Pleiochaeta brown spot disease was assessed at two time points in a single experiment. Line T1765 performed significantly better than all other transgenic lines and Kalya in this test. Lines T824 and T363 that had intermediate disease scores at eight DAI were severely infected by the brown spot disease at 12 DAI, similar to other lines (Table 3) Figure S4 shows an example of the best performing line T1765 compared to the empty-vector control line T764.

Nodulation of *p35-NLL* lines

We compared nodule numbers and fresh and dry weights on the *p35*-NLL lines with colonization data for conventional cultivars. All *p35*-NLL lines were nodulated and none had significantly different numbers or dry weight of nodules from the parent cultivar Unicrop. However, the resistant cultivar Tanjil had significantly more dry weight of nodules than Unicrop or its derived p35-NLL lines (Table 3).

Discussion

Results of this study showed that the *p35* construct (Figure 1) was stably integrated into the NLL genome as single or multiple gene copies (Table 1, Figure S1), expressed in different organs of *p35*-NLL lines although at apparently varied abundances (Figure 2; Table 2), and inherited through to the T_4 generation (Figure S1b and data not shown). Of 14 events obtained, four lines (T363, T821, T824 and T1765) performed significantly better than the empty-vector control line T764 and susceptible parent Unicrop, when inoculated with one or more necrotrophic fungal pathogens (Table 3). Line T821 performed well when inoculated with anthracnose and *Pleiochaeta* root

rot (Table 3; Figure S2) while lines T824 and T363 showed improved tolerance only to *Pleiochaeta* root-rot disease (Table 3; Figure S3). Line T1765 consistently had reduced disease symptoms for both root and leaf infection caused by *P. setosa* (Table 3; Figures S3 and S4). These results follow similar improvements in resistance of *p35*-transformed tomato (Lincoln *et al.*, 2002) and confirm that *p35* provides a model for effective genetic control of a wide range of necrotrophic pathogens in the field. However, further work will be required to identify the molecular requirements for efficacy of this construct.

The lupin transformation efficiencies (3.3% at T_0 and 0.8% at T_1 generations) were comparable to or higher than previously reported, using different gene constructs. Babaoglu *et al.* (2000), Li *et al.* (2000) and Pigeaire *et al.* (1997) reported lupin transformation efficiencies at T_0 of 1.2% (pearl lupin), 0.91% (yellow lupin) and 2.8% (NLL cv. Unicrop), respectively; and at T_1 generation of about 0.3% (Li *et al.*, 2000), over the total inoculated explants. These comparisons indicate that in our research, the modifications to the lupin transformation method (i.e. tissue culture media and the rooting step) had a positive effect and/or that the construct itself was favourable. For example, *p35* transgenic passion-fruit has improved herbicide tolerances (Scandiucci de Freitas *et al.*, 2007); this effect might reinforce the selectable marker (herbicide resistance) in dual *p35*, *bar* transformants.

As previously reported (Pigeaire *et al.*, 1997; Li *et al.*, 2000), there is low gene transmission between T_0 and T_1 using this transformation methodology. Reduced transmission is probably because the initial T_0 shoot was a chimera of transgenic and non transgenic cells (data not shown; Dong and McHughen, 1993). Our T_1 segregation results support this interpretation (Table 1). For example, Southern blot data showed that Line T821 has two independent inserts that are probably closely linked (T_2 PCR data). However, a 1 : 1 ratio was observed in the T_1 generation ($P = 1.0$) rather than the expected ratios of 15 : 1 ($P = 0.000$) or 3 : 1 ($P = 0.014$) ratio suggesting that the T_0 plant had a significant sector of non-transgenic tissue from which some of the T_1 progeny were derived. Further research is required to determine how to overcome this limiting technical issue that complicates the derivation of fixed transgenic lines for subsequent analysis and eventual commercialization. The observed segregation ratios of *p35*-NLL lines at the T2 generation were consistent with the estimated *p35* copy number from the Southern blot (Table 1), which supports the conclusion that the transgene was integrated into the nuclear genome and transmitted in a normal Mendelian fashion. However, our data also emphasize the importance of checking adequate numbers of progeny for statistically meaningful conclusions about 'homozygosity' of apparently fixed lines.

