The *Botrytis cinerea* hexokinase, Hxk1, but not the glucokinase, Glk1, is required for normal growth and sugar metabolism, and for pathogenicity on fruits

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INTRODUCTION

Hexose kinases play a central role in the initiation of sugar metabolism of living organisms and have also been implicated in carbon catabolite repression in yeasts and plants. In this study, the genes encoding glucokinase (Glk1) and hexokinase (Hxk1) from the plant-pathogenic ascomycete *Botrytis cinerea* were isolated and functionally characterized. Glk1-deficient mutants were indistinguishable from the wild-type in all growth parameters tested. In contrast, Δhxk1 mutants lacking Hxk1 showed a pleiotropic growth defect. On artificial media, vegetative growth was retarded, and conidia formation strongly reduced. No or only marginal growth of Δhxk1 mutants was observed when fructose, galactose, sucrose or sorbitol were used as carbon sources, and fructose inhibited growth of the mutant in the presence of other carbon sources. *B. cinerea* mutants containing Δhxk1 alleles with point mutations leading to enzymically inactive enzymes showed phenotypes similar to the Δhxk1 disruption mutant, indicating that loss of hexose phosphorylation activity of Hxk1 is solely responsible for the pleiotropic growth defect. Virulence of the Δhxk1 mutants was dependent on the plant tissue: on leaves, lesion formation was only slightly retarded compared to the wild-type, whereas only small lesions were formed on apples, strawberries and tomatoes. The low virulence of Δhxk1 mutants on fruits was correlated with their high contents of sugars, in particular fructose. Heterologous expression of Hxk1 and Glk1 in yeast allowed their enzymic characterization, revealing kinetic properties similar to other fungal hexokinases and glucokinases. Both Δglk1 and Δhxk1 mutants showed normal glucose repression of secreted lipase 1 activity, indicating that, in contrast to yeast, *B. cinerea* hexose kinases are not involved in carbon catabolite repression.
Similar to yeast hexokinases, the A. niger hexokinase is strongly inhibited by trehalose 6-phosphate, which plays a role in the regulation of hexokinase activity also in vivo (Arisan-Atac et al., 1996; Panneman et al., 1998). In A. nidulans, the possible involvement of hexokinases in catabolite repression was indicated by the analysis of mutants defective in either hexo- or glucokinase or in both enzymes (Flipphi et al., 2003).

Botrytis cinerea is a necrotrophic plant-pathogenic fungus with a wide spectrum of host plants (Van Kan, 2006). It prefers to infect soft and senescing tissues, causing serious damage in fruits and vegetables (Droby & Lichter, 2004). Spore germination and infection are strongly stimulated in the presence of sugars, in particular fructose (Blakeman, 1975; Benito et al., 1998). Recently, a transporter specific for fructose has been characterized, but its physiological role remains unclear (Doehlemann et al., 2005). After successful invasion, the plant tissue is killed and macerated by the release of toxic metabolites and lytic enzymes (Van Kan, 2006). Catabolite repression in the presence of glucose has been described; however, no data regarding the molecular mechanisms of catabolite repression are available yet (Wubben et al., 2000; Reis et al., 2005).

This paper is believed to be the first report of the molecular and functional characterization of hexose kinases in a plant-pathogenic fungus. By phenotypic characterization of knockout mutants and heterologous gene expression in yeast, it was shown that in B. cinerea, the glucokinase is dispensable for growth, while the hexokinase is absolutely required for normal growth, sporulation and infection, in particular in the presence of hexoses.

**METHODS**

Fungal growth conditions and transformation. Botrytis cinerea strain B05.10 was grown as described by Doehlemann et al. (2006a). To test growth of B. cinerea mutants on various carbon sources, minimal medium containing Gamborg’s B5 basal salt mixture (Duchefa Biochemie), a carbon source (1 % unless otherwise stated), and 1.5 % Bacto agar was used. Transformation of A. niger was performed either with 10 μg/ml of the plasmid pDR196 (Rentsch et al., 1995). Site-directed mutagenesis of the hexokinase-deficient Ahxk1Ahxk2Agk1 mutant of S. cerevisiae (strain CEM.1S14-4D) was performed using the CLUSTAL W algorithm (Thompson et al., 1994), with manual corrections.

Expression of gkl1 and hxx1 cDNAs in yeast. The coding regions of the gkl1 and hxx1 genes were amplified from B. cinerea cDNA using primers cGKL-1/cGLK-2 and cHXK-1/cHXK-2, and cloned into the yeast expression vector pDR196 (Rentsch et al., 1995). Site-directed mutagenesis of the hxx1 expression construct was performed as described above. Plasmids were transformed into a hexokinase-deficient Ahxk1Ahxk2Agk1 mutant of S. cerevisiae using the CLONING system (CLONING systems). The extracts were desalted by passage through Sephadex G-50 columns, and protein contents determined according to Bradford (1976). For preparation of yeast extracts, cells were grown in SC-Gal medium (Sherman et al., 1986) to mid-exponential growth phase (OD600nm−1), harvested by centrifugation and washed with 50 mM phosphate buffer, pH 7.0; 5 mM MgCl2; 0.5 mM EDTA; 5 mM 2-mercaptoethanol; 1 mM Pefabloc (Roche Molecular Biochemicals).

