

Use of a non-homologous end-joining-deficient strain (*delta-ku70*) of the biocontrol fungus *Trichoderma virens* to investigate the function of the laccase gene *lcc1* in sclerotia degradation

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Abstract The aim of this study was to apply a generated $\Delta ku70$ strain with increased homologous recombination efficiency from the mycoparasitic fungus *Trichoderma virens* for studying the involvement of laccases in the degradation of sclerotia of plant pathogenic fungi. Inactivation of the non-homologous end-joining pathway has become a successful tool in filamentous fungi to overcome poor targeting efficiencies for genetic engineering. Here, we applied this principle to the biocontrol fungus *T. virens*, strain I10, by deleting its *tku70* gene. This strain was subsequently used to delete the laccase gene *lcc1*, which we found to be expressed after interaction of *T. virens* with sclerotia of the plant pathogenic fungi *Botrytis cinerea* and *Sclerotinia sclerotiorum*. *Lcc1* was strongly upregulated at early colonization of *B. cinerea* sclerotia and steadily induced during colonization of *S. sclerotiorum* sclerotia. The $\Delta ku70\Delta lcc1$ mutant was altered in its ability to degrade the sclerotia of *B. cinerea* and *S. sclerotiorum*.

Interestingly, while the decaying ability for *B. cinerea* sclerotia was significantly decreased, that to degrade *S. sclerotiorum* sclerotia was even enhanced, suggesting the operation of different mechanisms in the mycoparasitism of these two types of sclerotia by the laccase LCC1.

Keywords *ku70* · Gene targeting · *Trichoderma virens* · Laccase · Sclerotia · Mycoparasitism

Introduction

The scopes of this study were to enhance gene targeting efficiency for generation of knockout strains in the mycoparasite *Trichoderma virens* in order to facilitate biocontrol research and further to exploit this tool for studying the involvement of laccases in mycoparasitism of sclerotia of the plant pathogenic fungi *Sclerotinia sclerotiorum* and *Botrytis cinerea*.

Sclerotia are a compact mass of rigidified, aggregated fungal hyphae that are an essential feature of the life cycle of many asco- and basidiomycetes including plant pathogenic taxa such as *B. cinerea* and *S. sclerotiorum* (Alexopoulos and Mims 1995). They usually consist of a continuous layer of pseudoparenchymatous, melanized cells, which develop on the outer surface and enclose a broad medulla of interwoven hyphae. Melanized pigments are deposited in large amounts in sclerotial cell walls, and contribute to resistance against negative environmental factors including irradiation and biological degradation (Willettts and Bullock 1992). However, species of the mitosporic fungal genus *Trichoderma* are mycoparasites and can also effectively antagonize sclerotial phytopathogenic fungi (Vannacci et al. 1989, 1991; Benhamou and Chet 1996; Sarrocco et al. 2006). Mycoparasitism is a

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complex process that involves sequential events, including recognition, attack and subsequent penetration and killing of the host. The mycoparasitic attack by *Trichoderma* spp. of the mycelia of its prey fungi was already investigated in some detail and was shown to be an interplay of different mechanisms, including a plethora of different lytic enzymes (glucanases, chitinases, proteases) and secondary metabolites (Benitez et al. 2004). However, *Trichoderma* spp. are also able to parasitize the sclerotia of its prey fungi, but which enzymes or other mechanisms are involved in this attack has not been addressed yet. The ability to degrade melanin may be an important trait for antagonistic fungi used in the biocontrol of plant pathogenic fungi (Goméz and Nosanchuk 2003; Butler et al. 2005). Laccases are multicopper phenol-oxidases and are the most likely candidates for melanin degrading enzymes of ascomycetes because of their ability to attack condensed aromatic structures (Baldrian 2006; Giardina et al. 2010). Their presence and involvement in *Trichoderma* biocontrol strains have not been studied as yet.

A bottleneck of studying gene functions in biocontrol strains of *Trichoderma*—such as *T. harzianum*, *T. virens* or *T. atroviride*—is the low frequency of homologous integration events that is required for generation of gene knockout strains. This obstacle was recently successfully overcome in filamentous fungi using strains impaired in one of the genes involved in the non-homologous end-joining pathway (Krappmann 2007), which was shown to enhance homologous recombination of transformed DNAs in a wide variety of fungal taxa (Ninomiya et al. 2004; da Silva Ferreira et al. 2006; Krappmann et al. 2006; Nayak et al. 2006; Pöggeler and Kück 2006; Takahashi et al. 2006; Meyer et al. 2007; Chang 2008; Choquer et al. 2008; Haarmann et al. 2008; Levy et al. 2008; Snoek et al. 2009; Yamada et al. 2009; Hoff et al. 2010), including *Trichoderma reesei* (Guangtao et al. 2009).

In this study, we therefore identified the *ku70* homologue of the biocontrol fungus *T. virens*—*tku70*—and used it to generate a Δ *tku70* strain. The improved gene targeting efficiency of this strain was then tested and exploited to delete a laccase gene, which was identified to be specifically upregulated during attack of sclerotia from *B. cinerea* and *S. sclerotiorum*, in order to investigate its role in sclerotial mycoparasitism of *T. virens*.

Methods

Fungal strains

T. virens I10 (CBS 116947), a strain with high antagonistic activity (Vannacci and Pecchia 2000), was used

throughout this study as wild-type strain (WT). *B. cinerea* (SAS 405) was kindly provided by Dr. Pollastro (Department of Plant Biology and Pathology, University of Bari, Italy) and *S. sclerotiorum* de Bary strain 2048 was isolated in 1992 from the aerial part of tomato plants cultivated in an experimental greenhouse in Pisa, Italy. *Rhizoctonia solani* strain RB4 (AG4), isolated from diseased tobacco plants, was kindly provided by Dr. Nicolotti (C.R.A. Tobacco Research Institute, Scafati, Italy). Unless otherwise stated, all fungi were maintained on potato dextrose agar (PDA; BD Difco, Franklin Lakes, NJ, USA).

