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Beginning to Understand the Role of Sugar Carriers in *Colletotrichum lindemuthianum*: the Function of the Gene *mfs*1

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Fungi of the *Colletotrichum* genus are among the most prominent phytopathogens that cause diseases with a considerable economic impact, such as anthracnose. The hemibiotrophic fungus *Colletotrichum lindemuthianum* (teleomorph *Glomerella cingulata* f. sp. *phaseoli*) is the causal agent of the anthracnose of the common bean; and similarly to other phytopathogens, it uses multiple strategies to gain access to different carbon sources from its host. In this study, we examine *mfs*1, a newly identified *C. lindemuthianum* hexose transporter. The *mfs*1 gene is expressed only during the necrotrophic phase of the fungus' interaction within the plant and allows it to utilize the available sugars during this phase. The deletion of *mfs*1 gene resulted in differential growth of the fungus in a medium that contained glucose, mannose or fructose as the only carbon source. This study is the first to describe a hexose transporter in the hemibiotrophic pathogen *C. lindemuthianum* and to demonstrate the central role of this protein in capturing carbon sources during the necrotrophic development of the plant/pathogen interaction.

Keywords: *Colletotrichum lindemuthianum*, hemibiotrophic lifestyle, anthracnose, MFS transporters, *Sugar Porter* family

Introduction

The fungus, *Colletotrichum lindemuthianum* (teleomorph *Glomerella cingulata* f. sp. *phaseoli*), causes anthracnose, one of the most significant and severe diseases of the common bean (*Phaseolus vulgaris* L.). This disease is highly devastating, especially when infected seeds are used and environmental conditions are favorable for the pathogen development. The presence of such factors during the culture period may lead to a 100% production loss (Rava *et al.*, 1994). In addition to decreasing crop yield and causing economic losses, the disease reduces the quality of the product because it generates spots on the bean pods rendering them unfit for consumption. The earlier the disease appears in the field, the greater the damage will be (Sartorato and Rava, 1994).

Along with other species of the *Colletotrichum* genus, *C. lindemuthianum* is a hemibiotrophic fungus. The fungus is biotrophic at the beginning of its infectious phase when it obtains nutrients from living plant tissues through the development of specialized infection structures, such as appressorium, vesicles and primary hyphae. By contrast, it is necrotrophic at the end of colonization when the secondary hyphae penetrate the host tissue to acquire essential nutrients (Perfect *et al.*, 1999; Münch *et al.*, 2008). In this phase enzymes such as pectin liases, endo-polygalacturonases and α-arabinofuranosidases degrade the structurally diverse polymers that compose the plant host cells, resulting in the release of a wide variety of sugars for the fungus (Wijesundera *et al.*, 1989; Carpita *et al.*, 2001; Herbert *et al.*, 2004). This stage features the tissue necrosis that is typical of anthracnose. Consequently, the fungus must respond to changes in the available carbon sources during the different stages of infection by establishing different transport systems in the plasma membrane that allow it to adjust the uptake of these carbon sources. Therefore, genes encoding mono- and oligosaccharide transporters that have different substrate specificities are differentially expressed during the biotrophic and necrotrophic phases of phythopathogenic fungi. This differential expression represents a key factor in the development of the pathogen within its host (Lingner *et al.*, 2011).

In fungi, members of the MFS (Major Facilitator Superfamily) play a key role in membrane transport (Del Sorbo *et al.*, 2000). These proteins are ubiquitous and widely expressed throughout the three domains of life. MFS proteins are able to transport multiple substrates through different kinetic mechanisms, including uniport (transport of a single substrate using energy from the substrate gradient), symport (simultaneous translocation of two or more substrates in the same direction using energy from the electrochemical gradient) and antiport (membrane transport of two or more substrates in opposite directions). Some members of the MFS superfamily have extremely stringent specificity, while others transport a wide variety of substrates such as ions, sugars, drugs, neurotransmitters, nucleosides, amino acids and peptides (Law *et al.*, 2008). The transport of these compounds is driven by the proton-motive force; therefore, the transporters of this superfamily are characterized as a secondary transport system (Lewis, 1994). MFS proteins may be composed of 400 to 600 amino acids, and an analysis of

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their primary structures reveals a high level of sequence similarity within individual families. Regardless of having some sequence divergence between members belonging to different families, MFS transporters almost always have the same three-dimensional structure and functional identity (Law *et al*., 2008). Currently, 58 MFS transporter families have been described, and approximately 10,000 sequences are available in the transporter classification database (www.tcdb.org). The sugar transporters family *SP* (*Sugar Porter*) is one of the most representative (Stergiopoulos *et al*., 2002). A MFS transporter may have 6, 12, 14 or even 24 transmembrane domains (TMDs) that are connected by hydrophilic loops with the C- and N-terminal domains located in the cytoplasm (Pao *et al*., 1998; Yin *et al*., 2006). Current studies of carbohydrate transport in filamentous fungi have illustrated the importance of these proteins in maintaining cell viability and establishing interactions between organisms. A sugar transporter has been found in models such as the ascomycete fungi *Neurospora crassa* (Madi *et al*., 1997), *Aspergillus nidulans* (Forment *et al*., 2006), and *Metarhizium robertsii* (Fang and Leger, 2010); the basidiomycete fungi *Uromyces fabae* (Voegele *et al*., 2001) and *Ustilago maydis* (Wahl *et al*., 2010), and the mycorrhizal fungus *Amanita muscaria* (Nehls *et al*., 1998), as well as the glomeromycete fungus *Geosiphon pyriformis* (Schüssler *et al*., 2006). However, little is known about the use and transport of carbon sources by pathogenic fungi, specifically of the hemibiotrophic ones such as *C. lindemuthianum* in different stages of infection; only one study of hexose transporters in *Colletotrichum graminicola* has been conducted (Lingner *et al*., 2011).

Because the monosaccharides that are available to the fungus at different stages of its interaction with the plant are the main sources of both carbon and energy, playing also a key role in regulating gene expression (Forment *et al*., 2006), it is necessary to study these transporters to understand the developmental stages of the fungus during the interaction between the pathogen and its host. This study is the first to describe a hexose transporter in the hemibiotrophic fungus *C. lindemuthianum*, and it represents a new way to understand the role of these proteins in phytopathogenic fungal species.

Materials and Methods

Fungal strains and culture conditions

Targeted gene knockouts were created in the wild-type *C. lindemuthianum* LV49 isolate (race 89) that belongs to the mycological collection of the Plant Resistence Laboratory, Department of Biology, Federal University of Lavras (UFLA). The pathogenicity of this strain was confirmed by periodically testing susceptible bean plants for infection (Rosinha cv.).

The wild-type and mutant *C. lindemuthianum* (Δ*mfs*1, this study) strains were both stored at -80°C as concentrated suspensions of conidia (10^8 conidia/ml) in 25% (v/v) glycerol. To obtain the conidia, the strains were cultured in bean pods set on previously sterilized water-agar (1.5% agar w/v) in test tubes and incubated at 22°C for 15 days, with a photoperiod of 16 h of light and 8 h of darkness. For total DNA extraction, the strains were cultured for seven days at 22°C in pH 6.8 PDA medium (potato dextrose agar) (Himedia) or liquid GPYECH (20 g glucose, 5 g peptone, 1 g yeast extract, and 1 g hydrolyzed casein/L), with a photoperiod of 16 h of light (166 μE/sec/m^2) and 8 h of darkness.

