

Requirement for either a host- or pectin-induced pectate lyase for infection of *Pisum sativum* by *Nectria hematococca*

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Fungal pathogens usually have multiple genes that encode extracellular hydrolytic enzymes that may degrade the physical barriers in their hosts during the invasion process. *Nectria hematococca*, a plant pathogen, has two inducible pectate lyase (PL) genes (*pel*) encoding PL that can help degrade the carbohydrate barrier in the host. *pelA* is induced by pectin, whereas *pelD* is induced only in planta. We show that the disruption of either the *pelA* or *pelD* genes alone causes no detectable decrease in virulence. Disruption of both *pelA* and *pelD* drastically reduces virulence. Complementation of the double disruptant with *pelD* gene, or supplementation of the infection droplets of the double disruptant with either purified enzyme, PLA, or PLD, caused a recovery in virulence. These results show that PL is a virulence factor. Thus, we demonstrate that disruption of all functionally redundant genes is required to demonstrate the role of host barrier-degrading enzymes in pathogenesis and that dismissal of the role of such enzymes based on the effects of single-gene disruption may be premature.

The role of fungal extracellular enzymes that can degrade physical barriers during the invasion of the host has been controversial. A variety of lines of evidence for their role in pathogenesis, including the correlation between the level of such enzymes and virulence, immunocytochemical evidence for the secretion of the enzyme during infection, protection of the host by inhibition of the enzymes with selective inhibitors or specific antibodies, and enhancement of virulence by gene transfer, have been challenged on the basis of the results of disruption experiments on genes that encode cutinase and cell wall-degrading enzymes (1–14). In the case of animal pathogens, single and double proteinase gene disruption failed to show a clear decrease in virulence (15–17). The inability to demonstrate a drastic decrease in virulence by such gene disruption has been interpreted as casting doubt on the role of extracellular enzymes that degrade physical barriers of the host in pathogenesis (18). However, highly pathogenic fungi usually have multiple genes encoding enzymes that degrade the polymer barriers of the host (5–7, 9, 14, 19), and some may be expressed only in the host (20). Therefore, the results from gene disruption experiments may not be able to reveal the true role of the extracellular enzymes in pathogenesis unless the genes that can encode all of the functionally redundant enzymes are disabled. Here we report the first example of loss of virulence by disrupting genes encoding both of the two functionally redundant enzymes, either of which can function alone as virulence factor.

A plant pathogen, *Nectria hematococca*, which infects pea plants, produces several pectin-degrading enzymes, presumably to help penetrate cell-wall carbohydrate barriers in the host during infection. Such enzymes include endopectate lyases that are encoded by at least four pectate lyase (PL) genes (*pel*) that belong to a new class of lyases, based on the lack of the consensus signature sequences of other PL and the inability to fit the structure-based alignments of other PL (20). Two are inducible: *pelA* by pectin (21) and *pelD* only by the host (20), whereas *pelB* and *pelC* are only constitutively expressed and only at very low levels. Thus, the inducible *pelA* and *pelD* genes were selected for

study as candidates as virulence factors. Here we report that disruption of either *pelA* or *pelD* alone does not reduce virulence, but disruption of both *pelA* and *pelD* reduces virulence drastically. Complementation of the double gene disruptant with a *pelD* gene or supplementation of the infection droplets containing the double disruptant with either of the two purified enzymes, PLA or PLD, causes a recovery of virulence, demonstrating that PL is a virulence factor.

Materials and Methods

Fungal Strain and Growth Conditions. *N. hematococca* (*Fusarium solani* f. sp. *pisi*) was a field isolate, T8, obtained from H. D. VanEtten (University of Arizona, Tucson, AZ) (22).

Vectors, Enzymes, and Chemicals. The pSV40/Zeo and zeocin were from Invitrogen. Plasmids were propagated in *Escherichia coli* DH5 α . Restriction and modifying enzymes, *Taq* polymerase, DH5 α cells, 123-bp DNA ladder, and TRIZol reagent were from Life Technologies (Gaithersburg, MD). Expand High Fidelity *Taq* polymerase was from Boehringer Mannheim, Novozyme 234 from InterSpex Products (Foster City, CA), Driselase and α -glucuronidase were from Sigma and hygromycin, from Calbiochem. Primers were from Integrated DNA Technologies (Coralville, IA). Rediprime random-primed labeling kit and enhanced chemiluminescence Western blotting detection system were from Amersham International.