Plasmid construction and DNA techniques

For deletion of *T. virens tku70*, 1.2 kb of the 5'-non-coding and 1.8 kb of the 3'-non-coding regions of *T. virens tku70* (protein ID 83371 in the *T. virens* Gv29-8 genome sequence database v2.0, http://genome.jgi-psf.org/TriviGv29_8_2/TriviGv29_8_2.home.html) were amplified using the primer pairs A/B and C/D, respectively (Table 1) and cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). The 5'-*ku70* fragment was excised with *NotI* and the 3'-*ku70* fragment with *ApaI/NsiI* and both were ligated into a pBluescript SK (+) vector that contained the *A. nidulans amdS* gene, which encodes an acetamidase and can be used as a dominant selection marker and enables growth on acetamide as the sole nitrogen source (e.g. Seiboth et al. 1997). The correct orientation of the fragments was checked by control restriction digests. From the resulting plasmid pVCKU70, the 6.1 kb linear *tku70* deletion cassette was amplified with primers E/F (Table 1) using the Long Template Expand PCR System (Roche, Indianapolis, IN, USA) and PCR conditions according to the manufacturer's instructions. This cassette was used for deletion of the *tku70* gene in *T. virens* I10. For disruption of *T. virens lcc1* (protein ID 48916), 1.2 kb of its 5'-non-coding and 1.3 kb of its 3'-non-coding regions were amplified with the primer pairs G/H and I/J, respectively (Table 1). Vector pBS31 contains the *hph*-cassette from pRLMEX30 (Mach et al. 1994), conferring resistance to hygromycin B. The PCR fragments were digested with *HindIII/SpeI* and *Acc65I/XhoI* and ligated into vector pBS31 using the respective restriction sites, resulting in the *lcc1* knockout vector. The final 5.3 kb *lcc1* deletion cassette was again amplified with the primer pair G/J (Table 1) using Long Template Expand PCR system (Roche) and used for deletion of the *lcc1* gene in *T. virens* strains I10 and Δ *tku70*.

Fungal genomic DNA isolation was performed according to Hartl and Seiboth (2005). Standard methods (Sambrook and Russell 2001) were used for DNA electrophoresis and blotting. Hybridization and labeling of probes were performed with the DIG nonradioactive

Table 1 Primers used in this study

Primer	Sequence
A: Ku70_5'_rv	5'-GCCGAGTTAGCCGAAATTGGGCAATTGCCGACAGCCTTATTG-3'
B: Ku70_5'_fw	5'-AACTTCAGTCGCTCCATCAC-3'
C: Ku70_3'_rv	5'-AGAGTCTGGCTCAACACCC-3'
D: Ku70_3'_fw	5'-ATTGGCTGGGTTGATGGCTGCTTCGAGCGTATACAAAACCACCG-3'
E: Ku70_3'_rv_nst	5'-ACAGCGGCAGTATCCTGAGAAC-3'
F: Ku70_5'_fw_nst	5'-TGAACGGTGGCGGCAAC-3'
G: Lcc1_Prom_rv	5'-GTACA <u>CTAGT</u> GACAGGAGTAGAGGCGTT-3' ^a
H: Lcc1_Prom_fw	5'-GTACA <u>AAGCTT</u> AGACTGAAACATAGCGAGA-3' ^a
I: Lcc1_Term_rv	5'- <u>CTCGAGGAGCCCA</u> ACTTGAGATAA-3' ^a
J: Lcc1_Term_fw	5'-AGCTGGTACCCTTGTTCATTCT-3' ^a
K: amdSinAR	5'-GGGACAGAGTCTTGACGTAG-3'
L: hph_fw2	5'-TGAATGAGGATACACGGG-3'
M: Tef-F	5'-GGTACTGGTGAGTTCGAGGCTG-3'
N: Tef-R	5'-GGGCTCAATGGCGTCAATG-3'
O: RTFI-Lcc1	5'-TACGCTCTTTGCCGATTG-3'
P: RTRI-Lcc1	5'-TGTTACTATTCTTGCCACC-3'

^a Restriction sites (see “Methods”) are underlined

system (Roche). For analysis of the $\Delta tku70$ transformants, the 3.4 kb *tku70* probe was amplified with primers E/K (Table 1) and genomic DNA was digested with *EcoRI*. $\Delta tku70\Delta lcc1$ transformants were analysed using a 1.9 kb *lcc1* probe that was amplified with primers G/L (Table 1) and restriction digests of the genomic DNA were performed with *XbaI*.

Transformation of *T. vires*

Transformation was performed with *T. vires* protoplasts, essentially as previously described by Gruber et al. (1990) but with minor modifications outlined in Lopez-Mondejar et al. (2009). Briefly, 7.5 mg ml⁻¹ lysing enzymes (Sigma-Aldrich, St Louis, MO, USA) were used to generate protoplasts. Mycelia were incubated in the lysing solution for 2 h at 30°C with gentle agitation and mycelial clumps were gently separated with sterile tweezers every 30 min.

Following transformation with the *amdS* marker, protoplasts were plated out on plates containing *amdS*-selective medium (1 g l⁻¹ MgSO₄, 10 g l⁻¹ KH₂PO₄, 10 g l⁻¹ glucose, 20 ml l⁻¹ trace elements solution, Seidl et al. 2004; 15 g l⁻¹ agar, 10 ml l⁻¹ 1 M acetamide), supplemented with 1 M D-sorbitol, and incubated at 28°C. Similarly, after transformation with the hygromycin B-resistance marker (*hph*), protoplasts were plated out on PDA containing 1 M D-sorbitol and 50 µg ml⁻¹ hygromycin B and incubated at 28°C. Colonies emerging from the transformation plates were subcultivated on respective selective media and subsequently purified by single spore isolation on plates containing additionally 0.1% Triton X-100 for restriction of colony growth.

RNA isolation and RT-PCR

RNA isolation was performed on *B. cinerea* and *S. Sclerotinia sclerotia* colonized by *T. vires* and on control *T. vires* mycelium. The guanidinium isothiocyanate/phenol–chloroform method (Chomczynski and Sacchi 1987) was used, modified by the addition of 1% (w/v) soluble polyvinylpyrrolidone (40 kDa) in the extraction buffer. The quantity and concentration of RNAs obtained were measured spectrophotometrically (Gene Quant II, Pharmacia Biotech, Freiburg, Germany) and their integrity was verified by formaldehyde gel electrophoresis. RNAs were stored at –80°C until use.

RT-PCR analysis was carried out using the Access RT-PCR Kit (Promega, Madison, WI, USA). Primers used for RT-PCR were M/N for the *tef* gene (encoding translation elongation factor 1 alpha) as reference gene and O/P for the *lcc1* gene (Table 1).

