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Effects of introgressions from *Festuca pratensis* on winter hardiness of *Lolium perenne*

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Abstract Previous studies reported that some genotypes with introgressed *Festuca* chromosome segment(s) in *Lolium* genome showed enhanced winter hardiness compared to *Lolium.* The aim of this study was to search comprehensively for the *Festuca pratensis* chromosome regions affecting winter hardiness-related traits when introgressed into the *Lolium perenne* genome. Association between *F. pratensis* introgression and winter hardiness-related traits (fall and winter hardiness indexes, early-spring dry matter yield, and freezing tolerance) were screened in the diploid introgression populations ($n = 203$) that had some *F. pratensis* chromosome segments introgressed. Eighty-four intron markers corresponding to unique rice genes randomly distributed across the genome were used for genotyping. Winter hardiness of almost all plants in the introgression populations was lower than that of the *F. pratensis* and triploid hybrid parents, but the average was higher than that of *L. perenne*. A significant positive effect of *F. pratensis* introgression on early-spring dry matter yield was detected on chromosome 7. This quantitative trait locus (QTL) was confirmed by linkage analysis using a backcross population with *F. pratensis* introgression in the target region of chromosome 7. However, the contribution of the newly identified QTL was rather small (6.7–9.6%), suggesting that superior winter hardiness of *F. pratensis* compared to *L. perenne* is conferred by multiple small-effect QTLs. We also detected a previously unreported negative effect of *Festuca* introgression on winter hardiness. Newly obtained QTL information in this study would contribute to the design of *Festuca/Lolium* hybrid breeding.

Keywords *Festuca* · Introgression · *Lolium* · Quantitative trait loci · Winter hardiness

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Introduction

Perennial ryegrass (*Lolium perenne* L.) is one of the most widely used temperate forage grasses because it has good forage quality, palatability, and aftermath tillering (Thomas et al. 2003). However, its poor

tolerance to abiotic stress (especially low winter hardiness, including freezing tolerance and snow mold resistance) limits its use under harsh environmental conditions (Abe 1986). For example, in Hokkaido, Japan, where the snow cover period exceeds 4 months and the lowest temperature goes down below −20 °C in winter, perennial ryegrass may suffer severe damage during winter; therefore, the use of meadow fescue (*Festuca pratensis* Huds.) instead of perennial ryegrass is often recommended.

Lolium and *Festuca* species can be hybridized to generate *Festulolium*, and their homoeologous chromosomes pair and recombine (Jauhar 1975; Thomas et al. 1994). Introgression breeding, that is, the transfer of segments of *Festuca* chromosome(s) into *Lolium* by backcrossing, could improve winter hardiness of *Lolium* while preserving its good forage characteristics (Thomas et al. 2003). Several studies have reported physical or genetic mapping of *Festuca*-derived genetic regions introgressed in *Lolium* involved in winter hardiness, especially freezing tolerance (Grønnerød et al. 2004; Kosmala et al. 2006, 2007; Humphreys et al. 2007). These studies used selected plants with high winter hardiness or freezing tolerance and their progeny; therefore, they may not necessarily have analyzed the effects of the introduction of *Festuca* chromosomal segments into the *Lolium* genome comprehensively. Quantitative trait loci (QTLs) for these traits were searched for over the whole genomes using linkage mapping populations of *L. perenne* (Yamada et al. 2004) and *F. pratensis* (Alm et al. 2011). However, such analysis in *Festulolium* is difficult because F_1 diploid hybrids between *Festuca* and *Lolium* are generally sterile. Using an association mapping approach, Bartos et al. (2011) analyzed the relationship between polymorphisms in DArT markers and freezing tolerance in the high- and low-freezing-tolerance genotypes of *Festulolium*, and identified several markers significantly associated with freezing tolerance. However, it remained unclear whether the detected associated polymorphisms were between *Lolium* and *Festuca* or within each species.

Extensive comparative genomics studies have revealed the genomic relationship (genomic structure and colinearity) between forage grasses (*Lolium* and *Festuca*) and model crop grasses (e.g., rice, barley, and *Brachypodium*) (King et al. 2007; Pfeifer et al. 2013; Byrne et al. 2015). Therefore, genomic information available for model grasses can be used for forage grasses, for which genomic information is insufficient. For genetic analysis of *Festulolium*, intron markers showing polymorphisms between *Lolium* and *Festuca* orthologs were developed from sequences of single copy rice gene (Tamura et al. 2009, 2012). Genomic positions of these markers could be estimated using comparative genomic information; this approach has produced a set of markers randomly distributed across the genome.

The aim of this study was to identify the genomic regions involved in the effects of *F. pratensis* chromosome introgression into *L. perenne* on winter hardiness-related traits. Based on the hypothesis that survey on the association between presence or absence of *Festuca* allele and winter hardiness over the genome of the *Festuca*/*Lolium* introgression populations could identify the causal regions; first, we comprehensively screened diploid introgression populations with the *L. perenne* background and introgressed *F. pratensis* chromosome segments using DNA markers developed from rice genomic information. Next, we conducted linkage analysis using a backcross population with *F. pratensis* introgression in this region. This study revealed that the introduction of *Festuca* genomic regions either negatively or positively affects winter hardiness traits in the *Lolium* background.