Construction of Gene Replacement Vector pPELA::hph. pG3S2 (21) carrying a 4.7-kb *SstI* fragment of the *pelA* gene was digested with *SacII* to generate a 895-bp fragment that included 312 bp of the 3' end of the *pelA* open reading frame (ORF) and a 6.69-kb fragment containing the pBluescript vector flanked by 3.1 kb of the 5' end of the *pelA* gene (including 417 bp of the ORF) and 625 bp of the 3' noncoding region. The 6.69-kb fragment was filled in with Klenow and dNTPs. The 2.5-kb *hph* construct carrying *hyg* gene with a *Cochliobolus heterostrophus* promoter (23) was restricted from a pBluescript vector with *BamHI*, filled in with Klenow, and ligated to the blunt-ended *pelA* fragment to yield the *pelA*-disrupted construct pS2H2 with *hph* in the opposite orientation to *pelA*. To extend the 3' flanking region of the *pelA* gene in the construct pS2H2, a 2.75-kb *SstI* and *XbaI* fragment flanking the 3' noncoding region of *pelA* was ligated to the *BamHI/SstI*-digested pS2H2, yielding the 8.5-kb *pelA* disruption construct, pPELA::hph (Fig. 1A).

Abbreviations: *pel*, pectate lyase gene; PL, pectate lyase; RT-PCR, reverse transcription-PCR; ORF, open reading frame.

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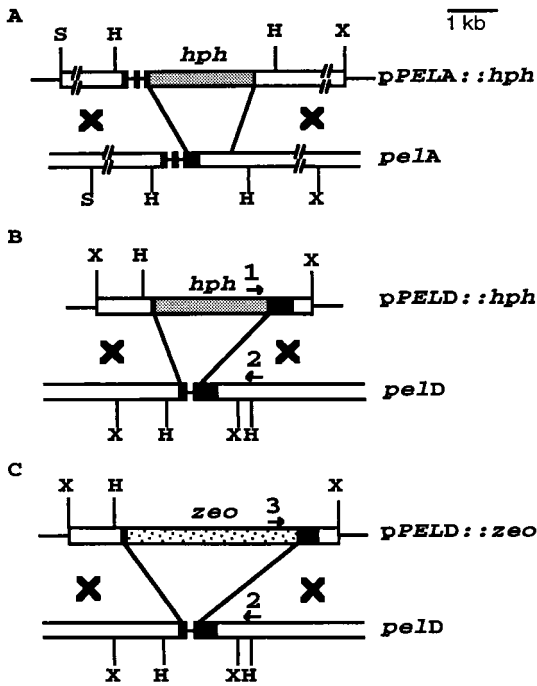


Fig. 1. The replacement vectors for the *pel* genes in *N. hematoococca*. Open boxes, the 5' and 3' flanking regions of the *pel* genes; black boxes, the *pel* gene exons; restriction sites, H, *HindIII*; X, *XbaI*; and S, *SstI*. Primers 1 and 2 were used for detecting homologous recombination of *hph*-disrupted *pelD* and primers 2 and 3 for *zeo*-disrupted *pelD*.

Construction of Gene Replacement Vector pPELD::hph. The *EcoRI* site in the *pelD* gene clone (20), pX55, was eliminated, and a fragment of pX55 with a deletion of 250 bp from the coding region of *pelD* was amplified by an inverse PCR with a sense primer, 5'-GCG CGC GAA TTC GTC TTC ATC CTC GAG GAG-3', and an antisense primer, 5'-GGC CGG ACT AGT CGA GGA CAG TGA CGA TGC-3', with Expand High Fidelity *Taq* DNA polymerase. The 5-kb PCR product was treated with T4 DNA polymerase and dNTP to remove any overhang A-nucleotide, and a *SalI* fragment carrying the *hyg* gene with *Aspergillus nidulans trpC* promoter from pCSN43 (24) was ligated to generate pPELD::hph (Fig. 1B).

Construction of Gene Replacement Vector pPELD::zeo to Produce *pelA*, *pelD* Double Disruptant. A sense primer 5'-GGA GGG CCA CCA TGG CCA-3' and an antisense primer 5'-TGC CAT GGG GTC GGC GTC GGT CAG TC-3' were used to amplify the zeocin resistance gene with *Pfu* polymerase and pSV40/*Zeo* vector as a template. The product was cut with *NcoI* and cloned into the *NcoI* site of pNOM102 (25) to generate pNOM102::zeo, placing the expression of the *zeo* gene under the control of the *Aspergillus nidulans gpd* promoter. pNOM102::zeo was cut with *HindIII*, partially filled in with Klenow in the presence of dATP and dGTP, and then cut with *EcoRI* to release the zeocin cassette. The 5-kb *pelD* PCR fragment obtained above by inverse PCR was cut with *SpeI* and partially filled in by Klenow in the presence of dCTP and dTTP, then cut with *EcoRI* and the zeocin cassette was ligated to generate pPELD::zeo (Fig. 1C).