Our results extend the information available about the efficacy and genetic structure of twin T-DNA transformed legumes. Although several lines were identified as *bar*+ but without the *p35* T-DNA, we did not detect a *p35*+, *bar*- line. This may be the consequence of the lengthy time period (6 months at least) in which putative transgenic material remains on selection media. In research on soybean aimed to manipulate the fatty acid profile of seeds and also using a twin T-DNA approach with the *bar* gene as the selectable marker, 29 of 55 primary twin T-DNA transformants had both T-DNA inserts and 17 of these produced T_1 progeny that had the desired fatty acid expression profile. However, some rearrangements and at least one chimeric line were observed. For example, four of eight lines that segregated for *bar* gene function still had *bar* gene sequences. Only one desirable transgenic line was obtained that had a single locus insert with a simple genetic structure (Sato *et al.*, 2004). Those results support our experience that a robust starting number of transgenic events are required to ensure the outcome of a selectable marker-free, genetically simple, agronomically improved line, using the twin T-DNA approach as described by Komari *et al.* (1996). Significant improvement of transformation efficiency may facilitate incorporation of genetic transformation more broadly into legume breeding programmes.

P35 transformation also has been achieved in tomato and *Arabidopsis* (Lincoln *et al.*, 2002), tobacco (del Pozo and Lam, 2003) and passion-fruit (Scandiucci de Freitas *et al.*, 2007). Although blocking PCD may improve transformation efficiencies, one could expect that *p35* expression would affect plant growth by inhibiting the PCD that normally occurs during plant development (Hansen, 2000; Gray, 2004). For example, some *p35* transgenic tomato plants exhibited varying degrees of sterility to full fertility, even though all primary transgenics were phenotypically normal (Lincoln *et al.*, 2002). However, in this study the phenotype of the *p35* transgenic lupin plants was indistinguishable from the control plants, in terms of normal growth and seed production (data not shown). Efforts to ameliorate fungal pathogen symptoms in a crop legume would not be of value if the genetic change also negatively impacted beneficial symbioses. NLL is nodulated but not mycorrhizal and this study showed no adverse impact on the bacterial symbiont by the presence of *p35* (Table 3). However, as we did not investigate cell specificity of *p35*

expression, the role of apoptosis in nodulation cannot be inferred from this study. Some tomato, arabidopsis, tobacco and passionfruit *p35* transformants also were reported to develop normally (Lincoln *et al.*, 2002; del Pozo and Lam, 2003; Scandiucci de Freitas *et al.*, 2007).

In most of the NLL lines there was a positive association between apparent gene expression and disease level with those lines having the least symptoms generally exhibiting the strongest signal in the *p35* gene expression analysis. For example, line T821, the only one to have significantly reduced anthracnose symptoms, also appeared to have much more *p35* RT-PCR product amplified from stem extract relative to other lines. Similarly, line T1765 performed well against *Pleiochaeta* root rot and brown spot and appeared to have abundant *p35* gene expression in the root and leaf tissues. However, an apparent high level of gene expression did not always correlate with a better phenotype expression of transgenic lines (e.g. lines T1562 and T1563, Tables 2 and 3). RT-PCR analyses (Figure 2; Table 2) assessed total RNA from a particular organ and did not provide conclusive evidence of *p35* gene expression in the infected tissues of that organ, or of its presence or quantity as a functional protein product, for which a direct assessment should be performed, as discussed by Jansen *et al.* (2002). Future investigations into cell-type specific expression and accumulation of P35 protein may clarify reasons for differences between transgenic lines.

The NLL cultivar Unicrop was used for *p35* transformation because it is very susceptible to a range of lupin fungal diseases including anthracnose, root rot and brown spot, enabling the extent of any *p35* effect to be assessed without confounding effects from endogenous NLL resistance genes. Crossing the transgenic lupin lines generated here to other lupin cultivars will test whether expression of *p35* can complement or enhance the 'conventional' resistance of cultivars like Tanjil. NLL cultivar Wonga is a sister line to Tanjil that also carries resistance to anthracnose (Cowling, 1999). Wonga was consistently ranked in the top three out of 10 lupin cultivars for anthracnose resistance, based on lesion severity on leaves, petioles, stems and pods, in eight international glasshouse or field experiments (Cowling *et al.*, 2000). This resistance is not a 'hypersensitive' type resistance or immunity, but a quantitative resistance that results in a reduction in lesion size and severity and an increase in latent period, compared with susceptible varieties (Thomas *et al.*, 2008). The *p35* transformed line T821 had an anthracnose disease phenotype similar to Tanjil with significantly smaller lesions than susceptible cultivar Unicrop and empty-vector negative control T764 (Table 3). Smaller lesions in T821 and Tanjil are consistent with suppression of the plant response to pathogen signals triggering cell death, thereby limiting the food base and pathogen growth. This raises the interesting possibility that Tanjil or Wonga, and T821 have functionally similar resistance mechanisms, which culminate in a suppression of pathogen induced programmed cell death in host cells, thereby limiting disease severity.

