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ORIGINAL PAPER

Genetic mapping of DArT markers in the *Festuca–Lolium* complex and their use in freezing tolerance association analysis

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Abstract Species belonging to the *Festuca–Lolium* complex are important forage and turf species and as such, have been studied intensively. However, their out-crossing nature and limited availability of molecular markers make genetic studies difficult. Here, we report on saturation of *F. pratensis* and *L. multiflorum* genetic maps using Diversity Array Technology (DArT) markers and the DArTFest array.The 530 and 149 DArT markers were placed on genetic maps of *L. multiflorum* and *F. pratensis*, respectively, with overlap of 20 markers, which mapped in both species. The markers were sequenced and comparative sequence analysis was performed between *L. multiflorum*, rice and *Brachypodium*. The utility of the DArTFest array was then tested on a Festulolium population FuRs0357 in an integrated analysis using the DArT marker map positions to study associations between markers and freezing tolerance. Ninety six markers were significantly associated with freezing tolerance and five of these markers were genetically mapped to chromosomes 2, 4 and 7. Three genomic loci associated with freezing tolerance in the FuRs0357 population co-localized with chromosome segments and QTLs previously indentified to be associated with freezing tolerance. The present work clearly confirms the potential of the DArTFest array in genetic studies of the *Festuca–Lolium* complex. The annotated DArTFest array resources could accelerate further studies and improvement of desired traits in *Festuca–Lolium* species.

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Introduction

The *Festuca–Lolium* species complex includes some of the world's most important forage grasses. Even though these species are closely related to major cereal crops and share many characteristics with wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and other well studied crops, the species of the *Festuca–Lolium* complex possess a number of biological and genomic features unique to this group; for example, the perenniality of many forage grasses as compared to the annuality of temperate cereals, and chromosome rearrangements specific for the *Festuca–Lolium* complex (Alm et al. 2003). The species within the

complex possess a range of complementary characteristics, which are often explored in grass hybrid breeding. Italian ryegrass (*Lolium multiflorum* Lam.) and perennial ryegrass (*L. perenne* L.) are two of the most important forage grasses of temperate regions. Italian ryegrass is especially valued for its high dry matter yield and its excellent forage quality. Although primarily used for hay and silage production in temporary leys, it is also a substantial component of permanent grassland (Peter-Schmid et al. 2008). On the contrary, perennial ryegrass is cultivated mainly for grazing. It forms the main component of productive permanent grassland mainly due to its incomparable perenniality. However, *Lolium* species often suffer from limited persistence under abiotic stress.

On the other hand, *Festuca* species generally have a better tolerance to abiotic stress when compared to *Lolium* species. Meadow fescue (*Festuca pratensis* Huds.) is a forage grass which constitutes a significant component of species-rich permanent pastures and hay fields in alpine and eastern regions of Europe. In Scandinavia, it is also a major component of intensively managed swards cut for silage (Rognli et al. 2010). Because of their close evolutionary relationships, introgression of *Festuca* genes into *Lolium* genomes through the development of *Festuca* × *Lolium* hybrids may allow to improve forage grass stress resistance (Humphreys et al. 2005). In this respect, *F. pratensis* is often used to improve freezing tolerance in *Festuca* × *Lolium* hybrids (Kosmala et al. 2006). Genome-wide molecular markers are essential for characterization of such hybrids and identification of introgressed chromosome segments.

*D*iversity *A*rrays *T*echnology (DArT, Jaccoud et al. 2001) is a high-throughput sequence independent genotyping method based on the reduction of genome complexity and DNA microarray hybridization. Through the past decade, it has become a valuable source of markers for genomes with limited sequence information. DArT arrays have been developed for crop species like banana (*Musa acuminata* Colla), cassava (*Manihot esculenta* Crantz), cereals as well as for model plants such as *Arabidopsis*, rice (*Oryza sativa* L.) and sorghum (*Sorghum bicolor* L.) (for a complete list of species see http://www.diversityarrays.com/genotypingserv.html). DArT markers were often used for diversity studies, and for construction and saturation of genetic maps (Wenzl et al. 2006; Tinker et al. 2009). DArT markers have also been used in the analysis of important agricultural traits, for example in an association study of tan spot resistance in hexaploid wheat (Singh et al. 2010).

Recently, Kopecký et al. (2009) developed a DArTFest array for the *Festuca–Lolium* complex. This printed microarray contains 7,680 probes derived from methyl-filtered genomic representations of three *Festuca* (*F. arundinacea* Schreb., *F. glaucescens* Boiss. and *F. pratensis*) and two *Lolium* (*L. multiflorum* and *L. perenne*) species. The DArTFest array has a potential to provide important insights into the genome structure and evolution of the species within the *Festuca–Lolium* complex. Many of the DArT markers cross-hybridize with several different species, which enables comparative genomic studies. DArT markers can also be sequenced and physically mapped to genomes of fully sequenced model species to further understand the syntenic relationships between chromosomes of forage grasses and model plants. This may in turn lead to more efficient identification of genes underlying important agricultural traits. To date, DarTFest array has been used to study genetic diversity (Kopecký et al. 2009) and to determine genomic constitution of Festulolium cultivars (Kopecký et al. 2011).

Following the breeding priorities, molecular dissection of agronomic traits in *L. multiflorum* has so far mainly been focused on resistance to diseases such as crown rust or bacterial wilt (Studer et al. 2006, 2007). In this context, a genetic linkage map based on 306 F_1 individuals has been established (*Xtg*-ART) and more recently been used for construction of a consensus linkage map in *Lolium* using EST-derived SSR markers (Studer et al. 2010). Alm et al. (2003) established a *F. pratensis* mapping population (HF2/7 × B14/16) consisting of 138 F_1 individuals and mapped 446 RFLP, AFLP and SSR markers. This population has been used for the analysis of freezing and drought tolerance, winter hardiness, and vernalization sensitivity (Alm et al., unpublished; Ergon et al. 2006). In this study, we (1) saturated and improved these genetic maps of *F. pratensis* and *L. multiflorum* with DArT markers, (2) sequenced the mapped markers to investigate the genomic origin of DArT markers and the syntenic relationships to model genomes, and (3) used these new DArTFest array resources in an integrated analysis of agriculturally important trait freezing tolerance in a *L. perenne* × *F. pratensis* hybrid population.

Materials and methods

Plant material

The HF2/7 × B14/16 mapping family consisting of 138 F_1-offspring was used for the construction of the *F. pratensis* map (Alm et al. 2003). For the *L. multiflorum* map, 288 F_1 individuals of the *Xtg*-ART mapping population (Studer et al. 2006) were used. In the association study, the Festulolium population (FuRs0357) was used. The FuRs0357 population (*L. perenne* × *F. pratensis*) originates from a wide genetic pool from several initial hybrids made from either *Festulolium* cv. Prior (LpFp, 4*x*) crossed with *L. perenne* (2*x*) or crosses between *L. perenne* (4*x*)

and *F. pratensis* (2*x*). The initial hybrids were backcrossed twice onto diploid *L. perenne* to obtain BC_2 progenies and then put through two generations of seed propagation. Twenty one plants belonging to either a high freezing tolerance (HFT) group (11 plants) or a low freezing tolerance (LFT) group (10 plants) were used in the study.

