Constitutive Expression of Pea Defense Gene DRR206 Confers Resistance to Blackleg (*Leptosphaeria maculans*) Disease in Transgenic Canola (*Brassica napus*)

Yaping Wang,¹ Goska Nowak,³ David Culley,² Lee A. Hadwiger,² and Brian Fristensky¹

¹Department of Plant Science, University of Manitoba, Winnipeg, R3T 2N2, Canada; ²Department of Plant Pathology, Washington State University, Pullman 99164-6430, U.S.A.; ³NRC Plant Biotechnology Institute, 110 Gymnasium Place, Saskatoon, SK S7N 0W9, Canada
Accepted 26 January 1999.

To identify genes effective against the blackleg fungus *Leptosphaeria maculans* (*Phoma lingam*), we have transformed canola (*Brassica napus*) with four pea (*Pisum sativum*) genes under constitutive control by the cauliflower mosaic virus 35S promoter: PR10.1, chitinase, DRR206, and defensin. Transgenic lines containing single-copy T-DNA insertions for each gene were screened for both seedling (cotyledonary) and adult plant resistance. Lines for which pea DRR206 mRNA was expressed showed decreased disease scores, compared with non-expressing transgenic lines. Transgenic plants expressing pea defensin showed a slight enhancement of resistance, while for PR10 and chitinase transgenics there was little or no enhancement of resistance. Resistance to *L. maculans* cosegregated with DRR206 transgenes. Extracts from DRR206 and defensin transgenic plants inhibited fungal germination *in vitro*. DRR206 transgenic plants also demonstrated decreased hyphal growth at inoculation sites. While the precise function of DRR206 remains to be determined, these results suggest that it does play an important role in defense against fungi.

Additional keywords: genetic engineering, pathogen.

The simplest means for genetic engineering of resistance to fungal diseases entails the constitutive expression of one or more defense proteins in transgenic plants. Defense proteins with clearly demonstrated antifungal activities have been effective in several cases. Broglie et al. (1991) have shown that constitutive expression of bean chitinase protects transgenic tobacco or canola seedlings from *Rhizoctonia solani*. Transgenic tobacco plants expressing PR1a showed decreased or delayed symptoms of both blue mold (*Peronospora tabacina*) and black shank (*Phytophthora parasitica*; Alexander et al. 1993). In potato, constitutive expression of osmotin delayed

the onset of symptoms of potato late blight (*Phytophthora infestans*; Liu et al. 1994). Terras et al. (1995) demonstrated that constitutive expression of radish defensin protects transgenic tobacco from the foliar pathogen *Alternaria longipes*. In Arabidopsis, constitutive expression of the toxic thionin viscotoxin A3 from mistletoe provided resistance to the clubroot pathogen *Plasmodiophora brassicae* (Holtorf et al. 1998). While no direct antifungal activity has been demonstrated for PR10, constitutive expression of pea PR10 in transgenic potato confers resistance to potato early drying disease (*Verticillium dahliae*; Chang et al. 1993). In a rare example in which an inducible gene was used, Hain et al. (1993) demonstrated that a genomic copy of the grape stilbene synthase gene confers resistance to *Botrytis cinerea* in transgenic tobacco.

In some cases, genes other than defense genes have been useful in plant protection. Constitutive expression of the fungal elicitor protein β -caryogein from *Phytophthora cryptogea* conferred resistance to *Phytophthora parasitica* in transgenic tobacco (Tepfer et al. 1998). In potato, constitutive expression of the fungal glucose oxidase gene, which generates H₂O₂ from glucose, resulted in resistance to both *Phytophthora infestans* and the bacterium *Erwinia carotovora* (Wu et al. 1995).

While such results are encouraging, there is no guarantee that a protein that was effective in one host against one pathogen will be effective in a different host against a different pathogen. For example, the same osmotin construct that conferred resistance to *P. infestans* in transgenic potato had no effect on *P. parasitica* in transgenic tobacco (Liu et al. 1994). Similarly, constitutively expressed potato PR10 had no effect on resistance to the late blight pathogen *Phytophthora infestans* (Constabel et al. 1993). We have therefore tested four pea genes for their effectiveness against the blackleg fungus, *Leptosphaeria maculans*, in transgenic canola (*Brassica napus*). Three of the genes, PR10.1, chitinase, and defensin, belong to classes of proteins that have previously demonstrated effectiveness in other plant/pathogen systems, as discussed above. The fourth gene, DRR206, has not previously been used in transgenic plant experiments, although this gene is strongly induced in pea in response to both fungal and bac-

Corresponding author: B. Fristensky; E-mail: frist@cc.umanitoba.ca

35S constructs and DRR206 antisera were made in the laboratory of Lee A. Hadwiger. Transformation of *Brassica napus* was performed by Goska Nowak. All other work was done by Yaping Wang and Brian Fristensky.

terial pathogens and elicitors (Riggleman et al. 1985; Fristensky et al. 1985; Daniels et al. 1987; Hadwiger et al. 1992; Daniels et al. 1988). We now demonstrate that constitutive expression of pea DRR206 can confer substantial resistance to blackleg, caused by *L. maculans*, in transgenic *B. napus*. Transgenics expressing defensin also showed limited resistance to blackleg. Neither PR10.1 nor chitinase had a significant impact on blackleg resistance, in parallel experiments.

RESULTS

Transformation of pea DNA constructs into *B. napus* and selection of single-copy lines.

Genomic or cDNA coding sequences for pea PR10 (DRR49) (Culley et al. 1995a), chitinase (Chang et al. 1995), DRR206 (GenBank AF115574), or defensin DRR230 (Chiang

and Hadwiger 1991) were cloned into T-DNA-based binary vector pBI121 downstream from the constitutive cauliflower mosaic virus (CaMV) 35S promoter, replacing the β -glucuronidase (GUS) gene. Constructs were directly transfected into *Agrobacterium tumefaciens*, and cotyledonary petioles were transformed by cocultivation, as described in Materials and Methods.

