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

Valentina Catalano · Mariarosaria Vergara · Jasmin R. Hauzenberger · Bernhard Seiboth · Sabrina Sarrocco · Giovanni Vannacci · Christian P. Kubicek · Verena Seidl-Seiboth

Abstract The aim of this study was to apply a generated Δku70 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 Δku70Δlcc1 mutant was altered in its ability to degrade the sclerotia of *B. cinerea* and *S. sclerotiorum*.

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 (Willett 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...
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 Δtku70 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* tka70, 1.2 kb of the 5′-non-coding and 1.8 kb of the 3′-non-coding regions of *T. virens* tka70 (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 *Apa*I/*Nsi*I 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 tka70 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 tka70 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 *Hind*III/*Sph*I and *Acc65I*/*Xho*I 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 Δtka70.

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
Transformation was performed with T. virens. Transformation of formed with XbaI and restriction digests of the genomic DNA were performed with XbaI.

**Table 1** Primers used in this study

| Primer                | Sequence                                      |
|-----------------------|-----------------------------------------------|
| A: Ku70_5’_rv         | 5’-GCCGAGTTACCGGAAATGGGGCAATTGCAGCCTATTG-3’  |
| B: Ku70_5’_fw         | 5’-AACCTACGTCGCCCATCAC-3’                     |
| C: Ku70_3’_rv         | 5’-AGAAGCTTGTCAACACCC-3’                      |
| D: Ku70_3’_fw         | 5’-ATTGGCTGTTGGATGCGCTTCGAGGCTATACAAACCAC-3’ |
| E: Ku70_3_ry_nst      | 5’-ACACGGCAGATGTCCCTGAAC-3’                   |
| F: Ku70_5_ry_nst      | 5’-TGACCGGTGCGGCAAC-3’                        |
| G: Lccl1 PROM_rv      | 5’-GTGACCTAGTGACAGGGAGTAGGGCGTT-3’a          |
| H: Lccl1 PROM fw      | 5’-GTACAAAGCTTTAGCTGAACATACGCGAG-3’a         |
| I: Lccl1 Term_rv      | 5’-CTGGAGGACCCAACTTGAGATAA-3’a               |
| J: Lccl1 Term Rw      | 5’-AGCTGGATCCCTGTATTTCTATTCT-3’a             |
| K: amdSinAR           | 5’-GGGACAGAGGCTCTGACGTAG-3’                   |
| L: hph-fw2            | 5’-TGAATGAGGATACGGG-3’                        |
| M: Tef-F              | 5’-GGTACTGGTGGAGTTCGAGGCTG-3’                |
| N: Tef-R              | 5’-GGGCTCTAATGGGCTCAATG-3’                    |
| O: RTFI-Lccl1         | 5’-TACGGCTTTTGCCGATTG-3’                     |
| P: RTRI-Lccl1         | 5’-TGTTACTATTCTTGGCCACC-3’                   |

* Restriction sites (see “Methods”) are underlined.

Transformation of T. virens

Transformation was performed with T. virens 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 α-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 α-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.

RNAs were also subjected to variance analysis of regression

**RNA isolation and RT-PCR**

RNA isolation was performed on *B. cinerea* and *S. sclerotiorum* sclerotia colonized by *T. virens* and on control *T. virens* 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 \( \mu \)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 ± 2°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 (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...
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 Δtku70 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 Δtku70 strain from here on).

Physiological properties of T. virens Δtku70

In order to be able to use the T. virens Δtku70 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 Δtku70 strains (P_slope = 0.53). Efficiency of germination, considered to be another property of prime importance, was also not statistically altered in the Δtku70 strain in comparison to the WT (Table 2).

Further, the susceptibility of the Δtku70 strain to different treatments with DNA damaging agents was tested. Growth on agar plates containing the chemical agents phleomycin and MMS up to 200 µg 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 Δtku70 strain (data not shown). Growth on MMS was delayed at elevated concentrations (>100 µg ml⁻¹) in the Δtku70 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 Δtku70 strain (data not shown) using the...
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. virens* Δku70 strain was detectable (data not shown). Therefore, we conclude that the antagonistic properties of *T. virens* have not been altered by deletion of the *tku70* gene, and the respective Δku70 strain is thus an appropriate parental strain for knocking out genes of putative relevance for mycoparasitism.