DArT screening

Genomic DNA was isolated from individual plants from both mapping populations and from the Festulolium population FuRs0357 using Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany). The previously developed DArTFest array (Kopecký et al. 2009) was hybridized with fluorescently labelled genomic representations of individual plants, which were prepared from genomic DNA by the same *Pst*I/*Taq*I complexity reduction method as used for preparing the array (for details see Akbari et al. 2006). Hybridization signals were converted into 0–1 scores using the DArTsoft software package developed at Diversity Arrays Technology Pty Ltd (DArT P/L, Yarralumla, Australia).

Genetic mapping

Genetic maps of *F. pratensis* and *L. multiflorum* were constructed in JoinMap 4.0 using the Kosambi mapping algorithm. As DArT markers are dominant, marker files were coded for double haploid populations (DH) and maps were calculated for each parent separately. Only markers segregating close to the expected 1:1 ratio (>0.4 and <0.6) were used. Maps were then combined using bridging markers present in both parents. The inclusion of markers was decided by the following procedure. First, all markers were grouped into putative linkage groups based on Likelihood Ratio Odds (LOD) grouping. Next, initial maps were calculated and all markers with a Chi-square value of >3 (highly distorted segregation) were removed. Subsequent recalculation of maps was performed until no markers had a Chi-square score of >3. Finally, the two parental maps for each LG were combined into one. All final linkage maps were edited and finalized using the MapChart software (Voorrips 2002).

Association of DArT markers with freezing tolerance

Freezing tolerance was measured with the re-growth method (Larsen 1978). In brief, 300 genotypes from FuRs0357 were put through controlled freezing stress which resulted in differential freezing induced damage and survival. Plants were grouped randomly in five boxes using six replicates per genotype. The resulting HFT (high freezing tolerance) and LFT (low freezing tolerance) plants belonged to the 10% of phenotypes with the highest and lowest freezing tolerance, respectively. The HFT group had an average freezing tolerance score of 7.06 (±0.22) and the LFT group had an average freezing tolerance score of 2.59 (±0.61) [range; 0 (dead) to 9 (no damage)]. Associations between DArT marker genotypes and freezing tolerance groups were tested using Fisher's Exact test. The null hypothesis was that the DArT marker genotypes were not associated with freezing tolerance; hence we expected random distribution of genotypes in HFT and LFT groups. We calculated the corresponding q values from the Fisher Exact test p values (Storey 2002) to correct for multiple testing and false positives. A significance threshold of $q < 0.05$ was used. Statistical analyses were carried out in R (R Development Core Team 2009) using the "fisher.test" and "q value" functions in the q-value package.

Sequencing of selected clones/markers

All DArT markers, which were placed on the genetic maps of *F. pratensis* and *L. multiflorum* and/or significantly associated with freezing tolerance, were sequenced. Reaction mix for cycle sequencing was prepared using standard BigDye chemistry (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems). The dilution of reaction components was scaled to a final volume of 10 µl containing 3.2 pmol of universal M13 (forward or reverse) primer and 20 ng of sequence-ready (DArT clones) template. The reaction products were purified using the CleanSEQ kit (Agencourt Bioscience Corp., Beckman Coulter Comp., Beverly, MA, USA) and analyzed on an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA). The raw sequence data were assembled and edited using the DNA Baser software v.2 (Heracle-Software, http://www.DnaBaser.com). Vector and adaptor sequences were removed prior to further analysis. All sequences were deposited in GenBank under accession numbers HN266254-HN266937.

Sequence analysis

All mapped markers were compared to each other to determine the extent of redundancy in DArT clones. Stand alone blastn software was used for the search with E value set to e-10. Only reciprocal blast hits were taken into account. The map position of markers with significant hits was checked and markers which mapped to different map positions were not considered redundant. Groups of markers, which shared significant homology to each other and mapped to the same genetic position, are hereafter referred to as 'bins'. The sequences were further compared to known plant repeat sequences. To analyze repeat content, TREP Release 10 (http://wheat.pw.usda.gov/ITMI/Repeats/) was merged with

TIGR Plant Repeat Databases for Brassicaceae, Fabaceae, Poaceae and Solanaceae (Ouyang and Buell 2004). This database contains 8,432 repetitive elements from 18 different genera. Repeat analysis was performed using RepeatMasker software (http://repeatmasker.org) with CrossMatch search engine (http://www.phrap.org/phredphrapconsed.html) and default settings. We compared DArT sequences with non-redundant protein sequences (nr) and non-human, non-mouse ESTs database (est_others) at GenBank in order to estimate the number of DArT markers derived from expressed loci. The protein search (blastx) was performed using blastcl3 with default settings and BLOSUM62 scoring matrix. Nucleotide search (blastn) was also performed by blastcl3 with default settings but reward for a nucleotide match was set to 2. Only the best blast hits (with lowest *E* value) were taken into account.

Comparison of mapped DArTs to model genomes

The sequences of the mapped DArT markers were compared to the *Oryza sativa* ssp. *japonica* cv. 'Nipponbare' genome and the *Brachypodium distachyon* Bd21 genome using blastn with *E* value set to e-10. Twelve pseudomolecules for the rice chromosomes (Build5) were downloaded from the IRGSP website (http://rgp.dna.affrc.go.jp/E/IRGSP/Build5/build5.html). The Bd21 genome was downloaded from Brachybase.org (http://files.brachypodium.org/). Only the best blast hits with alignment lengths of at least 50 bp were taken into account.

GISH analysis of Festulolium plants

Twelve individuals from the Festulolium population FuRs0357 (six with high freezing tolerance, HFT, and six with low freezing tolerance, LFT) were used for the analysis of genomic constitution using genomic in situ hybridization (GISH). The other plants suffered under conditions optimal for preparation of chromosome spreads (root growth and accumulation of cells in metaphase). GISH was performed as described by Kopecký et al. (2005). Briefly, total genomic DNA of *F. pratensis* was labeled with digoxigenin using DIG-Nick Translation Kit (Roche Applied Science, Indianapolis, IN, USA) and used as a probe. Genomic DNA of *L. perenne* was sheared to ~200 bp fragments and used as blocking DNA. Sites of probe hybridization were detected by anti-DIG-FITC conjugate (Roche). Chromosomes were counterstained with 1.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) prepared in Vectashield antifade solution (Vector Laboratories, Burlingame, USA). Observations were made using an Olympus AX70 microscope equipped with epi-fluorescence and SensiCam B/W camera. ScionImage and Adobe Photoshop software were used for processing of color pictures.

Results

Genetic mapping

DArTFest array contains 2,257 probes positively scored in *F. pratensis* and 2,761 probes positively scored in *L. multiflorum* (Kopecký et al. 2009). In total we placed 659 markers on genetic maps, 149 markers in the *F. pratensis* population HF2xB14, and 530 markers in the *L. multiflorum* population *Xtg*-ART (Table 1; Fig. 1). The effectiveness of DArT mapping was 6.6 and 19.2% for *F. pratensis* and *L. multiflorum*, respectively. Only 20 markers could be mapped in both species. Out of them, 17 mapped to homologous linkage groups and three markers D355658, D557428, D558076 mapped to Fp4 and Lm1, Fp5 and Lm7, Fp1 and Lm7, respectively.