Measurement of fungal growth rates

This was performed in 96-well microplates (PBI International, Milano, Italy). Each well was inoculated with 150 µl of a potato dextrose broth (PDB; BD Difco) spore suspension (10⁶ conidia ml⁻¹) prepared from PDA cultures grown for 1 week at 24 ± 2°C with 12 h/12 h cycles of darkness/light. Three replicates with 6 wells each were used. PDB without spores was used as a control. Fungal growth was monitored for a period of 72 h by reading the absorbance of the suspensions at 595 nm (OD) every 8 h in a microplate reader 680 (Bio-Rad Laboratories, Hercules, CA, USA). OD values were used to create growth curves. Data were also subjected to variance analysis of regression

in order to compare the slope of both curves, assuming $P < 0.05$ as a significant level.

The effect of DNA damaging agents was evaluated on PDA plates supplemented with 0–200 $\mu\text{g ml}^{-1}$ phleomycin or MMS (methyl methanesulfonate). Sensitivity to UV exposure was tested as described in Guangtao et al. (2009).

Growth assays under osmotic stress conditions were carried out on PDA plates and in PDB medium supplemented with either NaCl or glucose at concentrations of up to 0.8 M. Radial growth on agar plates was measured daily for 6 days. Shake flask cultures were grown for 30 h at 25°C and 250 rpm; biomass was harvested by filtration, washed with tap water and dried at 80°C for mycelial dry weight measurements.

At least two independent experiments were carried out for measurement of fungal growth rates and agar plates for each condition were always set up in triplicates.

Measurement of conidial germination

For each strain, three spore suspensions (10^3 conidia ml^{-1}) were prepared from cultures grown on PDA, as described above. Each spore suspension was streaked on three microscope slides and incubated in a petri dish at $24 \pm 2^\circ\text{C}$ with 12 h/12 h darkness/light cycles. Two layers of Whatman No. 1 filter paper, soaked with 2 ml of sterilized distilled water, were also put into the petri dish to maintain constant humidity. After 24 h, spore germination was stopped by incubating the slides at 50°C for 24 h. The slides were then stained with Cotton Blue-Sudan III and examined under the microscope. Germination ability was expressed as a percentage of germinated conidia and number of germ tubes for each conidium (40 conidia per slide) based on a total of nine slides for each strain. Percentages of germinated conidia (after angular transformation) and number of germ tubes were submitted to one-way ANOVA, assuming $P < 0.05$ as a significant level.

In vitro confrontation tests

The ability to antagonize plant pathogenic fungi was tested on plates by confronting *T. virens* and the mutants created in this study with *S. sclerotiorum*, *B. cinerea* and *R. solani*. PDA disks of 6 mm diameter, cut from the edge of an actively growing colony of each antagonist and pathogen, were placed at the opposite sides on PDA plates or on a sterile cellophane membrane laid on water agar medium (20 g l^{-1} , Bacteriological Agar, BD Difco). Assays of each antagonist/pathogen combination were set up in triplicates. Plates were incubated as described above. On PDA plates, radial growth of each pathogen in the direction of the antagonist (Ra) and in a control direction (Rc) was monitored until the colonies of the two fungi came in contact.

Values were used to create growth curves. Further, radial growth data were subjected to variance analysis of regression in order to compare the slope of curves in the presence/absence of the antagonist, assuming $P < 0.05$ as a significant level. On the same plates, after 21 days, overgrowth and sporulation of the antagonists on pathogens' colonies were assessed. Further, on water agar plates, interaction zones and overlapping regions for each antagonist/pathogen combination were analysed by microscopic investigations and searching for coiling of *T. virens* around the host hyphae.

Production of sclerotia and analysis of their degradation

Sclerotia of *B. cinerea* were produced by inoculating malt extract agar plates (MEA: 2% malt extract, Oxoid, Cambridge, UK, and 2% agar, Oxoid) with 8-mm diameter mycelial plugs cut from the colony grown on PDA. Plates were incubated for 2 days at 21°C and then for 30 days at 15°C in the dark. Production of *S. sclerotiorum* sclerotia was performed by inoculating flasks containing sterilized barley seeds and water with 8-mm diameter mycelial plugs derived from colonized PDA plates. Flasks were incubated in static cultures for 1 month at 24°C, under a cycle of 16 h light/8 h darkness. After incubation, the mature sclerotia of both *B. cinerea* and *S. sclerotiorum* were collected by sieving, dried under sterilized air flow at room temperature for 24 h and stored at 4°C.

The ability of the *T. virens* WT and recombinant strains to degrade sclerotia was evaluated in 24-well microplates. Each well was half-filled with PDA and inoculated with *T. virens*. After 7 days, two sclerotia from each pathogen (*B. cinerea* or *S. sclerotiorum*) were sown into each well. Each microplate contained two replicates, consisting of two rows of six wells. 2×12 sclerotia were tested for each isolate. After 4, 9, 13, 17, 21 days of incubation for *S. sclerotiorum* and 3, 6, 9, 12, 15 days for *B. cinerea*, the firmness of the sclerotia was evaluated by pressing them between sterile tweezers; sclerotia were classified as soft or hard as an yes/no decaying parameter. Percentages of decaying sclerotia of the WT and recombinant strains were expressed as vertical bar charts. Data were then linearized into logit ($\text{logit}_{(p)}: \ln(p/100 - p)$) and subjected to linear regression. Comparison between regression lines was performed by the program GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). $P < 0.05$ was assumed as a significant level.