Nucleic acid manipulation

Total DNA was extracted according to Specht *et al.* (1982). The DNA-DNA hybridizations were performed by Southern blot technique according to Sambrook and Russell (2001). The DNA was digested with restriction endonucleases and the resulting fragments were transferred to nylon membranes (Amersham Hybond™ - N^+ - GE Healthcare®) and fixed by UV light (UV Crosslink UVC 500). Probe labeling, hybridization and washings were performed using the Dig High Prime DNA Labeling and Detection Starter Kit II and Dig Probe Synthesis Kit (Roche®) kits according to the manufacturer's instructions.

Total RNA from *C. lindemuthianum* was extracted for *mfs*1 gene expression analysis using the TRIzol (Invitrogen™) reagent and treated with DNase (Promega). Single-stranded cDNA was synthesized using the ImProm-IITM Reverse Transcription System (Promega) kit according to the manufacturer's instructions.

Other routine procedures (e.g., bacterial transformation) were performed according to the standard protocols described by Sambrook and Russell (2001).

Isolation and characterization of the *mfs*1 gene

The gene encoding the MFS transporter was isolated by DNA hybridization using the plaque lift method (Benton and Davis, 1977), utilizing a *C. lindemuthianum* genomic DNA library in a λEMBL3 vector (Soares, 2007). A DNA fragment of 408 bp that encoded a partial sequence of the gene was

Table 1. Primers used in this study

Primer	Sequence (5′→3′)
MFS1L	GAGCTCTGGAACACCCACTC
MFS1R	AAGGCTATGGTGGTGTTTGC
MFS1cDNAL	ATTCAGCCGTCCAGATTGC
MFS1cDNAL	TTTGTGTCGCCTTCCAGGT
MFS2qF	TTGGAAGTGTCGCTGCTGTCT
MFS2qR	GTGCCGACGATGACAATGG
CLGpDq1	CCGCACTGCTGCTCAGAAC
CLGpDq2	GGACATGCCGGTGAGCTT
M13R	AGCGGATAACAATTTCACACAGGA
M13F	CGCCAGGGTTTTCCCAGTCACGAC
NLC37	GGATGCCTCCGCTCGAAGTA
NLC38	CGTTGCAAGACCTGCCTGAA
FP1	GCATCGTCTTTGCCATGTAA
RP1	TCCTGTGTGAAATTGTTATCCGCTAGCTGTACG ATTGGGTGGTC
FP2	GTCGTGACTGGGAAAACCCTGGCGTAGATGCG CATGTGCTTACC
RP2	GCGCTAAACACTTCCTCCAG
CIR	GTGCCCTCAATGGCTATGAC
CIF	TTCAAGCCGGTTTTTCTTTG
HPH1	CAGCGAGAGCCTGACCTATTG
HPH2	GCCATCGGTCCAGACGGCCGCGC

used as *mfs*1 probe (Oliveira, 2006). This fragment was amplified from the *C. lindemuthianum* genomic DNA using the MFS1L and MFS1R primers (Table 1). The Go Taq™ DNA polymerase (Promega) enzyme was used according to the manufacturer's instructions. Thus, a DNA fragment of approximately 6.5 kb/*Sal*I containing the gene of interest was obtained from a single recombinant vector. This fragment was subcloned into the pBluescript II KS⁻ (Stratagene®) vector and both strands were sequenced with the primer walking method (Macrogen INC., Korea) using the 3730XL sequencer (Applied Biosystems). The MFS1cDNAR and MFS1cDNAL primers (Table 1) which correspond to the 5′ and 3′ gene flanking regions were designed to confirm the presence and number of introns in the open reading frame (ORF). The 1,623 bp sequence that corresponded to the cDNA obtained from the *C. lindemuthianum* total RNA was cloned into the pGEM®-T Easy vector (Promega). The DNA sequences were analyzed, assembled and edited using the DNAMAN (Lynnon Corporation) program, with the aid of BLAST (Altschul *et al*., 1997) tools and the National Center for Biotechnology Information database (NCBI: http://www.ncbi.nlm.nih.gov/). Protein sequences were aligned by using the CLUSTAL W algorithm (Thompson *et al*., 1994) with manual corrections. The deduced protein analysis was performed using the NCBI search tool for conserved domains, along with the BLASTP tool. We used the program Toppred® (bioweb.pasteur.fr/seqanal/interfaces/toppred.html) to identify the typical transmembrane domains of MFS transporters. The genomic sequence of *mfs*1 was deposited in GenBank (NCBI) under accession number HQ541322.1.

*mfs*1 gene structure in the *C. lindemuthianum* genome

The *mfs1* gene structure and copy number in the *C. lindemuthianum* genome were examined by Southern blot (Sambrook and Russel, 2001). The total DNA of the wild- type was cleaved with *Bam*HI, *Eco*RI, *Kpn*I, *Not*I, and *Xba*I restriction enzymes, which do not have restriction sites in the DNA fragment that was used as probe (Probe 1), and with *Hin*dIII, which has a unique restriction site in the fragment corresponding to the probe. The hybridization was conducted as described above and the membranes were exposed to the BioMax Light X Ray Film (Kodak®).

Phylogenetic analysis of the *mfs*1 gene

The phylogenetic analysis was conducted using amino acid sequences of representative proteins of different families of MFS transporters; these sequences were available in the GenBank (NCBI) and BROAD Institute (www.broadinstitute.org) databases. Initially, the MEGA 4.0 (Tamura *et al*., 2007) program was used to align the selected sequences. A maximum parsimony tree was designed, and the tests were performed by the PAUP * 4.0b10 (Swo Vord, 2002) program. The heuristic search method chosen for the tree design was tree bisection and reconnection (TBR). The phylogenetic tree reliability was assessed using a bootstrap of 1,000. The Tree View (Page, 1996) program was used to visualize the tree obtained by the maximum parsimony method.

*mfs*1 gene expression analysis by semi-quantitative RT-PCR and qRT-PCR

A preliminary analysis of *mfs*1 gene expression was accomplished using semi-quantitative RT-PCR, using fungi from susceptible bean leaves (Rosinha cv.) at different stages of infection. The bean leaves were excised from seedlings of the Rosinha cv., and their abaxial surfaces were inoculated with the conidial suspensions obtained from the wild-type and mutant strains at a final concentration of 10^6 conidia/ml. The inoculated leaves were kept in separate Petri dishes and incubated at 22°C with a photoperiod of 16 h of light (166 μE/sec/m^2) and 8 h of darkness. The leaves were collected and frozen every 24 h for seven days as described in Dufresne *et al*. (1998). The symptoms of anthracnose were first observed six days after the inoculation. Inoculation with autoclaved, ultrapure water, on the abaxial surface of the bean leaves served as a negative control. An infection cycle that corresponded to the fungus' biotrophic and necrotrophic phases was observed during the period of study.

The first-strand cDNA was synthesized from the total RNA extracted from the infected *C. lindemuthianum* leaves as previously described, using the ImProm-IITM Reverse Transcription System (Promega) according to the manufacturer's instructions.

The semi-quantitative RT-PCR amplification parameters were established using the CLGpDq1 e CLGpDq2 (Table 1) primers to create an internal control; these primers amplify a 100 bp fragment that corresponds to *C. lindemuthianum* glyceraldehyde-3-phosphate dehydrogenase (accession number AF000310). The number of cycles that corresponded to the logarithmic amplification phase of the endogenous control was identified by PCR under the following amplification conditions: 94°C for 2 min (1), 94°C for 30 sec (2), 50°C for 45 sec (3), 72°C for 30 sec (4) and a final extension at 72°C for 7 min, with aliquots being removed after 20, 25, 30, and 35 cycles. From these analyses, 30 cycles of amplification (steps 1, 2, 3, and 4) were established.

