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Genetic linkage mapping in an F2 perennial ryegrass population using DArT markers.

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Keywords

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Abstract

Perennial ryegrass is the principal forage grass species used in temperate agriculture. In recent years significant efforts have been made to develop molecular marker strategies to allow cost effective characterisation of a large number of loci simultaneously. One such strategy involves using DArT markers, and a DArT array has recently been developed for the *Lolium-Festuca* complex. In this study, we report the first use of the DArTFest array to generate a genetic linkage map based on 326 markers in a *Lolium perenne* F_2 population, consisting of 325 genotypes. For proof of concept, the map was used to identify QTL associated with differences in crown rust susceptibility, caused by the fungal biotroph, *Puccinia coronata*.

Introduction

Lolium perenne (L.) is the principle forage used in temperate agriculture (Hannaway et al. 1999) and traditional breeding programs have been successful in developing varieties fit for purpose. By targeting genetic improvement through the use of molecular markers, there is potential to rapidly assist traditional ryegrass breeding programs. However, this will require availability of high throughput marker systems.

Existing *L. perenne* genetic linkage maps are primarily based on Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP) and Simple Sequence Repeat (SSR) markers (Bert el al. 1999, Armstead et al. 2002, Jones et al. 2002a and b, Faville et al. 2004, Gill et al. 2006, Anhalt et al. 2008). These marker systems suffer from being relatively low throughput. Single Nucleotide Polymorphism (SNP) marker systems are one high throughput option but require DNA sequence information prior to array development. An alternative high throughput marker system is DArT (Diversity Array Technology), which allows simultaneous screening of thousands of anonymous loci on microarrays. DArT arrays have already been used for genotyping in *Festuca pratensis, Lolium multiflorum, Triticum aestivum* and *Arabidopsis thaliana* among others (Bartoš et al. 2011; Akbari et al. 2006; Wittenberg et al. 2005). A DArT array, named DArTFest, was recently developed for the *Lolium-Festuca* complex of plants containing 7680 probes derived from methyl filtered genomic fragments (Kopecky et al. 2009).

In this study we describe the first use of the DArTFest array for genetic linkage mapping in the economically important species *Lolium perenne*. An existing F_2 perennial ryegrass mapping population was utilised and 325 genotypes were hybridized on to the DArTFest array. The utility of the resulting genetic linkage map for QTL analysis was demonstrated by identification of QTL associated with moderate crown rust resistance.

Materials and Methods

Genetic Linkage Map Construction

A genetic linkage map primarily based on SSR markers has previously been created for this population (Anhalt et al. 2008) and consisted of 75 markers in total. Since then, a number of additional SSR markers have also been added to the population and are included in this study. In order to develop a higher density map we have utilised the DArTFest array developed for Lolium/Festuca by Kopecký et al. (2009). The DArtFest array contains 7,680 probes and was developed from five species within the Lolum-Festuca complex (*Lolium perenne, Lolium multiflurum, Festuca pratensis, Festuca arundinacea, and Festuca glaucescens*). The parents of the F_2 biomass population were used in the establishment of the DArTFest array, together with the parents of other *Lolium* and *Festuca* mapping populations. After array development, individual genotypes were hybridised and scored for the presence and absence of each marker (Kopecky et al. 2009) and the information has been deposited on a publicly accessible website (http://bioinf.scri.ac.uk/germinate_grasse/). Additionally, we developed three markers to map putative disease resistance genes belonging to the NBS-LRR gene family EST1, EST7 and EST45 previously identified in ryegrass (Xing et al. 2007). EST1 was sequenced in the parental material of the F_2 population using the primers described in (Xing et al. 2007) and a hybridization probe was designed in a region of nucleotide diversity (FP: CGGTAAACACTTTCCGTGCT, RP: GTGGTTTGGGCTCTGTTCAT, probe 1: TGAAGCCCGTTTGTGCGATGGT-Fluorescein, probe 2: LC Red 640-CCACCATTCTTAATCTTGAGCTACTTGGCTCCAGCCGTA) and genotyping was performed after melt curve analysis on the Roche LightCycler® 480 System II . EST7 and EST45 were mapped using the “Blind” mapping approach as described in (Studer et al. 2009). Primers were designed to amplify a 200bp and 216bp product in EST7 (FP: TGTGTGCATCTTCCACCAAT, RP: AAGGACCACTCCTCCCAGTT) and EST45 (FP: TGTCCCCATCCTCAATTCTC, RP: CAGGAGATCCTCCTCTGTGC) respectively. High resolution melt analysis was carried out on the Roche LightCycler® 480 System II using LightCycler® 480 High Resolution Melting Master (Cat. No. 04909631001, Roche).