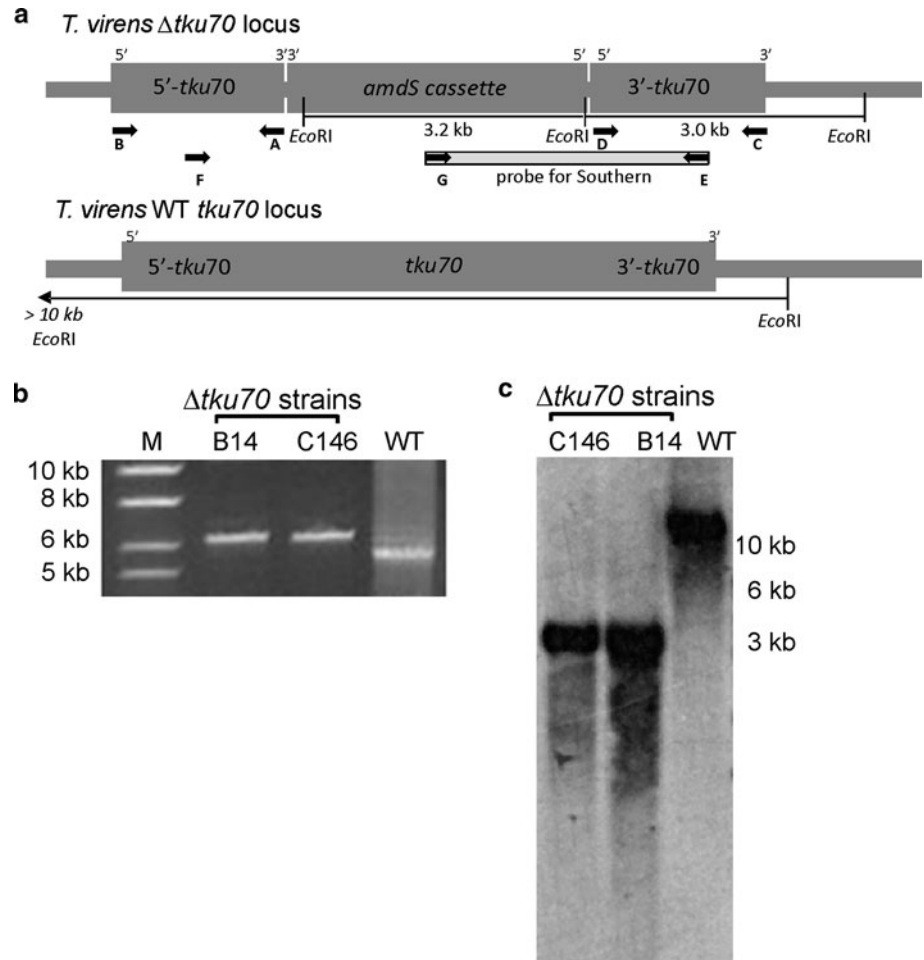
Results

Identification and deletion of the *T. virens* *tku70* gene

Using the *T. reesei* TKU70 protein sequence as a query in a BLASTP search, we identified the corresponding *T. virens*

Fig. 1 Generation and analysis of $\Delta tku70$ knockout strains.

a Schematic presentation of the $\Delta tku70$ and $tku70$ -WT loci, indicating the location of the primers and restriction sites used for analysis of the transformants. **b** PCR analysis of the $\Delta tku70$ strains and the WT with primers E/F (Table 1), showing a 5.1 kb band for the WT and a 6.1 kb band for the knockout strains B14 and C146. *M* molecular weight marker (1 kb ladder, Fermentas, St. Leon-Rot, Germany). **c** Southern analysis of the $\Delta tku70$ strains. Genomic DNA was digested with *EcoRI* and hybridization with the probe (amplified with primers E/K) showed a >10 kb band for the WT strain and a 3.0/3.2 kb double band for the knockout strains



TKU70 homologue in the *T. virens* genome sequence database (protein ID 83371). The *T. virens* TKU70 protein exhibits 91% similarity to the *T. reesei* TKU70 and 60% to the *N. crassa* MUS51 protein. To knock out the *tku70* gene in *T. virens*, a deletion vector (pVCKU70) was constructed in which the *A. nidulans* acetamidase gene *amdS* was flanked by 1.2 and 1.8 kb of the 5'- and 3'-non-coding regions of *T. virens tku70*, respectively (see “Methods” and Fig. 1a). After transformation and purification of 32 transformants, two of them (B14 and C146) were shown to be deleted in the *tku70* gene by PCR with primers E/F (see Table 1) by giving a 6.1 kb PCR product in contrast to 5.1 kb in the WT (Fig. 1b). Southern analysis confirmed the deletion (shift from >10 kb for the WT to a double band at 3.2 and 3.0 kb for $\Delta tku70$ strains B14 and C146), and further showed that both transformants had undergone a single integration event (Fig. 1c). Strain B14 was chosen for further investigations (called *T. virens* $\Delta tku70$ strain from here on).

Physiological properties of *T. virens* $\Delta tku70$

In order to be able to use the *T. virens* $\Delta tku70$ strain to create further gene knockouts for studying its biocontrol

properties, we first had to rule out that deletion of the *tku70* gene had not introduced any undesirable traits that would interfere with its antagonistic properties. Growth of both strains, expressed as OD, is shown in Fig. 2a. Variance analysis of regression performed on OD values showed no significant difference between the WT and $\Delta tku70$ strains ($P_{\text{slope}} = 0.53$). Efficiency of germination, considered to be another property of prime importance, was also not statistically altered in the $\Delta tku70$ strain in comparison to the WT (Table 2).

Further, the susceptibility of the $\Delta tku70$ strain to different treatments with DNA damaging agents was tested. Growth on agar plates containing the chemical agents phleomycin and MMS up to $200 \mu\text{g ml}^{-1}$ was evaluated as well as sensitivity towards UV irradiation. No difference in growth on agar plates containing increasing concentrations of phleomycin was detected between the WT and $\Delta tku70$ strain (data not shown). Growth on MMS was delayed at elevated concentrations ($>100 \mu\text{g ml}^{-1}$) in the $\Delta tku70$ strain (Fig. 2b). With respect to UV irradiation, in contrast to the findings in *T. reesei*, we were not able to find a statistically significant difference between the WT and $\Delta tku70$ strain (data not shown) using the

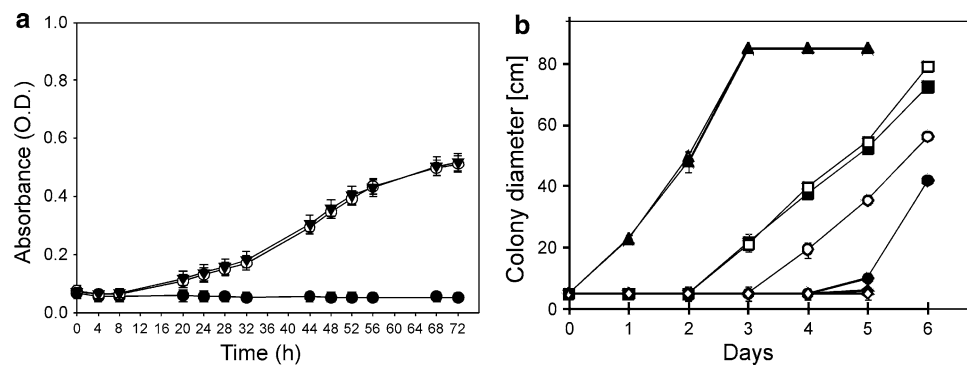


Fig. 2 Growth rate analysis of the *T. vires* WT strain and the $\Delta tku70$ strain. **a** Growth in PDB. Values are expressed as absorbance at 595 nm (WT: open circles, $\Delta tku70$ strain: filled triangles, uninoculated PBD: filled circles). **b** Growth on PDA plates (WT: open

triangles, $\Delta tku70$: filled triangles) and PDA supplemented with MMS; $50 \mu\text{g ml}^{-1}$ (WT: open squares, $\Delta tku70$: filled squares), $100 \mu\text{g ml}^{-1}$ (WT: open circles, $\Delta tku70$: filled circles) and $200 \mu\text{g ml}^{-1}$ MMS (WT: open diamonds, $\Delta tku70$: filled diamonds)

