Analysis of two pathogenicity genes of the blackleg fungus *Leptosphaeria maculans*

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INTRODUCTION

The fungus *Leptosphaeria maculans* (anamorph = *Phoma lingam*) causes blackleg of oilseed Brassicas, including canola (*Brassica napus*) and Indian mustard (*B. juncea*). The lifecycle and infection pathway of the fungus have been described (Hammond *et al.*, 1985; Hammond and Lewis, 1987). Wind-borne ascospores germinate on the leaf and resultant hyphae enter via stomata or wounds on the leaf or cotyledon surface, without the use of specialized infection structures like appressoria. The fungus initially grows *in planta* as a biotroph and later becomes necrotrophic. Knowledge of the molecular basis of pathogenicity in *L. maculans* is at a preliminary stage and few genes have been characterized (Howlett *et al.*, 2001). In other plant pathogenic fungi, genes essential for disease development (pathogenicity genes) are being identified at an exponential rate and such discoveries are revealing new aspects of plant-pathogen interactions (reviewed by Idnurm and Howlett, 2001). One successful approach to identify novel pathogenicity genes has been restriction enzyme mediated insertional mutagenesis (REMI) - a reverse genetics approach whereby a plasmid conferring hygromycin resistance inserts randomly in the fungal genome in the presence of high levels of restriction enzymes. In this paper we describe the application of REMI to isolating pathogenicity genes of *L. maculans*.

MATERIALS AND METHODS

Culturing and transformation of L. maculans

L. maculans isolate M1 was chosen for analysis. It causes cotyledonary and stem lesions on all cultivars of *B. napus* and *B. juncea* tested and its ability to attack *B. juncea* is controlled by a single locus (Cozijnsen *et al.*, 2000). Gene targets sought by insertional mutagenesis included this host-specificity locus, as well as pathogenicity genes. Protoplasts of isolate M1 were transformed to hygromycin resistance using plasmid pUCATPH (Lu *et al*., 1994) in the presence of restriction enzyme (10-40 U of *Hin*dIII, *Kpn*I or *Sac*I) as described by Idnurm and Howlett (2002). Transformants were visible within two to three weeks, and were subjected to two rounds of hyphal tip culture onto 10 % Campbells V8 juice agar plates containing hygromycin (50 μg/ml) before transfer to non-selective media.

Pathogenicity tests

Transformants (516) were screened for the ability to infect cotyledons of 14 day old seedlings of *B. juncea* cv. Stoke as described by Purwantara *et al.* (1998). Transformants with reduced pathogenicity were retested on *B. juncea* and also on *B. napus* cv. Westar, and those that still had reduced pathogenicity were denoted loss-of-pathogenicity (LOP) mutants.

Nucleic acid manipulations

Genomic DNA and total RNA from *L. maculans* was isolated, resolved by gel electrophoresis and hybridized (Sexton and Howlett, 2000). Regions of *L. maculans* DNA flanking the pUCATPH sequence were obtained by inverse PCR and were radiolabelled and used to isolate wild-type copy of each of the mutated gene from a cosmid library of isolate M1 DNA. The inserts from hybridizing cosmids were subcloned and resultant fragments sequenced. Vectors for complementing the mutations were constructed by ligating subclones of the cosmid insert into the *Xba*I site of pAN8-1, a plasmid that confers phleomycin resistance (Mattern *et al*., 1988). Protoplasts of the mutants were transformed with these constructs as described above

and resultant transformants were selected for growth on 20 µg/ml phleomycin (Cayla, France). These putative complemented strains were inoculated onto *B. napus* cv. Westar cotyledons.