Measurements of hexose kinase activities and sugar contents. Hexose kinase activities were measured with either B. cinerea or S. cerevisiae crude extracts. For preparing B. cinerea extracts, 30 ml of 1 % malt extract in Erlenmeyer flasks were inoculated with mycelium scraped from non-sporulating, 4 day old tomato malt agar cultures, and shaken at 140 r.p.m. for 36 h at 20 °C. The mycelium was washed (2500 g, 5min) twice with 10 ml 50 mM potassium phosphate buffer (pH 7.0). About 500 mg of fresh mycelium was homogenized in liquid nitrogen with a mortar and resuspended in 2 ml of extraction buffer by vortexing [extraction buffer: 50 mM potassium phosphate buffer, pH 7.0; 5 mM MgCl2; 0.5 mM EDTA; 5 mM 2-mercaptoethanol; 1 mM Pefabloc (Roche Molecular Biochemicals)]. The extracts were desalted by passage through Sephadex G-50 columns, and protein contents determined according to Bradford (1976). For preparation of yeast extracts, cells were grown in SC-Gal medium (Sherman et al., 1986) to mid-exponential growth phase (OD600−1), harvested by centrifugation and washed with 50 mM phosphate buffer, pH 7.0; 5 mM MgCl2; 0.5 mM EDTA; 5 mM 2-mercaptoethanol; 1 mM Pefabloc (Roche Molecular Biochemicals).
Cells were disrupted in a buffer containing 20 mM Tris/HCl, pH 7.9; 10 mM MgCl₂; 1 mM EDTA; 5% (v/v) glycerol; 1 mM DTT; 0.3 M ammonium sulfate and 1 mM PefaBlock (Ausubel et al., 1999), with 0.5 mm glass beads and 5–30 s bead beater pulses (Mini-Beadbeater; Biospec Products), and desalted as described above. For measurement of hexose kinase activity, B. cinerea or yeast extracts containing 50–70 mg protein was added to the reaction buffer (50 mM PIPES, pH 7.5; 5 mM MgCl₂; 2 mM ATP; 0.5 mM NADP⁺; 2 U glucose-6-phosphate dehydrogenase; and 4 U phosphoglucose isomerase for determination of fructokinase activity) to a final volume of 1 ml. The reactions were started by addition of either 1 mM glucose or 10 mM fructose, and hexose kinase activity was determined photometrically at 434 nm. Kinetic constants were calculated by nonlinear regression analysis with the Origin 6.0 software (OriginLab Corporation).

For determination of sugar contents in tomato leaves and in fruits, tissues were homogenized in a mortar with liquid nitrogen, and extracted with 80% ethanol at 65 °C for 2 h. Ethanolic extracts were dried, and redissolved in distilled H₂O. Separation and quantification of d-fructose, d-glucose and sucrose was done by HPLC, using an EC250/4 Nucleosil column (Macherey & Nagel) and refractometric detection.

### RESULTS

**Cloning of glk1 and hxl1 genes**

Starting from two B. cinerea EST sequences (Viaud et al., 2003) showing high similarity to fungal hexo- and glucokinase genes, respectively, gene-specific primers were designed, and used for the amplification of hxl1 and glk1 gene fragments from genomic DNA of B. cinerea strain B05.10. Using these PCR fragments as hybridization probes, genomic clones containing each of the genes were isolated from a B. cinerea genomic bacteriophage library. After subcloning into plasmids, restriction fragments

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
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<tr>
<td>GLK-1</td>
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<tr>
<td>GLK-2</td>
<td>TCCCACCCATTTCTCCTTG</td>
</tr>
<tr>
<td>GLK-3</td>
<td>CCGACCACTGATGACCTCT</td>
</tr>
<tr>
<td>GLK-KO1</td>
<td>GCCATTAGCCAATGGAAGCAAA</td>
</tr>
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</tr>
<tr>
<td>GLK-KO4</td>
<td>TTATCAACGACAGAGGTTT</td>
</tr>
<tr>
<td>HXK-S158A-1</td>
<td>GAGCTCTGTTTACCTGGCCACCCAGCTACTC</td>
</tr>
<tr>
<td>HXK-S158A-2</td>
<td>GATGAGGTGGGTTGGGCAAGTCACGACTCT</td>
</tr>
<tr>
<td>HXK-D211S-1</td>
<td>GCTTACAGCCTTTGATCACAGCTACTG</td>
</tr>
<tr>
<td>HXK-D211S-2</td>
<td>GCAATCAAGTTGAGTACAGCTGCTACTC</td>
</tr>
<tr>
<td>cGLK1-PST</td>
<td>GTCCTGACGCTACAAGAGCGCAATTC</td>
</tr>
<tr>
<td>cGLK2-SAL</td>
<td>CTTCGACGACGCTAGGCTGAGCTACTCT</td>
</tr>
<tr>
<td>chHXK1-ECO</td>
<td>GGAATTCAAGCTTCATTTCCAGATCTCC</td>
</tr>
<tr>
<td>chHXK2-SAL</td>
<td>CTTCGACGACGCTAGGCTGAGCTACTC</td>
</tr>
<tr>
<td>chHXK3-ECO</td>
<td>TGCATATGTTCACTTTATGGCTAGTGC</td>
</tr>
<tr>
<td>HXK-EX1</td>
<td>TGATGAGAAGTCCAGATGGTG</td>
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<td>HXK-EX2</td>
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<td>GLX-EX2</td>
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<tr>
<td>ACT-2</td>
<td>GTAAGAATCGGAGGAGGCGGAGGAGG</td>
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Table 1. Oligonucleotide primers used in this work