Table 2 Germination of conidia of the *T. vires* WT strain (I10) and the $\Delta tku70$ strain (B14)

Strain	% ^a	Germ tubes ^b
WT	91.87 ± 7.03*	0.98 ± 0.08**
$\Delta tku70$	92.12 ± 4.99*	0.95 ± 0.05**

All values represent the average of 9 slides, 40 conidia/slides

* $P = 0.345$ (analysis of variance of I10 vs. B14)

** $P = 0.383$ (analysis of variance of I10 vs. B14)

^a % of germinated conidia

^b Number of germ tubes for each conidium

same experimental setup as described in Guangtao et al. (2009).

A recently reported *ku70* knockout strain from *Penicillium chrysogenum* showed increased sensitivity to osmotic stress and an upregulation of the genes from the HOG pathway (Hoff et al. 2010). Therefore, we also included growth assays under osmotic stress conditions in our phenotypic analysis. We investigated radial growth on agar plates containing 0.4 and 0.8 M NaCl or glucose, respectively, and under these conditions biomass formation in shake flask cultivations was also measured, but neither on agar plates nor in submerged cultivations a growth reduction of the *T. vires* $\Delta tku70$ strain was detectable (data not shown).

The ability of the $\Delta tku70$ strain to antagonize the mycelial growth of *B. cinerea*, *S. sclerotiorum* and *R. solani* was checked in plate antagonism assays in order to test whether a *tku70* knockout would interfere with any other property that is relevant for mycoparasitism. Data in Fig. 3 show that both the WT and the $\Delta tku70$ strain reduced growth of all the three tested plant pathogens. This behaviour was confirmed by the variance analysis of regression where differences between slopes of curves in the presence/absence of the antagonists were all statistically significant ($P_{\text{slope}} \leq 0.0015$). Finally,

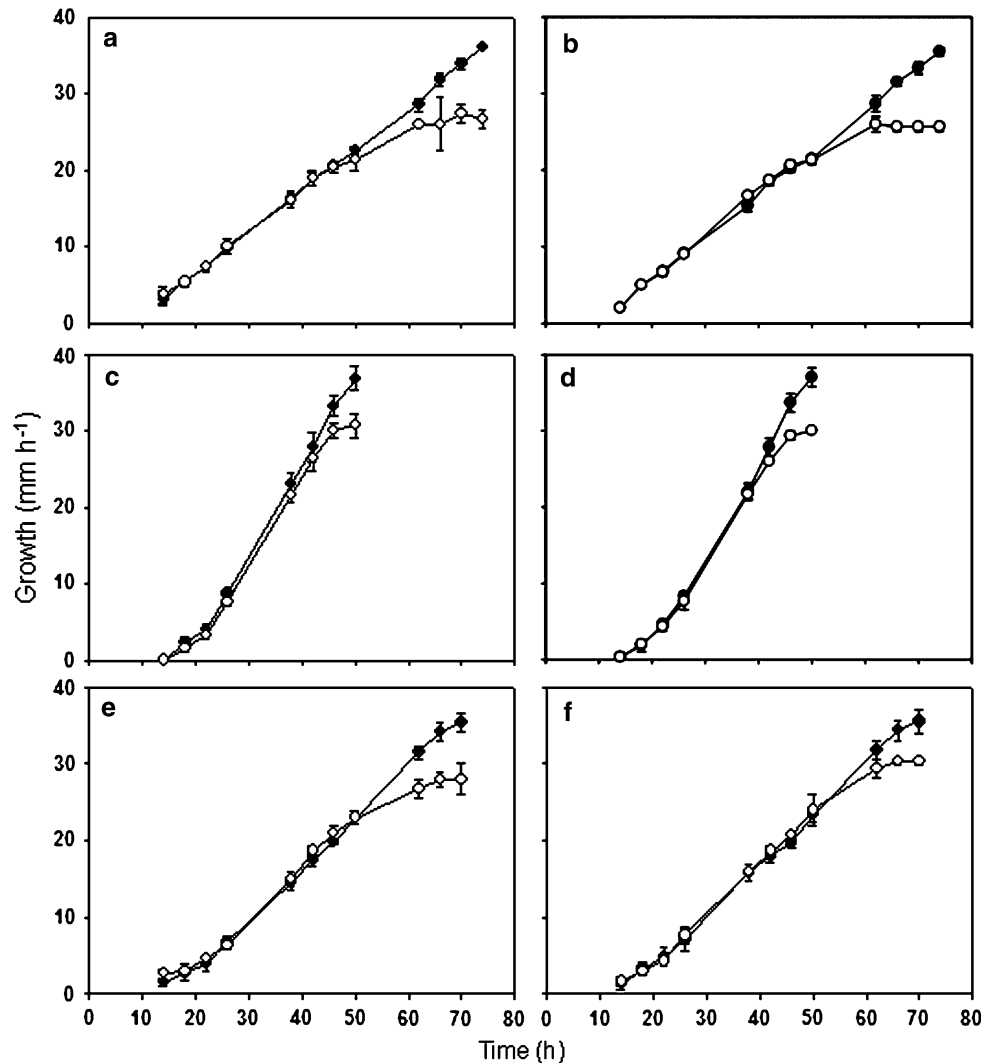
we investigated whether *T. vires* would coil around hyphae of the three pathogens, which is considered a step preceding invasion of the prey. During this study, coiling around hyphae of *B. cinerea* and *S. sclerotiorum* was observed neither with the WT strain nor with the $\Delta tku70$ strain, while it occurred at moderate frequency with *R. solani* for both strains (data not shown). Therefore, we conclude that the antagonistic properties of *T. vires* have not been altered by deletion of the *tku70* gene, and the respective $\Delta tku70$ strain is thus an appropriate parental strain for knocking out genes of putative relevance for mycoparasitism.

