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Isocitrate Lyase Is Essential for Pathogenicity of the Fungus *Leptosphaeria maculans* to Canola (*Brassica napus*)

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A pathogenicity gene has been identified in *Leptosphaeria maculans*, the ascomycetous fungus that causes blackleg disease of canola (*Brassica napus*). This gene encodes isocitrate lyase, a component of the glyoxylate cycle, and is essential for the successful colonization of *B. napus*. It was identified by a reverse genetics approach whereby a plasmid conferring hygromycin resistance was inserted randomly into the *L. maculans* genome. Twelve of 516 transformants tested had reduced pathogenicity on cotyledons of *B. juncea* and *B. napus*, and 1 of these 12 had a deletion of the isocitrate lyase gene, as well as an insertion of the hygromycin resistance gene. This mutant was unable to grow on fatty acids, including monolaurate, and the isocitrate lyase transcript was not detected. When the wild-type gene was reintroduced into the mutant, growth on monolaurate was restored and pathogenicity was partially restored. *L. maculans* isocitrate lyase is produced during infection of *B. napus* cotyledons, while the plant homologue is not. When 2.5% glucose was added to the inoculum of the isocitrate lyase mutant, lesions of sizes similar to those caused by wild-type isolate M1 developed on *B. napus* cotyledons. These findings suggest that the glyoxylate pathway is essential for disease development by this plant-pathogenic fungus, as has been shown recently for a fungal and bacterial pathogen of animals and a bacterial pathogen of plants. Involvement of the glyoxylate pathway in pathogenesis in animals and plants presents potential drug targets for control of diseases.

Fungal plant pathogens employ diverse strategies to infect their hosts. Mechanisms underlying these strategies are being revealed because of the increasing amenity of many fungi to molecular genetic analysis. In particular, techniques including insertional mutagenesis have identified fungal pathogenicity genes crucial to disease. Such genes are defined on the basis of mutagenesis techniques; disruption of a pathogenicity gene results in significant or complete loss of disease symptoms when mutants are inoculated onto hosts that are susceptible to the wild-type pathogen. The types of pathogenicity genes expressed depend on the infection process of a particular fungus, for instance, whether hydrolytic enzymes, specialized infection structures, or toxins are required, as well as the nutritional status of the pathogen (for reviews, see references 8 and 22). Pathogenicity genes may be valuable as fungicide targets for disease control. If such genes are specific to the fungus, inhibitors may be designed to bind to their products and block the growth of the fungus without affecting the plant.

Leptosphaeria maculans (anamorph = *Phoma lingam*) causes blackleg disease of canola (*Brassica napus*) worldwide. Its infection pathway is well characterized (for a review, see reference 7). Hyphae enter the host through stomata without the aid of specialized infection structures such as appressoria. The fungus initially grows in planta as a biotroph and later becomes necrotrophic. The fungus grows down the stem and produces a dark canker girdling the base of the plant, hence the name blackleg. In spite of the economic importance of blackleg disease, *L. maculans* has not been studied in great detail at a molecular genetic level. This paper describes an insertional mutagenesis approach to identifying pathogenicity genes of *L. maculans* and the characterization of one such gene encoding isocitrate lyase.

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MATERIALS AND METHODS

Fungal culturing and transformation. *L. maculans* isolate M1, which has well-defined host specificity, was mutated. This isolate causes cotyledonary and stem lesions on all of the *B. napus* and *B. juncea* (Indian mustard) cultivars tested, and its ability to attack *B. juncea* is controlled by a single locus (3, 15). Isolate M1 was transformed to hygromycin resistance by using plasmid pUCATPH (11) and restriction enzyme-mediated integration in the presence of 10 to 40 U of *Hin*dIII, *Kpn*I, or *Sac*I as previously described (17). Transformed protoplasts were plated onto regeneration medium (1.2 M sucrose, 10% Campbell's V8 juice, 1.5% agar) containing hygromycin (50 μg/ml; Sigma). Hyphae were visible after 2 to 3 weeks and were subjected to two rounds of hyphal-tip culturing onto 10% V8 juice agar plates containing hygromycin before transfer to nonselective medium. For Northern blot analysis, mycelia were grown in complete medium (17.5 g of Czapek-Dox per liter, 0.005% yeast extract) in still culture (50 ml) in petri dishes (15-cm diameter) for 15 to 25 days, washed in sterile water, and transferred to complete medium or minimal medium (14) containing no carbon or nitrogen source, ammonium acetate (0.5%) only, or ammonium acetate (0.5%) and glucose (0.25%). Mycelia were harvested 7 h later.