Complementation of *pelA*, *pelD* Double Disruptant with *pelD* Gene. The *SalI* fragment (2.0 kb) released from pX55 (20), which contains 1.0 kb of 5'-flanking region, coding region, and 300 bp of 3'-flanking region of *pelD*, was ligated to *XhoI*-digested pCSN43, and the resulting vector was used to transform *pelA*,

pelD double disruptant. Because the double disruptant used does not show a high degree of hygromycin resistance, complemented transformants could be selected on hygromycin (200 μ g/ml)-containing PDA. Transformants were tested for intact *pelD* by PCR with *pelD* gene primer: 5'-GCT CCG CAG CAA TCT CCA G-3' and a primer from *hph* gene: 5'-CTA GCT CCA GCC AAG CCC-3'. *pelD* gene expression was tested by reverse transcription-PCR (RT-PCR) with *pelD*-coding region primers.

Transformation of Protoplasts. Protoplast preparation and transformation were performed as before (22). For the double disruptant, the *pelA* disruptant was transformed with the *pelD* disruption construct pPELD::zeo. DNA and PEG4000-treated protoplasts (10^5) in 200 μ l of STC (1.2 M sorbitol/10 mM Tris, pH 7.5/10 mM $CaCl_2$) were mixed with 10 ml of regeneration medium (mineral medium containing 1.2 M sorbitol/2% glucose/1% agar) at 45°C and overlaid on a previously poured layer (12 ml) of regeneration medium containing 300 μ g/ml zeocin. Transformants were regrown on selection medium, and conidia were produced on nonselective medium.

PCR Screening Directly on Spores as the Template Source. Spores (10^4 - 10^5) were mixed with PCR buffer/1.5 mM $MgCl_2$ /200 μ M of each dNTP/4% DMSO/25 ng of each primer in a total volume of 48 μ l and heated at 94°C for 15 min; 2.5 units of *Taq* DNA polymerase was then added to each reaction mixture (36 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, and polymerization at 72°C for 45 s, final extension reaction at 72°C for 10 min). The primers used for the identification of *pelA* disruptants were sense, 5'-GTT CAC TGC TGC TTT CGT-3' from the 5' end of the ORF of *pelA*, and antisense, 5'-GGA GCC AGT AAC AGT GCA G-3', from the 3' end of the *pelA* ORF that was deleted in making the construct. For *pelD* disruption with *hph*, a sense primer from *hph* gene, 5'-GAG CTC TCC AAC ATT GAT G-3', and an antisense primer from the *pelD* gene, 5'-GAG CTC TCC AAC ATT GAT G-3' (outside the region used for gene replacement construct), were used to amplify an expected junction PCR product of 995 bp; for *pelD* disruption with *zeo*, a sense primer from *zeo* gene, 5'-CTT CAA GTC AGC CAA CTG C-3', and an antisense primer from *pelD* gene, 5'-GAG CTC TCC AAC ATT GAT G-3' (outside the segment used for gene replacement construct), were used to amplify an expected junction PCR product of 980 bp.

Genomic Southern Analysis. Genomic DNA of wild-type and single or double disruptants of *pelA* and *pelD* was prepared (26), digested with *HindIII*, and subjected to Southern blot analysis (27).

Gene-Specific RT-PCR with RNA from Pea Stems Infected with *N. hematoococca*. The preparation of pea stem and infection with conidia of *N. hematoococca* were performed as described (28). Total RNA was isolated from the infected plant tissue as described (20). RNA (1 μ g) was treated with amplification-grade RNase-free DNase and reverse transcribed into cDNA with SuperScript II H⁻ reverse transcriptase with oligo (dT) in a 40- μ l reaction mixture. A 5- μ l aliquot of the first-strand cDNA was used for PCR in a 50- μ l reaction mixture. The sense and antisense primers used were 5'-CAA GAC TCT CCC CAA GAG-3' and 5'-GGA GCC AGT AAC AGT GCA-3' for *pelA* (expected PCR product, 654 bp); 5'-TAC CGT TCT TCC CGC TTC-3' and 5'-ACC GGA AGA GCG GTC GAT-3' for *pelB* (expected PCR product, 208 bp); 5'-GCA GCA AGT CTC TGA GCG-3' and 5'-GCA GTG CCG CTG CCA AGG-3' for *pelD* (expected PCR product, 332 bp). Aliquots of the PCR products were analyzed on 2% agarose gels. In all cases, the identities of the PCR products were verified by DNA blot hybridization.