Restriction sites are underlined. Mutated nucleotides are indicated in bold italics.
carrying *hxk1* and *glk1* were sequenced (not shown). The *B. cinerea* *glk1* gene (accession no. EF156463) contains a 1677 bp coding region interrupted by two introns. The predicted Glk1 protein is 559 amino acids long, and shares 55 and 38 % identity, respectively, with glucokinases from *A. niger* (GlkA) and *S. cerevisiae* (Glk1). The complete coding sequence of the *hxk1* gene (EF156464) could be determined after release of the *B. cinerea* strain B05.10 genome sequence on the website of the Broad Institute. This was because exon 1, encoding amino acids M1–S15, was not on the genomic insert used for *hxk1* sequencing, but separated from exon 2 by an unusually long intron of 747 bp. The *hxk1* coding sequence has a size of 1473 bp, and contains five introns. The predicted 491 amino acid Hxk1 protein shares 74 and 54 % identity with *A. niger* HxkA and with *S. cerevisiae* Hxk2, respectively, but only 34 % identity with *B. cinerea* Glk1. Expression analysis via semiquantitative RT-PCR was performed with ungerminated conidia, 16 h old germlings and 48 h old mycelium grown in 1 % malt extract, and with infected tomato leaf tissue (4 days p.i.). Similar to the actin gene *actA*, which was included as a putatively constitutively expressed control gene, transcript levels for *hxk1* and *glk1* were similar during all stages of fungal development (Fig. 1). Whether or not the *hxk1* expression in germlings and mycelium was significantly increased relative to the other stages was not analysed further.

**Construction of Δ*glk*, Δ*hxk1* and Δ*hxk1*-mut mutants**

In order to examine the role of hexokinases in *B. cinerea*, disruption mutants of both *glk1* and *hxk1* genes were created (Fig. 2A, B). After transformation with the PCR fragments carrying the disruption constructs, hygromycin-resistant colonies were isolated, and their DNA analysed. By this means, five isolates deleted in *hxk1* (Δ*hxk1*) and three isolates deleted in *glk1* (Δ*glk1*) were confirmed by PCR (Fig. 2B). Further confirmation of the targeted deletions was obtained, for one Δ*hxk1* and for one Δ*glk1* isolate, by means of RT-PCR with primers amplifying fragments within the deleted regions of both genes. As expected, cDNAs for *hxk1* and *glk1* could not be amplified from the Δ*hxk1* and the Δ*glk1* mutant, respectively (Fig. 2C). All Δ*glk1* mutants were indistinguishable from the wild-type in all growth parameters tested. In contrast, all Δ*hxk1* mutants revealed a pleiotropic phenotype, showing reduced growth on all media tested as well as other defects (see below). In *A. nidulans* and *S. cerevisiae*, hexokinase-deficient mutants have not been reported to be impaired in their growth, except on fructose-containing media (Walsh et al., 1991; Ruijter et al., 1996; Flippin et al., 2003). We therefore considered the possibility that the growth defect of the *B. cinerea* hexokinase mutants might be due to an additional, non-enzymic function of the Hxk1 protein. In order to test this possibility, we constructed two derivatives of the *hxk1* coding region with mutations leading to enzymically inactive proteins. In the *hxk1*-S158A mutant, a serine residue was removed which has been shown in yeast and *Arabidopsis thaliana* hexokinase to be essential for the phosphoryl transfer during catalysis. In the *hxk1*-D211S mutant, an aspartate residue essential for yeast HXK2 catalytic function was replaced (Kraakman et al., 1999; Moore et al., 2003). The mutant *hxk1* genes were introduced by transformation into *B. cinerea*, replacing the wild-type *hxk1* gene by homologous recombination (Fig. 3A). From the hygromycin-resistant transformants tested, one (*hxk1*-S158A) and three (*hxk1*-D211S) were shown by PCR to have replaced in their genomes the wild-type *hxk1* gene by the mutant alleles (Fig. 3B).