Expression of *T. vires* laccase genes during sclerotia colonization

T. vires strain I10 was previously shown to be particularly efficient in the degradation of sclerotia of phytopathogenic fungi (Sarrocco et al. 2006). In order to investigate the improved gene targeting efficiency of the *T. vires* $\Delta tku70$ strain by knocking out additional genes, we looked for laccase candidate genes in the genome of *T. vires* that would possibly be involved in sclerotia degradation. To this end, first the NCBI/DDBJ/EMBL database was screened for laccases from other asco- and basidiomycetes, and then these were used for a BLASTP search of the *T. vires* Gv29-8 genome database to detect putative homologues. Six genes encoding putative laccases (multi-copper oxidases, type 1) were identified and the corresponding protein IDs in the *T. vires* database v2.0 are 48916, 30456, 174487, 66278, 194054, 91146.

Transcript abundance of the respective putative laccase genes during the interaction with sclerotia derived from *B. cinerea* and *S. sclerotiorum* was tested by means of RT-PCR analysis, to study whether one or more of these genes were preferentially expressed during sclerotia colonization. Only one gene, *lcc1* (protein ID 48916), showed a specific and high induction in the presence of sclerotia

Fig. 3 Antagonism of the *T. virens* WT and the $\Delta tku70$ strain. Growth of the pathogenic fungi *B. cinerea*, *S. sclerotiorum* and *R. solani* is shown in the absence (filled circles) and in the presence (open circles) of the *T. virens* WT or $\Delta tku70$ strain. **a** *B. cinerea* versus WT; **b** *B. cinerea* versus $\Delta tku70$; **c** *S. sclerotiorum* versus WT; **d** *S. sclerotiorum* versus $\Delta tku70$; **e** *R. solani* versus WT; **f** *R. solani* versus $\Delta tku70$



(Fig. 4). Its transcript was abundantly produced during the early phases of contact with *B. cinerea* sclerotia (days 1, 2 and 3), while it was steadily expressed at a lower level during all colonization steps of *S. sclerotiorum* sclerotia after day 3 (Fig. 4). These data indicated that LCC1 could be involved in degradation of sclerotia by *T. virens*, although the different expression profiles pointed to a different role of LCC1 in the interaction with sclerotia of the two pathogens.

Efficient *lcc1* gene replacement in the *T. virens* $\Delta tku70$ strain

Consequently, *lcc1* was chosen as candidate for a gene knockout to test the effect of the *tku70* deletion on homologous gene targeting efficiency in *T. virens*. A *lcc1* knockout vector was constructed (pVCLAC) that contained the *hph* gene (encoding hygromycin B phosphotransferase) and 1.2 and 1.3 kb of the 5'- and 3'-non-coding regions of *lcc1* (see “Methods” and Fig. 5a).

After purification of positive transformants, 32 strains of the $\Delta tku70$ and 28 strains of the wild-type were examined for their gene targeting by diagnostic PCR. Seven double knockout strains out of the 32 transformants are shown in Fig. 5b. The results from the PCR analysis showed that efficiency of gene targeting in the $\Delta tku70$ strain was 88%, whereas it was only a 15% in the WT strain. Double mutants ($\Delta tku70\Delta lcc1$) were further subjected to Southern analysis, which showed a hybridization signal at the expected size of 3.2 kb in the transformants and 1.4 kb in the WT corresponding to the native *lcc1* locus (Fig. 5c; the same seven strains as in Fig. 5b are shown). Thus, these molecular weight shifts confirmed the deletion event and a single-copy integration of the cassette into the genome.

Having proven the deletion event, one of the $\Delta tku70\Delta lcc1$ double knockout strains (K36) was randomly chosen for further physiological investigations. Growth rate, conidial germination and antagonistic properties of the $\Delta tku70\Delta lcc1$ strain were assessed as described above

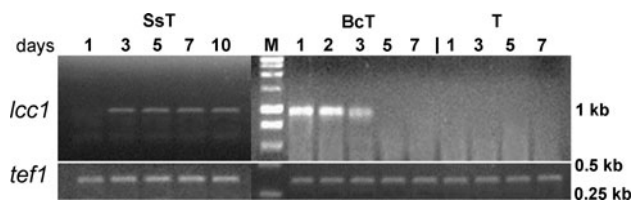


Fig. 4 Gene expression of *lcc1* during colonization of sclerotia by *T. virens*. *SsT*, *S. sclerotiorum* sclerotia after 1, 3, 5, 7, 10 days with *T. virens*; *BcT*, *B. cinerea* sclerotia after 1, 2, 3, 5, 7 days with *T. virens*; *T*, 110 *T. virens* at 1, 3, 5, 7 days alone as control. *M* molecular weight marker. *tef1* (encoding translation elongation factor 1 α) was used as reference gene

for the $\Delta tku70$ strain. In addition, growth on osmotic stress media and on DNA damaging agents was tested. None of these parameters was statistically significantly different from the WT strain and the $\Delta tku70$ strain, respectively, in the case of MMS (data not shown). Therefore, *T. virens* $\Delta tku70\Delta lcc1$ did not display any alterations in its phenotypic properties and antagonistic abilities to inhibit the mycelial growth of plant pathogenic fungi.

Effect of the deletion of *T. virens lcc1* on the degradation of sclerotia

Next, the effect of the *lcc1* deletion on the degradation of sclerotia from the two pathogenic fungi was evaluated. As control, the WT and $\Delta tku70$ mutant were used in these

experiments. The results (Figs. 6, 7) showed that *T. virens* WT and $\Delta tku70$ strains displayed the same decaying activities against sclerotia derived from both pathogens, indicating that this ability had not been changed by the *tku70* deletion. Degradation of sclerotia by the $\Delta tku70\Delta lcc1$ strain was clearly altered but interestingly the results showed the opposite effect against *B. cinerea* sclerotia in comparison to *S. sclerotiorum* sclerotia. Consistent with our hypothesis, *T. virens* $\Delta tku70\Delta lcc1$ exhibited a significantly reduced ability to degrade *B. cinerea* sclerotia (Fig. 6). However, the ability to degrade *S. sclerotiorum* sclerotia was even increased in this strain $\Delta tku70\Delta lcc1$ (Fig. 7). This behaviour was statistically confirmed by regression analysis, shown in Table 3, where for each strain regression line comparisons are displayed as statistical significance of slopes and elevation differences.