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

*T. virens* 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. virens* Δku70 strain by knocking out additional genes, we looked for laccase candidate genes in the genome of *T. virens* 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. virens* 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. virens* 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. 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 Δku70 strain

Consequently, lcc1 was chosen as candidate for a gene knockout to test the effect of the Δku70 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 Δku70 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 Δku70 strain was 88%, whereas it was only a 15% in the WT strain. Double mutants (Δku70Δlcc1) 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 Δku70Δlcc1 double knockout strains (K36) was randomly chosen for further physiological investigations. Growth rate, conidial germination and antagonistic properties of the Δku70Δlcc1 strain were assessed as described above.
for the Δtku70 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 Δtku70 strain, respectively, in the case of MMS (data not shown). Therefore, T. virens Δtku70Δlcc1 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 Δtku70 mutant were used in these experiments. The results (Figs. 6, 7) showed that T. virens WT and Δtku70 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 Δtku70Δlcc1 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 Δtku70Δlcc1 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 Δtku70Δlcc1 (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 Δtku70 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
T. virens sclerotia. Decaying ability (% of decayed sclerotia) by WT versus T. virens strains against B. cinerea sclerotia. Decaying ability (% of decayed sclerotia) by T. virens WT (black), Δku70 (light grey) and Δku70Δlcc1 (dark grey).

![Fig. 6 Mycoparasitic activity of T. virens strains against B. cinerea sclerotia. Decaying ability (% of decayed sclerotia) by T. virens WT (black), Δku70 (light grey) and Δku70Δlcc1 (dark grey)](image)

Table 3 Linear regression analysis of sclerotia decaying ability of the T. virens WT strain (I10), the Δku70 strain (B14) and the Δku70Δlcc1 strain (K36)

| Strains                        | $y_0$  | $a$  | $R^2$ | $P^*$  | $P_{slope}$ | $P_{elevation}$ |
|-------------------------------|--------|------|-------|--------|-------------|-----------------|
| Botrytis cinerea sclerotia    |        |      |       |        |             |                 |
| WT                            | -7.33  | 0.85 | 0.89  | <0.0001|             |                 |
| Δku70                         | -6.89  | 0.74 | 0.87  | <0.0001| 0.295       | 0.467           |
| Δku70Δlcc1                    | -6.66  | 0.63 | 0.82  | <0.0001| 0.047       |                 |
| Sclerotinia sclerotiorum      |        |      |       |        |             |                 |
| WT                            | -7.42  | 0.52 | 0.95  | <0.0001|             |                 |
| Δku70                         | -7.49  | 0.53 | 0.96  | <0.0001| 0.820       | 0.928           |
| Δku70Δlcc1                    | -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 Δku70Δlcc1 double knockout strains which were subsequently used to study the involvement of lcc1 in sclerotia penetration by T. virens. 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. virens, which has not been investigated yet and will be an interesting topic for further studies on the biocontrol potential of T. virens with respect to sclerotial fungi.

The KU70 protein of T. virens is—as in other fungi—involved in non-homologous DNA recombination, and its deletion enhanced the frequency of homologous integration in T. virens. 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 Δku70 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 Δku70 strain. As already discussed in Guantao 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 (Guantao 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 Δku70 strain showed no phenotypic constraints and T. virens 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 Δku70 mutant of T. virens. Our interest in laccases resulted from previous evidence about sclerotial mycoparasitism by T. virens (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 (Goméz 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 *S. sclerotiorum* against the degradation of the cell wall polysaccharides by *S. sclerotiorum* grown in association with *B. cinerea* sclerotia (Fig. 4), implying a specific function of the *lcc1* deletion mutant against *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 induced 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.

Acknowledgments We thank Monika Schmoll for kindly providing pBS31. The authors thank the Scuola Normale Superiore di Pisa, Italy, for partially funding the research and Italian Ministry of University and Research (MIUR) for supporting the PhD student Valentina Catalano. This work was also supported by the Austrian Science Fund (research grants P20559 and T390 to V.S.). The work conducted by the US Department of Energy Joint Genome Institute is supported by the Office of Science of the US Department of Energy under Contract No. DE-AC02-05CH11231.