Pathogenicity tests. Transformants were screened for the ability to infect cotyledons of 14-day-old seedlings of *B. juncea* cv. Stoke wounded with a 26-gauge needle as previously described (15). *B. juncea* was chosen for the initial screen so that target pathogenicity genes in *L. maculans* would include the host specificity gene (3). Lesion development was assessed between 10 and 14 days postinoculation and compared visually to lesions caused by isolate M1. Transformants with reduced pathogenicity were rescreened on *B. juncea* cv. Stoke and *B. napus* cv. Westar, and those with confirmed reduced pathogenicity were characterized further.

Nucleic acid manipulations and analysis of a nonpathogenic mutant. Chromosomal and genomic DNA or total RNA from *L. maculans* was isolated, resolved by gel electrophoresis, blotted onto nylon membranes, and hybridized as previously described (17). Regions of *L. maculans* DNA flanking the pUCATPH plasmid were obtained by inverse PCR, and the resultant products were cloned into pGEM T-Easy (Promega). The inverse PCR product derived from a non-

pathogenic mutant, which has a single insertion of pUCATPH, was radiolabeled and used to probe a cosmid library (pWEB vector; EpiCentre Technologies) containing 30- to 40-kb inserts of DNA from isolate M1 to obtain the wild-type copy of the mutated *L. maculans* gene. Insert DNA from a hybridizing cosmid was subcloned, and resultant inserts were sequenced by using ABI dye-primer cycle reactions and an automated sequencer (Applied Biosystems model 373A). The DNA sequence obtained was compared to those in the GenBank database by using BLAST (1), and genes were predicted by using FGENESH software (http://www.softberry.com) and GENSCAN (www.bionavigator.com). One subclone contained an open reading frame with a high degree of sequence similarity to that of isocitrate lyase (*icl1*). A 6-kb *Xba*I fragment containing *icl1* was ligated into the *Xba*I site of pAN8-1, a plasmid that confers phleomycin resistance (12). Protoplasts of the nonpathogenic mutant (Δ*icl1*) were transformed with this construct or pAN8-1 as described above, and resultant transformants were selected for growth on phleomycin at 20 μg/ml (Cayla).

Putative complemented strains were inoculated onto *B. napus* cv. Westar cotyledons and pycnidiospores germinated on water agar plates. Strains were also grown on minimal medium agar supplemented with monolaurate (0.25% Tween 20, polyoxyethylene sorbitan monolaurate) or glucose (0.25%) as the carbon source. Additionally, growth on this carbon source was tested in the presence of inhibitors of isocitrate lyase, i.e., 0.5 or 1 mM 3-nitropropionate (an analogue of succinate) and 0.5 or 1 mM 3-bromopyruvate (an analogue of glyoxylate) (19). After 9 days, colony diameters were compared to those of the wild type (M1) and the Δ*icl1* mutant. These inhibitors were also added to inoculum and tested for the ability to affect disease development on *B. napus* cotyledons. The infection patterns of the wild-type and Δ*icl1* strains were investigated by light microscopy. Fourteen days after inoculation, cotyledon tissue was placed in boiling 95% ethanol for 15 min, cleared in saturated chloral hydrate for 5 days, mounted in Hoyer's medium, and examined with an Olympus light microscope (BH-2) as previously described (18).

To investigate transcription of isocitrate lyase during infection, cotyledons were harvested over the course of infection with isolate M1 and total RNA was examined by Northern blot analysis. The *L. maculans icl1* gene and the *B. napus* isocitrate lyase gene which was cloned by PCR with primers 5′-GAAGGGAG ATTCGAGGCG-3′ and 5′-GCCACACTTCTTGGTGAC-3′ (4) were radiolabeled and hybridized to the RNA blot. Control RNA samples were purified from germinated seed, uninfected cotyledons and leaves, and *L. maculans* mycelia.

