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Detection and quantification of three distinct *Neotyphodium lolii* endophytes in *Lolium perenne* by real time PCR of secondary metabolite genes

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ABSTRACT

Perennial ryegrass (*Lolium perenne*) is a widely used pasture grass, which is frequently infected by *Neotyphodium lolii* endophytes that enhance grass performance but can produce alkaloids inducing toxicosis in livestock. Several selected endophyte strains with reduced livestock toxicity, but that confer insect resistance, are now in common use. Little is known regarding the survival and persistence of these endophytes when in competition with common toxic endophytes. This is mainly because there are currently no assays available to easily and reliably quantify different endophytes in pastures or in batches of seeds infected with multiple strains. We developed real time PCR assays, based on secondary metabolite genes known to differ between *N. lolii* endophyte strains, to quantify two selected endophytes, AR1 and AR37, and a common toxic ecotype used in New Zealand. A duplex PCR allowed assessment of endophyte:grass DNA ratios with high sensitivity, specificity and precision. Endophyte specific primers/probes could detect contamination of AR37 seeds with other endophytes down to a level of 3–25%. We demonstrated that it is possible to quantify different endophyte strains simultaneously using multiplex PCR. This method has potential applications in management of endophytes in pastures and in fundamental research into this important plant-microbe symbiosis.

Introduction

Perennial ryegrass (*Lolium perenne* L.) is a widely used pasture grass that is often infected with *Neotyphodium lolii* (*N. lolii*), an asexual fungal endophyte that grows systemically in the intercellular spaces of aboveground grass tissues (Bacon 1993). The perennial ryegrass/*N. lolii* association is widely regarded as mutualistic (Latch *et al.* 1984; Christensen *et al.* 2002; Hahn *et al.* 2008). Infection with this endophyte can enhance the growth and survival of the host perennial ryegrass plant when grown under environmental stress, in particular when subject to attack from insect pests (Thom *et al.* 2012).

Endophytes, such as New Zealand common toxic *Neotyphodium lolii* (NZCT), that infect perennial ryegrass produce a range of bioactive compounds: peramine, ergot alkaloids, and indole-diterpenes such as lolitrem B, as well as other compounds whose bioactive potential has not yet been fully investigated (Christensen *et al.* 1993; Lane *et al.* 2000; Cao *et al.* 2008). These three main classes of alkaloids all provide the host grass with protection from a wide range of

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invertebrate pests, while the ergot alkaloids and indole-diterpenes are also toxic to livestock (Young *et al.* 2009; Thom *et al.* 2012).

In order to utilize the benefits of endophytes, while excluding toxic effects on livestock, several selected endophyte strains which produce fewer, or no, animal toxic alkaloids have been selected and used in pasture grasses in the last decade. The AR1 strain, which produces peramine but not ergovaline or lolitrem B, has been widely used in New Zealand since 2001(Thom *et al.* 2012). The effects of AR1 on perennial ryegrass agronomy and the health and productivity of grazing livestock have been intensively studied. It was shown that AR1 increases grass resistance to insects (e.g. Argentine stem weevil), enhances pasture herbage yield compared with endophyte-free, reduces fly strike, increases livestock weight gain and milk solids compared to NZCT, and does not cause ryegrass staggers (Fletcher 1999; Popay *et al.* 1999; Popay & Baltus 2001). Another selected endophyte strain AR37, named Lp14 in scientific studies (Tanaka *et al.* 2005; Young *et al.* 2009; Fleetwood *et al.* 2011; Schardl *et al.* 2012), does not produce peramine, lolitrem B or ergovaline, but is known to produce epoxy-janthitrems. Testing to date has shown that AR37 increases grass resistance to a wide range of pests including Argentine stem weevil, black beetle, root aphid, pasture mealybug, and porina (Popay & Thom 2009). It is also more agronomically robust than NZCT and has similar animal performance levels to AR1, although it can cause ryegrass staggers, due to production of epoxy-janthitrems (Hume *et al.* 2007; Thom *et al.* 2014).

