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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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, $\Delta h x k 1$ 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 $\Delta h x k 1$ 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 *hxk1* alleles with point mutations leading to enzymically inactive enzymes showed phenotypes similar to the $\Delta h x k 1$ disruption mutant, indicating that loss of hexose phosphorylation activity of Hxk1 is solely responsible for the pleiotropic growth defect. Virulence of the $\Delta h x k 1$ 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 $\Delta h x k 1$ 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 $\Delta g l k 1$ and $\Delta h x k 1$ 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.

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INTRODUCTION

Hexose kinases are crucial enzymes for most living cells, by catalysing the intracellular trapping and feeding into metabolism of the hexoses glucose and fructose after their uptake. In addition, hexokinases have been shown in yeast, plants and mammals to participate in glucose signalling (De Winde *et al.*, 1996; Moore *et al.*, 2003; Efrat *et al.*, 1994). *Saccharomyces cerevisiae* has three enzymes catalysing hexose phosphorylation, namely hexokinases (EC 2.7.1.1) Hxk1 and Hxk2, and glucokinase (EC 2.7.1.2) Glk1. Each of these enzymes can support growth on glucose, while either of the two hexokinases is required for growth on fructose (Lobo & Maitra, 1977; Gancedo *et al.*, 1977). Hxk2, in addition, is mainly required for

catabolite repression, which prevents the expression of genes involved in catabolism of less preferred carbon sources in the presence of a more favourable nutrient such as glucose (Entian, 1980). In the presence of glucose, Hxk2 interacts with the carbon catabolite repressor Mig1 and thereby is translocated into the nucleus. In the nucleus, the Hxk2–Mig1 complex seems to form a repressor complex that binds to the promoters of carbon-catabolite-repressible genes (Ahuatzi *et al.*, 2004, 2007).

In filamentous fungi studied so far, a functional hexokinase and a functional glucokinase have been identified. In *Aspergillus nidulans* and *Hypocrea jecorina*, double mutants in both genes were unable to grow on either glucose or fructose (Flippin *et al.*, 2003; Hartl & Seiboth, 2005). In *Aspergillus niger*, the hexokinase and the glucokinase have been purified and biochemically characterized. Both enzymes seem to contribute similarly to the rate of glucose phosphorylation *in vivo*, dependent on the pH and the glucose concentration (Panneman *et al.*, 1996, 1998).

Abbreviations: EST, expressed sequence tag; p.i., post-inoculation.

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences determined in this work are EF156463 (*glk1*) and EF156464 (*hxk1*).

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 *B. cinerea* was performed as described previously (Reis *et al.*, 2005). For selection of $\Delta h x k 1$ and *h x k 1-mut* transformants, the plating agar (SH agar) was supplemented with 100 mM glycerol ($\Delta h x k 1$ mutants do not grow on sucrose).

Germination and plant infection tests. Conidial germination assays and leaf infection tests were performed as described by Doehlemann *et al.* (2006a). Prior to inoculation of tomato and strawberry fruits, the tissues were wounded with a pinprick from a syringe. Before inoculation of apples, they were surface-sterilized by immersion in 75% ethanol, and small plugs (approx. 4 mm diameter) were removed using a cork borer. Inoculation of the fruits was performed either with 10 μ l droplets of spore suspensions (10^5 conidia ml^{-1} ; tomatoes), or with mycelial agar plugs (apples).

Cloning and disruption of the *B. cinerea glk1* and *h x k 1* genes, and site-directed mutagenesis of *h x k 1*. Starting with two EST sequences of *B. cinerea* strain T4 encoding parts of putative hexokinase and glucokinase genes (*h x k 1*: AL110892; *glk1*: AL114821; Viaud *et al.*, 2003), gene-specific PCR fragments were generated using

