

Molecular markers for quantification of bioactive fungal strains associated with *Pinus radiata*

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Abstract *Trichoderma cf. atroviride* is an endophytic soil fungus, which has been the target of much research due to its plant growth promoting effects and use as a biocontrol agent. For specific bioactive strains of this species to be used with long-lived forest trees such as *Pinus radiata*, they must persist over the long term in the host root system. To investigate the persistence of specific *T. cf. atroviride* strains in the roots of *P. radiata*, unique regions were identified in the genomes of strains that were more than 99.7% identical by using next generation sequencing. Based on these unique regions, probe-based, strain-specific quantitative-PCR assays were developed. The assays can be used to test strain persistence in nursery and forest-grown seedlings inoculated with mixtures of *T. cf. atroviride* strains, as well as in laboratory experiments to determine the effect of these strains on plant metabolism and defence.

Keywords *Trichoderma*, *Pinus radiata*, quantitative PCR, next-generation sequencing, bioinoculants

INTRODUCTION

Trichoderma spp. are common soil fungi that have become targets of research in bioprotection because strains of some species exhibit biocontrol activity, with some marketed as biopesticides (Harman 2011). Protection against pathogens in hosts inoculated with *Trichoderma* can result from direct antagonism and antibiosis within the rhizosphere, or indirect protection via the enhancement of host resistance to pathogen attack (Harman 2011). Certain species of *Trichoderma* have also been shown to promote plant growth.

For example, both growth and health benefits were shown for *Pinus radiata* seedlings treated with *T. hamatum* (Hohmann et al. 2011).

A decisive factor before deploying specific strains of *Trichoderma* spp. in forests is whether they are able to persist and become established in the soil and roots, potentially providing long-term protection and possibility other benefits. Because *Trichoderma* spp. are ubiquitous and commonly found in soil, to answer this question it is first necessary to develop

molecular tools that can identify and quantify specific strains shown to have a bio-protective effect amongst the *Trichoderma* population.

The aim of this study was to develop sensitive and specific probe-based, real-time PCR assays for two strains of *Trichoderma cf. atroviride* (R33 and R84) that were shown to promote growth in *P. radiata* (Reglinski et al. 2012) and to trial the use of these assays with *P. radiata* root samples. To achieve specificity, primers tested in this assay were designed to target unique single nucleotide polymorphisms that were detected using genome sequencing of *T. cf. atroviride* strains.

MATERIALS AND METHODS

Sequencing and identification of single nucleotide polymorphisms (SNPs)

T. cf. atroviride strains R33 and R84, along with two other strains of the same species, R32 and R40 (Lincoln University, New Zealand; Lange 2015) were grown in potato dextrose broth (Difco) for 3 days at 25°C and genomic DNA extracted using a Gentra®Puregene™ Tissue kit (Qiagen, Netherlands). Genomic DNA was sequenced using an Illumina GAI platform (Massey University Genome Service, New Zealand). The reads (>11 million per strain) were mapped to the reference genome of *T. atroviride* IMI206040 (Joint Genome Institute; <http://genome.jgi-psf.org/Triat2/Triat2.home.html>)

using the Burrows-Wheeler transform (BWT) algorithm and the program bwa v.0.5.8 (Li & Durbin 2009). The strains were sequenced with a mean of 15-35 reads per nucleotide position and 73-87% representation of the reference genome. Amongst the mapped reads, SNPs unique to R33 or R84 were identified using SAMtools (Li et al. 2009) and custom code, using a threshold of at least eight reads at those sites in every sequenced strain.

PCR conditions

Primers and fluorescent probes for real-time PCR were designed to the selected SNP regions using AlleleID® software (<http://www.premierbiosoft.com>) (Table 1). The 5' and 3' ends of R33- and R84-specific probes were labelled with 6-carboxy-fluorescein (FAM) and quencher dye BHQ-1 respectively. For a normalisation control, the 5' and 3' ends of the *Pinus radiata* cinnamyl alcohol dehydrogenase (CAD) gene probe were labelled with HEX (5') and BHQ-1 (3'), respectively (Chettri et al. 2012). Real-time PCR was carried out using the quantitative PCR (qPCR) protocol of Chettri et al. (2012). The reaction was performed with a LightCycler® 480 Probes system (Roche Applied Science, Penzberg, Germany) where each 10 µl of reaction contained: 5 µl of 2× Probe master mix, 1 µl 10× primer probe mix (containing 2 µM of the appropriate probe and 4 µM of each specific primer), 2 µl water and 2 µl DNA template. PCR

Table 1 Primer and probe sequences.

Target	Primer/probe	Sequence (5' to 3')
<i>P. radiata</i> CAD gene ¹	CAD918	CAGCAAGAGGATTTGGACCTA
	CAD1019	TTCAATACCCACATCTGATCAAC
	Probe945	HEX-TGTGAACCATGACGGCACCC-BHQ1
<i>T. cf.</i> <i>atroviride</i>	RM5Ka	GCAAGTTGGATACAGTTG
	RM6Ka	CCAGTAACTAGAATCGCA
R33	ProbeTrichKa	6FAM-TTGATATTAGTCCCACTCTATCAAG-BHQ1
<i>T. cf.</i> <i>atroviride</i>	RM1	CACTTCCAACCTTATTTTCTGCC
	RM2	CCGTACATGATGCCTCACA
	R84	ProbeLu584Pc

¹ Chettri et al. 2012

was conducted at 95°C for 10 min followed by 55 cycles of: denaturation at 95°C for 10 s, annealing at 58°C for 15 s, extension at 72°C for 20 s, followed by 40°C for 10 s. All real-time PCR runs included negative and positive controls, with no template or purified target template, respectively.