Because unlinked loci assort independently, genetic analysis of traits governed by more than one locus is complex. Also, previous studies have shown that multiple T-DNA insertions can lead to gene silencing (Flavell 1994; Taylor 1997). For these reasons, DNA of T₀ plants (i.e., the initial transformants), was screened by Southern blot hybridization to identify transformants bearing single T-DNA insertions. Transgene copy numbers ranged from 1 to 11 as estimated by DNA gel blot analysis (data not shown). The ratios of plants containing

Fig. 1. Disease scores and transgene mRNA expression in T₁ transformed lines. For each gene (patterned bars), an independent control was done with untransformed cv. Westar (white bars). Each bar represents the average of 20 plants inoculated with *Leptosphaeria maculans* PG2 plus or minus the standard error of the mean. Disease scores are shown for both cotyledons and adult leaves. Transgene mRNA expression was assayed by hybridization to Northern (RNA) blots of RNA extracted from pooled tissue samples of five of the 20 plants scored in the disease assays. For each pea gene indicated at top, bands hybridizing with the corresponding pea probe are shown. To verify equal loading between lanes, total RNA in gels was visualized by ethidium bromide staining. For transgenic lines GN3-4#22 and GN4-2#15, data for T₂ plants are also included.

single-copy inserts to the total number of transgenic lines were as follows: PR10.1, 10/28; chitinase, 3/22; DRR206, 7/28; defensin, 2/24. Seeds were collected separately from each T₀ individual, and these seeds constituted the T₁ generation. All further work was carried out on single-copy lines.

Disease resistance is correlated with strong expression of pea DRR206 and defensin.

Progeny from individual T₀ plants (i.e., the T₁ generation) were tested for blackleg resistance in cotyledons and in adult stems, and for expression of mRNA for pea defense genes. For each T₀ parent, leaf tissue from five T₁ plants was pooled and expression of transgenes assayed by RNA gel blot analysis. In parallel experiments, 20 8-day seedlings were grown for each line, and cotyledons inoculated with compatible blackleg isolate PG2. After being scored at 13 days post inoculation, cotyledons were removed to prevent colonization of stems. For the cotyledon assay, plants showing no disease symptoms at all were excluded from scoring to control for the possibility of escapes. Also, two of four lobes were inoculated per cotyledon, and the lobe showing the highest disease score was used for scoring. Cotyledon assay scores must therefore be considered conservatively high. At the 5- to 6-leaf stage, stems of the same plants were inoculated with *L. maculans*, and symptoms scored 5 weeks after inoculation. The results of this experiment are presented in Figure 1 and Table 1.

Transgenic lines containing PR10.1 exhibited disease scores similar to those of the Westar controls in cotyledon and adult plant assays, while mRNA levels for this gene ranged from

high to undetectable. Among three independently transformed lines containing chitinase constructs, GN2-2#13 and GN-2-2#31 most strongly expressed chitinase mRNA. These lines showed only a small decrease in adult plant disease. The strongest effect was seen with DRR206. Five lines exhibiting DRR206 expression (GN3-1#5, GN3-4#22, GN3-5#23, GN3-5#24, and GN3-5#26) showed significantly lower disease scores in adult plant assays, compared with lines with low or no detectable expression (Table 1). GN3-1#5 and GN3-4#22 also had lower cotyledon disease scores. Finally, of two lines carrying pea defensin, the one line that exhibited mRNA expression for this gene (GN4-2#15) had significantly lower disease scores in adult and cotyledon assays (Table 1).

Verification of transgene effects in T₂ and T₃ plants.

Transgenic lines with the lowest adult plant disease scores, GN3-4#22 (DRR206) and GN4-2#15 (defensin), were chosen for further study. T₂ plants for these lines expressed DRR206 and defensin mRNAs (Fig. 1), as well as blackleg resistance. For DRR206 line GN3-4#22, T₂ cotyledon scores were significantly lower than those seen in the T₁ generation (Table 1). One possible explanation is that the T₁ generation represented the genetic variability of 20 T₁ individuals, while T₂ scores resulted from only three T₁ parents. However, for defensin line GN4-2#15, disease scores were similar to those seen in the T₁ generation (Table 1).

The effects of the pea DRR206 and defensin on blackleg resistance in transgenic *B. napus* are shown in Figure 2. On untransformed Westar cotyledons, brown lesions with black pycnidia are evident (Fig. 2A). In T₂ plants bearing the defensin gene, browning is limited to a smaller region near the inoculation site, and no pycnidia are in evidence. In T₂ DRR206 transgenic plants, little browning is seen outside of the site of inoculation. Adult plant resistance phenotypes for T₁ plants are shown in Figure 2B. Stem-inoculated, untransformed Westar plants die after blackleg inoculation, exhibiting almost complete girdling of the stem. Stem lesions are reduced in defensin transformants, and plants remain viable. Stem lesions are not evident on DRR206 transformants.

To more precisely characterize resistance in DRR206 line GN3-4#22, T₃ offspring from six T₂ parents homozygous for DRR206 were scored (Table 2). T₃ plants carrying DRR206 exhibited much less severe symptoms than Westar controls in both cotyledon and adult plant assays. These results indicate that resistance in this line is stable to at least the T₃ generation.

