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ORIGINAL PAPER

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Introgression-mapping of genes for drought resistance transferred from *Festuca arundinacea* var. *glaucescens* into *Lolium multiflorum*

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Abstract Procedures for the transfer of genes for drought resistance from *Festuca glaucescens* ($2n = 4x = 28$) into *Lolium multiflorum* ($2n = 2x = 14$) are described. Following the initial hybridisation of a synthetic autotetraploid of *L. multiflorum* ($2n = 4x = 28$) with *F. glaucescens*, the F_1 hybrid was backcrossed twice onto diploid *L. multiflorum* ($2n = 2x = 14$) to produce a diploid *Lolium* genotype with a single *F. glaucescens* introgression located distally on the nucleolar organiser region arm of chromosome 3. The transmission of *F. glaucescens*-derived amplified fragment length polymorphisms and a sequence-tagged-site (STS) marker was monitored throughout the breeding programme. Those genotypes of a mapping population of backcross 3 that survived combined severe drought and heat stress all contained the *F. glaucescens*-derived markers. The STS marker provided a prototype for a PCR-based system for high-throughput screening during cultivar development for the presence of the *F. glaucescens*-derived genes for drought resistance. The frequency of intergeneric recombination between *L. multiflorum* and *F. glaucescens* is described. During the initial stages of the breeding programme, preferential intraspecific chromosome pairing between *Lolium* homologues and *Festuca* homoeologues dominated with low frequencies of intergeneric chromosome associations. However, these increased in the backcross 1 due to the absence of opportunities for intraspecific chromosome pairing between homoeologous *Festuca* chromosomes following the loss of half of the *Festuca* chromosomes. Once transferred to *Lolium*, *F. glaucescens* sequences recombined with *Lolium* at high frequencies, thereby enabling the loss of potentially deleterious gene combinations that might reduce the forage quality of *Lolium*.

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Introduction

Demands for the development of multifunctional and sustainable grassland systems require the development of more persistent high-quality grasses. In a European context, the two ryegrass (*Lolium*) species, *L. perenne* and *L. multiflorum*, are considered to offer farmers the best options for safe animal fodder since they provide high yields and forage of good quality and digestibility. *L. multiflorum* in particular has excellent establishment, spring growth and good forage quality, but its use is restricted, due primarily to its poor ability at withstanding summer and winter stresses. Fescue (*Festuca*) species, which are related closely to *Lolium* and hybridise readily, have a greater resistance to abiotic stresses and through hybridisation offer opportunities for combining the attributes of the two genera (Humphreys et al. 2003). However, the efficacy of breeding programmes aimed at gene transfer between *Lolium* and *Festuca* spp. will in hybrids rely on the extent of intergeneric chromosome pairing and recombination.

We describe here how a backcross breeding programme was constructed that enabled gene transfer from *Festuca arundinacea* var. *glaucescens* ($2n = 4x = 28$) (henceforth referred to as Fg) into *L. multiflorum* (Lm) ($2n = 2x = 14$). Procedures led to improved persistency of Lm under combinations of severe drought and heat. These included an introgression-mapping approach that effectively "dissected" the *Festuca* genome and enabled the physical location of regulatory and/or major *Festuca* genes that in Lm contributed consistently over generations to enhanced drought resistance. Any Fg-specific molecular markers recovered in a Lm introgression line with only a single alien *Festuca* chromosome segment would be expected to be located within that introgressed

segment. In this way, molecular markers were selected that targeted Fg genes transferred into Lm. We report the development of a Fg-derived sequence-tagged-site (STS) that may be used as a diagnostic test in PCR-based marker screens designed to detect the presence of Fg genes for drought resistance over generations in the development of drought-resistant Lm varieties.

Materials and methods

The scheme for the breeding programme used here was as the one described by Morgan et al. (2001) but was more extensive and employed alternative plant genotypes. The objectives were: (1) to measure the frequency of intergeneric Lm-Fg chromosome pairing and gene exchange in order to test the suitability of the procedures for introgression-mapping and (2) to test whether Fg genes transferred to Lm led to improvements in drought resistance.

The breeding programme

A *Festuca arundinacea* var. *glaucescens* genotype, accession Bn 354-4 ($2n = 4x = 28$), taken from a collection made in Portugal was hybridised onto Bb 2264-2, a genotype from a synthetic autotetraploid Lm cultivar, Roberta ($2n = 4x = 28$). The F_1 hybrid P188/48-1 as male was then backcrossed onto Lm Bb2205-15 cv. Atalja ($2x$) to generate backcross-1 (BC_1F_1) plants for use in the cytological study (Table 1). Nine of the BC_1F_1 plants were subsequently backcrossed once more onto Lm cvs. Atalja, AberComo, Trajan and Meribel (all $2n = 2x = 14$) to produce nine backcross-2 (BC_2F_1) populations for inclusion in tests for drought resistance. Procedures used for generating, selecting and mapping Fg genes for drought tolerance are summarised in Fig. 1. A BC_2F_1 plant, P194/208 genotype 19 (P194/208-19), was derived from the backcross between Lm Bb2042-12 cv. AberComo and BC_1F_1 P193/206-4. P194/208-19, which was more drought resistant than its Lm parents, was diploid with 14 Lm chromosomes and carried a clearly identifiable single Fg recombinant, was backcrossed further onto an AberComo genotype (Lm Bb2042-13) to produce a backcross-3 (BC_3F_1) population, P201/32 ($2n = 2x = 14$). The BC_3F_1 population, P201/32, was screened once again for drought resistance and for the presence of Fg-specific amplified fragment linkage polymorphism (AFLP) and STS markers that would identify plants among the BC_3F_1 that carried the Fg-derived chromosome recombinant.

