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Functionally associated molecular genetic marker map construction in perennial ryegrass (*Lolium perenne* L.)

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Abstract A molecular marker-based map of perennial ryegrass (*Lolium perenne* L.) has been constructed through the use of polymorphisms associated with expressed sequence tags (ESTs). A pair-cross between genotypes from a North African ecotype and the cultivar Aurora was used to generate a two-way pseudo-testcross population. A selection of 157 cDNAs assigned to eight different functional categories associated with agronomically important biological processes was used to detect polymorphic EST–RFLP loci in the $F_1(NA_6 \times AU_6)$ population. A comprehensive set of EST–SSR markers was developed from the analysis of 14,767 unigenes, with 310 primer pairs showing efficient amplification and detecting 113 polymorphic loci. Two parental genetic maps were produced: the NA_6 genetic map contains 88 EST–RFLP and 71 EST–SSR loci with a total map length of 963 cM, while the AU_6 genetic map contains 67 EST–RFLP and 58 EST–SSR loci with a total map length of 757 cM. Bridging loci permitted the alignment of homologous chromosomes between the parental maps, and a sub-set of genomic DNA-derived SSRs was used to relate linkage groups to the perennial ryegrass reference map. Regions of segregation distortion were identified, in some instances in common with other perennial ryegrass maps. The EST-derived marker-based map provides the basis for in silico comparative genetic mapping, as well as the evaluation of co-location between QTLs and functionally associated genetic loci.

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Introduction

The first-generation molecular marker-based genetic maps for agronomically important plant species have been largely based on anonymous genetic markers. In the case of the key temperate pasture grass species perennial ryegrass (*Lolium perenne* L.), the reference genetic map contains large numbers of amplified fragment length polymorphism (AFLP) and genomic DNA-derived simple sequence repeat (SSR) markers (Jones et al. 2001, 2002b), as well as heterologous restriction fragment length polymorphism (RFLP) markers, some of which correspond to unannotated cDNA sequences from other Poaceae species (Jones et al. 2002a). Although the current set of framework genetic markers provides the means to anchor maps across different pedigrees, to establish linkage with genes for agronomic traits and to detect quantitative trait loci (QTLs), they are not in general closely associated with variations in genes controlling phenotypic traits. Molecular variation based on functionally defined genes underlying specific biochemical or physiological functions will provide the next generation of molecular markers for forage species. The advantage of such markers, often described as 'candidate gene-based', is the promise of very close association with loci controlling variation for the trait in question, allowing the development of 'perfect markers' which may be used for linkage disequilibrium (LD) based mapping studies (Prioul et al. 1999; Thornsberry et al. 2001) and the direct selection of genotypes with superior allele content (Sorrells and Wilson 1997).

Functionally associated variation may be detected as RFLP, SSR polymorphism or single nucleotide polymorphism (SNP). In principle, any partial or full-length cDNA corresponding to a functionally defined gene may be used to detect RFLP between genotypes, through the judicious choice of one or more informative restriction endonucleases. RFLP is usually attributable to changes in non-coding sequences flanking the gene or to internal intron variation, and may be associated with DNA rearrangements generated by transposable genetic elements. A proportion of the expressed gene sequences represented in cDNA also contain SSR arrays, which may be located in the 5′- or 3′-untranslated regions (UTRs) as well as the coding sequence (CDS) (Cho et al. 2000; Scott et al. 2000; Cordeiro et al. 2001). Although SSRs derived from cDNAs may show lower levels of intraspecific polymorphism than those derived from non-coding genomic regions (Cordeiro et al. 2001; Eujayl et al. 2002), they provide the potential advantage of close linkage to significant gene variants.

The generation of large-scale gene sequence resources, especially collections of expressed sequence tags (ESTs) derived from randomly selected cDNA sequences, provides the opportunity for intensive development of functionally associated markers. A resource of 44,636 ESTs based on 5′-single pass sequencing has been established for perennial ryegrass. Cluster analysis based on a minimum criterion of 100 bp overlap has allowed the definition of approximately 14,767 'unigenes', including contigs with >2 sequences and singletons. Approximately 40% of the unigenes have been tentatively assigned to functional categories through BLASTN and BLASTX analysis (Sawbridge et al. 2003).

This resource is being utilised for RFLP-based mapping using functionally annotated cDNA probes identified by EST analysis. Complementary to this, the EST database has been exploited as an SSR marker development resource. We report here the development of a functionally associated marker-based genetic linkage map for perennial ryegrass, using EST–RFLP and EST–SSR marker technologies. A two-way pseudo-testcross family based on the cross of genotypes from two phenotypically divergent base populations has been used for construction of parental genetic maps. These maps have been aligned through the use of common loci and with the reference genetic map for this species through the use of genomic DNA-derived SSRs.

Materials and methods

Plant material

The perennial ryegrass population used for the genetic mapping of candidate genes was an F_1 progeny set derived from a reciprocal pair cross between the heterozygous parental genotypes North African$_6$ (NA_6), obtained from a Moroccan ecotype, and Aurora$_6$ (AU_6), obtained from a high water soluble carbohydrate (WSC) cultivar developed in the United Kingdom from a Swiss ecotype collected near Zürich (Tyler and Jones 1982). The cross was performed at DPI-Hamilton, and 157 progeny individuals were germinated and clonally propagated for DNA extraction and genetic analysis. Genomic DNA was extracted by the 1× CTAB method of Fulton et al. (1995).

RFLP analysis

Polymorphism was detected by Southern hybridisation. Genomic DNA (10 μg) from NA_6 and AU_6 and six F_1 progeny was digested with each of four different restriction enzymes (*Dra*I, *Eco*R I, *Eco*RV and *Hin*dIII). RFLP analysis was performed using isotopic detection with the oligolabelling method of Feinberg and Vogelstein (1984) and the hybridisation conditions of Sharp et al. (1988). Polymorphic probes were screened on a progeny set of up to 157 individuals.

Genomic DNA-derived SSR analysis

Perennial ryegrass genomic DNA-derived SSR (LPSSR) primer pairs ($n=151$) were used to evaluate genetic

polymorphism and for genetic mapping as described by Jones et al. (2001, 2002b).

EST–SSR discovery and primer design

Dinucleotide to pentanucleotide-based SSRs were identified in EST unigenes using the SSR detection programme *Sputnik* (http://rast.abajian.com/sputnik/), the core algorithm of which was re-implemented in Java 1.2 programming language to take advantage of file parsing and sequence manipulation features of the Biojava libraries (http://www.biojava.org). Primers for the identified SSRs were designed using *Primer 1.0* (http://www genome.wi.mit.edu/ftp/distribution/software/), to meet the following criteria: 18–26 bp in length, optimal melting temperature 60°C, G + C% of 40–60%, PCR product length 100–250 bp. Primer design excluded sections of ambiguous sequence, and SSRs located less than 30 nucleotides from one or the other ends of a unigene were also eliminated due to difficulty in the design of suitable primer pairs. For each SSR-containing EST unigene, BLASTN was used to find nucleotide homologues in the non-redundant GenBank/EMBL databases as well as in the TIGR *Arabidopsis* and rice tentative consensus (TC) sequences. BLASTN parameters were: e×1^{-10}, v×10, b×10.

EST–SSR analysis

A set of 480 primer pairs was selected, primarily on the basis of SSR array length, for screening in the $F_1(NA_6 \times AU_6)$ mapping population. Primer pairs were evaluated for amplification efficiency and polymorphism between the parental genotypes. In instances of indeterminate polymorphism type (e.g. one primer pair detecting a single locus of segregation type AB × AB, or two monomorphic loci with the allelic structures AA × AA and BB × BB), further evaluation of segregation was performed using a set of 12 progeny genotypes. Polymorphic primer pairs detected in screening were subsequently used to generate genotypic data for 157 F_1 individuals.

Primers were synthesised by Invitrogen (Carlsbad, Calif., USA). Forward primers were synthesised with a 21 nucleotide M13 tail sequence at the 5′-terminus (5′-TGTAAAACGACGGCCAGT-3′), to facilitate the universal labelling of PCR products by a fluorophore-labelled M13 primer (Schuelke 2000). Reverse primers were synthesised with the sequence 5′-GTTTCTT-3′ at the 5′-terminus end to promote non-templated adenylation at the 3′-terminus end of PCR product (Brownstein et al. 1996). PCR amplifications were conducted in a 10 µl reaction volume (96-well format) for polymorphism screening, and in an 8 µl reaction volume (384-well format) for genetic mapping. A Biomek 2000 liquid handling robot (Beckman Coulter Instruments, Fullerton, Calif., USA) was used to consolidate the PCR reactions. A 10 µl reaction volume contained 10 ng of genomic DNA, 1.5 mM magnesium chloride, 1× PCR buffer (Invitrogen), 0.05 mM of each dNTP, 0.0375 µM forward primer, 0.15 µM reverse primer, 0.15 µM of fluorescent-labelled M13 primer and 0.3 U of Platinum *Taq* DNA polymerase (Invitrogen). An 8 µl reaction contained the same components with volumes scaled by a factor of 0.8. The fluorophores used were 6-FAM, NED, VIC and PET (Applied Biosystems, Foster City, Calif., USA). PCR was carried out using iCyclers (Bio-Rad, Hercules, Calif., USA) with the following profile: (1) 94°C for 4 min, (2) 30 cycles of: 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, (3) 8 cycles of: 94°C for 30 s, 53°C for 30 s and 72°C for 30 s, (4) 72°C for 30 min (after Schuelke 2000).

The PCR products were analysed on an ABI 3100 Genetic Analyser using a 22 cm capillary array with POP-6 polymer (Applied Biosystems). In the screening phase, only one fluorophore was used for labelling PCR products and no pooling of products was conducted. In the mapping phase, PCR products labelled with each of the four fluorophores were quadruplexed using a Quadra 96 SV liquid handling system (Tomtec, Hamden, Conn., USA). Electropherograms were analysed using ABI Prism GeneScan (v 3.7, Applied Biosystems), and genotype data was scored using ABI Prism Genotyper (v 3.7, Applied Biosystems).

Linkage analysis

The $F_1(NA_6 \times AU_6)$ population was analysed as a two-way pseudo-testcross (Grattapaglia and Sederoff 1994). Genetic linkage maps were established for NA_6 and AU_6 using segregation data generated for up to 157 mapping population progeny, with polymorphic genomic DNA-derived SSR, EST–SSRs, EST–RFLP and functionally defined gene-based RFLP markers which could be derived as dominant features. Marker segregation ratios were checked for deviation from Mendelian expectation (1:1) by χ^2 analysis. Map construction was conducted using MAPMAKER 3.0 (Lander et al. 1987). Data sets were inverted and merged with the normally coded data in order to detect repulsion phase linkages. Using the *group* command, markers were grouped at a LOD threshold of 6.0, and were subsequently ordered within groups at LOD > 2.0 using the *order* command. Final marker orders were confirmed using the *ripple* command. Map distances in centimorgans were calculated using the Haldane mapping function (Haldane 1919). Polymorphic loci detected by the same SSR primer pair at similar locations on the maps of both parents were used to identify and align the homologous linkage groups in the NA_6 and AU_6 parental maps.