RESULTS AND DISCUSSION

A total of 516 hygromycin-resistant transformants were screened on *B. juncea* and *B. napus,* and 12 caused either no lesions or lesions less than 30% of the diameter of those caused by isolate M1. The host specificity of these 12 mutants indicates that none of them had an insertion of pUCATPH in the locus conferring host specificity on *B. juncea* (3). Nine of these mutants had multiple insertions of pUCATPH, while three had single copies; one of the latter mutants was characterized further. Flanking DNA obtained by inverse PCR was used to screen a cosmid library to obtain the wild-type gene. A sequence including the isocitrate lyase (EC 4.1.3.1) gene (*icl1*) was isolated (GenBank AY118108), and the intron positions in this gene were determined by sequencing fragments of cDNA of *L. maculans* amplified by PCR with appropriate primers (Fig. 1). A predicted gene is located downstream of *icl1* and transcribed in the opposite direction. This gene has high sequence similarity to that which encodes ribose-phosphate pyrophosphokinase (EC 2.7.6.1; *prs5*) of *Neurospora crassa* and *Saccharomyces cerevisiae* and is predicted to be involved in nucleotide metabolism (6). Pulsed-field gel electrophoresis showed that the *L. maculans icl1* gene is located on one member of a chromosome doublet (6/7; size, 2.15 Mb) of isolate M1 (data not shown). Southern blot analysis of isolates M1 and Δ*icl1* showed that deletion of the *icl1* gene and an unknown amount of DNA occurred during integration of pUCATPH (Fig. 1 and data not shown).

Isocitrate lyase is one of two enzymes in the glyoxylate pathway that are involved in the metabolism of two-carbon compounds such as acetate and fatty acids. Accordingly, growth of the Δ*icl1* mutant was tested on such carbon sources. This mutant was unable to grow on a C_{12} fatty acid (monolaurate; 0.25% Tween 20) or ammonium acetate (0.5%) as the sole carbon source, in contrast to the wild-type isolate (Fig. 2). Both the mutant and the wild type grew on 0.25% glucose. An analogue of glyoxylate, 3-bromopyruvate, at a concentration of 0.5 or 1 mM did not inhibit growth of isolate M1 specifically on 0.25% Tween 20 (data not shown). However, 3-nitropropionate (an analogue of succinate) at a concentration of 0.5 or 1 mM prevented growth on Tween 20 when it was the sole carbon source (Fig. 2). Both molecules were toxic to *L. maculans* at concentrations of greater than 5 mM.

No transcript of *icl1* was observed in the Δ*icl1* mutant, while in isolate M1, the gene was induced by starvation conditions and by 0.5% ammonium acetate. Glucose (0.25%) did not affect the induction of transcription by acetate (Fig. 3). Glucose causes catabolite repression of isocitrate lyase in fungal species. However, in *Aspergillus nidulans*, addition of glucose to acetate-containing medium at concentrations similar to those used here for *L. maculans* did not result in reduced transcription of isocitrate lyase (2). Transcription of *icl1* in isolate M1 was detected in cotyledons of *B. napus* 14 days after infection (data not shown). Transcription of β-tubulin, a constitutively expressed gene that is an indicator of fungal biomass during infection, was also detected at this stage. A wild-type copy of the *icl1* gene was reintroduced into the Δ*icl1* mutant, and eight transformants (Δ*icl1*+*icl1*) that grew on 0.25% Tween 20 were tested on *B. napus* (Fig. 3). The resultant lesions for all strains developed more slowly than those caused by the wild-type isolate; however, they were larger than those produced by the Δ*icl1* mutant (Fig. 4).

Pycnidiospores of the Δ*icl1* mutant had an extremely low germination rate, compared to that of the wild type or complemented strains, on water agar plates. Microscopic examination of the wounded infection site revealed limited hyphal growth of the Δ*icl1* mutant in planta (Fig. 5). This suggests that, rather than a defect in spore germination leading to reduced pathogenicity, a lack of carbon utilization in planta or on the plant surface (in the form of fatty acids) 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 Δ*icl1* mutant, lesions of sizes similar to those caused by isolate M1 developed (Fig. 4).