Our results extend previous reports of other plant systems where *p35* reduced symptoms caused by a range of pathogens (Mitsuhara *et al.*, 1999; Dickman *et al.*, 2001; Lincoln *et al.*, 2002). This supports the hypothesis that symptom development in many so-called necrotrophic plant diseases is the result of pathogen-induced PCD and involves caspase-like activity (Lincoln *et al.*, 2002). No other target or catalytic function has been reported for p35 (Zhou *et al.*, 1998). Although no sequence homologues of animal caspases have been characterized in plants, there are recent claims of the presence of proteases in plants that cleave caspase-specific substrates (Uren *et al.*, 2000; Hatsugai *et al.*, 2004; Watanabe and Lam, 2006). It is possible that the genomes of plants will have functional equivalents of cell death-related proteins that share little or no sequence homology to their animal counterparts (Gilchrist, 1998; Uren *et al.*, 2000; Elbaz *et al.*, 2002), providing that the functional domains are conserved such as the ability to interact with the *p35* product.

In conclusion, our results with *p35* provide an alternative molecular explanation for 'necrotrophy' in these fungal diseases of NLL, in which the fungal pathogens induce not necrosis but PCD in host cells during infection. This supports proposals that regulating PCD in plants with plant genome-derived anti-PCD genes is a potential strategy for engineering broad-spectrum necrotrophic disease resistance (Lincoln *et al.*, 2002; van Kan, 2006). Our results underscore the need for functional screens to identify anti-PCD plant genes as was reported recently from a tomato cDNA library screen in transgenic tissue (Harvey *et al.*, 2008).

Experimental procedures

Regulatory approval

Prior approval for this research was obtained from the Office of the Gene Technology Regulator (OGTR), Australia under application number NLRD 162/2002.

Transfer of the p35 gene cassette to a vector suitable for NLL transformation

The anti-apoptotic baculovirus *p35* gene cassette (CaMV *35S* promoter-*p35* ORF-*Nos* terminator) was recovered by PCR from pBILW/*Escherichia coli* DH5-α (provided by J.E. Lincoln, UC Davis, CA, USA). Oligonucleotides used for PCR amplification were 5′-C**TTAATTAA**AGATTAGCCTTTTCAATTTC-3′ (forward) and 5′-C**TTAATTAA**GATCTAGTAACATAGATGAC-3′ (reverse), containing a *Pac*I restriction site (indicated in bold letters). The high fidelity *pfu* DNA polymerase (Promega Corporation, Madison, USA) was used to amplify the *p35* gene cassette. An 'A'- base tailing step (Promega Corporation's Technical manual No. 042) was performed on the purified PCR product, to enable cloning the fragment into an intermediate pGEM®-T Easy vector (Promega Corporation). Subsequently the ligated vector was electroporated into competent *E. coli* cells. The integrity of the *p35* gene cassette was confirmed by sequencing analysis.

The plant transformation plasmid vector pRM66 (courtesy R.L. Morton and T.J. Higgins, CSIRO, Australia) was modified by addition of a multiple cloning site (*mcs*) in the 'empty' T-DNA and named pRM66-mcs (S. Wylie, unpublished data). The *Pac*I-digested *p35* gene cassette fragment was inserted in the unique *Pac*I site of the *mcs,* and the resultant plasmid (pTW35) was electroporated into *Agrobacterium tumefaciens* strain *AgL*0 (Lazo *et al.*, 1991).

Agrobacterium-mediated lupin transformation and generation of putative-transformed shoots

Procedures for explant preparation, explant transformation and transgenic shoot development essentially followed the method of Pigeaire *et al.* (1997) with some modifications routinely used in the CLIMA transgenic laboratory at UWA (Atkins *et al.*, 1998; S. Chapple, personal communication, detailed below).