DNA sequence information and BLAST annotation data (Altschul et al. 1997) for functionally associated markers detected as RFLPs on the NA_6 and AU_6 genetic maps (as summarised in Table 2) is freely available on request from the corresponding author.

Results

Lolium perenne SSR evaluation and mapping data

A total of 151 *Lolium perenne* SSR (LPSSR) primer pairs were used to screen for genetic polymorphism using the parents and six F_1 progeny from the $F_1(NA_6 \times AU_6)$ mapping population. This included the 101 primer pairs previously demonstrated to detect segregating loci in the p150/112 reference population (Jones et al. 2002b), and an additional 50 primer pairs selected on the basis of simple monomorphic amplification patterns in the reference family. Ninety-two primer pairs (61%) detected segregating loci, with 55 primer pairs (36%) detecting monomorphic loci and the remainder failing to produce clear amplification products. The proportion of primer pairs detecting polymorphism varied between the LPSSRH library (68%), which is predominantly enriched for long, perfect dinucleotide repeat types and the LPSSRK library (58%), which is predominantly enriched for short, imperfect and trinucleotide repeat types (Jones et al. 2001), consistent with results from the reference population. Of the 55 primer pairs detecting monomorphic loci, 23 detected loci previously mapped in the reference population, and the majority (73%) were derived from the LPSSRK library.

The genotypic dataset for map construction was obtained using 72 of the original 92 primer pairs. Of these, 58 produced single locus amplification products while 14 primer pairs detected multiple polymorphic products defined as separate loci by subsequent genetic map analysis: ten primer pairs detected two loci, three primer pairs detected three loci and one primer pair detected four loci. The dataset consequently contained 91 segregating markers. Seven markers were ungrouped following map construction, with a total of 84 polymorphic loci detected by 65 primer pairs. A total of 53 loci were assigned to eight linkage groups (LGs) in the NA_6 parental genetic map and 54 loci were assigned to the AU_6 parental genetic map, with 23 loci detected by the same primer pair and assigned to the same LGs, providing the basis for map alignment (data not shown). The LGs of the parental maps were numbered in accordance with the reference map nomenclature (Jones et al. 2002b) through comparison of conserved SSR locus location. The most parsimonious solution was based on loci detected by 38 primer pairs, permitting assignment of LGs 1–6 of the NA_6 and AU_6 parental maps. The seventh LGs could not be readily assigned, as the majority of the primer pairs detecting loci on LG7 in the reference map failed to detect polymorphism in $F_1(NA_6 \times AU_6)$. This LG was consequently designated as LG7 by default.

A sub-set of 18 LPSSR loci were used in combination with the EST–RFLP and EST–SSR data for the construction of the combined genetic map (Table 1). The majority of these markers detected common loci between the two parental maps and conserved loci with the perennial ryegrass reference map. The LPSSR loci showed a range of allelic segregation types, and 12 of 18 (66%) exhibited at least one null allele giving rise to dominant-type segregation patterns.

RFLP evaluation and mapping data

Perennial ryegrass cDNAs from EST analysis were mapped as RFLP loci. These ESTs were selected on the basis of functional annotation by BLASTN/BLASTX and were classified in terms of core biochemical and physiological processes. In parallel, the same set is in use

Table 1 Properties of the LPSSR loci assigned to the parental genetic maps of NA_6 and AU_6. Nomenclature is as described in Jones et al. (2002b) and locus segregation type is as described in Table 5

SSR locus	SSR motif	Linkage group p150/112	Linkage group $F_1(NA_6 \times AU_6)$	Segregation type F_1 $(NA_6 \times AU_6)$	
				NA_6	AU_6
xlpssrk10f08	$(CAA)_{12}$	1	1 (NA_6 and AU_6)	AB	CD
xlpssrk15h05	$(TA)_4(CTA)_6$	1	1 (NA_6 and AU_6)	AB	CD
xlpssrh12g03	$(AC)_{21}$	1	1 (NA_6 and AU_6)	AB	AC
xlpssrk09f06.1	$(CT)_{15}$	2 and 4	2 (NA_6 and AU_6)	A0	B0
xlpssrh06h02	$(GT)_{10}$	3	3 (AU_6)	A0	B0
xlpssrhxx242	$(TG)_{12}$	3	3 (NA_6 and AU_6)	AB	CD
xlpssrh02d12	$(CA)_{12}$	3	3 (NA_6 and AU_6)	A0	BC
xlpssrk15f05.2	$(CAA)_{11}$	4 and 7	4 (NA_6)	A0	00
xlpssrk05a11.1	$(GAG)_6$	4	4 (NA_6)	AB	AC
xlpssrk03c05	$(GT)_9$	4	4 (NA_6)	AB	AA
xlpssrk02d08.2	$(GT)_{28}$	4	4 (AU_6)	00	A0
xlpssrk14c12	$(GT)_5$	5	5 (NA_6 and AU_6)	AB	AC
xlpssrk03b03.1	$(CA)_6$	5	5 (NA_6 and AU_6)	AB	C0
xlpssrh02h05	$(CA)_{25}$	6	6 (NA_6 and AU_6)	A0	BC
xlpssrk11g12	$(TC)_6..(TC)_{11}$	6	6 (NA_6 and AU_6)	AB	C0
xlpssrk05h01	$(CA)_8$	6	6 (NA_6)	AB	AC
xlpssrk10b07	$(TC)_7..(TC)_3$	6	6 (NA_6 and AU_6)	AB	CD
xlpssrh03a08.3	$(TG)_7..(TG)_6$	2	7 (NA_6 and AU_6)	A0	00

Table 2 Properties of the EST–RFLP loci mapped in the $F_1(NA_6 \times AU_6)$ cross and detected by functionally annotated cDNAs. The functional annotation classes are as follows: *1* cell wall metabolism, *2* carbohydrate metabolism. *3* floral development, *4* plant defence, *5* abiotic stress tolerance, *6* metal handling enzyme, *7* saline stress tolerance, *8* flavonoid biosynthesis. Genetic polymorphism in the parental genotypes is indicated as present (Y) or absent (N) with the following restriction endonucleases: *D* = *Dra*I, E_1 = *Eco*RI, E_5 = *Eco*RV; *H* = *Hin*dIII. Probes indicated with mapping enzymes but the entry *N/A* (not applicable) were not scored for the final genotypic set. Multiple genetic loci detected by single probes are indicated with the suffixes .1, .2 or .3, corresponding to dominant markers

EST name	Class	Putative function	Polymorphism in NA_6				Polymorphism in AU_6				Mapping enzyme	Number of loci	Map location NA_6	Map location AU_6
			D	E_1	E_5	*H*	*D*	E_1	E_5	*H*				
*Lp*4CLJa	1	4-Coumarate CoA ligase	N	N	N	Y	Y	N	N	Y	*Hin*dIII	1	4	4
*Lp*4CLJb	1	4-Coumarate CoA ligase	–	N	N	Y	Y	N	N	Y	*Hin*dIII	1	1	1
*Lp*4CLJc	1	4-Coumarate CoA ligase	Y	Y	N	N	Y	Y	N	–	*Dra*I	1	N/A	N/A
*Lp*C4H	1	Cinnamate-4 hydroxylase	Y	Y	Y	Y	Y	Y	Y	N	*Eco*RI	1	3	3
*Lp*CADc	1	Cinnamyl alcohol dehydrogenase	Y	Y	Y	Y	N	N	Y	N	*Eco*RV	1	1	–
*Lp*CADd	1	Cinnamyl alcohol dehydrogenase	Y	Y	Y	N	N	N	N	N	*Dra*I	1	3	–
*Lp*CADe	1	Cinnamyl alcohol dehydrogenase	Y	N	N	N	Y	N	N	N	*Dra*I	1	8	8
*Lp*CCoAOMT1	1	Caffeoyl-CoA O-methyl transferase	N	N	Y	N	N	N	N	N	*Eco*RV	1	7	–
*Lp*CCoAOMT2	1	Caffeoyl-CoA O-methyl transferase	N	N	Y	N	N	N	N	N	*Eco*RV	1	N/A	N/A
*Lp*CCoAOMTb	1	Caffeoyl-CoA O-methyl transferase	Y	–	Y	N	Y	Y	Y	Y	*Hin*dIII	1	N/A	N/A
*Lp*CCRa	1	Cinnamoyl CoA-reductase	Y	–	Y	Y	N	–	Y	Y	*Hin*dIII	1	6	6
*Lp*CCRb	1	Cinnamoyl CoA-reductase	Y	N	N	N	Y	N	N	N	*Dra*I	1	–	7
*Lp*CELL	1	Cellulase	–	Y	Y	Y	Y	Y	N	N	*Eco*RI	1	4	–
*Lp*F5H	1	Ferulate-5-hydroxylase	–	N	Y	N	N	N	N	N	*Eco*RV	2	3 (*Lp*F5H.1)	–
													1 (*Lp*F5H.2)	–
*Lp*OMT2	1	Caffeic acid O-methyltransferase	Y	Y	Y	Y	Y	N	N	–	*Dra*I	1	6	6
*Lp*OMT3	1	Caffeic acid O-methyltransferase	Y	Y	N	Y	Y	Y	Y	Y	*Dra*I	1	4	7
*Lp*PALa	1	Phenylalanine lyase	N	Y	N	Y	Y	N	N	Y	*Hin*dIII	1	N/A	N/A
*Lp*PALb	1	Phenylalanine lyase	Y	Y	Y	Y	Y	Y	Y	–	*Eco*RV	1	–	6
*Lp*Per1	1	Peroxidase	Y	Y	Y	Y	N	N	Y	Y	*Hin*dIII	1	2	2
*Lp*Per2	1	Peroxidase	N	Y	N	N	N	Y	N	N	*Eco*RI	1	N/A	N/A
*Lp*FFTa	2	Fructan fructan 1-fructosyltransferase	Y	Y	N	Y	Y	N	N	–	*Dra*I	2	4	4 (*Lp*FFTa.1)
													–	6 (*Lp*FFTa.2)
*Lp*FEH	2	Fructan exohydrolase	Y	Y	Y	Y	N	N	N	N	*Hin*dIII	1	3	3
*Lp*CWinv	2	Cell wall invertase	Y	N	N	N	N	Y	Y	Y	*Eco*RI	1	–	6
*Lp*INVa	2	Invertase	N	Y	N	N	Y	Y	N	N	*Eco*RI	1	N/A	N/A
*Lp*INVc	2	Invertase	Y	N	Y	Y	N	N	N	N	*Eco*RV	1	3	–
*Lp*INVe	2	Invertase	N	N	N	N	N	N	N	N	–	–	–	–
*Lp*INVf	2	Invertase	N	N	N	N	N	N	N	N	–	–	–	–
*Lp*INVg	2	Invertase	Y	N	Y	N	Y	N	N	–	*Dra*I	1	3	3
*Lp*SFTa	2	Sucrose fructan fructosyltransferase	N	N	N	Y	N	N	Y	Y	*Hin*dIII	2	–	3 (*Lp*SFTa.1)
													–	3 (*Lp*SFTa.2)
*Lp*SPSa	2	Sucrose phosphate synthase	N	N	N	N	Y	N	Y	N	–	–	–	–
*Lp*SPSb	2	Sucrose phosphate synthase	N	N	N	N	N	N	N	N	–	–	–	–
*Lp*SPSc	2	Sucrose phosphate synthase	N	N	N	N	N	N	N	N	–	–	–	–
*Lp*SPSd	2	Sucrose phosphate synthase	Y	N	N	Y	Y	N	N	Y	*Dra*I	1	N/A	N/A
*Lp*SPSe	2	Sucrose phosphate synthase	N	N	N	–	N	N	N	N	–	–	–	–
*Lp*SPSf	2	Sucrose phosphate synthase	Y	N	N	–	Y	N	N	N	*Dra*I	1	6	6
*Lp*SucSyn	2	Sucrose synthase	Y	Y	Y	Y	Y	Y	Y	N	*Dra*I	1	7	7
*Lp*SSa	2	Sucrose synthase	Y	Y	Y	Y	–	Y	N	Y	*Eco*RV	2	3 (*Lp*SSa.1)	–
													1 (*Lp*SSa.2)	–
*Lp*SSb	2	Sucrose synthase	Y	N	Y	Y	N	N	N	N	–	–	–	–

