Exploring endophyte compatibility in perennial ryegrass

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Abstract

The association between perennial ryegrass (PRG) and fungal endophyte is an important factor in the protection of New Zealand pastures from insect pests, and the genetic background of the host plant significantly affects endophyte performance. Quantitative trait locus (QTL) analysis of phenotypic data from herbage harvested from a mapping population in autumn 2005 identified loci in the PRG genome influencing three traits: (1) endophyte mycelial mass; (2) ergovaline level; (3) peramine level. Three QTL were identified for each trait. For mycelial mass two QTL accounted for more than 75% of the variation (PV) for in the population and have considerable potential for development as pre-inoculation marker-assisted selection (MAS) tools for endophyte colonisation in PRG breeding programmes. The largest-effect QTL for peramine level (18% PV) coincided with a major mycelial mass QTL, implying peramine concentration is partly determined by endophyte colonisation. All other alkaloid QTL, however, were independent of mycelial mass, indicating it may be possible to develop MAS that will facilitate breeding for alkaloid phenotype, independent of the degree of endophyte colonisation.
Keywords: DIMS, ELISA, *Lolium*, map, metabolic profiling, *Neotyphodium*, SSR

Introduction

The perennial ryegrass (PRG, *Lolium perenne*) - endophyte (*Neotyphodium lolii*) mutualistic association is successfully exploited in commercial PRG improvement by inoculating cultivars with selected endophyte strains conferring alkaloid phenotypes that deter feeding by insect pests, but are non-toxic to grazing mammals. For example, the commercial PRG endophyte AR1 produces the insect-feeding deterrent pyrrolopyrazine alkaloid, peramine, but neither ergovaline nor lolitrem B, toxins implicated in mammalian mycotoxicoses. Recent research in forage grasses confirms that variation in endophyte alkaloid levels, as well as the quantity of the endophyte present in the plant is influenced by the genetic background provided by the host plant and is highly heritable (Adcock *et al*. 1997; Easton *et al*. 2002). Potential, therefore, exists for the manipulation of alkaloid phenotype and endophyte colonisation by optimising host plant genetics through breeding.

The objective of the current research is to develop marker-assisted selection (MAS) for endophyte compatibility – as determined by endophyte quantity and concentration of key endophyte alkaloids *in planta*. MAS describes breeding strategies whereby DNA marker tests are used to select indirectly for phenotype, and is likely to be of particular value where the trait under selection is genetically-complex, with low heritability, or where it is difficult or costly to obtain reliable phenotypic data. In the current context, MAS would allow for low cost and genetically-precise selection of superior host genotypes in PRG breeding programmes and, crucially, it could be employed early in the breeding cycle, before endophyte inoculation. MAS strategies are being pursued elsewhere as a means of exploiting host plant genetic control of specific fungal metabolites, primarily in the context of fungal disease resistance. For example, quantitative trait locus (QTL) analysis has been used to identify genomic regions in corn that influence accumulation of aflatoxin, a toxic metabolite produced by invasive *Aspergillus* spp. fungi (Paul *et al*. 2003; Widstrom *et al*. 2003; Busboom & White 2004). Similarly, QTL were located in barley and wheat for accumulation of the mycotoxin deoxynivalenol (DON), produced by the fungal pathogen *Fusarium graminearum* (Somers *et al*. 2003; Smith *et al*. 2004; Horsely *et al*. 2006).

As a first step towards development of MAS for endophyte compatibility in PRG, we are using rapid metabolite profiling (Koulman *et al*. 2006) and enzyme-linked immunosorbent assay (ELISA) technology, to undertake QTL analysis of endophyte alkaloid concentration and endophyte quantity in a PRG mapping population. These data will also facilitate further genetic and metabolomic dissection of host plant control of endophyte performance. We present the results of a QTL analysis of autumn 2005 phenotypic data collected for mycelial mass, ergovaline and peramine in a PRG mapping population.

Materials and Methods

Mapping population

Mapping population IxS is the F_1 full-sibling progeny (n=188) of a pair cross between heterozygous individuals from cultivars 'Grasslands Impact', which carries a common toxic endophyte, and 'Grasslands Samson', which carries a novel endophyte strain (AR6). Population IxS is derived from seed harvested only from the 'Grasslands Impact' parent, and therefore all individuals

Table 1 Parental ('Grasslands Impact' and 'Grasslands Samson') and population IxS mean values, range of values, and P-values (ANOVA for within-population variation) for mycelial mass ELISA (immunoreactive equivalents, IRE), peramine and ergovaline (normalised intensity units, niu) measured as the mean of two replicates in leaf material harvested in autumn 2005. Note: 'Grasslands Samson' parent contains endophyte AR6, whilst a common toxic endophyte is present in all other genotypes.

