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Transgenic canola lines expressing pea defense gene DRR206 have resistance to aggressive blackleg isolates and to *Rhizoctonia solani*

Yaping Wang and Brian Fristensky*
*Department of Plant Science, University of Manitoba, Winnipeg, R3T 2N2, Canada; *Author for correspondence; e-mail frist@cc.umanitoba.ca)*

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Abstract

We have previously demonstrated that transgenic *Brassica napus* plants expressing pea DRR206 constitutively are resistant to the hemibiotrophic blackleg fungus, *Leptosphaeria maculans* isolate PG2. The present work seeks to determine whether DRR206 is effective against a wider range of fungi. Transgenic plants expressing DRR206 exhibit decreased severity of stem canker in adult plants inoculated with aggressive *L. maculans* isolates PG3 and PG4. Decreased seedling mortality with the biotrophic root pathogen *Rhizoctonia solani* is also seen. Finally, leaves of DRR206 transgenic plants inoculated with the necrotroph *Sclerotinia sclerotiorum* show smaller lesions at 48 h after inoculation, leading to a delay, but not a prevention, of disease development. These results demonstrate the effectiveness of DRR206 against several fungal species with three distinct modes of pathogenicity. Although its precise function remains to be determined, a recent report shows that pea DRR206 shares strong amino acid sequence similarity with 'dirigent proteins' which couple monolignol radicals to form the lignan (+) pinoresinol.

Introdcution

The terms 'basic resistance' or 'non-host resistance' refer to mechanisms by which plants exclude most of the fungi, bacteria and viruses present in their immediate environment. Some types of microorganisms learn to evade or suppress basic resistance, allowing them to cause disease. Specific resistance refers to resistance targeted at specific genotypes of pathogenic microbes. Basic resistance has proven difficult to study, largely because it is controlled by many genes, compared to specific resistance which is regulated by single genes.

For genetic engineering purposes, resistance genes have the advantage that their specificity makes it possible to target a particular disease. Their effects are precise and predictable. Their main disadvantage is that they are only effective against genotypes of a pathogen having the corresponding allele for avirulence.

Generally, resistance (R) genes function by activating numerous defense genes, which carry out various defense responses, such as the production of antifungal, antiviral or antibacterial compounds, strengthening the cell wall, papilla formation, or cell death to contain the pathogen. Use of defense genes for genetic engineering seeks to bypass R gene regulation, either by constitutive expression of one or more defense genes, or expression under the control of a pathogen-inducible promoter. The disadvantage of this strategy is that one can not always predict the range of pathogens against which a particular defense gene may be effective.

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 previously shown that constitutive expression of pea defense gene DRR206 confers resistance to *Leptosphaeria maculans* pathogenicity group PG2 (Wang et al. 1999). DRR206 is strongly induced in pea (*Pisum sativum*) in response to both fungal and bacterial pathogens and elicitors (Riggleman et al. 1985;

Fristensky et al. 1985; Daniels et al. 1987; Cody et al. 1988; Hadwiger et al. 1992). Although the exact biochemical function for the DRR206 gene product remains to be determined, previous work provides some clues. In non-transformed *Brassica*, compatible isolates of *L. maculans* invade tissue through stomates or wounds and grow intercellularly in an asymptomatic biotrophic phase (Hamond and Lewis 1987). Subsequently, necrotic cell death occurs, which is required for sporulation of the fungus. In DRR206 transgenic *Brassica*, necrosis is limited to the site of inoculation and no sporulation is seen. Microscopic examination shows that little biotrophic hyphal growth is seen even at the inoculation site (Wang et al. 1999). We have also shown that water-soluble extracts from DRR206 transgenic plants have inhibitory activity towards germination of *L. maculans* pycnidiospores (Wang et al. 1999).

To evaluate the potential of DRR206 for use in genetic engineering of disease resistance, as well as to learn more about its mode of action, we have tested DRR206 transgenic plants for resistance to *L. maculans* pathogenicity groups PG3 and PG4, and to *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. In the previous study, resistance of DRR206 transgenic plants was only tested using blackleg isolate PG2 which is common in Canada, and not to PG3 and PG4 which are found in other parts of the world. At present, canola cultivars grown in Canada have resistance to PG2, but most do not have resistance to PG3. No resistance has yet been identified to PG4.