*Lp*SSc	2	Sucrose synthase	N	Y	Y	Y	Y	N	N	N	*Eco*RV	N/A	N/A	N/A
*Lp*SSTas	2	Sucrose sucrose fructosyltransferase	N	–	–	–	N	N	Y	N	–	–	–	–
*Lp*AP2b	3	Apetala-2	Y	Y	Y	Y	Y	Y	N	Y	*Hin*dIII	3	–	1 (*Lp*AP2b.1)
													–	1 (*Lp*AP2b.2)
													–	2 (*Lp*AP2b.3)
*Lp*CENa	3	Centroradialis	Y	N	N	N	N	N	N	N	*Dra*I	1	5	–
*Lp*HBa	3	Homeobox protein	–	–	Y	N	N	N	N	N	*Eco*RV	2	6 (*Lp*HBa.1)	–
													7 (*Lp*HBa.2)	–
*Lp*HBb	3	Homeobox protein	Y	Y	N	Y	N	Y	N	Y	*Eco*RI	1	5	–
*Lp*HBc	3	Homeobox protein	N	–	N	N	Y	N	N	N	–	–	–	–
*Lp*HBd	3	Homeobox protein	–	Y	Y	Y	–	–	N	Y	*Hin*dIII	1	6	6
*Lp*MADS1	3	MADS-box gene	Y	Y	Y	Y	N	N	N	N	*Eco*RV	1	3	–
*Lp*MADS2-1	3	MADS-box gene	Y	Y	Y	Y	Y	Y	N	–	*Dra*I	1	N/A	N/A
*Lp*MADS3	3	MADS-box gene	N	Y	Y	Y	N	Y	N	N	*Eco*RV	1	7	5
*Lp*MADS4	3	MADS-box gene	Y	Y	Y	Y	N	Y	N	N	*Eco*RI	2	7(*Lp*MADS4.1)	–
													7(*Lp*MADS4.2)	–
*Lp*MADS5	3	MADS-box gene	N	N	N	Y	N	Y	N	N	–	–	–	–
*Lp*MADSa	3	MADS-box gene	N	Y	N	Y	N	Y	N	Y	–	–	–	–
*Lp*MADSb	3	MADS-box gene	Y	Y	N	Y	Y	Y	N	Y	–	–	–	–
*Lp*MADSc	3	MADS-box gene	N	Y	N	N	N	N	N	N	–	–	–	–
*Lp*MADSd	3	MADS-box gene	Y	N	N	N	Y	N	N	N	–	–	–	–
*Lp*MADSf	3	MADS-box gene	N	Y	N	N	N	N	N	Y	–	–	–	–
*Lp*MADSh	3	MADS-box gene	N	N	N	N	N	N	N	N	–	–	–	–
*Lp*MADSk	3	MADS-box gene	N	N	Y	Y	N	Y	N	N	–	–	–	–
*Lp*DEFa	4	Proteinase inhibitor	Y	N	Y	Y	Y	N	N	N	*Dra*I	1	6	6
*Lp*DEFb	4	Anti-fungal protein	N	N	N	N	N	N	N	N	–	–	–	–
*Lp*DEFc	4	Flavonol 4-sulphotransferase	Y	N	Y	Y	Y	N	Y	–	*Eco*RV	1	5	5
*Lp*DEFd	4	Flavonol 4-sulphotransferase	Y	Y	Y	Y	N	Y	Y	Y	*Hin*dIII	1	N/A	N/A
*Lp*DEFe	4	Flavonol 4-sulphotransferase	Y	Y	Y	Y	Y	N	Y	Y	*Hin*dIII	1	N/A	N/A
*Lp*DEFf	4	Flavonol 4- sulphotransferase	Y	–	Y	–	Y	–	Y	–	*Dra*I	1	5	5
*Lp*ERa	4	Elicitor-responsive gene	Y	Y	Y	Y	Y	N	N	Y	*Eco*RV	1	2	2
*Lp*THa	4	Thionin	–	Y	N	–	–	Y	N	–	*Eco*RI	1	N/A	N/A
*Lp*THb	4	Thionin	Y	N	N	–	Y	N	N	–	*Dra*I	1	–	1
*Lp*THc	4	Thionin	Y	N	Y	Y	Y	N	N	Y	*Hin*dIII	2	7	7 (*Lp*THc.1)
													7	7 (*Lp*THc.2)
*Lp*TLa	4	Thaumatin-like	Y	Y	N	Y	Y	N	–	Y	*Dra*I	1	5	–
*Lp*TLb	4	Thaumatin-like	Y	N	N	–	Y	N	N	–	*Dra*I	1	–	5
*Lp*CHIa	4	Endochitinase	Y	Y	Y	Y	Y	N	Y	Y	*Eco*RV	1	6	–
*Lp*CHIe	4	Endochitinase	Y	Y	N	Y	Y	Y	Y	Y	*Hin*dIII	1	4	4
*Lp*CHIhs	4	Endochitinase	Y	Y	Y	Y	N	Y	Y	Y	*Eco*RI	1	1	–
*Lp*CHIJa	4	Endochitinase precursor	N	N	N	N	N	N	N	N	–	–	–	–
*Lp*CHIJb	4	Endochitinase precursor	Y	N	Y	Y	Y	N	N	N	*Dra*I	1	8	8
*Lp*CHIJc	4	Endochitinase precursor	N	N	N	N	N	N	N	N	–	–	–	–
*Lp*CHIJg	4	Endochitinase precursor	N	Y	N	N	Y	N	N	N	*Dra*I	1	N/A	N/A
*Lp*Glucb	4	1,4-glucanase	Y	Y	N	N	Y	N	N	N	*Dra*I	1	N/A	N/A
*Lp*Gluce	4	1,4-glucanase	N	N	N	N	Y	Y	N	Y	*Dra*I	1	N/A	N/A
*Lp*Glucf	4	1,4-glucanase	N	N	Y	N	N	N	N	N	*Eco*RV	1	N/A	N/A
*Lp*Glucg	4	1,4-glucanase	Y	N	Y	N	Y	Y	Y	Y	*Eco*RV	1	N/A	N/A
*Lp*Gluck	4	1,4-glucanase	Y	N	Y	N	Y	N	Y	Y	*Eco*RV	1	–	5
*Lp*Glucm	4	1,4-glucanase	Y	N	N	N	Y	Y	N	N	*Dra*I	1	N/A	N/A
*Lp*PIb	4	Proteinase inhibitor	Y	N	Y	Y	Y	N	N	N	*Dra*I	1	3	–
*Lp*PIc	4	Proteinase inhibitor	N	N	N	N	N	N	N	N	–	–	–	–

Table 2 (Contd.)

EST name	Class	Putative function	Polymorphism in NA_6				Polymorphism in AU_6				Mapping enzyme	Number of loci	Map location NA_6	Map location AU_6
			D	E_1	E_5	*H*	*D*	E_1	E_5	*H*				
*Lp*PId	4	Proteinase inhibitor	Y	Y	N	N	Y	N	N	N	*Dra*I	1	8	8
*Lp*PIf	4	Proteinase inhibitor	N	Y	Y	Y	Y	Y	N	Y	*Eco*RI	1	1	1
*Lp*PIh	4	Proteinase inhibitor	Y	Y	Y	Y	Y	Y	Y	Y	*Hin*dIII	1	3	5
*Lp*ASRa1	5	Abscisic stress ripening protein	Y	N	N	N	N	N	N	N	*Eco*RI	1	N/A	N/A
*Lp*ASRa2	5	Abscisic stress ripening protein	Y	Y	N	N	N	N	N	N	*Dra*I	1	4	–
*Lp*A22a	5	ABA and stress-inducible gene	Y	Y	Y	Y	Y	Y	Y	Y	*Eco*RV	2	7 (*Lp*A22a.1)	–
													7	7 (*Lp*A22a.2)
*Lp*A22b	5	ABA and stress-inducible gene	Y	Y	N	Y	Y	N	Y	N	*Dra*I	1	8	2
*Lp*A22c	5	ABA and stress-inducible gene	Y	Y	Y	N	N	N	N	N	*Eco*RV	1	4	–
*Lp*CYSa	5	Cysteine proteinase precursor	N	N	–	N	Y	Y	Y	Y	*Dra*I	1	–	7
*Lp*CYSme	5	Cystatin precursor	Y	N	N	–	Y	N	Y	N	*Hin*dIII	1	3	–
*Lp*LEAa	5	Late embryogenesis abundant protein	N	N	N	N	Y	Y	N	Y	*Eco*RI	1	–	7
*Lp*DHNa	5	Dehydrin	N	N	Y	N	N	Y	Y	Y	*Hin*dIII	2	6 (*Lp*DHNa.1)	–
													6 (*Lp*DHNa.2)	–
*Lp*PKABAa	5	ABA-inducible protein kinase	N	N	N	N	N	N	Y	N	*Eco*RV	1	N/A	N/A
*Lp*PKABAb	5	ABA-inducible protein kinase	Y	Y	Y	Y	Y	Y	Y	N	*Dra*I	1	2	2
*Lp*BCBa	6	Blue copper protein precursor	N	N	N	N	N	N	N	N	–	–	–	–
*Lp*CCHa	6	Copper transport protein	Y	N	Y	Y	Y	N	N	Y	*Hin*dIII	1	7	7
*Lp*CCHb	6	Copper transport protein	Y	N	Y	Y	Y	N	N	Y	*Hin*dIII	1	N/A	N/A
*Lp*CCHd	6	Copper transport protein	Y	N	Y	Y	Y	N	N	Y	*Hin*dIII	1	N/A	N/A
*Lp*CIa	6	Cadmium-induced protein	Y	Y	Y	Y	Y	N	Y	Y	*Eco*RI	1	7	7
*Lp*CTAa	6	Copper-transporting ATPase	N	N	Y	N	N	N	Y	N	*Hin*dIII	1	6	–
*Lp*CTRa	6	Copper transporter	N	Y	N	Y	N	N	N	N	*Eco*RI	1	N/A	N/A
*Lp*HAKa	6	High affinity potassium transporter	Y	N	Y	N	Y	N	Y	N	*Dra*I	1	4	–
*Lp*HAK1	6	High affinity potassium transporter	Y	Y	Y	N	Y	Y	Y	N	*Dra*I	1	3	3
*Lp*HISH3	6	Histone H3	Y	Y	N	N	N	N	Y	N	*Dra*I	1	2	2
*Lp*MTb	6	Metallothionein	N	N	N	N	N	N	N	N	–	–	–	–
*Lp*MTc	6	Metallothionein	Y	N	Y	Y	Y	Y	Y	Y	*Dra*I	1	3	3
*Lp*MTd	6	Metallothionein	Y	Y	Y	Y	Y	N	Y	Y	*Hin*dIII	1	1	1
*Lp*MTe	6	Metallothionein	Y	Y	Y	N	Y	Y	N	N	*Dra*I	–	–	–
*Lp*MTf	6	Metallothionein	N	N	N	N	N	N	N	N	–	–	–	–
*Lp*MTg	6	Metallothionein	Y	Y	Y	Y	Y	Y	Y	Y	*Dra*I	1	3	3
*Lp*MTh	6	Metallothionein	Y	Y	N	N	N	Y	Y	Y	*Hin*dIII	–	–	–
*Lp*MTi	6	Metallothionein	N	N	N	N	N	N	N	N	–	–	–	–
*Lp*MTj	6	Metallothionein	Y	Y	Y	Y	Y	N	Y	N	*Dra*I	2	3 (*Lp*MTj.1)	–
													8 (*Lp*MTj.1)	–
*Lp*MTl	6	Metallothionein	Y	Y	Y	Y	N	N	N	N	*Eco*RV	2	–	3 (*Lp*MTl.1)
													3	3 (*Lp*MTl.2)
*Lp*MTn	6	Metallothionein	Y	Y	Y	Y	Y	Y	Y	Y	*Eco*RV	1	3	3
*Lp*MTo	6	Metallothionein	Y	N	Y	Y	Y	Y	Y	N	*Dra*I	1	N/A	N/A
*Lp*PCSa	6	Pentose isoprimeverose cation-symporter	Y	N	Y	N	Y	Y	Y	Y	*Eco*RV	1	–	1
*Lp*PCSb	6	Pentose isoprimeverose cation-symporter	Y	N	Y	N	Y	Y	Y	Y	*Dra*I	–	–	–
*Lp*WALIa	6	Wound-induced protease	N	N	Y	Y	Y	N	Y	Y	*Hin*dIII	–	–	–
*Lp*WALIb	6	Wound-induced protease	Y	Y	Y	Y	Y	N	Y	Y	*Dra*I	1	2	–
*Lp*WALIc	6	Wound-induced protease	Y	N	N	Y	N	N	N	N	*Hin*dIII	1	1	–
*Lp*WALId	6	Wound-induced protease	Y	N	Y	Y	N	N	N	N	*Hin*dIII	–	–	–
*Lp*WALIe	6	Wound-induced protease	N	Y	Y	Y	Y	N	Y	Y	*Hin*dIII	1	1	–