To determine whether resistance cosegregated with pea DRR206 or defensin, seeds from a single T₁ selfed parent from each line were grown, and these T₂ plants were screened by polymerase chain reaction (PCR) with primers specific for pea DRR206 or defensin. T₂ progeny segregated 3:1 (presence:absence of the gene) based on PCR results. T₂ segregants testing positive for DRR206 uniformly exhibited significantly improved resistance to *L. maculans* (25 progeny, cotyledon: 2.6 ± 0.5, adult: 1.4 ± 0.6) when compared with null segregants (i.e., no DRR206 band, 8 progeny, cotyledon: 8.6 ± 0.5; adult: 4.5 ± 0.2). In contrast, T₂ segregants testing positive for defensin (24 progeny, cotyledon: 7.2 ± 0.5, adult: 3.4 ± 0.9) showed little difference in disease scores from null segregants (8 progeny, cotyledon: 8.5 ± 0.6; adult: 4.6 ± 0.2). Cosegregation of the DRR206 PCR band and low adult and cotyledon resistance scores helps to rule out the

Table 1. Summary of data from T₁ and T₂ generations^x

Brassica napus line	Gene	RNA ^y	Disease scores ^z	
			Cotyledon	Adult plant
Westar	—	—	9.0 ± 0.0 a	4.8 ± 0.2 a
GN1-2#4	PR10.1	—	8.8 ± 0.3 a	4.6 ± 0.1 a
GN1-4#51-1	PR10.1	+	8.9 ± 0.2 a	4.7 ± 0.2 a
GN1-5#18	PR10.1	+	8.8 ± 0.4 a	4.4 ± 0.1 a
GN1-5#22	PR10.1	+	8.8 ± 0.3 a	4.2 ± 0.5 a
GN1-5#53-1	PR10.1	+	8.9 ± 0.1 a	4.6 ± 0.2 a
GN1-5#54-1	PR10.1	+	8.7 ± 0.4 a	4.8 ± 0.1 a
GN1-5#54-2	PR10.1	+	8.8 ± 0.3 a	4.6 ± 0.2 a
GN2-4#32-2	Chitinase	—	8.9 ± 0.4 a	4.9 ± 0.4 a
GN2-2#13	Chitinase	+	8.9 ± 0.1 a	3.8 ± 0.8 ab
GN2-2#31	Chitinase	+	8.6 ± 0.2 a	3.6 ± 0.9 ab
GN3-2#11-1	DRR206	—	8.9 ± 0.4 a	4.5 ± 0.3 a
GN3-2#11-2	DRR206	—	8.8 ± 0.2 a	4.8 ± 0.2 a
GN3-5#26	DRR206	+	8.8 ± 0.1 a	3.4 ± 1.1 b
GN3-5#23	DRR206	+	8.6 ± 0.1 a	3.0 ± 1.2 bc
GN3-1#5	DRR206	+	7.8 ± 0.5 b	2.9 ± 1.3 bc
GN3-5#24	DRR206	+	8.4 ± 0.2 a	2.6 ± 1.3 c
GN3-4#22	DRR206	+	7.9 ± 0.5 b	2.5 ± 1.4 c
GN3-4#22 (T ₂)	DRR206	+	2.5 ± 0.3 c	1.4 ± 0.4 d
GN4-1#12	Defensin	—	9.0 ± 0.1 a	4.7 ± 0.1 a
GN4-2#15	Defensin	+	7.9 ± 0.1 b	3.5 ± 1.0 b
GN4-2#15 (T ₂)	Defensin	+	7.4 ± 0.4 b	3.4 ± 0.5 b

^x All plants are T₁ transformants, except where indicated in parentheses.

^y +, mRNA expressed; —, no detectable mRNA expression.

^z Means ± SE (standard error of the mean). For the T₁ generation, 20 progeny were tested per T₀ parent. For the T₂ generation, 25 T₂ plants pooled from three T₁ parents were inoculated with *Leptosphaeria maculans* and scored for GN3-4#22 (three replicates, for a total of 75 plants) and GN4-2#15 (two replicates for a total of 50 plants). Scores with the same letter are not significantly different at *P* = 0.05, according to Duncan's multiple range test. For each line, a separate Westar check of at least five plants was done. Westar scores were pooled.

possibility that observed resistance is an artifact due to somaclonal variation.

The growth of *L. maculans* in cotyledons was compared among resistant T₂ plants bearing defensin or DRR206 genes, and untransformed Westar plants. As shown in Figure 2, Westar and defensin transgenics both exhibit substantial intercellular hyphal growth at the site of inoculation. DRR206 transgenic plants show a marked reduction in the density of hyphae visible in tissue.

To further investigate the inhibition of fungal growth, extracts of water-soluble components from leaves of Westar or

T₂ plants transgenic for either defensin or DRR206 were inoculated with *L. maculans* PG2. After incubation for 30 h, extracts from either defensin or DRR206 transgenic plants showed significant decreases in germination, compared with extracts from untransformed Westar plants (Table 3).

The expression of the DRR206 protein was verified by immunoblots (Fig. 3) with polyclonal antibodies from rabbits injected with DRR206 expressed in *Escherichia coli* (Culley et al. 1995b; Allaire 1993). As a positive control, proteins were extracted from pea pods 48 h after inoculation with *Fusarium solani* f. sp. *phaseoli*. Under these conditions, pea

Fig. 2. Infection phenotypes of transgenic (T₂) and untransformed canola inoculated with *Leptosphaeria maculans* PG2. Left to right: Untransformed Westar; GN4-2#15; GN3-4#22. **A**, Cotyledons 12 days after pinprick inoculation. **B**, Close-ups of adult plants 30 days after stem inoculation. **C**, Hyphal growth at inoculation sites in cotyledons 8 days after pinprick inoculation. Bar equals 20 μ m.

expresses a basic resistance to *F. solani* f. sp. *phaseoli*, along with strong induction of defense transcripts, including DRR206 mRNA (Fristensky et al. 1985). In pea tissue, two major bands at apparent molecular mass classes 25 and 22 kDa, along with a minor band at 28 kDa, were detected. The molecular mass of the DRR206 polypeptide, inferred from the complete sequence of the genomic clone, is 20.4 kDa, while the estimated molecular mass of the purified protein from a previous paper was 23 kDa (Culley et al. 1995b). The 28-kDa band was also faintly visible in proteins from untransformed Westar and is therefore presumably an unrelated protein that also cross-reacts with the antisera. Neither the 25 nor 22 kDa proteins were visible in Westar-derived protein extracts. Proteins from T₁ and T₂ DRR206 transgenic plants exhibited bands that comigrated with the 25 and 22 kDa bands from pea.