Root induction and plant culture

Root induction on NLL shoots was performed *in vitro* by transfer to rooting medium (Atkins *et al.*, 1998). Shoots with developed roots were moved to growth medium in a controlled environment room (CER) maintained at 22°C day/18°C night. The growth medium was a mixture of potting mix (1/3) and washed river sand (2/3). A small amount of lupin rhizobial inoculant strain WU425 (Nodulaid 100, Bio-Care Tech. Pty. Ltd, Somersby, Australia) was added before covering the washed, agar-free roots with growth medium. The medium was then watered and finally the shoot was covered with a clear plastic tub. When the plants showed healthy growth, the plastic tub was gradually opened over a number of days. The tub was replaced with an aerated plastic bag that covered the plant. Gradual opening of the plastic bag (over about 2 weeks) was again performed until the plant was ready to withstand the normal CER conditions. Regular watering every second day and fortnightly fertilising with Phostrogen™ (Debco, Tyabb, Australia) were conducted to ensure healthy growth.

Segregation of transgenes in the progeny

PCR screening of putative transformed plants, T_1 and T_2 progenies was conducted to determine the presence and segregation of transgenes (*p35* and *bar*) and to help eliminate non-transgenic lupin plants. Depending on the number of seeds available, at least 10 seeds of each putative transgenic line were sown. Leaf samples from germinated seedlings were removed and DNA extracts were prepared, based on a high-throughput method of Xin *et al.* (2003), to obtain genomic DNA amplification templates. Primers used for *p35* gene amplification were: p35CD-F (5′-TGTGTAATTTTTCCGGTAGAAATCGAC-3′) and p35CD-R (5′-GGCAATAAATTTTAACATTTATTTAATTGTG-3′).

Primers for the *bar* gene amplification were: SHBAR1 (5′-TCTGCACCATCGTCAACCAC-3′) and SHBAR1R (5′-ACTTCAGCAGGTGGGTGTAG-3′). The predicted PCR products were ~900 bp and ~300 bp in size for *p35* and *bar* genes, respectively. The PCR was carried out using the 'PCR Express Thermal Cycler' (Thermo Hybaid, Franklin, USA) in a 25 μL reaction containing 1× PCR buffer, 200 μM of each dNTP, 10 pmol of each primer, 50 ng genomic DNA and 1 unit of Taq DNA polymerase (Qiagen, Valencia, USA). Cycler conditions were: 94°C for 3 min; 30 cycles of 30 s at 94 °C, 30 s at 55°C and 1 min at 72°C; 72°C for 10 min; 4°C hold. Reactions were subjected to gel electrophoresis in 2% agarose, 0.5× TBE and visualized with ethidium bromide.

Southern blot analysis of p35-NLL lines

Purified pTW35 plasmid DNA was used as a positive control on the Southern blots. The calculation for the reconstruction was based on *L. angustifolius* genome size of 2C = 1.89 pg (Naganowska *et al.*, 2003). For 10 μg of lupin genomic DNA loaded per lane, the *Hin*dIII-digested pTW35 plasmid DNA equivalent to one and two inserts per genome was loaded. A preliminary experiment with one, 25 and 75 insert equivalents showed that less than 25 copies of *p35* were present in these lines (data not shown).

Genomic DNA of lupin leaf was purified using a slight modification of Pallotta *et al.* (2000). The extraction buffer was modified by the addition of 100 mM Na_2SO_3 and pH adjusted to 8.5, and the precipitated nucleic acids were resuspended in 40 μg/mL RNAase A in 10 mM Tris–HCl pH 8.0, 1 mM EDTA. DNA (20 μg) was digested with *Hin*dIII for 4 h, ethanol precipitated, electrophoresed (along side the plasmid controls) in a 1× TAE, 1% agarose gel at 45 V for ~14 h and transferred to nylon membrane (Hybond-N^+, Amersham Biosciences, Fairfield, USA). Membranes were hybridized with labelled probe derived either from PCR-amplified fragment (the 685-bp probe) or from *HindIII*-digested pTW35 plasmid DNA (the 900-bp probe) as shown in Figure 1. The probe was radioactively labelled with α-^{32}P dCTP (Easytides®, Perkin-Elmer™ Pty Ltd, Waltham, USA), following the 'Prime-a-Gene' kit method (Promega Corporation). Hybridization steps were carried out according to the standard method without formamide (Sambrook *et al.*, 1989). Post hybridization, the membrane was rinsed with 2× SSC, washed with 2× SSC, 0.1% SDS solution at room temperature (25°C), and washed with pre-heated (65°C) 0.2× SSC, 0.1% SDS. The signal was detected by overnight room temperature exposure of membranes to a phosphor imaging plate and read using a Phosphorimager (Bioimaging Analyzer BAS-2500 FUJIFILM Corp., Tokyo, Japan).