Sequencing and sequence analysis of mapped markers

Out of the 659 mapped markers, 620 yielded 302,808 bp of sequence with an average length of 488.4 bp. Sequencing of the remaining 39 DArT markers failed due to technical reasons. A total of 398 DArT markers (64.2%) were found to be singletons, while the 222 remaining markers were redundant and were assigned to 90 marker bins. The biggest bin consisted of six markers. Hence, 489 non-redundant DArT markers/bins were mapped when considering each bin a unique locus (Table 2). Nevertheless, markers belonging to one bin can slightly differ in their precise map position (see Fig. 1). This could be due to inaccuracy during the array hybridization leading to genotyping errors and variation in estimated numbers of recombinants, inaccuracies in the consensus map estimation, or duplicated genes in tandem arrays.

Table 1 Distribution of genetic markers among linkage groups

	Festuca pratensis		*Lolium multiflorum*	
	Non-DArT markers[a]	DArT	Non-DArT markers[b]	DArT
LG1	61	23	63	67
LG2	26	10	43	73
LG3	34	22	49	73
LG4	68	22	61	106
LG5	70	12	43	71
LG6	49	27	35	63
LG7	65	32	68	76
Total	373	148	352	529

[a] AFLP, RFLP, SSR markers and isozymes, see Alm et al. (2003) for details

[b] SSR and AFLP markers, see Studer et al. (2006) for details

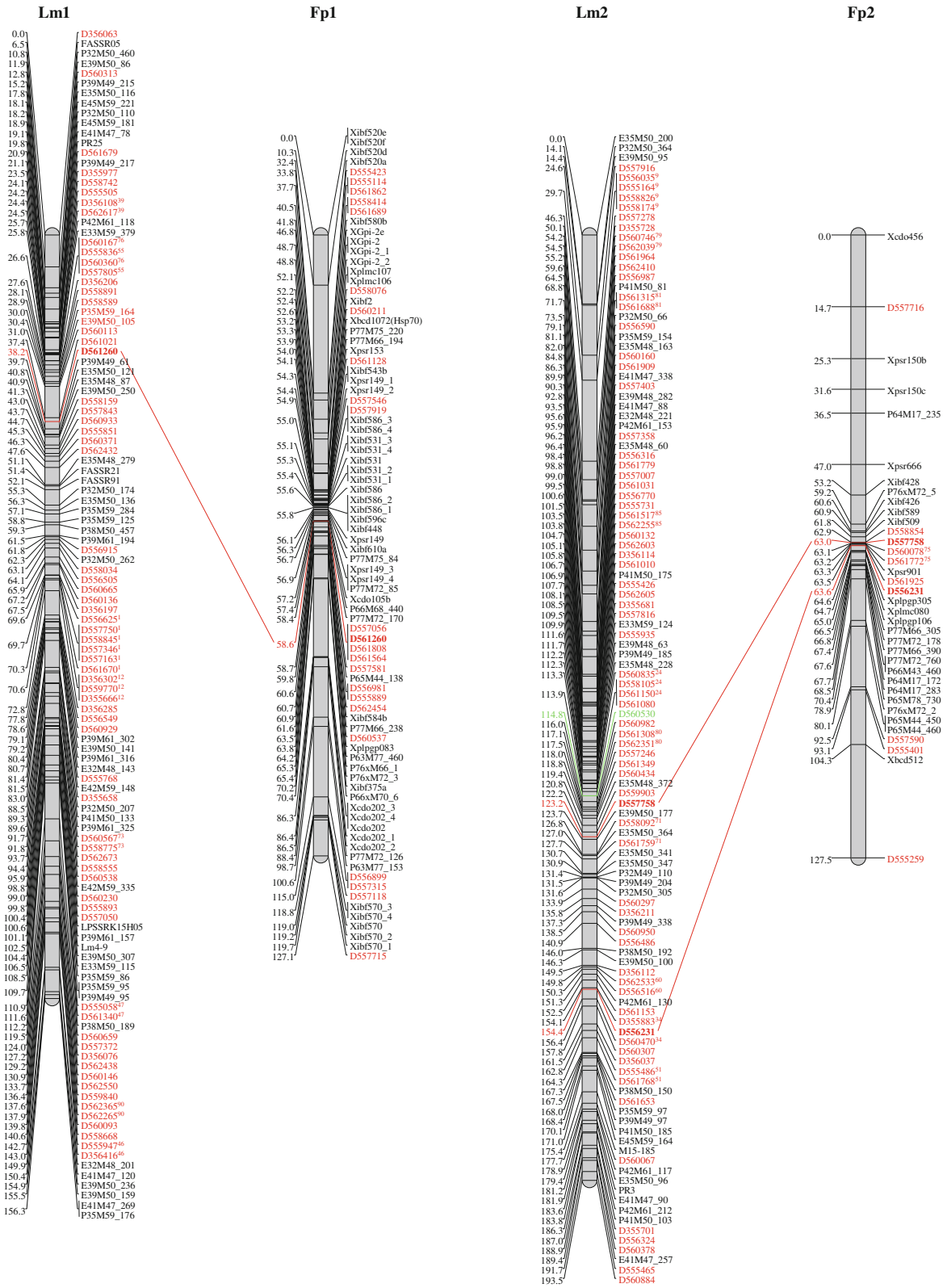

Fig. 1 Genetic maps of seven *F. pratensis* and *L. multiflorum* chromosomes. DArT markers are labeled with D and clone number (six digits) and are indicated by *red color*. Markers belonging to one redundant marker bin are indicated by the same *superscript*. Markers associated with freezing tolerance are indicated by *green color*