*Lp*WALIf	6	Wound-induced protease	Y	Y	N	N	Y	N	N	Y	*Dra*I	–	–	–
*Lp*WALIg	6	Wound-induced protease	Y	N	N	Y	N	N	N	N	*Dra*I	1	N/A	N/A
*Lp*WALIh	6	Wound-induced protease	Y	Y	N	N	N	N	N	Y	*Eco*RI	1	2	–
*Lp*WALIi	6	Wound-induced protease	N	Y	Y	Y	Y	N	Y	Y	*Hin*dIII	–	–	–
*Lp*ZBa	6	Zinc-binding protein	Y	Y	Y	Y	Y	Y	Y	Y	*Eco*RV	1	4	4
*Lp*ZBb	6	Zinc-binding protein	Y	N	N	Y	Y	N	N	Y	*Hin*dIII	1	–	7
*Lp*ZTa	6	Zinc transporter	Y	Y	N	Y	N	N	N	N	*Dra*I	1	3	–
*Lp*ALDPa	7	Fructose biphosphate aldolase	N	Y	N	N	Y	Y	N	N	*Eco*RI	1	–	4
*Lp*CSAa	7	Glutathione peroxidase	N	Y	Y	N	N	N	N	N	*Eco*RI	–	–	–
*Lp*CSAb	7	Glutathione peroxidase	Y	N	N	N	N	N	N	N	*Dra*I	–	–	–
*Lp*ESI3a	7	Low temperature-induced protein	Y	Y	N	N	N	N	N	N	*Eco*RI	1	N/A	N/A
*Lp*ESI3b	7	Hydrophobic protein	Y	Y	N	N	N	Y	Y	Y	*Eco*RI	1	–	5
*Lp*ESI3c	7	Hydrophobic protein	N	N	N	N	N	N	N	N	–	–	–	–
*Lp*ESI3d	7	Low temperature-induced protein	N	N	N	Y	N	N	Y	Y	*Hin*dIII	1	N/A	N/A
*Lp*ESI3e	7	Hydrophobic protein	Y	N	Y	Y	N	Y	N	Y	*Hin*dIII	1	1	1
*Lp*ESI3f	7	Low temperature-induced protein	Y	N	Y	Y	N	N	Y	Y	*Eco*RV	1	1	1
*Lp*ESI3 g	7	Hydrophobic protein	Y	Y	N	N	N	Y	Y	Y	*Eco*RI	1	5	5
*Lp*ESI3 h	7	Hydrophobic protein	Y	N	N	N	N	Y	Y	N	*Dra*I	1	5	–
*Lp*LT16Aa	7	Hydrophobic protein	Y	N	N	N	Y	N	Y	Y	*Dra*I	1	1	1
*Lp*LT16Ab	7	Hydrophobic protein	Y	N	N	Y	Y	N	Y	Y	*Hin*dIII	1	1	1
*Lp*LT16Ba	7	Hydrophobic protein	Y	N	N	Y	N	N	Y	N	*Eco*RV	1	2	–
*Lp*LT16Bb	7	Hydrophobic protein	Y	Y	Y	Y	N	N	N	N	*Eco*RV	1	2	–
*Lp*SALTa	7	Salt stress-induced protein	Y	N	N	Y	Y	N	N	N	*Dra*I	2	1 (*Lp*SALTa.1) 6 (*Lp*SALTa.2)	1 –
*Lp*SALTb	7	Salt stress-induced protein	N	Y	N	Y	Y	N	N	Y	*Hin*dIII	1	–	5
*Lp*WESR5a	7	Glucose-phosphate-1-dehydrogenase	Y	Y	Y	Y	N	Y	Y	Y	*Eco*RI	1	–	2
*Lp*WESR5b	7	Glucose-phosphate-1-dehydrogenase	Y	Y	Y	Y	Y	Y	Y	Y	–	–	–	–
*Lp*WESR5c	7	Glucose-phosphate-1-dehydrogenase	Y	Y	Y	Y	Y	Y	Y	Y	–	–	–	–
*Lp*DFRb	8	Dihydroflavonol 4-reductase	N	Y	N	Y	N	N	N	Y	*Hin*dIII	1	6	6
*Lpp*F3Ha	8	Flavanone 3-beta-hydroxylase	N	N	N	N	N	N	N	N	–	–	–	–

for a number of functional genomic screens including microarray-based expression profiling and transgenic modification (Sawbridge et al. 2003). A total of 157 partial or full-length cDNAs from the following functional categories were screened for RFLP (Table 2): cell wall metabolism (including lignin biosynthesis), carbohydrate metabolism (including fructan biosynthesis), floral development (including homeotic genes), plant defence (including chitinases, proteinase inhibitors, defensins etc.), abiotic stress tolerance (including dehydrins and LEAs), metal handling enzymes (including metallothioneins), salt stress protection and flavonoid biosynthesis (including chalcone synthase and dihydroflavone reductase genes). A total of 144 probes detected polymorphism in the $F_1(NA_6 \times AU_6)$ cross (92%), with 137 detecting heterozygosity in NA_6 (87%) and 121 detecting heterozygosity in AU_6 (77%). Variable levels of heterozygosity were detected with different restriction enzymes in each parent: for NA_6, the values were 64% for *Dra*I, 48% for *Eco*RI, 49% for *Eco*RV and 52% for *Hin*dIII, while for AU_6 the corresponding values were 52% for *Dra*I, 33% for *Eco*RI, 38% for *Eco*RV and 41% for *Hin*dIII. A substantial number of probes detected small multigene families, with several potential polymorphic loci segregating from each parent.

In addition to the EST–RFLPs, two previously described functionally defined genes involved in carbohydrate biosynthesis [*Lp*FT1 (fructosyltransferase), Lidgett et al. 2002 and *Lp*FT2 (invertase), Johnson et al. 2003] that had been assigned to map positions in the p150/112 reference population were mapped in the $F_1(NA_6 \times AU_6)$ cross using the restriction enzymes *Eco*RV and *Eco*RI respectively.

From the 144 EST–cDNA probes that detected genetic polymorphism, 116 were selected as hybridisation probes for genetic mapping in the full $F_1(NA_6 \times AU_6)$ population. The probes were selected on the basis of polymorphism, clarity of signal and non-redundant hybridisation pattern. From this group, some probes were further excluded due to low hybridisation signal. The final data set for genetic map construction contained 96 EST–RFLP loci segregating from NA_6 and 70 EST–RFLP loci segregating from AU_6. Following genetic map construction (see below), 85 loci were assigned to the NA_6 genetic map and 67 loci were assigned to the AU_6 genetic map. When a single probe detected a segregating locus assigned to the same linkage group in each parental map, the loci were assumed to be homologous. A total of 40 loci were identified in this category. The proportions of mapped loci corresponding to each functional class were determined (Table 3). Twelve probes detected multiple loci (Tables 2, 3).

EST–SSR evaluation and mapping data

Of the 14,767 EST unigenes defined in the perennial ryegrass EST database (Sawbridge et al. 2003), 1,591 (11%) were identified as SSR-containing (EST–SSR), and primer pairs were successfully designed for 1,175 (74%) of these (representing 8% of EST unigenes).

In contrast to the development of the RFLP marker set, selection of EST–SSRs for genetic mapping analysis was based primarily on polymorphism, with putative gene function not being considered. A total of 480 EST–SSR primer pairs, ranking highest in terms of SSR array length, were screened for amplification efficiency and polymorphism in the $F_1(NA_6 \times AU_6)$ population, and of these 310 (65%) obtained clear and efficient PCR amplification profiles. A significant majority (67%) of the 480 EST–SSR loci used for initial screening contained trinucleotide repeat arrays, reflecting the overall trend in the full set of 1,175 unigenes (Table 4). Similarly, most (62%) of the screened EST–SSRs had a repeat number of less than six, although selection bias towards longer array lengths ensured that this proportion was significantly lower than that observed in the full EST–SSR set (mean repeat number 4.7 ± 1.88 SD) (Table 4). Of the 310 EST–SSR primer pairs that showed efficient amplification, 130 (42%) detected polymorphic loci (heterozygous in one or both parents) in the $F_1(NA_6 \times AU_6)$ cross. Although there were large disparities in the absolute numbers of EST–SSRs in each class, polymorphism was apparently highest amongst EST–SSRs with a dinucleotide repeat motif, and was positively associated with increasing motif repeat number (Table 4).

