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Genome-scale investigation of phenotypically distinct but nearly clonal *Trichoderma* strains

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Abstract

Biological control agents (BCA) are beneficial organisms that are applied to protect plants from pests. Many fungi of the genus *Trichoderma* are successful BCAs but the underlying mechanisms are not yet fully understood. *Trichoderma cf. atroviride* strain LU132 is a remarkably effective BCA compared to *T. cf. atroviride* strain LU140 but these strains were found to be highly similar at the DNA sequence level. This unusual combination of phenotypic variability and high DNA sequence similarity between separately isolated strains prompted us to undertake a genome comparison study in order to identify DNA polymorphisms. We further investigated if the polymorphisms had functional effects on the phenotypes. The two strains were clearly identified as individuals, exhibiting different growth rates, conidiation and metabolism. Superior pathogen control demonstrated by LU132 depended on its faster growth, which is a prerequisite for successful distribution and competition. Genome sequencing identified only one non-synonymous single nucleotide polymorphism (SNP) between the strains. Based on this SNP, we successfully designed and validated an RFLP protocol that can be used to differentiate LU132 from LU140 and other *Trichoderma* strains. This SNP changed the amino acid sequence of SERF, encoded by the previously undescribed single copy gene "small EDRK-rich factor" (*serf*). A deletion of *serf* in the two strains did not lead to identical phenotypes, suggesting that, in addition to the single functional SNP between the nearly clonal *Trichoderma cf. atroviride* strains, other non-genomic factors contribute to their phenotypic variation. This finding is significant as it shows that genomics is an extremely useful but not exhaustive tool for the study of biocontrol complexity and for strain typing.

Introduction

The filamentous fungi *Trichoderma atroviride*, and the closely related *Trichoderma cf. atroviride* (Braithwaite M et al., unpublished), are species of economic and ecological importance to horticulture and agriculture for biological control of a variety of plant pathogens. However, there is considerable strain-specific variation in biocontrol characteristics, exemplified by *T. cf. atroviride* biocontrol strains LU132 (formerly C52) and LU140 (formerly D73). These strains were isolated three years apart from the same New Zealand onion field but extensive trials revealed differences in their biocontrol abilities with pathogenic fungi (Dodd-Wilson 1996; Harrison & Stewart 1988; Kay 1991; Kay 1994; Kay & Stewart 1994; Lange 2015; McLean 1996; McLean 2001). The two strains are propagated asexually by mycelial growth and the production of conidia. Both strains were found to be antagonistic towards the onion pathogen *Sclerotium cepivorum* (Harrison & Stewart 1988; Kay & Stewart 1994; McLean 1996). As they consistently grew more rapidly than *S. cepivorum* and showed no evidence of antibiotic production or direct antagonism, their chief biocontrol mechanism was suggested to be competition for nutrients and space (Harrison & Stewart 1988; Kay 1994). However, LU132 was substantially more efficient than LU140 in inhibiting *S. cepivorum* on agar plates and in the glasshouse (Kay 1994; McLean 1996; McLean 2001), as well as in proliferation and establishment in soil (McLean 1996), promoting onion seedling emergence (McLean & Stewart 2000) and growth promotion of rye grass (Chohan et al. 2010). Due to the excellent biocontrol of *S. cepivorum* and *Botrytis cinerea* demonstrated by LU132, it was formulated into the commercial biocontrol products Trichopel® Ali 52, Sentinel® and Tenet® (Agrimm Technologies Ltd., Lincoln, New Zealand) (Card et al. 2009; McLean et al. 2012; McLean et al. 2005).

In order to release a new biocontrol product on the European market, authorities require a molecular identification method to monitor the development and distribution of the organism in the environment. Attempts were made to develop an LU132-specific molecular marker so its ecology could be monitored within commercial systems. Markers are commonly designed on the basis of individual fingerprinting profiles. However, in the case of LU132, this approach was not sufficient, as the profiles could distinguish LU132 from all tested strains except the less effective strain LU140. Fingerprinting profiles, a SCAR approach (Cordier et al. 2007; Hermosa et al. 2001; Naeimi et al. 2011) and sequencing of a number of marker genes and regions (the translation elongation factor 1α (*tef1α*) (Shoukouhi & Bissett 2009), the 42 kDa endochitinase (*ech42*) (Lieckfeldt et al. 2000), the Internal Transcribed Spacer regions 1 and 2 (ITS1 and ITS2) of rRNA genes (Kuhls et al. 1997; Rehner & Samuels 1994) and the mitochondrial cytochrome oxidase subunit 1 encoding gene *cox1* (Hamari et al. 2003)) could not identify any DNA sequence differences between LU132 and LU140 (Dodd-Wilson 1996; McLean 2001 and unpublished data from within our research group). Therefore a genome-wide search for polymorphisms was required.

Much genetic research has focussed on fungi as eukaryotic model organisms due to their relatively small genome sizes and simplistic lifestyles (van der Klei & Veenhuis 2006). However, many fungi have interesting traits in their own right. The genus *Trichoderma* comprises more than 200 species (Atanasova, Druzhinina & Jaklitsch 2013), of which many form endophytic relationships with higher plants (Harman et al. 2004). This close relationship promotes plant growth and activates the plant's resistance to pests. In addition, they parasitise plant pathogens (mycoparasitism) and produce bioactive secondary metabolites and enzymes,

which makes them valuable in agriculture as BCA and in industry as sources of hydrolytic enzymes (Harman et al. 2012; Harman et al. 2004; Lorito et al. 2010).

To understand what makes a good *Trichoderma* BCA, many studies have focussed on mycoparasitism related genes (e.g. cell wall degrading enzymes) and genes that promote plant growth or induce systemic resistance in the plants (Harman et al. 2012). A recent genome comparison between one hypercellulolytic species (*Trichoderma reesei*) and two biocontrol species (*T. atroviride* and *T. virens*) found that the two biocontrol species contained more mycoparasitism-relevant genes, such as genes coding for chitinolytic enzymes, antibiotics and toxins, than the hypercellulolytic species (Kubicek et al. 2011). However, the complexity of biocontrol interactions between the pathogen, plant and antagonist makes it difficult to link the phenotype of a good BCA to a specific genetic origin. The high genetic similarity, but phenotypic differences, between *T. cf. atroviride* LU132 and LU140 presented a rare opportunity to compare nearly identical biocontrol fungi in order to study biocontrol-specific gene variants.

The aim of this research was to search for genetic differences (SNPs) between LU132 and LU140 on a whole genome scale to enable a strain-specific DNA-based distinction. We further investigated whether the genetic differences influence the distinct phenotypes. Initially, LU132 and LU140 phenotypes were compared directly to each other to confirm their individuality, to describe and quantify the different characteristics and to predict what target genes might be important. Then the whole genomes of LU132 and LU140 were sequenced to identify SNPs and an LU132-specific molecular marker was designed. Target genes whose function might be affected by the SNPs were identified and the effects of changes in target genes on the phenotypes of LU132 and LU140 were examined by gene deletion experiments.

Materials and Methods

Fungal strains

We refer to LU132 and LU140 as *T. cf. atroviride* because a recent five gene phylogeny of *Trichoderma* spp. from New Zealand (*Tef1α*, *ACLA1*, *Calm1*, *LAS1* and *RPB2*) resulted in the definition of this new species, closely related to *T. atroviride* (Braithwaite M et al., unpublished).