Open Access This article is distributed under the terms of the Creative Commons Attribution Noncommercial License which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

References

Alexopoulos CJ, Mims CW (1995) Introductory mycology, 4th edn. Lubrecht and Cramer Ltd., Port Jervis, NJ

Baldrian P (2006) Fungal laccases—occurrence and properties. FEMS Microbiol Rev 30:215–242

Benhamou N, Chet I (1996) Parasitism of sclerotia of *Sclerotium rolfsii* by *Trichoderma harzianum*: ultrastructural and cytotoxic aspects of the interaction. Phytopathology 86:405–416

Benitez T, Rincon AM, Limon MC, Codon AC (2004) Biocontrol mechanisms of *Trichoderma* strains. Int Microbiol 7:249–260

Butler MJ, Gardinter RB, Day AW (2005) Degradation of melanin or inhibition of its synthesis: are these a significant approach as a biological control of phytopathogenic fungi? Biol Control 32:326–336

Butler MJ, Gardiner RB, Day AW (2009) Melanin synthesis by *Sclerotinia sclerotiorum*. Mycologia 101:296–304

Chang PK (2008) A highly efficient gene-targeting system for *Aspergillus parasiticus*. Lett Appl Microbiol 46:587–592

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 162:156–159
Choquer M, Robin G, Le Pecheur P, Giraud C, Levis C, Viala M (2008) Ku70 or Ku80 deficiencies in the fungus Botrytis cinerea facilitate targeting of genes that are hard to knock out in a wild-type context. FEMS Microbiol Lett 289:225–232

da Silva Ferreira ME, Kress MR, Savoldi M, Goldman MH, Hartl A, Heinekamp T, Brakhage AA, Goldman GH (2006) The akaB(KU80) mutant deficient for nonhomologous end joining is a powerful tool for analyzing pathogenicity in Aspergillus fumigatus. Eukaryot Cell 5:207–211

Eisenman HC, Mues M, Weber SE, Frases S, Chaskes S, Gerfen G, da Silva Ferreira ME, Kress MR, Savoldi M, Goldman MH, Hartl A, Choquer M, Robin G, Le Pecheur P, Giraud C, Levis C, Viaud M Curr Genet (2011) 57:13–23

Haarmann T, Lorenz N, Tudzynski P (2008) Use of a nonhomologous end-joining element-binding protein Seb1 is involved but not essential for glycerol dehydrogenase (gld1) gene expression and glycerol accumulation in Trichoderma atroviride during osmotic stress. Fungal Genet Biol 41:1132–1140

Seidl V, Seiboth B, Karaffa L, Kubicek CP (2004) The fungal STRE‐element‐binding protein Seb1 is involved but not essential for glycerol dehydrogenase (gld1) gene expression and glycerol accumulation in Trichoderma atroviride during osmotic stress. Fungal Genet Biol 41:1132–1140

Seidl V, Gamauf C, Druzhinina IS, Seiboth B, Hartl L, Kubicek CP (2008) The Hypocrea jecorina (Trichoderma reesei) hypercel-lulolytic mutant RUT C30 lacks a 85 kb (29 gene-encoding) region of the wild-type genome. BMC Genomics 9:327

Snoek IS, van der Kroot ZA, Touw H, Kerkmann R, Pronk JT, Bovenberg RA, van den Berg MA, Daran JM (2009) Construction of an hfdA Penicillium chrysogenum strain impaired in non-homologous end-joining and analysis of its potential for functional analysis studies. Fungal Genet Biol 46:418–426

Takahashi T, Masuda T, Koyama Y (2006) Enhanced gene targeting frequency in ka70 and ku80 disruption mutants of Aspergillus sojae and Aspergillus oryzae. Mol Genet Genomics 275:460–470

Vannacci G, Pecchia S (2000) Discovery of Trichoderma I252 and Glidioladum I10, components of the biofungicide GT10/252. In: European Cost 830 workshop “Selection strategies for plant beneficial microorganisms”, 3–5 April 2000, Nancy

Vannacci G, Pecchia S, Resta E (1989) Presence of Sclerotinia minor Jagger antagonists in soils from different Mediterranean areas II. Sclerotia decaying fungi. Acta Hortic 255:293–302

Vannacci G, Pecchia S, Mallegni C, Cortellini W, Fascini F (1991) Biocloture of Sclerotinia lettuce drop. Petria 1:140–141

Willetts HJ (1971) The survival of fungal sclerotia under adverse environmental conditions. Biol Rev 46:387–407

Willetts HJ, Balloch S (1992) Developmental biology of sclerotia. Mycol Res 96:801–816