**Phenotypic characterization of *glk1* and *hxk1* mutants**

On rich media such as tomato malt agar, radial growth of all *hxk1* mutants was significantly retarded, whereas growth of the Δ*glk1* mutant was similar to that of the wild-type (Table 2). Furthermore, sporulation of the Δ*hxk1* mutants was strongly reduced. For instance, only 9.7 ± 3.0 × 10^5 conidia (mean ± SE) were produced per tomato malt agar plate after 14 days of growth by the Δ*hxk1* mutant, in comparison to 1.8 ± 0.5 × 10^6 conidia of the wild-type. Very low sporulation was also observed for the mutants with mutated *hxk1* alleles, while normal sporulation occurred in the Δ*glk1* mutant (not shown). Furthermore, the *hxk1* conidia formed were smaller (8.4 ± 1.0 μm) than those of the wild-type (10.3 ± 0.7 μm) and the Δ*glk1* mutants (10.5 ± 1.3 μm). On minimal media, the degree of growth impairment was strongly dependent on the carbon source used (Table 2). With acetate and xylose, colony expansion of the Δ*hxk1* mutants was only moderately

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**Fig. 1.** Expression of *glk1* and *hxk1* at different growth stages of *B. cinerea*. Expression analysis was done with RT-PCR, using *actA* as a constitutively expressed reference gene. RNA was extracted from ungerminated conidia (lane 1), 16 h old germlings (lane 2), 48 h old mycelium (lane 3), and tomato leaf tissue 4 days p.i. (lane 4). In lane 5, PCR was performed with genomic *B. cinerea* DNA, which yielded larger fragments than the cDNAs (*glk1*and *hxk1*), or no fragment (*actA*), because of primers flanking or overlapping introns. The following primers were used: *glk1*, GLK-EX1, GLK-EX2; *hxk1*, HXK-EX1, HXK-EX2; *actA*, ACT-1, ACT-2.
reduced, while with glucose, it was less than 50% of the wild-type. With glycerol, growth of the Δhxk1 mutants was about 30% of the wild-type. Only marginal growth of the Δhxk1 mutants was observed with sucrose, galactose and sorbitol, and no growth at all with fructose. These data indicate that Hxk1 provides the only source of fructokinase activity in B. cinerea, and that the metabolism of sucrose, galactose and sorbitol is connected to that of fructose.

When fructose was supplied at 0.1% or higher concentrations together with carbon sources that are able to support growth, the Δhxk1 mutants could not grow either, demonstrating that fructose strongly inhibits growth of the Δhxk1 mutant.

Germination assays with conidia were performed under different conditions. In distilled water on hydrophobic polyethylene surfaces, conidia of the wild-type and both mutants showed similar germination rates (data not shown; Doehlemann et al., 2006a). On hydrophilic glass surfaces, germination of B. cinerea conidia is usually nutrient dependent (Doehlemann et al., 2005; Blakeman, 1975). On glass slides in solutions containing glucose, acetate or 1% malt extract, germination rates were also similar for all strains. Although they did not support mycelial growth, fructose and galactose also stimulated conidial germination of the Δhxk1 mutants, but germination rates were significantly lower than those of wild-type and Δglk1 conidia (Fig. 4A). However, in fructose the Δhxk1 germlings formed only very short germ tubes (Fig. 4B). These data indicate that the early stages of germination are less sensitive to the inhibitory effects of fructose.

To check the pathogenic performance of the mutants, infection tests with different plant tissues were performed (Fig. 5A). On leaves, little difference in lesion formation was observed between wild-type and mutants, although lesions induced by the Δhxk1 mutants developed somewhat more slowly those induced by the wild-type or the Δglk1 mutant. However, while the wild-type and the Δglk1 mutant lesions developed conidiophores and conidia above the infected tissue, sporulation was never observed in any of the Δhxk1 mutants. In contrast, lesion formation by the...
B. cinerea

Verification of B. alleles (for construction see Methods), PCR fragments were homologous recombination. From plasmids carrying the mutated B. cinerea and their new restriction sites into the genome of strains, and digested with Apa B. cinerea were amplified from total DNA of wild-type and mutant alleles. PCR fragments containing parts of arrows; introns in crossed dotted lines. Coding regions are indicated by black hxk1 the wild-type genomic region (not shown) are indicated by black arrows; introns in hxk1 are shown by open bars. S, Sall. (B) Verification of B. cinerera transformants carrying mutated hxk1 alleles. PCR fragments containing parts of the hxk1 coding region were amplified from total DNA of wild-type and mutant B. cinerera strains, and digested with Apal (A; for hxk1-S158A) or PvuII (P; for hxk1-D211S). Note that the Apal digest of the hxk1-S158A fragment was incomplete, but sequencing confirmed the presence of the Apal site. nd, Non-digested DNA.