The MFS1cDNAL and MFS1cDNAR (Table 1) primers were designed from the complete sequence of the *mfs*1 gene to amplify a 1,623 bp fragment of the second cDNA strand of the transcript that corresponded to the MFS transporter gene. The following cycles of semi-quantitative RT-PCR were used: an initial denaturation at 94°C for 2 min, 30 cycles of 94°C for 1 min, 56°C for 45 sec, 72°C for 2 min, and 15 sec and a final extension step at 72°C for 7 min. The aliquots were removed after 20, 25, 30, and 35 cycles. The number of cycles used for the *mfs*1 gene analysis was also 30. The reactions were performed in triplicate to ensure reliability and reproducibility of the results.

For quantitative analysis of *mfs*1 expression (qRT-PCR), total RNA was extracted from infected bean leaves 1, 2, 3, 4, 5, 6, and 7 days after the inoculation, as described previously. Total RNA (2 μg) was DNAse-treated according to standard techniques (Sambrook and Russel, 2001) and was used for cDNA synthesis. Real-time PCR reactions were carried out in a final volume of 25 μl, using a SYBR® Green PCR Master Mix (Applied Biosystems) and the CFX384 Real-Time PCR Detection System (Bio-Rad) following manufacturer's instructions. Cycling parameters were as follows: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min (DNA polymerase ac-

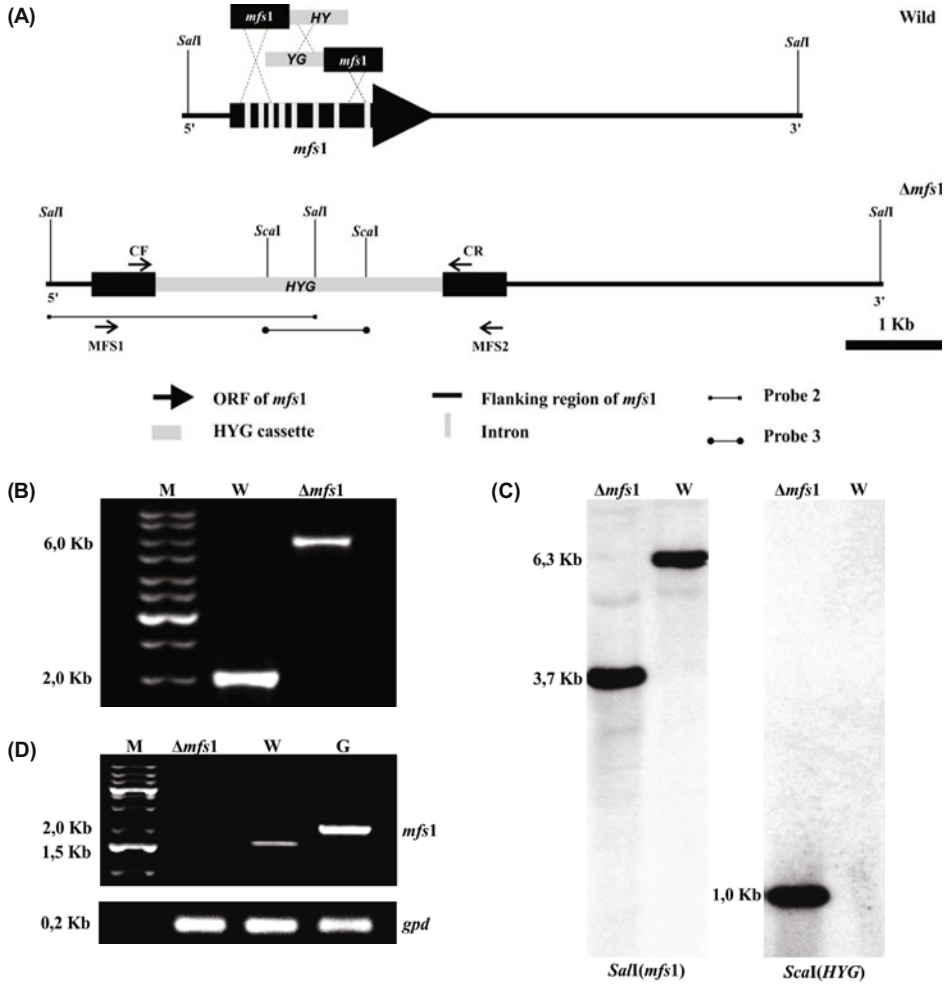

Fig. 1. The *mfs*1 gene characterization, knockout design and confirmation of the *C. lindemuthianum* mutant Δ*mfs*1. (A) The *mfs*1 gene structure, cassettes designed and used for knockout via PCR (wild type) and structure of the truncated gene *mfs*1 (Δ*mfs*1). (B) Confirmation of the specific integration of the mutant construct by PCR. (C) Auto-radiography of wild-type and mutant *C. lindemuthianum*. The total DNA was hybridized and cleaved with *Sal*I (Probe1/760 bp) and *Sca*I (Probe2/712 bp). The profile of specific integrations and the corresponding sizes of the hybridized DNA fragments are shown. (D) The wild-type and Δ*mfs*1 mutant *mfs*1 gene expression. The *mfs*1 transcript was detected by RT-PCR using specific primers. The *gpd* transcript was used as an endogenous control. M, GeneRuler™ 1 kb DNA Ladder Plus marker.

tivation), and 40 cycles, each consisting of 15 sec at 95°C (denaturation) and 1 min at 60°C (annealing and extension). Each sample was analyzed in triplicate. Three biological replications of the experiment were carried out.

Primers for all target sequences of the qRT-PCR (MFS2qF/MFS2qR and CLGpDq1/CLGpDq2) were designed using Primer Express software (Applied Biosystems). The specificity of the amplicons was checked by melting-curve analysis and by 2% agarose gel electrophoresis. Expression levels were normalized to the glyceraldehyde-3-phosphate dehydrogenase gene and relative quantification was performed using the comparative cycle threshold method (ΔΔCT) (Livak and Schmittgen, 2001).

Knockout cassette design and transformation of the fungus

The split-marker technique was used to knock out the mfs1 gene and all the procedures were performed according to Catlett *et al.* (2003). The hygromycin phosphotransferase cassette (HYG) was amplified from the pAN7.1 (Punt *et al.*, 1987) using the M13R/NLC37 and M13F/NLC38 primers (Table 1). The mfs1-specific sequences were amplified from *C. lindemuthianum* total DNA using the FP1/RP1 and FP2/RP2 primers (Table 1). The second round of the PCR was done with the products from the first set of reactions and the FP1/NLC37 and RP2/NLC38 primers.

C. lindemuthianum protoplasts were obtained and subsequently transformed with the previously designed knockout cassette according to procedures described previously by Redman and Rodriguez (1994). The mitotic stability of the transformants was assessed by transferring the transformant colonies to selective and non-selective media for five generations. The CF and CR primers (Table 1) were used to confirm the site-specific knockout and selection of the transformants. The Platinum® Taq DNA polymerase High Fidelity (Invitrogen™) enzyme was used according to the manufacturer's instructions in all the PCR analyses.

Molecular characterization of the transformants and selection of *C. lindemuthianum mfs*1 mutant

The total DNA preparations of wild-type and transformant fungi were subjected to digestion by *Sal*I and *Sca*I enzymes, chosen based on the restriction sites located in the *mfs*1 and *hyg* gene sequences. Because *Sal*I does not have a restriction site inside the *mfs*1 gene, a 6,352 bp DNA fragment should result from the wild-type fungus. *Sca*I was used to verify the integration of the *hyg* gene and its copy number into the transformant genome. There are two *Sca*I recognition sites that flank the *hyg* gene, which should generate a DNA

fragment of 1,089 bp (Fig. 1).