A total of 325 genotypes were hybridised to the DArTFest array and used for map

construction in JoinMap 4.0 (Van Ooijen, 2006). Genotyping data from the DArTFest array was combined with genotyping data from SSR markers previously mapped in this population to generate a new genetic linkage map. Severely distorted markers were removed by retaining only markers with a chi-squared value of less than 25 (this included the removal of some SSR markers previously mapped in the population). Markers were grouped into seven linkage groups based on Logarithm of Odds (LOD) groupings. In the first instance all dominant markers were placed into two sets depending on the segregation, A/C and B/D scores, and co-dominant markers were in both sets. A preliminary genetic linkage map was constructed for both sets using the multipoint Maximum Likelihood (ML) algorithm of JoinMap 4.0. The resulting map order of the longest map was then used as a fixed order file in a subsequent round of ML mapping using the complete set of markers (A/C, B/D and co-dominant). This resulted in an initial map based on 653 DArT markers and 29 co-dominant markers (Supporting Information 1). The *plausible positions* tab was then utilised to identify uncertainties in marker order. In areas where marker order was difficult to resolve, markers were removed and re-calculated until a final map was obtained, in which we had a high confidence in marker order. Diagrams were prepared with MapChart 2.2 (Voorrips 2002).

Crown Rust Assessment

The experimental mapping population used in the study consisted of genotypes from an F_2 biomass population previously used to identify biomass QTL (Anhalt et al. 2008; Anhalt et al. 2009). The genotypes were planted in the field (Carlow, Ireland) in 2006 as mini swards in an α-lattice design with two-fold replication (described in detail in Anhalt et al. 2009). Crown rust was visually assessed on an entire plant basis on four occasions between May and July in 2007 (time point 1 to 4), and once in May 2008 (time point 5). Disease severity was recorded at each assessment using a scale of 0 (no visible infection) to 9 (highly infected) (Studer et al. 2007).

QTL Analysis

The disease scores (mean of block 1 and 2) from each time point individually were used for QTL modeling, which was performed using R/QTL (Browman et al. 2003). A genome wide significance level of 5% was calculated after 1000 permutations (Churchill and

Doerge 1994). The suite of functions for multiple QTL modelling was used as described in Browman and Sen (2009). Genotype probabilities were calculated using the function *calc.genoprob.* An initial QTL model was developed by adding significant QTL after performing a single QTL analysis employing standard interval mapping with the function *scanone.* A QTL object was prepared with *makeqtl* and *fitqtl* was used to fit the initial model. *T*o get improved estimates of QTL position *refineqtl* was used, whilst *addint* and *addqtl* were employed to test for QTL interactions and scan for additional QTL to be added to the model, respectively. When interactions or new QTL were added to the model the process was repeated with the newly developed model as a starting point.

Results and Discussion

We have generated a robust genetic linkage map using ML mapping that has a map length of 966 cM and consists of 297 DArT markers and 29 co-dominant markers (Figure 1). The inclusion of SSR markers allowed the assignment of DArT markers to seven linkage groups. The average spacing between markers has been reduced from 7.5 cM in the original map (Anhalt et al. 2008) to 1.54 cM, which should improve its utility for mapping applications. Only markers not showing severe segregation distortion in the original map were retained for use in the generation of the current DArT based map. The initial map generated was based on 653 DArT markers showing polymorphism in the population. However, this was reduced to 297 DArT markers that we could unambiguously order to generate a map suitable for QTL analysis. The putative order of all 653 markers is shown in Supporting Information 1. The DArTFest array contains 1,725 probes positively scored in perennial ryegrass (Kopecky et al. 2009) and we have assigned 37.8% to putative positions and 17.2% have been ordered with high confidence.

This is the first report of mapping DArT markers using the DArTFest array in *L. perenne*. However, the array has been used recently in the creation of *F. pratensis* and *L. multiflorum* genetic maps (Bartoš et al. 2011), and markers in common between maps are shown in supporting information 1. The DArT markers mapped in the study of Bartoš et al (2011) have been sequenced and 64.2% were found to be unique, with the majority of markers originating from expressed regions of the genome. The sequencing of markers allowed the establishment of syntenic relationships with rice and Brachypodium. The

sequencing of additional markers from the DArT array is possible, including those mapped in the current study.

In future the DArT array may be applied to the genotyping of association mapping populations. In this case the relative order of markers provided by these genetic linkage maps will be very helpful. Perennial ryegrass is an outbreeding species and Linkage Disequilibrium (LD) is expected to decay over short distances in the genome (Brazauskas et al., 2011), which would require a much larger number of markers than is available on the DArTFest array. However, within individual perennial ryegrass breeding programs, LD may extend over much longer distances (Brummer and Casler, 2009). A high throughput marker system such as the DArT array may prove useful for such association analysis performed within the breeding material, requiring a moderate number of markers. Alternative marker systems that could be used for this purpose are SSR and SNP based markers. SSR marker systems have the advantage of being co-dominant, however they are not very amenable to high throughput analysis. SNP arrays developed by Illumina (GoldenGate, Infinium HD Assay) on the other hand represent very high throughput marker system, and arrays have been developed to interrogate up to 1 million SNPs in humans. However, to date no public SNP array resource for perennial ryegrass has yet been developed.