The finding that isocitrate lyase is involved in pathogenesis of *L. maculans* is consistent with recent findings that this gene is necessary for the full virulence of *Mycobacterium tuberculosis* and *Candida albicans* in mice (10, 13). *L. maculans icl1* is transcribed during infection of *B. napus*, which is consistent with the induction of transcription of this gene in *C. albicans* cells grown in the presence of mouse macrophages (10). Vereecke et al. have reported that, in the plant-pathogenic bacterium *Rhodococcus fascians*, the second enzyme in the glyoxylate pathway, malate synthase, is essential for pathogenicity on tobacco (25). These authors proposed that glyoxylate buildup in the bacterium inhibits further growth after colonization of the plant.

Unlike animals, plants utilize the glyoxylate pathway, so

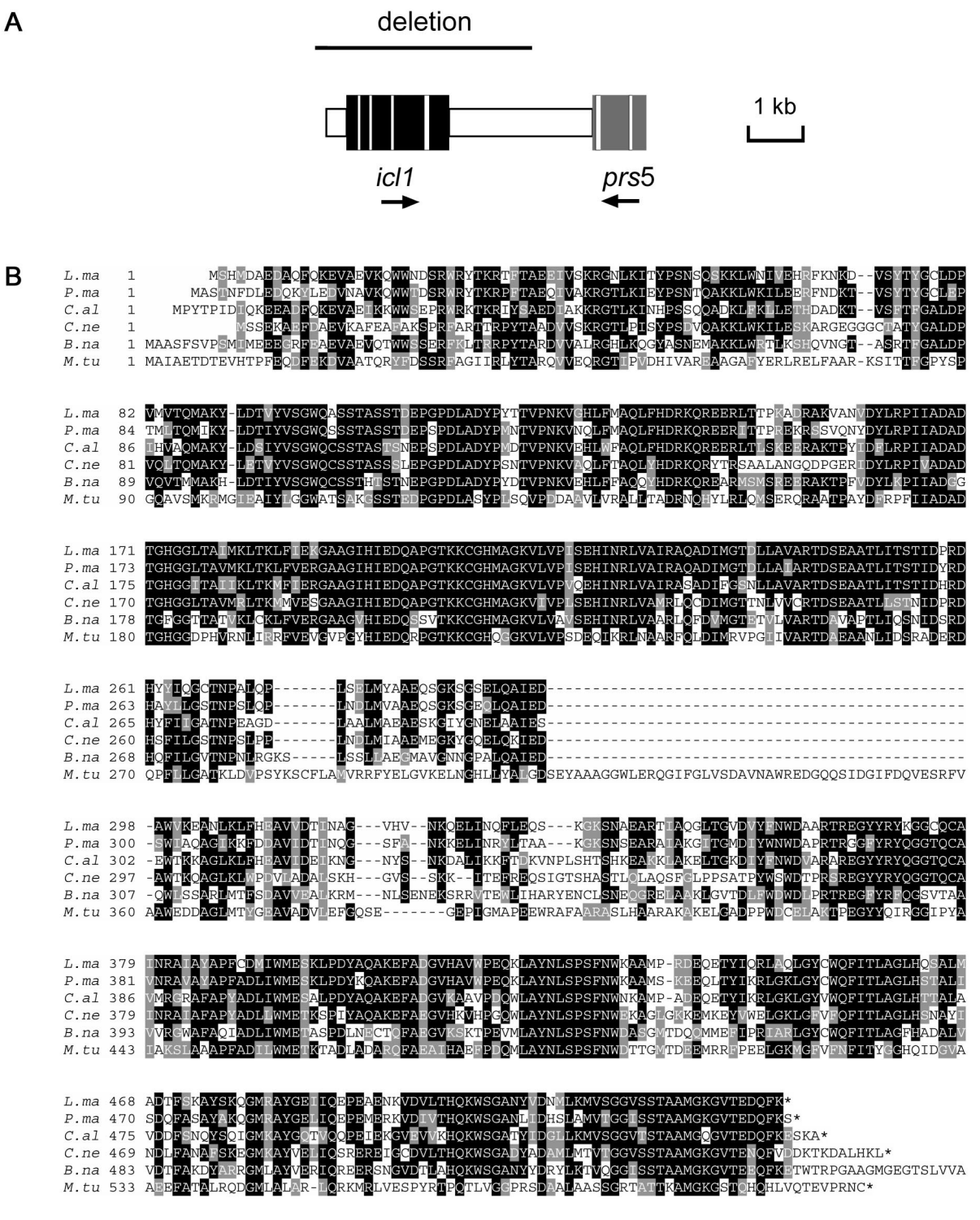

FIG. 1. Alignment of the *L. maculans* isocitrate lyase gene (*icl1*) with homologues. (A) Structure of a 5.8-kb region of *L. maculans* DNA including *icl1* (exons are black) and an open reading frame with high sequence similarity to ribose-phosphate pyrophosphokinase (*prs5*) (exons are