The DNA fragments from *Sal*I cleavage were transferred to a membrane and hybridized with a 760 bp probe (Probe 2) that represented an *mfs*1 gene fragment amplified by the FP1 and RP1 primers (Table 1). Another DNA-containing membrane cleaved by *Sca*I was hybridized with a 712 bp probe (Probe 3) that was constructed from the *hyg* gene sequence in the pAN7.1 plasmid by amplification with the

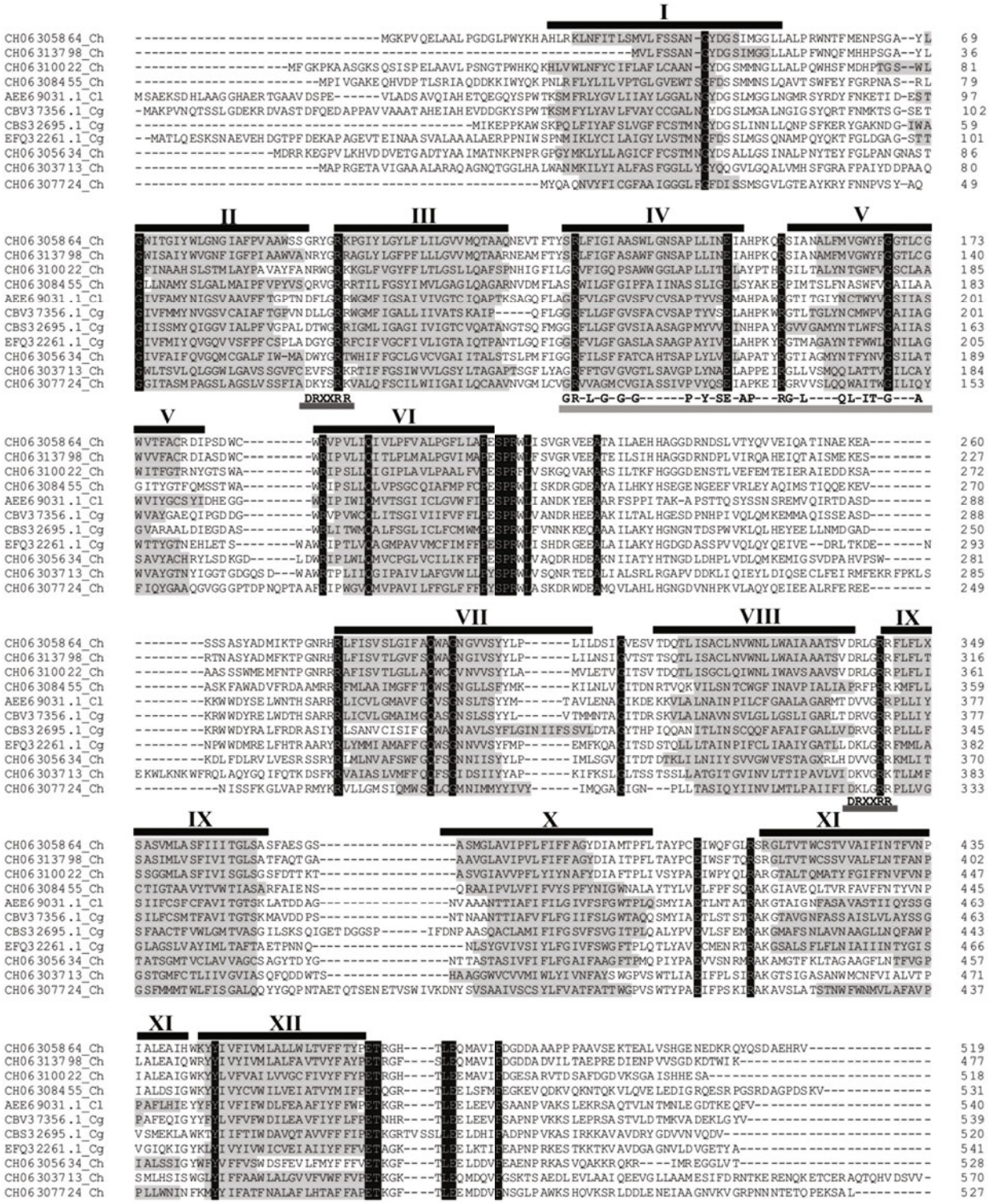

Fig. 2. Alignment of various hexose transporters. The alignment of the following deduced protein sequences of *C. graminicola* (Cg), *C. higginsianum* (Ch), and *C. lindemuthianum* (Cl) shows that amino acids residues are conserved in all sequences (black shaded boxes). The bold bars above the sequences indicate the positions of the 12 putative transmembrane domains. The blue bars indicate the conserved motifs of the MFS superfamily. The gray bar indicates the signature of the *Sugar Porter* family. The sequences were obtained from GenBank and BROAD Institute databases.

HPH1 and HPH2 primers (Table 1). The hybridizations were conducted at a temperature of 48°C, and the membranes were exposed to the BioMax Light X Ray Film (Kodak®).

Phenotypic characterization of the Δ*mfs*1 mutant

The Δ*mfs*1 mutant was analyzed to check whether the putative phenotypic changes resulted from the absence of this transporter in the fungus. Both its applied fungicide susceptibility profile and its virulence and efficiency in assimilating different sugars were assessed.

The antifungal susceptibility of *C. lindemuthianum* was examined by incorporating the fungicides into the PDA medium (Edginton *et al*., 1971). The medium was poured into Petri dishes, and 6-mm plaques of mycelium from the wild-type and **Δ***mfs*1 fungi were placed in the center of the dishes, which were then incubated at 22°C with a photoperiod of 16 h of light and 8 h of darkness. Independent triplicates of the wild-type and Δ*mfs*1 mutant were assessed.

The fungicides used were Dithane (mancozeb), Folicur (tebuconazole), and Copper Sandoz (cuprous oxide) in five concentrations (1, 10, 100, 500, 1,000 ppm); PDA medium with no fungicide was used as a control. The mycelial diameter (in mm) was measured daily throughout the 10-day incubation period. The effect of the different treatments was compared using Tukey's test at a significance level of 0.05%.

The pathogenicity of the Δ*mfs*1 mutant fungus was examined using the pathogenicity test that has been described above.

To examine the Mfs1 transporter functionality, wild-type and Δ*mfs*1 mutant *C. lindemuthianum* were cultured in Petri dishes with 25 ml of a minimal medium (MM) (Pontecorvo *et al*., 1953) supplemented with different sugars as carbon sources. The sugars used were glucose, fructose, xylose, mannose, myo-inositol, sorbitol, lactose, and sucrose, all of which purchased from manufacturers (Sigma or Merck), that had a high level of purity and used at a 5% (w/v) concentration.