Immunoblot Analysis. Multiple droplets of conidial suspension of the wild-type and *pel* gene-disrupted *N. hematococca* (5×10^4 per $5 \mu\text{l}$) were placed on sections of etiolated pea stem as described (28). After 4 days, the infected pea stems were ground in liquid N_2 , resuspended in an equal volume of H_2O , and centrifuged at $12,000 \times g$ for 10 min. The supernatants were dialyzed against H_2O and concentrated 10-fold with a Centricon 10 concentrator. Aliquots were subjected to SDS/PAGE with 12% resolving gel for Western blot by using antibody raised against pectate lyase C; the four PL are immunologically cross-reactive (20, 29).

PL Induction and Assay. Spores (5×10^7) in water were incubated with sterile pea stems for 4 days at 24°C . Mycelia and infected stems were frozen and ground in liquid N_2 , and the extract supernatant prepared as above was dialyzed, concentrated, and assayed for PL activity (30).

Virulence Assays. Bioassay on etiolated pea stem segments was performed as described (28). Where indicated, the conidial suspension also contained purified enzyme, either PLA or PLD. Degree of penetration was assessed by microscopic examination of stained cross section of the tissue from the middle of the lesion. A hand-sectioned slice fixed in 50% ethanol containing 5% glacial acetic acid and 13% formalin for 20 min was stained with cotton blue-lactophenol for 10 min followed by removal of excess stain by washing with lactophenol. The number of cell layers penetrated in at least 30 sections was scored under the microscope, and the average is shown for each assay.

The second bioassay involved a modification of the whole-plant assay (31). In the modified-growth assay, sterile soil was used in place of vermiculite, and the pea seeds were soaked in aerated water for 15 h, germinated for 24 h at 24°C , and then planted in the spore-impregnated soil.

Results

Disruption of Only *pelA*. *N. hematococca* contains a pectin-inducible *pelA* gene (25) and a host-inducible *pelD* gene (20). *pelA* was disrupted by using a construct containing about 3 kb each of 5'- and 3'-flanking regions of *pelA* gene with a 895-bp internal segment replaced with a hygromycin resistance gene (*hyg*) (27). The transformants were screened by PCR with the spores directly as the source of the template. One of the primers used was from the 3' end of the ORF of *pelA* that was replaced with *hph* gene in the disruption construct and therefore the disruptant did not give the 848-bp PCR product expected from the native *pelA* gene (data not shown). PCR with *hph* gene-specific primers gave a product at 880 bp, as expected (data not shown). Gene disruption was confirmed by Southern hybridization that showed the 2.2-kb band from *HindIII* digestion of the wild type was shifted to ≈ 4.0 kb in the disruptant as expected (Fig. 2A Left). RT-PCR of RNA isolated from infected pea stems showed the absence of *pelA* transcripts and presence of *pelD* transcripts in the fungus growing on the host (Fig. 2B). No PL activity or immunologically cross-reacting protein could be detected when the *pelA*-disrupted mutant was cultured in the presence of pectin. Furthermore, on growth on the pea epicotyl tissue that induces both *pelA* and *pelD* in the wild type, the expected 23-kDa PLD and 26-kDa PLA were detected by immunoblot whereas the *pelA* disruptant showed only 23 kDa (PLD) (Fig. 3A). PL B and C were not seen in these immunoblots, probably because of very low levels of expression. To test whether *pelA* disruption affected virulence, lesion formation caused by the conidia of the *pelA* disruptant on pea epicotyl segments was assessed (28). The *pelA* disruptant and the wild type showed identical time course of formation of equally severe lesions (Fig. 3B). Inclusion of antibodies prepared against PLA in the inoculation droplet protected the host from infection by

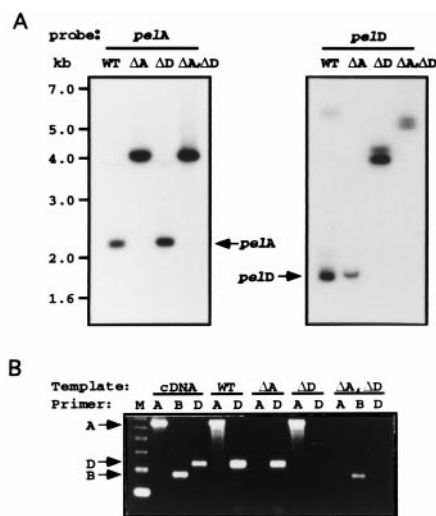


Fig. 2. (A) Genomic Southern blots showing the absence of native genes and appearance of new fragments from the disrupted genes in the mutants. Genomic DNA ($10 \mu\text{g}$ each) was digested with *HindIII* and probed with cDNAs of *pelA* (Left) and *pelD* (Right). Size markers shown on the left apply for both panels. (B) RT-PCR showing the absence of transcripts from the disrupted genes and the presence of transcripts from the native genes. Gene-specific RT-PCR amplification of *pelA*, *pelB*, or *pelD* was done by using gene-specific primers (20) and RNA isolated from *N. hematococca*-infected pea epicotyl. WT, wild type; ΔA , *pelA* disruptant; ΔD , *pelD* disruptant; and ΔA , ΔD : *pelA* and *pelD* double-gene disruptant. Position of the expected products from the three genes are shown by the arrows. Molecular size marker (M), 123-bp DNA ladder.