Cytological analyses

The F_1 hybrid P188/48-1 from Lm ($4x$) (♀) × Fg ($4x$) and 18 BC_1F_1 plants derived from the backcross breeding programme described above were analysed cytologically. Inflorescences were fixed in Carnoy's solution 6:3:1 (ethanol:acetic acid:chloroform) and anthers squashed in 1.5% acetocarmine. Chromosome pairing and chiasma frequency were determined from 25 pollen mother cells (PMCs) per genotype following staining in alcoholic hydrochloric acid-carmine (Snow 1963).

Root-tip chromosome spreads were also made in order to determine the chromosome number of the

Table 1 Analysis of chromosome pairing[a] in the F_1 P188/48-1 derived from *Lolium multiflorum* ($4x$) × *Festuca glaucescens* ($4x$) and BC_1F_1 plants derived from *L. multiflorum* ($2x$) × F_1 P188/48-1. Scores of the chromosome configurations and chiasma are means of 25 cells per genotype

Genotype no.	$2n =$	IV:**LLFFLLLF**	III:**LLFFF**LLLL	II:LLFF**LF**	I:LF	Total:*Xta*	**L/F *Xta* (GISH)**
F_1P188/48-1	28	**1.28**	**0.4** (no LLL)	9.64 (**0.57L/F**)	2.40	23.00	**2.25**
BC_1							
P193/206-2	21	0.24	3.0	3.80	4.40	17.40	–
P193/206-3	22	0.28	2.24	4.56	5.04	16.48	–
P193/206-4	21		3.08	3.88	4.00	18.28	–
P193/206-5	21		1.88	5.20	4.96	15.08	–
P193/206-6	22	0.32	2.24	4.92	4.12	18.12	–
P193/206-7	21		2.92	4.20	3.84	16.28	–
P193/206-8	21		**3.52** (no LLL)	3.48 (**1.56L/F**)	3.48	18.96	**5.08**
P193/206-9	21		**3.72** (no LLL)	3.40	3.04	19.72	**3.72**
P193/206-10	21		2.28	4.68	4.80	14.84	–
P193/206-11	21		2.28	4.72	4.72	14.80	–
P193/206-12	21	**0.08**	**3.12** (no LLL)	3.72 (**0.55L/F**)	3.88	17.76	**3.75**
P193/206-13	21		**3.52**–(0.70 LLL)	3.52 (**0.65L/F**)	3.40	16.00	**3.47**
P193/206-14	21		**2.64**–(0.42 LLL)	4.24 (**0.42L/F**)	4.60	17.88	**2.64**
P193/206-15	21		3.24	3.60	4.08	16.08	–
P193/206-16	21		2.64	4.36	4.36	17.32	–
P193/206-17	21		2.28	4.72	4.72	17.60	–
P193/206-18	21	0.12	3.52	3.36	3.24	20.08	–
P193/206-19	21		3.16	3.96	3.60	16.60	–

[a]Numbers of chromosome associations and chiasma (*Xta*) frequency are scored using Snows (1963) stain; numbers of intergeneric *Lolium-Festuca* (LF) associations (in bold) are scored by genomic in situ hybridization (GISH) (in bold)