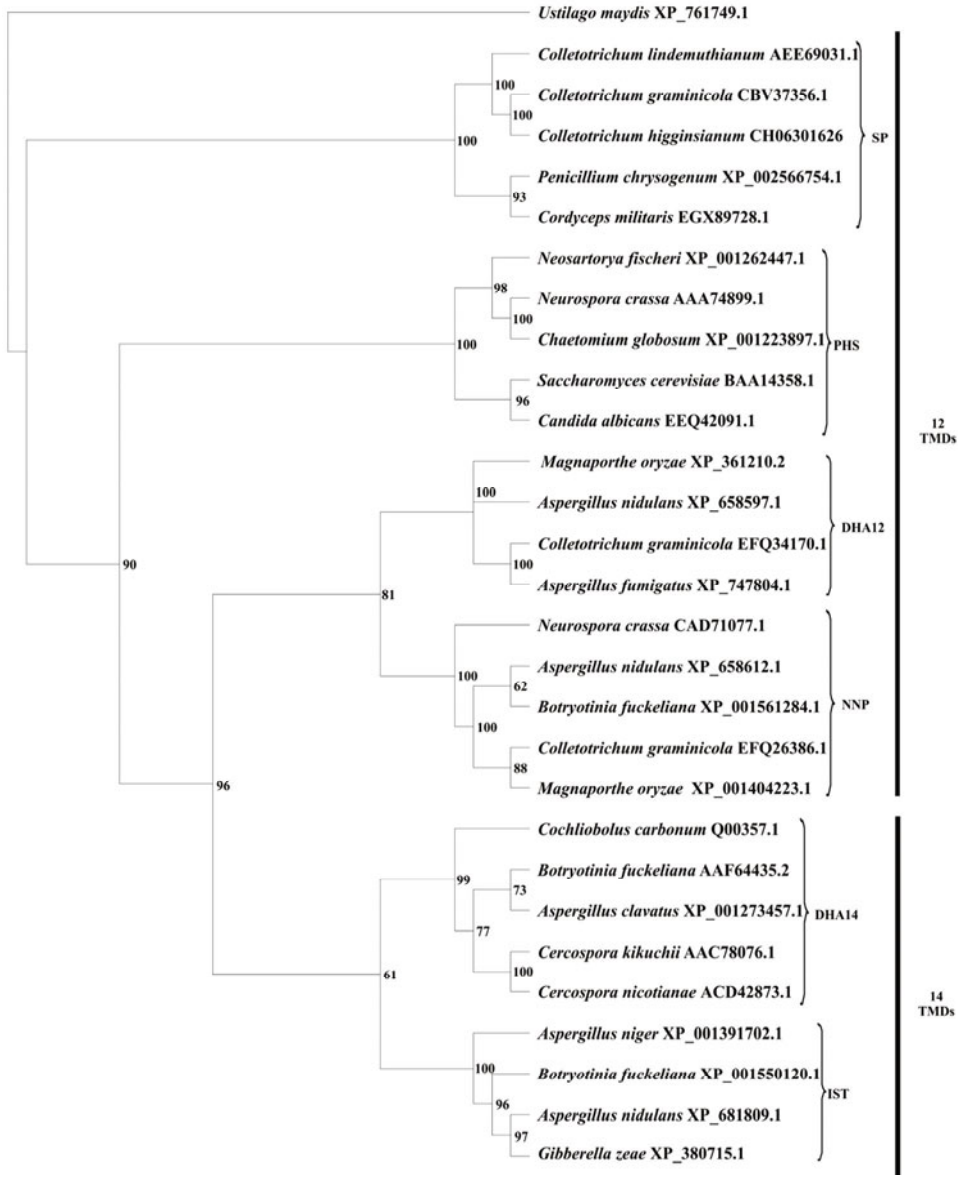

Fig. 3. A phylogenetic tree of MFS members that belong to the *Ascomycota* phylum including the *C. lindemuthianum* Mfs1 protein (AEE69031.1). The length of the horizontal branches is proportional to the genetic distance between the species. The tree is rooted in the *Ustilago maydis* Basidiomycota fungus and was designed by the maximum parsimony method using the PAUP * 4.0b10 program. The numbers next to the branches indicate the percentage of bootstrap analysis repetitions in which the branchings were observed, based on 1,000 replicates. DHA14, drug: H^+ antiporter 14-spanner; IST, Iron-siderophore transporters; NNP, nitrate-nitrite porter; DHA12, drug: H^+ antiporter 12-spanner; PHS, Phosphate: H^+symporter; SP, *Sugar Porter*.

After the medium had solidified, 6-mm diameter plaques of wild-type and mutant fungal colonies were inoculated at the center of the Petri dishes, which were then incubated for 10 days at 22°C with a photoperiod of 16 h of light (166 μE/sec/m^2) and 8 h of darkness. The mycelial diameter in millimeters (mm) was measured daily. The effect of the different treatments was compared using Tukey's test at a significance level of 0.05%.

Assessment of *C. lindemuthianum* conidia germination

The conidia obtained from the wild-type and Δ*mfs*1 mutant strains were germinated from a suspension at a concentration of 10^6 conidia/ml. The spore suspension was added to MM medium supplemented with glucose, mannose or sucrose 5% (w/v). Erlenmeyer flasks were also kept at 22°C with rotary shaking at 80 rpm. After 12, 24, and 48 h, the samples were observed under a light microscope (Olympus BX-50).

Assessment of *C. lindemuthianum* conidiation

Mycelium fragments of identical age and size from the wild-type and Δ*mfs*1 mutant strains were inoculated in bean pods fixed on previously sterilized water-agar (1.5% agar w/v) in test tubes and incubated at 22°C for 15 days. Three independent tests were conducted to provide biological replicates.

Results

A single copy of the *mfs*1 gene is found in the *C. lindemuthianum* genome and it encodes a sugar transporter of the *Sugar Porter* family

A partial sequence corresponding to an MFS transporter was identified in a previous study that had been investigated by Random Amplified Polymorphic DNA (RAPD) in different races of *C. lindemuthianum*. This sequence, which is specifically found in the LV 49 isolate, was a DNA fragment distinct from previously studied isolates of this species (Oliveira *et al*., 2006). Therefore, to assess the contribution of this gene to the pathogenicity of *C. lindemuthianum,* we used a partial sequence as probe to screen a fungal genomic library and isolated a λEMBL *mfs*1 recombinant phage, which had a 6.5 kb fragment that hybridized with the probe. This DNA fragment was sequenced and the *mfs*1 gene was characterized (Fig. 1A). The complete *mfs*1 gene sequence contains 3,134 bp and has an ORF that is interrupted by eight introns (of 61, 57, 55, 64, 57, 59, 55, and 60 bp each). The putative protein has 541 amino acids (1,623 bp) and a molecular mass of approximately 59.3 kDa, which is consistent with previous studies of MFS transporters. An alignment of the amino acid sequence obtained with available sequences in the GenBank database (NCBI) indicated a similarity at amino acid level between the Mfs1 protein and MFS sugar transporters found in other fungi of the *Ascomycota* phylum. Twelve transmembrane domains (TMDs) were identified by protein analysis using the Toppred® program. Most previously characterized MFS transporters have a uniform topology of 12 transmembrane α-helices that are connected by hydrophilic loops with their N- and C-termini located in the cytoplasm. Using the NCBI conserved domains search tool and the BLASTP tool, the MFS superfamily domains were confirmed to be typical of the SP (*Sugar Porter*) family of transporters. In Fig. 2, an alignment of the sequences of eleven hexose transporters of the genus *Colletotrichum* is shown. In accordance with other hexose transporters, Mfs1 transporter also contains the *Sugar Porter* signatures located between TMD IV and TMD V (Pao *et al*., 1998).

A Southern blot analysis of the *C. lindemuthianum* genomic DNA digested with various restriction enzymes revealed that a single copy of the *mfs*1 gene is present in the *C. lindemuthianum* genome (data not shown).