Materials and methods

Plant materials

Three populations (IPA12, IPA12-24, and IPA18-2) of diploid *L. perenne* with introgressed *F. pratensis* chromosome segments were generated as described in Fig. 1. The triploid F_1 hybrid plants A12 and A18 with high pollen fertility were selected from progeny of a cross between a diploid *F. pratensis* 'Makibasakae' plant as a maternal parent and a tetraploid *L. perenne* 'Pokoro' plant as a paternal parent. BC_1 progeny of A12 and A18 were generated by crossing with diploid *L. perenne* 'Yatsugatake D12' plants as maternal parents. Nuclear DNA content was estimated by using flow cytometry (PA, Partec, Munster, Germany); DNA was stained with DAPI (CyStain UV Precise P High Resolution DNA Staining Kit, Partec). Among the BC_1 plants derived from A12, those with a nuclear DNA content corresponding to a diploid were named

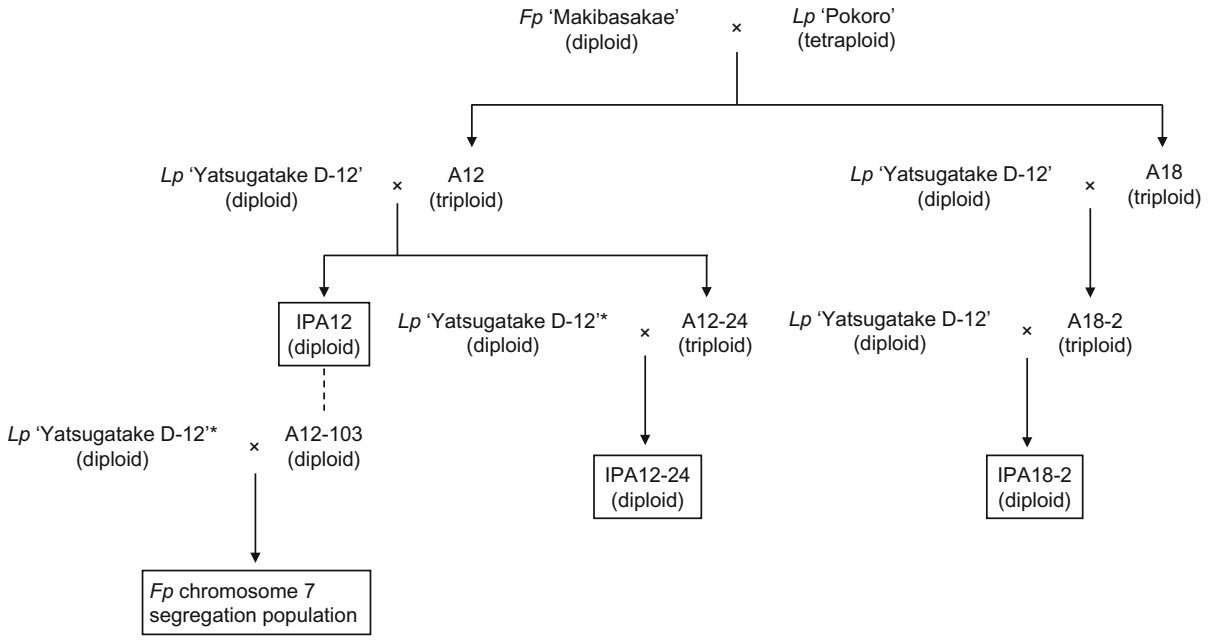

Fig. 1 A schematic diagram of the breeding process of introgression populations used in this study. *Lp* and *Fp* mean *Lolium perenne* and *Festuca pratensis*, respectively. *Boxes* indicate populations, and the others are individuals. All *Lp* 'Yatsugatake D12' individuals used for backcrossing were different genotypes. Ploidy level was estimated from the nuclear DNA content

the 'IPA12' population ($n = 47$). All A18 BC_1 progeny ($n = 5$) had a nuclear DNA content corresponding to a triploid. The BC_1 plants A12-24 (generated from A12) and A18-2 (generated from A18) with a nuclear DNA content corresponding to a triploid were crossed with 'Yatsugatake D12' plants as maternal parents. BC_2 progeny of A12-24 and A18-2 with a nuclear DNA content corresponding to a diploid were designated 'IPA12-24' ($n = 79$) and 'IPA18-2' ($n = 77$). For the linkage analysis of the winter hardiness QTL on chromosome 7, a BC_2 population ($n = 186$) derived from the A12-103 plant (from IPA12), which carried a *F. pratensis* chromosome 7 segment, was generated by backcrossing with a 'Yatsugatake D12' plant as a maternal parent. Five plants from 'Yatsugatake D12' used in the five backcrossing are different genotypes, respectively. Flowers of all maternal parents were emasculated and enclosed in bags.

Genotyping and linkage mapping

To investigate the introgression and segregation of *F. pratensis* alleles, DNA markers polymorphic between *L. perenne* and *F. pratensis* were used for genotyping. For introgression mapping, 84 *Lolium/Festuca* intron-flanking markers (Supplementary material 1) were used to genotype 203 individuals. Many of these markers were previously described by Ishikawa et al. (2007, 2009) and Tamura et al. (2009, 2012); primer sequences for some markers were modified (Supplementary material 1). Eight markers were newly developed using the previously described method (Tamura et al. 2009, 2012). Genotyping was performed as in Tamura et al. (2009, 2012). Markers were named based on the Institute for Genomic Research (TIGR) locus names of corresponding single-copy rice genes (Supplementary material 1). The chromosomal location of each marker was previously determined using seven *Lolium/Festuca* monosomic substitution plants (Tamura et al. 2009; Harper et al. 2011) or estimated using *L. perenne* GenomeZipper data (Byrne et al. 2015).