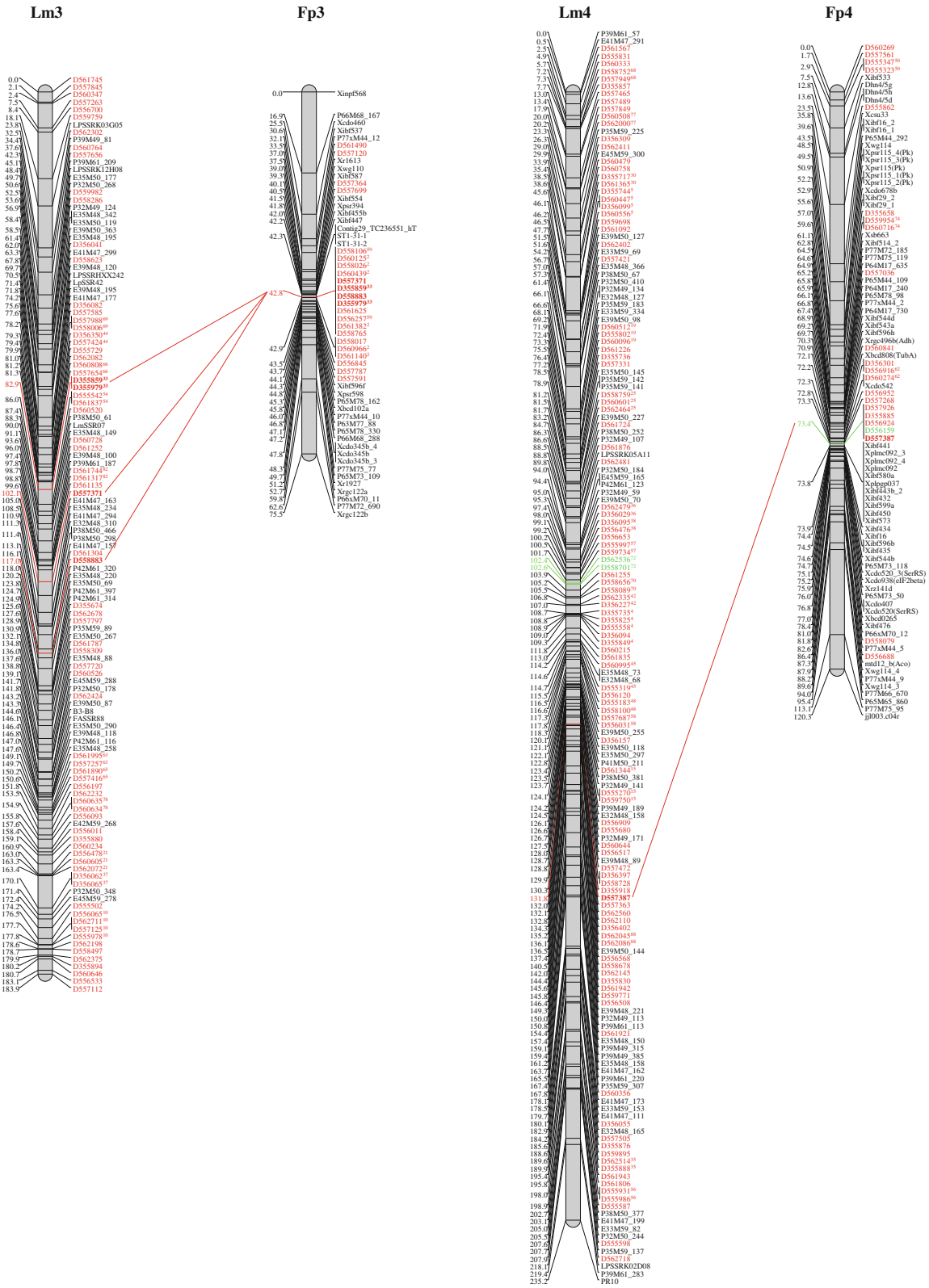

Fig. 1 continued

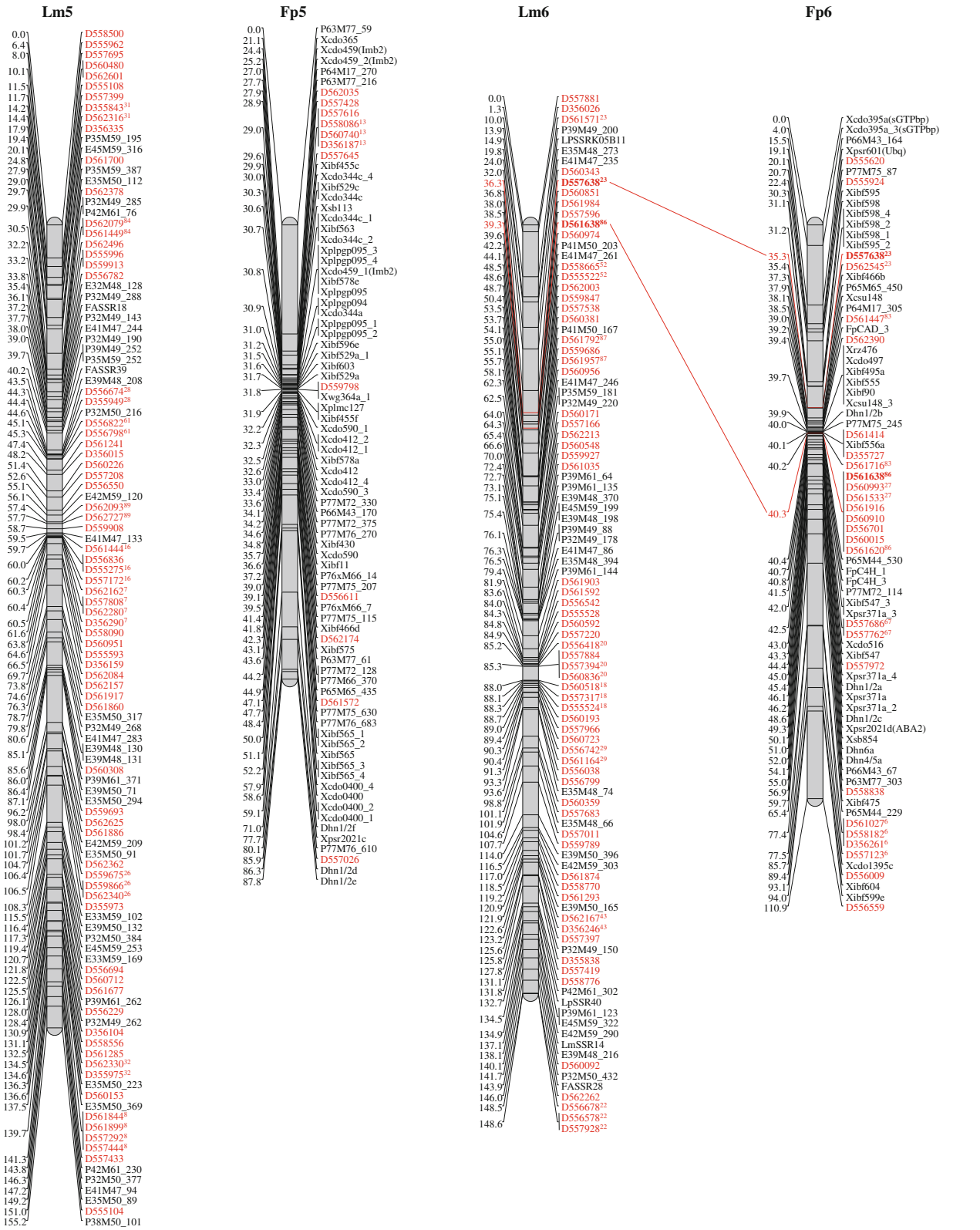

Fig. 1 continued

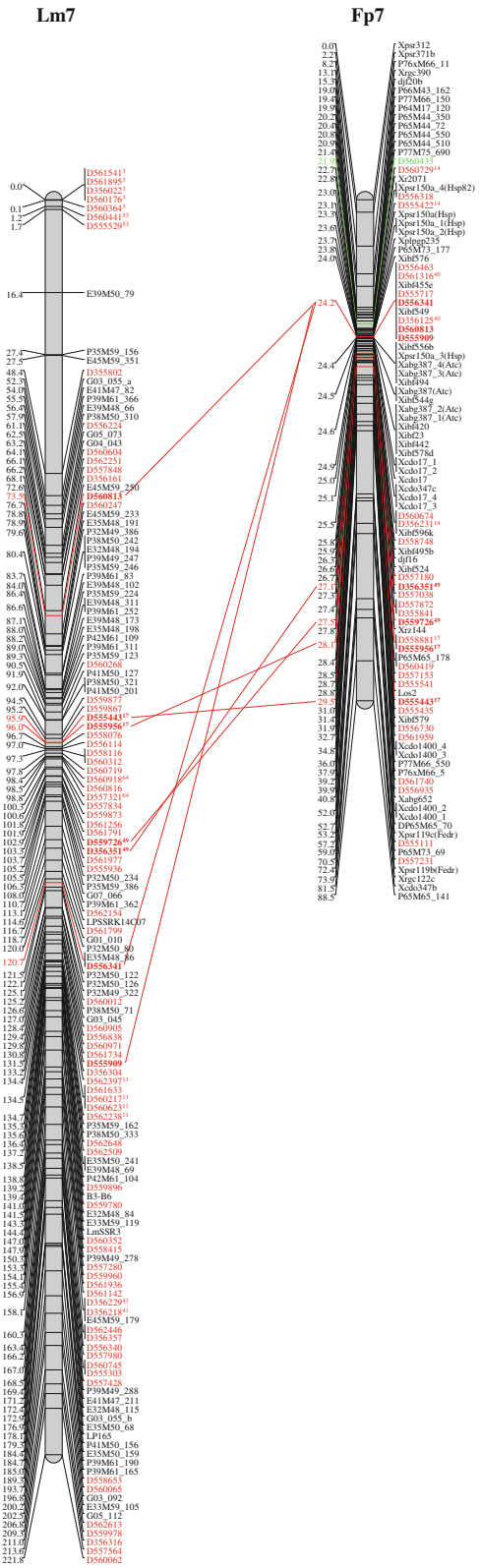

Fig. 1 continued

Table 2 Redundancy of sequenced DArT markers

Extent of redundancy	Number of bins	Number of markers	Frequency of markers (%)
Unique	399	399	64.3
Duplicates	64	128	20.6
Triplicates	15	45	7.2
4/bin	8	32	5.2
5/bin	1	5	0.8
6/bin	2	12	1.9
Total	489	621	100

To estimate the number of DArT markers derived from repetitive DNA elements, we performed blast search against an in-house built composite plant repeat database. Only 44 (7.1%) of the DArT markers contained repetitive elements supporting the notion that DArT markers represent hypo-methylated low copy genomic regions (Wenzl et al. 2006). In the repeat containing DArT sequences, the retrotransposons (27) dominated DNA-transposons (13). The remaining four hits in the plant repeat database were unclassified. To estimate the impact of repeat elements on marker redundancy, we compared DArT markers derived from repetitive elements with the marker bins. Interestingly, repetitive elements could explain the presence of redundant markers only for seven (7.8%) of the marker bins. This indicates that the majority of redundant markers in bins are either due to multiple cases of cloning the same sequence during the random marker development procedure, or they come from tandem duplicated genes.

In order to estimate the proportion of expressed sequences among our mapped DArT markers, we performed a blastn search against GenBank "est_others" database. This identified 368 (59.4%) DArT markers with significant homology to expressed sequences. Blastx search against non-redundant protein sequences (nr) revealed 162 (26.1%) DArT markers with significant homology to known and hypothetical proteins, and of these 152 were also identified as expressed sequences in blastn search. In total, 378 DArT markers (293 non-redundant bins) were identified as potentially gene-derived sequences.

Comparison of mapped DArTs to model genome(s)

The genetically mapped DArT markers were compared to the rice and *Brachypodium* genomes in order to map the sequences in silico and investigate the synteny between the model genomes and those of the *Festuca–Lolium* complex. Using the defined criteria for blast search, mapped DArT

markers in *L. multiflorum* produced 227 (162 non-redundant bins) and 299 (227) hits against rice and *B. distachyon* genomes, respectively. Of the markers mapped in *F. pratensis*, 50 (40) and 70 (54) produced blast hits in rice and *Brachypodium* genomes, respectively. Due to the low number of hits of the *F. pratensis* markers, the analysis of syntenic relationships was limited to comparisons between *L. multiflorum* and rice (Table 3) and *L. multiflorum* and *Brachypodium* (Table 4). All significant homologies are listed in Supplementary Tables 1 and 2.