Trichoderma atroviride strain IMI206040 was obtained from Alfredo Herrera-Estrella, (Langebio, Mexico) and *T. cf. atroviride* strains LU132 and LU140 from the Lincoln University Culture Collection (LUCC, New Zealand), where single spore isolations had been stored at -80°C since the original strain isolations. *Trichoderma* inocula were prepared by growing the strains on Potato Dextrose Agar (PDA; Difco) at 25°C with constant light for 8 d. The conidia were then suspended in water, filtered through two layers of Miracloth (Calbiochem®); the conidial suspension was adjusted to 1 X 10^9 conidia per mL and aliquots stored in 25% glycerol at -80°C. Fresh cultures of *Botrytis cinerea* (BC106) and *Sclerotium cepivorum* (LU360) were obtained from the LUCC, sub-cultured onto PDA and incubated at 20°C with constant light for 14 d. The resulting sclerotia were stored in 25% glycerol at -80°C. Agar plugs of *Pythium ultimum* spp. were sub-cultured onto PDA containing ampicillin, chloramphenicol and streptomycin (50, 100 and 50 µg/mL respectively) and incubated at 20°C in complete darkness. Agar plugs were stored in 25% glycerol at 4°C.

Growth Rate and Conidiation

Radial mycelial growth rates and colony morphologies of LU132 and LU140 were determined on standard 90 mm Petri dishes containing either buffered or un-buffered media in the pH range

2.4 to 6.0. The media were PDA (Difco); Malt Extract Agar (MEA, 30 g Malt extract (Difco), 5 g Peptone (Difco) 15 g agar per L water); or Minimal Medium agar with 0.2% glucose (MMA, (Carsolio et al. 1994; Steyaert et al. 2004)). Where indicated, the pH was adjusted as described previously (McIlvaine 1921; Steyaert, Weld & Stewart 2010) (Table S1). Agar plates were inoculated centrally with 2 µL conidial suspensions or with agar plugs containing one colony derived from a single conidium. Plates were incubated at 25°C in total darkness or with constant light for up to 7 d. Conidial yield was determined for one treatment in Exp. 3 (Holder & Keyhani 2005). The experimental design is outlined in Table S1.

Dual Culture Assay with Plant Pathogens

The plant pathogens *Sclerotium cepivorum* (ascomycete), *Botrytis cinerea* (ascomycete) and *Pythium ultimum* (oomycete) were selected to study the antagonistic activity of LU132 and LU140 on dual culture plates using methods described elsewhere (McLean 1996). Five replicate plates were inoculated with 5 mm mycelial plugs from 3 d old cultures of the pathogens and *Trichoderma* test strains, 60 mm apart from each other.

Metabolic Profiling

Phenotype MicroArrays™ for Filamentous Fungi (Biolog FF, Biolog Inc., Hayward, CA) were used to compare metabolic profiles of LU132 and LU140, utilising 95 single compounds. Assimilation of compounds was reflected by mycelial growth and quantified by measuring the optical density (OD) in the wells at 750 nm, the wavelength at which hyaline mycelium has its maximum absorbance. To quantify catabolism, the wells contain a tetrazolium dye that turns into a purple insoluble precipitate when reduced due to mitochondrial activity. This was measured at the maximum absorbance of the reduced tetrazolium salt of 490 nm (Bochner, Gadzinski &

Panomitros 2001). Conidia of LU132 and LU140 were generated on PDA plates at 25°C in constant light. The FF plate procedure was carried out essentially as per manufacturer's instructions and as described elsewhere (Bochner, Gadzinski & Panomitros 2001) with modifications. Three replicate plates with separately prepared inocula were incubated at 25°C under constant light. Cluster analysis was carried out as described elsewhere (Druzhinina et al. 2006). Absorbance readings over 2.0 and wells that contained conidia were excluded from the absorbance analysis as they would skew the data. In addition to absorbance measurements, conidiation was assessed using a scoring system from 0 to 5 (Friedl, Kubicek & Druzhinina 2008).

Genome Sequencing

Genomic DNA (gDNA) of LU132 and LU140 were prepared from mycelia using the Gentra® Puregene™ Tissue Kit (Qiagen) and further purified using the DNeasy® Plant Mini Kit (Qiagen) as per manufacturers' instructions. DNA samples were sequenced with an Illumina GAII (Solexa) machine by the Massey University Genome Service, New Zealand. The data were analysed to identify Single Nucleotide Polymorphisms (SNPs) by mapping LU132 and LU140 reads against the unmasked genome sequence of the closest available reference genome of *T. atroviride* strain IMI206040 (Genome build v. 2.0, May 2010, Joint Genome Institute, http://genome.jgi.doe.gov/Triat2/Triat2.home.html) using the Burrows-Wheeler transform (BWT) algorithm implemented in the program bwa v.0.5.5 (Cox, Peterson & Biggs 2010; Li & Durbin 2009). Polymorphisms were identified using *SAMtools* (Li et al. 2009) and custom in-house software. At least 8 reads confirming a mutant allele were required to call a SNP at any given position. Unmapped reads were assembled *de novo* using *ABySS* (version 1.2.0) and *Phrap*

(version 1.090518) and reads of one strain were mapped against the other's assembled contigs (*bwa* version 0.5.8).

SNP Confirmation

Regions that encompassed putative SNPs were PCR-amplified from gDNA of IMI206040, LU132 and LU140, then sequenced (primers are shown in Table S2). All PCR amplifications were performed in a Bio-Rad Icycler™ (Bio-Rad Laboratories) using the Expand long template PCR system (Roche) according to manufacturer's instructions. SNP-containing genes (target genes) were identified using the reference genome annotation (IMI206040). The SNP-containing sequences were subjected to BLAST analysis on GenBank® to identify putative gene homologs in other organisms and possible biological functions.

Protein Structure Predictions

Deduced amino acid sequences that were altered as a consequence of a SNP were analysed to predict protein structures functional motifs using PSIPRED v3.3 (http://bioinf.cs.ucl.ac.uk/psipred/), SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/), TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP/), NetSurfP 1.1 (http://www.cbs.dtu.dk/services/NetSurfP/), Phyre2 (http://www.sbg.bio.ic.ac.uk/servers/phyre2/html/page.cgi?id=index), SAM-T08 (http://compbio.soe.ucsc.edu/SAM_T08/T08-query.html) and ELM (http://elm.eu.org/search/).

Development and validation of a strain-specific molecular marker for LU132

The molecular test for the *T. cf. atroviride* biocontrol strain LU132 was developed based on PCR amplification of the genomic region containing an LU132-specific SNP, followed by restriction

fragment length polymorphism (RFLP) analysis of the amplified fragment. The PCR amplification from gDNA of LU132, LU140, IMI206040 and 39 other *T. atroviride* and *T. cf. atroviride* strains from New Zealand, Europe and Asia was carried out as described above. The sequences from LU132, LU140 and IMI206040 were subjected to *in silico* restriction analysis using DNAMAN (v. 4.0a, Lynnon Corporation, Quebec, Canada) to identify SNP-specific restriction sites. The PCR fragments were digested to completion with the identified enzyme according to manufacturer's instructions. Digested fragments were size fractionated by 2% agarose TAE gel electrophoresis for confirmation of strain-specific banding patterns.