both the wild type and *pelA* disruptant (Fig. 3B). This result suggested that, even in the absence of PLA, another immunologically similar PL probably was produced in sufficient amounts to achieve penetration through the pectinaceous barrier of the host. In fact, an extract of pea stem infected by a *pelA* disruptant showed 60% of the lyase activity observed with the wild type.

Disruption of Only *pelD*. The *pelD* gene was disrupted by protoplast transformation with a construct made from a 2.7-kb *pelD* gene fragment with the replacement of a 250-bp internal coding segment of *pelD* with the *hyg* gene (24). The hygromycin-resistant transformants were screened by PCR with spores directly as the source of the template. PCR, by using a sense

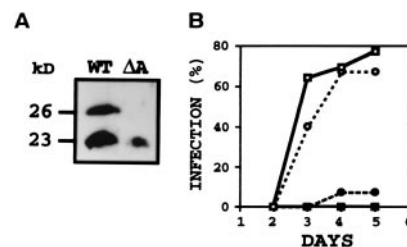


Fig. 3. (A) Immunoblot analysis of proteins from *N. hematococca* wild type (WT) or *pelA* disruptant (ΔA) grown on pea epicotyl sections showing products of *pelA* (26 kDa) and *pelD* (23 kDa) in WT and only *pelD* product in the *pelA* disruptant. After 5 days of growth at 24°C (28), the soluble proteins were subjected to immunoblot by using anti-PLC (29) and enhanced chemiluminescence detection system. (B) Time course of infection of pea epicotyl sections by WT or *pelA* disruptant in the presence of antibody to PLA or preimmune IgG. Assay was done with 10^4 conidia and $10 \mu\text{g}$ of IgG (28, 31). Infection in the presence of water only was identical to that in the presence of preimmune IgG. (○) WT, preimmune; (□) ΔA , preimmune; (●) WT, anti-PLA; and (■) ΔA , anti-PLA. % infection, % of epicotyl sections that showed lesion.

primer from the *hph* gene and an antisense primer from the 3'-side of the *pelD* gene from a region outside that which was used for making the gene replacement construct, yielded a 995-bp product expected from homologous recombination (data not shown). When the *Hind*III digestion product of the genomic DNA from the putative disruptant was subjected to Southern hybridization, the *pelD*-containing fragment was found at 4 kb, whereas the native *pelD* gene was found at 1.7 kb, showing *pelD* disruption (Fig. 2A Right). RT-PCR of RNA isolated from infected pea stems did not show *pelD* transcripts with the *pelD*-disrupted mutant, whereas the *pelA* transcript was readily detected; with wild type, both *pelA* and *pelD* transcripts were found (Fig. 2B). These results showed that the disruptant did not express *pelD* in the host.

Double Disruption of Both *pelA* and *pelD*. To disrupt both *pelA* and *pelD*, *pelA* disruptant was transformed with a *pelD* disruption construct containing a zeocin resistance gene under the control of *gpd* promoter from *A. nidulans* (25) that replaced a 250-bp internal coding segment of a 2.7-kb *pelD* gene segment. The transformants resistant to zeocin were screened for double disruptants by PCR on the conidia of the transformants. With primers representing the *zeo* gene and a segment of *pelD* gene outside that used for making the gene replacement construct, the 980-bp product, expected from homologous recombination, was found in the disruptant (data not shown). Southern hybridization of the genomic DNA of the *pelA*, *pelD* double disruptant showed the expected absence of hybridization bands representing the genomic fragments containing the native *pelA* and *pelD* (Fig. 2A). To test for the expression of the *pel* genes, spores of the disruptants were used to inoculate pea epicotyl tissue and the extracted RNA was subjected to RT-PCR with gene-specific primers (20). The double disruptant showed the lack of a 654-bp product expected from native *pelA* gene and lack of a 332-bp product expected from native *pelD* gene (Fig. 2B), but showed the expected low level of product (at 208 bp) from a constitutively expressed *pelB* (32) (Fig. 2B). Immunoblots of proteins from host tissue incubated with the double disruptant showed the absence of both *pelA* and *pelD* products (data not shown).