We identified 11 syntenic regions with more than 5 markers shared between particular *L. multiflorum* and rice chromosomes. The highest degree of synteny was found between the chromosomes/linkage groups Lm3 and Os01, where 71.4% of markers presented in Lm3 had a homologous sequence on Os01. In comparison, 12 syntenic regions with more than 5 shared markers were found between the genomes of *L. multiflorum* and *Brachypodium*. The most conserved syntenic relationships were found between Lm5-Bd4 and Lm3-Bd2, with 76.9 and 76.5% of the *L. multiflorum* markers having homologues in corresponding *Brachypodium* chromosome, respectively.

Table 3 Synteny of *L. multiflorum* and rice as revealed by mapped DArT markers

	Lm1	Lm2	Lm3	Lm4	Lm5	Lm6	Lm7
Os01	1	1	**15**	1	1	1	–
Os02	2	–	1	3	2	**16**	–
Os03	–	1	–	**18**	–	–	2
Os04	2	**10**	2	2	2	1	–
Os05	**7**	–	1	1	–	–	–
Os06	–	–	–	2	–	–	**15**
Os07	–	**7**	–	1	–	2	–
Os08	–	2	–	–	–	–	**7**
Os09	–	–	–	–	**7**	1	–
Os10	**7**	–	–	–	1	1	–
Os11	1	–	1	**6**	1	2	1
Os12	–	–	1	–	3	–	1
Total	20	21	21	34	17	24	26

Numbers correspond to non-redundant bins mapped to each rice chromosome. Chromosome relationships with highest synteny are bold

Table 4 Synteny of *L. multiflorum* and *Brachypodium* as revealed by mapped DArT markers

	Lm1	Lm2	Lm3	Lm4	Lm5	Lm6	Lm7
Bd01	1	**11**	3	**30**	1	3	**22**
Bd02	**12**	1	**26**	2	4	2	1
Bd03	**10**	2	1	4	1	**20**	**8**
Bd04	1	1	3	**9**	**20**	**5**	1
Bd05	1	**15**	1	1	1	1	2
Total	25	30	34	46	27	31	34

Numbers correspond to non-redundant bins mapped to each *Brachypodium* chromosome. Chromosome relationships with highest synteny are bold

Analysis of markers associated with freezing tolerance

Out of 1,868 DArT markers segregating in the Festulolium FuRs0357 population, about 5% (96) had a significantly different distribution of genotype scores among HFT and LFT plants ($q < 0.05$). Unfortunately, only five markers (four non-redundant bins) could be mapped. These five markers were mapped to linkage groups Fp4 (D556159) and Fp7 (D560433) in *F. pratensis* and Lm2 (D560530) and Lm4 (D558701, D562536) in *L. multiflorum* (Table 5; Fig. 1). The FuRs0357 population was in fact derived from a cross between *L. perenne* and *F. pratensis*. Nevertheless, one can expect that map positions of DArT markers in *L. perenne* to be highly co-linear with their position in *L. multiflorum* due to the close evolutionary relationship (Catalán et al. 2004, Studer et al. 2010). Seventy two of the freezing tolerance-associated markers were sequenced for the purpose of studying their putative genomic origin and localization in model genomes in silico. Several markers associated with freezing tolerance were found to map Bd1, and nine of these markers were flanking *Vrn1* gene paralogs on Bd1 (Fig. 2). This further supports the presence of a freezing tolerance QTL on chromosome 4 in *L. perenne* and *F. pratensis* as Bd1 has syntenic relationship to chromosome 4 of species in the *Festuca–Lolium* complex. We performed functional analysis of all DArT markers associated with freezing tolerance using blast2go (Conesa et al. 2005). For 18 of these markers a gene functional class could be assigned based on the homology to known proteins and transcription is the most frequent one (4 markers). All markers associated with freezing tolerance are listed in Supplementary Table 3.

