Molecular Plant Microbe Interactions 12:410-418, 1999

Constitutive expression of pea defense gene DRR206 confers resistance to blackleg (*Leptosphaeria maculans*) disease in transgenic canola (*Brassica napus*)

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35S constructs and DRR206 antisera were made in the laboratory of LAH. Transformation of *B. napus* was performed by GN. All other work was done by YW and BF.

ABSTRACT

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 CaMV 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 to 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* co-segregated 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.

Addiontional 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 both to 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 ß-crytogein 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 H_2O_2 from glucose, resulted in resistance to both *Phytophthora infestans*, as well as to 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 bacterial pathogens and elicitors (Riggleman et al., 1985, Fristensky et al., 1985, Daniels et al., 1987, Hadwiger et al., 1992, Cody 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 CaMV 35S promoter, replacing the GUS gene (see Methods). Constructs were directly transfected into *A. tumefaciens*, and cotyledonary petioles were transformed by cocultivation, as described in 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 (ie. 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 single copy inserts to the total number of transgenic lines were, PR10.1: 10/28; chitinase: 3/22; DRR206: 7/28; defensin: 2/24. Seeds were collected separetely 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 (ie. 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 5 T1 plants was pooled and expression of transgenes assayed by RNA gel blot analysis. In parallel experiments, twenty 8-day seedlings were grown for each line, and cotyledons inoculated with compatible blackleg isolate PG2. After scoring 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 5-6 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 similar disease scores to the Westar controls in cotyledon and adult plant assays, while mRNA levels for this gene ranged from high to undetectable. Among three independenty-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 to lines with low or no detectible expression (Table 1). GN3-1#1 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 (Figure 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 3 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 *Brassica* are shown in Figure 2. On untransformed Westar cotyledons, brown lesions with black pycnidia are evident (Figure 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 co-segregated with pea DRR206 or defensin, seed from a single T1 selfed parent from each line were grown, and these T2 plants were screened by PCR using 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 to null segregants (ie. 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 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 hours, extracts from either defensin or DRR206 transgenic plants showed significant decreases in germination, compared to extracts from untransformed Westar plants (Table 3).

The expression of the DRR206 protein was verified by immunoblots (Figure 3) using polyclonal antibodies from rabbits injected with DRR206 expressed in *E. coli* (Culley et al., 1995b, Allaire, 1993). As a positive control, proteins were extracted from pea pods 48h after inoculation with *F. solani* f. sp. *phaseoli*. Under these conditions, pea 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 weight classes 25 and 22 kDa, along with a minor band at 28 kDa, were detected. The molecular weight of the DRR206 polypeptide, inferred from the complete sequence of the genomic clone, is 20.4 kDa, while the estimated molecular weight 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 crossreacts 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 which comigrated with the 25 and 22 kDa bands from pea.

One possible explanation for presence of two bands in pea and transgenic *Brassica* is that DRR206 undergoes postranslational modifications. Alternatively, Culley et al. (1995b) found that at least 5 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 weights. However, these bands were also seen in transgenic *Brassica*, even though only one pea DRR206 gene was present the transgenic lines. Since the 22 kDa and 25 kDa bands were not seen in untransformed *Brassica*, we can rule out the possibility that they were products of *Brassica* 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 *Brassica* 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 *Verticillium dahlie* showed an almost 50% reduction in lesion size, compared to untransformed controls (Chang et al., 1993). In *Verticillium*-infested soil, PR10 transgenic potatoes showed a decrease in wilting, accompanied by about a 2-fold increase in tuber yield, compared to 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 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 to suppression by PR3 type chitinases than *R. solani*.

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 hours post-inoculation (h.p.i.). In gene-specific incompatible interactions with *P. syringae*, DRR206 is induced early and expression remains strong for at least 30 hour post-inoculation (Daniels et al., 1987). In *F. oxysporum*-infested soil, DRR206 is strongly expressed in resistant isoline Vantage, and only weakly induced in susceptible isoline M410 (Hadwiger et al., 1992).

Despite the strong difference in expression seen with DRR206 in resistant, compared to 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 (Figure 2C). Resistance to blackleg can occur both in cotyledons (Figure 2A) and in adult stems (Figure 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 (eg. inhibiting spore germination or hyphal growth) rather than later (eg. 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, Figure 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 (Figure 2A). Defensin transgenics also exhibited smaller stem lesions compared to the Westar control (Figure 2B) but allowed a similar degree of hyphal growth in cotyledons (Figure 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.