A total of 122 of the 130 polymorphic EST–SSR primer pairs detected 128 loci that could be coded as

Table 3 Distribution of mapped EST–RFLP loci by functional annotation class. The eight categories are as described in legend for Table 2

Annotation class	Number of mapped loci NA_6	Number of mapped loci AU_6	Number of common mapped loci	Number of multiple mapped loci
1	14	10	6	1
2	8	9	5	3
3	9	5	1	3
4	15	14	10	1
5	9	5	2	2
6	19	13	10	1
7	10	10	5	1
8	1	1	1	0
Total	85	67	40	12

Table 4 Properties of EST–SSR primer pairs, based on all designed primer pairs ($n = 1{,}175$), evaluated primer pairs ($n = 480$) and proportion of evaluated primer pairs detecting genetic polymorphism in the $F_1(NA_6 \times AU_6)$ cross ($n = 130$)

EST–SSR type	All primer pairs (% of total)	Evaluated primer pairs (% of total)	Primer pairs detecting polymorphism (% EST–SSR type)
Dinucleotide motif	132 (11)	49 (10)	18 (37)
Trinucleotide motif	748 (64)	321 (67)	85 (26)
Tetranucleotide motif	163 (14)	60 (13)	14 (23)
Pentanucleotide motif	132 (11)	50 (10)	13 (26)
Repeat number < 6	920 (78)	300 (62)	71 (24)
Repeat number $\geq 6 < 10$	238 (21)	163 (34)	52 (32)
Repeat number ≥ 10	17 (1)	17 (4)	7 (41)
Total EST–SSRs	1,175	480	130 (27)

Table 5 Properties of the EST–SSR loci mapped in the $F_1(NA_6 \times AU_6)$ cross, based on the number and proportion of different segregation types (after Maliepaard and Van Ooijen 1994), and the type, number and proportion of variant structures with one or more null alleles

Locus segregation type F_1 ($NA_6 \times AU_6$)		Number (% total)	Null allele subclasses		Number (% segregation type)
NA_6	AU_6		NA_6	AU_6	
AA	AB	23 (20)	00	A0	5 (22)
AB	AA	30 (27)	A0	00	3 (10)
AA	BC	6 (5)	00	AB	1 (17)
BC	AA	14 (12)	AB	00	2 (14)
			A0	BB	2 (14)
AB	AC	24 (21)	AB	A0	2 (8)
			A0	AB	3 (13)
AB	CD	17 (15)	AB	C0	4 (24)
			A0	BC	5 (29)
Total		114 (100)			27 (24)

dominant genetic markers. The remaining eight EST–SSR primer pairs detected loci of the segregation type AB × AB, which cannot be represented as dominant markers, and were consequently not used further for genetic linkage analysis. Fourteen of the 122 polymorphic EST–SSRs were not used due to the presence of putative introns in one or more amplicon alleles.

A residual group of 108 EST–SSR primer pairs were used for the construction of the genetic linkage map. When derived as dominant markers, this sub-set detected a total of 114 loci: 85 EST–SSR loci segregated in the NA_6 gametes, 72 in the AU_6 gametes, with 43 of those loci being common to both parents (Table 5). One hundred and three of the EST–SSR primer pairs detected only one marker locus, with three (pps0023, pps0411 and pps0439) detecting three loci each and one (pps0098) detecting two loci. For 24% ($n = 27$) of all loci detected, the presence of one or more putative null alleles was inferred (Table 5). Primer DNA sequences for a selection of mapped EST–SSR loci are provided in Table 6.

Amongst the mapped EST–SSRs, 47% were derived from ESTs with functionally annotated BLASTN hits ($e < 1^{-10}$) in other plant species, predominantly *Arabidopsis* (18%), followed by rice (16%) and other Poaceae species (10%). A further 21% yielded non-annotated hits in either *Arabidopsis* or rice, and the remaining 32% showed no homology to sequences in the databases interrogated.

Construction of the NA_6 and AU_6 genetic linkage maps

Separate genetic linkage maps were constructed for the partially heterozygous parental genotypes NA_6 and AU_6, using EST–SSR and EST–RFLP markers. Maps for both parents were obtained at LOD 6.0, with the NA_6 map containing 85 EST–RFLP and 71 EST–SSR marker loci, and the AU_6 map comprising 67 EST–RFLP and 59 EST–SSR marker loci (Fig. 1, Table 7). For NA_6, 11 EST–RFLP and 14 EST–SSR markers did not link to any other marker at the LOD threshold applied, and for AU_6 three EST–RFLP and 13 EST–SSR markers remained unlinked. Overall, the NA_6 map contained 27% more marker loci than AU_6 (Table 7), indicating a higher level of heterozygosity in this genotype.

On both maps, seven major linkage groups and a significantly smaller eighth linkage group were detected, giving a total map length in NA_6 of 963 cM, and in AU_6 of 757 cM (Fig. 1). The variation in map lengths was reflected in map distances between non-overlapping pairs of bridging loci (data not presented), which were greater in NA_6 for 16 out of 21 pairs (Fig. 1), by a mean of 6.1 cM ± 2.23 SEM. Additionally, six of the eight linkage groups were longer in NA_6 than AU_6 (Table 7). The difference in total map length combined with the smaller number of markers located on the AU_6 map ensured that the mean marker density for the two maps was similar, with one locus every 5.5 cM in NA_6 and every 5.4 cM for the AU_6 map (Table 7). Irregular distribution of marker loci was observed within and between linkage groups in each parental map, and between parental homologues (Table 7).

Homologous linkage groups from NA_6 and AU_6 were identified and aligned using common allelic bridges, provided by SSR marker loci that were heterozygous in both genotypes (segregation types AB × AC and AB × CD) (Fig. 1). The position of allelic bridges was predominantly conserved between the parental genotypes, except on LG1 where the position of two LPSSR loci was confounded by ambiguous ordering (Fig. 1). Linkage group assignments were based tentatively on those defined in the perennial ryegrass reference genetic linkage map (Jones et al. 2002a, b) through the use of the LPSSR loci, of which 18 are indicated in Fig. 1. In addition, the RFLP loci detected by the

Table 6 Primer sequences and locus map position data for 20 EST–SSR markers mapped in the $F_1(NA_6 \times AU_6)$ population. The top sequence of each primer pair is the forward primer, and the bottom sequence is the reverse primer

SSR	SSR motif	Linkage group	Amplicon size (bp)		Forward and reverse primers (5′ → 3′)
			Predicted	Observed range	
pps0094	[CGC]	1 (NA_6 and AU_6)	101	104–114	GGTATTCCTCTCTCGCCCTCTCGAATCGGATAAACAACCC
pps0286	[GGA]	1 (NA_6 and AU_6)	167	162–168	CGAAGAGGCGGTAGATCTTGAGCAAGCCAAGCAGAGAG
pps0174	[TTTTC]	1 (NA_6)	250	249–250	CGTACGTTGCTATTGAGGCAATTGTTCATTTGGTACGTTCCC
pps0080	[CATCC]	2 (NA_6 and AU_6)	210	195–207	GCAAGAAGACGTGGAGGAAGAAACCAAATCAACAGGCGAG
pps0223	[CCCTG]	2 (NA_6 and AU_6)	247	228–246	CCTGACCTGAAGATTCTCGCCTCTTTCCTCTTTCGTCTCTCG
pps0259	[TGC]	2 (NA_6 and AU_6)	192	190–198	GCGTAGTCGTCGAAGAGGTCCATCGACTACGACCACATGG
pps0007	[TGAT]	3 (NA_6 and AU_6)	224	208–220	CACCAAGTACATCACCGTCGCGAACACATTCATGAGTCGG
pps0164	[TC]	3 (NA_6 and AU_6)	179	180–185	GGCTTGCTTCTTCCTTCCTTACATGACTTCTCTTGAGGAAATCC
pps0213	[AT]	3 (NA_6)	184	167–185	CTGATTCTCCCAAAACTTACCGTGTAGCAGAGCCCAGGCTAT
pps0006	[AG]	4 (NA_6 and AU_6)	218	222–248	GGAATTTTCTGGGTGGGATTATCGGAGTATATAGACGAGTGTTGC
pps0040	[ATG]	4 (NA_6 and AU_6)	243	239–248	AAGAAGGTACAAGAAGCATTGACCCCTCTTCTTCTTTTAGCACAAGC
pps0433	[TC]	4 (NA_6 and AU_6)	163	162–169	GCGACTCTCGTTTCCTTGTCTGCAATCAGAGTTTCATCACG
pps0397	[GTA]	5 (NA_6 and AU_6)	246	226–253	GGAGGAGGATTCAGGTAGGGACTTAAATCCATCCACCCACC
pps0199	[AGA]	5 (NA_6)	230	228–231	CAGTAAGAAGAAGGGATGGGCACAACTAGATCTTTCACCTCCAGG
pps0210	[GCT]	6 (NA_6 and AU_6)	187	183–186	GGTGAGTGGGCGTTAGCTAGAATAGATGGAAATCGTGCGC
pps0299	[GT]	6 (NA_6 and AU_6)	189	189–191	CTGCGACTCTGTAAATGACAGGACAGAAAGCTAGGACTTGGCC
pps0374	[GGT]	6 (NA_6 and AU_6)	202	203–207	GATTCGAGCAAGTTGATGCAGCCTGGACTACGCTCAAGAC
pps0049	[AGGCG]	7 (NA_6 and AU_6)	141	135–144	CTCCCCATTTACTCCAAACGGGTAGTAGTGCTGCACGAAGG
pps0376	[GCT]	7 (NA_6 and AU_6)	191	181–189	GGTCATGATACGCTGGACCTCGAAAACAAAACCCTGTCGT
pps0447	[CA]	7 (NA_6)	215	212–218	ACACAAGGTTGTCATGGCAATGTTCCAGTTTGTGTTTGTTCC

*Lp*FT1 and *Lp*FT2 carbohydrate biosynthesis genes were assigned to the distal regions of LG7 and LG6 on the NA_6 parental map, respectively. The alignments between the corresponding LGs of the NA_6, AU_6 and p150/112 maps are shown in further detail in Fig. 2, including the locations of an additional set of informative genomic DNA-derived SSR loci that showed lower confidence of ordering during final map construction.

On both maps there were a number of markers that could not be ordered at the applied threshold of LOD > 2.0, and these were distributed across all linkage groups except for NA_6 groups LG5 and LG8 and AU_6 group LG8 (Fig. 1). On the NA_6 map, 22 regions contained one or more unordered markers. These regions covered map distances of 3.1–26.0 cM, accounting for 303 cM (31%) of the total map length. For AU_6, there were 16 such regions, ranging in length from 0.9 to 33 cM, and covering 226 cM (30%) of the map in total.

Distorted segregation ratios were evident for both EST–SSR and EST–RFLP marker loci, with 41 loci (26%) mapped in NA_6 and 20 loci (16%) mapped in AU_6 exhibiting distorted ratios at $P < 0.05$ (Fig. 1). Segregation distortion at $P < 0.01$ occurred for 25 (16%) and 16 (12%) loci mapped in NA_6 and AU_6, respectively, and at $P < 0.001$ eight (5%) loci in NA_6 showed segregation distortion compared with four (3%) in AU_6. Segregation distortion was not substantially influenced by marker type, with 24% of all SSR loci (LPSSRs or EST–SSRs) having distorted segregation ratios at $P < 0.05$, compared with 20% of all EST–RFLP loci. The majority of the distorted markers were localised to regions on NA_6 linkage groups LG2, LG3 and LG4 (73%), and AU_6 linkage group LG5 (40%), with the remainder distributed randomly throughout the genome (Fig. 1). Within each segregation distortion region, distortion was unidirectional, favouring alleles exclusively from one phase (data not presented).

