Abstract AFLP markers have been successfully employed for the development of a high-density linkage map of ryegrass (*Lolium perenne* L.) using a progeny set of 95 plants from a testcross involving a doubled-haploid tester. This genetic map covered 930 cM in seven linkage groups and was based on 463 amplified fragment length polymorphism (AFLP) markers using 17 primer pairs, three isozymes and five EST markers. The average density of markers was approximately 1 per 2.0 cM. However, strong clustering of AFLP markers was observed at putative centromeric regions. Around these regions, 272 markers covered about 137 cM whereas the remaining 199 markers covered approximately 793 cM. Most genetic distances between consecutive pairs of markers were smaller than 20 cM except for five gaps on groups A, C, D, F and G. A skeletal map with a uniform distribution of markers can be extracted from this high-density map, and can be applied to detect and map QTLs. We report here the application of AFLP markers to genome mapping, in *Lolium* as a prelude to quantitative trait locus (QTL) identification for diverse agronomic traits in ryegrass and for marker-assisted plant breeding.

**Key words** Genetic mapping · AFLP · *Lolium perenne*, Plant breeding

**Introduction**

The genus *Lolium* includes eight species (Terrell 1968). Among them, two major fodder crops are cultivated for temperate grassland agriculture: perennial ryegrass (*L. perenne*) for grazing and turf and Italian ryegrass (*L. multiflorum*) for hay and silage making. *L. perenne* is one of the most important forage grasses commonly cultivated in Europe, New Zealand, Japan and Australia for permanent pasture and amenity grassland. Until recently, forage plant breeding was carried out with limited insight into the genotypic breeding value. Grass breeders have recognised the potential of DNA markers to enable events at the genome level to be understood and monitored in a much more precise way (Hayward et al. 1994). As genetic maps facilitate the study of genome structure and evolution, as well as the identification of monogenic traits or Mendelian components of quantitative traits (QTLs), they can be very useful in current breeding programs and are also the basis for future positional gene cloning. Substantial effort is currently being directed towards the production of genetic maps in many grass species. Genetic mapping of *Lolium* species (2n=14) has been relatively little developed compared to the cereals such as wheat (Chao et al. 1989; Devos and Gale 1993) or rice (McCouch et al. 1988; Maheswaran et al., 1997) probably because of its lower economical importance and its relatively large genome size (2 C=4.16 pg, Hutchinson et al. 1979; 1660 Mbp, Forster 1999). Nevertheless, a *Lolium* linkage map based on an interspecific cross (*L. perenne* x *L. multiflorum*) has already been constructed (Hayward et al.1994) and recently extended to include 106 markers distributed over 692 cM (Hayward et al. 1998).

Restriction fragment length polymorphisms (RFLPs; Botstein et al. 1980) have been extensively used for the construction of genetic linkage maps in plants. However, they require large amounts of genomic DNA, are time consuming and expensive. Since the development of the polymerase chain reaction (PCR) for amplifying DNA fragments (Saiki et al. 1988), several PCR-based technologies have been developed. Random amplified polymorphic DNA (RAPD; Williams et al. 1990) and microsatellite (Litt and Luty 1989; Roder et al. 1998) markers overcome many of the technical limitations of RFLPs but have their own limitations: RAPDs have the major...
disadvantage of being dominant and very sensitive to the reaction conditions, while microsatellites are expensive to produce. More recently, a new PCR-based-technology has been developed by Vos et al. (1995), termed amplified fragment length polymorphism (AFLP), which uses a PCR system to detect RFLP indirectly. AFLP methodology takes advantage of the polymerase chain reaction but differs from classical PCR and single primer-based methods such as RAPD, AP-PCR (arbitrary primed PCR; Welsh and McClelland 1990) and DAF (DNA amplified fragments; Caetano-Anolles et al. 1991) by generating highly reproducible multi-banded profiles which can reveal extensive polymorphism, resulting in reduced work loads and increased speed of data generation. The AFLP technique has the capacity to reveal a much greater number of polymorphic loci than other currently available techniques (a virtually unlimited number of fragments can be detected) and is particularly amenable for genetic mapping and profiling studies (Karp et al. 1996).

The objective of the present study was to extend the genetic map for ryegrass exploiting an intraspecific population and the use of isozyme, EST (expressed sequence tags; Adams et al. 1991) and AFLP markers. The population involved is one being used by the International Lolium Genome Initiative (ILGI, Forster 1999) as a common basis for a coordinated international programme on genome analysis in the genus Lolium. The linkage map reported in this paper contributes to our knowledge of the genome structure of ryegrass and may be exploited as a potential reference map for QTL analysis as part of the ILGI programme.