It is worth mentioning that database searches reveal that DRR206 has no known homologues in any other plant species. In view of the enormous amount of sequence data now available from both dicots and monocots, this is noteable. There are many examples of proteins commonly found to be associated with defense responses, including proteins of the phenylpropanoid pathway, those associated with the oxidative burst, and other previously described as pathogenesis-related proteins. These appear to be conserved across many plant families. In contrast, it is possible that DRR206 falls into a second category of defense proteins, which arise independently in different plant genera, probably in short evolutionary timescales.

MATERIALS AND METHODS

Transgenic Canola Plants

35S-constructs were made by replacing the ß-glucuronidase protein coding sequence from pBI121 (Clonetech Inc.) with pea defense cDNA or genomic sequences. Inserts were excised using restriction enzymes compatible with recipient sites in pBI121, or, where needed, restriction sites were blunt-ended using *E. coli* DNA polymerase I Klenow fragement. 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 *XbaI* and *SacI* sites of pBI121 to give plasmid pDC49-4. A 1035 bp *BsmAI/HincII* fragment containing the protein coding sequence from the genomic pea chitinase (L37876: 265..1299), Chang et al, 1995) was recloned from pBluescript between the *XbaI* and *SacI* sites of pBI121 to give pDC-CHIT-26. The 801bp DRR206.3 *DdeISspI* genomic fragment containing the protein sequence and terminator (AF1155574: 1025..1825) was recloned between the *XbaI* and *SacI* 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 *SmaI* and *SacI* sites of pBI121 to give pDC230-12. 35S-defense gene plasmid constructs were transfected into *Agrobacterium tumefaciens* strain MP90 by the freeze-thaw method (Holtorf et al. 1978). Transformation of *Brassica 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 ºC and 16/8 hour photoperiod.

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 minutes. Fifteen l'g samples 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 ^{32}P-labelled random-primed probe in 0.5 M Na_2HPO_4, (pH 7.2) and 7% SDS at 65ºC. A 0.5kb fragment containing the *Npt*II gene from pBI121 was used as 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 Na_2HPO_4 (pH 7.2) and 5% SDS at 65ºC for 30 minutes, and twice with 40 mM Na_2HPO_4 (pH 7.2) and 1% SDS at 65ºC for 30 minutes according to the manual from Bio-Rad laboratories. 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, (Dr. 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 minutes, and then placed on V-8 juice agar medium containing 0.75% $CaCO_3$, 0.1% streptomycin sulphate and 0.04% rose bengal. The plates were incubated under constant light at 22-24ºC for one week until sporulation. Agar strips containing the infected tissue were transferred to fresh V-8 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 92039 USA) and centrifuged. The spore suspension was stored in a -80 ºC freezer. The spore concentration as adjusted to 2×10^7 spores/ml for inoculation.

Plant Inoculation

a) Cotyledon inoculation - Cotyledons of eight-day old seedlings grown as described above were wounded with a syringe needle and 10 l'l of the pycnidiospore suspension was dropped onto the lesion. After the drops were dried for 4 hours under light at 24 ºC, 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). Briefly, 0 - no symptoms; 1 - necrosis around wounding site, 0.5 - 1.5 mm lesion, faint chlorotic halo around lesion; 3 - dark necrotic lesion 1.5 - 3.0 mm., chlorotic halo may be present around lesion; 5 - lesions 3 - 6 mm delimited by dark necrotic margins, may show gray green tissue collapse; 7 - 3 - 5 mm lesions, gray green tissue collapse, sharply delineated non-darkened margin; 9 - gray lesion > 5mm with diffuse margins; black pycnidia evident throughout lesion. For microscopy (Figure 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, v/v). The fixed solution was changed twice daily for 3 days. Inoculation sites without staining were examined by light microscopy for the presence of hyphae (Figure 2C).

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

c) 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 N_2 and 1 ml of sterile distilled water was added per gram of fresh weight. Insoluble components were removed by centrifugation at 4000 g at 4 °C for 20 minutes. Supernatant was filtered through a 0.2 l'M cellulose acetate filter. *L. maculans* pycnidiospores were added to the extract to a concentration of 1 x 10^6 spores/ml (approx. 80 ml per 250 ml flask) and incubated on a shaker at 120 rpm at room temperature for 30 hours. Germination was quantified using a hemocytometer, using 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 7 lines with PR10.1, 3 lines with chitinase, 7 lines with DRR206 and 2 lines with defensin. 20 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 using at least 5 Westar plants. For T2 plants listed in Table 1, seed was planted from 3 T1 parents, and seedlings were inoculated with *L. maculans* and scored at both cotyledon and adult stages. For GN3-4#22, 3 replicates of 25 plants each, for a total of 75 plants, were scored. For GN4-2#15, 2 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 7 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 (ie. 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 6 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 l'l of extraction buffer (100 mM Tris-HCl (pH 8.0)), 50 mM EDTA, 500 mM NaCl, 1.25% SDS) and incubated for 20 minutes at 65°C. Next, potassium acetate was added to a final concentration of 1 M. The solution was kept on ice for 20 minutes and then extracted with chloroform:isoamyl alcohol (24:1), followed by ethanol precipitation. For the PCR reactions,