For the segregation mapping of a *F. pratensis* segment of chromosome 7, ten *Lolium/Festuca* intron-flanking markers and six *L. perenne* EST-derived SSR markers located on *L. perenne* linkage group 7 (Studer et al. 2010) were used. The presence or absence of a *F.*

pratensis allele was regarded to indicate a heterozygote or homozygote, respectively. Linkage analysis was performed using the program JoinMap version 4.0 (Van Ooijen 2006) with the population type code "BC1". Marker order and genetic distance were calculated using a regression mapping algorithm with Kosambi's mapping function.

Genomic in situ hybridization (GISH)

The experiments were performed as described in Kubota et al. (2015).

Field experiment with introgression populations

Plants of the three introgression populations and their parents grown in plastic nursery pots (5 cm × 5 cm × 5 cm) in a greenhouse were transplanted to the field nursery of the Hokkaido Agricultural Research Center, National Agriculture and Food Research Organization (HARC/NARO) in Sapporo, Japan (43°00′N, 141°42′E) on 16 July 2010. Three clones of each genotype were placed in 80 × 50 cm with a randomized block design. The environmental conditions of the 2010/2011 and the 2011/2012 winter are indicated in Supplementary material 2. The snow cover period in the second winter (2011/2012; 136 days) was longer than that in the first winter (2010/2011; 114 days). Cutting at a height of 7 cm above the ground was performed when the plant length reached 30–40 cm, followed by application of chemical fertilizer (21 kg N ha^{-1}, 16 kg P_2O_5 ha^{-1} and 21 kg K_2O ha^{-1}). In 2011, the first cutting was conducted on 5 May and was followed by six cuttings until 28 September. In 2012, the first cutting was conducted on 18 May. Phenotyping was performed on three replicates before the first cutting in 2011, after that was performed on two replicates. Dry matter in the first cutting (early-spring dry matter) was measured after drying at 70 °C for 72 h. Winter hardiness was scored on a scale of 1–9 according to the vigor of sprouting after snowmelt (20 April 2011 and 2 May 2012), with 1 corresponding to no sprouting and 9 corresponding to very vigorous sprouting: Score 5 corresponded to moderate sprouting across the stub but with some injured parts. Plant vigor in fall (5 December 2010 and 10 December 2011) was evaluated as growth on a scale of 1–9, with 1 corresponding to very little growth and 9 corresponding to extensive growth: Score 5 was intended to be scored as median of all genotype. Dates of field operations were summarized in Supplementary material 3.

Field experiment with a *Festuca pratensis* chromosome 7 segregation population

For the phenotyping, of 186 genotypes used for linkage mapping, we randomly selected 52 of 55 recombined plants, 25 of 40 non-recombined heterozygous (*Festuca*/*Lolium*) plants and 21 of 91 of non-recombined *L. perenne* homozygous plants, respectively, because our nursery did not have enough area to investigate all genotypes, and the population have a significant segregation distortion with a lower frequency of *F. pratensis* alleles. Three clones of each genotype grown in a plastic nursery pots (5 cm × 5 cm × 5 cm) in a greenhouse were placed in 80 × 50 cm with a randomized block design at the field nursery of HARC/NARO on 13 September 2012. The environmental condition of the 2012/2013 winter is indicated in Supplementary material 2. The snow cover period was 151 days. Indexes of fall vigor, winter hardiness, and spring vigor were scored on 13 November, 23 April, and 13 May, respectively. Spring vigor was evaluated similar to fall vigor as described above. Overhead digital images of each plant were taken on 23 April. Using Photoshop CS6 (Adobe Systems, San Jose, CA, USA), the area of whole plant and its green parts were manually picked out from the background using the Magic Extractor tool and their pixels were counted. Proportion of green parts was calculated as a percentage ratio of the number of green part pixels to that of the whole plant. Heading date (ear emergence) was measured as days after 1 June 2013. Dry matter was measured at the heading stage as described above. Dates of field operations were summarized in Supplementary material 3.

Freezing tolerance test

Freezing tolerance of crown tissues was evaluated according to Moriyama et al. (1995) with some modifications. Three clones per genotype were transplanted in plastic pots (4 cm × 4 cm × 4 cm) on 23 July 2010 and were kept in a greenhouse. Plants were transferred outside at HARC/NARO on 8 October 2010. Freezing treatment was performed on 13–15 December 2010. Three clones were bulked, their tillers were divided, and leaves and roots were cut off.

On average, seven crowns were wrapped together in a moist absorbent cotton sheet, covered with aluminum foil, and placed in a programmable freezer (LU-112, Tabai ESPEC, Osaka, Japan). After ice nucleation at −3.0 °C for 6 h, the temperature was reduced by 1 °C h^{-1} to −12.5 °C or −14 °C and then maintained for 3 h; after freezing, the crowns were transferred to 6 °C overnight. Crowns were transplanted into vermiculite in a plastic box and grown in a greenhouse for 4 weeks. Each divided tiller was scored, and average Larsen's visual score was calculated for each genotype according to Kosmala et al. (2006) with some modifications: 1 = dead, no sign of leaf elongation; 2 = dead but leaves having previously elongated < 0.5 cm; 3 = dead, but leaves having previously elongated 0.5–2 cm; 4 = likely to die, but at least one new root elongated; 5 = likely to survive, but badly damaged; 6 = plant survived, but with severe damage to approximately 50% of leaves; 7 = plant survived but with visual signs of freezing injury; 8 = minimum freezing injury; 9 = no visible signs of injury.