Expression analyses

Accumulated *p35* transcripts were detected by Northern blot hybridization (data not shown) and RT-PCR. Total RNA was obtained from 500 mg of young leaves of non-transformed and transformed lupin plants. Additionally, total RNA was also extracted from stem and root of lupin plants for RT-PCR. RNA extraction was carried out using TRIzol® reagent (Invitrogen,™ Carlsbad, USA) according to the manufacturer's instructions. RNA concentration (μg/mL) was quantified using a spectrophotometer (UV-1201 Spectrophotometer, Shimadzu, Kyoto, Japan).

RNA manipulations and gel electrophoresis for Northern blot were based on those of Sambrook *et al.* (1989). PCR-generated full-length *p35* coding sequence was labelled and used as probe. Probe labelling, hybridization and signal detection were with the 'Gene Images AlkPhos Direct Labelling and Detection System' and CDP-Star™ following the manufacturer's instructions (Amersham Bioscience).

For RT-PCR analysis, purified total RNA was first DNase-treated (Roche Diagnostics, Basel, Switzerland) to avoid false-positive results. First strand cDNA was synthesized from DNased-RNA (~0.6 μg) and 30 pmol of oligo-dT$_{(15)}$ (Roche Diagnostics), incubated at 65°C for 10 min. This reaction was then used in an RT reaction (Expand RT of Roche diagnostics) following the manufacturer's instructions.

PCR of the RT product used the same oligonucleotides and thermal cycle conditions, as described for genomic DNA PCRs. The PCR was carried out in a 25 μL reaction containing 1× PCR buffer, 200 μM of each dNTP, 10 pmol of each primer DNA, 3 μL (~100 ng) RT/cDNA template and 1 unit of Taq DNA polymerase. A PCR mixture was also prepared for each DNased-RNA as template (0.5 μL each, ~150 ng), to check for DNA contamination.

Assessment of nodulation

Nodulation was assessed in three replications (pots) of six plants per pot. Growth medium was 70% washed river-sand and 30% potting mix in a 150-mm pot. A small amount of lupin rhizobial inoculant strain WU425 (Nodulaid 100) was added with the germinated seeds. Plants were grown for 5 weeks before harvesting in a CER set at 18°C day/12°C night; no fertilizers added during growth; watered as necessary. At harvesting, growth medium was freed and the root systems were washed clean. Nodule numbers per plant were counted and were averaged for the six plants in a pot. All nodules (from each pot) were then separated from the roots, air-dried and weighed (fresh weight, data not shown). For dry weight (DW), the nodules were dried in an oven at 70°C for 48 h before being weighed. The averages of nodule DW from the six plants in each pot were determined. Statistical analyses of these data were performed by one-way analysis of variance using Excel.

Fungal disease tests

Six replicated pots of six seeds per treatment (NLL genotypes) were used for each fungal disease experiment. Two experiments were conducted for anthracnose and root-rot disease, but only one experiment for brown spot disease due to limited seed availability. Fungi were standard isolates used by the Department of Agriculture and Food, Western Australia (DAFWA) (courtesy of Mr Geoff Thomas). The anthracnose isolate used was previously designated as *C. gloeosporioides* isolate 96A4 (IMI375715) VCG-2 (Yang and Sweetingham, 1998). *Pleiochaeta setosa* isolates for root-rot and brown spot disease inoculation were WHRS, EDRS, Meckering, Kellerberrin, Avondale and Goomalling. Procedures for the inoculum production, soil inoculation and disease inoculation generally followed the standard protocols of the National NLL Breeding Program at DAFWA (Yang and Sweetingham, 2002; Thomas and Sweetingham, 2003), with necessary adjustments as described below.