mixture contained 1.5 mM MgCl, 0.2 mM dNTP, 0.5 l'M of the specific primer mix and 2.5 units of Taq DNA polymerase. Gene-specific primers were as follows: for DRR206: oC206+1 (5'aattccaaacaagagaaagcc3') and oC206-2 (5'cttgatataaacaccaagtcg3'); for defensin: oS39b+3(5'caagaaatagtggtgagtgaa3') and oS39b-4 (5'gcgacaaccacgtgattttg3'). PCR cycles were as follows, cycle 1: 94°C for 3 minutes, 55°C for 45 seconds, 72°C for 1 minutes; cycles 2-29: 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 1 minute.

RNA Extraction and Northern Blotting

RNA was extracted using a procedure modified from Logemann *et al* (1987). Leaves (0.5 g) were added to a tube containing 750 l'l of extraction buffer (4 M guanidine isothiocyanate, 0.1 M Tris-HCl, pH 7.5, 10 mM EDTA, 1% methcaptoethanol), mixed well and centrifuged for 20 minutes at 4 °C. The supernatant was extracted using 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 hours, then washed with 3 M sodium acetate (pH 4.8) and 70% ethanol. Dried RNA was resuspended in DEPC-treated sterile distilled water, and 20 l'g samples were denatured and electrophoresed on 1.2% agarose 1 x MOPS 2.2M formaldehyde gels as described in (Ausubel et al., 1998). Hybridization and washing were done using 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 N_2 and extracted with 6 ml extraction buffer (50 mM Tris-HCl (pH 7.5), 1 mM phenylmethylsulfonyl fluoride, 2 mM thiourea, and 1.5% (w/v) polyvinylpyrrolidone) for 2 h at 4 °C under continuous stirring. Following centrifugation, $(NH_4)_2SO_4$ was added to the supernatant to a 75% saturation. Proteins were precipitated over night, 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.p.i. were run through 12% polyacrylamide Tricine-SDS-polyacrylamide gels using Bio-Rad low MW markers (66.0 kDa-14.2 kDa). One gel was stained in 0.25% Coomassie Brilliant Blue R 250 in 45% methanol and 10% acetic acid, and then destained 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 Peijun Zhang for early screening of transgenics, and to Joe Hammerlindl and Wilf Keller for help 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.

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Table 1. Summary of data from T1 and T2 generations. All plants are T1 transformants, except where indicated in parentheses.

B. napus line	Gene	RNA*	Disease scores**	
			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(T2)	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(T2)	Defensin	+	7.4±0.4 b	3.4±0.5 b

*+ : mRNA expressed; - : no detectible mRNA expression

** 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 3 T1 parents were inoculated with *L. maculans* and scored for GN3-4#22 (3 replicates, for a total of 75 plants) and GN4-2#15 (2 replicates for a total of 50 plants). Scores with the same letter are not significantly different at p=0.05. (Duncan's Multiple Range Test). For each line, a separate Westar check of at least 5 plants was done. Westar scores were pooled.

Table 2. Disease scores in DRR206 T3 transgenic progeny.

	Disease score*		
	Cotyledon	Cotyledon	Adult
B. napus line	9 d.p.i	13 d.p.i.	30 d.p.i.
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

*GN3-4#22 T2 plants were screened by PCR using DRR206-specific primers, as described in Methods. Seed (ie. T3) was collected from six T2 progeny testing positive for pea DRR206, and were scored in cotyledon and adult assays. Scores were pooled from 3 experiments, for a total of 323 plants, from all six parents. Westar checks are from a total of 55 plants taken from three separate inoculations.

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

B. napus line	% germination*
Westar	30.9±1.2 a
GN4-2#15 (Defensin)	15.6±1.5 b
GN3-4#22 (DRR206)	5.8±1.0 c

*plus or minus standard error of the mean. Results are from four replicates, each using water-soluble leaf extracts prepared as described in Methods from at least 5 plants per line. Four grids were counted per replicate. Letters denote measurements that are significantly different at p=0.05 (Duncan's Multiple Range Test).