In contrast to the hemibiotrophic ascomycete *L. maculans*, *R. solani* is a basidiomycete which grows saprophytically in the soil and infects the roots and hypocotyls, rather than the leaves and stems. *Rhizoctonia* causes wilting of seedlings at the cotyledon stage. *Sclerotinia sclerotiorum* is distinct both in its mode of action and its broad host range. *Sclerotinia* is a necrotrophic ascomycete that kills tissue as it spreads, and proliferates in necrotic tissue. Its mode of pathogenicity includes the production of oxalic acid, which is toxic to most plants, and polygalacturonase, which breaks down plant cell walls. Whereas most plant pathogens are highly specialized for their hosts, *Sclerotinia* can parasitize a very wide range of species. Despite its ability to infect most parts of a plant, *Sclerotinia* preferentially invades petals and leaves in *B. napus*.

Pathogens tested in this study also differ with respect to host range. *L. maculans* tends to be highly specific for its host, with one exception being that one aggressive isolate of *L. maculans* taken from *B. napus* was able to cause disease in the crucifer *Raphanus raphanistrum* (Chen and Séguin-Schwartz 1999). Yang et al. (1996) have shown that *R. solani* isolated from canola can also cause disease on cauliflower and flax. Finally, *S. sclerotiorum* is well known for its extremely wide host range, spanning 408 species (Boland and Hall 1994).

We now demonstrate that transgenic *B. napus* lines expressing pea DRR206 exhibit decreased severity of stem canker in adult plants inoculated with aggressive *L. maculans* PG3 and PG4. Decreased seedling mortality is also seen with the soil-borne pathogen *Rhizoctonia solani*. Finally, leaves of DRR206 transgenic plants inoculated with *Sclerotinia sclerotiorum* show smaller lesions at 48 h after inoculation, leading to a delay, but not a prevention, of disease development.

Materials and methods

Plants and pathogens

Transgenic lines derived from canola cultivar Westar were described previously (Wang et al. 1999). Plants were grown in Metromix in a growth chamber at 20/16 °C for day/night and at a 16 h photoperiod. Seedlings were replanted in a 6:2:3 mixture of soil, sand and peat moss supplemented with 17-20-0 fertilizer (N 17%, P_2O_5 20%, S 15%) at the same temperature and photoperiod conditions.

T_2 plants from DRR206-transformed line GN3-4#22 were screened for transgene homozygosity. For each T_2 parent, at least 20 progeny were screened by PCR for the presence of the DRR206 insert using methods described previously (Wang et al. 1999). DNA from non-transgenic Westar plants was used as a negative PCR control. For plants that did not exhibit DRR206-specific PCR products, new DNA extractions were performed, and PCR repeated as a check. Parents for which all progeny tested positive for DRR206 were deemed homozygous.

Leptosphaeria maculans PG3 and PG4 (Rimmer and van den Berg 1992), and *Rhizoctonia solani* isolate RC6 were obtained from Dr R. Rimmer, University of Manitoba. *Sclerotinia sclerotiorum* clone 38 is a common clone in western Canada and was supplied by Dr L. M. Kohn, Department of Botany, University of Toronto.

Leptosphaeria maculans*: cotyledon inoculation*

Pycnidiospore suspensions were prepared from infected tissue as described (Williams 1985). Cotyledons of 8-day old seedlings were wounded with a syringe needle and 10 μl of the pycnidiospore suspension at 2×10^7 spores per ml was dropped onto the wound. 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 after inoculation according to Williams and Delwiche (Williams and Delwiche 1980). Briefly, 0 stands for no symptoms, 1 for necrosis around wounding site (0.5–1.5 mm lesion, faint chlorotic halo around lesion), 3 for dark necrotic lesion (1.5–3.0 mm lesion, chlorotic halo may be present around lesion), 5 for lesions 3–6 mm delimited by dark necrotic margins (may show gray green tissue collapse), 7 for 3–5 mm lesions, gray green tissue collapse, and sharply delineated non-darkened margin, and 9 for gray lesions >5 mm with diffuse margins, black pycnidia evident throughout lesion.