Table 5 Mapped markers having significantly (Fisher Exact test) different distributions among HFT (high freezing tolerance) and LFT (low freezing tolerance) plants

DArT ID	*q* value	Presence	LG	cM
D560433	0.028	LFT	LG7 *F. pratensis*	21
D556159	0.042	HFT	LG4 *F. pratensis*	73
D558701	0.042	LFT	LG4 *L. multiflorum*	103
D562536	0.042	LFT	LG4 *L. multiflorum*	102
D560530	0.044	HFT	LG2 *L. multiflorum*	115

"Presence" denotes if a marker presence signal is associated with the LFT or HFT group

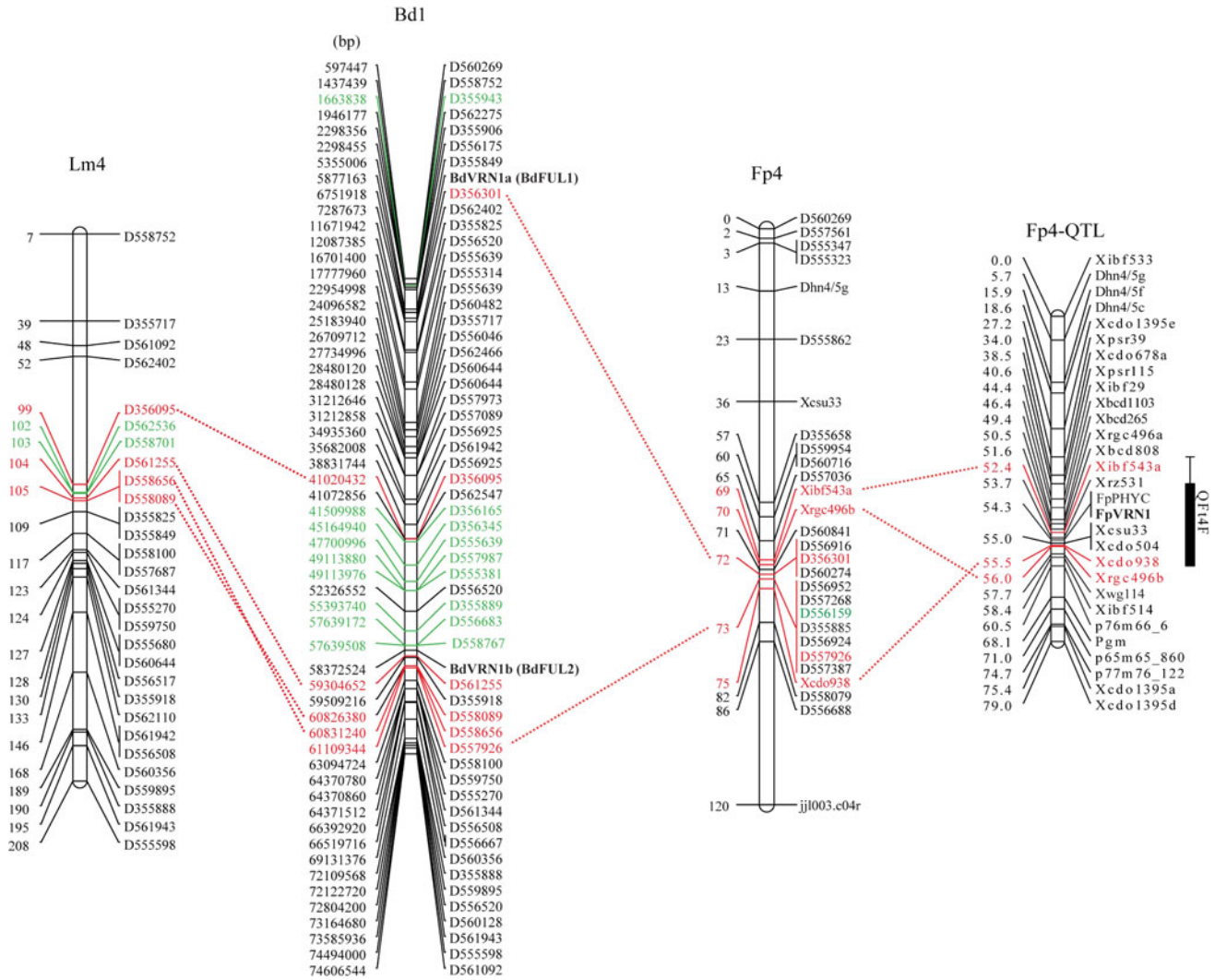

Fig. 2 Syntenic relationships between *F. pratensis*, *L. multiflorum*, and *Brachypodium* chromosome 4. Comparative map shows the syntenic relationships between the freezing tolerance QTL in *F. pratensis* and the DArT markers associated with freezing tolerance phenotype closely linked to VRN1 paralogs in the *Brachypodium* genome. The position of the DArT markers on Bd1 are extrapolated from the best blastn hit in the Bd genome. *Red color text* denotes bridging markers between maps. *Green color text* denotes DArT markers significantly associated with freezing tolerance phenotype flanking FUL1 and FUL2. For the Bd1 chromosome, the *left side numbers* are physical position in base pair. *Left side numbers* for all other maps are genetic distances in cM. The rightmost map is reproduced from Alm et al. (unpublished results) where the *black bar* represents the 95% confidence interval for a freezing tolerance QTL

GISH analysis of Festulolium plants

Microscopic analysis of mitotic metaphase plates of six LFT and six HFT plants revealed that the FuRs0357 Festulolium population contained plants with different ploidy levels. Four of the LFT plants were tetraploid, with the remaining two diploid, while all six HFT plants were diploid. Such a large difference is not likely to occur by chance (Fisher Exact test; $P = 0.045$), which indicates that the polyploidy levels could be functionally linked to freezing tolerance. The higher ploidy level could affect the proportion of markers which cannot be called as "0" or "1" when converting fluorescence signal since it increases signal to noise ratio. Exactly this pattern was observed in the FuRs0357 population. The average numbers of not called DArT marker genotypes per plant were 14 in HFT and 127 in LFT (*T* test; $P = 0.0003$), reflecting the higher ploidy level in the LFT group.