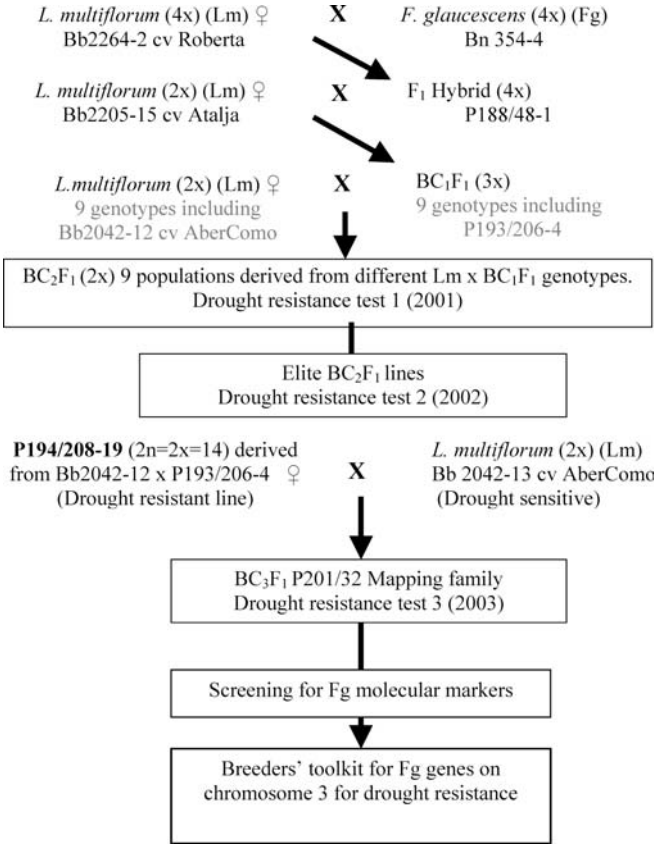

Fig. 1 A schematic diagram of the breeding programme and procedures used for the transfer of genes for drought resistance from *Festuca glaucescens* (*Fg*) to *Lolium multiflorum* (*Lm*)

BC_1F_1 plants and, subsequently, of the BC_2 plants selected for their drought resistance. Only diploid ($2n = 2x = 14$) BC_2F_1 plants were chosen for genetic and physiological screens. Genomic in situ hybridisation (GISH) was employed to discriminate between Lm and Fg chromosomes and to locate any sites of intergeneric chromosome recombination. For GISH analysis, inflorescences of the 28-chromosome F_1 hybrid and five 21-chromosome BC_1F_1 plants were selected to assess chromosome pairing behaviour. All tissues for GISH analysis were fixed in ethanol:acetic acid (3:1) and chromosome preparations made from squashes in 45% acetic acid. The numbers of intergeneric Lm/Fg recombinants obtained from the GISH analysis were incorporated into the interpretation of the meiotic analysis made earlier on the same genotypes using the conventional staining procedures described above (Snow 1963). For GISH, total genomic DNA of Fg was used as a probe and labelled with FluoroLink Cy3-dCTP (Amersham, UK); Lm DNA was used as a blocker. The procedures were those described by Morgan et al. (2001). For fluorescent in situ hybridisation (FISH), an 18S-26S rDNA probe, pTa71 (Gerlach and Bedbrook 1979), was applied to mitotic chromosome preparations of BC_2F_1 plant P194/208-19 (as in Thomas et al. 1996).

Tests for drought resistance

Three screens for drought resistance were made in consecutive years (2001–2003). Procedures for drought testing followed well-established methodologies used at the Institute of Grassland and Environmental Research (IGER) (see, for example, Thomas and Evans 1989). Ramets of approximately equal tiller number and size were planted and spaced evenly at 7-cm intervals within polythene-lined brick-built bins (1.3×0.95 m; 0.9 m deep) containing a 10-cm bottom layer of gravel beneath a 70-cm layer of sterilised soil (silty loam of the Rheidol series) and an upper 10-cm layer of soil-based potting compost. The plants were irrigated regularly and allowed 2 months to establish. They were provided with supplementary nutrients (Sulphurcut: 34 g per bin) 2 weeks prior to the start of the drought treatment. It was established that all of the genotypes used in the drought experiments had headed prior to or during the drought-treatment.

Drought test 1 (2001)

Nine BC_2F_1 populations, each consisting of 30 plants derived from nine of the BC_1F_1 plants described in Table 1 were included in a trial for drought resistance together with their parent genotypes. Two replicates of each genotype were drought-stressed and two replicates irrigated as controls. The initial screen of the nine BC_2F_1 populations commenced on 16 May 2001 and continued for 14 weeks. Water was withheld from the two drought-stressed replicates until 90–95% of the plants appeared to have died. At the end of the drought period, all plants were cut to 4 cm from the soil surface (the standard height used at IGER to simulate cattle grazing) and re-growth assessed as dry matter yield (DMY) following 4 weeks of growth under irrigated conditions. Dry weight after a 1-month recovery period was determined following defoliation to 4 cm and the off-cut dried overnight for 16 h at 80°C in accordance with standard IGER procedures.

Drought test (2002)

The plants used were selected on the basis of their performance in 2001 and included genotypes that, compared with their parent genotypes, were either superior or inferior with respect to their recovery growth subsequent to the drought treatment. Twenty-one BC_2F_1 plants were selected for inclusion in the 2002 drought trial as well as all of the relevant parent genotypes. Procedures for drought testing were as described for the 2001 trial except that the drought treatment commenced on 22 June 2002 and progressed for 12 weeks. Recovery growth was assessed as dry matter production over 4 weeks, as described previously.

Drought test (2003)

The final drought trial involved the BC_3F_1 population P201/32 that derived from the drought-resistant BC_2F_1 genotype, P194/208-19. From a population of 122 BC_3F_1 plants, two replicates were drought-stressed together with their BC_2F_1 parent, P194/208-19, and their Lm and Fg parent genotypes as controls. In this trial, no irrigated control was used. The drought treatment commenced on 22 June, 2003. Unlike the previous drought tests, the summer of 2003 had days of high temperature which led to exceptional glasshouse temperatures that required the plants to withstand combined drought and extreme heat stress. This necessitated a reduction in the duration of the drought treatment compared with the earlier tests. Consequently, the BC_3F_1 population was subjected to 7 weeks of continuous drought. Over this period the maximum temperature was higher than 40°C on 20 days and higher than 50°C on seven other days. Recovery growth was measured over 4 weeks, as described previously.