Trait	Population mean	Population range	'Impact' parent	'Samson' parent	P-value
Mycelial mass (IRE mg/g)	4.7	2.0 - 7.9	6.3	2.0	<0.001
Peramine (niu)	18.4	6.6 - 36.9	18.3	13.1	<0.001
Ergovaline (niu)	6.9	2.1 - 13.9	7.4	6.5	<0.001

Figure 1 Perennial ryegrass genetic linkage map showing QTL detected for endophyte mycelial mass, ergovaline and peramine. Linkage groups LG 1 to LG7 (assignments consistent with other ryegrass maps; Faville *et al.* 2004) show marker names at left of bars, QTL positions (2-LOD confidence intervals) at right of the bars, and length of linkage groups in centimorgans (cM) at the foot of the bars.
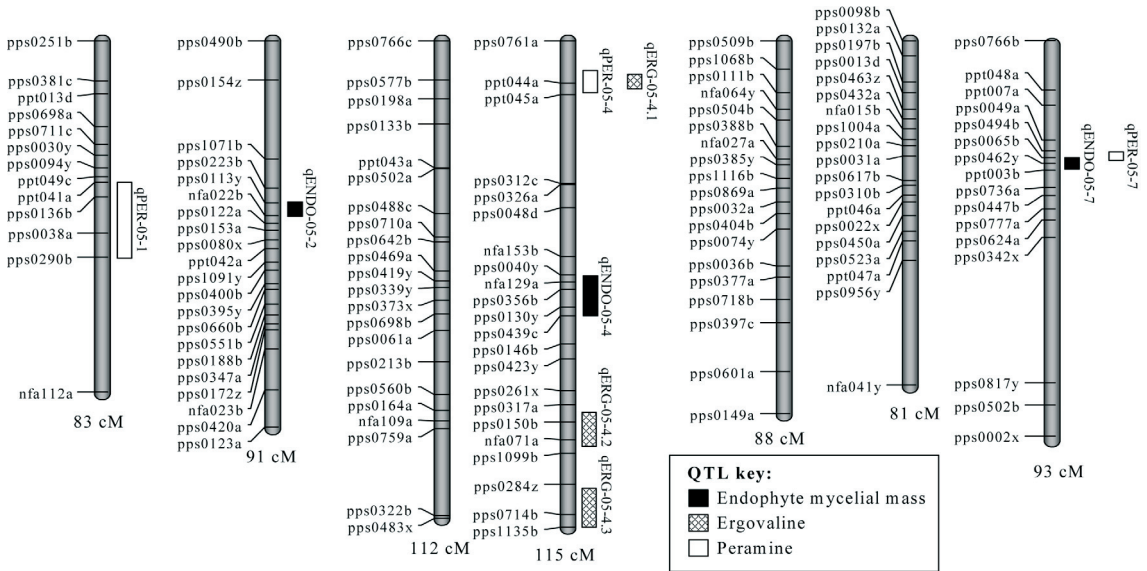
contain the same common toxic endophyte. Clonal replicates (n=3) of the population were grown in 10 cm diameter pots and arranged in a randomised complete block design, outdoors at the Grasslands Research Centre in Palmerston North. Herbage material (leaf lamina and sheath) was harvested from two replicates in April 2005, 2 weeks post-cutting, and freeze-dried and milled before metabolic profiling and ELISA analysis.

Metabolic profiling

Semi-quantitative metabolite data was generated from herbage of two replicates for all 188 sampled mapping population genotypes, using direct-infusion mass spectrometry (DIMS). Processed leaf material (50 mg) was extracted with 50% isopropanol:H_2O. A Thermo Surveyor HPLC pump and autosampler were used, aligned with a linear ion trap mass spectrometer (Thermo LTQ) using electrospray ionisation (ESI) in positive mode. Infusion solvent was H_2O:MeCN:HCO_2H (50:50:0.1), flow rate 250 µL min^{-1} and split into a 12 µL min^{-1} to the autosampler and a 238 µL min^{-1} to a T-junction just in front of the ESI source. A 40 µL aliquot of each sample was injected in the low flow stream in the auto-sampler. The sample joined the T-junction just in front of the ESI source. The flow rate was kept constant for 9 min, after which it was increased to 500 µL min^{-1} for several wash steps.

From 1.0 to 1.5 min only MS1 spectra were recorded; from 1.5 to 9.0 min data dependent mode was used to collect a MS1 spectrum, followed by the isolation and fragmentation (35% CE) of the most intense ion from MS1, followed by the isolation and fragmentation of the most intense ion from MS2, and this was repeated for the 25 most intense ions in MS1 after which the system started again with a MS1 spectrum. Averaged MS1 spectra were exported to MS Excel and aligned and normalised. Data were presented as normalised intensity units (niu) for QTL analysis.