**Table 2.** Radial growth of B. cinerea strains on different agar media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Wild-type [mm day⁻¹]</th>
<th>Hxk1 [mm day⁻¹] (%) of wt</th>
<th>hxk1-D211S [mm day⁻¹] (%) of wt</th>
<th>hxk1-D211S [mm day⁻¹] (%) of wt</th>
<th>Aglk1 [mm day⁻¹] (%) of wt</th>
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</thead>
<tbody>
<tr>
<td>Tomato malt agar</td>
<td>23.8 ± 1.2</td>
<td>17.3 ± 1.3 (73 ± 3)</td>
<td>15.7 ± 0.6 (66 ± 4)</td>
<td>17.7 ± 1.6 (74 ± 3)</td>
<td>25.1 ± 0.3</td>
</tr>
<tr>
<td>MM glucose</td>
<td>19.3 ± 1.4</td>
<td>9.1 ± 2.8 (47 ± 3)</td>
<td>9.5 ± 1.8 (49 ± 3)</td>
<td>9.0 ± 1.5 (47 ± 3)</td>
<td>19.9 ± 2.4</td>
</tr>
<tr>
<td>MM fructose</td>
<td>19.4 ± 2.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>18.1 ± 1.6</td>
</tr>
<tr>
<td>MM Glic/Fru*</td>
<td>22.1 ± 0.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>22.5 ± 0.6</td>
</tr>
<tr>
<td>MM glyceral</td>
<td>22.3 ± 0.0</td>
<td>7.0 ± 0.3 (31 ± 3)</td>
<td>8.2 ± 0.7 (37 ± 3)</td>
<td>10.3 ± 1.4 (46 ± 4)</td>
<td>19.9 ± 5.5</td>
</tr>
<tr>
<td>MM acetate</td>
<td>12.6 ± 0.8</td>
<td>7.5 ± 2.0 (60 ± 4)</td>
<td>ND</td>
<td>ND</td>
<td>13.1 ± 1.0</td>
</tr>
<tr>
<td>MM sucrose</td>
<td>20.3 ± 1.0</td>
<td>0.4 ± 0.1</td>
<td>(2 ± 0)</td>
<td>0.3 ± 0.1 (1 ± 0)</td>
<td>0.4 ± 0.2 (2 ± 1)</td>
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<tr>
<td>MM sorbitol</td>
<td>21.1 ± 3.5</td>
<td>0.4 ± 0.6</td>
<td>(2 ± 0)</td>
<td>0.4 ± 0.2 (2 ± 1)</td>
<td>0.9 ± 0.3 (4 ± 1)</td>
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<tr>
<td>MM galactose</td>
<td>16.1 ± 4.9</td>
<td>1.3 ± 0.5</td>
<td>(8 ± 2)</td>
<td>0.4 ± 0.2 (2 ± 1)</td>
<td>0.9 ± 0.4 (6 ± 2)</td>
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<tr>
<td>MM xylose</td>
<td>11.8 ± 0.4</td>
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<td>ND</td>
<td>12.1 ± 0.6</td>
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<tr>
<td>MM Xyl/Fru*</td>
<td>21.6 ± 1.6</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>19.8 ± 1.2</td>
</tr>
</tbody>
</table>

*0.1% Fructose (Fru) plus 1% glucose (Glc) or 1% xylose (Xyl).

hxk1 mutants in fruits was strongly retarded (Fig. 5B and data not shown). To investigate the possible reason for the tissue-specific infection defect of the hxk1 mutants, sugar measurements were performed. As shown in Fig. 5(C), the concentrations of glucose, fructose and sucrose in leaves were much lower than those in tomatoes and apples.

**Biochemical characterization of Glk1, Hxk1 and mutated Hxk1 enzymes**

For biochemical characterization of Glk1 and Hxk1, hexose kinase activities were first measured in extracts prepared from B. cinerea wild-type as well as from Δhxk1 and Δglk1 mutants (Fig. 6). Glucose kinase activity was highest in the wild-type (0.26 ± 0.03 mmol glucose min⁻¹ mg⁻¹), and significantly lower but present both in the Δglk1 mutant (0.17 ± 0.06) and in the Δhxk1 mutant (0.08 ± 0.02). Treatment of wild-type extracts with 1 mM of the hexokinase-specific inhibitor trehalose 6-phosphate (Thevelein & Hohmann, 1995) resulted in approximately 50% inhibition of the glucose kinase activity. Extracts of the Δglk1 mutant were inhibited by 85%, and no significant inhibition was observed in extracts of the Δhxk1 mutant. Regarding fructose kinase, similar activities were measured in wild-type and Δglk1 mutant extracts, which were strongly inhibited by trehalose 6-phosphate. In contrast, no significant fructose kinase activity was found in the Δhxk1 mutant.