Application of AFLP and STS markers and genetic mapping of translocated sequences

The cytological analysis carried out on the BC_2F_1 plant P194/208-19 (see Results) indicated the presence of a Fg alien chromosome introgression on chromosome 3 of Lm. Primers were used to screen for AFLP markers in order to target specifically the introgressed Fg sequences using procedures described by King et al. (2002a). All of the parental genotypes (Fig. 1) used to produce P194/208-19 were used in the initial screens for Fg markers. Procedures for the gel-based AFLP screens were as described by Vos et al. (1995) and King et al. (2002a). Combinations of 64 selective primers were used for the gel-base analyses: M50, M51, M52, M54 and M55 in combination with H31–H37 and H40–H44, and M50, M51, M52 and M54 in combination with H46. The AFLP bands were visualized by silver staining using a Promega Silver Sequence kit (Madison, Wis.) and protocols supplied by the manufacturer. Further AFLP screens involved the use of an ABI 3100 Genetic Analyser (Applied Biosystems, Foster City, Calif.) (Skøt et al. 2004) according to the manufacturer's instructions. The data were collected using GENSCAN software and analysed using GENOTYPER software according to the manufacturers' instructions (Applied Biosystems). The 36 AFLP primer pairs used with the ABI 3100 Genetic Analyser were E31–E34 with M48; E35 with M47, M49–M62; E36 with M48, M54–56; E38 with M47–50, M57–62; E41 with M48, M57–59.

STS marker Fg71673, which has been shown previously to map terminally to the NOR (nucleolar organiser region) arm of chromosome 3 in *L. perenne* (I.P. Armstead, unpublished), was used to screen for a Fg-specific polymorphism using primer sequences Fg71673.F (CAGATCCCAACGGTCAGTA) and Fg71673.R (TAGGTAGACCGGACCTGC) and PCR amplification conditions consisting of an initial denaturation at 96°C for 2 min; ten cycles of 96°C for 15 s, 60°C for 30 s (decreasing the temperature by 1°C per cycle), 72°C for 1 min; 30 cycles of 96°C for 15 s, 50°C for 30 s, 72°C for 1 min; one 7-min cycle at 72°C; a final hold at 4°C. The STS allelic variants were separated by electrophoresis on a 1% agarose gel and visualised with ethidium bromide under UV light.

Markers chosen to map the Fg introgression on BC_2F_1 plant P194/208-19 were selected on the basis of: (1) their consistent presence in plants containing Fg DNA and consistent absence in Lm parent genotypes; (2) their presence over all the generations described in Fig. 1; (3) clear size differences between Fg and Lm allelic variants. The Fg markers were mapped on 96 genotypes used in the 2003 drought treatment from BC_3F_1 population P201/32 using JOINMAP 3.0 (Van Ooijen and Voorrips 2001).

Results

The frequency of intergeneric *Lolium* × *Festuca* chromosome recombination

The meiotic analysis of the F_1 P188/48-1 Lm-Fg hybrid revealed predominantly Lm-Lm and Fg-Fg intraspecific chromosome pairing (Table 1). The mean number of chiasma involving Lm-Fg associations in the F_1 hybrid was low at 2.25 compared with the combined mean of 20.75 for intraspecific Lm-Lm and Fg-Fg chiasma associations. Incidents of pairing between Lm-Fg chromosomes were found among quadrivalents (Lm-Lm-Fg-Fg, or Lm-Lm-Lm-Fg), trivalents (Lm-Lm-Fg, or Fg-Fg-Lm) and bivalents (Lm-Fg), the latter at a low frequency of around 0.6 per cell. Most of the BC_1F_1 plants had 21 chromosomes, but two of the 18 scored carried an additional chromosome. In the BC_1F_1, multivalents comprised mostly intergeneric chromosome associations, but some intraspecific Lm-Lm-Lm trivalents were also observed at a low frequency. Bivalent chromosome associations in the BC_1F_1 were primarily, but not exclusively, Lm-Lm; Lm-Fg associations ranging from 0.4 to 1.5 per cell were also observed in different genotypes. Univalents in the BC_1F_1 were primarily Fg chromosomes, but unpaired Lm chromosomes were also found (Fig. 2a). The number of chiasma involving Lm and Fg chromosomes increased in the BC_1F_1 compared with the F_1, and in one genotype, P193/206-8, the number more than doubled to five per cell. Although GISH was not used to interpret the meiosis of BC_1F_1 plant P193/206-4—the parent of BC_2F_1 P194/208-19 that was selected subsequently for its drought resistance—its chromosome pairing and chiasma frequency (Table 1) were very similar to those of the genotypes used in the GISH analysis and would not be expected to differ from them significantly in this respect. Moreover, a GISH analysis of the mitosis of BC_1F_1 P193/206-4