Statistical analysis

Mean phenotypic values for each trait were compared between the presence and absence of a *F. pratensis* allele at each locus in the three bulked introgression populations by *t* test using JMP 9 (SAS Institute, Cary, NC, USA). To eliminate the background effect attributed to the different parents among three populations, values of the IPA12-24 and IPA18-2 populations were standardized by multiplication by the ratio of the mean value of IPA12 to the mean value for the respective population. Populations with no *F. pratensis*–derived introgressed allele at a locus were excluded from the comparison at that locus. Significance level was set at P ($-\log_{10}$) > 3.2 based on the Bonferroni correction at $\alpha = 0.05$. QTL analysis using a population segregating for a *F. pratensis*–derived segment of chromosome 7 was conducted using a simple interval mapping procedure of MapQTL 6 (Van Ooijen 2009). Analysis was performed only for linkage group 7. Permutation tests (1000 iterations) were performed to determine the LOD threshold, and loci with a LOD score above the threshold ($P < 0.05$) were considered as significant QTLs.

Results

Genetic characterization of introgression populations

A total of 203 diploid progeny of triploid *L. perenne*/*F. pratensis* hybrids backcrossed with diploid *L. perenne* were genotyped using 84 intron-flanking markers randomly distributed across the genome. Among the 203 plants, 138 (68%) had a *F. pratensis*-specific allele at least in one locus: 41 of 47 (85%) in IPA12, 45 of 79 (57%) in IPA12-24, and 52 of 77 (68%) in IPA18-2 (Supplementary materials 4–6). The mean and maximum numbers of introgressed loci per plant were 6.8 and 22 in IPA12, 3.0 and 15 in IPA12-24, and 3.8 and 32 in IPA18-2. Based on the genotyping data, it was assumed that 41% ($n = 84$), 18% ($n = 36$), 6% ($n = 13$), 2% ($n = 3$) and 1% ($n = 2$) of the plants in the three populations had *F. pratensis* introgression(s) in one, two, three, four and five chromosome(s), respectively. Genomic in situ hybridization analysis with probes for genomic DNA of *L. perenne* and *F. pratensis* also revealed that several individuals had partial or whole chromosome(s) from *F. pratensis* (Supplementary material 7). Introgression frequency for each chromosome is shown in Table 1. Mean introgression frequency for all 84 loci was 8.1% in IPA12, 3.7% in IPA12-24, and 4.6% in IPA18-2 (Table 1). For some loci, the introgression frequency differed significantly among the three populations; in particular, the introgression frequency of chromosome 1 was much higher in IPA12 than in the other populations ($P < 0.0001$, Chi-square test; Table 1). The hybrid parents A12-24 and A18-2 had no *F. pratensis* allele at 7 and 19 loci, respectively (e.g., on chromosome 2 in A18-2); at such loci, backcross progeny naturally had no *F. pratensis* alleles (Supplementary materials 5, 6). *Festuca pratensis*-derived alleles were confirmed at all investigated loci except Os02g17870 on chromosome 6 (Supplementary materials 4–6); the latter locus was excluded from association analysis.

Winter hardiness and freezing tolerance in the introgression populations

Fall vigor index, winter hardiness index, and early-spring dry matter were evaluated over 2 years. After both winters, disease caused by snow molds, especially *Typhula ishikariensis*, was detected in most

Table 1 *Festuca pratensis* allele frequency in the three introgression populations

Chromosome[a]	Number of markers	Mean (minimum–maximum) allele frequency (%)				
		IPA12 ($n = 47$)	IPA12-24 ($n = 79$)	IPA18-2 ($n = 77$)	Total	Total used for association analysis[b]
1	10	37.2 (25.5–42.6)	3.3 (2.5–5.1)	4.9 (2.6–6.5)	11.8 (8.4–13.3)	11.8 (8.4–13.3)
2	13	3.6 (2.1–6.4)	3.0 (0.0–11.4)	1.8 (0.0–9.1)	2.7 (0.5–6.4)	4.2 (2.1–9.5)
3	9	3.8 (2.1–8.5)	4.9 (1.3–9.0)	5.5 (0.0–14.3)	4.9 (1.0–9.9)	5.1 (1.6–9.9)
4	17	3.8 (2.1–8.5)	4.5 (0.0–7.6)	5.7 (0.0–13.0)	4.8 (0.5–6.4)	5.9 (2.1–11.7)
5	9	4.7 (2.1–10.6)	2.8 (1.3–7.6)	5.2 (0.0–18.2)	4.2 (1.0–8.9)	4.5 (1.6–8.9)
6	14	2.6 (0.0–8.5)	2.5 (0.0–7.6)	4.8 (0.0–11.7)	3.2 (0.0–9.4)	3.5 (2.0–9.4)
7	12	7.3 (4.3–12.8)	4.7 (0.0–7.6)	4.8 (0.0–9.1)	5.3 (1.5–7.9)	6.9 (4.8–12.8)
Total	84	8.1 (0.0–42.6)	3.7 (0.0–11.4)	4.6 (0.0–18.2)	5.1 (0.0–13.3)	5.9 (1.6–13.3)

[a] Chromosome location of some marker loci was estimated using *Lolium perenne* GenomeZipper (Byrne et al. 2015)

[b] For each marker locus, a population without *F. pratensis* alleles was excluded from the calculation of allele frequency

plants. *Festuca pratensis* and hybrid parents of the introgression populations showed superior winter hardiness and higher dry matter in early spring than *L. perenne* in both years (Table 2). Mean values of winter hardiness index and early-spring dry matter of the three introgression populations were lower than those of the corresponding hybrid parents and higher than those of *L. perenne*, except for the winter hardiness index after the first winter in IPA12-24 (Table 2). Only one plant died (i.e., winter hardiness index was 1.0) in IPA18-2 after the first winter; a total of 31 plants died after the second winter (7 in IPA12, 5 in IPA12-24, and 19 in IPA18-2). Introgression populations showed a wide range of winter hardiness, but only two plants showed greater winter hardiness than that of hybrid parents after the first winter (one in IPA12-24 and the other in IPA12-18), and no plant did after the second winter (Table 2).