MFS proteins are highly conserved and share structural features; therefore, a phylogenetic analysis was performed to confirm the relationship between the Mfs1 protein and several other proteins from different MFS transporter families. The amino acid sequences of only the MFS transporter proteins from fungi belonging to the Ascomycota phylum were aligned, and a phylogenetic tree was constructed (Fig. 3). Six distinct transporter families were identified, and the Mfs1 protein was in a group with members of the SP family, which included monosaccharide transporters that have been previously described in *C. higginsianum* and *C. graminicola*. The analysis also revealed the presence of two distinct groups; one contained members of the drug: H^+ antiporter 14-spanner (DHA14) and Iron Siderophore (IST) transporter families, both of which feature 14 TMDs, and the second group (which included the Mfs1 protein) contained only families with 12 TMDs.

The *mfs*1 gene is expressed during the necrotrophic phase of the *C. lindemuthianum* infection cycle

Semi-quantitative RT-PCR was used to assess the *mfs*1 gene expression. The *mfs*1 gene transcription was analyzed in detached bean leaves infected with spores of wild-type *C. lindemuthianum* fungus. *mfs*1 expression was significantly

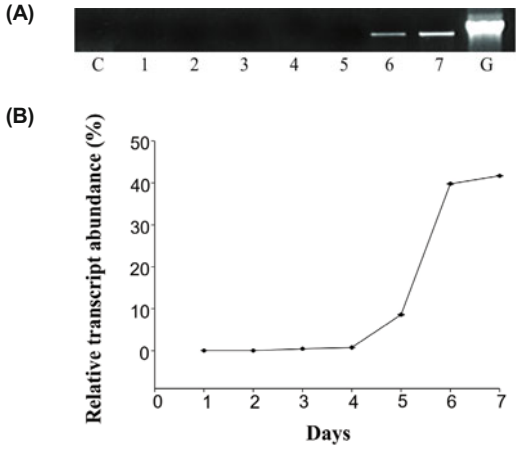

Fig. 4. *mfs*1 expression levels in *C. lindemuthianum*-infected bean leaves. (A) Semiquantitative RT-PCR analyses of *mfs*1. The PCR products were loaded onto a 2% agarose gel. (B) Quantitative RT-PCR. mRNA levels of *mfs*1 were studied at 1–7 dpi. Gpd transcript levels served as endogenous control. RT-pCRs were performed on total RNA isolated infected bean leaves at the indicated times. (C) Control-uninfected leaves, (G) Genomic DNA. Absence of bars indicates that errors were smaller than symbols.

higher in the bean leaves during the sixth and seventh days of infection, a period that represents the necrotrophic phase of the infectious cycle of *C. lindemuthianum* in susceptible *Phaseolus vulgaris* (Fig. 4). The temporal expression of the *mfs*1 gene is highly significant because it suggests that the fungus can express a different set of membrane proteins during each phase of its development. This finding also emphasizes the role of membrane transport proteins, specifically MFS members, in the adaptive physiological processes of a pathogenic fungus.

Characterization of the Δ*mfs*1 mutant

The function of the *mfs*1 gene was assessed by examining knockout mutants obtained by the split-marker method. At the end of the second round of the PCR analysis, we obtained two knockout cassettes of 3,862 and 2,192 bp that contained part of the region flanking the *mfs*1 gene and part of the hygromycin resistance gene, respectively. These cassettes were transformed into wild-type *C. lindemuthianum* protoplasts. The transformants obtained were randomly selected and monosporically purified. A mitotic stability test demonstrated that after five generations in nonselective culture medium, these transformants were all able to grow in a selective medium containing hygromycin.

The previously selected hygromycin-resistant transformants were analyzed by PCR using the CF and CR gene-specific primers (Table 1). In the PCR analysis, we observed a 2,015 bp DNA fragment in the wild-type genome, while the Δ*mfs*1 mutant produced a fragment of approximately 6,234 bp (Fig. 1B). One transformant was selected because produced a pattern of bands consistent with a specific *mfs*1 gene knockout; this transformant was subsequently characterized by hybridization to confirm the site-specific recombination and to assess the number of copies of the hygromycin cassette that had integrated into the genome (Figs. 1B and 1C). This characterization ensured that the phenotypic changes found in Δ*mfs*1 mutants were due to the loss of the *mfs*1 gene alone and not for other mutations.

The molecular characterization of the Δ*mfs*1 mutant was performed by Southern blot analysis using probes that consists of a 760 bp DNA fragment (initial portion of the *mfs*1 gene) and a 712 bp DNA fragment (hygromycin resistance gene). This analysis was performed with both the wild-type and Δ*mfs*1 mutant strains. Using the *mfs*1 probe and the *Sal*I restriction enzyme, which does not cleave within the *mfs*1 gene, but has a cleavage site both in its flanking region and in the hygromycin resistance gene, we confirmed the integration of the Δ*mfs*1 mutant that had been initially demonstrated by the PCR analysis.

To examine the integration profile of the hygromycin resistance gene, a Southern blot was performed using a probe containing the hygromycin resistance gene and the *Sca*I restriction endonuclease, which does not cleave within the *mfs*1 gene but it has a cleavage site in the hygromycin resistance gene. A single DNA fragment of approximately 1 kb was detected in the transformant genome (Fig. 1B) but was absent in the wild-type genome, which confirmed that the Δ*mfs*1 mutant was specifically integrated into the *mfs*1 locus and a single copy of the knockout cassette was present in the *C. lindemuthianum* genome. This pattern suggested that the transformant was suitable for the functional analysis of the *mfs*1 gene. This conclusion was supported by RT-PCR analysis with the MFS1cDNAL and MFS1cDNAR gene-specific primers, confirming the absence of the *mfs*1 transcript in the mutant during the sixth and seventh days of infection in

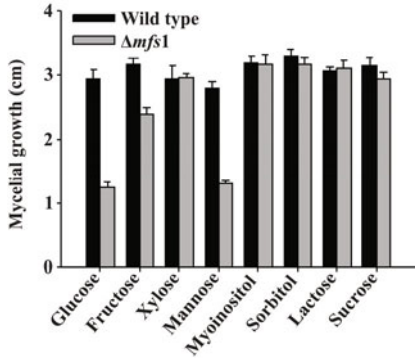

Fig. 5. Mycelial growth of wild-type and Δ*mfs*1 mutant *C. lindemuthianum* in different sugar sources. Wild-type and Δ*mfs*1 mutant strains were cultured for 10 days in a minimum media containing the following sugars as the only carbon source: glucose, fructose, xylose, mannose, myoinositol, sorbitol, lactose, and sucrose.

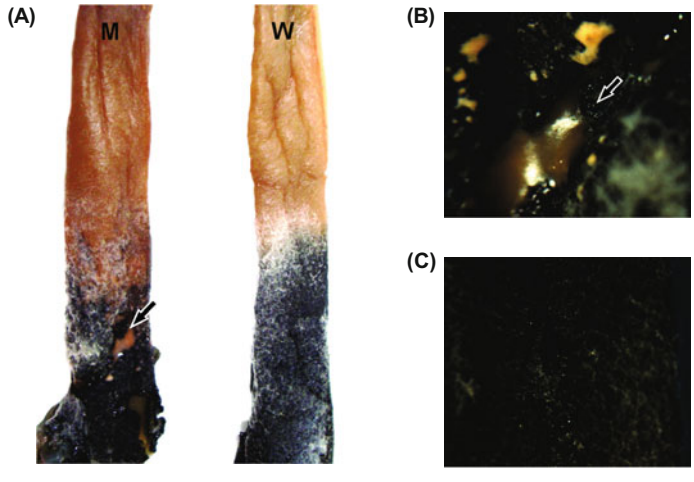

Fig. 6. Wild-type and Δ*mfs*1 mutant *C. lindemuthianum* conidiation. (A) The Δ*mfs1* mutant (M) is sporulating on the surface of a common bean pod 10 days after inoculation, while the wild-type fungus (W) shows no sign of sporulation. (B) A pod surface that was inoculated with the Δ*mfs*1 mutant is shown at 27× magnification; here, hyphae are present on the necrotic plant tissue and a dense layer of conidia can be seen. (C) A pod surface that was inoculated with wild-type fungi is shown at 27× magnification; the presence of hyphae on the necrotrophic plant tissue and the absence of a conidial mass can be observed. The arrows indicate the mucilaginous mass that is filled with conidia.