primers HXK-1/ HXK-2 and GLK-1/ GLK-2, respectively (Table 1). Hybridization screening of *B. cinerea* genomic libraries was done according to Reis *et al.* (2005). Genomic *B. cinerea* regions carrying *glk1* and *h x k 1* genes were subcloned into the vector pBSKS(+) lacking the intrinsic *EcoRI* site and sequenced. For *glk1* inactivation, a hygromycin-resistance cassette (Doehlemann *et al.*, 2006a) was inserted into the *glk1* genomic insert of pBGLK via two naturally occurring *EcoRI* sites, thus replacing 1367 bp (codons 29–484) of the *glk1* coding region (see Fig. 2A). To delete the central part of the *h x k 1* gene, inverse PCR was performed with pBHXX, using primers HXK-KO-1 and HXK-KO-2 (Fig. 2A). The amplification product, containing pBSKS(+) plus 608 bp and 2094 bp of *h x k 1* flanking sequences, was digested with *EcoRI* and ligated with a hygromycin-resistance cassette as described above. From the resulting plasmids, the $\Delta glk1$ and $\Delta h x k 1$ knockout constructs were amplified (Fig. 2A) and used for *B. cinerea* transformation.

Mutant alleles *h x k 1-S158A* and *h x k 1-D211S* were generated with the QuickChange Site-Directed Mutagenesis kit (Stratagene), with primer pairs HXK-S158A-1/ HXK-S158A-2 and HXK-D211S-1/ HXK-D211S-2, respectively, and a plasmid carrying the *h x k 1* gene as a PCR template. To allow selection for replacement of wild-type *h x k 1* by the mutated *h x k 1* alleles in the *B. cinerea* genome by homologous recombination, a hygromycin-resistance cassette was inserted via a *CpoI* site downstream of the *h x k 1* stop codon. For transformation of *B. cinerea* B05.10, PCR fragments amplified with primers HXK-5 and HXK-6 were used.

Nucleic acid manipulations and sequence data analysis. Total DNA from *B. cinerea* was isolated according to Möller *et al.* (1992). Total RNA from different stages of fungal development was isolated by a low-pH extraction method (Purescript, Gentra Systems). Semiquantitative RT-PCR, using the *B. cinerea actA* gene for calibration, was performed as described by Doehlemann *et al.* (2005). DNA sequences were confirmed and, if necessary, extended by sequences obtained from the *B. cinerea* genome sequence database of the Broad Institute (http://www.broad.mit.edu/annotation/genome/botrytis_cinerea/Home.html). Protein sequences were aligned using the CLUSTAL W algorithm (Thompson *et al.*, 1994), with manual corrections.

Expression of *glk1* and *h x k 1* cDNAs in yeast. The coding regions of the *glk1* and *h x k 1* genes were amplified from *B. cinerea* cDNA using primers cGLK-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 *h x k 1* expression construct was performed as described above. Plasmids were transformed into a hexokinase-deficient $\Delta h x k 1 \Delta h x k 2 \Delta glk 1$ mutant of *S. cerevisiae* (strain CEN.IS14-4D; provided by Peter Kötter, University of Frankfurt).

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, 5 min) two times 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 MgCl_2 ; 0.5 mM EDTA; 5 mM 2-mercaptoethanol; 1 mM PefaBlock (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 ($\text{OD}_{600\text{nm}} \sim 1$), harvested by centrifugation and washed with 50 mM

Table 1. Oligonucleotide primers used in this work

Restriction sites are underlined. Mutated nucleotides are indicated in bold italics.