Relative Expression

The expression of identified target genes, relative to the expression of the reference gene encoding translation elongation factor 1α (*tef1α*) (Bustin et al. 2009; Seidl, Druzhinina & Kubicek 2006), was studied under standard and specific inducing conditions. The standard culturing conditions were as follows: 100 mL PDB (Difco) were inoculated with 5 µL of LU132 and LU140 conidial suspensions in 250 mL conical flasks. The cultures were incubated at 25°C in constant light with shaking at 200 rpm for 3 d. To induce a mycoparasitic response, the pH of the PDB was adjusted to 4.75 with HCl (Moreno-Mateos et al. 2007) and 4 h before the end of incubation, N-acetyl-D-glucosamine (NAG) (Sigma-Aldrich®) was added to the culture to a final concentration of 0.5% (Mach et al. 1999). NAG is known to trigger the expression of target genes *nag*1 and *nag*2 and to induce biocontrol mechanisms (Brunner et al. 2003; Mach et al. 1999; Peterbauer et al. 1996; Ramot et al. 2004; Zeilinger et al. 1999). Mycelia from three replicates were harvested by filtration and snap frozen in liquid nitrogen. Total RNA was prepared using the Plant Total RNA Extraction Miniprep System (Viogene BioTek Corp.) and the RNA samples were treated with DNase using Turbo DNA-*free*™ Kit (Ambion®), as per

manufacturers' instructions. Intron-spanning primers were designed to amplify around 100 bp of transcript sequence (Table S2). Reverse Transcription quantitative real-time PCR amplification (RT-qPCR) and cycling conditions were based on the protocol from Holyoake et al. (2008), except using 10 ng RNA as template. All PCR reactions were done in duplicate and the whole experiment was repeated. The normalised gene expression data were expressed as $\Delta Cq = Cq\ (tg) - Cq\ (tef1\alpha)$ (Bustin et al. 2009).

Gene Transcript Sequencing

The mRNA transcripts of SNP-harbouring target genes, obtained from total RNA prepared as described above, were reverse transcribed into complementary DNA (cDNA) and sequenced using primers described in Table S2. First strand cDNA synthesis was carried out using SuperScript™ III Reverse Transcriptase (Invitrogen™), as per manufacturer's instructions, using the 3'UTR (or reverse) primers. Subsequent PCR amplifications were performed as above.

Mutational Analyses

Primers used for the construct and vector creation are listed in Table S2. The *serf* knock-out construct (SKO) contained the hygromycin B phosphotransferase gene (*hph*) under the control of the pyruvate kinase gene promoter (*pki*) (Mach, Schindler & Kubicek 1994), embedded in approximately 1 kb of each of the genomic 5' and 3' flanking sequences of *serf*. The knockout construct was then cloned into the binary vector pYT6 to create the new vector pSKO. The pSKO plasmid was electroporated to *A. tumefaciens* EHA105 cells using a MicroPulser™ Electroporator (Bio-Rad Laboratories) as per manufacturer's instructions. Transformation of LU132 and LU140 was based on standard protocols (de Groot et al. 1998; Zwiers & De Waard 2001) with modifications. pSKO-containing *A. tumefaciens* were selectively grown in media

with 25 µg/mL kanamycin and 25 µg/ml rifampicin. Co-cultivation of 3 d old *Trichoderma* conidia and *Agrobacterium* colonies was carried out on sterile cellophane discs on IMAS agar, without overlay, at 23°C. The cellophane disks were cut into three pieces, transferred onto separate PDA plates (Difco), containing 200 µg/mL hygromycin B and 300 µg/mL timentin (GlaxoSmithKline Plc.), and incubated at 25°C for up to 4 d in complete darkness. Transformants were transferred to fresh PDA (Difco) containing antibiotics as above and purified via single-spore isolation. Homologous recombination at the *serf* locus was confirmed by PCR (Table S2) and Southern hybridisation (Sambrook, Fritsch & Maniatis 1989). Mutants were characterised for growth, conidiation and metabolic activity as described above.

Statistical Analyses

All data were analysed using GenStat (v. 14, VSN International Ltd., Hemel Hempstead, UK). Unless mentioned otherwise, data were analysed using the General Analysis of Variance method. The least significant differences of means (l.s.d.) and multiple comparisons (using Fisher's Unprotected LSD algorithm) were determined at a significance level of 5% ($P<0.05$).

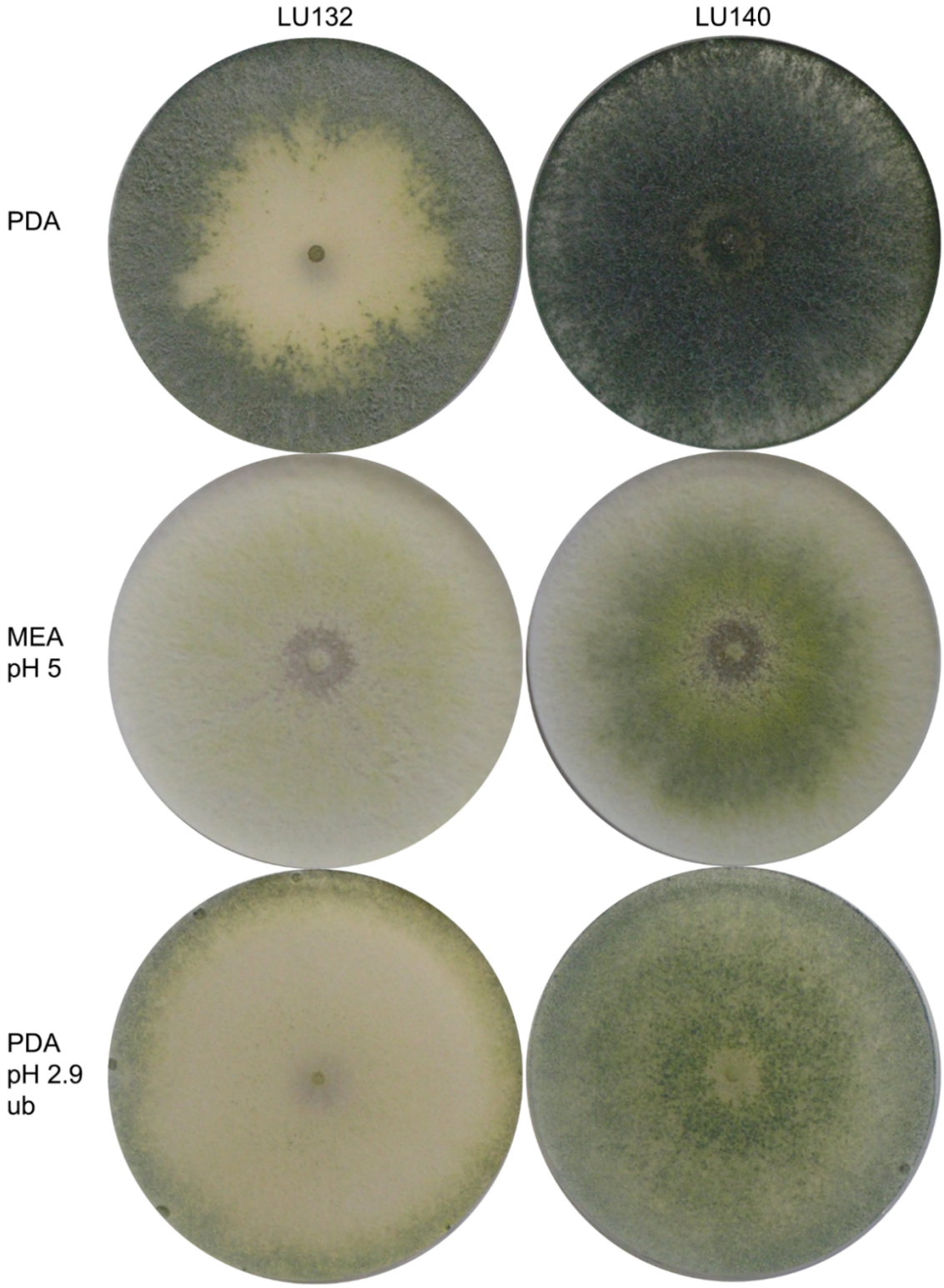
Results

Phenotype Comparison

Morphological Analysis. Morphological comparisons were done to assess the extent of phenotypic differences between the *T. cf. atroviride* strains LU132 and LU140. LU132 grew consistently significantly faster than strain LU140 in all treatments ($P<0.05$) (Table S3). For example, in experiment 2 (PDA, pH 5, unbuffered, dark) the average growth rates for LU132 and LU140 were 24.8 ± 0.45 mm/d and 21.0 ± 0.61 mm/d. The pH range that resulted in the fastest