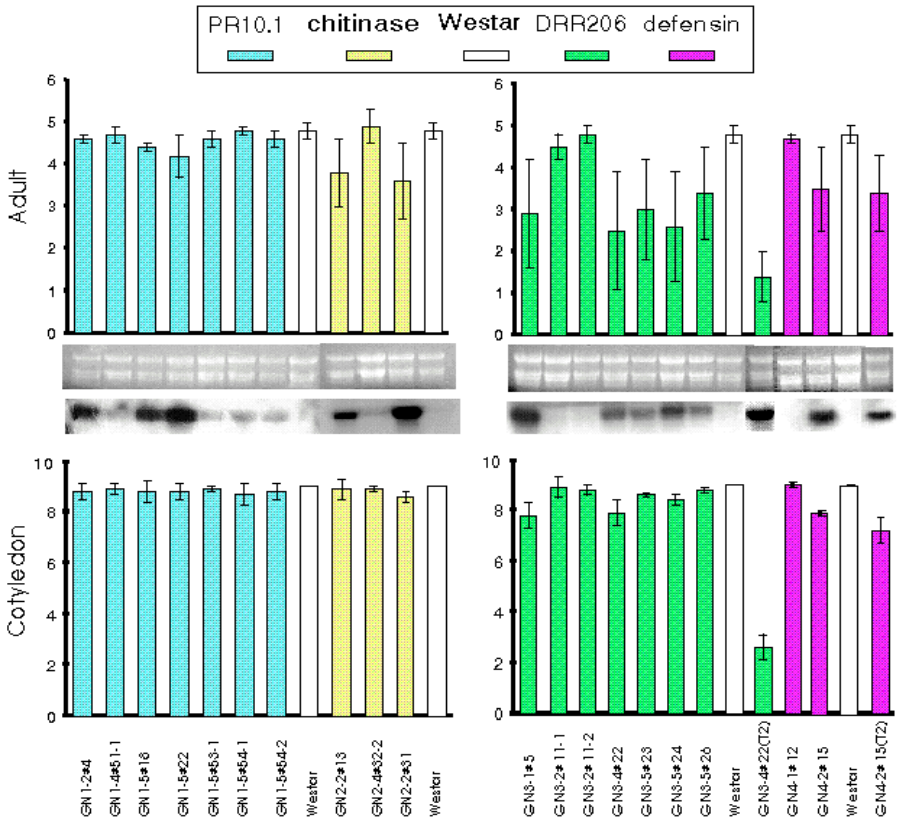

Figure 1. Disease scores and transgene mRNA expression in T1 transformed lines. For each gene (patterned bars), an independent control was done with untransformed cv. Westar (white bars). Each bar represents the average of 20 plants inoculated with *L. maculans* PG2 plus or minus the standard error of the mean. Disease scores are shown for both cotyledons and adult leaves. Transgene mRNA expression was assayed by hybridization to Northern blots of RNA extracted from pooled tissue samples of 5 of the 20 plants scored in the disease assays. Tissue samples were taken just prior to adult plant inoculation. 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. Note: For transgenic lines GN3-4#22 and GN4-2#15, data for T2 plants are also included.
Nomenclature: PR10, chitinase, DRR206, and defensin constructs were used in experiments GN1, GN2, GN3 and GN4, respectively. The names for transgenic lines are illustrated using 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 a one of two plantlets regenerated from non-contiguous sectors of that explant, and is therefore the product of a unique transformation event.