Primer	Sequence (5'→3')
GLK-1	GAGCTATGGATGGCATTGG
GLK-2	TCCCACCAATTCTCCTTG
GLK-3	CCGACACCAGATCCATCCTT
GLK-KO1	GCCATTAGCCAATCGAATCC
GLK-KO2	GGAGGTGGTGTGATATACTG
HXK-1	CACTTCGTCTCCGAGCTTAC
HXK-2	GATCCTCGCCATGATGGTG
HXK-3	AGACTCGTCAGCTTGATATG
HXK-4	ATGATACCCAGGGTTAAAGG
HXK-5	CCTCATCATCTCCAATTCTA
HXK-6	TGATGGAGTCGATGGCATTC
HXK-KO1	CTGAATTCATCGCAGTGAGAGGACACAA
HXK-KO2	GTGAATTCGAAGCGTGTCGAAGGCAGGTA
HXK-KO3	CCTCATCATCTCCAATTCTA
HXK-KO4	TTATCACCAGCACAGGAGTT
HXK-S158A-1	GAGCTTTGGTTTACCT G GGCCACCCAGCTACTC
HXK-S158A-2	GAGTAGCTGGGT G GGCCAGGTGAAACCAAGACTC
HXK-D211S-1	GCTTACAGCTTTGATCAA CAGCTG CACTGGTACCTTGATTGC
HXK-D211S-2	GCAATCAAGGTACCACTG CAGCTG TTGATCAAAGCTGTAAGC
cGLK1-PST	GTCCTGCAGCCTACAGAAGCGCCAATATC
cGLK2-SAL	CTCGTCGACATCGTGCAGCCATTACTCTC
cHXK1-ECO	<u>CGAATTC</u> TACCCTTCATTTACAGATCTCC
cHXK2-SAL	CTT <u>GTCGAC</u> AGAACTGCTATGCTTGTC
cHXK3-ECO	TCGAATTCGTTCTATGGCTGATGTCC
HXK-EX1	TGATGAGAAGTCCGAGTTTG
HXK-EX2	TTGATTGCCATTGGAGTGTC
GLK-EX1	GAGCCAAATTCACATACG
GLK-EX2	TGCTCTTGCCATCAATGTTC
ACT-1	TATCAACCATGGAAGAAGAAGTCG
ACT-2	GTAGAATCGAGGGGCGTTGAAG

potassium phosphate buffer (pH 7.0). 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 µg 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. Ethanol 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.

Determination of extracellular esterase activity. Esterase secreted by *B. cinerea* was measured according to Reis *et al.* (2005). Cultures

(30 ml) in 1 % malt extract medium, inoculated with fresh mycelium, were incubated for 48 h at 20 °C and 140 r.p.m. The mycelium was washed twice, weighed, and resuspended in 10 ml minimal medium. From this suspension, 2 ml aliquots were added to 10 ml of a medium containing a carbon source (0.5 or 2 %), the lipase inducer methyl oleate (0.3 %), the emulsifier taurocholic acid (0.06 %), and Gamborg's B5 basal salt mixture. The cultures were incubated for up to 4 days at 20 °C in 60 mm Petri dishes without shaking. Enzymic measurements were done as described by Reis *et al.* (2005).

RESULTS

Cloning of *glk1* and *hvk1* 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 *hxt1* 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

carrying *hxx1* 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 *hxx1* 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 *hxx1* sequencing, but separated from exon 2 by an unusually long intron of 747 bp. The *hxx1* coding sequence has a size of 1473 bp, and contains five introns. The predicted 491 amino acid Hxx1 protein shares 74 and 54 % identity with *A. niger* HxxA and with *S. cerevisiae* Hxx2, 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 *hxx1* and *glk1* were similar during all stages of fungal development (Fig. 1). Whether or not the *hxx1* expression in germlings and mycelium was significantly increased relative to the other stages was not analysed further.

Construction of Δglk , $\Delta hxx1$ and *hxx1-mut* mutants

In order to examine the role of hexokinases in *B. cinerea*, disruption mutants of both *glk1* and *hxx1* genes were

Fig. 1. Expression of *glk1* and *hxx1* 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 *hxx1*), or no fragment (*actA*), because of primers flanking or overlapping introns. The following primers were used: *glk1*, GLK-EX1, GLK-EX2; *hxx1*, HXX-EX1, HXX-EX2; *act1*, ACT-1, ACT-2.