Mycelial mass (MM) ELISA analysis

Extractions were made from two replicates of all 188 sampled mapping population genotypes, and the supernatant was analysed for MM using a semi-quantitative ELISA, based on the following immunoreagents: specific anti- *N. lolii* antibodies and *N. lolii* endophyte standard prepared by Ball *et al.* (1995); and *N. lolii* antigens gifted by R.G. Keogh (formerly AgResearch). The sandwich format described by Ball *et al.* (1995) was replaced by a competitive ELISA which was developed and validated for the current study. ELISA plates were coated with *N. lolii* antigen and 1% BSA was utilised as a blocking agent. Samples were quantified using standard curves prepared with endophyte standard. Presence of endophyte in sample extracts was indicated by inhibition of specific antibody binding to coating antigen which was determined using commercial anti-rabbit-horseradish peroxidise (HRP) conjugate. A commercial TMB (3,3',5,5' tetramethylbenzidine) substrate for HRP was also used. Curve fits of mean absorbance versus the logarithm of the analyte concentration were performed by four-parameter curve fit and a sample working range of 2- 50 mg/g (I_{20}-I_{80}) was achieved. Data were presented as immunoreactive equivalents (IRE) in mg/g for QTL analysis.

Genetic linkage analysis and QTL analysis

A genetic linkage map was constructed for population IxS in JoinMap 3.0 (http://www.kyazma.nl/), using 165 EST-SSR (simple sequence repeat markers derived from expressed sequence tags) and EST-STS (sequence-tagged site markers derived from ESTs). DNA isolation, marker PCR and genotyping were conducted as described in Faville *et al.* (2004). Metabolite and ELISA data (mean of two replicates) from population IxS were used for QTL analysis, which was executed using the MQM procedure in MapQTL 4.0 (http://www.kyazma.nl/). Permutation testing (n=1000) was used to establish a logarithm-of-odds ratio

(LOD) threshold for the declaration of a QTL at genome-wide significance of α=0.05.

Results and Discussion

Metabolic profiling of mapping population IxS was used to semi-quantify ca. 250 individual plant or endophyte-derived metabolites per genotype sample, based on MS2 spectra. From these profiles, data for the endophyte alkaloids peramine and ergovaline were identified on the basis of MS fingerprints, and mean genotype values were used for QTL analysis alongside MM data.

Data for each trait approximated a normal distribution (not presented) and ANOVA indicated the presence of genetic variation for all traits in the IxS population (Table 1). A weak correlation (r^2=0.10) was detected between peramine and MM, but there was no correlation amongst the other pairs of traits within population IxS. Levels of ergovaline detected were relatively low and occupied a narrow range compared with peramine, a result that may have been influenced by seasonal variation in herbage ergovaline concentration, with concentrations potentially low in leaf tissue in autumn (Woodburn *et al*. 1993).

Genetic linkage analysis located 164 EST-SSR loci on seven linkage groups, and this was reduced to 129 loci in order to decrease marker locus density in some regions of the map (Fig. 1). The final IxS map used for QTL analysis was 664 Kosambi cM long, with mean density of 5.1 cM/locus and six intervals >10 cM.

Three genomic regions influencing MM were identified using QTL analysis (Fig. 1), with two major QTL on linkage groups LG2 (qENDO-05-2) and LG7 (qENDO-05-7) together accounting for up to 76% of the phenotypic variation (PV) in the population. The magnitude of effect partitioned to these two QTL make them strong candidates for further development as DNA tests to facilitate pre-inoculation MAS for endophyte colonisation in PRG breeding.

Multiple QTL, individually of moderate effect (explaining 6-18% PV), were detected for both peramine and ergovaline. Three QTL associated with variation for peramine levels were located on LG1, LG4 and LG7, collectively explaining up to 43% of the PV for the trait. For ergovaline, despite being present at low levels in the harvested leaf tissue, three QTL accounting for up to 27% PV were identified, all occurring on LG4.

The peramine QTL of greatest effect (18% PV) located to an LG7 position (qPER-05-7) coincident with the major MM QTL, qENDO-05-7, and shares a common direction of allelic effect. Variation in peramine accumulation associated with this QTL is, therefore, likely based on variation in endophyte levels in the leaf tissue, and consequently undertaking MAS for increased endophyte colonisation using DNA markers at this locus would be expected to concomitantly select for increased peramine levels.

By contrast, the identification of peramine QTL at two genome positions independent of MM QTL supports earlier findings that alkaloid levels in PRG are determined only in part by endophyte distribution (Easton *et al*. 2002; Spiering *et al*. 2005). Similarly, although ergovaline QTL results may be compromised to some extent by the low levels of this compound detected, none of the detected QTL coincided with MM QTL, implying independence of host-mediated genetic control of ergovaline levels from the level of colonisation by the endophyte. These findings indicate that it should be possible to undertake development of MAS in PRG to facilitate breeding for controlled levels of alkaloid production, independent of the degree of endophyte colonisation.

Before being applied in a MAS context, marker:trait associations should be substantiated through validation of QTL effects across time, environments and/or alternative genetic backgrounds. Initial validation of the current results will be provided from analysis of herbage harvested in autumn 2006, which has been timed to capture peak ergovaline levels. QTL analysis of other endophyte-derived metabolites from both 2005 and 2006 harvests, and comparative genomics (Faville 2005) integrated with AgResearch gene discovery efforts, will provide further opportunities for the genetic dissection of the host PRG genotype-endophyte association.

ACKNOWLEDGEMENTS

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