Tests for Virulence of the *pel* Gene Disruptants. Virulence of the wild-type *N. hematococca* and its gene-disrupted mutants was tested in two different ways. When conidial spore suspension was placed on pea epicotyl segments (24), the wild-type fungus and the *pelA* or *pelD* single-gene disrupted mutants produced lesions on most epicotyl sections (Fig. 4 a, b, and c), suggesting that absence of one of the two inducible lyases, PLA or PLD, had no impact on the virulence. The *pelA*, *pelD* double gene disruptant showed very few mild lesions that did not extend deep into the tissue (Fig. 4d). Examination of the cross section of the inoculated areas after staining the fungus with lactophenol cotton blue revealed that the wild-type and *pelA* or *pelD* single gene-disrupted mutants penetrated extensively and deeply into the host, whereas the *pelA*, *pelD* double gene-disrupted mutant did not penetrate deep into the tissue (Fig. 4h). As infection advanced, a great amount of tissue degradation and maceration could be seen extending deep into the tissue, reaching the central vascular bundles with the wild-type and single gene disruptants. At the same time, the double-gene disruptant seldom reached more than one or two cells in depth into the tissue with little tissue damage.

In a second test for virulence, conidia were mixed with the soil before planting pea seeds, and the seedlings were examined for infection. With the wild type or *pelA* or *pelD* single-gene disruptants, severe lesions were visible, with stunted growth for those seedlings that emerged from the soil. The seedlings from the soil inoculated with *pelA*, *pelD* double disruptant showed the same degree of growth as the seedlings in the noninoculated soil

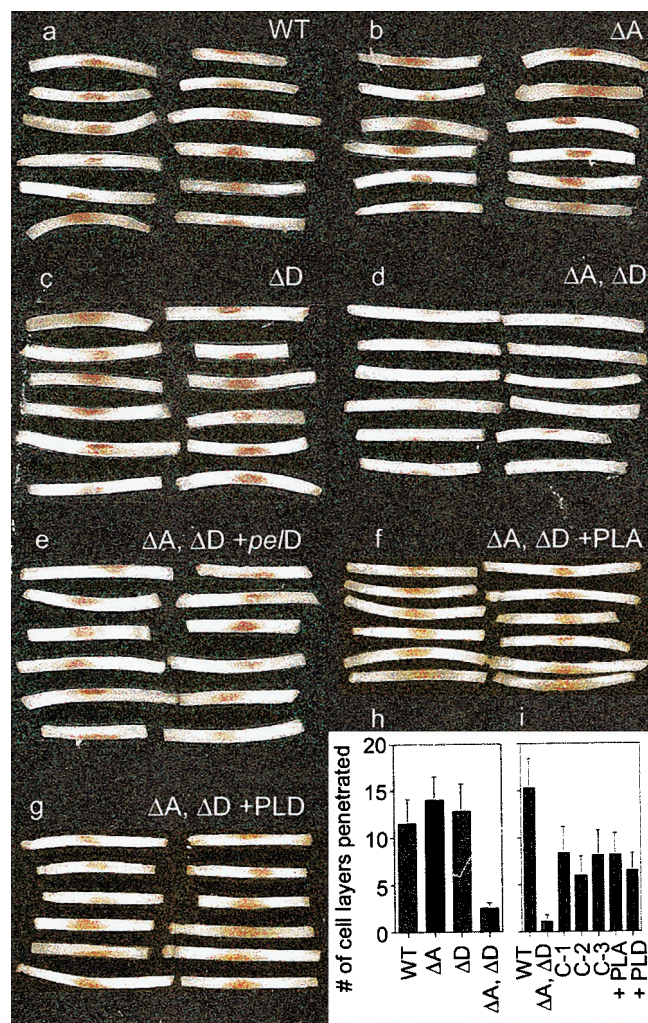


Fig. 4. Infection of pea epicotyl sections by (a) wild type; (b) *pelA* disruptant; (c) *pelD* disruptant; (d) *pelA*, *pelD* disruptant; (e) *pelD*-complemented *pelA*, *pelD* disruptant of *N. hematococca*; (f) infection droplets of *pelA*, *pelD* double-disruptant supplemented with PLA; or (g) double disruptant supplemented with PLD – lesion formation is seen in all cases except with *pelA*, *pelD* double-gene disruptant. (h) Mycelial penetration of pea epicotyl sections inoculated with the wild type or the indicated single and double disruptants. (i) Penetration by the wild-type, double-disruptant, three *pelD*-complemented double disruptants (C-1, C-2, and C-3), and double-disruptant spore suspension supplemented with purified PLA (100 ng) or PLD (40 ng). Assay with 10^6 conidia (28).

and had only a few very mild lesions (Fig. 5). Under conditions of more severe infection, no seedlings emerged from the soil when the wild-type or single-gene disrupted mutants were used, but *pelA*, *pelD* double disruptant allowed the growth of seedlings with some lesions (data not shown). Clearly, *pelA*, *pelD* double disruptant showed drastically decreased virulence with both tests for virulence.