growth was wider for LU132 than for LU140 (Table S3). Colonies incubated with constant light also differed in the distribution and density of conidia (Fig. 1); whereas LU140 produced conidia all over the plate on PDA, LU132 conidia were more concentrated at the edges. This distribution effect was most apparent on unbuffered PDA with the lowest pH (2.9), on which LU140 produced 3.6 times more conidia than LU132 (1.8×10^9 and 0.5×10^9 conidia per plate on average respectively, $P<0.05$).

Dual Culture with Plant Pathogens. LU132 and LU140 showed significant differences in biocontrol activity against pathogenic fungi. LU132 inhibited the growth of the plant pathogens *Botrytis cinerea*, *Sclerotium cepivorum* and *Pythium ultimum* significantly better than LU140 ($P<0.05$) (Table S4). As soon as the pathogen colonies met the *T. cf. atroviride* colonies, they stopped growing. All pathogens were completely overgrown by both *T. cf. atroviride* strains after 12 d. There were no interaction zones between pathogens and antagonists. On the pathogen-only control plates with *S. cepivorum* and *B. cinerea*, sclerotia were observed, whereas no sclerotia were produced on the dual culture plates with *T. cf. atroviride*.



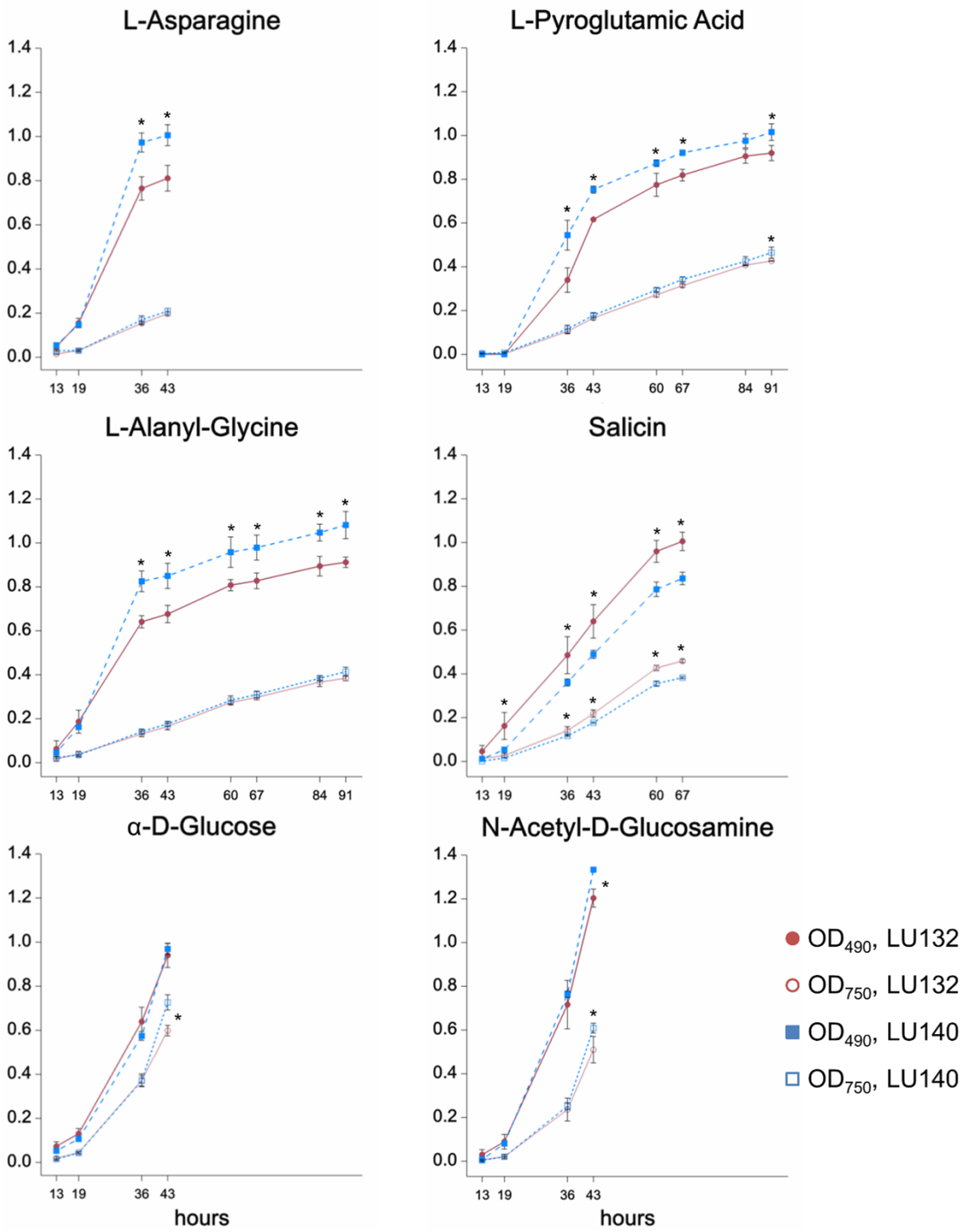
269

270 **Fig. 1 Colony appearance/ conidiation patterns of LU132 and LU140.**

LU132 conidiated less in the middle of the colony and more around the edges while LU140 conidiated over the whole plate on PDA. The most obvious conidiation difference between LU132 and LU140 was found on pH 2.9 PDA, where LU132 only conidiated around the edge of the plate (ub = unbuffered).

Metabolic Profiling. The phenotype microarray assay (Biolog FF) assessed the growth, metabolism and conidiation of LU132 and LU140 in the presence of 95 single compounds on 96 well plates. A cluster analysis was done to group compounds that were metabolised by each strain at high, medium or low rates; based on absorbance measurements at OD_{490} and OD_{750} (representing catabolism and mycelial growth respectively). The six compounds for which the LU132 and LU140 strains differed most in their growth and/or metabolism are shown in Fig. 2. The amount of conidia in the wells was scored from 0 to 5 (Friedl, Kubicek & Druzhinina 2008). The average conidiation score on all 95 compounds was significantly bigger for LU140 than for LU132 at each time point studied (60, 84 and 108 h). LU140 also produced conidia in more wells than LU132 throughout the experiment (Table S5). Apart from α-D-glucose, the compounds that were differentially metabolised by LU132 and LU140 (Fig. 2) did not result in altered conidiation. On α-D-glucose LU140 had a conidial mat covering the whole well at 60 h and LU132 at 84 h. The biggest conidiation score difference of 3 was found on D-galactose, D-raffinose, α-methyl-D-galactoside and stachyose at the end of the experiment (108 h).