Freezing tolerance was investigated in 152 plants arbitrarily selected from the three populations. The order of freezing tolerance in both (−12.5 and −14 °C) treatments was as follows: *F. pratensis* > hybrid parents > *L. perenne* parents (Table 2). Introgression populations showed a wide range of freezing tolerance, but the mean value was similar to that of *L. perenne* (Table 2). After −14 °C treatment, freezing tolerance of only three plants (two in IPA12 and one in IPA12-24) was higher than that of the hybrid parents.

All correlation coefficients among fall vigor index, winter hardiness index, and early-spring dry matter in both years were positive and significant (Table 3), especially those between winter hardiness index and early-spring dry matter (0.73 and 0.77 after the first and second winter, respectively). The correlation coefficient between freezing tolerance at −12.5 and −14 °C was 0.52. Correlation coefficients between freezing tolerance at −12.5 °C and winter hardiness index were small but significant in both years (0.24 in 2010/2011 and 0.17 in 2011/2012; Table 3). Freezing tolerance at −14 °C did not show significant correlation with any traits (Table 3).

Association between introgressed *F. pratensis* alleles and winter hardiness

Mean values of the winter hardiness-related traits with or without *F. pratensis* allele were compared at 83 loci in the bulked introgression population (mean *F. pratensis* allele frequency, 5.9%; Table 1). Significant differences in the fall vigor index assessed in the first year were detected on chromosome 5, with negative effects of *F. pratensis* alleles (Table 4). After the first winter, significant negative effects of *F. pratensis* alleles were also detected for winter hardiness index on chromosomes 4 and 6, and for early-spring dry matter on chromosome 4 (Table 4). After the second winter, there was no significant difference for fall vigor index and winter hardiness index at any locus, but significant differences in early-spring dry matter were detected at Os06g13810 and Os10g25360 loci on chromosome 7, with positive effects of *F. pratensis* alleles (Table 4). For freezing tolerance, no significant difference at P $(-\log_{10}) > 3.2$ was detected at any

Table 2 Fall vigor index (FVI), winter hardiness index (WHI), early-spring dry matter (ESDM), and freezing tolerance of the *Lolium–Festuca* introgression populations and their parents [mean ± standard deviation (minimum–maximum)]

Population	Field test in 2010/2011			Field test in 2011/2012			Freezing tolerance	
	FVI[a,b]	WHI[a,b]	ESDM (g/plant)[b]	FVI[a,b]	WHI[a,b]	ESDM (g/plant)[b]	−12.5 °C[a,c]	−14.0 °C[a,c]
Fp[d]	7.7 ± 0.8 (6.7–8.7)	6.7 ± 1.2 (5.7–8.7)	47.1 ± 19.4 (13.7–62.0)	6.6 ± 1.0 (5.0–7.5)	6.8 ± 1.4 (5.5–8.5)	18.4 ± 8.1 (8.2–25.2)	7.1 ± 1.1 (5.9–8.1)	6.1 ± 1.0 (5.1–6.9)
IPA12								
Progeny	5.1 ± 1.2 (2.0–7.3)	4.0 ± 1.2 (1.3–7.3)	6.0 ± 4.8 (0.0–20.3)	3.8 ± 1.8 (1.0–6.0)	2.8 ± 1.3 (1.0–5.0)	0.9 ± 1.0 (0.0–3.4)	3.9 ± 1.1 (2.1–6.0)	2.4 ± 0.7 (1.6–4.6)
Triploid parent	7.7	8.0	60.2	7.5	7.5	30.7	6.3	4.4
Lp[d]	3.0	2.3	0.5	2.0	1.5	0.0	4.3	2.0
IPA12-24								
Progeny	6.9 ± 1.3 (2.3–9.0)	4.0 ± 0.9 (1.7–6.0)	12.0 ± 8.2 (0.2–33.9)	4.7 ± 1.5 (1.0–8.0)	2.9 ± 1.1 (1.0–5.5)	1.0 ± 1.2 (0.0–4.9)	3.7 ± 1.3 (1.3–6.6)	2.5 ± 0.8 (1.0–5.5)
Triploid parent	5.3	5.7	17.0	3.0	5.5	2.6	5.0	5.5
Lp	4.0	5.0	4.6	3.0	4.0	0.8	5.0	2.3
IPA18-2								
Progeny	5.8 ± 1.8 (1.0–9.0)	3.4 ± 1.0 (1.0–7.3)	6.4 ± 5.7 (0.0–22.5)	4.0 ± 2.0 (1.0–8.5)	2.4 ± 1.0 (1.0–5.0)	0.6 ± 0.8 (0.0–3.2)	2.8 ± 1.1 (1.0–5.9)	2.0 ± 0.6 (1.1–3.9)
Triploid parent	6.8	5.5	24.1	6.3	6.0	16.0	3.9	4.8
Lp	5.7	2.3	2.0	2.5	1.5	0.0	2.6	1.9

