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Genome-wide analysis of pectate-induced gene expression in *Botrytis cinerea*: Identification and functional analysis of putative D-galacturonate transporters

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ABSTRACT

The fungal plant pathogen *Botrytis cinerea* produces a spectrum of cell wall degrading enzymes for the decomposition of host cell wall polysaccharides and the consumption of the monosaccharides that are released. Especially pectin is an abundant cell wall component, and the decomposition of pectin by *B. cinerea* has been extensively studied. An effective concerted action of the appropriate pectin depolymerising enzymes, monosaccharide transporters and catabolic enzymes is important for complete D-galacturonic acid utilization by *B. cinerea*. In this study, we performed RNA sequencing to compare genome-wide transcriptional profiles between *B. cinerea* cultures grown in media containing pectate or glucose as sole carbon source. Transcript levels of 32 genes that are induced by pectate were further examined in cultures grown on six different monosaccharides, by means of quantitative RT-PCR, leading to the identification of 8 genes that are exclusively induced by D-galacturonic acid. Among these, the hexose transporter encoding genes *Bchxt*15 and *Bchxt*19 were functionally characterised. The subcellular location was studied of BcHXT15-GFP and BcHXT19-GFP fusion proteins expressed under control of their native promoter, in a *B. cinerea* wild-type strain. Both genes are expressed during growth on D-galacturonic acid and the fusion proteins are localized in plasma membranes and intracellular vesicles. Target gene knockout analysis revealed that BcHXT15 contributes to D-galacturonic acid uptake at pH 5∼5.6. The virulence of all *B. cinerea* hexose transporter mutants tested was unaltered on tomato and *Nicotiana benthamiana* leaves.

1. Introduction

The plant cell wall is the first barrier to pathogen invasion, and consists mainly of polysaccharides that form a complex three-dimensional network together with lignin and proteins. The main components of plant cell wall polysaccharides are cellulose, hemicellulose, and pectin. The ability to decompose complex plant cell wall polysaccharides is an important aspect of the lifestyle of fungal pathogens. Necrotrophic fungal plant pathogens secrete large amounts of enzymes to decompose plant cell wall polysaccharides in order to facilitate the penetration, the subsequent maceration and the acquisition of carbon from decomposed plant tissues (Amselem et al., 2011). Hemi-biotrophic pathogens also produce polysaccharide decomposing enzymes during the late, necrotizing phase of infection (Gan et al., 2013; King et al., 2011; O'Connell et al., 2012). By contrast, many biotrophic pathogens and symbionts have a markedly lower content of genes encoding enzymes for cell wall decomposition in their genome (Baxter et al., 2010; Duplessis et al., 2011; Martin et al., 2010), presumably to reduce the damage to the host and avoid the plant defence responses triggered by the release of cell wall fragments.

Botrytis cinerea is a necrotrophic fungal plant pathogen infecting more than 200 host plants and causing severe economic damage to crops worldwide (Dean et al., 2012; Williamson et al., 2007). *B. cinerea* secretes large amounts of cell wall degrading enzymes for host tissue decomposition and nutrient acquisition (Zhang and van Kan, 2013b). The preference for infection of pectin-rich plants and tissues (ten Have et al., 2002) suggests that effective pectin degradation is important for virulence of *B. cinerea*. The genome of *B. cinerea* encodes 118 Carbohydrate Active enZymes (CAZymes, www.cazy.org) (Cantarel et al., 2009) associated with plant cell wall decomposition, of which a large proportion is involved in the decomposition of pectin (Amselem et al., 2011).

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Several *B. cinerea* pectin degrading enzyme activities have been detected during host infection, including pectin and pectate lyases, pectin methylesterase (PMEs), exo-polygalacturonases (exo-PGs), and endo-polygalacturonases (endo-PGs) (Cabanne and Doneche, 2002; Kars et al., 2005b; Kars and van Kan, 2004; Rha et al., 2001; ten Have et al., 2001). The importance of several pectinases for virulence of *B. cinerea* was investigated by targeted mutagenesis in endo-PG genes and PME genes. Knockout mutants Δ*Bcpg*1 and Δ*Bcpg*2 were reduced in virulence by 25% and >50%, respectively (Kars et al., 2005a; ten Have et al., 1998). A Δ*Bcpme*1 mutant in one *B. cinerea* strain showed reduction in virulence (Valette-Collet et al., 2003); in a different strain, however, mutants in the same *Bcpme*1 gene or the *Bcpme*2 gene, or even Δ*Bcpme*1/Δ*Bcpme*2 double knockout mutants, were not altered in virulence (Kars et al., 2005b). However, there is still a number of pectinolytic genes that remain to be functionally analysed.

The monosaccharide D-galacturonic acid is the most abundant component of pectic polysaccharides (Caffall and Mohnen, 2009; Mohnen, 2008) and is the final product released from pectin degradation. The D-galacturonic acid catabolic pathway is conserved in many filamentous fungi (Martens-Uzunova and Schaap, 2008; Richard and Hilditch, 2009). The pathway has been genetically and biochemically characterized in *B. cinerea*, and consists of three catalytic steps involving four genes: *Bcgar*1, *Bcgar*2, *Bclgd*1, and *Bclga*1 (Zhang et al., 2011). Their transcript levels were induced substantially when the fungus was cultured in media containing D-galacturonic acid, pectate or pectin as the sole carbon source (Zhang et al., 2011). Knockout mutants in each of the three catalytic steps (*ΔBcgar*1/*ΔBcgar*2, *ΔBclgd*1, and *ΔBclga*1) were affected in growth on D-galacturonic acid, pectate, or pectin as the sole carbon source (Zhang et al., 2011), and in virulence on *Nicotiana benthamiana* and *Arabidopsis thaliana* leaves (Zhang and van Kan, 2013a).