Leptosphaeria maculans*: adult plant inoculation*

The lower portion of the stem (close to the third node), was inoculated at the 5–6 leaf stage. Of the pycnidiospore suspension 10 μl was injected into the stem and the plants were kept in a humid environment for 24 h. The disease rating scale is based on length and cross-sectional area of infected stem regions (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).

Rhizoctonia solani *inoculation*

Inoculum was prepared according to Acharya et al. (1984) by growing the fungus on ca. 20 ml of Difco PDA in 8 cm petri dishes incubated at 20 °C for seven days. Plugs 8 mm in diameter cut from the growing margins were carefully placed next to the hypocotyl region just below the soil surface. Controls were inoculated with sterile 8 mm PDA plugs. Plants were incubated in a growth room at day/night temperatures of 20 °C/16 °C and day/night light of 16 h/8 h. The percentage of mortality of seedlings was scored at 8 days after inoculation.

Sclerotinia sclerotiorum *inoculation*

Inoculum preparation and inoculation were adapted from Fang (1993). Sclerotia were placed in a petri dish containing PDA for 3 days to allow germination, and agar plugs containing mycelia were cut and transferred to new PDA plates. Plates were incubated for 48 h at 24 °C. Mycelial plugs 3 mm in diameter were cut with a cork borer from the growing margin of colonies and placed on the middle of the first and the second leaves of each plant. Inoculated plants were incubated for 2 days in a mist chamber to provide continuous leaf wetness. The mist chamber was kept in the growth room and illuminated with ambient light. The diameter of necrotic lesions was measured at 48 h after inoculation.

Results

To ensure that resistance was not due to spurious transgene effects, T_2 plants were chosen from several independently transformed lines in which DRR206 expression was confirmed by RNA gel blots (Wang et al. 1999), and for which adequate seed was available. Although independent transformants had been previously screened using blackleg isolate PG2 (Wang et al. 1999), extensive screening had not been done with other pathogens. Figure 1 shows that in both stem-inoculated adult plants and in cotyledons, disease scores with both PG3 and PG4 were lower for DRR206 transgenics than for untransformed Westar plants. Two non-transgenic cultivars were included as checks. *B. napus* cv. Glacier, which is susceptible to PG3 and PG4, exhibited high cotyledon disease scores for both isolates. Cultivar Quinta, which carries a resistance gene to PG3, also showed low disease scores with PG3, but high disease scores with PG4.

Resistance to *Rhizoctonia solani* and *Sclerotinia sclerotiorum* was tested with the same transgenic lines. T_2 lines expressing DRR206 showed low mortality rates compared to untransformed Westar (Figure 2). Similarly, DRR206-expressing lines had reduced lesion sizes on leaves inoculated with *Sclerotinia sclerotiorum*, compared to untransformed Westar (Figure 3).

Resistance to aggressive blackleg isolates

DRR206 transformants used in this work were previously shown to have single T-DNA insertions, as determined both by DNA gel-blot analysis and by the fact that resistance to PG2 co-segregated 3:1 with the