[a] Scores (1–9) were assigned as described in the "Materials and methods"

[b] Fp, $n = 5$; IPA12 progeny, $n = 47$; IPA12-24 progeny, $n = 79$; IPA18-2 progeny, $n = 77$

[c] Fp, $n = 3$; IPA12 progeny, $n = 38$; IPA12-24 progeny, $n = 53$; IPA18-2 progeny, $n = 61$

[d] Fp, *Festuca pratensis* 'Makibasakae', used as a parent of triploid hybrids; Lp, *Lolium perenne* 'Yatsugatake D12', used as a parent in backcrossing

locus. At the locus Os02g03260 on chromosome 6, the *F. pratensis* allele increased freezing tolerance, although this effect was not significant: P ($-\log_{10}$) = 2.2 (−12.5 °C) and 2.6 (−14 °C).

Winter-hardiness QTLs on chromosome 7

To confirm the positive effect of *F. pratensis* alleles on chromosome 7 on winter hardiness, we performed QTL analysis using the progeny of A12-103, which had an introgression of a *F. pratensis* chromosome 7 segment including loci that had significant effects on early-spring dry matter, backcrossed with *L. perenne*. This backcross population showed significant segregation distortion, with a lower frequency of the *F. pratensis* alleles (average ratio of *L. perenne*/*L. perenne*:*F. pratensis*/*L. perenne* genotype = 1:0.53 [SD, 0.04], whereas the expected ratio was 1:1; $P < 0.0001$, Chi-square test). A genetic linkage map of the introgressed *F. pratensis* region in linkage group 7 was constructed; this map spanned 34.1 cM (Fig. 2). In the field experiment, snow mold disease was detected after the winter, and about 30% of the clones died. One week after the snow had melted, the average proportion of green parts (leaves that stayed green under the snow, or sprouts) in each plant was 1.8% (Table 5). Winter hardiness index, winter survival rate, the proportion of green parts, spring vigor index, and dry matter at heading showed high positive correlation (Table 6). Fall vigor index and heading date showed no significant correlation with winter hardiness-related traits, except for a negative correlation between fall vigor index and proportion of green parts (Table 6). QTLs for winter hardiness index,

Table 3 Pearson's correlation coefficients among winter hardiness-related traits in the bulked *Festuca/Lolium* introgression population

Year	Traits	2010/2011			2011/2012			2010	
		FVI	WHI	ESDM	FVI	WHI	ESDM	FT12.5	FT14
2010/2011	Fall vigor index (FVI)		****	****	****	****	****	****	NS
	Winter hardiness index (WHI)	0.52		****	****	****	****	**	NS
	Early-spring dry matter (ESDM)	0.66	0.73		****	****	****	**	NS
2011/2012	Fall vigor index (FVI)	0.79	0.47	0.62		****	****	****	NS
	Winter hardiness index (WHI)	0.49	0.42	0.46	0.61		****	*	NS
	Early-spring dry matter (ESDM)	0.40	0.33	0.45	0.42	0.77		NS	NS
2010	Freezing tolerance at −12.5 °C (FT12.5)	0.35	0.24	0.22	0.36	0.17	0.08		****
	Freezing tolerance at −14 °C (FT14)	0.10	0.08	0.11	0.13	0.04	0.04	0.52	

NS not significant

**** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$

Table 4 Association between winter hardiness-related traits and the presence or absence of *Festuca pratensis* alleles in the bulked *Lolium/Festuca* introgression population

Year	Trait	Marker	Chromosome	P (−log10)[a]	Mean value	
					Presence of *Festuca* allele	Absence of *Festuca* allele
2010/2011	Fall vigor index[b]	Os09g23650	5	4.0	3.4	5.2
		Os09g03610	5	3.5	3.5	5.2
		Os09g15820	5	3.5	3.6	5.2
	Winter hardiness index[b]	Os11g29380	4	4.0	2.9	4.1
		Os03g46410	4	3.7	2.9	4.1
		Os03g43760	4	3.7	2.9	4.1
		Os11g38020	4	3.5	2.8	4.1
		Os11g47710	4	3.5	2.9	4.1
		Os04g31210	4	3.4	2.9	4.1
		Os03g38980	4	3.4	2.9	4.1
		Os03g27320	4	3.4	2.9	4.1
		Os11g09280	4	3.2	3.0	4.1
		Os01g01080	6	3.7	2.5	4.1
		Os02g39570	6	3.7	2.5	4.1
		Os02g14730	6	3.4	2.5	4.1
	Early-spring dry matter (g/plant)	Os11g29380	4	3.4	1.5	6.7
2011/2012	Early-spring dry matter (g/plant)	Os10g25360	7	3.2	2.4	0.8
		Os06g13810	7	3.2	1.8	0.8

[a] Significance level was set at $P\ (-\log_{10}) > 3.2$ based on the Bonferroni correction at $\alpha = 0.05$, $n = 83$

[b] Scores (1–9) were assigned as described in the "Materials and methods"

winter survival rate, and proportion of green parts were detected around the Os08g33630 locus with R^2 6.7–9.6 (Table 5); the LOD 1.0 support interval included Os10g25360, which was associated with early-spring dry matter in the introgression population mentioned above (Fig. 2). For all these traits, *F.*