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 *hxx1* ($\Delta hxx1$) and three isolates deleted in *glk1* ($\Delta glk1$) were confirmed by PCR (Fig. 2B). Further confirmation of the targeted deletions was obtained, for one $\Delta hxx1$ and for one $\Delta glk1$ isolate, by means of RT-PCR with primers amplifying fragments within the deleted regions of both genes. As expected, cDNAs for *hxx1* and *glk1* could not be amplified from the $\Delta hxx1$ and the $\Delta glk1$ mutant, respectively (Fig. 2C). All $\Delta glk1$ mutants were indistinguishable from the wild-type in all growth parameters tested. In contrast, all $\Delta hxx1$ 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 Hxx1 protein. In order to test this possibility, we constructed two derivatives of the *hxx1* coding region with mutations leading to enzymically inactive proteins. In the *hxx1*-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 *hxx1*-D211S mutant, an aspartate residue essential for yeast HXX2 catalytic function was replaced (Kraakman *et al.*, 1999; Moore *et al.*, 2003). The mutant *hxx1* genes were introduced by transformation into *B. cinerea*, replacing the wild-type *hxx1* gene by homologous recombination (Fig. 3A). From the hygromycin-resistant transformants tested, one (*hxx1*-S158A) and three (*hxx1*-D211S) were shown by PCR to have replaced in their genomes the wild-type *hxx1* gene by the mutant alleles (Fig. 3B).

Phenotypic characterization of *glk1* and *hxx1* mutants

On rich media such as tomato malt agar, radial growth of all *hxx1* mutants was significantly retarded, whereas growth of the $\Delta glk1$ mutant was similar to that of the wild-type (Table 2). Furthermore, sporulation of the $\Delta hxx1$ mutants was strongly reduced. For instance, only $9.7 \pm 3.0 \times 10^5$ conidia (mean $\pm$ SE) were produced per tomato malt agar plate after 14 days of growth by the $\Delta hxx1$ mutant, in comparison to $1.8 \pm 0.5 \times 10^8$ conidia of the wild-type. Very low sporulation was also observed for the mutants with mutated *hxx1* alleles, while normal sporulation occurred in the $\Delta glk1$ mutant (not shown). Furthermore, the *hxx1* conidia formed were smaller ($8.4 \pm 1.0 \mu\text{m}$) than those of the wild-type ($10.3 \pm 0.7 \mu\text{m}$) and the $\Delta glk1$ mutants ($10.5 \pm 1.3 \mu\text{m}$). 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 $\Delta hxx1$ mutants was only moderately

Fig. 2. Construction of *B. cinerea* $\Delta glk1$ and $\Delta hxx1$ deletion mutants. (A) Constructs used for transformation. The remainders of the coding regions of *glk1* (left) and *hxx1* (right), and the *hph* genes are indicated by black arrows. The deleted regions of *glk1* and *hxx1* are shown with dashed lines. Restriction sites: B, *Bam*HI; E, *Eco*RI; S, *Sal*I. Primer positions are indicated by arrowheads, for the following primers. Left: P1, P2: GLK-KO1, GLK-KO2. Right: P1, P2: HXX-KO1, HXX-KO2; P3, P4: HXX-KO3, HXX-KO4. Left and right: H1, H2: HYG1-ECO, HYG2-ECO (Doehlemann *et al.*, 2006a). (B) PCR-based verification of *B. cinerea* *hxx1* and *glk1* disruption. PCR was performed with primers GLK-1/ GLK-3 ('nested' into GLK-KO1/GLK-KO2) and HXX-3/ HXX-4 ('nested' into HXX-KO1/HXX-KO2), using genomic DNA of the *B. cinerea* strains as templates, as follows. *glk1*: Δ1–3, verified $\Delta glk1$ strains. *hxx1*: Δ1–5, verified $\Delta hxx1$ strains. wt, Wild-type; ect, strain with ectopic $\Delta glk1$ or $\Delta hxx1$ insertion; c, control, cloned genomic DNA template. (C) Expression analysis by RT-PCR of *glk1* (left), *hxx1* (middle) and *actA* (right) genes in 16 h old *B. cinerea* germlings from the following strains: wild-type (wt), $\Delta glk1$ (Δg) and $\Delta hxx1$ (Δh). c, Genomic *B. cinerea* DNA. The same primers as in Fig. 1 were used.