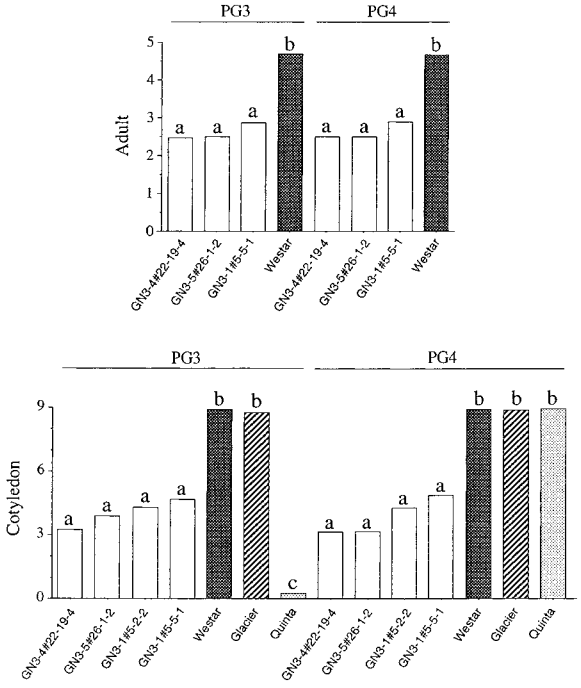

Figure 1. Mean blackleg disease scores in cotyledons ('Cotyledon') and adult stems ('Adult') of transgenic DRR206 T3 progeny (white bars) or untransformed Westar (darkly shaded bars). Plants were inoculated with *L. maculans* isolates PG3 or PG4 as indicated. For adult plant assays, a minimum of 15 plants were scored per line. For cotyledon assays, a minimum of 21 plants per line were scored. As additional checks, cotyledon assays included cv. Glacier (hatched bars), which is susceptible to PG3, and cv. Quinta (lightly shaded bars), which carries a resistance gene to PG3 but is susceptible to PG4. Scores with the same letter are not significantly different at $p = 0.05$ (Duncan's Multiple Range Test).

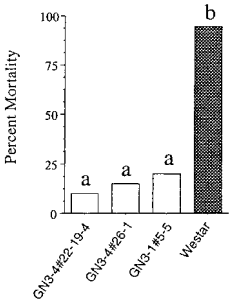

Figure 2. Seedling mortality rates in DRR206 T_3 progeny inoculated with *Rhizoctonia solani* RC6. Seedlings from DRR206 transformant lines (open bars) or untransformed Westar (dark bar) were inoculated 8 days after planting and scored 8 days after inoculation. At least 90 plants were tested per line. Scores with the same letter are not significantly different at $p = 0.05$ (Duncan's Multiple Range Test).

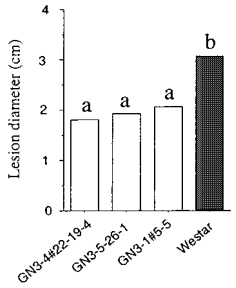

Figure 3. Lesion diameter (cm) in leaves of DRR206 T_3 progeny (open bars) or untransformed Westar (shaded bar) inoculated for 48 h with *Sclerotinia sclerotiorum*. At least 23 plants were tested per line. Scores with the same letter are not significantly different at $p = 0.05$ (Duncan's Multiple Range Test).

presence of the DRR206 insert (Wang et al. 1999). To allow for more consistent disease evaluation, lines homozygous for DRR206 were identified. To illustrate, seed from T_1 parent GN3-4#22-1 were planted, and progeny from selfed T_2 plants were tested for DRR206 homozygosity as described in Materials and methods. T_3 progeny from three homozygous T_2 parents, GN3-4#22-1-19, GN3-4#22-1-2 and GN3-4#22-1-35, were scored for cotyledonary and adult plant resistance to *L. maculans* PG3. Some of these T_3 individuals were selfed and used as parents for the T_4 generation. For example, GN3-4#22-1-19-3 and GN3-4#22-1-19-5 are T_3 progeny of GN3-4#22-1-19, and plants grown from their seed were part of the T_4 generation. In each subsequent generation, progeny from 7 individuals was used for each resistance assay.

As shown in Figure 4a, inoculation of cotyledons in DRR206 transgenic plants resulted in small lesions, with necrosis limited to the region near the inoculation site. In contrast, cotyledons of untransformed Westar plants exhibited large lesions covering much of the area of the cotyledon, usually spreading to the margins of the cotyledon. The presence of pycnidia were also evident. In general, small variation in resistance to PG4 was seen among DRR206 transgenic lines (Figure 5). In fact, phenotypic variability for resistance decreased in later generations. In the T_3, T_4 and T_5 generations, adult plant resistance scores fell into two distinct classes, although the differences between classes were small compared to the differences between DRR206 and untransformed Westar. Two lines with the lowest T_5 disease scores, GN3-4#22-1-19-5-3 and GN3-4#22-19-4-21, were selected for two additional generations of evaluation. Results in the T_6 and T_7 generations show single phenotypic classes in a narrow range of disease scores, indicating that