Nowadays, selected endophytes are widely used in pastures (Thom *et al.* 2012; Parish *et al.* 2013). However, relatively little is known about the survival and persistence of selected endophytes in pasture when in competition with common toxic strains (Hume & Barker 2005) and to what extent this may be influenced by stresses such as herbivory and climatic factors such as drought. Information on proportions of various endophyte strains in pastures could facilitate better use of the selected endophytes, for example, through the monitoring of toxic endophyte invasion in pastures. Another problem facing seed suppliers and farmers is contamination of commercial selected endophyte-infected seed with common toxic strains (Dombrowski *et al.* 2006). Detection and quantification of strains in pastures and grass seeds is possible through measurement of endophyte alkaloids or strain detection by endophyte-simple sequence repeats (SSRs); but these methods can be cumbersome, limited in their ability to detect multiple strains and can not easily analyse a single bulk grass or grass seed sample (Bluett *et al.* 2005; Briggs *et al.* 2007). In addition, concentrations of endophyte alkaloids are affected by plant genotype and environmental factors, and can be very dependent on time of the year (Easton *et al.* 2002; Easton 2007; Hahn *et al.* 2008; Brosi *et al.* 2011; Moate *et al.* 2012). Quantitative PCR and real time PCR have been applied to endophyte quantification in several studies (Panaccione *et al.* 2001; Young *et al.* 2005; Rasmussen *et al.* 2007; Hahn *et al.* 2008; Charlton *et al.* 2012). Some of these studies used a ratio of endophyte DNA to plant DNA to reflect endophyte biomass. However, their method did not distinguish between different types of endophytes and verify the degree of correlation between their measurements and endophyte biomass A fast, sensitive, reliable, and more versatile method that can directly quantify different types of endophyte would assist in the deployment and management of selected endophytes.

We therefore undertook to develop real time PCR assays which quantify and distinguish between the NZCT and the selected endophyte strains of AR1 and AR37.

Material and methods

Biological materials

Three types of endophytes were used in this study: NZCT, AR1, and AR37. All endophyte cultures were supplied by AgResearch (Palmerston North, New Zealand) and maintained on 2.4 % potato dextrose agar (PDA; Difco Laboratories, Detroit, Michigan, USA) under conditions described previously (Moon *et al.* 1999).

Mature plants of NZCT-infected, AR1-infected, AR37-infected or endophyte-free perennial ryegrass and clover (*Trifolium repens*) were provided by AgResearch, Palmerston North, New Zealand, and were grown in an unheated glasshouse without artificial illumination in Southern spring 2010. Nine clonal NuiD perennial ryegrass plants (the same clones as those used by Zhang *et al.* (2011) infected with Lp19 endophyte (French wild-type *Neotyphodium lolii*)) were used for endophyte biovolume measurement. These were grown from single tillers for 22 d in a controlled environment plant growth chamber (CAT630, Contherm Scientific Ltd, New Zealand), at 15 °C (13.7−17 °C) and with a light intensity about 700 μmol m^{-2} s for 12/24 h with a relative humidity of 50 %.

DNA isolation and quantification

Fresh fungal mycelium from PDA cultures, or plant tissue, was ground into a fine powder in liquid nitrogen with a mortar and a pestle. Genomic DNA was isolated from approximately 50 mg of ground tissue using a plant genomic DNA mini kit (Geneaid, Taipei, Taiwan) following the manufacturer's instructions. DNA concentration was determined with a Dyna Quant DQ200 Single-wavelength Fluorometer (Amersham Biosciences Corp, CA, USA), and calf thymus DNA (Invitrogen™, Catalogue Number: 15633-019) was used as standard.

Primer and probe design

Beacon Designer™ software (version 7.0, Premier Biosoft) was used in primer and probe design. Probes were custom synthesized by Biosearch Technologies (Novato, CA, USA) with fluorophores chosen according to the dye selection chart provided by the manufacturer (Biosearch-Technologies 2013).

Real time PCR cycling condition (simplex, duplex, and multiplex)

Real time PCR was performed on LightCycler® 480 (Roche, Germany). A LightCycler® 480 Probes Master mix kit (catalogue number: 04707494001, Roche) was used to perform the real time PCR. The simplex reaction mix contained 5 μL of probe master mix, and a final concentration of 200 nM of each