Complementation of the Double-Gene Disruptant with *pelD*. If the loss of virulence resulting from the double-gene disruption is in fact caused by the inability to produce PL needed for penetration, introduction of *pelD* gene into the double-gene disruptant should enable it to infect its host. In fact, expression of lyase in such complemented transformant clearly showed recovery of pathogenicity (Fig. 4e) and host penetration (Fig. 4i). The PL activity found in the extracts of pea stem infected with the wild-type and gene-disrupted and -complemented mutants

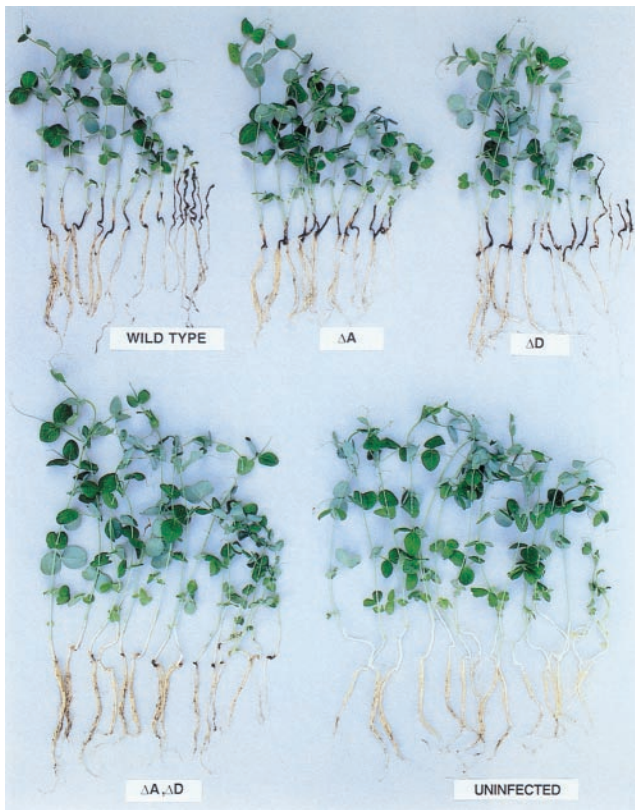


Fig. 5. Virulence of wild-type and *pel* gene disruptants as shown by the growth of pea seedlings raised from seed in soil mixed with the conidia; 3.7×10^5 conidia/g soil, 12 days of growth as in ref 30. Designations of disruptants as in Fig. 2.

strongly supports the conclusion that the observed loss and recovery of virulence resulted from the loss and recovery of the ability to produce lyase, respectively. For example, extracts of pea stem infected with either *pelA* or *pelD* disruptants showed 60 and 80%, respectively, of the activity observed with the wild type, whereas the double-gene disruptant did not show detectable lyase activity. On the other hand, *pelD*-complemented transformants showed 10–20% of the lyase activity observed with the wild type and a recovery of virulence because most stem segments inoculated with the complemented mutants showed lesions (Fig. 4e). Microscopic examination of tissue slices showed that the complemented double mutants penetrated to about half of the depth penetrated by the wild type, whereas the double disruptant hardly penetrated into the tissue (Fig. 4i). Because the complemented mutants did not produce PL levels anywhere near that of the wild type, it is not surprising that the observed degree of recovery of virulence was not complete. However, the observed recovery is a clear indication that the loss of virulence resulting from the double disruption was caused by the absence of the lyase.

Supplementation of the Infection Droplets of the Double-Gene Disruptant with Purified PLA or PLD. Inclusion of purified PLA or PLD in the infection droplet resulted in a recovery of virulence, as seen by lesion formation on virtually all host segments (Fig. 4f and g). Enzyme droplets without conidia showed no symptoms. With exogenous lyase, penetration of the double disruptant into the host tissue reached about 50% of that observed with the wild type (Fig. 4i), whereas the double disruptant failed to penetrate into the tissue or produce visible lesions in the absence of enzyme supplementation. Exogenous enzyme added into the

droplet in which the conidia are placed is not targeted specifically to the point of contact between the germ tube and the host wall and therefore cannot be expected to be as fully effective as the enzyme naturally secreted at the point of penetration by the germ tube.