GISH analysis revealed *F. pratensis* chromatin only in a small subset of plants, no matter if they were freezing tolerant or not. Two recombined chromosomes carrying terminal *F. pratensis* segments were found in one tetraploid LFT plant (Fig. 3a). *Festuca pratensis* chromatin was not detected in any other LFT plant. Two HFT plants contain

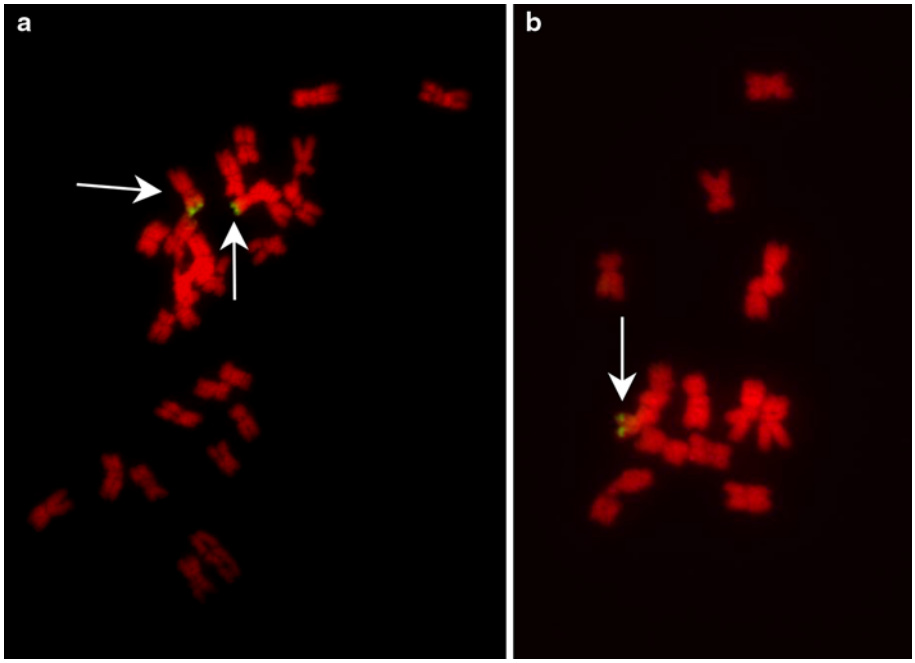

Fig. 3 Analysis of Festulolium plants with low (LFT) and high (HFT) freezing tolerance using genomic in situ hybridization (GISH). GISH in *L. perenne* × *F. pratensis* LFT149 (**a**) and HFT152 (**b**) plants. Total genomic DNA of *F. pratensis* was labeled with FITC and used as a probe (*green color*); genomic DNA of *L. perenne* was used to block hybridization of common sequences. Chromosomes were counterstained by DAPI (shown in red pseudocolor). Tetraploid LFT149 plant ($2n = 4x = 28$) has two recombined chromosomes with small terminal translocations of *F. pratensis* (*arrows*), while diploid HFT plant ($2n = 2x = 14$) has one recombined chromosome with small terminal *F. pratensis* translocation (*arrow*)

chromosomes carrying terminal *F. pratensis* segments (Fig. 3b). No *F. pratensis* chromatin was found in the remaining four HFT plants.

Discussion

Mapping of DArT markers

The DArT technology has become a valuable molecular tool for many plant species. More than thousand DArT markers have been genetically mapped in rye (*Secale cereale* L.) (Bolibok-Bragoszewska et al. 2009) and oat (*Avena sativa* L.) (Tinker et al. 2009), and more than 2,000 have been mapped in barley (Wenzl et al. 2006). Here we have used the recently developed DArTFest array (Kopecký et al. 2009) to map genetically hundreds of DArT markers in the *Festuca–Lolium* complex. Different numbers of markers could be mapped in the two species and compared to *F. pratensis*, four times more markers were mapped in *L. multiflorum*. Among the 2,257 probes detected in *F. pratensis*, 1,078 (47.8%) were previously found polymorphic within this species. Similarly, 2,184 out of 2,761 probes (79.1%) were found polymorphic among the same number of *L. multiflorum* accessions (Kopecký et al. 2009). Based on this, we expect significantly lower number of markers to be mapped in *F. pratensis*. The discrepancy in the number of markers mapped in *L. multiflorum* compared to *F. pratensis* could be also due to low genetic variation between the parents of the *F. pratensis* mapping population. A higher level of polymorphism in *L. multiflorum* ecotypes compared to those of *F. pratensis* was also observed when using co-dominant SSR markers (Peter-Schmid et al. 2008). Based on studies of cpDNA variation, Fjellheim et al. (2006) concluded that meadow fescue in Europe went through a bottleneck during or after the last glaciations. Thus, the number of genetically mapped markers may reflect the overall diversity within a particular species.

Unexpectedly, only 20 markers could be mapped in both species. To understand this, we used data collected by Kopecký et al. (2009) and examined markers mapped in *L. multiflorum* for their presence and polymorphism among *F. pratensis* accessions and vice versa. The detailed analysis revealed that the low number of shared markers was not due to the absence of markers in the other species but due to the lack of polymorphism in the other mapping population. These 20 shared markers represent 7.0% of markers mapped in *L. multiflorum* and present in *F. pratensis* and at the same time 18.3% of markers mapped in *F. pratensis* and present in *L. multiflorum*. Consequently, these findings are in agreement with overall performance of markers available at DarTFest array (for details see Table 6).

Table 6 Extent of DArT markers' polymorphism and effectiveness of genetic mapping

	Fp present[a]	Fp polymorphic	Fp mapped	Lm present[a]	Lm polymorphic	Lm mapped
DArTFest[b]	2,257	1,078 (47.8%)	149 (6.6%)	2,761	2,184 (79.1%)	530 (19.2%)
Fp mapped	–	–	–	109	77 (70.6%)	20 (18.3%)
Lm mapped	287	146 (50.9%)	20 (7.0%)	–	–	–

[a] Number of markers detected in at least one accession of particular species. Based on data collected by Kopecký et al. (2009)

[b] DArTFest array contains 7,680 probes derived from five species of *Festuca–Lolium* complex (Kopecký et al. 2009)

Sequence analysis of DArT markers

Among the sequenced DArT markers, 64.2% were found to be unique. In similar studies, Wittenberg et al. (2005) and Tinker et al. (2009) found 56.3 and 48.1% unique markers among DArTs developed for *Arabidopsis* and oat, respectively. The levels of redundancy are strikingly similar despite the great difference in genome size, ranging from 157 Mbp for *A. thaliana* to 12,961 Mbp for oat (Bennett and Leitch 2005), and contrasting repeat content in the three species. Sequence analysis revealed that only about 7% of the mapped DArT markers were of repetitive origin and the effect of repetitive DNA on marker redundancy seems to be negligible. Sources of DArT marker redundancy are most likely related to the PCR amplification involved in array development and possibly also the presence of duplicated genes in tandem arrays which are frequent in plant genomes (Rizzon et al. 2006; Hanada et al. 2008).

DArT markers are derived from genomic representations prepared with methyl-sensitive restriction enzymes and hence should represent low-copy genomic regions. As expected, a majority of the sequences were found to be derived from expressed parts of the genomes. About 60% of all markers had homology to the NCBI EST databases and one fourth had homology to the NCBI protein database. These groups are overlapping extensively, resulting in slightly more than 60% expressed non-redundant markers/bins. This is similar to the finding of Tinker et al. (2009) in oat. Interestingly, also most of the DArT markers with repetitive origin had blast hits to ESTs and proteins. This further supports the tendency of DArT markers to originate from expressed genomic loci.