Collectively, the functional analyses on the *B. cinerea* endo-PG genes and the D-galacturonic acid catabolic pathway genes indicate that a concerted action of the appropriate pectin depolymerising enzymes and catabolic enzymes is important for complete D-galacturonic acid utilization by *B. cinerea*. Previous studies showed that several genes involved in pectin decomposition and D-galacturonic acid catabolism are induced *in vitro* by D-galacturonic acid and are expressed at high levels during infection in the stage of lesion expansion, when plant cell wall degradation occurs (Wubben et al., 2000; Zhang et al., 2011; Zhang and van Kan, 2013a).

In order to get further insight into the *B. cinerea* genes that participate in pectin decomposition and D-galacturonic acid utilization, we have exploited the next generation RNA-sequencing (RNA-seq) technology (Wang et al., 2009) to perform a genome-wide transcriptome analysis in *B. cinerea* grown in media containing either pectate or glucose as sole carbon sources. We identified a set of genes that are significantly altered in gene expression, including two hexose transporter genes that are up-regulated in a culture with pectate as the sole carbon source, as compared to cultures with glucose. Their expression was further investigated by quantitative RT-PCR during growth on different carbon sources and during infection on different plants. Subcellular localization of these hexose transporters was determined in a *B. cinerea* wild-type strain and their function was studied by generating *B. cinerea* knockout mutants, which were tested *in vitro* and *in planta*.

2. Materials and methods

2.1. Fungal strain and growth conditions

B. cinerea wild-type strain B05.10 and the mutant strains used in this study (Table 1) were routinely grown on Malt Extract Agar (Oxoid, Basingstoke, UK; 50 g/L) in the dark at 20 °C for 3–4 days. The plates were placed for one night under near-UV light (350–400 nm) to promote sporulation, and were subsequently returned to darkness. Conidia were harvested 4–7 days later in 10–20 ml of water, and the suspension was filtered over glass wool to remove mycelium fragments. The conidia suspension was centrifuged at 1200 rpm for 5 min. The supernatant was discarded and the conidia in the pellet were resuspended at the desired density.

For radial growth assays, conidia of the strains were inoculated on Gamborg's B5 (Duchefa, Haarlem, The Netherlands) agar medium supplemented with 10 mM $(NH_4)H_2PO_4$ and as carbon source either 50 mM D-glucose or 50 mM D-galacturonic acid at the appropriate pH as indicated. Cultures were grown at 20 °C and the colony diameter was measured after 4 days of incubation.

2.2. RNA extraction

The conidia of the wild-type strain B05.10 were incubated in Gamborg's B5 liquid culture supplemented with 10 mM $(NH_4)H_2PO_4$ and 50 mM glucose at 20 °C, 150 rpm. After 16 h of growth, the mycelium was harvested and transferred into fresh Gamborg's B5 medium supplemented with 10 mM $(NH_4)H_2PO_4$ and a carbon source. For the cultures used for RNA-seq analysis, either 50 mM glucose or 0.5% sodium polygalacturonate (pectate) was added; mycelium was harvested from these cultures at 6 h post-transfer and freeze-dried. For the cultures used for quantitative RT-PCR, either glucose, D-galacturonic acid, L-arabinose, L-rhamnose, D-galactose, or L-xylose was added at 50 mM final concentration; mycelium was harvested from these cultures at 3 h post-transfer and freeze-dried.

For *in planta* gene expression analysis, six discs containing the infected lesions in the centre (30 mm in diameter) from three leaves of three plants were sampled at 2 and 3 days post-inoculation (dpi) as a pool for RNA isolation.

Total RNA was isolated using the Nucleospin® RNA plant kit (Machery-Nagl, Düren, Germany), according to the manufacturer's instructions. First strand cDNA was synthesized from 1 μg total RNA with SuperScript® III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions.

2.3. RNA-sequencing and data analysis

Twenty micrograms of total RNA for each RNA sample were prepared as described above. cDNA synthesis, library preparation and Illumina sequencing (100 bp paired-end reads) were performed at Beijing Genome Institute (BGI, Hong Kong). The obtained Illumina RNA-seq reads were trimmed to remove the first 12 nucleotides using fastx trimmer (http://hannonlab.cshl.edu/fastx_toolkit/) and mapped to annotated genes on version 2 of the *B. cinerea* genome (Staats and van Kan, 2012) using Tophat (version 2.0.6) with default settings (Trapnell et al., 2009). Differentially expressed genes were then determined using Cuffdiff (version 2.0.2) with default settings and cut-offs (Trapnell et al., 2013).

Table 1
Botrytis cinerea strains used in this study.

Strain	Description	Reference
B05.10		van Kan et al. (1997)
*ΔBchxt*15-4, 10	B05.10 *ΔBchxt*15::*HPH*	This study
*ΔBchxt*19-2, 3	B05.10 *ΔBchxt*19::*NAT*	This study
*ΔBchxt*15/*ΔBchxt*19-4, 7	B05.10 *ΔBchxt*15::*HPH* *ΔBchxt*19::*NAT*	This study

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