grey). Arrows under the genes indicate directions of transcription. The region deleted in the mutant (Δ*icl1*) is indicated. (B) Alignment of *L. maculans icl1* (*L.ma*) with isocitrate lyase genes from selected organisms: the ascomycetes *Penicillium marneffei* (*P.ma*; accession no. AF373018) and *C. albicans* (*C.al*; accession no. AF222905), the basidiomycete *C. neoformans* (*C.ne*; accession no. AF455253), the plant *B. napus* (*B.na*; accession no. L08482), and the bacterium *M. tuberculosis* (*M.tu*; accession no. AE007051).

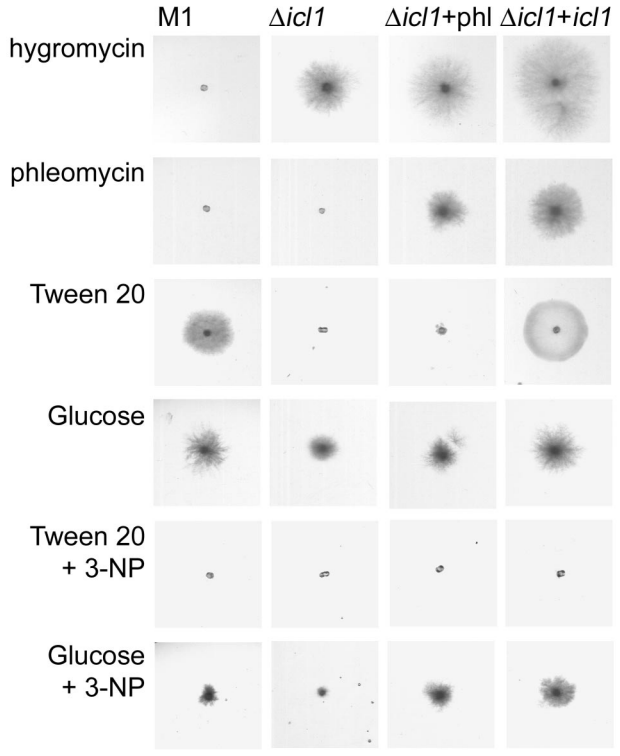

FIG. 2. Growth of *L. maculans* isolates on complete medium supplemented with hygromycin or phleomycin and on minimal medium supplemented with glucose or monolaurate (Tween 20). A wild-type isolate (M1), an isocitrate lyase mutant (Δ*icl1*), a Δ*icl1* isolate transformed with phleomycin resistance gene only ((Δ*icl1*+phl), and a Δ*icl1* isolate transformed with a wild-type copy of *icl1* and the phleomycin resistance gene (Δ*icl1*+*icl1*) were tested. Addition of 3-nitropropionate (3-NP; 1 mM) inhibited the growth of all isolates on monolaurate as the sole carbon source.