The metabolism of the glucoside salicin was significantly better in LU132; however, the salicin metabolism could not be associated with a specific pathway. N-acetyl-D-glucosamine (NAG) was the only compound that induced differential metabolism in LU132 and LU140 and that could be associated with pathways implicated in biocontrol (Brunner et al. 2003; Lopez-Mondejar et al. 2009; Seidl, Druzhinina & Kubicek 2006; Zeilinger et al. 1999). *Trichoderma* spp. have two genes encoding N-acetyl-β-D-glucosaminidases, *nag1* and *nag2* (Mach et al. 1999; Peterbauer et al. 1996; Ramot et al. 2004), which were selected as target genes for further analyses.



296

297 **Fig. 2 Compounds that were differentially metabolised by LU132 and LU140.**
298 Values with * are significantly different (P<0.05) between LU132 and LU140 at the respective wavelengths and time
299 points. Time points at which conidia were present in the wells were not analysed.

The results clearly identified LU132 and LU140 as two individuals. The strains exhibited distinct growth, conidiation and metabolism. LU132's better pathogen control could be attributed to its faster growth.

Genome Comparison

Whole genome sequences of LU132 and LU140 were mapped to the closest available reference genome of *T. atroviride* IMI206040 (Genome build v. 2.0, May 2010, Joint Genome Institute, http://genome.jgi.doe.gov/Triat2/Triat2.home.html) to identify single nucleotide polymorphisms (SNPs). The size of the reference genome is 36.1 Mb. It has 29 contigs and 11,863 gene models. Given the coverage threshold (the SNP calling requirement of at least 8 reads per allele) a read coverage of 87% and 73.5% was achieved for LU132 and LU140 respectively, with an average of 35 and 15 reads per nucleotide position for LU132 and LU140 respectively. Both *T. cf. atroviride* strains were found to have a sequence divergence rate of 2.5% compared to the reference strain *T. atroviride* IMI206040.

All identified 659 putative SNPs were validated empirically. By visualising the sequencing reads and the reference genome in IGV (v.1.5.65, Broad Institute), false positives were detected when putative SNPs occurred at the end of a read where the sequencing accuracy was low and deletions or insertions were introduced incorrectly by the mapping program. Some putative SNPs occurred within microsatellites or homopolymers and were therefore unreliable. Sanger sequencing of the SNP-encompassing regions confirmed two SNPs, both transition polymorphisms: SNP1, specific to LU132 (IMI206040 v. 2.0, contig_21:1,174,857, C→T) and SNP2, specific to LU140 (IMI206040 v. 2.0, contig_15:842,707, A→G). The remaining 657 putative SNPs were false positives, and there were no other SNPs in non-coding regions.

De novo assembly of unmapped reads was done to determine whether LU132 and LU140 differed in genome content, such as insertions or deletions that could account for their phenotypic differences. The assembly revealed that both LU132 and LU140 contained DNA sequences that were not present in the reference genome but none were specific for one strain. The contigs containing assembled sequences that mapped to LU132/LU140, but not the reference genome, were small and either matched to the φX174 genome (which is used as control DNA in the Illumina sequencing process) or had high similarities to human and bacterial genes, suggestive of contamination.

Development and validation of a strain-specific molecular marker for LU132

A PCR to amplify a 660 bp region, which encompassed the LU132-specific SNP1, was carried out with LU132, LU140, IMI206040 and 39 other *T. atroviride* and *T. cf. atroviride* strains from New Zealand, Europe and Asia. Sequence analysis of the PCR products from LU132, LU140 and IMI206040 showed that LU132 contained three *Hph*I restriction sites while the products of LU140 and IMI206040 contained two. The additional *Hph*I site in LU132 was conferred by the LU132-specific SNP1 (Table 1).

Table 1 LU132-specific *Hph*I restriction site created by SNP1.

	Sequence (5' → 3')
IMI204060	TAAAGGCGAAGGTAGAAGCGAAAAT
LU132	TAAAGGTGAAGGTAGAAGCGAAAAT
LU140	TAAAGGCGAAGGTAGAAGCGAAAAT
***Hph*I restriction sequence**	....GGTGANNNNNNNN/.....

SNP1 (underlined) generated a third *Hph*I restriction site in the PCR fragment that was only present in LU132 (at 350 bp).

All PCR products were digested to completion with *Hph*I (New England Biolabs). Fig. 3 shows the banding patterns for LU132, LU140 and IMI206040. The SNP1-containing region could also

be amplified from five *T. atroviride* strains from Europe and Asia and three *T. atroviride* strains from New Zealand but the digest banding pattern resembled that of LU140 and IMI206040. Of 34 tested *T. cf. atroviride* strains from New Zealand, five could not be amplified and the remaining showed the LU140 and IMI206040 banding pattern (see strain identities and RFLP results in Table S6). These results confirmed the strain-specificity of the developed LU132-specific molecular RFLP marker.

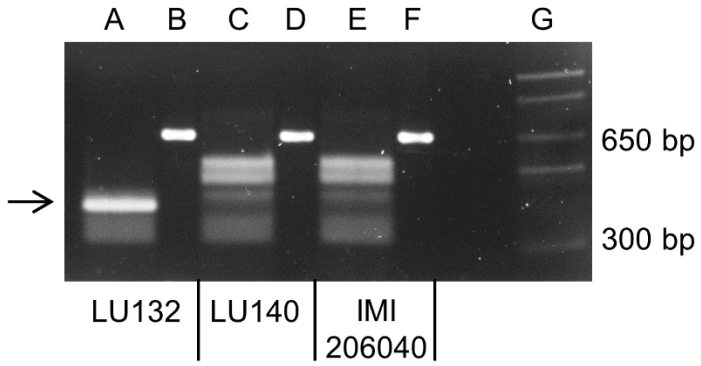


Fig. 3 Electrophoretic separation of *Hph*I digested PCR fragments.
Lanes B, D and F represent the undigested 660 bp PCR fragments from LU132, LU140 and IMI204060, respectively. Lanes A, C and E represent the *Hph*I digested PCR fragments for LU132, LU140 and IMI204060, respectively. The arrow indicates the 350 bp restriction fragment that was only found in LU132. Size standard was 1 Kb Plus DNA Ladder™ (Invitrogen™) in lane G.

Linking Phenotype and Genotype

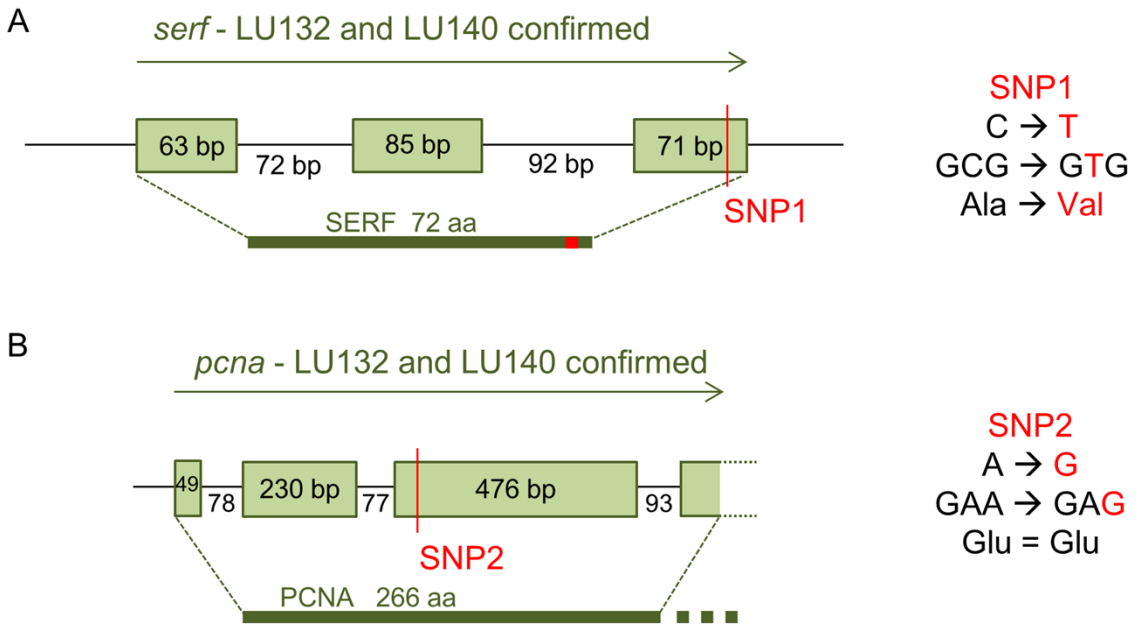
The high genetic similarity in combination with the phenotypical distinctness of LU132 and LU140 led us to investigate functional relationships between the SNPs and phenotypic characteristics.

