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Mutations to *LmIFRD* affect cell wall integrity, development and pathogenicity of the ascomycete *Leptosphaeria maculans*

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ABSTRACT

Maintaining cell wall integrity is essential for fungal growth and development. We describe two mutants with altered expression of a gene, *LmIFRD*, from the ascomycete *Leptosphaeria maculans*. Truncation of the *LmIFRD* transcript in a T-DNA insertional mutant led to slower germination, less sporulation and loss-of-pathogenicity towards *Brassica napus,* whereas silencing of the *LmIFRD* transcript led to increased germination, sporulation and earlier infection. The increased tolerance to cell wall lysing enzymes and cell wall-disrupting compounds of the T-DNA mutant contrasts with decreased tolerance of the silenced mutant and suggests altered cell wall integrity and accessibility to 1,3-linked glucan and chitin. Lectin binding experiments and monosaccharide analysis revealed altered polysaccharide content and structure within the cell wall of the *LmIFRD* mutants, notably increased 1,3-linked galactose and chitin within the cell wall of the T-DNA mutant. This is the first analysis of monosaccharide linkage composition of cell walls of spores and mycelia for any dothideomycete.

1. Introduction

Cell walls provide structural support as well as mediating exchanges between the cell and its environment. Despite its importance, little is known about the composition and structure of fungal cell walls with the exception of a few euascomycetes including *Aspergillus fumigatus* and *Penicillium janczewskii* and the hemiascomycete *Saccharomyces cerevisiae* (Latgé, 2007; Pessoni et al., 2005).

Although the fungal cell wall varies in composition and structure between different taxonomic groups, glucose comprises between 50% and 60% of the total monosaccharides (Guest and Momany, 2000; Pessoni et al., 2005). The stability or integrity of the cell wall is achieved via various cross-linkages between polysaccharides, including glucan, chitin and mannan, and cell wall associated glycoproteins, some of which contain a glycosylphosphatidylinositol (GPI) anchor (for review see Bowman and Free (2006), Latgé (2007)). Mutations in genes encoding polysaccharide synthases, particular glycoproteins, transcription factors, and mitogen activated protein (MAP) kinases can result in changes in cell wall integrity due to altered cell wall content or assembly of the various components. This can affect hyphal growth and development, production of asexual/sexual fruiting bodies and pathogenicity (Odenbach et al., 2007; Valiante et al., 2008; Werner et al., 2007).

Mutants with altered cell wall integrity can be identified by screening for changes in tolerance towards cell wall-disrupting compounds such as Congo Red (CR) and Calcofluor White (CFW) (Ram and Klis, 2006). These dyes have affinity for fibrillar molecules and they can interact specifically with polysaccharides that have β1-4 and β1-3 linkages such as chitin and cellulose (Kopecka and Gabriel, 1992; Ram et al., 1994; Wood, 1980). However, the precise molecular basis of the dye-glucan interaction remains unclear (Ram and Klis, 2006). Although changes in sensitivity to cell wall-disrupting compounds indicate altered cell wall integrity, a range of mutations can lead to this phenotype. For instance, a large scale study of *S. cerevisiae* mutants with altered sensitivity to CFW revealed genes related to cell wall assembly as well as genes not previously associated with cell wall integrity or ones with unknown function (Lussier et al., 1997). In the pathogenic ascomycete *Fusarium oxysporum*, isolates mutated in *Rho1*, encoding a Rho-type GTPase protein, displayed dramatically restricted growth in the presence of CR. The mutations to *Rho1* led to increased and decreased activity of chitin and glucan polysaccharide synthases, respectively, which altered cell wall integrity and reduced virulence of this fungus towards tomato plants (Martinez-Rocha et al., 2008).

Changes in cell wall integrity can affect the development of important penetration structures such as appressoria, which are essential for some fungi to invade plants. In *Magnaporthe oryzae*, mutations to a MAP kinase kinase kinase, MCK1, altered the integrity of the appressorial cell wall, presumably affecting the level of turgor pressure, thus reducing the ability of the pathogen to penetrate the cells of rice plants (Jeon et al., 2008). The dothideomycete,

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Leptosphaeria maculans is a pathogen of oil seed rape, *Brassica napus*, causing significant yield losses annually (Fitt et al., 2006). Unlike pathogens such as *M. oryzae*, *L. maculans* does not use specialized infection structures such as appressoria. Instead, germ tubes of asexual and sexual spores gain entry to *B. napus* plant tissue via stomata or wounds (Hammond and Lewis, 1987). The role of cell wall integrity in virulence of *L. maculans* towards *B. napus* is unknown.

In this paper we describe mutations of a particular gene, *LmIFRD*, that affect germination, production of asexual fruiting bodies and virulence of *L. maculans* towards *B. napus*. Additionally, mutations to *LmIFRD* affect monosaccharide content and linkage within the cell walls of germinating spores and mycelia, and are associated with changes in tolerance towards cell wall lysing enzymes and cell wall-disrupting compounds.