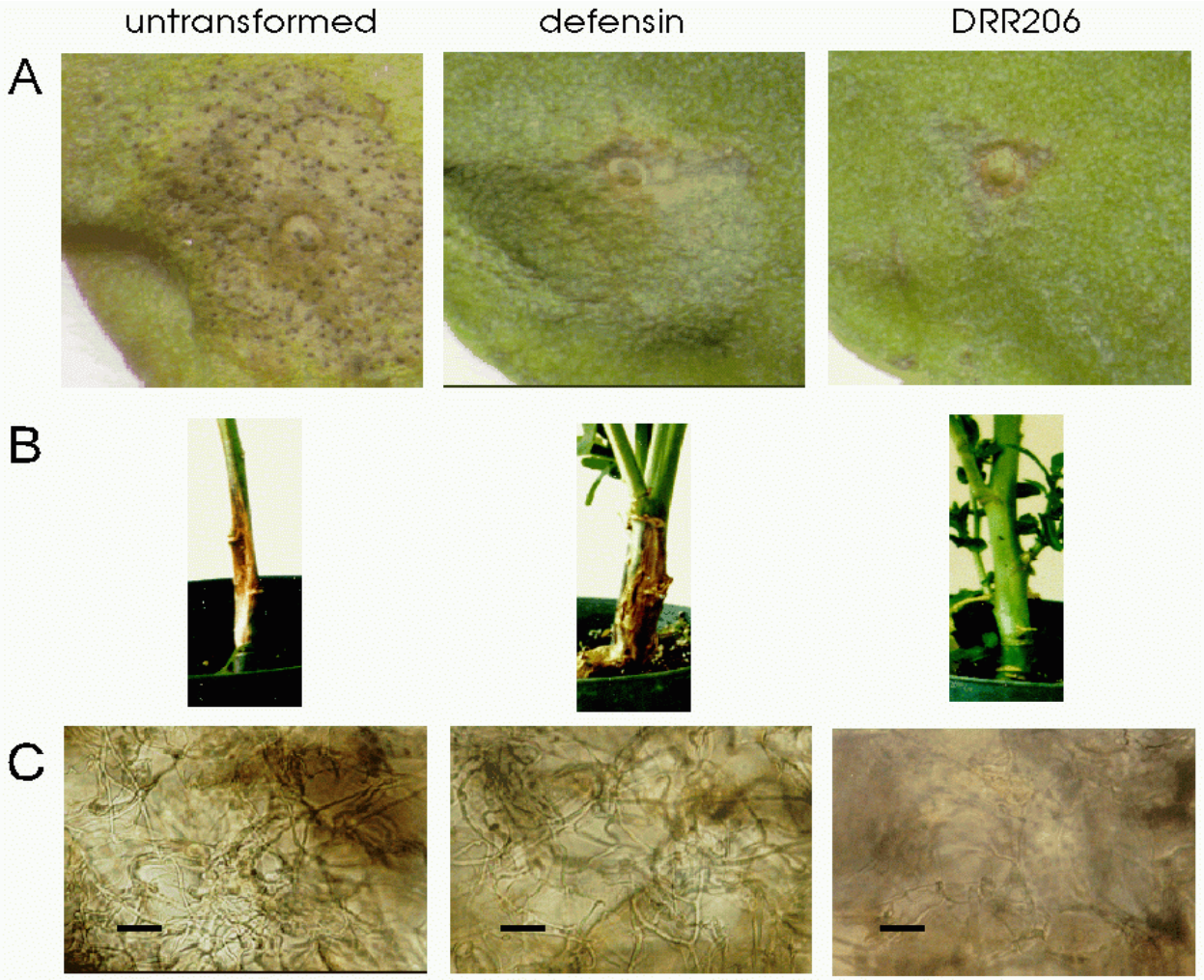

Figure 2. Infection phenotypes of transgenic (T2) and untransformed canola inoculated with *Leptosphaeria maculans* PG2. Left to right: Untransformed Westar; GN4-2#15; GN3-4#22. **A**. Cotyledons 12 days after pinprick inoculation. **B**. Closeups of adult plants 30 days after stem inoculation. **C**.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

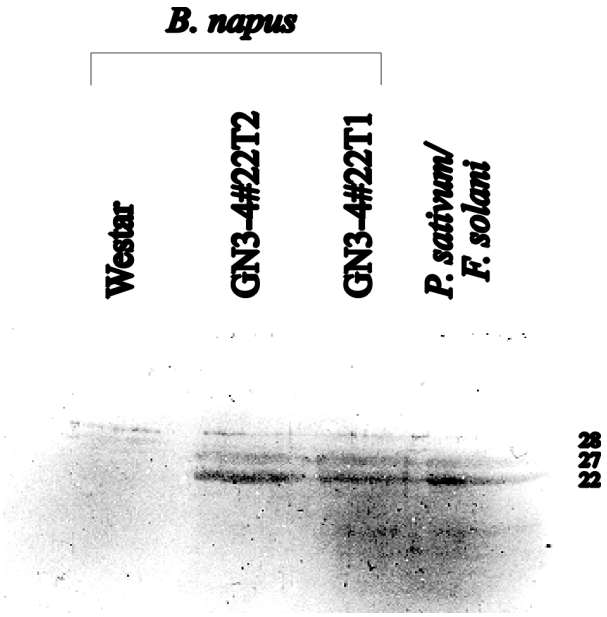

Figure 3. Immunoblot of proteins from *B. napus* transgenic line GN3-4#22 T1 and T2 plants using polyclonal antibodies for pea DRR206. Proteins from pea pods inoculated with *Fusarium solani* f. sp. *phaseoli*, and from *B. napus* cv. Westar serve as positive and negative controls, respectively. Estimated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