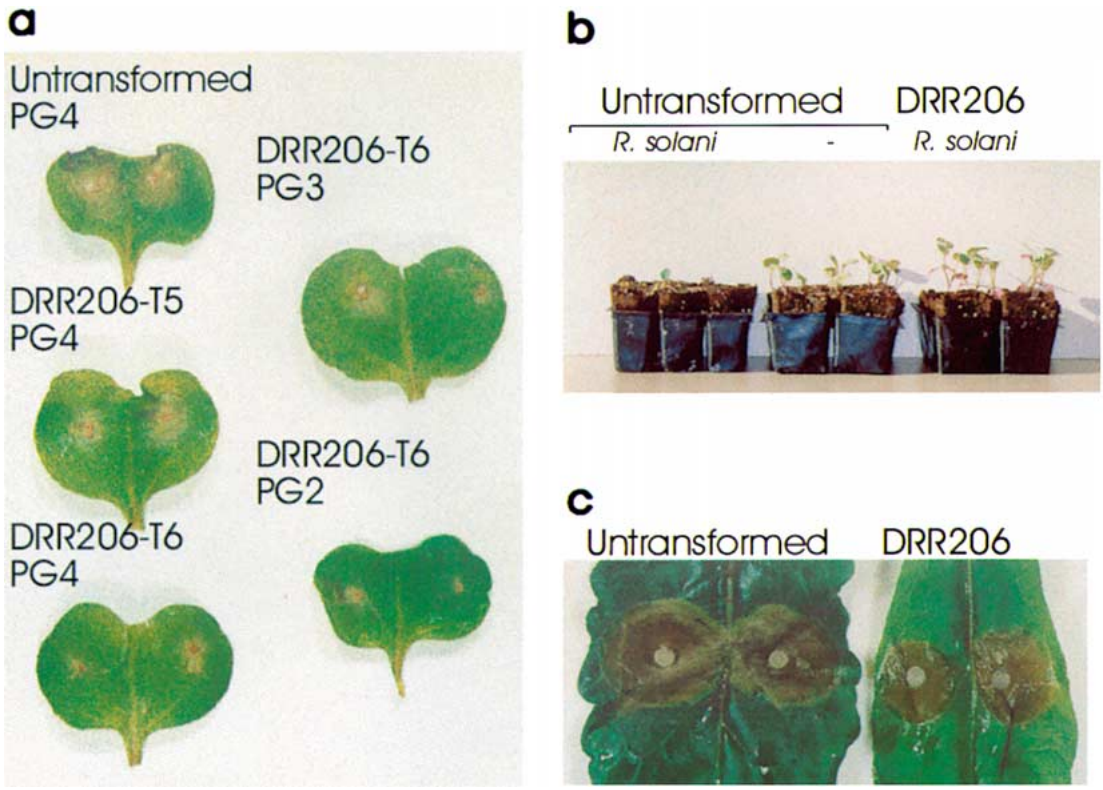

Figure 4. Infection phenotypes in *B. napus* cultivar Westar, transformed with DRR206, or untransformed plants. A. Cotyledons at 10 days after inoculation with *L. maculans*. Left: plants inoculated with *L. maculans* PG4; top to bottom: untransformed Westar; Westar transformed with DRR206 in T_5 and T_6 generations. Right: Westar transformed with DRR206 GN3-4#22-1-19-5-3-12 at T_6 generation, inoculated with *L. maculans* PG3 (top) or PG2 (bottom). B. *B. napus* 17-day seedlings. Untransformed Westar inoculated with *R. solani* (left), uninoculated (middle) or DRR206 GN3-4#22-1-19-5-3-6 (T_6 generation) inoculated with *R. solani* (right). Inoculations were done 8 days after planting. C. Leaves of untransformed Westar (left) or DRR206 GN3-4#22-1-19-5-3-12 T6 (right) plants. Leaves of 2-month old plants were inoculated with *S. sclerotiorum*, and results are shown 48 h after inoculation.

in a uniform genetic background, DRR206-mediated resistance to blackleg PG4 is stable and uniform.