reduced, while with glucose, it was less than 50 % of the wild-type. With glycerol, growth of the $\Delta hxx1$ mutants was about 30 % of the wild-type. Only marginal growth of the $\Delta hxx1$ 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 $\Delta hxx1$ mutants could not grow either, demonstrating that fructose strongly inhibits growth of the $\Delta hxx1$ 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 *hxx1* mutants, but germination rates were significantly lower than those of wild-type and $\Delta glk1$ conidia (Fig. 4A). However, in fructose the $\Delta hxx1$ 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 *hxx1* mutants developed somewhat more slowly those induced by the wild-type or the $\Delta glk1$ mutant. However, while the wild-type and the $\Delta glk1$ mutant lesions developed conidiophores and conidia above the infected tissue, sporulation was never observed in any of the *hxx1* mutants. In contrast, lesion formation by the

Fig. 3. Construction of *B. cinerea* mutants with mutated *hxx1* alleles. (A) Integration of *hxx1*-S158A and *hxx1*-D211S alleles and their new restriction sites into the genome of *B. cinerea* by homologous recombination. From plasmids carrying the mutated *hxx1* alleles (for construction see Methods), PCR fragments were amplified using primers 1 (HXK-5) and 2 (HXK-6), and used for *B. cinerea* transformation. The sites of expected recombination with the wild-type genomic *hxx1* region (not shown) are indicated by crossed dotted lines. Coding regions are indicated by black arrows; introns in *hxx1* are shown by open bars. S, *Sal*I. (B) Verification of *B. cinerea* transformants carrying mutated *hxx1* alleles. PCR fragments containing parts of the *hxx1* coding region were amplified from total DNA of wild-type and mutant *B. cinerea* strains, and digested with *Ap*I (A; for *hxx1*-S158A) or *Pvu*II (P; for *hxx1*-D211S). Note that the *Ap*I digest of the *hxx1*-S158A fragment was incomplete, but sequencing confirmed the presence of the *Ap*I site. nd, Non-digested DNA.

hxx1 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 *hxx1* 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 Glxk1 and Hxk1, hexose kinase activities were first measured in extracts prepared from *B. cinerea* wild-type as well as from $\Delta h x k 1$ and $\Delta g l k 1$ mutants (Fig. 6). Glucose kinase activity was highest in the wild-type (0.26 ± 0.03 mmol glucose $\text{min}^{-1} \text{mg}^{-1}$), and significantly lower but present both in the $\Delta g l k 1$ mutant (0.17 ± 0.06) and in the $\Delta h x k 1$ 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 $\Delta g l k 1$ mutant were inhibited by 85 %, and no significant inhibition was observed in extracts of the $\Delta h x k 1$ mutant. Regarding fructose kinase, similar activities were measured in wild-type and $\Delta g l k 1$ mutant extracts, which were strongly inhibited by trehalose 6-phosphate. In contrast, no significant fructose kinase activity was found in the $\Delta h x k 1$ 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

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

MM: minimal medium containing 1 % of the indicated carbon source. Data are the means $\pm$ SE of at least three independent experiments, with two replicates each. The percentage of the wild-type value is shown in parentheses. ND, Not determined.