Discussion

We have exploited an EST database to generate a functionally associated marker-based genetic linkage map for perennial ryegrass. Expressed sequences from perennial ryegrass that had been functionally classified based on sequence analysis were used to develop EST–RFLP markers, and a set of EST–SSR markers was derived from the same resource in a complementary SSR discovery initiative. These markers were located in the perennial ryegrass genome by genetic linkage analysis in the $F_1(NA_6 \times AU_6)$ two-way pseudo-testcross population.

EST functional categories

The partial and full-length cDNAs from EST analysis were selected for EST–RFLP development on the basis

of a number of functional categories that are related to important agronomic traits in perennial ryegrass. Each annotated EST represents a gene in a biosynthetic pathway, and co-location of these ESTs with QTLs for targeted traits provides a means for the association of phenotype with molecular variation in functionally defined gene sequences. The composition of cell walls, particularly the content and cross-linking of lignin, is an important determinant of herbage digestibility (Buxton and Russell 1988), while the biosynthesis of soluble oligosaccharides such as fructans is of key importance for the energy provision to the grazing animal (Michell 1973; Jones and Roberts 1991). The study of floral development is of significance for the seasonal variation in yield associated with reproductive maturity in pasture grasses, as well as offering the potential for the regulation of transgene spread (Giddings 2000) through facultative control of flowering. Biotic stresses due to infection with fungal pathogens such as crown rust (*Puccinia coronata* Corda f.sp. *lolii* Brown) and viral pathogens such as ryegrass mosaic virus (RMV) reduce the yield, nutrient quality and palatability of herbage (Plummer et al. 1990; Potter 1987; Potter et al. 1990; Price 1987). Tolerance to abiotic stresses such as drought, salinity and toxic metals limit the extent and efficiency of perennial ryegrass cultivation in many temperate regions. For each functional category, EST–RFLP loci were assigned to the genetic map, with the largest number contributed by the metal handling enzyme class and the lowest number contributed by the flavonoid biosynthesis class. There was little apparent evidence for clustering of ESTs from the same functional category, except for a preponderance of metalllothionein EST–RFLP loci in the upper parts of LG3 on each parental map.

In contrast to the functional criteria used for the selection of cDNAs for RFLP mapping, the EST–SSRs were identified in unigenes on the basis of SSR array structure. Nevertheless, 47% of the SSR-containing ESTs that were mapped were capable of functional annotation through BLASTN analysis. The locations of those EST–SSR loci that do show significant similarity to annotated sequences will provide information for other functional categories in addition to those already defined. In addition, the present expansion of public domain biological information will permit the continuous reassessment of currently unannotated EST–SSR sequences.

EST–SSR development and mapping

The frequency of EST–SSRs identified in the perennial ryegrass EST database (11%) is low compared with the frequency of SSRs identified in enriched perennial ryegrass genomic libraries (39%, Jones et al. 2001), but exceeds the range reported for EST–SSR development in a number of Poaceae species, including barley, rye, rice, sorghum, sugarcane, wheat and maize (1.5–8.5%) (Cordeiro et al. 2001; Hackauf and Wehling 2002; Holton et al. 2002; Kantety et al. 2002; Thiel et al. 2003), as well as grape (2.5%, Scott et al. 2000). This figure is likely to be biased towards a higher value through the inclusion of a large number of perennial ryegrass EST–SSR arrays that contain fewer than six repeats (Table 4), as it is typical to exclude SSR arrays shorter than 20 bp in length from genetic mapping analysis (e.g. Temnykh et al. 2001). It is noteworthy that 24% of the EST–SSRs evaluated in the $n < 6$ repeat number class (96% with arrays shorter than 20 bp) detected polymorphism in the $F_1(NA_6 \times AU_6)$ population. This observation suggests that for SSR resources of limited size, discrimination on the basis of SSR array length should be avoided.

The majority of perennial ryegrass EST–SSR arrays contained trinucleotide repeat motifs, with low repeat numbers. The predominance of EST–SSRs with trinucleotide repeat motifs (64%) is consistent with observations in other plant species (Kantety et al. 2002; Morgante et al. 2002; Thiel et al. 2003; Barrett et al. 2004), and reflects the selection against frameshift mutations in coding regions caused by length changes in non-trinucleotide SSRs, and/or positive selection for single amino acid arrays (Metzgar et al. 2000; Morgante et al. 2002).

Effective use of the EST–SSR resource was limited by a high rate of attrition between the stages of primer design and successful PCR amplification, with 35% of the 480 primer pairs screened generating no clear amplicon. This figure is high compared with those from genomic DNA-derived SSRs developed in plant species (mean value 19.5%, Squirrel et al. 2003), including perennial ryegrass (19%, Jones et al. 2001), as well as EST–SSRs from maize (12–14%, Sharapova et al. 2002). However, it compares favourably with data for EST–SSR resources from rye (26%, Hackauf and Wehling 2002), barley (36%, Thiel et al. 2003) and sugarcane (40%, Cordeiro et al. 2001). The reduced number of primer pairs showing efficient amplification in the current study may be attributable to limitations of the selected primer design software, primer development from sequence of marginal quality, or the disruption of primer sites by intron-exon splice sites. A polymorphism rate amongst efficiently amplified EST–SSRs of only 42% eliminated a further subset of primer pairs. Although this figure is low compared with genomic DNA-derived SSR resources (e.g. 67% for perennial ryegrass, Jones et al. 2001), such a discrepancy in informative value between genomic- and EST-derived SSRs is typical for plant species, including rice (Cho et al. 2000), wheat (Eujayl et al. 2002) and tomato (Areshchenkova and Ganal 2002), and in the present study may be further attributed to the use of only two genotypes for screening.

Although the EST–SSR markers are less efficient than perennial ryegrass genomic DNA-derived SSRs in terms of frequency of SSR discovery, development of functional primer pairs, and polymorphism detection, these demerits are balanced by the relatively low expense

Fig. 1 Genetic linkage maps of the NA_6 and AU_6 parents of the $F_1(NA_6 \times AU_6)$ cross. LPSSR markers are indicated as xlpssr loci using the nomenclature described by Jones et al. (2002b). EST–RFLP markers are indicated with *xlp* (*Lolium perenne*) prefixes and gene-specific abbreviations as described in Table 2. The eight functional annotation classes are *colour-coded*. EST–SSR markers are indicated with *xpps* prefixes; suffix '*y*' indicates a codominant locus of segregation type AB × AC, '*x*' indicates segregation type AB × CD, *all other suffixes* indicate a dominant (single dose) locus. Codominant SSR loci used as bridging loci to align LGs are joined by *black lines*. Common EST–RFLP loci between homologous groups are shown as *grey lines* between LGs. Loci showing segregation distortion are shown with *asterisks* to indicate level of significance. *Bracketed regions* indicate equivocal ordering at LOD > 2.0

associated with EST–SSR development as a by-product of a genomics database. In addition, the value of EST–SSRs compared to genomic DNA-derived SSRs is enhanced by: (1) superior transferability across taxon boundaries, as demonstrated in grape (Arnold et al. 2002; Decroocq et al. 2003), sugarcane (Cordeiro et al. 2001) and white clover (Griffiths et al. 2002), and (2) by their potential as 'perfect markers' for functionally defined genes involved in determining agronomic traits.

Genetic linkage map

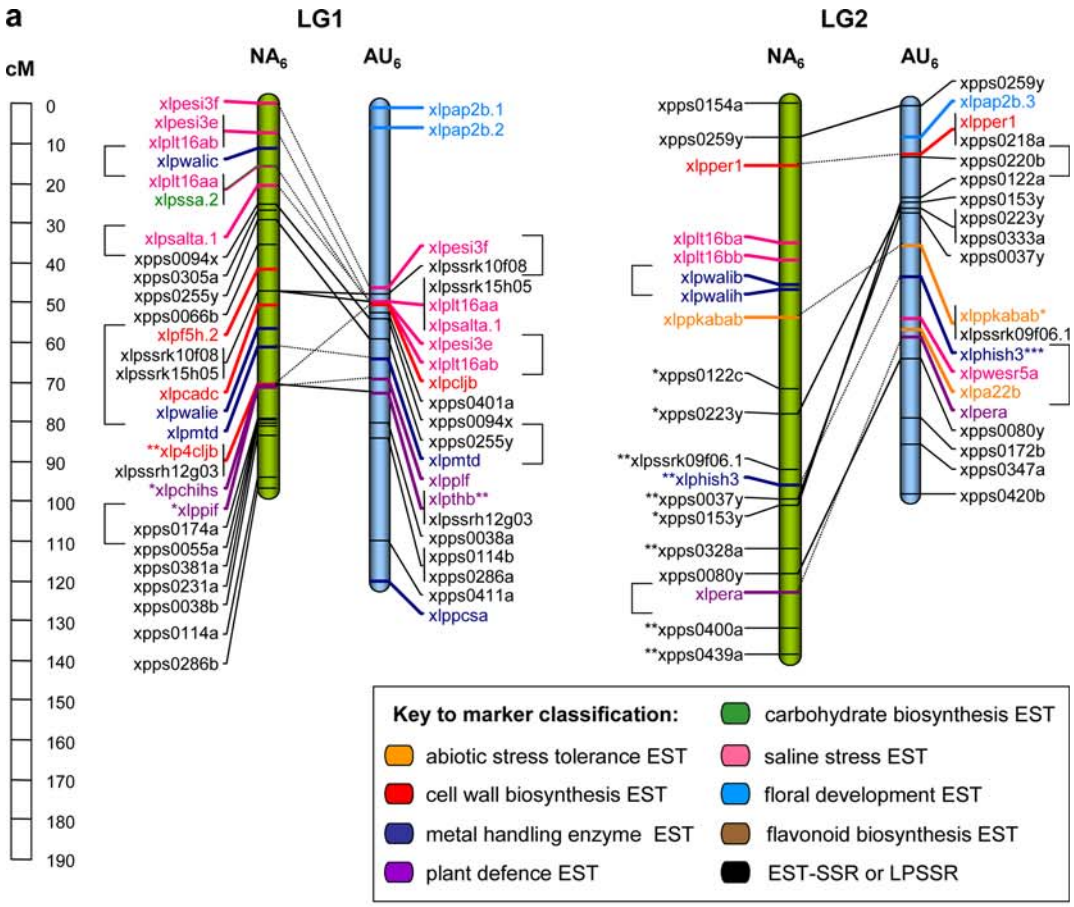
Separate maps were developed for the parental genotypes NA_6 and AU_6, with the total map length for the NA_6 parent (963 cM) being 27% longer than that of AU_6 (757 cM), and 20% longer than the perennial ryegrass reference map (approximately 800 cM, Jones et al. 2002a, b). This may reflect either structural polymorphism in the NA_6 genome, or a higher recombination frequency in NA_6, as suggested by differences between NA_6 and AU_6 in centimorgan map distances between pairs of bridging loci. Variation in recombination frequency between different genetic backgrounds, either genome-wide or in specific chromosomal regions (recombinational hot spots), has been demonstrated for several plant species (Säll 1990; Hadad et al. 1996; Sebastian et al. 2000; Barth et al. 2001). An elevated recombination frequency in the NA_6 parent might be attributable to a higher level of heterozygosity in that genotype, such that more efficient chiasma formation is achieved (Rees and Thompson 1956; Barth et al. 2001). The presence of regions of segregation distortion on the NA_6 map, the possibility of genotyping errors (Knox and Ellis 2002; Hackett and Broadfoot 2003), and the lower number of markers mapped to AU_6 may also have contributed to the discrepancy in estimated map lengths.