The perennial ryegrass DArT linkage map was initially used to identify QTL for crown rust resistance. The incidence of disease varied depending on the five time points and the highest disease pressure occurred in May 2007 (Supporting Information 2). The distribution of disease scores in the F2 population is also shown in supporting information 2. QTL modelling was performed at each time point (Table 1, ANOVA for each time point in Supporting Information 3). The percentage variation explained by QTL models varied between 18 and 35% depending on time-point and all QTL identified were located on linkage groups 2, 3, 4 and 7. There was no evidence of interactions between QTL at any of the time points. A QTL was identified on LG2 at all time points. The QTL identified on LG2 at 66.5 cM with scores from time point 1 was the largest effect QTL identified, explaining 12.76% of the phenotypic variance. This is also the time point that had the greatest disease pressure. The QTL on LG2 identified at the remaining four time points were

located between 103.7 and 110.2 cM and accounted for between 5 and 11% of the phenotypic variation. One of the NBS-LRR genes (EST1) mapped in this study was located on LG2 at 71 cM, proximal to the largest effect QTL and it is possible that this gene is part of a larger NBS-LRR cluster in this region. In recent years a number of studies have reported on the identification of Quantitative Trait Loci (QTL) for crown rust resistance in ryegrass (Dumsday et al., 2003; Muylle et al., 2005, Studer et al., 2007, Sim et al., 2006, Thorogood et al., 2001; Schejbel et al., 2007). To date, the genes underlying these resistance loci have not been cloned. Although many potential QTL regions have been identified, the most consistent detection has been on linkage group two (Dumsday et al. 2003; Muylle et al. 2005; Sim et al. 2007; Studer et al. 2007). However a lack of common polymorphic markers makes comparisons difficult. The value of the QTLs identified in the current study as potential sources of resistance is uncertain given the relatively low phenotypic variation explained by the individual QTL, and the possiblity for differences in resistance due to field environment and local *P. coronata* populations (Dracatos et al., 2010). However, further investigation is warranted. The success of any molecular markers identified within experimental populations can be uncertain, since in order for a QTL to be valuable it must be segregating with the breeding material. However, presuming the identified loci represent valuable sources of resistance, the markers flanking these QTL may be used in a marker assisted backcrossing strategy to transfer the QTL to elite breeding material.

In this study we have generated the first genetic linkage map of *Lolium perenne* using the DArTFest array. In a preliminary study we have used it to map QTL associated with natural crown rust infection. It is envisaged that in the future more studies will utilise the DArTFest array for genotyping of both within family populations and association populations.

Supporting Information

Additional supporting information may be found in the online version of this article and genotyping data is available from the authors on request.

Supporting Information 1. Excel sheet showing the grouping of 682 markers initially mapped in the F_2 genetic linkage map of *Lolium perenne*. Numbers in first row refer to Linkage Group (LG) number. All DArT markers begin with D. Common markers between

the *L. perenne* map and the maps of *F. pratensis* and *L. multiflorum* were highlighted in blue and red respectively. Markers common to the three maps are indicated in green. The markers in italic did not map to homologous linkage group.

Supporting Information 2. Figure 1 showing the disease scores at each of the five time points for parents, F_1 and the mean of the F_2 population. Figure 2, histogram showing the distribution of disease scores across the F_2 population.

Supporting Information 3. Full ANOVA model showing results of QTL analysis at each of the five scoring time points.

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Tables

Table 1: Summary of QTL modelling for each time point where disease incidence was measured. LOD and % variance explained (VE) describe the fit of the full model with all QTL relative to the null model. The position in cM of each QTL in the model is shown where at maximum LOD score.

Time Point	LOD and % VE by QTL model	QTL in Model		LOD	% VE
		LG	Position (cM)		
1 (08/05/2007)	29.79 (35.31%)	2	66.5	12.31	12.76
		3	102.5	3.39	3.28
		4	48.3	6.51	6.46
		4	142.4	2.59	2.50
		7	49.5	9.28	9.40
2 (29/06/2007)	14.41 (18.63%)	2	110.2	6.33	7.71
		3	59.4	3.85	4.61
		7	89.6	3.89	4.65
3 (19/07/2007)	15.83 (20.26%)	2	103.7	9.03	10.99
		4	52.2	3.37	3.93
		7	100.0	2.24	2.59
4 (25/07/2007)	13.95 (18.09%)	2	103.7	7.00	8.63
		7	105.1	6.22	7.62
5 (19/05/2008)	25.22 (30.59%)	2	110.2	5.18	5.41
		3	80.8	7.92	8.42
		4	13.7	7.66	8.14
		7	136.4	3.51	3.62

Figures

Figure 1: F_2 genetic linkage map of *Lolium perenne* with subset of DArT markers. Numbers above refer to Linkage Group (LG) number. All DArT markers begin with D. EST markers relate to three markers developed to map NBS-LRR related genes in the current study. Descriptions of the other markers utilised can be found in Anhalt et al., (2008). The numbers to the left of the linkage groups refer to the genetic distances (Kosambi cM).