For a more detailed analysis of Glk1 and Hxk1, the coding regions of glk1 and hxk1 were cloned into the yeast expression vector pDR196 and transformed into the hexose-kinase-deficient yeast strain CEN.IS14-4D. In addition to the wild-type hxk1 sequence, hxk1 derivatives
encoding the mutated Hxk1-S158A and Hxk1-D211S proteins, and an N-terminal deletion derivative (Hxk1-Δ1-15) were used for transformation. Functional expression of these sequences was tested by growing the yeast transformants on agar media containing hexoses as carbon sources. While all transformants grew well on galactose (which can be metabolized by strain CEN.IS14-4D), only transformants expressing wild-type \textit{glk1} and \textit{hxk1} grew well on glucose, whereas the mutated \textit{hxk1} sequences did not support growth (Fig. 7A). On fructose media, only the transformant expressing wild-type \textit{glk1} and \textit{hxk1} grew well on glucose, whereas the mutated \textit{hxk1} sequences did not support growth (Fig. 7A). On fructose media, only the transformant expressing wild-type \textit{glk1} and \textit{hxk1} grew well on glucose, whereas the mutated \textit{hxk1} sequences did not support growth (Fig. 7A). On fructose media, only the transformant expressing wild-type \textit{glk1} and \textit{hxk1} grew well on glucose, whereas the mutated \textit{hxk1} sequences did not support growth (Fig. 7A). On fructose media, only the transformant expressing wild-type \textit{glk1} and \textit{hxk1} grew well on glucose, whereas the mutated \textit{hxk1} sequences did not support growth (Fig. 7A). On fructose media, only the transformant expressing wild-type \textit{glk1} and \textit{hxk1} grew well on glucose, whereas the mutated \textit{hxk1} sequences did not support growth (Fig. 7A). On fructose media, only the transformant expressing wild-type \textit{glk1} and \textit{hxk1} grew well on glucose, whereas the mutated \textit{hxk1} sequences did not support growth (Fig. 7A).

Fig. 4. Nutrient-dependent germination of \textit{B. cinerea} conidia. (A) Germination rates (16 h p.i.) on glass slides of wild-type (black bars), Δglk1 (white bars) and Δhxk1 (grey bars) strains, in 10 mM glucose (Glc), fructose (Fru), galactose (Gal), galactose plus fructose (Gal/Fru), acetate (Ace), or in 1 % malt extract (1 % ME). Data are the means ± SE of two experiments with two replicates. (B) Photographs of \textit{B. cinerea} germlings (16 h p.i.) on glass slides with fructose (Fru), acetate (Ace), and fructose plus acetate (Fru/Ace). Bars, 25 μM.

Fig. 5. Infection tests with \textit{B. cinerea} wild-type and hexose kinase mutants. (A) Photographs showing infected leaf and fruit tissues (72 days p.i.). (B) Lesion expansion of wild-type and mutant strains on tomato leaves (white bars) and apples (black bars). (C) Contents of glucose (black bars), fructose (white bars) and sucrose (grey bars) in the plant tissues used for infection tests. Data are the means ± SE of three experiments with at least two measurements performed in parallel.

Fig. 6. Role of \textit{B. cinerea} hexokinases in catabolite repression

To test whether Hxk1 and/or Glk1 are involved in catabolite repression, we analysed the expression of the similar for Glk1 and Hxk1, but Glk1 showed a higher affinity ($K_m$ 48 ± 12 μM) for glucose than Hxk1 ($K_m$ 132 ± 19 μM). While Glk1 did not phosphorylate fructose, the affinity of Hxk1 for fructose ($K_m$ 700 ± 46 μM) was lower than that for glucose.

Role of \textit{B. cinerea} hexokinases in catabolite repression

To test whether Hxk1 and/or Glk1 are involved in catabolite repression, we analysed the expression of the
secreted lipase, Lip1, which is specifically induced by methyl oleate, but strongly repressed by glucose (Reis et al., 2005). *B. cinerea* mycelium was pre-cultivated in rich medium, and mycelial aliquots were transferred into minimal media containing methyl oleate and various carbon sources. After 3–4 days of incubation, glucose consumption and esterase activities were determined in the supernatants. When the wild-type strain was cultivated in 0.5 % glucose, the glucose concentration rapidly decreased to very low levels within 48 h, and high esterase activities were observed after 72 h. In contrast, when it was cultivated in 2 % glucose, very low esterase activity was measured after 72 h, and glucose was still present in the medium (Table 3 and data not shown). Similar results were obtained in the presence of fructose. In contrast, mannitol allowed similar induction of Lip1 activity at both 0.5 % and 2 % concentration. The Δglk1 mutant showed essentially the same behaviour as the wild-type, except for slightly lower induction levels. Similarly, the Δhxk1 mutant showed high levels of esterase activity in 0.5 % glucose, but no activity at all in 2 % glucose (Table 3). No data with the Δhxk1 mutant could be obtained with fructose and mannitol because they did not support growth. Thus, the glk1 and hxk1 mutants seemed to be unaffected in carbon catabolite repression.