Yamada T, Makimura K, Hayakawa T, Ishihara Y, Umeda Y, Abe S (2009) Enhanced gene replacements in Ku80 disruption mutants of the dermatophyte, Trichophyton mentagrophytes. FEMS Microbiol Lett 298:208–217

Meyer V, Arendshorst M, El-Ghezal A, Andrews AC, Kooistra R, van den Hondel CA, Ram AF (2007) Highly efficient gene targeting in the Aspergillus niger kasA mutant. J Bacteriol 189:770–775

Nakay T, Szewczyk E, Oakley CE, Osmani A, Ukil L, Murray SL, Hynes MJ, Osmani SA, Oakley BR (2006) A versatile and efficient gene-targeting system for Aspergillus nidulans. Genetics 172:1557–1566

Ninomiya Y, Suzuki K, Ishii C, Inoue H (2004) Highly efficient gene replacements in Neurospora strains deficient for nonhomologous end-joining. Proc Natl Acad Sci USA 101:12248–12253

Poggeler S, Kück U (2006) Highly efficient generation of signal transduction knockout mutants using a fungal strain deficient in the mammalian ku70 ortholog. Gene 378:1–10

Rey P, Le Floch G, Benhamou N, Salerno ML, Thullier E, Tirilly Y (2005) Interactions between the mycoparasite Pythium oligandrum and two types of sclerotia of plant-pathogenic fungi. Mycol Res 109:779–788

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Lab. Press, Plainview, NY

Sarrocco S, Mikkelsen L, Vergara M, Jensen DF, Lukeb M, Vannacci G (2006) Histopathological studies of sclerotia of phytopathogenic fungi parasitized by a GFP transformed Trichoderma virens antagonistic strain. Mycol Res 110:179–187

Seiboth B, Hakola S, Mach RL, Suominen PL, Kubicek CP (1997) Role of four major cellulases in triggering of cellulase gene expression by cellulose in Trichoderma reesei. J Bacteriol 179:5318–5330

Seidl V, Seiboth B, Karaffa L, Kubicek CP (2004) The fungal STRE‐element‐binding protein Seb1 is involved but not essential for glycerol dehydrogenase (gld1) gene expression and glycerol accumulation in Trichoderma atroviride during osmotic stress. Fungal Genet Biol 41:1132–1140

Seidl V, Gamauf C, Druzhinina IS, Seiboth B, Hartl L, Kubicek CP (2008) The Hypocrea jecorina (Trichoderma reesei) hypercel-lulolytic mutant RUT C30 lacks a 85 kb (29 gene-encoding) region of the wild-type genome. BMC Genomics 9:327

Snoek IS, van der Kroot ZA, Touw H, Kerkmann R, Pronk JT, Bovenberg RA, van den Berg MA, Daran JM (2009) Construction of an hfdA Penicillium chrysogenum strain impaired in non-homologous end-joining and analysis of its potential for functional analysis studies. Fungal Genet Biol 46:418–426

Takahashi T, Masuda T, Koyama Y (2006) Enhanced gene targeting frequency in ka70 and ku80 disruption mutants of Aspergillus sojae and Aspergillus oryzae. Mol Genet Genomics 275:460–470

Vannacci G, Pecchia S (2000) Discovery of Trichoderma I252 and Glidioladum I10, components of the biofungicide GT10/252. In: European Cost 830 workshop “Selection strategies for plant beneficial microorganisms”, 3–5 April 2000, Nancy

Vannacci G, Pecchia S, Resta E (1989) Presence of Sclerotinia minor Jagger antagonists in soils from different Mediterranean areas II. Sclerotia decaying fungi. Acta Hortic 255:293–302

Vannacci G, Pecchia S, Mallegni C, Cortellini W, Fascini F (1991) Biocloture of Sclerotinia lettuce drop. Petria 1:140–141

Willetts HJ (1971) The survival of fungal sclerotia under adverse environmental conditions. Biol Rev 46:387–407

Willetts HJ, Balloch S (1992) Developmental biology of sclerotia. Mycol Res 96:801–816

Yamada T, Makimura K, Hayakawa T, Ishihara Y, Umeda Y, Abe S (2009) Enhanced gene replacements in Ku80 disruption mutants of the dermatophyte, Trichophyton mentagrophytes. FEMS Microbiol Lett 298:208–217