Medium	Wild-type [mm day ⁻¹]	$\Delta h x k 1$ [mm day ⁻¹] (% of wt)	$h x k 1$ -D211S [mm day ⁻¹] (% of wt)	$h x k 1$ -D211S [mm day ⁻¹] (% of wt)	$\Delta g l k 1$ [mm day ⁻¹] (% of wt)
Tomato malt agar	23.8 ± 1.2	17.3 ± 1.3 (73 ± 3)	15.7 ± 0.6 (66 ± 4)	17.7 ± 1.6 (74 ± 3)	25.1 ± 0.3
MM glucose	19.3 ± 1.4	9.1 ± 2.8 (47 ± 3)	9.5 ± 1.8 (49 ± 3)	9.0 ± 1.5 (47 ± 3)	19.9 ± 2.4
MM fructose	19.4 ± 2.4	0	0	0	18.1 ± 1.6
MM Glc/Fru*	22.1 ± 0.6	0	0	0	22.5 ± 0.6
MM glycerol	22.3 ± 2.0	7.0 ± 0.3 (31 ± 3)	8.2 ± 0.7 (37 ± 3)	10.3 ± 1.4 (46 ± 4)	19.9 ± 5.4
MM acetate	12.6 ± 0.8	7.5 ± 2.0 (60 ± 4)	ND	ND	13.1 ± 1.0
MM sucrose	20.3 ± 1.0	0.4 ± 0.1	(2 ± 0)	0.3 ± 0.1 (1 ± 0)	0.4 ± 0.2 (2 ± 1)
MM sorbitol	21.1 ± 3.5	0.4 ± 0.6	(2 ± 0)	0.4 ± 0.2 (2 ± 1)	0.9 ± 0.3 (4 ± 1)
MM galactose	16.1 ± 4.9	1.3 ± 0.5	(8 ± 2)	0.4 ± 0.2 (2 ± 1)	0.9 ± 0.4 (6 ± 2)
MM xylose	11.8 ± 0.4	7.3 ± 0.6 (62 ± 2)	ND	ND	12.1 ± 0.6
MM Xyl/Fru*	21.6 ± 1.6	0	0	0	19.8 ± 1.2

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

Fig. 4. Nutrient-dependent germination of *B. cinerea* conidia. (A) Germination rates (16 h p.i.) on glass slides of wild-type (black bars), $\Delta glk1$ (white bars) and $\Delta hxx1$ (grey bars) strains, in 10 mM of glucose (Glc), fructose (Fru), galactose (Gal), galactose plus fructose (Gal/Fru), acetate (Ace), or in 1% malt extract (1% ME). Data are the means $\pm$ SE of two experiments with two replicates. (B) Photographs of *B. cinerea* germlings (16 h p.i.) on glass slides with fructose (Fru), acetate (Ace), and fructose plus acetate (Fru/Ace). Bars, 25 μ M.

encoding the mutated Hxk1-S158A and Hxk1-D211S proteins, and an N-terminal deletion derivative (Hxk1- Δ 1-15) 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 *glk1* and *hxx1* grew well on glucose, whereas the mutated *hxx1* sequences did not support growth (Fig. 7A). On fructose media, only the transformant expressing wild-type *hxx1* was able to grow (data not shown). These data confirmed that *glk1* and *hxx1* encode functional hexose kinases, and indicated that the amino acid exchanges and the N-terminal deletion lead to the inactivation of Hxk1. The mutated Hxk1 derivatives were largely inactive, with only low residual activity being detectable with Hxk1- Δ 1-15. Kinetic parameters of the heterologously expressed enzymes were determined (Fig. 7B). Maximal activities (V_{max}) for glucose phosphorylation were

Fig. 5. Infection tests with *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 $\pm$ SE of three experiments with at least two measurements performed in parallel.

similar for Glk1 and Hxk1, but Glk1 showed a higher affinity (K_m 48 ± 12 μ M) for glucose than Hxk1 (K_m 132 ± 19 μ M). While Glk1 did not phosphorylate fructose, the affinity of Hxk1 for fructose (K_m 700 ± 46 μ M) was lower than that for glucose.

Role of *B. cinerea* hexokinases in catabolite repression

To test whether Hxk1 and/or Glk1 are involved in catabolite repression, we analysed the expression of the

Fig. 6. Hexose phosphorylation rates (A, D-glucose; B, D-fructose) in extracts of *B. cinerea* wild-type and hexose kinase mutant strains. Assays were performed in the absence (black bars) and in the presence (grey bars) of the hexokinase inhibitor trehalose 6-phosphate. Data are the means $\pm$ SE of two experiments with two replicates.