2. Materials and methods

2.1. Fungal isolates, crossing and culturing

L. maculans isolates were maintained on 10% Campbell's V8 juice (Australia) agar at 22 °C with a 12 h photoperiod. *L. maculans* wild type isolate IBCN18 was transformed with plasmid pPK2 (Covert et al., 2001) using *Agrobacterium tumefaciens*-mediated transformation (Gardiner and Howlett, 2004). Plasmid pPK2 contains a gene encoding hygromycin resistance under transcriptional control of an *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter flanked by the right and left border transfer DNA (T-DNA) sequences of *A. tumefaciens* (Covert et al., 2001). This plasmid was randomly integrated into the *L. maculans* genome resulting in a bank of insertional mutants. One mutant (A9, subsequently referred to as the T-DNA mutant) showed reduced virulence on cotyledons of *B. napus* (Fig. 4) and was characterized further. Crosses were performed between isolate Lm691 and the T-DNA mutant, which harbor different idiomorphs, *MAT1-1* and *MAT1-2*, respectively, at the mating type (*MAT*) locus, (Cozijnsen and Howlett, 2003). Random ascospore progeny were recovered as described previously (Cozijnsen et al., 2000).

2.2. Pathogenicity assays

Seventy *L. maculans* random T-DNA insertional mutants were screened for their ability to infect cotyledons of 14-day old seedlings of *B. napus* cv. Monty (a moderately susceptible variety) that had been wounded with a 26-gauge needle, as described previously (Purwantara et al., 1998). Lesions were visually assessed using a disease severity scale of 0 (no darkening around wounds) to 9 (large gray-green lesions > 7 mm diameter, with profuse sporulation) (Koch et al., 1991). Plants were inoculated with either a single isolate (four inoculation points per plant, two per cotyledon) or two isolates (one isolate on the left inoculation point and the other isolate on the right inoculation point on each cotyledon). Mean pathogenicity scores were calculated from two or four inoculation points on the cotyledons of at least 10 replicate plants. Data were analyzed using one-way ANOVA comparisons.

2.3. Gene prediction and expression analysis

Genomic DNA was prepared from mycelia of the T-DNA mutant and digested with an enzyme that cut once within the T-DNA and with an enzyme that did not cut in the T-DNA. Southern analysis showed that a single copy of the T-DNA was integrated in the genome but that part of the T-DNA was truncated. Thermal asymmetric interlaced (TAIL)-PCR identified sequences flanking the T-DNA insertion as previously described (Mullins et al., 2001) with the following modifications. An 840 bp PCR product was amplified from the T-DNA left border (LB) using primers LB1, LB2 and LB3 in combination with redundant primer AD2 (Specht et al., 1996) and cloned into plasmid pCR2.1 (Invitrogen, USA), which was then sequenced. However, TAIL-PCR failed to amplify sequence flanking the right border (RB) of the T-DNA, probably due to truncation of the T-DNA sequence. A 600 bp *Sac*I/*Xho*I fragment of the left border PCR product was radiolabeled with [α-^{32}P] dCTP and used to screen a cosmid library of isolate IBCN18 to obtain the wild type copy of the mutated gene. A 42 kb hybridizing cosmid was identified and 3.4 kb flanking the T-DNA insertion was sequenced. All primers used in this study are listed in Table 1. The coding region of a gene flanking the T-DNA insertion (subsequently referred to as *LmIFRD*) and a gene downstream of the T-DNA (subsequently referred to as LmcABH-like) were predicted using FGENESH software (http://www.softberry.com/berry.phtml). A full length cDNA of *LmIFRD* was amplified with primers Exon1F and Exon3R and then sequenced. Intron positions were confirmed by comparison with the genomic sequence. Transcriptional start and stop sites in the wild type *LmIFRD* gene were determined via 5′- and 3′ Rapid Amplification of cDNA Ends (RACE) (Invitrogen) using primers 5′ RACE and 3′ RACE (Accession number: GQ183869).

Table 1
Sequences of oligonucleotides used in this study.

Primer name	Sequence (5′–3′)	Use
LB1	GTGTAAAGCCTGGGGTGCCTAATGAGTG	Tail PCR LB1
LB2	AGCTAACTCACATTAATTGCGTTGCG	Tail PCR LB2
LB3	CGGGGAGAGGCGGTTTG	Tail PCR LB3
AD2	AGWGNAGWANCAWAGG	Redundant primer for tail PCR
Exon1F	GCTCACGCATTGTCTCAACT	RT-PCR, Probe 1, sequencing
Exon 2R	GCACCTCGATCTCGTCTTTC	
Exon3F	CAGCAGTAGAAGCCGACGAT	RT-PCR and Probe 2
Exon3R	TTCGTTGTCTTGACCCGTCT	
Exon 2F	GGAAGGCTGAGCTCAATACG	RT-PCR
LmIFRDqF	GGAAGGCTGAGCTCAATACG	qRT-PCR, sequencing
LmIFRDqR	CACACTGGACTCGGCTGATA	
5′ RACE	GCGCCTTGCGAGAAACTGTCTTGTG	5′ RACE
3′ RACE	AGGAGGACGCTTCAAGGTGGCTTCA	3′ RACE
attB1LmIFRD	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGCGATCTTCGTAGACAGG	Silencing vector
attB2LmIFRD	GGGGACCACTTTGTACAAGAAAGCTGGGTTTACGTTGTCTCCTGTTTGATGC	Silencing vector
cABH1F	AGCGGTCTCGTGTCTTCTTC	RT-PCR and Probe 3
cABH1R	CCAGCGTTTGCCATACTCA	
ActinF	TTGGTCTTGAAAGCGGTGGTAT	RT-PCR and qRT-PCR
ActinR	CATCACTGTCCCACGAATTG	