One possible explanation for the presence of two bands in pea and transgenic *B. napus* is that DRR206 undergoes post-translational modifications. Alternatively, Culley et al. (1995b) found that at least five bands in pea genomic DNA hybridize with pea DRR206, indicating that DRR206 is a multigene family in pea. In pea, the presence of two or three distinct DRR206 protein bands could indicate that different DRR206 family members encode DRR206 protein variants of different molecular masses. However, these bands were also seen in transgenic *B. napus*, even though only one pea DRR206 gene was present in the transgenic lines. Since the 22 and 25 kDa bands were not seen in untransformed *B. napus*, we can rule out the possibility that they were products of *B. napus* DRR206 genes. Finally, screening of *B. napus* Southern blots and a genomic library failed to detect any sequences that cross-hybridize with the pea probe (data not shown). In light of these observations, if *B. napus* DRR206 genes do exist, they must be substantially diverged from those

in pea. Therefore, it is unlikely that antisera specific for pea DRR206 would cross-react with putative *B. napus* DRR206 proteins.

DISCUSSION

For a single, constitutively expressed foreign gene to confer disease resistance to a susceptible plant, there are three broad categories of possible mechanisms. Firstly, the gene product itself, or an enzymatic product produced by it, might be directly active against the pathogen. Secondly, the gene could influence the expression of resistance in the host, either through direct (epistatic) effects on defense regulatory pathways, or as an indirect (pleiotropic) effect leading to the induction of defense pathways. Lastly, the gene product could provide some function that is compatible with the host's defenses, but is normally lacking in the incompatible interaction.

Neither pea PR10 nor basic chitinase appears to meet any of these criteria in the interaction of transgenic *B. napus* with *L. maculans*. In transgenic potatoes containing the same pea PR10 gene used in this paper, tubers inoculated with *V. dahliae* showed an almost 50% reduction in lesion size, compared with untransformed controls (Chang et al. 1993). In *V. dahliae*-infested soil, PR10 transgenic potatoes showed a decrease in wilting, accompanied by about a twofold increase in tuber yield, compared with untransformed controls. Since our experiments employ a different host/pathogen system, there may be several reasons why PR10 did not enhance resistance to blackleg.

The pea chitinase (Pissa;Chia1;2) used in this paper is a PR3 endochitinase in the same subfamily as the bean chitinase (Phavu;Chia1;3) used by Broglie et al. (1991). These proteins share 87% amino acid identity. Constitutive expression of the

Table 2. Disease scores in DRR206 T₃ transgenic progeny

<i>Brassica napus</i> line	Disease score ^z		
	Cotyledon		Adult plant
	9 dpi	13 dpi	30 dpi
GN3-4#22 T3	1.23 ± 0.49	3.50 ± 0.77	1.29 ± 0.46
Westar	6.93 ± 0.26	9.00 ± 0.00	4.97 ± 0.11

^z GN3-4#22 T₂ plants were screened by polymerase chain reaction with DRR206-specific primers, as described in Materials and Methods. Seeds (i.e., T₃) were collected from six T₂ progeny testing positive for pea DRR206, and were scored in cotyledon and adult assays. Scores were pooled from three experiments, for a total of 323 plants, from all six parents. Westar checks were from a total of 55 plants taken from three separate inoculations. dpi = days post inoculation.

Table 3. Percent germination of *Leptosphaeria maculans* pycnidiospores 30 h after treatment with water-soluble leaf extracts from transgenic or untransformed *Brassica napus* leaves

<i>B. napus</i> line	Germination (%) ^z
Westar	30.9 ± 1.2 a
GN4-2#15 (Defensin)	15.6 ± 1.5 b
GN3-4#22 (DRR206)	5.8 ± 1.0 c

^z Plus or minus standard error of the mean. Results are from four replicates, each with water-soluble leaf extracts prepared as described in Materials and Methods from at least five plants per line. Four grids were counted per replicate. Letters denote measurements that are significantly different at *P* = 0.05 according to Duncan's multiple range test.

Fig. 3. Immunoblot of proteins from *Brassica napus* transgenic line GN3-4#22 T₁ and T₂ plants with polyclonal antibodies for pea DRR206. Proteins from pea pods inoculated with *Fusarium solani* f. sp. *phaseoli* and from *B. napus* cv. Westar serve as positive and negative controls, respectively. Estimated molecular masses of major bands are indicated in kilodaltons.

bean chitinase decreased seedling mortality in both transgenic tobacco and *B. napus* cv. Westar to *Rhizoctonia solani* (Broglie et al. 1991). In our work, the same cultivar of *B. napus* was used. The simplest explanation therefore is that *L. maculans* is less sensitive than *R. solani* to suppression by PR3 type chitinases.

While the strongest enhancement of resistance to blackleg was seen in DRR206 transgenic lines, the function of this gene remains unknown. In pea, DRR206 is induced strongly by an incompatible race of *F. solani*, and more weakly by a compatible race (Fristensky et al. 1985). Purified DNase from *F. solani* also induces this gene (Hadwiger et al. 1995). With compatible isolates of *P. syringae*, an early induction of DRR206 is seen, which subsides after 12 h post inoculation. In gene-specific incompatible interactions with *P. syringae*, DRR206 is induced early and expression remains strong for at least 30 h post inoculation (Daniels et al. 1987). In *F. oxysporum*-infested soil, DRR206 is strongly expressed in resistant isolate Vantage, and only weakly induced in susceptible isolate M410 (Hadwiger et al. 1992).