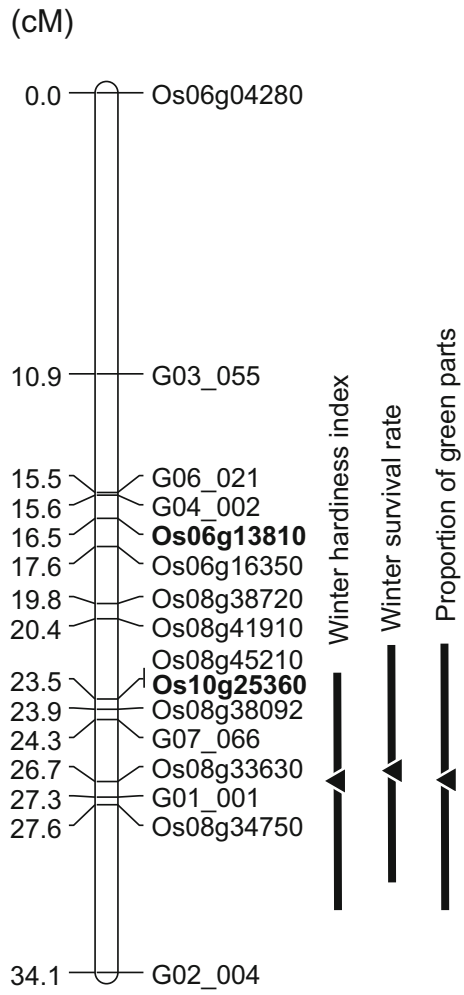

Fig. 2 Winter hardiness-related QTLs in linkage group 7 in the backcross progeny of *Lolium/Festuca* introgression genotype A12-103. *Bars* 1.0-LOD support intervals, *triangles* LOD peaks. Loci significantly associated with early-spring dry matter in the *Lolium/Festuca* introgression populations are shown in bold

pratensis alleles had positive effects (Table 5). No QTLs were detected for spring vigor index, heading date, or yield at heading.

Discussion

In this study, we used association analysis of introgression populations followed by linkage analysis of a backcross population, and identified a QTL on *F. pratensis* chromosome 7 with a positive effect on winter hardiness in the field. We detected no QTLs for heading date or dry matter yield at the heading stage in the backcross population, which indicates that the identified QTL does not affect growth rate in spring. In the field nursery where this study was performed, snow depth was sufficient to prevent soil freezing during winter; this is consistent with the absence of correlation between winter-hardiness index and freezing tolerance in the introgression population. In this field nursery, a major factor of winter injury is snow mold disease (Sanada et al. 2007), which was caused mainly by *T. ishikariensis* in this study. Therefore, the QTL detected on chromosome 7 might be involved in snow mold resistance. The absence of an association between winter hardiness and *F. pratensis* chromosome 7 introgression in the first winter (2010/2011) is likely attributable to a shorter period of snow cover than in winters 2011/2012 and 2012/2013, because the duration of snow cover is the most critical factor for snow mold disease (Matsumoto and Hoshino 2013). Previously, no QTL for winter hardiness in the field was detected in linkage group 7 in *L. perenne* or *F. pratensis* (Yamada et al. 2004; Alm et al. 2011; Paina et al. 2016), although Alm et al. (2011) detect a frost tolerance QTL and a drought tolerance QTL on chr 7 in *F. pratensis*. It might be because these studies did not use intergeneric hybrids. Further studies of snow mold infection and comparative genomic studies would reveal the function of this QTL.

Triploid hybrids between *F. pratensis* and *L. perenne* showed winter hardiness and freezing tolerance clearly superior to those of *L. perenne*, although they were not as tolerant as *F. pratensis*. Winter hardiness of almost all backcross progeny of the triploid hybrids was lower than that of the parents, but the average was still higher than that of *L. perenne*. However, we identified only one QTL with the *F. pratensis* allele having a small positive effect in the association analysis. These data suggest that superior winter hardiness of *F. pratensis* compared to *L. perenne* is conferred by multiple small-effect QTLs, most of which were not detected in this study. A small number of genotypes with *F. pratensis* introgression (mean *F. pratensis* allele frequency, 5.9%) might have prevented the detection of QTLs with small effects. Analysis of backcross progeny of plants carrying introgressed segments of each *F. pratensis* chromosome (similar to the analysis performed in our study for chromosome 7) would be more precise because many genotypes with recombined *Festuca* alleles

Table 5 Values of and quantitative trait loci for winter hardiness-related traits in the population segregating for a *Festuca pratensis*-derived segment of chromosome 7 in the *Lolium* background

Trait	Mean ± SD	Range	Broad-sense heritability	QTL				
				Max. LOD position (cM)	1.0 LOD-support interval (cM)	Max. LOD value	R^2 (%)	Weight[a]
Fall vigor index[b]	5.9 ± 0.8	3.0–7.3	0.54	ND				
Winter hardiness index[b]	2.6 ± 1.2	1.0–6.7	0.35	26.7	22.4–31.7	1.7	7.7	0.7
Winter survival (%)	72.5 ± 32.2	0.0–100	–	26.3	21.4–30.7	2.2	9.6	20.8
Proportion of green parts (%)	1.8 ± 3.6	0.0–23.0	0.26	26.7	21.4–32.7	1.5	6.7	1.9
Spring vigor index[b]	3.7 ± 1.9	1.0–8.3	0.41	ND				
Dry matter yield at heading (g/plant)	14.0 ± 14.2	0.0–65.3	0.40	ND				
Heading date[c]	10.7 ± 2.6	6.0–17.0	–	ND				