Target Genes Directly Associated with SNPs. The loci with which the two SNPs were associated were determined by analysis of their positions in the reference genome. SNP1 was

363 located in the coding region of a predicted gene (protein ID 306899) with the putative conserved
364 domain 4F5 (pfam04419) and similarities to the predicted small EDRK-rich factor (*serf*) that is
365 conserved in fungi, protozoa and animals. 4F5 protein family members are short proteins with
366 unknown function (Marchler-Bauer et al. 2011). Because SNP1 was a non-synonymous change,
367 it altered the deduced amino acid sequence of the SERF protein from alanine to valine at position
368 64 in LU132 (Fig. 4 - A). The amino acid change had the potential to change the protein
369 structure. Phyre2 and SAM-T08 predicted four helices for the LU132 and three helices for the
370 LU140 protein (Fig. S1) (Karplus 2009; Karplus et al. 2005; Kelley & Sternberg 2009). ELM
371 identified a mitogen-activated protein kinase docking motif (DOC_MAPK_1) with the amino
372 acid pattern KKRxxKxxxxLxV created by SNP1 in LU132 but absent in LU140 (Dinkel et al.
373 2013) that could potentially change the function of SERF.

374 SNP2 was located in the third exon of a gene (protein ID 212486) containing a putative
375 conserved vezatin superfamily domain (pfam12632). Vezatin is a peroxisome transmembrane
376 receptor that is involved in membrane-membrane and cell-cell adhesions (Marchler-Bauer et al.
377 2011). The protein had low level similarities (50-60%) to proliferating cell nuclear antigen
378 (*pcna*) from *Beauveria bassiana* (XP_008593672), *Colletotrichum orbiculare* (ENH88404) and
379 *Togninia minima* (XP_007918421), but its function in these organisms was not known. SNP2
380 was located in the 3rd exon of *pcna* but was a synonymous change that did not alter the deduced
381 amino acid sequence for the PCNA protein (Fig. 4 - B).

382 The identified two SNP-related target genes (*serf* and *pcna*) were selected for further analyses to
383 determine whether their gene expression or functions were likely to be affected by the SNPs.



384

385 **Fig. 4 Confirmed annotation of SNP-containing *serf* and *pcna*.**
386 The gene models were corrected compared to those of the reference based on cDNA sequences. (A) The *serf* gene
387 was 21 bp longer than predicted by the IMI206040 annotation, contained three exons and SNP1 resided at bp
388 position 191 in the 3rd exon of *serf*. SNP1 changed the amino acid sequence of SERF in LU132 compared to other
389 strains. (B) The sequenced part of the *pcna* transcript was 3 bp shorter than the IMI206040 annotation as a result of
390 different intron-exon boundaries. SNP2 was a synonymous change in *pcna* in LU140 compared to other strains. aa =
391 amino acid.
392

393 **Relative Expression of Target Genes.** The relative expression of the two SNP-related target
394 genes (*serf* and *pcna*) and the two metabolism-related target genes (genes encoding N-acetyl-β-
395 D-glucosaminidases *nag1* and *nag2*) was assessed under standard culturing and mycoparasitism-
396 inducing conditions. Gene expression, normalised to the expression of the reference gene (*tef1α*),
397 were similar in LU132 and LU140 (Table S7). Induction with N-acetyl-D-glucosamine (NAG)
398 resulted in significantly higher relative expression than without NAG of *nag1*, *nag2* and *pcna* in
399 LU132 and of *nag2* and *pcna* in LU140**.** The results of this experiment showed that the different
400 phenotypes of LU132 and LU140 were not caused by differential expression of the two SNP-
401 harbouring genes or the two NAGase-encoding genes.

Functional Analysis of *serf*. Functional analysis of the *serf* gene was carried out by gene replacement in LU132 and LU140 with the aim of generating identical mutant strains. Three monokaryotic mitotically stable hygromycin B resistant Δ*serf* transformants for LU132 (mutants A, B and C) and LU140 (mutants D, E and F) were generated. PCR and Southern analysis confirmed that both LU132 WT and LU140 WT contained a single copy of *serf* at the predicted position in the genome and that *serf* was replaced by a single copy of the knock-out construct in all mutants (Figs. S2 and S3). The average growth rates (grand mean) of the LU140 Δ*serf* mutants were not significantly different from LU140 WT ($P<0.05$) (Table 2). The average growth rates of the LU132 Δ*serf* mutants varied significantly from the LU132 WT in all treatments. Two LU132 Δ*serf* mutants (B and C) displayed a growth rate reduction of 3% while one mutant (A) had a reduction of 9% compared to the LU132 WT. However, even the slowest growing LU132 mutant (A) grew on average significantly faster than LU140 WT.

Table 2 Average radial growth rates (mm/d) of WT and Δ*serf* mutants.

Strain	Actual pH 5.0 *		Actual pH 2.7 *	Grand mean †
	Dark	Light	Light	
LU132 WT	21.77 a	21.04 a	16.39 a	19.73 a
LU132 A	21.15 b	18.68 c	14.28 c	18.04 c
LU132 B	21.27 b	20.20 b	15.90 b	19.12 b
LU132 C	21.23 b	20.12 b	15.89 b	19.08 b
LU140 WT	20.54 c	18.31 cd	13.27 de	17.37 d
LU140 D	20.57 c	17.58 e	12.99 e	17.05 d
LU140 E	20.62 c	17.91 de	13.39 d	17.31 d
LU140 F	20.70 c	18.03 d	13.18 de	17.30 d
l.s.d. ‡	0.219	0.404	0.337	0.337