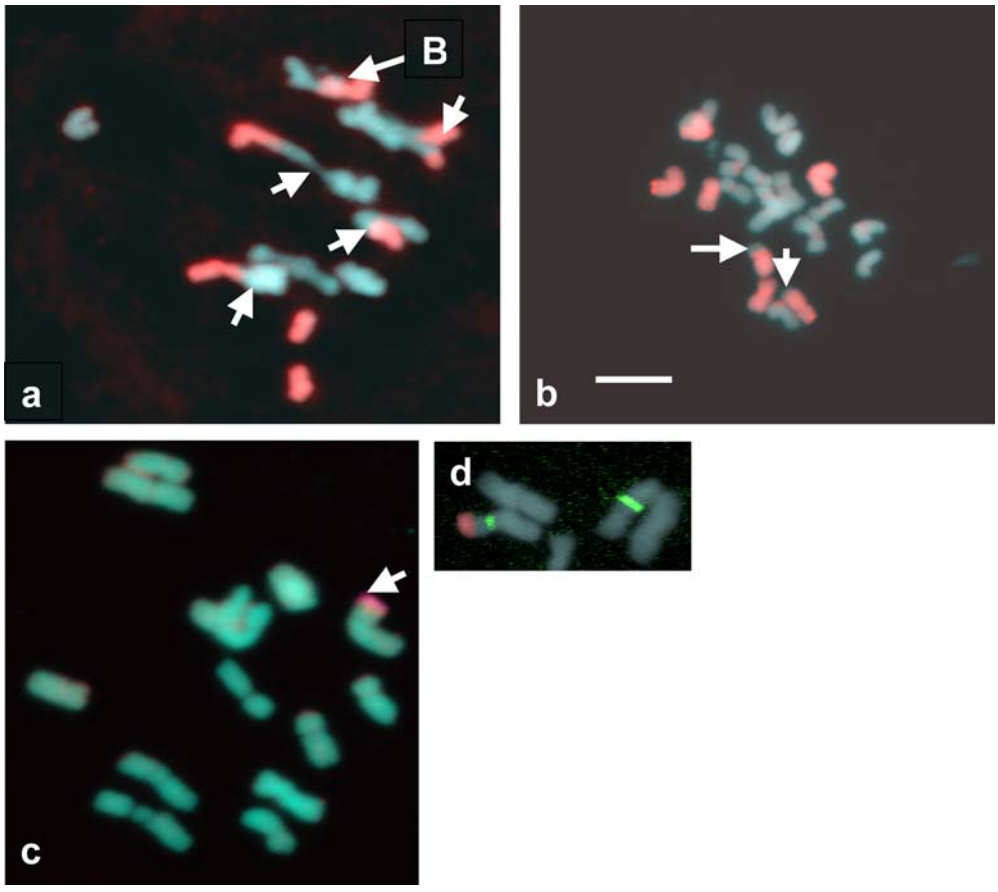

Fig. 2 **a** Chromosome associations at metaphase 1 in a backcross-1 (BC_1F_1) ($2n = 3x = 21$) genotype derived from the *L. multiflorum* ($2x$) (Lm) × (*L. multiflorum* × *F. glaucescens* (Fg) F_1 hybrid ($4x$) cross. GISH reveals chromosomes of Lm (*blue*) and Fg (*pink*). Four Lm-Lm-Fg trivalent associations (*arrows*) and a single Lm-Fg bivalent (*B*) are identified. Univalents are primarily but not exclusively Fg. **b** GISH analysis of a mitotic chromosome spread of the backcross-1 (BC_1F_1) ($2n = 3x = 21$) P193/206-4 parent of drought-resistant elite genotype P194/208-19. The chromosome constitution is 14 Lm chromosomes (*blue*) and 7 Fg chromosomes (*pink*). Two small terminal Lm introgressions are indicated by *arrows* on two Fg chromosomes. *Bar*: 10 μm. **c** GISH analysis of a mitotic chromosome spread of drought-resistant elite genotype backcross-2 (BC_2F_1) P194/208-19 ($2n = 2x = 14$) comprising 14 Lm chromosomes and a terminal Fg introgression (*arrow*) on the NOR arm of the Lm chromosome (chromosome 3). **d** GISH and FISH analysis of part of a mitotic chromosome spread of P194/208-19 ($2n = 2x = 14$), including the Fg recombinant on Lm chromosome 3. A rDNA probe (*green*) reveals the location of the 18S-26S rDNA site and aids chromosome identification

revealed the expected 14 Lm and 7 Fg chromosomes (Fig. 2b). Two of the Fg chromosomes in P193/206-4 carried a single Lm recombinant chromosome segment located terminally; no Fg recombinant segments on Lm chromosomes were visible. The BC_2F_1 plant P194/208-19 derived from P193/206-4 had 14 Lm chromosomes, including one Fg chromosome segment on a large Lm chromosome (Fig. 2c). The absence of this recombinant in P193/206-4 indicated that it arose at meiosis in the BC_1F_1 and not earlier in the F_1 hybrid. The FISH analysis involving rDNA probe pTa71 demonstrated that the Lm chromosome carrying the Fg introgression also carried the rDNA sequence at its NOR region (Fig. 2d). The Fg introgression was located terminally on the chromosome satellite. On the basis of chromosome karyotyping and the earlier localisation of the 18S-26S rDNA site (for example, Thomas et al. 1996), the chromosome was identified as Lm chromosome 3. This chromosome, referred to previously by Humphreys and Pašakinskienė (1996) as chromosome 2, is now numbered in accordance with current *Lolium* molecular maps (Jones et al. 2002).