Despite the strong difference in expression seen with DRR206 in resistant, compared with susceptible, interactions in pea, no homologues of this gene have yet been cloned in studies of other plant/pathogen interactions. Therefore, no clues to its function can be found from other systems.

Our results do show that DRR206 transgenic plants produce water-soluble antifungal activities (Table 3). Decreased hyphal growth is also seen at infection sites (Fig. 2C). Resistance to blackleg can occur in both cotyledons (Fig. 2A) and adult stems (Fig. 2B), indicating that DRR206-mediated resistance is not limited to a single developmental stage or tissue. The almost complete lack of symptoms beyond the site of pinprick inoculation in cotyledons indicates that DRR206 must act early in the infection process (e.g., inhibiting spore germination or hyphal growth) rather than later (e.g., inhibiting subsequent colonization). Regardless of the precise mechanism of its action, the ability of DRR206 to confer blackleg resistance in transgenic canola substantiates a role for this gene in plant defense.

Pea defensin is less effective than DRR206 in conferring resistance to *L. maculans*. Extracts from defensin-transgenic plants inhibit spore germination and growth. This is consistent with previous reports showing that radish defensin can cause hyperbranching and decreased growth of *Alternaria longipes* in vitro (Terras et al. 1995). Inhibition of fungal growth or germination by extracts from defensin transgenic plants is less pronounced than that seen with DRR206 (Table 3). This is consistent with the fact that DRR206 transgenics also showed lower disease scores (Table 1, Fig. 1). In the cotyledon assay, defensin plants allowed infection to spread to a wider area than in DRR206 plants. One potentially important difference between defensin transgenics and the Westar control was the absence of pycnidia in lesions of defensin plants (Fig. 2A). Defensin transgenics also exhibited smaller stem lesions, compared with the Westar control (Fig. 2B), but allowed a similar degree of hyphal growth in cotyledons (Fig. 2C). Finally, defensin transgenics did not exhibit the kind of chlorosis seen in DRR206 transgenics. Taken together, these comparisons suggest that the resistance mediated by DRR206 is probably qualitatively different from defensin-mediated resistance.

MATERIALS AND METHODS

Transgenic canola plants.

35S constructs were made by replacing the GUS protein coding sequence from pBI121 (Clontech, Palo Alto, CA) with pea defense cDNA or genomic sequences. Inserts were excised with restriction enzymes compatible with recipient sites in pBI121, or, where needed, restriction sites were blunt-ended with *E. coli* DNA polymerase I Klenow fragment. The 712-bp pea PR10.1 (pI49) cDNA (GenBank X13383:23..734; Fristensky et al. 1985) containing 43 bp of 5' leader and the entire 3' untranslated sequence was cloned as a blunt-ended fragment between the *Xba*I and *Sac*I sites of pBI121 to give plasmid pDC49-4. A 1,035-bp *Bsm*AI/*Hinc*II fragment containing the protein coding sequence from the genomic pea chitinase (L37876:265..1299; Chang et al. 1995) was recloned from pBluescript between the *Xba*I and *Sac*I sites of pBI121 to give pDC-CHIT-26. The 801-bp DRR206.3 *Dde*I/*Ssp*I genomic fragment containing the protein sequence and terminator (AF1155574:1025..1825) was recloned between the *Xba*I and *Sac*I sites of pBI121 to give pDC206-13. The defensin 392-bp fragment pI230 cDNA (L01578:77..468; Chiang et al. 1991), beginning 1 bp upstream from the start codon and containing the 3' untranslated region, was recloned between the *Sma*I and *Sac*I sites of pBI121 to give pDC230-12. 35S-defense gene plasmid constructs were transfected into *A. tumefaciens* MP90 by the freeze-thaw method (Holsters et al. 1978). Transformation of *B. napus* cv. Westar was carried out by cocultivation of transfected *A. tumefaciens* with cotyledonary petioles and regeneration of plantlets as described by Moloney et al. (1989). Seeds from Westar and from transformed plants were grown in Metro-mix in a growth chamber with a day/night regime of 22/15°C and 16/8 h photoperiod.

PR10, chitinase, DRR206, and defensin constructs were used in experiments GN1, GN2, GN3, and GN4, respectively (Fig. 1). The names for transgenic lines are illustrated with GN3-2#11-1 as an example. GN3 indicates the experiments in which the DRR206 constructs were used, GN3-2 represents a specific transformation procedure on a single day, GN3-2#11 refers to a specific cotyledonary petiole-explant, and GN3-2#11-1 is one of two plantlets regenerated from non-contiguous sectors of that explant, and is therefore the product of a unique transformation event.

Estimation of transgene copy number.

DNA extraction was the same as described for PCR (Dellaporta et al. 1983) with an additional two phenol (pH 8.0) extractions and RNase treatment for 30 min. Samples (15 µg) of genomic DNA were restricted with *Hind*III and *Sst*I (PR10.1) or *Hind*III and *Eco*RI (chitinase, defensin, and DRR206). The UV cross-linked Zeta probe blotting membrane was hybridized with ³²P-labeled, random-primed probe in 0.5 M Na₂HPO₄ (pH 7.2) and 7% sodium dodecyl sulfate (SDS) at 65°C. A 0.5-kb fragment containing the *Npt*II gene from pBI121 was used as a probe. The region encompassed by this probe does not contain sites for the enzymes used, but all enzymes are present in the T-DNA multiple cloning site. Thus, each band should be derived from one site internal to the T-DNA, and one distal site near the point of genomic insertion. Filters were washed twice with 40 mM Na₂HPO₄ (pH 7.2) and 5% SDS at 65°C for 30 min, and twice with 40 mM Na₂HPO₄

(pH 7.2) and 1% SDS at 65°C for 30 min according to the manual from Bio-Rad Laboratories (Hercules, CA). T₀ plants showing a single band in Southern blots were considered to have single T-DNA insertions.