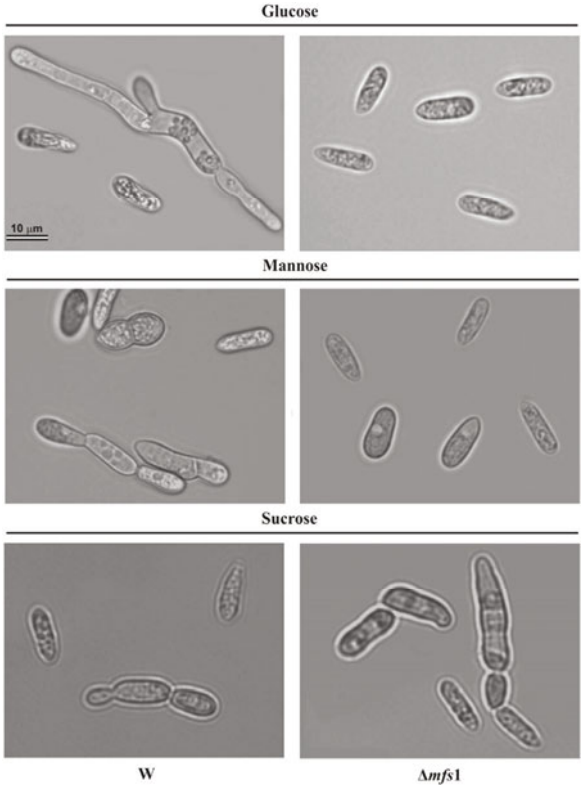

Fig. 7. Germination of wild-type and Δ*mfs*1 mutant *C. lindemuthianum* conidia. The wild-type conidia germinated earlier in the MM supplemented with glucose and the MM supplemented with mannose; by contrast, the Δ*mfs*1 mutant conidia did not germinate in media with glucose and mannose after 48 h. (Sucrose) positive control.

the bean plants. Taken together, these results indicated that the Δ*mfs*1 mutant did not express a functional copy of the *mfs*1 gene, while transcriptional levels were high in the wild-type fungus during the seventh day of the pathogenicity test described above (Fig. 1D).

The Δ*mfs*1 mutant displays decreased growth in the presence of glucose, mannose, and fructose

The ability of both the wild-type and mutant strains to utilize different sugars in the culture medium as their sole carbon source was analyzed to confirm the possible sugar-transport role of MFS members. Wild-type and Δ*mfs*1 mutant *C. lindemuthianum* mycelial growth was significantly different in the presence of different types of sugar.

The mutant strain grew significantly less than the wild-type when cultured in a mineral medium containing glucose, fructose or mannose as the sole carbon source (Fig. 5). The greatest variation in mean growth occurred in the media containing glucose and mannose, in which the wild-type fungus had a 50% larger colony diameter than did the Δ*mfs*1 mutant. The other examined sugars showed no difference in growth between the wild-type and mutant strains. Taken together, these results suggest that the *mfs*1 gene encodes a membrane transport protein that is specific for monosaccharides, such as glucose, mannose and fructose, which are essential for the primary metabolism of the fungus.

The Δ*mfs*1 mutant displays altered conidiation and germination

To investigate whether the *mfs*1 gene product is essential for *C. lindemuthianum*, the efficiency of wild-type and Δ*mfs*1 mutant strains in producing and germinating fungal conidia was examined. No signs of conidia release from any of the inoculated pods were observed ten days after sterilized pods were inoculated with wild-type mycelium. By contrast, all of the pods (n=10) that were inoculated with the Δ*mfs*1 mutant showed a dense mucilaginous orange mass full of conidia (Fig. 6). The conidiation of the wild-type fungus was observed only 14 days after the inoculation. This observation was verified in three independent experiments.

To evaluate spore germination, conidia of the wild-type and mutant strains were germinated in media that contained different sugars as carbon sources. The germination was examined only in the glucose and mannose media because these sugars had the greatest effect on the growth of the mutant fungus. After 48 h incubation at 22°C, the wild-type fungi conidia showed a significant percentage of germinated conidia (30%) in MM supplemented with mannose, glucose or sucrose. During the observation period, the germination of Δ*mfs*1 mutant in the glucose and mannose media was not observed (Fig. 7). Thus, our results suggest that the *mfs*1 gene product is involved in the physiology of *C. lindemuthianum* because when grown in pods, the conidiation of the mutant was earlier than that of the wild-type. While the spore germination was slower, this process was sharply affected when the mutant fungus was grown in media containing only glucose (MM + 5%) or mannose (MM + 5%) as carbon sources.

The *mfs*1 gene is not required for *C. lindemuthianum* pathogenicity in the bean plant

The role of *mfs*1 in *C. lindemuthianum* pathogenicity was determined by infecting susceptible bean leaves with both the wild-type and mutant strains. The severity of anthracnose symptoms on the inoculated leaves gradually increased during the experiment and became evident on the fifth day after inoculation, which is consistent with the onset of the necrotrophic phase. On the seventh day of the pathogenicity test, both sets of leaves showed signs of necrosis, mainly on the surface of the conductive vessels (Fig. 8). These results indicate that the *mfs*1 gene is not essential for *C. lindemuthianum* pathogenicity.

The *mfs*1 gene is not involved in resistance to fungicides that are commonly employed to control anthracnose in bean plants

The fungicide activity of Dithane (mancozeb), Folicur (tebuconazole), and Copper Sandoz (cuprous oxide) was similar between the wild-type and Δ*mfs*1 mutant strains at all the concentrations tested. This result indicates that the *mfs*1 gene is not involved in the efflux sensitivity or resistance to the tested drugs.

Discussion

In this work we describe the identification and characteriza-

tion of a new MFS transporter encoding gene in *C. lindemuthianum*. This is the first functional study of a hexose transporter that is involved in transporting photoassimilates during the necrotrophic phase of the hemibiotrophic fungus.

The deduced amino acid sequence showed 12 transmembrane domains, a typical organization for some subfamilies of MFS transporters and high similarity with other MFS proteins. At the superfamily level, individual MFS members share a low level of nucleotide sequence identity and are linked only by a conserved motif with the sequence DRXXRR, which is found in equivalent positions on the N- and C-termini of the proteins and in the loops joining TM2 to TM3 and TM8 to TM9 (Maiden, 1987). Subsequent studies have revealed that this motif can even be identified in members of the MFS that display extensive sequence divergence. Two sequences resembling those conserved motifs of individual MFS members were identified in the *mfs*1 gene at the precise positions described above (Fig. 2).

Although MFS proteins have strong structural conservation, each gene has a sequence specificity that allows it to be differentiated from other MFS genes (Vardy *et al*., 2004). Given that MFS transporter families are phylogenetically related according to their function (Paulsen and Skurray, 1996; Pao *et al*., 1998), each family must recognize and transport a distinct class of structurally related compounds determined by conserved motifs that are specific to each family (Del Sorbo *et al*., 2000; Law *et al*., 2008). A phylogenetic analysis performed by PAUP of the Mfs1 amino acid sequence revealed that it clustered with other sugar transport proteins, although the identity between the nucleotide sequences was not high (Fig. 3).