ND not detected

[a] Defined as the additive effect of *F. pratensis* alleles

[b] Scores (1–9) were assigned as described in the "Materials and methods"

[c] Days after 1 June 2013

Table 6 Pearson's correlation coefficients among winter hardiness-related traits in the population segregating for a *Festuca pratensis*–derived segment of chromosome 7 in the *Lolium* background

	FVI	WHI	WS	GP	SVI	HDM	HD
Fall vigor index (FVI)		NS	NS	**	NS	NS	NS
Winter hardiness index (WHI)	−0.10		****	****	****	****	NS
Winter survival (WS)	0.07	0.81		**	****	****	NS
Proportion of green parts (GP)	−0.31	0.68	0.30		****	****	NS
Spring vigor index (SVI)	0.00	0.94	0.82	0.54		****	NS
Dry matter at heading (HDM)	0.11	0.84	0.68	0.45	0.90		NS
Heading date (HD)	0.12	−0.21	−0.07	−0.03	−0.21	−0.11	

NS not significant

**** $P < 0.0001$; ** $P < 0.01$

would be available. A high frequency of the allele introgressed from *Festuca* would also enable detection of epistatic effects among multiple introgressed regions, which were found in the populations used in this study, for winter-hardiness related traits. This study used DNA markers derived from ESTs homologous to rice genes distributed over the whole genome. GenomeZipper between *L. perenne* and model grasses reported by Byrne et al. (2015) shows that some regions of the *L. perenne* genome do not correspond to any rice genes, and additional markers would be needed to identify small-effect QTLs that might be located in such regions. In this study, we did not detect the QTLs for freezing tolerance on chromosomes 2 and 4 reported by Kosmala et al. (2006, 2007). In addition to the low introgression frequency mentioned above, this discrepancy might be due to the difference in the background genomes: *L. multiflorum* was used by Kosmala et al. (2006, 2007), whereas we used *L. perenne*. Generally, winter hardiness and freezing tolerance of *L. multiflorum* are inferior to those of *L. perenne*; therefore, the positive effect of *Festuca*

introgression could be clearer in *L. multiflorum* than in *L. perenne*.

Little information on the negative effects of *Festuca* introgression into *Lolium* is available, because materials used in previous studies often originated from selected genotypes with superior stress tolerance (Grønnerød et al. 2004; Kosmala et al. 2006, 2007). We detected a negative effect of the introgression of *F. pratensis* segments of chromosomes 4 and 6 on winter hardiness index after the first winter. In these loci, genotypes with *F. pratensis* alleles tended to show lower scores of fall vigor index in the year of transplantation (data not shown). Fall vigor index was positively correlated with winter hardiness in both years. Therefore, the negative effects of *Festuca* introgression on winter hardiness might be due to reduced plant vigor.

King et al. (2013) revealed the presence of directional selection pressure for both the transmission of *F. pratensis* chromosomes and *L. perenne*/*F. pratensis* recombination in the backcross progeny of monosomic substitution lines. They reported that 72 of 73 backcross progeny with *F. pratensis* chromosome 1 substitution in the *L. perenne* background had recombined *L. perenne*/*F. pratensis* chromosomes. We confirmed positive selection for the transmission of recombinant *L. perenne*/*F. pratensis* chromosome 1 in the IPA12 population but not in IPA12-24 or IPA18-2. The difference in the transmission frequency of *F. pratensis* alleles among the populations might be caused by cytoplasmic type of the triploid parent: the triploid parent of IPA12 has the *F. pratensis* cytoplasm, whereas those of IPA12-24 and IPA18-2 have the *L. perenne* one. In a triploid hybrid of *F. pratensis* and *L. multiflorum*, the frequency of intergeneric translocations was higher in the *Festuca*-derived cytoplasm than in the *Lolium*-derived one (Zwierzykowski et al. 1999; Naganowska and Zwierzykowska 2001). King et al. (2013) also reported neutral selection pressure for the transmission of recombinant *L. perenne*/*F. pratensis* chromosomes, including chromosome 7. However, in this study, the progeny of a plant carrying a fragment of *F. pratensis* chromosome 7 backcrossed with *L. perenne* showed significant segregation distortion with a lower frequency of the *F. pratensis* allele. Further investigation of selection pressure for the transmission of *Festuca* chromosomes in the *Lolium* background using various genotypes is needed.

Conclusion

In this study, unlike previous studies of *Festulolium*, association between the introgression and winter hardiness were comprehensively surveyed over the genome using introgression populations. As a result, we identified a QTL on chromosome 7 with a positive effect of the *F. pratensis* allele on winter hardiness in the *L. perenne* genomic background, although multiple QTLs with small effects seem to be involved in the improvement of winter hardiness-related traits in *Festulolium* in comparison with *L. perenne*. We also found negative effects of *F. pratensis* alleles on winter hardiness in some genomic regions. Although the improvement of the winter hardiness of *Lolium* by introgression of a few *Festuca* genomic regions might be limited, even a minor improvement in winter hardiness with preservation of the advantageous traits of *Lolium* (e.g., good forage quality and rapid initial growth) would be useful. In addition to the previously reported QTLs, QTL information obtained in this study would improve the selection efficiency. Meanwhile, for a major improvement in winter hardiness-related traits, the use of F_1 hybrids such as amphidiploids is a better approach in *Lolium*/*Festuca* breeding (unpublished data). QTL information obtained here might also be useful for the fine tuning of winter hardiness-related traits in amphidiploid *Festulolium* breeding.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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