Inoculum.

Pycnidiospores of *L. maculans*, pathogenicity group 2 (PG2) from isolate 86-12, (R. Rimmer, University of Manitoba) were initially obtained from infected cotyledons, according to the modified method from Williams (1985). Diseased tissue was surface sterilized in 2% sodium hypochlorite for 5 min, and then placed on V8 juice agar medium containing 0.75% CaCO₃, 0.1% streptomycin sulfate, and 0.04% rose bengal. The plates were incubated under constant light at 22 to 24°C for 1 week until sporulation. Agar strips containing the infected tissue were transferred to fresh V8 plates and incubated for another week to maximize sporulation. Spores were collected by scraping the surface of a plate with a sterile glass slide to release pycnidiospores in 6 ml of sterile, distilled water. This suspension was then filtered through sterile Miracloth (Miracloth-Novabiochem Corporation, La Jolla CA) and centrifuged. The spore suspension was stored in a -80°C freezer. The spore concentration as adjusted to 2×10^7 spores per ml for inoculation.

Plant inoculation.

Cotyledon inoculation. Cotyledons of 8-day-old seedlings grown as described above were wounded with a syringe needle and 10 µl of the pycnidiospore suspension was dropped onto the lesion. After the drops were dried for 4 h under light at 24°C, the plants were moved back to the growth chamber. Disease scores were evaluated at 9 and 13 days post inoculation according to Williams and Delwiche (1980), on a scale in which 0 = no symptoms, 1 = necrosis around wounding site (0.5 to 1.5 mm lesion, faint chlorotic halo around lesion), 3 = dark, necrotic lesion (1.5 to 3.0 mm; chlorotic halo may be present around lesion), 5 = lesions 3 to 6 mm delimited by dark necrotic margins (may show gray-green tissue collapse), 7 = 3 to 5 mm lesions, gray-green tissue collapse, sharply delineated, nondarkened margin, 9 = gray lesion > 5 mm with diffuse margins (black pycnidia evident throughout lesion). For microscopy (Fig. 2C) cotyledons from Westar or transgenic plants were injected by syringe with the spore suspension and the samples were collected post inoculation each day for 8 days. Tissue was fixed in glacial acetic acid and absolute ethanol (1:2, vol/vol). The fixed solution was changed twice daily for 3 days. Inoculation sites without staining were examined by light microscopy for the presence of hyphae (Fig. 2C).

Adult plant inoculation. The lower portion of the stem (close to the third node), was inoculated at the 5- to 6-leaf stage. Ten microliters of pycnidiospore suspension was injected into the stem and the plants were kept in a humid environment for 24 h. The 0 to 5 disease rating scale is based on lesion length (L) and area of stem cross section showing necrosis (W) as follows: 0, no infection; 1, L < 10 mm, W < 25%; 2, L = 10 to 19 mm, W = 25 to 50%; 3, L = 20 to 29 mm, W = 51 to 75%; 4, L ≥ 30 mm, W ≥ 76 to 100%; 5, plant dead. This protocol was modified from Cargeeg and Thurling (1980).

Bioassays for antifungal activity. Uninoculated young leaves from the 5-true-leaf stage of *B. napus* cv. Westar or

transgenic lines carrying DRR206 or defensin, grown as described above, were ground in liquid N₂ and 1 ml of sterile, distilled water was added per gram of fresh weight. Insoluble components were removed by centrifugation at $4,000 \times g$ at 4°C for 20 min. Supernatant was filtered through a 0.2-µm cellulose acetate filter. *L. maculans* pycnidiospores were added to the extract to a concentration of 1×10^6 spores per ml (approximately 80 ml per 250-ml flask) and incubated on a shaker (Junior Orbit Shaker; Lab-Line Instruments, Melrose Park, IL) at 120 rpm at room temperature for 30 h. Germination was quantified with a hemacytometer, with four replicate grids per extract. Three hundred pycnidiospores were counted in each grid. Four replicate extracts from five or more plants per line were assayed.

Experimental design and data analysis for disease resistance evaluation.

As listed in Table 1, 19 T₁ independently transformed lines were tested, which included seven lines with PR10.1, three lines with chitinase, seven lines with DRR206, and two lines with defensin. Twenty progeny from each T₀ transformant were assayed by Northern blot and at both cotyledon and adult plant stages, as described above. For each of the four constructs, an independent check was done with at least 5 Westar plants. For T₂ plants listed in Table 1, seed was planted from three T₁ parents, and seedlings were inoculated with *L. maculans* and scored at both cotyledon and adult stages. For GN3-4#22, three replicates of 25 plants each, for a total of 75 plants, were scored. For GN4-2#15, two replicates of 25 plants, for a total of 50 plants, were scored. For the T₃ generation (Table 2), T₂ progeny from 13 T₁ individuals derived from DRR206 transgene GN3-4#22 were scored for cotyledon and adult plant resistance. For seven of the 13 lines with the lowest disease scores, 25 T₂ progeny from the same T₁ parents were screened by PCR for the presence of DRR206 to identify DRR206 homozygotes (i.e., plants not segregating for the PCR band). Six of the seven lines appeared to be homozygous by these criteria. For each of the homozygous lines, a single T₂ individual was chosen and T₃ progeny from these plants were tested for blackleg resistance in adult and cotyledon assays. Scores for a total of 323 plants from these six lines were pooled. Significant differences between means were determined by Duncan's multiple range test.