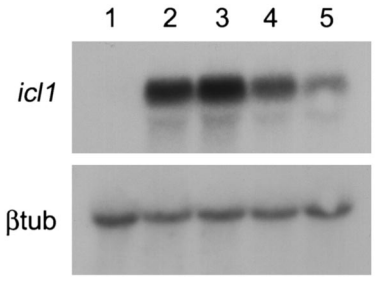

FIG. 3. Transcriptional regulation of the isocitrate lyase (*icl1*) gene of *L. maculans*. Northern analysis of RNA from isolate M1 or an isocitrate lyase mutant (Δ*icl1*) where mycelia were grown in complete medium and then transferred to minimal medium with ammonium acetate (lane 1, Δ*icl1*; lane 2, M1), ammonium acetate and glucose (lane 3, M1), or no carbon or nitrogen (lane 4, M1) or to complete medium (lane 5, M1). The blot was probed with *icl1*. No transcript is observed in the Δ*icl1* mutant. The gene is induced under starvation conditions and by acetate but is not repressed by glucose. The blot reprobed with a fragment of β-tubulin (βtub; bp 8 to 1648 of the nucleotide sequence with GenBank accession no. AF257329) shows equal loading and transfer of RNA.

inhibitors of fungal isocitrate lyase and malate synthase would only be effective for disease control if they did not bind the plant homologues (which is unlikely, given the high degree of amino acid sequence similarity between isocitrate lyase genes; Fig. 1) or if the plant homologues were not induced during infection. We cloned a fragment of the *B. napus* isocitrate lyase gene to investigate its transcription during infection. This gene was transcribed at high levels in germinating seeds, as seen previously (4). However, transcription was not detected at 3, 6, 9, and 12 days postinoculation or in uninfected leaves or cotyledons, although a control plant gene (that which encodes actin) was detected (data not shown). Although inhibitors of *icl1* might be expected to lead to a decrease in lesion size caused by the wild type on *B. napus* cotyledons, this did not occur when 1 mM 3-nitropropionate was added to inoculum (data not shown). Higher concentrations of 3-nitropropionate (>5 mM) caused necrosis, suggesting that this compound is phytotoxic at high concentrations.

The glyoxylate pathway occurs in the peroxisome, an organelle involved in various other functions, including β-oxidation of fatty acids and metabolism of reactive oxygen species. The role of peroxisomal proteins in plant-pathogenic fungi for the formation of infection structures such as appressoria has been demonstrated by disruption of the *ClaPEX6* gene in *Colletotrichum lagenarium*, whereby the mutant does not make appressoria and only colonizes wounded tissue (9). Appressorial turgor in the rice blast fungus *Magnaporthe grisea* depends on lipid reserves to make glycerol (24), an activity probably

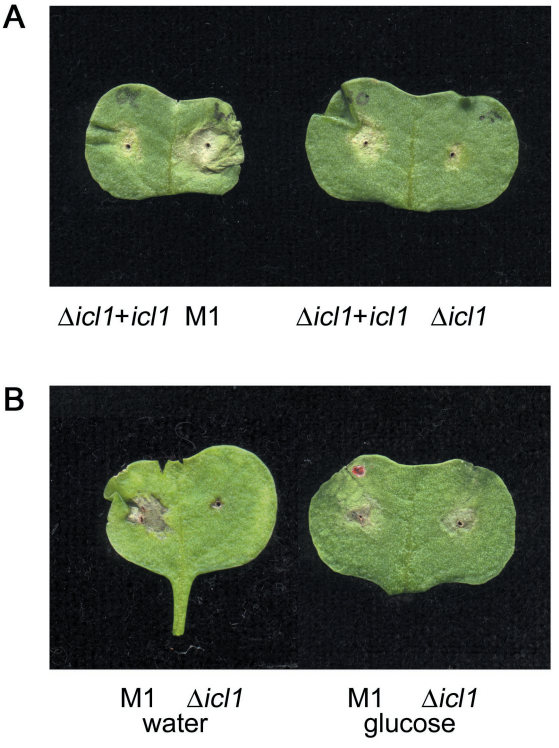

FIG. 4. Pathogenicity of *L. maculans* isolates, including wild-type isolate M1, an isocitrate lyase mutant (Δ*icl1*), and a strain where Δ*icl1* was transformed with a wild-type copy of *icl1* (Δ*icl1*+*icl1*), to cotyledons of *B. napus* cv. Westar. (A) Reintroduction of the wild-type copy of *icl1* restores the ability of Δ*icl1* to form lesions. (B) Pathogenicity is also partially restored by addition of glucose (2.5%) to inoculum of Δ*icl1*. Pen marks are present on the cotyledons to distinguish inoculations.