RESULTS AND DISCUSSION

Analysis of L. maculans *transformants*

In several independent experiments, hygromycin-resistant transformants of *L. maculans* were obtained at frequencies of 20-1000 per µg of plasmid. These frequencies did not correlate with concentrations or identities of restriction enzyme, or with plasmid quantity. To examine the pattern of integration of pUCATPH, DNA of 47 transformants was digested with the enzyme used in transformation, and one enzyme that cut only once in the plasmid. Southern blots showed the presence of pUCATPH sequences in all transformants, and no correlation between integration pattern (number of inserted copies) and amount of plasmid or identity of restriction enzyme. Only four transformants had a single copy of pUCATPH, another 12 had a single integration event with several copies of pUCATPH in tandem, and the other 31 transformants had multiple insertions. There was no evidence for 'perfect' REMI events, i.e. where the restriction site was restored upon integration of the plasmid. Of 516 hygromycin-resistant transformants, 12 consistently had reduced pathogenicity with either no lesions or lesions less than 30 % of the diameter of those caused by isolate M1 on *B. juncea* or *B. napus*. Growth rates of these mutants on minimal media and on 10 % V8 juice agar were comparable to those of the wild-type isolate M1. Because all 12 LOP mutants were unable to attack both *B. juncea (*Indian mustard) and *B. napus*, this indicates that in no case had the hygromycin resistance gene integrated into the locus conferring host-specificity on Indian mustard (Cozijnsen *et al.*, 2000). This is not surprising as not enough transformants were analysed to have a high probability of knocking out a particular single gene. The multiple insertion patterns of pUCATPH in nine of the LOP mutants discouraged us from characterizing *L. maculans* sequences flanking the insertions (Idnurm and Howlett 2003).

Characterization of two loss-of-pathogenicity mutants

Two mutants, LOP-B and LOP-F, which had single copies of pUCATPH were characterized further. Flanking DNA obtained by inverse PCR was used to screen a cosmid library to obtain the wild-type genes from isolate M1. For LOP-B, a 6088 bp sequence was obtained (GenBank AF525231) and an open reading frame of 529 amino acids was predicted with FGENESH software (www.softberry.com) with no database matches. There are no regions within this predicted protein with a targeting sequence or conserved domain. The pUCATPH sequence integrated into the promoter, 220 bp away from the predicted start codon of the gene. For LOP-F, a sequence including the isocitrate lyase gene (denoted *icl*1) was isolated (GenBank accession AY118108) and Southern blot analysis of isolate M1 and the *icl*1 mutant showed that deletion of the *icl*1 gene and an unknown amount of DNA occurred during integration of pUCATPH.

Isocitrate lyase is one of two enzymes in the glyoxylate pathway that is involved in the metabolism of two-carbon compounds such as acetate and fatty acids. Accordingly, growth of the *icl*1 mutant was tested on such carbon sources. The mutant was unable to grow on monolaurate (0.25 % Tween 20) or ammonium acetate (0.5 %) as sole carbon sources, in contrast to the wildtype isolate. Both the mutant and the wildtype grew on 0.25 % glucose. No transcript of *icl*1 was observed in the *icl*1 mutant, whilst in isolate M1 the gene was induced by starvation conditions and by 0.5% ammonium acetate. Transcription of *icl*1 in isolate M1 was detected in cotyledons of *B. napus* 14 days after infection, a time at which β-tubulin, a constitutively expressed gene that is an indicator of fungal biomass during infection, was detected. Pycnidiospores of the *icl*1 mutant had an extremely low germination rate, compared to that of wildtype or complemented strains, on water agar plates. This suggests that rather than a defect in spore germination leading to reduced pathogenicity, a lack of carbon (in the form of fatty acids) utilization *in planta* may be responsible for limiting growth; this hypothesis is supported by the finding that when 2.5 % glucose was added to the inoculum of the *icl*1 mutant, lesions of similar sizes to those caused by wildtype isolate M1 developed.

Microscopy of cleared cotyledon tissue of *B. napus* infected via wounding showed that both mutants (LOP-B - unknown gene and LOP-F - isocitrate lyase) colonised tissue, but grew much more slowly than isolate M1 *in planta*. When the wild-type copy of each gene was reintroduced into the appropriate mutants, the resultant

transformants tested formed larger lesions on cotyledons of *B. napus* than on those of the mutants.

Concluding Remarks

Inertional mutagenesis techniques such as REMI are useful in identifying pathogenicity genes. We have used it to identify two pathogenicity genes of *L.maculans* - one, isocitrate lyase with a role in fungal nutrition in planta, and the other, a gene with no database match. Several drawbacks to REMI including the generation of deletions and multicopy rather than single insertions were observed, as has been noted when this technique has been applied to other fungi (Kahmann and Basse, 1999). New mutagenesis methods including *Agrobacterium tumefaciens*-mediated transformation (Mullins *et al.* 2001) avoid problems associated with REMI, and are now being applied to analyse pathogenicity genes of *L.maculans*.

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