Identification of transgenic plants by PCR.

DNA extraction was performed according to a modified protocol from Dellaporta et al. (1983). Leaves (0.5 g) were added to 500 µl of extraction buffer (100 mM Tris-HCl [pH 8.0], 50 mM EDTA, 500 mM NaCl, 1.25% SDS) and incubated for 20 min at 65°C. Next, potassium acetate was added to a final concentration of 1 M. The solution was kept on ice for 20 min and then extracted with chloroform:isoamyl alcohol (24:1), followed by ethanol precipitation. For the PCRs, a mixture contained 1.5 mM MgCl₂, 0.2 mM dNTP, a 0.5 µM concentration of the specific primer mix, and 2.5 U of *Taq* DNA polymerase. Gene-specific primers were as follows. For DRR206: oC206+1 (5'aattccaacaagagaaagcc3') and oC206-2 (5'cttgatataaacaccaagtcg3'); for defensin: oS39b+3 (5'caagaaatagtggtgagtga3') and oS39b-4 (5'gcgacaaccagctgattttg3'). PCR cycles were as follows. Cycle 1: 94°C for 3 min, 55°C for 45 s, and 72°C for 1 min; cycles 2 to 29: 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min.

RNA extraction and Northern blotting.

RNA was extracted by a procedure modified from Logemann et al. (1987). Leaves (0.5 g) were added to a tube containing 750 µl of extraction buffer (4 M guanidine isothiocyanate, 0.1 M Tris-HCl, pH 7.5, 10 mM EDTA, 1% methcapto-ethanol), mixed well, and centrifuged for 20 min at 4°C. The supernatant was extracted with phenol and chloroform: isoamyl alcohol (24:1) three times. RNA was precipitated with 0.7 volume of cold 95% ethanol and 0.2 volumes 1 M acetic acid at -70°C for 2 h, then washed with 3 M sodium acetate (pH 4.8) and 70% ethanol. Dried RNA was resuspended in diethylpyrocarbonate (DEPC)-treated, sterile, distilled water, and 20-µg samples were denatured and electrophoresed on 1.2% agarose 1× morpholinepropanesulfonic acid (MOPS) 2.2 M formaldehyde gels as described in Ausubel et al. (1998). Hybridization and washing were done by the same procedure as that for DNA blots, as described above.

Protein extraction and Western blotting.

Three grams of uninfected leaves was ground in liquid N₂ and extracted with 6 ml of extraction buffer (50 mM Tris-HCl [pH 7.5], 1 mM phenylmethylsulfonyl fluoride, 2 mM thiourea, and 1.5% [wt/vol] polyvinylpyrrolidone) for 2 h at 4°C under continuous stirring. Following centrifugation, (NH₄)₂SO₄ was added to the supernatant to a 75% saturation. Proteins were precipitated overnight, then dialyzed against 5 mM Tris-HCl buffer, and lyophilized (Terras et al. 1992). The crude proteins from DRR206 transformants, from untransformed Westar, and from pea pods at 48 h post inoculation were run through 12% polyacrylamide Tricine-SDS-polyacrylamide gels with Bio-Rad low molecular mass markers (66.0 to 14.2 kDa). One gel was stained in 0.25% Coomassie brilliant blue R 250 in 45% methanol and 10% acetic acid, and then de-stained in 7.5% acetic acid and 10% methanol (Schagger and Gebhard 1987). A duplicate gel was electroblotted onto nitrocellulose membrane and detected by the Immun-Blot colorimetric assay kit (GAR-AP; Bio-Rad).

ACKNOWLEDGMENTS

This work was supported by Research Grant OGP0105628 from the Natural Sciences and Engineering Research Council of Canada, the Western Grains Research Foundation, and the NSERC/AAFC Research Partnership Program Grants 695-023-93 and 661-01996. Thanks to Pei-jun Zhang for early screening of transgenics, to Joe Hammerlindl and Wilf Keller for helpful guidance on transformation procedures, and to Roger Rimmer and Mario Keri for helpful comments. Special thanks to Kari-Lynne McGowan for assistance in lab and growth room work.

LITERATURE CITED

Alexander, D., Goodman, R. M., Gut-Rella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox, D., Ahl-Goy, P., Luntz, T., Ward, E., and Ryals, J. 1993. Increased resistance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. *Proc. Natl. Acad. Sci. USA* 90:7327-7331.

Allaire, B. S. 1993. Antisera development against and localization of pea disease resistance response proteins. M.Sc. thesis. Washington State University, Pullman, WA.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. 1998. *Current Protocols in Molecular Biology*. John Wiley & Sons, New York.

Brogie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C. J., and Brogie, R. 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science*

254:1194-1197.

Cargeeg, L. A., and Thurling, N. 1980. Seedling and adult plant resistance to blackleg (*Leptosphaeria maculans* (Desm.) Ces et de Not.) in spring rape *Brassica napus* L.. *Aust. J. Agric. Res.* 31:37-46.

Chang, M.-M., Chiang, C. C., Martin, W. M., and Hadwiger, L. A. 1993. Expression of a pea disease resistance response gene in the potato cultivar Shepody. *Am. Potato J.* 70:635-647.

Chang, M.-M., Horovitz, D., Culley, D., and Hadwiger, L. A. 1995. Molecular cloning and characterization of a pea chitinase gene expressed in response to wounding, fungal infection and the elicitor chitosan. *Plant Mol. Biol.* 28:105-111.

Chiang, C. C., and Hadwiger, L. A. 1991. The *Fusarium solani*-induced expression of a pea gene family encoding high cysteine content proteins. *Mol. Plant-Microbe Interact.* 4:324-331.