Resistance to Rhizoctonia solani

Resistance to *Rhizoctonia solani* was also evaluated in the T_6 and T_7 generations, as the percentage of seedlings that were dead within 8 days after inoculation (Figure 6). Results showed greater variation than was seen with blackleg. In the T_6 generation, DRR206 lines ranged from 5% to 32% mortality, with resistant control Quinta showing 16% mortality. A more narrow range of disease was seen in the T_7 generation, with DRR206 transformant lines showing between 15% to 23% mortality, and Quinta at 32% mortality. Some experimental variability in results is indicated by the fact that susceptible control Westar had 94% mortality in the T_6 generation, and 63% mortality in T_7.

As shown in Figure 4b, *R. solani*-inoculated DRR206 transformants grew as vigorously as uninoculated Westar plants. In all cases where plants were killed by the fungus, seedlings had lost all turgor. Complete girdling of the hypocotyl was seen, and in many cases the root was completely detached from the stem. Although some percentage of the DRR206 plants died in all cases, those which did not die by 8 days typically grew into healthy adult plants. Although Figure 6 shows that many untransformed Westar plants were still alive at 8 days after inoculation, all Westar plants were dead after 1 month.

Decreased leaf lesion size with Sclerotinia scleortiorum

Disease in DRR206 transformed and untransformed plants inoculated with *Sclerotinia* was measured on the basis of lesion diameter at 48 hours postinocu-

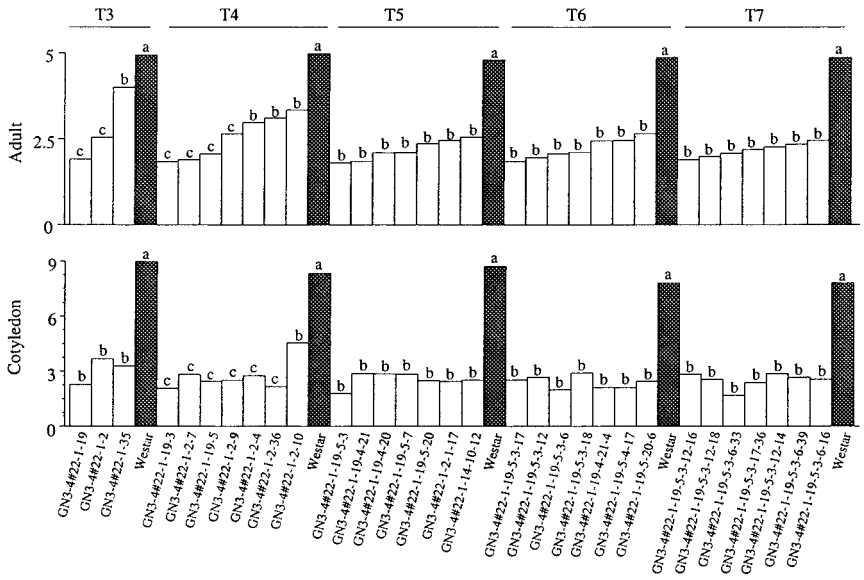

Figure 5. Mean blackleg disease scores in cotyledons ('Cotyledon') and adult stems ('Adult') of transgenic DRR206 progeny (white bars) or untransformed Westar (shaded bars). All experiments were carried out with *L. maculans* isolate PG4 as inoculum, except the T_3 generation, which was inoculated with PG3. Three replicates of 25 plants were done for each transgenic line in each generation. Within each generation, transgenic lines are sorted left to right from lowest to highest mean adult plant disease score. Scores with the same letter are not significantly different at $p = 0.05$ (Duncan's Multiple Range Test).