The SP family of sugar transporters is the most representative family within the MFS, and the genes encoding these proteins have diverse sequences and functions (Leandro *et al*., 2009). The multiple sequence alignment of 11 representative members of the SP family (Fig. 2) reveals the 47 residue segment signature of the SP family.

The genes encoding these proteins are widely distributed among fungal genomes. Differences in the number of genes that encode transporters may be related to the distinct needs of the efflux and influx systems, which vary according to the environment where these organisms are found (Nagata *et al*., 2008). Although the genes that encode MFS proteins are ubiquitous in the genome of living organisms, a single copy of the *mfs*1 gene is present in the *C. lindemuthianum* genome, which is similar to other MFS protein-coding genes in filamentous fungi (Van den berg *et al*., 2008; Lévesque *et al*., 2010).

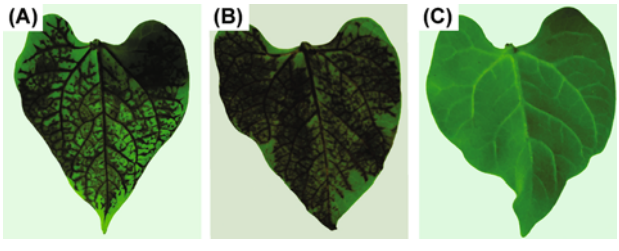

Fig. 8. The *mfs*1 gene is not involved in *C. lindemuthianum* pathogenicity. The pathogenicity assay was performed by adding 10^6 spores/ml to susceptible leaves that had been detached from the Rosinha bean plant. The leaves corresponding to the seventh day of contact were used to compare the effects of the wild-type (A) and Δ*mfs*1 mutant *C. lindemuthianum* fungi (B). Bean leaves inoculated with autoclaved ultrapure water, served as a negative control (C).

Analyzing *mfs*1 gene expression, we observed that the transcript levels of this gene were significantly higher on the seventh day after the infection of the bean leaves, which coincides with the onset of the necrotrophic phase of *C. lindemuthianum* (Fig. 4). During the necrotrophic phase, which begins 48 to 72 h after inoculation, the fungus undergoes a rapid growth of its hyphae, which then invades and kills plant cells; the hyphae also secrete enzymes that degrade plant polysaccharides and release large amounts of sugars that can be absorbed by the fungus. Therefore, the fungus must respond to this change in environmental carbon sources during this stage of infection. One adaptation by the fungus is the different transport systems in its plasma membrane that allow it to adjust its consumption of organic carbon sources based on changes in their concentration and composition in the environment (Krijger *et al*., 2008; O'Connell *et al*., 2012).

The characterization of mutant phenotypes is an essential element in the identification of gene function. The split-marker method was efficient at knocking out the *mfs*1 gene in *C. lindemuthianum*, generating mutants with site-specific integration and free of ectopic integrations (Fig. 1). In phytopathogens, knocking out genes that encode MFS transport proteins often results in a loss of drug resistance. An MFS protein knockout in the *Botrytis cinerea* fungus has been found to result in loss of tolerance to both toxic compounds that are present in the natural environment and fungicides (Hayashi *et al*., 2002). By contrast, the loss of *mfs*1 gene in *C. lindemuthianum* did not significantly alter the sensitivity profile of any of the evaluated fungicides compared to the wild-type.

Furthermore, in many cases, knocking out a gene that encodes a MFS protein in pathogenic fungi causes loss of pathogenicity, as has been observed in the phytopathogen *Ustilago maydis* (Wahl *et al*., 2010). However, a study of the phytopathogen *Metarhizium robertsii* has revealed that despite the importance of transport proteins for pathogen survival, they do not always directly relate to pathogenicity (Fang and Legar, 2010). A similar result has been found for *C. lindemuthianum*, the Δ*mfs*1 mutant that was not associated with host symptom reduction during a pathogenicity test (Fig. 8).

Many sugar transporters allow facilitated diffusion, while others only function when a sugar is scarce in the medium (Leandro *et al*., 2009). The SP transporters responsible for hexose (HXT) transport comprise 20 different SP families (Boles and Hollenberg, 1997). Previous studies have indicated that a single HXT transporter protein is responsible for transporting more than one hexose; in *Saccharomyces cerevisiae* six major glucose transporters (Hxt1-4, -6, and -7) can also transport fructose and mannose (Reifenberger *et al*., 1995; Wieczorke *et al*., 1999). Additional evidence of this multiple specificity has also been found in the CgHXT5 gene, which encodes a hexose transporter in *C. graminicola*. The specificity of this transporter has been inferred from an expression analysis, which confirmed its preference for

glucose and mannose (Lingner *et al.*, 2011). The wild-type and mutant *C. lindemuthianum* mycelial growth profiles showed significant differences when the fungi were cultured in media containing glucose, fructose or mannose, suggesting that Mfs1 transporter has multiple specificity (Fig. 5). This result confirmed the *in silico* analysis that connected the Mfs1 protein to MFS proteins of the SP family and related the *mfs*1 gene product to the transport of hexoses.

The Δ*mfs*1 mutant conidiation was premature (Fig. 6) and similar results have been obtained for knockouts of *Rco3*, a gene that encodes a glucose transporter in the filamentous fungus *N. crassa*. In this mutant, the induction of the sporulation process is related to a decrease in glucose uptake, which suggests that the RCO3 protein acts as a transporter and as a sensor of high and low glucose concentrations. Therefore, the mutant that lacked the *Rco3* gene was not able to suppress glucose transport when the fungus was grown in a medium containing low or high concentrations of this sugar, which caused earlier conidiation (Madi *et al.*, 1997).

The Δ*mfs*1 mutant conidia germinated slower than those of the wild-type fungus in the MM supplemented with glucose and the MM supplemented with mannose, which was likely a result of losing the *mfs*1 gene (Fig. 7). Previous studies in *A. nidulans* and *B. cinerea* have suggested that a link exists between hexose transporters that are sensitive to low concentrations of glucose and conidial germination (Doehlemann *et al.*, 2005; Forment *et al.*, 2006).

Taken together, these data suggest that the *mfs*1 gene encodes a membrane protein transporter with 12 TMDs that is involved in transporting monosaccharides, particularly hexoses. This transporter is responsible for the uptake of glucose, mannose, and fructose from the environment. Although it is not involved in pathogenicity, the MFS transporters are directly involved in balancing this interface between microorganisms and a continually varying environment, given that sugars are the primary source of energy in multicellular organisms.

The study of MFS transporters is still in its early stages, but it appears that the functions and specificities of these proteins are diverse. Although the role of some of these transporters has already been elucidated experimentally, most of these proteins are still only understood at the level of sequence homologies (*in silico* analysis) and expression patterns. Many questions remain unanswered, including the mechanisms by which these MFS transporters efficiently recognize and transport compounds of different chemical classes. Regardless of the organism, genome sequencing results have shown that these genes are extremely abundant, play a role in key cellular functions and represent a black box that has yet to be opened. Because of the functional relevance, studying the genes encoding these transporter proteins advances the understanding of MFS transporters. The regulation of these genes is also not yet fully understood, especially in phytopathogenic fungi. These investigations may also play a role both in discovering new disease control agents and in understanding the regulation and dynamics of the physiology of the organism. This data underscores the relevance of studying MFS sugar transporters for elucidating the mechanisms of phytopathogenic fungi development and host interaction.

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