**DISCUSSION**

Similar to the situation in *Aspergillus* spp., *B. cinerea* probably contains two functional hexokinases. This conclusion is based on the two following observations. Firstly, hexokinase Hxk1 and glucokinase Glk1 are the only proteins that share high similarities with the known counterpart hexose kinases from *Aspergillus* spp. and yeast. Secondly, glucose kinase activity in the wild-type was found to be equivalent to the sum of the activities in the Δglk1 and Δhxk1 mutants. Similar fructose kinase activities were measured in the wild-type and in the Δglk1 mutant, while no activity was detected in the Δhxk1 mutant. In *A. nidulans*, a gene (*xprF*) encoding a hexokinase-like protein has been identified. Analysis of *xprF* mutants indicated that this protein probably has no hexokinase activity, but seems to be involved in the regulation of expression of extracellular proteases (Katz et al., 2000). An *xprF* hexokinase-like sequence has also been found in other fungi, including *B. cinerea*, but its role remains to be determined. The Δglk1 and Δhxk1 genes were found to be expressed at all stages of development tested. Based on enzyme measurements with wild-type and mutant extracts, both Glk1 and Hxk1 contribute significantly to glucose phosphorylation in the cells. In *A. niger*, hexokinase HxkA and glucokinase GlkA were found to contribute similarly to glucose phosphorylation. However, this was strongly dependent on both the intracellular pH and the glucose concentration (Panneman et al., 1998).

Normal growth was observed for the *B. cinerea* Δglk1 mutant, while the Δhxk1 mutant showed a pleiotropic growth phenotype. In *A. nidulans*, a mutant lacking glucokinase (glkA4) was also found to grow normally; a hexokinase (hxkA1) deficient mutant was unable to grow on fructose but not reported to have other growth defects (Flipphi et al., 2003). In the *B. cinerea* Δglk1 mutant, *in vitro* glucokinase activity was reduced by only approximately 35 %, which is probably not rate-limiting *in vivo*. Nevertheless, we cannot rule out that there are situations in which Glk1 is important in addition to Hxk1. With regard to the Δhxk1 mutant, it was expected not to grow on fructose or on mannitol, which is metabolized via the mannitol cycle, involving the phosphorylation of fructose (Hult et al., 1980; Velez et al., 2007). However, the sensitivity of the Δhxk1 mutant to fructose was not expected. The fructose sensitivity of the Δhxk1 mutant explains its inability to grow on sucrose, which is probably split by invertases into glucose and fructose prior to uptake. The inability of the Δhxk1 mutant to grow on galactose indicated that galactose metabolism also involves a fructose intermediate. The classical pathway of galactose metabolism, via galactose 1-phosphate and glucose 1-phosphate, in most organisms proceeds via the Leloir pathway (Frey, 1996). Recently, an alternative (reductive) D-galactose metabolic pathway was described in *A. nidulans* and *H. jecorina*, which proceeds via sorbose and fructose (Fekete et al., 2004; Seiboth et al., 2004). In the genome sequence of *B. cinerea*, all putative components of the
alternative pathway are present (not shown). This indicates that \textit{B. cinerea} metabolizes galactose to a major extent by the reductive pathway. In addition to its inability to grow on fructose and fructose-related carbon sources, the \textit{Dhxk1} mutant also showed strongly reduced growth in the presence of other carbon sources such as glycerol and glucose. Irrespective of the growth medium, the mutants formed reduced aerial mycelium with very few conidia or no conidia at all. Nevertheless, the few conidia produced by the \textit{Dhxk1} mutant showed similar germination rates to wild-type and \textit{Dglk1} conidia in most media. Fructose also induced germination of \textit{Dhxk1} conidia, albeit with lower efficiency than with wild-type conidia. This indicates that the non-phosphorylated fructose can still be sensed by the \textit{Dhxk1} conidia, and germ tubes can appear but they fail to elongate. Fructose is taken up by \textit{B. cinerea} conidia by a fructose transporter (Frt1) and probably by a variety of hexose transporters (Doehlemann \textit{et al.}, 2005). Nevertheless, germinating conidia also use endogenous carbon sources such as lipids, glycogen and trehalose (Thines \textit{et al.}, 2000; Doehlemann \textit{et al.}, 2006b). The preferential metabolism of the storage compounds might be the reason for the higher fructose tolerance of germinating spores as compared to the growing mycelium.

\textbf{Table 3.} Esterase activities [nmol \textit{p}-nitrophenol butyrate min$^{-1}$ (mg dry wt)$^{-1}$] of \textit{B. cinerea} culture supernatants in the presence of 0.5\% or 2\% glucose (Glc), fructose (Fru) or mannitol (Man)

Data are the means ± se of three experiments performed in triplicate.