Both genomic DNA-derived SSR and EST–SSR loci in this study show evidence for null alleles, as revealed by segregation structures with dominant markers. The incidence for EST–SSRs is relatively low (23%), as may be expected from this class of sequences (Mogg et al. 2002), due to conservation of SSR flanking sequences targeted by the primers. By contrast, the incidence for LPSSR loci is considerably higher for this cross (approximately 60% of the total). While the incidence of null alleles should be higher in SSRs associated with non-transcribed regions (Mogg et al. 2002), this value is well in excess of the frequency of null-containing segregation patterns detected by LPSSR markers in the p150/112 reference population (15%; Jones et al. 2002b). This discrepancy may be attributable to the added complexity of marker analysis in a two-way pseudo-testcross structure, and also possibly to a high degree of genetic divergence between the parental base populations (Forster et al. 2001).

The proportions of markers exhibiting segregation distortion at $P < 0.05$, 26 and 15% for NA_6 and AU_6 respectively, are similar to values reported previously for perennial ryegrass (16.4–36%, Hayward et al. 1998; Bert et al. 1999; Armstead et al. 2002; Jones et al. 2002a, b), and both values lie in the middle of the range reported for intraspecific crosses in other plant species (2.6–44%, Jenczewski et al. 1997). The clustering of the majority of distorted markers to specific regions on the NA_6 and AU_6 maps may be attributed to linkage between markers and distorting genetic factors (Zamir and Tadmor 1986) for which there is selectable variation. The preponderance of regions with segregation distortion in the NA_6 map compared with the AU_6 map may reflect the undomesticated nature of the former genotype, with a higher proportion of recessive sub-lethal mutations capable of expression during gametogenesis or in the gametophyte. There is some correspondence between the regions of segregation distortion detected on the current maps with those on previous perennial ryegrass maps, most notably regions on LG3, but also on LG4 and LG5 (Bert et al. 1999; Armstead et al. 2002; Jones et al. 2002a, b). The consistency of segregation distortion on LG3 across different maps strongly suggests the presence in this genomic region of genes(s) affecting gametophytic or sporophytic survival in perennial ryegrass, and may correspond to an LG3 genetic factor identified by Thorogood et al. (2002) that interacts with the self-incompatibility *S* locus on LG1.

The use of LPSSR loci that had previously been assigned to the p150/112 reference map (Jones et al. 2002b) has allowed the unequivocal identification of six of the seven LGs of perennial ryegrass for each parental genetic map. The relative orientation and alignment of the maps of each LG between different mapping populations has also been enabled through the use of the LPSSR loci, with particularly clear relationships for LGs 1, 3, 4 and 6. However, the tendency of LPSSR loci to show centromeric clustering (Jones et al. 2002b) limits the degree of coverage of each LG.

The seventh LGs of both parental maps have been largely attributed to LG7 of the reference map by exclu-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cell wall biosynthesis EST
metal handling enzyme EST
plant defence EST
carbohydrate biosynthesis EST
saline stress EST
floral development EST
flavonoid biosynthesis EST
EST-SSR or LPSSR
b
LG3
LG4
cM
NA6
AU6
xlppih
**xlpmtn
*xlpmtg
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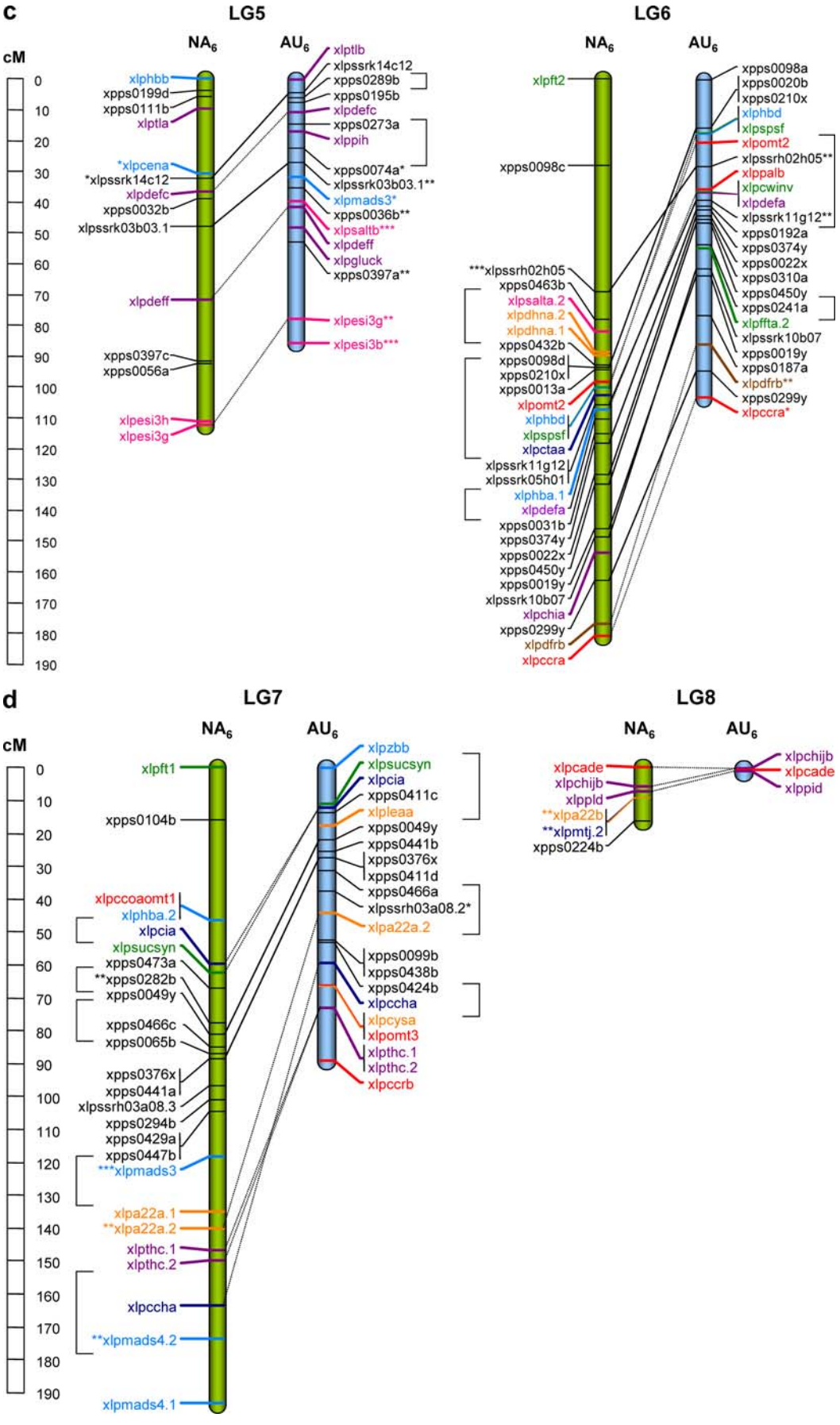
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AU6
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AU6
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xlpcade
xlppid

Fig. 1 (Contd.)

sion. However, the location of the *Lp*FT1 carbohydrate biosynthesis gene locus in a distal position of NA_6 LG7 provides further support for this assignment, as this gene probe detected an RFLP locus in the equivalent position on LG7 in the reference genetic map. The location of *Lp*FT2 in a distal position on NA_6 LG6 is also consistent with its position on LG6 in the reference map (Johnson et al. 2003). The relationship of LG8, detected in both parents, to the reference map LGs is problematic. The occurrence of this group in both parental maps suggests that it is not an artefact of map construction. Presumably the LG8 groups correspond to highly distal chromosomal regions and would coalesce with a major LG given sufficiently high marker density. Regional variation in chiasma frequency, with incidence of recombinational hot spots, may also contribute to this effect.

In the cases in which the same cDNA probe detected an EST–RFLP locus on the same putative linkage group in both parental maps, these loci have taken to be homeologous and to provide common loci to align the maps. However, a substantial number of the cDNAs detected multiple bands by Southern hybridisation, and in some instances (12 probes) duplicate or triplicate loci were detected by the same probe in different genomic locations. When multiple loci were detected on homeologous linkage groups, they were assumed to arise from variation in the same gene copy. However, the possibility of variation between paralogous DNA sequences on the same chromosome cannot be eliminated. Similarly, when a single probe detected polymorphic loci assigned to different LGs on the two parental maps (such as *Lp*OMT3 on NA_6 LG4 and AU_6 LG7 respectively), polymorphism in different paralogous sequences is the most likely explanation. The development of gene-specific PCR-based markers such as EST-SNPs will provide the means to further explore these homology/paralogy issues.

Value for comparative genetic mapping

Comparative genetic mapping in perennial ryegrass has been so far based on the use of heterologous RFLP anchor probes, many of which are anonymous cDNAs from wheat, barley, rice and oat, or *Pst*I-generated genomic clones (Jones et al. 2002a). This analysis revealed conserved syntenic relationships between the genome of perennial ryegrass and those of the Triticeae cereals (wheat and barley), oat and rice. Each of the seven LGs of perennial ryegrass shows a predominant correspondence to one of the homeologous groups of the Triticeae cereals, although some evolutionary translocations have been inferred (Yamada et al. 2004). This study has been extended through the genetic mapping of functionally defined genes for lignin and fructan biosynthesis. The map positions of the RFLP loci detected by the *Lp*CCR1 (McInnes et al. 2002) and *Lp*FT1 (Lidgett et al. 2002) genes are consistent with the locations of the putative ortholoci based on conserved synteny in rice and barley, respectively. The development of a comprehensive functionally associated marker-based map of perennial ryegrass will allow the confirmation and refinement of these comparative relationships through in silico analysis. Sequence alignment based on BLASTN and TBLASTX may be used to detect orthologous sequences in EST collections in wheat, many of which are being assigned to map locations based on deletion bins (Endo and Gill 1996; Qi et al. 2003; Sorrells et al. 2003), or in map-ordered BAC/PAC clones in rice (Chen et al. 2002).