Assessment of specificity, sensitivity and efficiency

To assess primer specificity, genomic DNA was extracted from a range of *T. cf. atroviride* and *T. atroviride* strains, including R32 and R40 (Lange 2015) as well as from other common forest and soil fungi including *Cyclaneusma minus*, *Lophodermium pinastri*, *Nectria fockeliana*, *Sclerotinia sclerotiorum* and *Rhizoctonia solani*. The quality of template DNA was checked by PCR using universal ribosomal spacer (ITS) primers as described previously (Bradshaw et al. 2000), then used in duplicate real-time PCR reactions with the R33- and R84-specific primer/probe combinations.

To assess the sensitivity of the probe-based PCR method, and to provide standard curves for quantification, qPCR reactions were carried out with a 5-fold dilution series of template DNA ranging from 0.1 pg to 10 ng for each of R33 and R84. For normalisation with pine DNA, a standard curve was similarly prepared using from 32 pg to 100 ng *P. radiata* DNA extracted from freeze-dried root samples using a Genomic DNA Mini Plant Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan). Reactions were run in triplicate. Root tissue was obtained from clones (cuttings) of *P. radiata* that had been treated by flooding the soil at the potting stage with either a mixed spore suspension of *T. cf. atroviride* R33 and R84 (treatment), or with water (control). Abundance of the specific *T. cf. atroviride* DNA in pine roots, as determined by qPCR (5 biological and 2 technical replicates), was normalised by the amount of *P. radiata* DNA by using both fungal (R33 or R84) and plant (CAD) primers and probes. Statistical analyses involved regression analysis of the relationship between log DNA concentration and quantification cycle (C_q) in the standard curves, and a two-tailed Student's t-test to compare levels of *T. cf. atroviride*

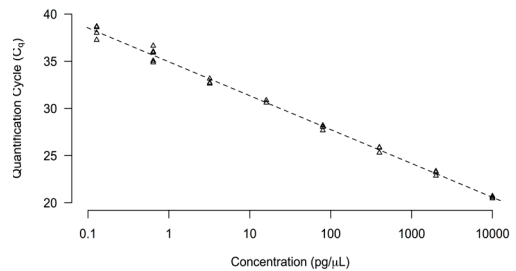


Figure 1 A standard curve for quantifying *T. cf. atroviride* strain R33, showing the concentration of serially diluted R33 DNA against the PCR quantification cycle (C_q) required to amplify the fluorescent signal above a fixed threshold. The lowest limit of detection within the linear range was ~0.1 pg per reaction.

in treated and control *P. radiata* roots.

RESULTS AND DISCUSSION

Primer development and specificity

Genome sequence data suggested that R33 and R84 were >99.7% identical to each other and to the other two *T. cf. atroviride* strains sequenced, with estimated genome sizes of 36 Mb. From among the variable regions, more than 16,000 putative SNPs were identified for each of R33 and R84. From these, two regions were selected for the development of diagnostic markers. Primers and probes were designed to one region specific for each of R33 and R84 (Table 1). Cross-template PCR reactions (i.e., R84 template with R33 primers/probe and vice-versa) did not show any amplification, indicating strain-specificity of the primers and probes. Specificity of the R33 or R84 primer/probe sets was further shown by a lack of PCR amplification with template DNA from either *P. radiata* or other fungal samples, including R32 and R40.

Assay sensitivity and PCR amplification efficiency

Standard curves prepared by amplifying a range of R33 and R84 template DNA concentrations

showed amplification efficiencies of 1.93 for both strains, close to the expected value of 2.0 (the amount of target DNA is doubled in each round of PCR). An example standard curve is shown in Figure 1. Regression (R^2) values of 0.995, 0.986 and 0.988 for R33, R84 and pine CAD targets, respectively, showed a linear correlation between quantification cycle (C_q) and log concentration over five orders of magnitude in each case. The lower limit of detection of R33 and R84 within the linear range of the standard curves was 0.1 pg of target DNA per reaction; based on a genome size of 36 Mb, this corresponds to just under 3 nuclei.

P. radiata root samples treated with *T. cf. atroviride* were shown to have 0.79 ± 0.36 pg/ μ l R33 and 0.73 ± 0.12 pg/ μ l R84, and normalised ratios to pine DNA of 1.7×10^{-5} and 1.5×10^{-5} , respectively, significantly higher than untreated controls with 0.00 pg/ μ l ($P < 0.01$) and well above the lower limit of detection.

CONCLUSIONS

Using an approach based on whole-genome sequencing to identify polymorphisms between two strains of *T. cf. atroviride*, a strain-specific, probe-based quantitative real-time PCR assay was developed. This assay will underpin the development of a testing system to check the durability of *Trichoderma* bioinoculants in the forest environment, and will be used in laboratory studies that aim to determine how pine defence is induced by these strains.

ACKNOWLEDGEMENTS

Robert Hill (Lincoln University) and Rebecca Ganley (Scion) are thanked for providing samples. Ruby Roach (Massey University) is thanked for laboratory assistance. This work was funded by AGMARDT and the Forest Owners Association of New Zealand.

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