The tests for drought resistance

From the nine populations tested for drought resistance in 2001, 16 BC_2F_1 plants were selected on the basis of their good survival and recovery compared with their siblings and parents. Each selected plant was analysed cytologically by GISH. All plants were diploid with 14 chromosomes which, except in two genotypes, were all Lm-derived. The two exceptions were chromosome substitution lines, each containing a single Fg-derived chromosome. However, in both of these plants a Lm introgression was present on the Fg chromosome. Of the 16 BC_2F_1 plants selected for good drought resistance, eight contained a single—or in one genotype, two—Fg

introgression on Lm chromosomes; the remaining eight BC_2F_1 plants had no visible Fg introgression, but these derived generally from Lm parents with comparatively good drought resistance. Among the nine BC_2F_1 populations tested in 2001, the genotype having the best recovery growth was P194/208-19 (Fig. 3a). Recovery under irrigated conditions after drought yielded 1.2 g of dry matter from the Fg parent Bn354-4. Under the same conditions, the Lm BC_2F_1 parent, Bb2042-12, died in one replicate and produced only 0.15 g dry matter in the second replicate experiment. While nine of the 30 P194/208 BC_2F_1 plants tested yielded more forage than either the Lm or Fg parent genotypes, P194/208-19, with a mean of 6 g of re-growth, produced twice or more dry matter than all of the other BC_2F_1 plants within the population.

The second drought test in 2002 included some genotypes that had performed well in 2001 and others that had performed poorly. The results from the 2002 test were in good agreement with those in 2001. The recovery of growth specific to BC_2 P194/208-19 and its parent genotypes is summarised in Fig. 3b. In the 2002 trials, the Fg parent, Bn354-4, and the Lm parent, Bb2042-12, used to produce the BC_2F_1 population P194/208 both died during the drought stress. P194/208-19 produced more than threefold the re-growth of both the surviving parent genotypes and an additional Fg genotype (Fg13) included as a control.

The BC_3F_1 mapping population P201/32, which comprised 122 plants derived from a pair cross between P194/208-19 and Bb2042-13 cv. AberComo, was assessed for drought resistance in 2003. Both BC_2F_1 and BC_3F_1 Lm parents performed poorly under the drought stress in 2001 and 2002. The stress in the glasshouse in 2003 was particularly severe, compounding the stress