Application to functional gene identification

The development of a functionally defined gene-based genetic map of perennial ryegrass provides the basis for the correlation of molecular variation associated

Table 7 Distribution of genetic markers within and between the NA_6 and AU_6 parental genetic maps, along with cumulative genetic map distances and mean locus density for each of the eight linkage groups

Genotype	LG1	LG2	LG3	LG4	LG5	LG6	LG7	LG8	Total
NA_6									
No. EST–RFLP loci	14	8	17	10	7	12	12	5	85
No. RFLP loci[a]	0	0	0	0	0	1	1	0	2
No. EST–SSR loci	11	10	10	11	5	12	11	1	71
No. LPSSR loci	3	1	2	3	2	4	1	0	16
Total no. loci	28	19	29	24	14	29	25	6	174
Length (cM)	97	138	129	96	113	181	193	16	963
Mean locus density (cM/locus)	3.5	7.3	4.4	4.0	8.1	6.2	7.7	2.7	5.5
AU_6									
No. EST–RFLP loci	12	7	11	5	9	9	11	3	67
No. EST–SSR loci	7	12	4	9	6	12	9	0	59
No. LPSSR loci	3	1	3	1	2	3	1	0	14
Total no. loci	22	20	18	15	17	24	21	3	140
Length (cM)	119	98	112	150	85	103	89	1	757
Mean locus density (cM/locus)	5.4	4.9	6.2	10	5.0	4.3	4.2	0.3	5.4

[a]Loci detected by the *Lp*FT1 and *Lp*FT2 carbohydrate biosynthesis genes

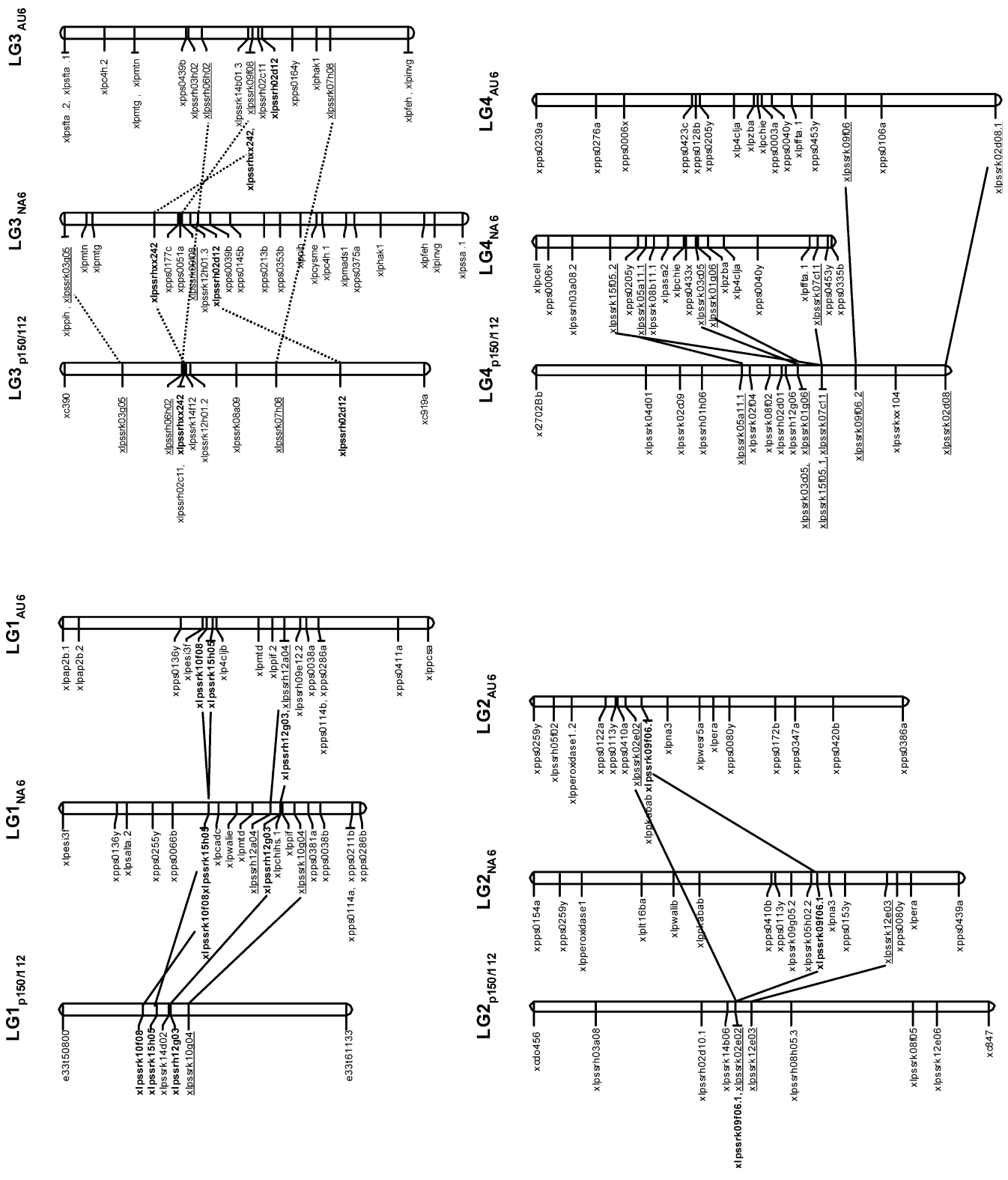

Fig. 2 Alignment of the genetic linkage maps for each LG of the NA_6, AU_6 and p150/112 reference genetic maps of perennial ryegrass. The relative locations of LPSSR loci are indicated, including the 18 represented in Fig.1 and others for which map order showed a lower degree of confidence during consolidated map construction. The xlpssr loci that are located on all three maps are shown in *bold* type, while those that are located on two of three maps are shown in *underlined* type. A framework set of other markers from the consolidated map is shown for each LG, and the positions of equivalent loci are connected by *dotted lines*

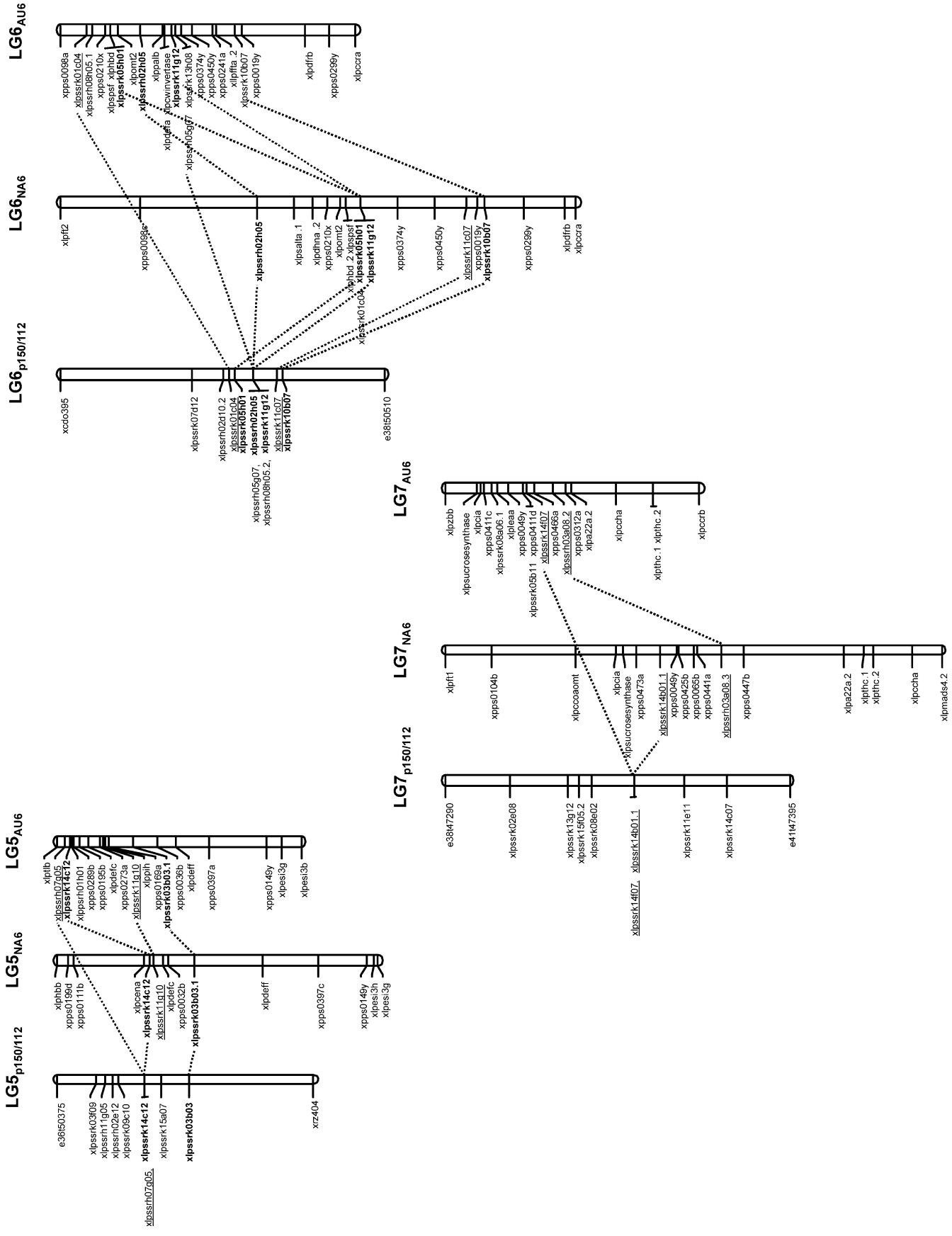
LG5p150/112
LG5NA6
LG5AU6
LG6p150/112
LG6NA6
LG6AU6
LG7p150/112
LG7NA6
LG7AU6

Fig. 2 (Contd.)

with functional sequences with the locations of QTLs for putatively related traits. QTL analysis in perennial ryegrass has so far been based on genetic maps constructed using anonymous genetic markers such as heterologous RFLPs, AFLPs and SSRs, permitting the identification of genomic regions controlling crown rust resistance (Dumsday et al. 2003), morphological and developmental traits (Yamada et al. 2004) and other characters (Forster et al. 2004). Genetic maps based on expressed sequences have been constructed in a variety of other agronomically important species such as rice (Kurata et al. 1994), maize (Chao et al. 1994), sugar beet (Schneider et al. 1999) and the Solanaceae species tomato and potato (Tanksley et al. 1992). For potato, a molecular function map of 85 loci detected by 69 gene-specific probes has been developed (Chen et al. 2001), with priority given to genes involved in carbohydrate metabolism and transport. Comparison of the map locations of such loci with QTLs for tuber starch content permitted the identification of several putative candidate genes for this trait. The $F_1(NA_6 \times AU_6)$ population shows high levels of phenotypic variation for a number of important traits such as crown rust resistance, root and shoot morphogenesis, pseudostem water soluble carbohydrate content and photosynthetic efficiency (Forster et al. 2004). QTL analysis using the EST–RFLP and EST–SSR markers described here may allow the identification of associations between functionally associated marker locations and QTLs for these and other agronomic traits. Successful demonstration of co-location may be extended through LD studies (Rafalski 2002) as well as transcriptome profiling, gene silencing (Vance and Vaucheret 2001) and induced mutagenesis (Li et al. 2001) approaches (Wilson et al. 2003). Such methods may be of particular value for ESTs of unknown classification, especially when used in concert with comparative genetic mapping at the macrosyntenic and microsyntenic levels.

Conclusion

In summary, we have constructed the first genetic linkage map of perennial ryegrass that is predominantly populated by functionally associated markers. This resource provides the means for developing 'perfect markers' associated with key QTLs, for comparative genomics and for reverse genetic analysis.

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