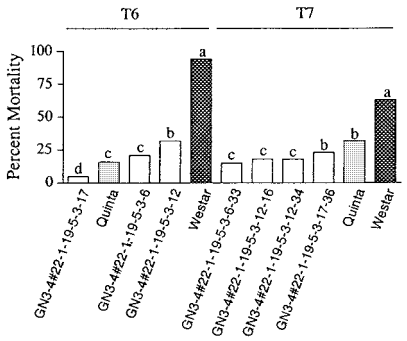

Figure 6. Seedling mortality rates in DRR206 transgenic lines inoculated with *Rhizoctonia solani* RC6. Seedlings from DRR206 transformant lines (open bars) (e.g. 'GN3-4#22-1-19-5-3-12') or susceptible and resistant controls, Westar (darkly shaded) and Quinta (lightly shaded), respectively, were inoculated 8 days after planting and scored 8 days after inoculation. Per line 78 to 111 progeny were tested. Within each generation, transgenic lines are sorted left to right from lowest to highest mean percent mortality. Scores with the same letter are not significantly different at $p = 0.05$ (Duncan's Multiple Range Test).

lation. As seen in Figure 4c, both transformed and untransformed plants exhibited large lesions at 48 h. However, as shown in Figure 7, the mean lesion diameter on DRR206 transformants was almost always at least 0.5 cm smaller than in Westar controls. Comparable results were seen over 5 generations, from T_3 to T_7.

In all cases where plants were allowed to incubate past the 48 h assay period, both DRR206 transformants and untransformed plants died. We conclude that while DRR206 appears to cause some decrease in the rate at which fungal necrosis propagates through leaf tissue, DRR206 transformants could not be considered resistant to *Sclerotinia*.

Discussion

While there was no *a priori* reason to assume that DRR206-mediated resistance would be limited to *L. maculans* PG2, it was necessary to learn something of the scope of the resistance provided by DRR206 to fungi with different modes of pathogenicity. Resistance to PG3 and PG4 was comparable to that observed previously with PG2. In particular, resistance to PG4 is significant because no good resistance genes to PG4 are currently available in *B. napus.* In contrast to the hemibiotrophic *L. maculans*, which grows intercellularly prior to the onset of necrosis, *Rhizoctonia* infection is characterized by growth of hyphae on the hypocotyl surface to form an 'infection cushion'. In susceptible interactions, hyphae penetrate the cuticle and ultimately invade vascular tissue, resulting in damping off of the seedling (Yang et al. 1992).

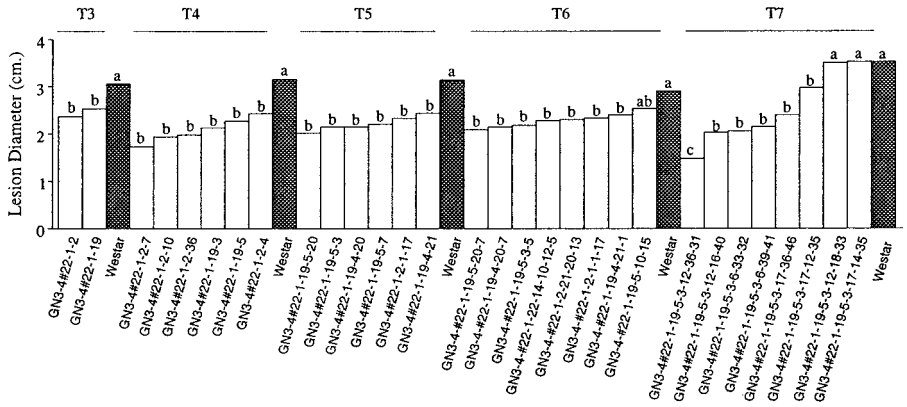

Figure 7. Lesion diameter (cm) in leaves of DRR206 transformant lines inoculated for 48 h with *Sclerotinia sclerotiorum*. Each Westar control included 15 plants and each transgenic line included 15 to 20 plants. Four replicates were done per line per generation. Within each generation, transgenic lines are sorted from left to right from lowest to highest mean lesion diameter. Scores with the same letter are not significantly different at $p = 0.05$ (Duncan's Multiple Range Test).

While *R. solani* is not at present a pathogen of major economic importance, the more broad the resistance, the more useful the gene. Resistance to *R. solani* has also been reported in transgenic *B. napus* carrying 35S-bean chitinase constructs (Broglie et al. 1991; Benhamou et al. 1993).