Discussion

The occurrence of multiple genes that encode functionally redundant enzymes that degrade the physical barriers in the host is a common feature of fungal pathogens that infect plants and animals. For example, *Candida* is known to have at least seven genes that encode secreted aspartic proteinases (9, 33). *Aspergilli* that cause invasive aspergillosis are known to express genes that encode serine proteinases (34–37), metalloproteinases (38–40), and aspartic proteinases (41). Disruption of one or two of these does not show a significant decrease in virulence (15–17). When one aspartic proteinase gene was disrupted, immunologically crossreactive proteinase(s) was found to be associated with the fungal wall (42), suggesting the presence of multiple aspartic proteinases. Multiple genes for cutinases and pectin-degrading enzymes are found in plant pathogenic fungi (5, 19, 43, 44). In such cases, single-gene disruption is not likely to reveal decreases in virulence. In addition, some genes that encode degradative enzymes such as the *pelD* of *N. hematococca* may be expressed only in the host (20), or different genes may be expressed on different organs or during different stages of growth or infection (45, 46). In such cases, even finding such enzymes may be difficult, as the commonly used culture conditions would not allow expression of such genes. The type of homology that allowed us to clone *pelD* does not have to hold true for functionally homologous enzymes. Another limitation of the gene disruption approach, in cases where multiple genes are involved, is the possibility of compensatory enhancement of expression of other genes accompanying disruption of a gene that encodes an enzyme with the same function. Disruption of serine proteinase caused an increase in metalloproteinase in *Aspergillus flavus* (47).

The presence of multiple genes that encode enzymes that can degrade the physical barriers of the host provides the pathogen an evolutionary advantage in that the differential regulation of expression of such genes would allow the pathogen to express the appropriate gene when the ecological need requires its expression. Usually, there is a low level of constitutive expression of the degradative enzymes and therefore the conidium would arrive at the host surface with a small amount of the enzyme. The cutinase and PL carried by *N. hematococca* are examples. From this organism, we have cloned *pelB* (32) and *cut2* (T. Sirakova and P. E. Kolattukudy, unpublished work) that are constitutively expressed. On contact with the polymeric barrier of the host, the constitutively expressed enzyme would generate small amounts of hydrolytic products. These products would then activate the transcription of the inducible gene, generating a high level of the enzyme that can assist the pathogen to penetrate through the physical barriers of the host. This concept has been demonstrated for cutinase, and the transcription factors involved in the expression of the induced gene (48, 49) and the constitutively expressed gene (T. Sirakova, D. Li, and P.E.K., unpublished work) have been cloned from *N. hematococca*. In this fungus, the constitutively expressed gene, *pelB*, was suggested to provide the oligomers that induce *pelA*, because antisense expression of *pelB* prevented induction of *pelA* (W. Guo and P.E.K., unpublished work). To penetrate through the polymeric barrier of the host to infect it or grow saprophytically on the polymer, the microbe can use the same strategy of using a low level of constitutively expressed enzymes to sense the nature of the polymeric nutrient present in the immediate environment. The monomers could then transcriptionally activate the inducible gene to produce the enzyme either for infection or saprophytic growth. Separate

polymer-degrading enzymes specializing in saprophytic growth or pathogenesis (45, 46) may also exist.

Even though the presence of multiple genes can make gene disruption ineffective in decreasing virulence, antibodies prepared against a barrier-degrading enzyme may show protection of the host against infection, if the products of the multiple genes are crossreactive as seen with both PL (20, 31) and cutinases (5, 6, 28) of *N. hematococca*. Enzyme-active site-directed inhibitors can show protection, if the multiple gene products use the same catalytic mechanism, as seen with cutinases that might assist penetration into plant hosts (5, 6) and with *Candida* aspartic proteinases that assist infection of animals (33). In such cases, inhibitors of the enzymes have been found to protect the plant or animal hosts from infection (5, 6, 33). However, disruption of one or more genes cannot reveal the role of the extracellular enzymes if other genes are expressed to produce functionally redundant enzymes. The results obtained with the single-gene disruptants illustrate this point (1–4, 10, 11, 16, 17, 38). There are cases where single-gene disruption caused some decrease in

virulence (43, 50). In other cases, multiple-gene disruption failed to significantly decrease virulence (8, 12, 13). Therefore, conclusive evidence for or against a role for any particular enzyme activity in any aspect of pathogenesis has been difficult to obtain (51). A major decrease in virulence would require disruption of genes that encode all of the functionally redundant barrier-degrading enzymes that can play an essential role in pathogenesis. The present results clearly show how multiple gene products that catalyze degradation of host barriers, including those that might be uniquely induced by the host, can play an important role in pathogenesis and how single-gene disruption experiments can fail to reveal the true role of the extracellular polymer-degrading enzymes in fungal pathogenesis (18). The extracellular enzymes that degrade the physical barriers of the host may be suitable targets for antifungal therapy.

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