For the anthracnose test, seeds were sown into a washed river-sand (70%) and potting mix (30%) pot (150 mm) and grown in the CER at 18°C day/12°C night. Twenty-one days after sowing, the young plants were spray inoculated to run-off (aiming towards the stem growing point) with approximately 1 mL of a spore suspension of 10^5 spores per mL (containing Wettasoil® at 0.1 mL/L). Both experiments involved six pots with six plants per pot (36 plants). About 2 weeks after inoculation, seedling disease ratings were scored. Each seedling was given a score based on the most severe disease symptom occurring on the stem, including on the growing point, using a 0–5 scale (0 = healthy stem, no infection; 1 = upper stem distinctly bending or pinpoint lesion less than 1 mm diameter, no sporulation; 2 = lesion less than 5 mm diameter, no sporulation; 3 = lesion less than 5 mm diameter with sporulation; 4 = large lesion covering more than half the circumference of the stem with abundant sporulation; 5 = large lesion with abundant sporulation severing the stem, stem dead) as described by Thomas and Sweetingham (2003).

Conidia of *P. setosa* for root-rot trials, produced as described by Yang and Sweetingham (2002), were incorporated uniformly at a rate of 800 conidia per gram into a free draining potting medium of coarse river sand in a rotating cement mixer for 2 min. Each 140-mm white plastic pot had approximately 8.8×10^5 spores. Pots were placed in the CER at 15°C day/12°C night and watered from below overnight by placing them in a watering tray filled with deionized water. The seeds were then sown and the surface of each pot was evenly covered with a thin layer of white sand. The pots were not watered again for the duration of the experiment. Experiment 1 involved six pots with six plants per pot (36 plants) and Experiment 2 involved three pots with six plants per pot (18 plants). After 21 days, the sand media were eased away from the roots that were then washed for rating. The upper six 1 cm segments of the taproot of each plant were rated for the most severe root disease on a 0–5 scale (0 = healthy root; 1 = pinpoint lesions present; 2 = lesion starts affecting the root (about half diameter); 3 = root severely infected; dead root-coat still attached; 4 = root severely infected and root-stele exposed; 5 = root snapped and completely dead). An average of the six scores represented the root-rot score for the individual seedling.

Growth medium and CER conditions for the brown spot trial were the same as for the anthracnose test. Fourteen days after sowing, when the third and fourth leaves were fully expanded, the young plants were spray inoculated to completely wet (aiming at the surface of the four leaves) with approximately 0.7 mL of a *Pleiochaeta* spore suspension of 17 500 spores per mL (containing Wettasoil® at 0.1 mL/L), equal to over 3000 spores per leaf. The experiment involved six pots with six plants per pot and four leaflets per plant (36 plants; 144 leaflets). Disease severity was mea-

sured seven and 10 days after inoculation for each of the four inoculated leaves on each plant, following the 0–5 scale of Yang and Sweetingham (2002). The 0–5 scale was 0 = healthy leaf, with up to two spots on one or two leaflets; 1 = a few single spots on each leaflet; 2 = more single spots and one or two larger spots on each leaflet; 3 = more large spots on each leaflet; 4 = one or more leaflets had dropped; 5 = defoliated, all leaflets had dropped. An average of the four scores represented the brown spot score for the individual seedling.

Fungal disease data analyses

Disease measures were discrete values (0–5 scale) recorded on individual plants. However, the data for analysis were averaged values (six plants per pot for anthracnose; six 1 cm tap-root segment from six plants per pot for *Pleiochaeta* root rot; and four leaflets on six plants per pot for brown spot), and approximated metric data. The averaged values therefore were assumed suitable for analysis of variance (Dr Berwin A. Turlach and Dr Nazim Khan of School of Mathematics and Statistics UWA, personal communication). Statistical analyses for these disease severity scores (and lesion length) therefore were performed by analysis of variance using GenStat Release 8.2 Software (Harpenden, UK) (Rothamsted Experimental Station). Lupin line (treatment) means were compared according to the LSD or Duncan test at the 5% probability level.

A second method of analysis was used to test the validity of our conclusions. All data for two disease assays were tested by pair-wise comparison of each line using the Kolmogorov–Smirnov test that is non-parametric and distribution free. An on-line calculator for this test was accessed at http://www.physics.csbsju.edu/stats/KS-test.n.plot_form.html. Identical conclusions about significance of differences in data between lines were found by these analyses (data not shown).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Southern blot analysis of *p35*-NLL insertion events. (a) Schematic diagram of possible simple arrangements of p35 T-DNA sequences at one insert site. (b) Example of a Southern blot hybridization analysis of some *p35*-NLL lines.

Figure S2 Anthracnose disease symptoms on *p35*-NLL.

Figure S3 *Pleiochaeta* root rot disease symptoms on *p35*-NLL.

Figure S4 *Pleiochaeta* brown spot infection symptoms on *p35*-NLL.

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