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 $\Delta glk1$ mutant showed essentially the same behaviour as the wild-type, except for slightly lower induction levels. Similarly, the $\Delta hxx1$ 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 $\Delta hxx1$ mutant could be obtained with fructose and mannitol because they did not support growth. Thus, the *glk1* and *hxx1* 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 Hxx1 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 $\Delta glk1$ and $\Delta hxx1$ mutants. Similar fructose kinase activities were measured in the wild-type and in the $\Delta glk1$ mutant, while no activity was detected in the $\Delta hxx1$ 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 $\Delta glk1$ and $\Delta hxx1$ 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 Hxx1 contribute significantly to glucose phosphorylation in the cells. In *A. niger*, hexokinase HxxA 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* $\Delta glk1$ mutant, while the $\Delta hxx1$ mutant showed a pleiotropic growth phenotype. In *A. nidulans*, a mutant lacking glucokinase (*glkA4*) was also found to grow normally; a hexokinase (*hxxA1*) deficient mutant was unable to grow on fructose but not reported to have other growth defects (Flippin *et al.*, 2003). In the *B. cinerea* $\Delta glk1$ 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 Hxx1. With regard to the $\Delta hxx1$ 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 $\Delta hxx1$ mutant to fructose was not expected. The fructose sensitivity of the $\Delta hxx1$ 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 $\Delta hxx1$ 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

Fig. 7. Expression of *B. cinerea* hexose kinases in yeast. (A) Growth of hexose kinase-deficient *S. cerevisiae* strain CEN.IS14-4D expressing *B. cinerea* Glk1 and different Hxk1 variants, on medium containing 1 % glucose (Glc) or galactose (Gal). (B) Kinetics of hexose phosphorylation: (a) Glk1, glucose; (b) Hxk1, glucose; (c) Hxk1, fructose. Hexose kinase activities are expressed as $\text{mmol glucose (a, b) or fructose (c) min}^{-1} (\text{mg protein})^{-1}$ and are the means $\pm$ SE of at least three experiments with two replicates each.

alternative pathway are present (not shown). This indicates that *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 $\Delta h x k 1$ 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 $\Delta h x k 1$ mutant showed similar germination rates to wild-type and $\Delta g l k 1$ conidia in most media. Fructose also induced germination of $\Delta h x k 1$ conidia, albeit with lower

efficiency than with wild-type conidia. This indicates that the non-phosphorylated fructose can still be sensed by the $\Delta h x k 1$ conidia, and germ tubes can appear but they fail to elongate. Fructose is taken up by *B. cinerea* conidia by a fructose transporter (Frt1) and probably by a variety of hexose transporters (Doehlemann *et al.*, 2005). Nevertheless, germinating conidia also use endogenous carbon sources such as lipids, glycogen and trehalose (Thines *et al.*, 2000; Doehlemann *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.

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

Data are the means $\pm$ SE of three experiments performed in triplicate.

	0.5 % Glc	2 % Glc	0.5 % Fru	2 % Fru	0.5 % Man	2 % Man
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
$\Delta g l k 1$	4.53 ± 2.47	0.32 ± 0.24	5.39 ± 1.39	0.57 ± 0.22	3.49 ± 0.42	3.63 ± 0.49
$\Delta h x k 1$	5.98 ± 2.34	0.01 ± 0.01	ND	ND	ND	ND

The pathogenicity of the $\Delta h x k 1$ 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* $\Delta h x k 1$ 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 $\Delta h x k 1$ 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 $\Delta h x k 1$ mutant because of the normal phenotype of the $\Delta g l k 1$ 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 $\Delta h x k 1$ 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 *h x k 1* 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 *h x k 1* encodes a hexokinase, with almost sixfold higher affinity for glucose than for fructose. These kinetic parameters, including the $V_{\max}$ values, are comparable to those (K_m , $V_{\max}$) 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* $\Delta t p s 1$ 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- $\Delta 1$ -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 *h x k 1* 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 *h x k 1 g l k 4* 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* $\Delta g l k 1 \Delta h x k 1$ double mutants. We failed to transform the $\Delta g l k 1$ mutant with a $\Delta h x k 1$ construct, because all of the more than 100 transformants tested turned out to be ectopic integration mutants outside of the *h x k 1* 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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