Discussion

This study showed that deletion of the *T. virens tku70* gene does not influence any of the properties relevant for mycoparasitism and biocontrol and that a *T. virens* $\Delta tku70$ strain can be used to study gene functions in this species. In combination with the availability of the *T. virens* genome sequence, this will greatly facilitate more intensive investigations of the molecular genetic principles of mycoparasitism and biocontrol in future. We were able to show

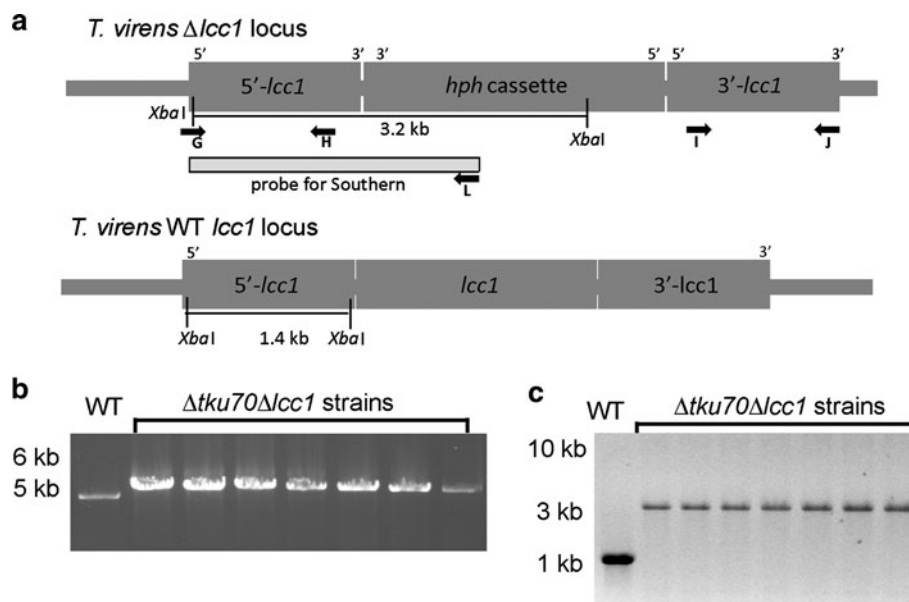


Fig. 5 Generation and analysis of $\Delta tku70\Delta lcc1$ knockout strains. **a** Schematic presentation of the $\Delta tku70\Delta lcc1$ and *lcc1*-WT loci, indicating the location of the primers and restriction sites used for analysis of the transformants. **b** PCR analysis results of seven $\Delta tku70\Delta lcc1$ strains and the WT with primers G/J (Table 1), showing a 5.0 kb band for the WT and a 5.3 kb band for the knockout strains

(only 7 out of the 32 $\Delta tku70\Delta lcc1$ strains are shown here). *M* molecular weight marker (1 kb ladder, Fermentas). **c** Southern analysis of seven $\Delta tku70\Delta lcc1$ strains. Genomic DNA was digested with *XbaI* and hybridization with the probe (amplified with primers G/L) showed a 1.4 kb band for the WT strain and a 3.1 kb band for the knockout strains

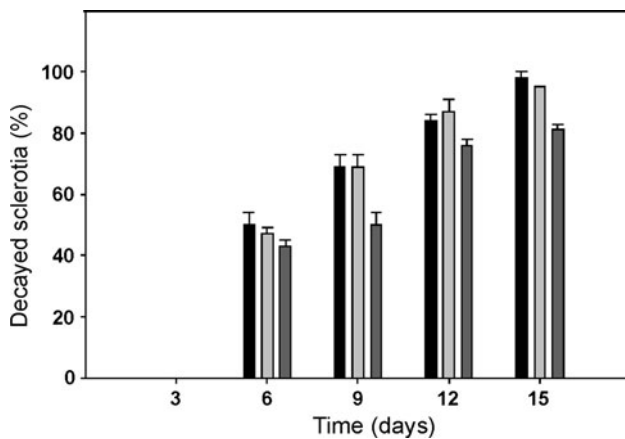


Fig. 6 Mycoparasitic activity of *T. vires* strains against *B. cinerea* sclerotia. Decaying ability (% of decayed sclerotia) by *T. vires* WT (black), $\Delta tku70$ (light grey) and $\Delta tku70\Delta lcc1$ (dark grey)

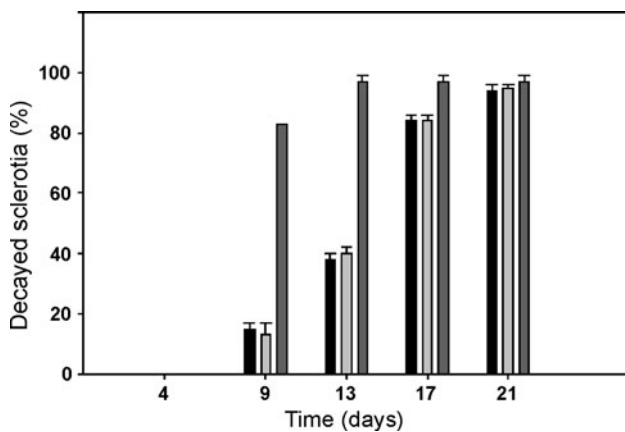


Fig. 7 Mycoparasitic activity of *T. vires* strains against *S. sclerotium* sclerotia. Decaying ability (% of decayed sclerotia) by *T. vires* WT (black), $\Delta tku70$ (light grey) and $\Delta tku70\Delta lcc1$ (dark grey)

Table 3 Linear regression analysis of sclerotia decaying ability of the *T. vires* WT strain (I10), the $\Delta tku70$ strain (B14) and the $\Delta tku70\Delta lcc1$ strain (K36)

Strains	y_0	a	R^2	P^*	P_{slope}	$P_{elevation}$
<i>Botrytis cinerea</i> sclerotia						
WT	-7.33	0.85	0.89	<0.0001		
$\Delta tku70$	-6.89	0.74	0.87	<0.0001	0.295	0.467
$\Delta tku70\Delta lcc1$	-6.66	0.63	0.82	<0.0001	0.047	
<i>Sclerotinia sclerotiorum</i> sclerotia						
WT	-7.42	0.52	0.95	<0.0001		
$\Delta tku70$	-7.49	0.53	0.96	<0.0001	0.820	0.928
$\Delta tku70\Delta lcc1$	-6.66	0.64	0.77	<0.0001	0.189	0.004

y_0 : intercept, a : slope, P^* : significance of regression line, P_{slope} : significance of differences between slopes of each strain versus WT, $P_{elevation}$: significance of differences between elevations of each strain versus WT

efficient gene targeting by creating $\Delta tku70\Delta lcc1$ double knockout strains which were subsequently used to study the involvement of *lcc1* in sclerotia penetration by *T. vires*. In agreement with the different transcription profiles of this gene during sclerotial degradation of the two plant pathogenic fungi *B. cinerea* and *S. sclerotiorum*, we found a reduced capability to parasitize the sclerotia of *B. cinerea* but not of *S. sclerotiorum*. The results from degradation indicated that the involvement of selected lytic enzymes may be strongly dependent on the sclerotial composition and structure. Our results provide, therefore, first insights in sclerotial mycoparasitism by *T. vires*, which has not been investigated yet and will be an interesting topic for further studies on the biocontrol potential of *T. vires* with respect to sclerotial fungi.