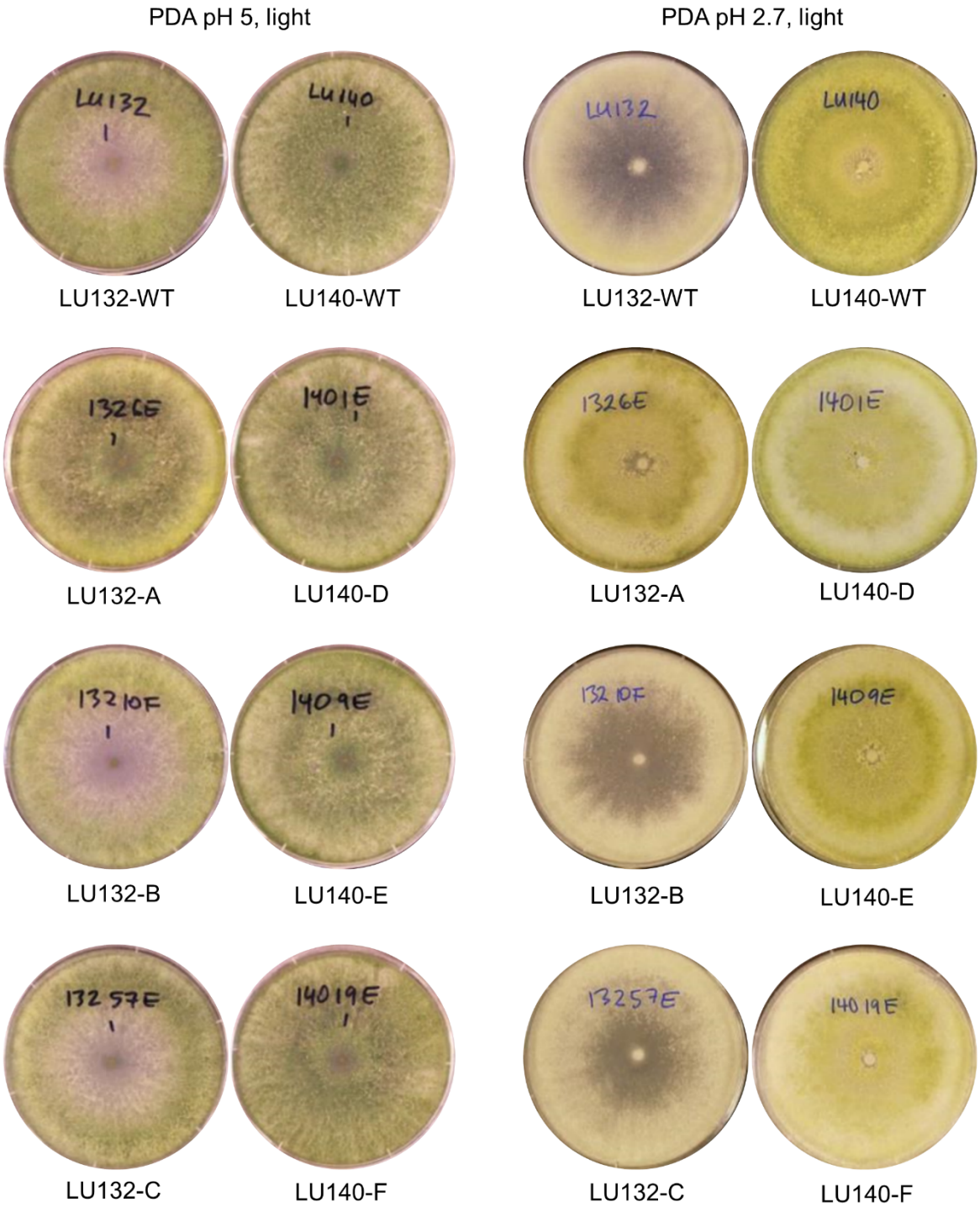
Growth rate data for LU132 and LU140 wild types (WT) and Δ*serf* mutants (A to F). Values are averages of two experiments with four replicates. Different letters within a column represent significantly different values ($P<0.05$). * The pH was adjusted to 5.0 and 2.4. Before inoculation, the actual pH of the plates was measured. † Grand mean is the average of the three conditions. ‡ The grand mean l.s.d. was determined using a split-plot design.

The colony appearances of the mutants that were incubated with constant light, are shown in Fig. 5. On PDA (pH 5) the conidia of LU140 and its mutants D, E and F and the LU132 mutant A covered the whole plate, while LU132 and its mutants B and C did not produce conidia in the

centre. On PDA pH 2.7, LU140 and its mutants D, E, F and the LU132 mutant A all produced yellow-green conidia while LU132 and its mutants B and C produced only immature white/light green conidia.

The phenotype microarray assay (Biolog FF) was used to group the mutants according to their metabolic profiles (Fig. S4). The cluster analysis of the OD_{750} data (mycelial growth) resulted in two main groups. One group contained LU132 WT and its Δ*serf* mutants B and C while the second group consisted of LU140 WT, all its mutants (D, E and F) and LU132 mutant A. The two groups separated at a similarity distance of 0.49 (a value of 1 represents complete similarity and 0 complete dissimilarity). Analysis of the conidiation resulted in the same two groups that separated at a similarity distance of 0.54. The OD_{490} data (catabolic activity) were not so clearly grouped. One strain (LU132 mutant C) was an outlier and the remaining strains were separated into two groups at a similarity distance of 0.67. One group contained LU132, its mutant B and the two LU140 mutants E and F, while the second group contained LU140, its mutant D and LU132 mutant A.

In summary these data show that the deletion of *serf* in LU132 and LU140 did not result in identical phenotypes. Despite the fact that SNP1 in *serf* was the only detectable functional genomic difference between LU132 and LU140, functional analysis with six independent Δ*serf* mutants suggested that this only partially accounted for the phenotypic differences.



442

443 **Fig. 5 Colony appearance/conidiation of mutants.**
444 LU132 mutants B and C always resembled the LU132 WT, while LU132 mutant A resembled the LU140 WT. The
445 conidiation characteristics of all LU140 mutants were similar to the 140 WT.

Discussion

The main aim of this study was to identify genetic differences between genetically highly similar *T. cf. atroviride* strains LU132 and LU140 by whole genome comparison. This research allowed the identification of two SNPs that distinguish the strains.

One SNP could be utilised for the successful development and validation of a molecular test for the commercial biocontrol strain *T. cf. atroviride* LU132. The marker screening technique is straightforward and affordable. The availability of the marker will enable the registration of LU132 biocontrol products internationally, thus opening them to additional markets. The molecular marker for LU132 will also enable more detailed research on this particular strain, as it will now be possible to identify this strain in the environment to study colonisation, competition and mycoparasitism.

The genomes of LU132 and LU140 were found to be nearly identical. Earlier molecular studies with the two strains indicated that there would only be a small number of genomic differences, but the identification of only 1 SNP per strain was very surprising. It has to be noted though that the inability to identify a SNP is not proof of absence and that SNPs in repetitive regions or in regions with missing coverage may have been missed in this exercise. However, *Trichoderma atroviride* contains only a small number of degenerate transposable elements (0.49% of the genome) and micro- or mini satellite DNA (0.94%) (Kubicek et al. 2011). The analysis of *de novo* assembled unmapped reads for LU132 and LU140 minimised the possibility that SNPs might have been missed because they were located in genomic regions with low coverage. In addition to that, the genomes of two other *T. cf. atroviride* strains from New Zealand were analysed at the same time, using the same methods, and were found to have on the order of

10,000 times more strain-specific SNPs than LU132 and LU140 (data not shown), confirming the appropriateness of the applied methods to identify SNPs. In fact, simple by-chance scrolling through these sequences in IGV enabled the identification of a SNP and a 1 bp insertion in these other strains (Figs. S5 and S6).

The revelation of the high sequence identity of LU132 and LU140 made it necessary to confirm the strains' individualities. The phenotype assays confirmed the strains to be individuals, exhibiting distinct growth rates, conidiation patterns and metabolism. The mode of action that makes LU132 a more successful BCA than LU140 under laboratory conditions appears to be its faster growth and pH adaptability. These attributes would make LU132 an especially successful competitor. Competition for nutrients has earlier been identified as one way in which LU132 controls *Botrytis cinerea* on strawberries (Card et al. 2009). The greater pathogen inhibition on dual culture plates by LU132 can be attributed to its faster growth alone. As no inhibition zones were formed between *Trichoderma* strains and pathogens, the inhibition was probably not caused by secreted antibiotics.