The necrotrophic fungus *Sclerotinia* is unique as a pathogen, both in terms of its unusually wide host range, as well as the rarity of resistance to this fungus among crop plants. At least two important factors contribute to pathogenicity. The most commonly-studied pathogenicity factor is the production of oxalic acid, which is toxic to host cells (Callahan and Rowe 1991). Hydrolytic enzymes such as polygalacturonate lyases have also been implicated in pathogenicity. Together, these mechanisms enable *Sclerotinia* to kill cells in advance of fungal growth, a strategy for which many hosts have not been able to evolve good defense mechanisms. In *B. napus*, only partial resistance to *Sclerotinia* has been found in chemically induced mutants in which resistance appeared to be associated with increased production of antifungal phenolic compounds (Mullins et al. 1995). In this context, it may not be surprising that DRR206-mediated resistance was limited to a small decrease in lesion diameter.

The results also indicate that DRR206-mediated resistance was not specific to a single blackleg pathogenicity group. In fact, differences from untransformed plants were seen with fungi with 3 distinct modes of pathogenicity. In many cases other workers have found that resistance mediated through constitutively expressed defense genes is often effective against more than one species of pathogen. Constitutive expression of a bean endochitinase in *B. napus* resulted in lower disease severity, under field conditions, with *Cylindorsporium concentricum*, *Phoma lingam* (*L. maculans*), and *Sclerotinia sclerotiorum* (Grison et al. 1996). 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 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).

Gene silencing often occurs in transgenic lines propagated over several generations, usually when sequences homologous to the transgene are present, or when multiple copies of a transgene are present (Flavel 1994). The fact that DRR206-mediated resistance was stable in these experiments at least through the T_7 generation, may be in part due to experimental conditions. First, all DRR206 transgenic lines were pre-screened to select lines with single-copy insertions (Wang et al. 1999). Secondly, the pea DRR206 gene used in these transgenic plants was unable to detect endogenous homologues of DRR206 in *B. napus*, when used as a hybridization probe (D.-H. He and B. Fristensky, unpublished results). This may indicate that either DRR206 is not present in *B. napus*, or that homologous DRR206 genes are widely diverged in sequence from the pea gene.

Finally, these results show that DRR206-mediated resistance is effective in stems and cotyledons against *L. maculans,* in roots and hypocotyls against *R. solani*,

and, to some extent, in leaves against *S. sclerotiorum*. There is therefore no evidence that DRR206 activity is limited to any specific organ. In this context, it is interesting to note that in pea roots, DRR206 is expressed in response to inoculation by mycorrhizal fungi such as *Glomus mosseae* (Ruiz-Lozano et al. 1999). Roots of Myc$^-$ mutants which resist mycorrhizal symbiosis show elevated levels of DRR206 and other defense transcripts, compared to wild type plants. Taken together with previous work (Wang et al. 1999), our results establish that constitutive expression of pea DRR206 can provide resistance to fungi with a range of modes of pathogenicity, in most parts of the plant, and that this resistance can be maintained stably over several generations. These data point to DRR206 as a promising tool for genetic engineering of disease resistance in a range of plant hosts against a range of plant pathogens.

While this work was nearing completion, Lewis and coworkers reported dirigent ('guide') proteins from *Forsythia* and other woody plants which direct the stereospecific coupling of two coniferyl alcohol radicals to form the lignan (+) pinoresinol (Gang et al. 1999). Members of this protein family share about 60% amino acid sequence identity with DRR206. While sequence identity strongly suggests that DRR206 may be involved in lignan synthesis, its precise function or functions in plant defense could fall in several areas. DRR206 may (1) produce (+) pinoresinol or other lignans, (2) produce some other phenolic product. In response to pathogenic attack, the product may be a precursor for lignin synthesis to strengthen cell walls. Alternatively, the product may drive the lignification of pathogen structures, inhibiting hyphal growth. Third, the product may have other fungitoxic or fungistatic properties. Finally, the product might have epistatic or pleiotropic effects which activate or enhance other defenses. All of these models are consistent with the results presented in this paper. Further work will be required to determine which of these models is correct.

Acknowledgments

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