Synteny with rice and Brachypodium

Genome-wide comparisons of *L. multiflorum* to rice and *Brachypodium* based on DArT markers revealed syntenic regions shared among the species. These regions corresponded to the established syntenic relationships between *L. multiflorum* and the model species for all chromosomes but Lm5 (see Tables 3, 4). Syntenic blocks between Lm5 and Os03 and Os12 could be expected (Bolot et al. 2009). We identified markers shared between Lm5 and Os12, however, not enough markers to meet our definition of a syntenic relationship, neither did we observe the expected syntenic relationship between Lm5 and Bd1 (The International Brachypodium Initiative 2010). The number of DArT markers used to infer syntenic regions in this paper is small, and many grass chromosomes have complex syntenic relationships (The International Brachypodium Initiative 2010). Thus, the lack of expected syntenic blocks in our study could be due to uneven and low marker coverage of *L. multiflorum* chromosomes. We also found an additional syntenic relationship between chromosomes Lm6 and Bd4, identified neither between wheat and *Brachypodium* nor between barley and *Brachypodium* (The International Brachypodium Initiative 2010). However, the number of markers shared is the lowest we have accepted and this syntenic relationship requires confirmation using additional markers.

The DArTFest resources and marker-trait analysis

As an example of how we can integrate genetic maps and DArT marker sequence information generated in this study, we performed a marker-trait association study in Festulolium populations with divergent freezing tolerance levels. We identified DArT markers associated with the freezing tolerance phenotype on chromosomes 2, 4 and 7, corresponding to QTLs and chromosome segments associated with freezing tolerance in previous studies.

One DArT marker (D556159) and one bin (D558701, D562536) associated with freezing tolerance mapped in the same genomic region of chromosome 4 in *F. pratensis* and *L. multiflorum*, respectively. This observation is in line with the results obtained by Kosmala et al. (2006) using GISH, who found that Festulolium hybrid plants carrying a large central part of *F. pratensis* chromosome 4 exhibited increased freezing tolerance. Three DArT markers mapped to a chromosomal region corresponding to a freezing tolerance QTL in *F. pratensis* closely linked to the *Vrn1* gene (Alm et al., unpublished). This QTL is also found in some species of *Triticeae* where it is referred to as Fr-1 (Galiba

et al. 2009). Figure 2 shows a comparative map between LG4 in forage grasses and the syntenic relationship to the *Brachypodium* chromosome 1. The existence of two *Vrn1* paralogs in grasses (Preston and Kellogg 2007) complicates the analysis of syntenic relationships. In rice, the two *Vrn1* paralogs (also referred to as FUL1 and FUL2) are situated on different chromosomes (Os03 and Os07), while both are found on Bd1 in *Brachypodium* (Higgins et al. 2010). Syntenic relationships between forage grass LG4 and both regions with *Vrn1* copies on Bd1 are revealed by blast analysis of the significant DArT markers (Fig. 2). Markers close to both *Vrn1* paralogs were significantly associated with freezing tolerance, and as many as nine significant DArT markers were clustering close to the BdVrn1b/FUL2. This strongly supports the notion that the significant markers on Lm4 and Fp4 reflect the same underlying QTL located close to the *Vrn1* gene.

It has been debated whether the gene underlying Fr-1 QTL is directly involved in freezing tolerance or if FR-1 QTL is a pleiotropic effect of the vernalization response gene *Vrn1*. *Vrn1* is a transcription factor involved in the transition from vegetative to reproductive phase in Pooideae grasses (Cattivelli et al. 2002; Trevaskis et al. 2007). Recently, polymorphisms in *Vrn1* were shown to exhibit a pleiotropic effect on freezing tolerance by directly or indirectly affected expression of genes involved in the cold acclimation pathway (e.g. CBF genes) (Dhillon et al. 2010). This might indicate that the DArT markers associated with freezing tolerance on LG4 in forage grasses are linked to differences in the vernalization control rather than differences in the ability to withstand freezing.

Another DArT marker associated with freezing tolerance (D560530) is mapped to central part of *L. multiflorum* chromosome 2. As in case of chromosome 4, also this chromosome was implicated to carry genes associated with freezing tolerance. Using GISH, Kosmala et al. (2006) identified hybrid Festulolium plants carrying terminal *F. pratensis* segment on the short arm of *L. multiflorum* chromosome 2 with increased tolerance. Moreover, Shinozuka et al. (2006) mapped a glycine-rich RNA binding protein putatively associated with freezing tolerance to the long arm of *L. perenne* chromosome 2, and this could also be a candidate QTL underlying the significant marker on Lm2. Because the genetic position of centromere on chromosome 2 is not known, we were unable to decide on which chromosome arm the marker D560530 resides. The last of DArT markers associated with freezing tolerance (D560433) are mapped to *F. pratensis* chromosome 7, about 1.5 cM proximal to the previously reported QTL on Fp7 (Alm et al., unpublished).

High freezing tolerance in Festulolium hybrids is usually explained by *F. pratensis* introgressions carrying desired alleles into *L. multiflorum* genomic background (Kosmala et al. 2006). We used GISH to investigate genomic constitution of Festulolium hybrids with the aim to of detecting *F. pratensis* introgressions in *L. perrenne* background. However, they were detected rarely in LFT and HFT plants. This observation indicated that the freezing tolerance is either not caused by *F. pratensis* introgressions, at least in this particular case, or that the introgressions were too small to be detected by GISH. This study, however, revealed that several LFT plants were tetraploid. A common notion is that polyploid plants have improved stress tolerance compared to their diploid progenitors (Jackson and Chen 2010, Zhang et al. 2010). However, the tolerance to physical stress as freezing may decrease. A tetraploid C2 population of *F. pratensis* was shown to be less freeze tolerant and display a lower genetic variation than the original diploid population (Larsen 1979, 1994). This is in agreement with our findings as the frequency of tetraploid individuals was significantly higher in (sub)population with low freezing tolerance. The Festulolium FuRs0357 population was developed from crosses between autotetraploid *L. perenne* and tetraploid or diploid *F. pratensis*, with further backcrossing to diploid *L. perenne* for two generations. This scheme should result in diploid hybrid plants with introgressed segments of *F. pratensis* chromosomes. Tetraploid hybrid plants among the BC2 progenies could have originated from unreduced gametes of tetraploid *L. perenne* parent, and if this is the case, they contain nearly pure *Lolium* genomes. It has been observed that autotetraploid *L. perenne* has lower cold tolerance than diploid *L. perenne* (Sugiyama 1998).

Conclusion

This work marks a significant advance in improving genetic maps of important grass species *F. pratensis* and *L. multiflorum*, which are also used frequently as parents to develop increasingly popular hybrid Festulolium cultivars. As the DArTFest array became available recently, we chose to use DArT markers, because of high-throughput and low cost per data point. Sequencing genetically mapped DArT markers not only revealed that a majority of them originated from transcribed low- and single-copy genomic regions, but also allowed to establish syntenic relations with sequenced genomes of rice and *Brachypodium*. All these resources and knowledge mark significant step forward in analyzing genome structure and evolution in important grass species and expand the range of molecular tools available for breeding. Identification of DArT markers associated with freezing tolerance may help in elucidating molecular mechanisms underlying this trait and contribute to the development of resistant varieties of forage grasses.

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