Constabel, C. P., Bertrand, C., and Brisson, N. 1993. Transgenic potato plants overexpressing the pathogenesis-related STH-2 gene show unaltered susceptibility to *Phytophthora infestans* and potato virus X. *Plant Mol. Biol.* 22:775-782.

Culley, D., Brown, S., Parsons, M. A., Hadwiger, L. A., and Fristensky, B. 1995a. Cloning and sequencing of disease resistance response gene Drr49 Ypr10.PS.1; pI49. from *Pisum sativum*. *Plant Physiol.* 109:772.

Culley, D. E., Horovitz, D., and Hadwiger, L. A. 1995b. Molecular characterization of disease-resistance response gene DRR206-d from (*Pisum sativum* L.). *Plant Physiol.* 107:301-302.

Daniels, C., Fristensky, B., Wagoner, W., and Hadwiger, L. A. 1987. Pea genes associated with non-host resistance to fungi are also active in race-specific resistance to bacteria. *Plant Mol. Biol.* 8:309-316.

Daniels, C. H., Cody, Y. S., and Hadwiger, L. A. 1988. Host responses in peas to challenge by wall components of *Pseudomonas syringae* pv. *pisi* races 1, 2, and 3. *Phytopathology* 78:1451-1453.

Dellaporta, S. L., Wood, J., and Hicks, J. B. 1983. A plant DNA mini-preparation. *Plant Mol. Biol. Rep.* 1:19-21.

Flavell, B. R. 1994. Inactivation of gene expression in plants as a consequence of specific sequence duplication. *Proc. Natl. Acad. Sci. USA* 91:3490-3496.

Fristensky, B., Riggleman, C. R., Wagoner, W., and Hadwiger, L. A. 1985. Gene expression in susceptible and disease resistant interactions of peas induced with *Fusarium solani* pathogens and chitosan. *Physiol. Plant Pathol.* 27:15-28.

Hadwiger, L. A., Chang, M.-M., and Parsons, M. A. 1995. *Fusarium solani* DNase is a signal for increasing expression of nonhost disease resistance response genes, hypersensitivity, and pisatin production. *Mol. Plant-Microbe Interact.* 8:871-879.

Hadwiger, L. A., Chiang, C. C., and Horovitz, D. 1992. Expression of disease resistance response genes in near-isogenic pea cultivars following challenge by *Fusarium oxysporum* race 1. *Physiol. Mol. Plant Pathol.* 40:259-269.

Hain, R., Reif, H.-J., Krause, E., Langebartels, R., Kindl, H., Vornam, B., Wiese, W., Schmelzer, E., Schreier, P. H., Stöcker, R. H., and Stenzel, K. 1993. Disease resistance results from foreign phytoalexin expression in a novel plant. *Nature* 361:153-156.

Holsters, M., de Waele, D., Depicher, A., Messens, E., van Montagu, M., and Schell, J. 1978. Transfection and transformation of *Agrobacterium tumefaciens*. *Mol. Gen. Genet.* 163:181-187.

Holtorf, S., Ludwig-Müller, J., Apel, K., and Bohlmann, H. 1998. High-level expression of a viscotoxin in *Arabidopsis thaliana* gives enhanced resistance against *Plasmidiophora brassicae*. *Plant Mol. Biol.* 36:673-680.

Liu, D., Raghothama, K. G., Hasegawa, P. M., and Bressan, R. 1994. Osmotin overexpression in potato delays development of disease symptoms. *Proc. Natl. Acad. Sci. USA* 91:1888-1892.

Logemann, J., Schell, J., and Willmitzer, L. 1987. Improved method for the isolation of RNA from plant tissues. *Anal. Biochem.* 163:16-20.

Moloney, M. M., Walker, M. J., and Sharma, K. K. 1989. High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. *Plant Cell Rep.* 8:238-242.

Riggleman, R. C., Fristensky, B., and Hadwiger, L. A. 1985. The disease resistance response in pea is associated with increased levels of specific mRNAs. *Plant Mol. Biol.* 4:81-86.

Schagger, H., and Gebhard, V. J. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166:368-379.

Taylor, B. C. 1997. Comprehending cosuppression. *Plant Cell* 9:1245-1249.

- Tepfer, D., Boutteaux, C., Vigon, C., Aymes, S., Perez, V., O'Donohue, M. J., Huet, J.-C., and Pernollet, J.-C. 1998. *Phytophthora* resistance through production of a fungal protein elicitor (β -cryptogein) in tobacco. *Mol. Plant-Microbe Interact.* 11:64-67.
- Terras, R. G. F., Eggermont, K., Kovaleva, V., Raikhei, V. N., Osborn, W. R., Kester, A., Rees, B. S., Torrekens, S., Leuven, V. F., Vanderleyden, J., Cammue, A. P. B., and Willem, F. B. 1995. Small cysteine-rich antifungal proteins from radish: Their role in host defense. *Plant Cell* 7:573-588.
- Terras, R. G. F., Schoofs, M. E. H., De Bolle, F. C. M., Leuven, V. F., Rees, B. S., Vanderleyden, J., Cammue, P. A. B., and Broekaert, F. W. 1992. Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) seeds. *J. Biol. Chem.* 267:15301-15309.
- Williams, P. H., and Delwiche, P. A. 1980. Screening for resistance to blackleg of crucifers in the seedling stage. *Eucarpia "Cruciferae 1979" Conference.* pp. 164-170.
- Williams, P. H. 1985. *Crucifer Genetics Cooperative (CrGC) Resource Book.* Department of Plant Pathology, University of Wisconsin Madison.
- Wu, G., Shortt, B. J., Lawrence, E. B., Levine, E. B., and Fitzsimmons, K. C. 1995. Disease resistance conferred by expression of a gene encoding H_2O_2 -generating glucose oxidase in transgenic potato plants. *Plant Cell* 7:1357-1368.