The metabolism and conidiation profiles of LU132 and LU140 could be distinguished using Phenotype Microarrays. N-acetyl-D-glucosamine (NAG) was one of the compounds that resulted in significantly different growth and catabolic activity of LU132 and LU140. NAG is a monomer of chitin, the main component of fungal cell walls. *Trichoderma* spp. contain two NAG-cleaving enzymes (N-acetyl-β-D-glucosaminidase 1 and 2) encoded by *nag*1 and *nag*2 (Mach et al. 1999; Peterbauer et al. 1996; Ramot et al. 2004). These enzymes are not only involved in chitin degradation of fungal cell walls (Brunner et al. 2003) and in mycoparasitism (Zeilinger et al. 1999) but also in mycelial growth on chitin (Lopez-Mondejar et al. 2009). However, LU132 and LU140 expressed these two genes at similar levels. By comparing NAGases activity to transcript

levels of *nag1* and *nag2* in *T. atroviride* P1 on Biolog FF plates, Seidl, Druzhinina & Kubicek (2006) found that both genes are regulated at the transcriptional level. This suggests that the similar expression levels of *nag1* and *nag2* in LU132 and LU140 would lead to similar NAGase activities in both strains and that their different growth rates are not likely to be affected by NAG metabolism. The difference in the biocontrol abilities of the two strains appears therefore to be more complex than originally assumed.

The two identified SNPs did not alter the expression of the associated target genes; however, SNP1 in *serf* was a non-synonymous change. Bioinformatics analyses predicted protein structure changes due to the amino acid change in SERF from LU132, which could have impacts on the protein function. A single change in the amino acid sequence could therefore potentially have effects on multiple processes (pleiotropy) and might lead to the altered phenotype of LU132, compared to LU140, in multiple complex ways. The more efficient biocontrol activity of LU132, compared to LU140, might therefore be a result of multiple changes caused by SNP1 rather than a result of SNP1 directly. This hypothesis correlates with the finding that a number of metabolic differences were found between LU132 and LU140 but the main biocontrol-related phenotypic difference was found in the growth rates.

To study the involvement of a particular gene in the development of a mutant phenotype, the mutation is usually introduced into the wild type to achieve targeted gene disruption, and the mutated gene then replaced with the wild type gene in the mutant. These processes include the introduction of selection markers that can also have phenotypic effects. For the analysis of a particular gene function this is generally not a problem. In contrast to this, our aim was to create genomically identical strains to see if their phenotypes would be identical. The only current

513 option to achieve this was to remove the SNP-containing gene in both strains and to replace it
514 with an identical knockout cassette, including an identical selection marker.

515 The deletion of *serf* in LU132 and LU140 did not result in identical mutant phenotypes, implying
516 that SNP1 was not the only reason for the phenotypic differences between LU132 and LU140.
517 Even though the change in *serf* was the only apparent functional genetic change, it did not
518 entirely account for the phenotypic differences. This result indicates that multiple factors must be
519 involved in the development of the dissimilar phenotypes. As the expected phenotypes (LU132
520 and LU140 being identical) could not be generated by deletion of *serf*, a complementation was
521 not attempted. Reinstating the gene might have confirmed the involvement of *serf* in the
522 development of the observed mutant phenotypes; however, it would not have provided more
523 information about the additional factors responsible for the different wild type phenotypes.

524 Epigenetic modification could contribute to development of the distinct phenotypes of LU132
525 and LU140. Interactions between pathogen, plant and the biocontrol agent are very complex and
526 are therefore difficult to associate with a genetic origin. This is attributed to multiple genetic
527 causes but also to epigenetic modifications, such as DNA methylation, histone modifications and
528 RNA interference. It has been shown for instance that the DNA methylation states of three
529 dimorphic fungi (*M. rouxii, Y. lipolytica* and *U. maydis*) differ between their mycelial and yeast
530 stages (Reyna-Lopez, Simpson & Ruiz-Herrera 1997), that chromatin-remodelling and DNA
531 methylation affect gene expression in *Neurospora* (Belden et al. 2011), that histone
532 modifications lead to transcriptional activation or repression in many fungi (Aghcheh & Kubicek
533 2015) and that non-coding micro-RNA like RNAs (milRNAs) could be potential regulators of
534 cellulase production or fungal growth in *T. reesei* (Kang et al. 2013). Genome comparisons
535 showed that *Trichoderma atroviride*, *T. virens* and *T. reesei* contain genes or homologs to the

536 genes known to be involved in epigenetic regulation of gene expression (Schmoll et al. 2016) but
537 the actual functionality of these processes have not yet been studied in *Trichoderma*.

538 Another reason for the different phenotypes of LU132 and LU140 could be that one or both
539 strains naturally contained an extra-chromosomal element, such as a mycovirus or a plasmid.
540 Mycoviruses are widespread in fungi (Ghabrial & Suzuki 2009) where they can affect virulence
541 and cause debilitation (McCabe, Pfeiffer & Van Alfen 1999; Preisig et al. 2000). Although the
542 function has not yet been studied, single-stranded RNA elements could be identified in one other
543 *T. cf. atroviride* strain from New Zealand but no extra-chromosomal elements have been found
544 in LU132 and LU140 (Lange 2015). To our knowledge, only double-stranded RNA (dsRNA)
545 elements have been reported for other *Trichoderma* species so far and their impact on the
546 phenotype is equally unknown (Antal et al. 2005a; Antal et al. 2005b; Jom-in & Akarapisan
547 2009). Circular plasmids have been identified in mitochondria of *Trichoderma viride*, *T.*
548 *harzianum* and *T. virens* (Antal et al. 2002; Meyer 1991). The plasmids appeared to have no
549 influence on the strain's morphology; however, it is known that a plasmid in *Neurospora* species
550 is responsible for senescence (Griffiths, Kraus & Bertrand 1986).

551 Epigenetic modification or extra-chromosomal elements could also explain the phenotypic
552 differences between the three LU132 Δ*serf* mutants. The phenotypic differences were intriguing,
553 as all mutants were confirmed to have identical sequences in the manipulated genomic region
554 and all contained exactly one copy of the knock-out cassette. LU132 might naturally harbour an
555 extra-chromosomal element, that only got lost from mutant A during the mutagenesis, with the
556 result that A's phenotype was more similar to LU140's phenotype. Epigenetic modifications
557 would not necessarily have been changed by the mutation process and could therefore have led
558 to the observed variable results.

Conclusion

The main goal to identify genetic differences between genetically highly similar *T. cf. atroviride* strains LU132 and LU140 by whole genome comparison was successful. A strain-specific molecular marker for *T. cf. atroviride* LU132 was successfully designed and validated. Further analysis of the polymorphic gene, containing the non-synonymous SNP1, highlighted that even apparently genetically identical strains (Δ*serf* mutants) can have different phenotypes and that natural strains with different phenotypes (LU132 and LU140) can be genetically extremely similar. Even though whole genome sequencing is an important tool for fundamental and applied research, the definition of an individual is not exclusively defined by its DNA sequence. In the microbiological context, this creates limitations for molecular strain typing to identify efficient biocontrol strains or pathogens and implies that these techniques should not be applied in isolation but should always be combined with phenotypic characterisation.

Data availability

Strains are available upon request. Gene sequence data are available at GenBank®, accession numbers: KR812141.1 (*serf* for LU132), KR812142.1 (*serf* for LU140), KR812145.1 (*pcna* for LU132), KR812146.1 (*pcna* for LU140) and EHK42777.1 (*tef1α*). Illumina raw data for LU132 and LU140 are available at the NCBI sequence read archive, accession SRP070858.

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