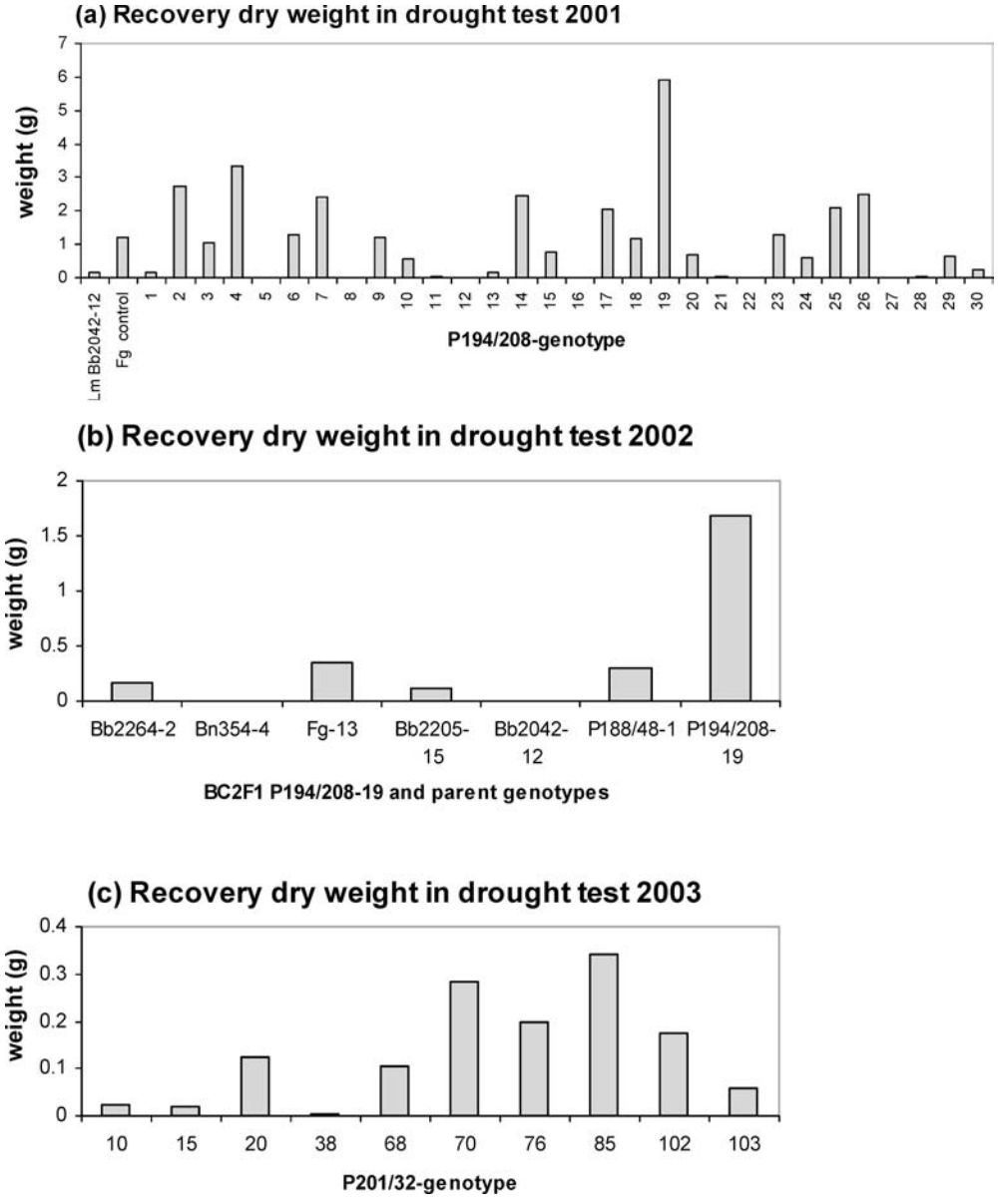

Fig. 3 Four-week recovery growth (in grams, dry weight) following drought treatments in the glasshouse. **a** 2001 drought test of backcross-2 (BC_2F_1) population P194/208 illustrating the superior regrowth of genotype P194/208-19 compared with its Lm parent, the Fg control and its sibling genotypes. **b** 2002 drought test of elite genotype P14/208-19 in comparison with its Lm and Fg parent genotypes. **c** 2003 survivors of the backcross-3 (BC_3F_1) P201/32 population derived from P194/208-19 following combined heat and drought stress

effects from the drought treatment. Ten (8%) plants of the BC_3F_1 population survived the combined drought and heat stress and were able to re-grow following irrigation during the 4-week recovery period (Fig 3c).

The genetic marker analysis

Sixty-four primer pairs were used for the initial gel-based screen and 36 primer pairs for the analysis with the ABI 3100 analyser and GENOTYPER software. The inclusion in the marker analysis of all parent genotypes (see Fig. 1) used to produce P194/208-19 and the choice of only markers that discriminated clearly between Lm- and Fg-derived sequences ensured that only reliable Fg markers are described. The selective primer pairs that gave discriminatory Fg-specific bands and PCR-amplified Fg marker sequences and sizes (in basepairs) were H42-M52-120; H41-M50-242; E35-M53-395; E38-M61-260; E38-M47-218; E35-M48-213; E38-M57-277; E35-M52a-170; E35-M52b-70.

The products of amplification with the STS marker Fg71763 primers consisted of up to three fragments and included a 411-bp Fg-specific band that co-segregated with the Fg introgression and the Fg-specific AFLP markers. The co-segregation of the Fg introgression with the Fg71673 marker confirmed its location to Lm chromosome 3. The results of the genetic mapping of the Fg-specific markers are presented in Fig. 4. Forty-seven percent of the BC_3F_1 population had no Fg-derived markers, 43% of the population had all ten of the Fg-derived markers and 10% were recombinants. Overall, the genetic distance along the introgressed Fg segment was estimated to be 32 cM. From a total of 122 plants tested, the ten BC_3F_1 plants (Fig 3c) that survived and recovered following the combined stress from drought and high temperature each contained the entire set of ten Fg markers.