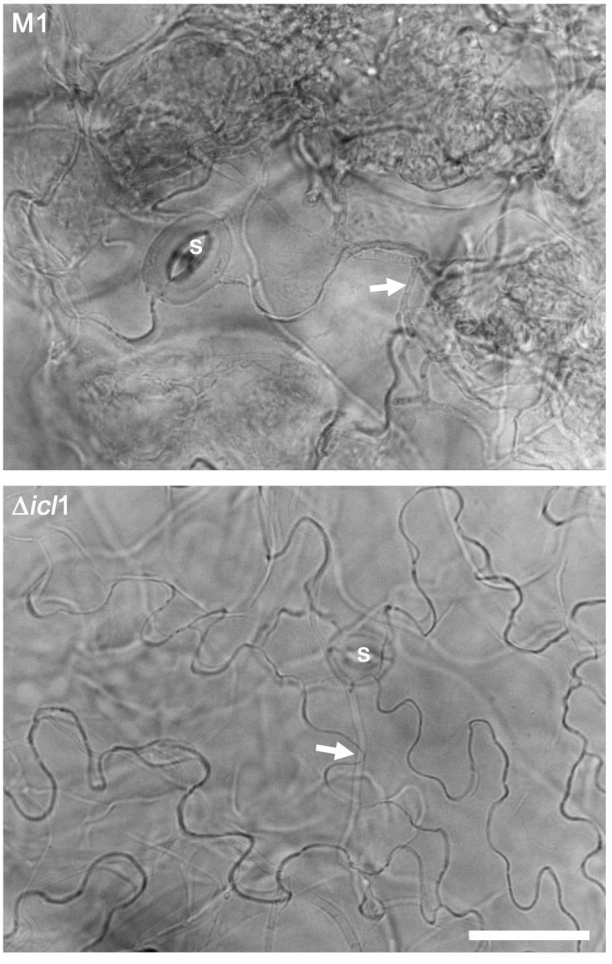

FIG. 5. Phenotypes of isocitrate lyase mutant (Δ*icl1*) and wild-type (M1) *L. maculans* strains in planta. Tissue of *B. napus* was cleared 14 days after inoculation. The plane of focus is just below the cotyledon surface (s, plant stoma; arrow, fungal hypha). Both isolates colonize the plant, but the Δ*icl1* mutant shows less hyphal branching and ramification of than does isolate M1. Bar = 10 μm.

reliant on peroxisomal function and the glyoxylate pathway. Recently, an isocitrate lyase mutant of *M. grisea* has been shown to have reduced pathogenicity (Nick Talbot, personal communication). An additional pathway for utilization of acetyl coenzyme A derived from β-oxidation of fatty acids involves carnitine acetyltransferases (23). A carnitine acetyltransferase gene, probably located in the peroxisomes and mitochondria, is required by *M. grisea* for full pathogenicity (21). Curiously, the transcription of another carnitine acetyltransferase gene, probably located in the cytoplasm, was induced even more highly than the glyoxylate pathway genes in *S. cerevisiae* during phagocytosis by macrophages (10). Unlike *M. grisea*, *L. maculans* does not make appressoria, so the role of fatty acid utilization in the latter fungus is not to provide molecules to promote turgor pressure for infection structures. These results indicate that the glyoxylate pathway functions in pathogenicity in two pathogenic plant fungi with very different modes of infection.

Although the glyoxylate pathway genes are involved in pathogenicity in several animal and plant pathogens, they are not universal pathogenicity factors. Isocitrate lyase is dispensable for disease development in an isolate of *S. cerevisiae* that is pathogenic to mammals (5), as well as in *Cryptococcus neoformans,* an opportunistic pathogen of animals (16). Glyoxylate pathway genes are absent from the plant-pathogenic bacterium *Xylella fastidiosa* (20).

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