\begin{table}[h]
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\begin{tabular}{|l|c|c|c|c|c|}
\hline
 & 0.5 \% Glc & 2 \% Glc & 0.5 \% Fru & 2 \% Fru & 0.5 \% Man & 2 \% Man \\
\hline
Wild-type & 7.00 ± 2.01 & 0.38 ± 0.01 & 6.41 ± 1.84 & 0.70 ± 0.13 & 4.36 ± 0.64 & 3.98 ± 0.51 \\
\textit{Dglk1} & 4.53 ± 2.47 & 0.32 ± 0.24 & 5.39 ± 1.39 & 0.57 ± 0.22 & 3.49 ± 0.42 & 3.63 ± 0.49 \\
\textit{Dhxk1} & 5.98 ± 2.34 & 0.01 ± 0.01 & ND & ND & ND & ND \\
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\end{tabular}
\end{table}
The pathogenicity of the Δhxk1 mutant was dependent on the inoculated plant tissue. On tomato leaves, lesion formation and expansion occurred with only a small delay compared to the wild-type. In contrast, infection was strongly reduced on tomato fruits and apples. This difference in virulence appeared to be due to the high concentration of sugars, in particular fructose, of the fruits. While tomato leaves contained only 0.4% fructose, the fructose content of the fruits was between 13.7% (of the dry weight) in tomatoes and 31.4% in apples. Thus, growth of the B. cinerea Δhxk1 mutant in fruits seems to be inhibited mainly by the high concentrations of fructose.

So far, we have no satisfactory explanation for the multiple growth defects of the Δhxk1 mutant, even in the presence of sugars which are not metabolized via Hxk1. Possibly, the fructose kinase activity of Hxk1 is required for normal metabolism also in the absence of fructose or fructose-related carbon sources. It seems unlikely that glucose kinase activity is growth limiting in the Δhxk1 mutant because of the normal phenotype of the Ahk1 mutant, in which glucose kinase levels are also reduced in vitro (albeit to a lower extent). As a third explanation, we reasoned that Hxk1 might perform functions in addition to its enzymic activity. However, two B. cinerea mutants encoding catalytically inactive Hxk1 derivatives, Hxk1-S158A and Hxk1-D211S, showed the same pleiotropic phenotype as the Δhxk1 deletion mutant. This result strongly indicated that loss of Hxk1 enzyme activity is responsible for the pleiotropic growth phenotype. However, it does not completely rule out the possibility that the correct structure of the catalytic centre is also required for a regulatory function.

Heterologous expression of glk1 and hxk1 in yeast was used for the characterization of both hexokinases. Enzyme assays with cell extracts confirmed that glk1 encodes a glucokinase, with high affinity for glucose, while hxk1 encodes a hexokinase, with almost sixfold higher affinity for glucose than for fructose. These kinetic parameters, including the Vmax values, are comparable to those (Km, Vmax) determined for A. niger and S. cerevisiae (Walsh et al., 1991; Panneman et al., 1996, 1998). Similar to the hexokinases of yeast and A. niger, Hxk1 was found to be sensitive to trehalose 6-phosphate. This implies that trehalose 6-phosphate plays a role in sugar metabolism also in B. cinerea. A B. cinerea Δtps1 mutant disrupted in trehalose-6-phosphate synthase was found to grow normally, but to be defective in fructose- and glucose-induced germination (Doehlemann et al., 2006b). This was interpreted as an indication that trehalose 6-phosphate is particularly important during germination for regulating the glycolytic flux via Hxk1. When expressed in yeast, both Hxk1-S158A and Hxk1-D211S proteins were enzymically inactive, while the Hxk1-D1-15 protein showed only 3–4% residual activity. The latter result was unexpected, since a very similar N-terminal deletion in the yeast Hxk2 protein did not result in impaired enzyme activity (Ma et al., 1989). In B. cinerea, the 15 N-terminal amino acids are encoded by exon 1, which is separated from exon 2 by an unusually long intron of 747 bp, but we do not know the reason for this.

The role of the hexose kinases in carbon catabolite repression was analysed by studying the effects of glk1 and hxk1 deletions. In the wild-type and in the mutant strains, induced Lip1 expression remained suppressed by glucose and fructose. These results do not indicate that catabolite suppression in B. cinerea occurs via hexose kinases, as has been described for yeast (Entian, 1980; Hohmann et al., 1999). In A. nidulans, evidence for a common role of hexokinase and glucokinase in catabolite repression was obtained (Flipphi et al., 2003). While single hexokinase and glucokinase mutants were unaltered in glucose repression, the hexokinase mutant showed a partial deregulation in the presence of fructose. In contrast, the hxkA1glkA4 double mutant was strongly impaired in catabolite repression. Thus, it is likely that hexose phosphorylation by either of the two hexose kinases, or the following metabolic flux, are required for catabolite repression in A. nidulans (Flipphi et al., 2003). Unfortunately, we were unable to generate B. cinerea Aglk1Ahxk1 double mutants. We failed to transform the Aglk1 mutant with a Δhxk1 construct, because all of the more than 100 transformants tested turned out to be ectopic integration mutants outside of the hxk1 gene (data not shown). It is therefore possible that a situation similar to A. nidulans occurs in B. cinerea, but this is difficult to test without a double mutant. Nevertheless, the situation in Aspergillus and Botrytis is clearly different from that in yeast, in which Hxk2 directly interacts with the transcriptional repressor Mig1 (Ahuatzi et al., 2004). Thus, it remains to be elucidated how catabolite repression is mediated in B. cinerea.

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REFERENCES


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