Materials and methods

Plant material

An intraspecific (L. perenne) population (coded p150/112) of 150 plants was constructed at the Institute of Grassland and Environmental Research, Aberystwyth. It derived from a cross between a di-haploid plant (DH290) and a hybrid F1 (a plant from a polycross of a North Italian collection of perennial ryegrass cv Melle). A subset of 95 plants, randomly selected, were used for marker screening and map construction.

Preparation of genomic DNA and AFLP reactions

Approximately 0.5 g of young leaves was harvested and ground in liquid nitrogen to obtain a fine powder. The CTAB method of Rogers and Bendich (1985) was used for the isolation of ryegrass genomic DNA. The AFLP protocol developed by Vos et al. (1995) with minor modifications. Genomic DNA (350 ng) was restricted with 5 units of EcoRI and Tru9I at 37 °C for 3 h according to the manufacturer’s instructions (Boehringer Mannheim). Tru9I is an isoschizomer of Msel. After complete digestion, 5 pmol of the EcoRI adaptor (5′-CTC GTA GAC TGC GTA CC; CTT ACG CAT GGT TAA-5′), 50 pmol of the Tru9I adaptor (5′-GAC CAT GAG TCC TGA G; TAC TCA GGA CTC AF-5′; Zabeau and Vos 1993), 1 unit of T4 DNA Ligase and ligation buffer (95% formamide, 10 mM NaOH, 0.05% each of xylene cyanol and bromophenol blue) were loaded after denaturation (5 min at 95 °C) onto a 5% denaturing polyacrylamide gel (8 M urea; 50 cmx0.4 mm) and electrophoresed at 2,000 V for 3.5 h in 1× TBE buffer (Tris 90 mM, pH 8.2, Borate 90 mM, EDTA 2 mM). PCR products were visualised using the silver-nitrate staining method as described by Tixier et al. (1997).

Isozyme and EST analysis

Three isozyme markers, phosphoglucomutase (PGM, EC 5.4.2.2), glutamic-oxaloacetic transaminase (GOT, EC 2.6.1.1) and acid phosphatase (ACP, EC 3.1.3.2), were analysed for mapping using the methods described by Hayward et al. (1995).

The five EST markers corresponding to OSE (Late Embryogenesis Abundant gene), OSRB (α-amylase), OSW (ADP-glucose glycosyl transferase), LP1 (pollen allergen) and MZE (triosephosphate isomerase) were analysed according to Lallemand et al. (1998). As for AFLPs the EST polymorphism was detected directly as a size difference.

Data analysis and map construction

AFLP markers were identified based on the primer-pair combination employed used and the estimated molecular size. The approximate size of each marker was expressed in nucleotides as estimated in comparison with the mobility of the bands of the 10-base ladder (Sequamark, Research Genetics). Clearly readable AFLP bands were scored as dominant genetic markers. The software package JOINMAP 2.0 (Stam 1993) was used to estimate segregation distortion and determine linkage groups, while the software program MAPMAKER 3.0b (Lander et al. 1987) was used to order loci and construct linkage maps. Analyses were performed with a LOD score threshold of 5.0 and a maximum recombination value of 25% (=0.25) for grouping and ordering markers. Kosambi’s mapping function was applied for the calculation of map distances (Kosambi 1944).

Results

DNA marker generation

An investigation of the reliability of markers was first determined through a reproducibility test at each step in the AFLP procedure from DNA extraction to final selective amplification. Variation in the banding pattern generated was found to be in the range of 1−2%, (i.e. 1−2 additional or missing amplified DNA fragments over the total number of DNA fragments amplified). As already

Gel electrophoresis

Two microliters of each sample together with 1 µl of loading buffer (95% formamide, 10 mM NaOH, 0.05% each of xylene cyanol and bromophenol blue) were loaded after denaturation (5 min at 95 °C) onto a 5% denaturing polyacrylamide gel (8 M urea; 50 cmx0.4 mm) and electrophoresed at 2,000 V for 3.5 h in 1× TBE buffer (Tris 90 mM, pH 8.2, Borate 90 mM, EDTA 2 mM). PCR products were visualised using the silver-nitrate staining method as described by Tixier et al. (1997).

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