The KU70 protein of *T. vires* is—as in other fungi—involved in non-homologous DNA recombination, and its deletion enhanced the frequency of homologous integration in *T. vires*. Using deletion constructs with flanking regions of the target gene with a length of >1 kb, we were able to strongly improve gene targeting efficiency and achieved homologous recombination at a frequency of 88%. Importantly, no phenotypic defects regarding growth, conidial germination or the antagonistic properties of the $\Delta tku70$ strain were detected. Interestingly, we also found no increased susceptibility towards osmotic stress, as reported for *P. chrysogenum* (Hoff et al. 2010), or towards UV light in the $\Delta tku70$ strain. As already discussed in Guangtao et al. (2009), UV irradiation results mainly in point mutations which are repaired via a KU70/80 independent pathway (Goldman et al. 2002) and phenotypic analyses of different fungal species deleted in KU70/KU80 homologues show an inconsistent picture with respect to their susceptibility to different treatments with DNA damaging agents. The increased sensitivity towards UV light that was reported for *T. reesei* (Guangtao et al. 2009) might be due to the fact that the strains used in research are already mutant lines. These strains were selected for increased cellulase production, but have also numerous additional mutations (Seidl et al. 2008; Le Crom et al. 2009) that possibly render these strains more susceptible to certain stresses such as DNA damage. The $\Delta tku70$ strain showed no phenotypic constraints and *T. vires* is thus the first biocontrol fungus for which a high-efficacy gene replacement system is available.

In this study, the laccase gene *lcc1* was deleted exploiting the $\Delta tku70$ mutant of *T. vires*. Our interest in laccases resulted from previous evidence about sclerotial mycoparasitism by *T. vires* (Sarrocco et al. 2006) and the hypothesis that a laccase activity, as phenol-oxidase, could be essential for degrading the external melanic layer (rind) of sclerotia (Gómez and Nosanchuk 2003). The expression pattern of *lcc1* showed that its transcription was strongly

upregulated upon contact of *T. virens* with *B. cinerea* sclerotia (Fig. 4), implying a specific function of the respective protein in early stages of sclerotia degradation. Further, *lcc1* was constantly induced when *T. virens* was grown in association with *S. sclerotiorum* sclerotia, also suggesting a function during colonization of these sclerotia. The other five laccases were not found to be induced under the tested growth conditions (data not shown). The rigid structures of sclerotia protect fungi against biotic and abiotic stresses, and are thus an important trait against attack by other fungi such as mycoparasites. Thereby, the melanization of sclerotia appears to provide special protection against the degradation of the cell wall polysaccharides by hydrolytic enzymes (Willettts 1971). The melanin produced by *S. sclerotiorum* has been identified as a dihydroxynaphthalene (DHN; Butler et al. 2009), and been shown to be susceptible to attack by oxidative enzymes of the white rot fungus *Phanerochaete chrysosporium* (Butler et al. 2009). As a soft rot saprotroph, *T. virens* does not have any lignin and manganese peroxidases, but it contains laccases which have also been proven to play important roles in the oxidative damage of white rot fungi (Lundell et al. 2010). We considered it thus likely that the *T. virens* laccases would be able to attack sclerotia melanins.

Our results demonstrated that there was indeed a reduction in decay of *B. cinerea* sclerotia upon deletion of *T. virens lcc1*, but that degradation of *S. sclerotiorum* sclerotia was even enhanced (Figs. 5, 6). Although this finding seems puzzling at first, it becomes less surprising when the composition of the sclerotia from these two fungi is considered. *S. sclerotiorum* and *B. cinerea* represent two different types of sclerotia, i.e. the tuberoid sclerotium in the former, and the plano-convexoid sclerotium in the latter. The latter contains breaches at the junction of rind cells, which correspond to gaps in melanin deposits, through which mycoparasites can enter because of the reduced thickness of melanin deposits (Rey et al. 2005). No such breaches are observed in the tuberoid sclerotia (Rey et al. 2005). Therefore, the different results could be due to differences in the accessibility of the sclerotia of *S. sclerotiorum* and *B. cinerea*. Further, sclerotia from these two fungi vary their biochemical composition and structural organization (Willettts and Bullock 1992). Finally, the *lcc1* expression pattern points out a dissimilar role of LCC1 in degrading the two types of sclerotia. *lcc1* was specifically upregulated by *B. cinerea* sclerotia during the early phases of degradation, as expected by a function involved in attacking the rind for starting sclerotia penetration, whereas it was induced later and evenly expressed in further stages of degradation of *S. sclerotiorum* sclerotia. Considering the differences between the two sclerotial systems, the different effect that was observed in the *lcc1* knockout mutant against the two pathogens was not unexpected. However, the strong

enhancement of *S. sclerotiorum* sclerotia remains somewhat puzzling. One possibility to explain this finding is that the *T. virens lcc1* deletion may have upregulated some other functions involved in *S. sclerotiorum* sclerotia degradation, but this will need further investigation. It would be interesting to know whether the expression of any of the other putative laccase genes is enhanced in the *lcc1*-deficient *T. virens* strain when exposed to *S. sclerotiorum*. A possible change of the expression pattern of the other laccase genes in *T. virens* under these circumstances (deletion of *lcc1* and exposure to *S. sclerotiorum* sclerotia) could possibly point at a species-specific activation of redundant laccases, but this remains to be tested. In addition, it should be mentioned that laccases do not only have the ability to oxidize melanins and therefore to initiate their degradation, but are also needed in some fungi for the synthesis of melanins, e.g. Eisenman et al. (2007). More information about the nature, structure and biosynthesis of the sclerotial melanins of *B. cinerea* and *S. sclerotiorum* will be necessary to be able to study their degradation in more detail.

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