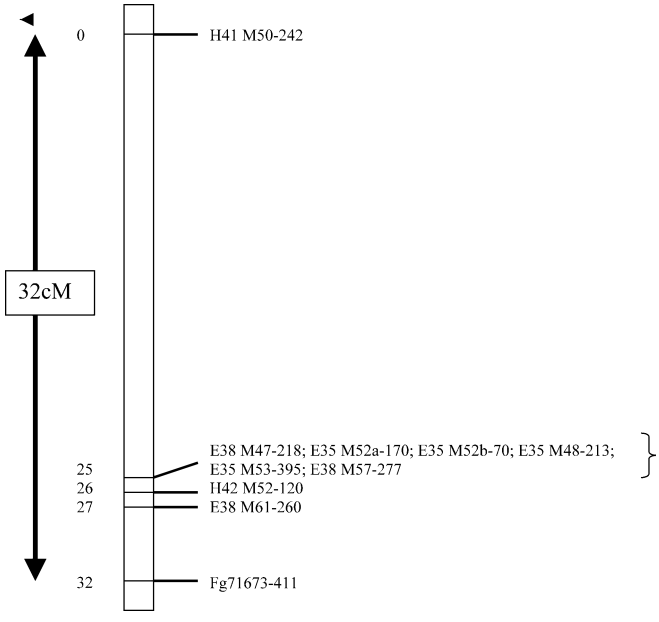

Fig. 4 Genetic linkage map of nine AFLP Fg-derived markers and one Fg-derived STS marker, Fg71673, representing a total 32 cM distance on the Fg introgression on the satellite arm of Lm chromosome 3. Genetic mapping on population P201/32 derived from drought elite BC_2F_1 genotype P194/208-19 and Lm Bb2042-13 was calculated using JOINMAP 3.0 (Van Ooijen and Voorrips 2001)

Discussion

Certain prerequisites have to be met before successful intergeneric introgression breeding programmes can be developed. Species should have sufficient homology to hybridise and allow chromosome pairing and genetic exchange at high frequencies. These hybrids should also be sufficiently fertile to allow their use in backcross breeding programmes. Efficient high-throughput molecular marker technologies should then be in place to screen for the presence of introgressed genes. Finally, these genes should be stable and expressed consistently in different genetic backgrounds and over generations. All these requirements are met in the investigation reported here.

The breeding programme involving Fg and Lm hybridisation has been described previously by Humphreys et al. (1998b) and Morgan et al. (2001). It involved the hybridisation between a synthetic autotetraploid of an Lm and an Fg ecotype, followed by two backcross generations involving diploid Lm as the female and the F_1 hybrid and the BC_1F_1 as the male parents. Humphreys et al. (1998a) demonstrated how two sets of homologous Lm chromosomes in the F_1 and in the BC_1F_1 encouraged preferential Lm chromosome pairing, the regular disjunction of Lm chromosomes, the retention of pollen fertility and the rapid restoration of diploid Lm-like genotypes in the majority of the BC_2F_1 plants. Genome recombination at meiosis between Lm and Fg has been reported previously (Humphreys and Ghesquière 1994; Humphreys et al. 1998a). In both examples, the intergeneric Lm × Fg chromosome recombination involved genomes of Fg derived from the hexaploid species *F. arundinacea*, which has two Fg genomes (Humphreys et al. 1995). Morgan et al. (2001), using a breeding programme equivalent to that described here, reported chromosome pairing in an individual BC_1F_1 plant that was very similar to that observed in the BC_1F_1 plants described within the more extensive study reported here. However, they did not use GISH and concluded that the bivalents were exclusively Lm and that the univalents were exclusively Fg, with intergeneric Lm-Fg recombination restricted to a limited number of trivalent associations. Our work herein demonstrated that this is not always the case. GISH analysis of the BC_1F_1 revealed Lm-Fg recombination events involving both quadrivalent and bivalent chromosome associations and also some intraspecific Fg bivalents and Lm trivalents. Lm univalents were also found, albeit at a low frequency.

While the homology between Lm-Lm and Fg-Fg chromosomes in the F_1 plants and between Lm-Lm chromosomes in the BC_1F_1 plants restricted the numbers of Lm-Fg associations, numbers did increase over generations. A mean of 2.25 Lm-Fg chiasma was found in the F_1, but this increased in the BC_1F_1 where, in one genotype, as many as 5 Lm-Fg chiasma were observed. The BC_1F_1 plants chosen for GISH analysis all carried 21 chromosomes with two homologous sets of Lm chromosomes and the Fg haplotype. In the absence of a Fg chromosome partner, intergeneric chromosome pairing and, presumably, recombination in the BC_1F_1 increased, which in turn reduced the homology between the Lm chromosomes in later generations and further encouraged intergeneric chromosome pairing. King et al. (2002a, b), in a backcross derivative of an *L. perenne* × *F. pratensis* hybrid, identified sites of intergeneric chromosome recombination along the entire length of chromosome 3 and a recombination hotspot proximal to the telomere of the NOR. This was approximately the same chromosome location as the Fg introgression reported here in the BC_1F_1 genotype P194/208-19. In BC_3F_1 mapping family P201/32 derived from P194/208-19, 11% of the plants that carried the Fg-introgressed chromosome segment had reduced numbers of Fg AFLP markers compared with the parental BC_2F_1 P194/208-19 genotype. These were the consequence of further recombination events between homoeologous Lm and Fg sequences on the NOR arm of chromosome 3 and are evidence of the close homology between these *Lolium* and *Festuca* species.

Chen and Sleper (1999) reported a breeding programme similar to the one described here that aimed to introgress genes for heat tolerance from the North African tetraploid species *F. mairei* into *L. perenne*. *F. mairei* is related closely to Fg and may well share a common ancestor, the diploid species *F. scariosa* (Harper et al. 2004). The frequency of intergeneric chromosome pairing in the *F. mairei* × *L. perenne* F_1 ($2n = 4x = 28$) hybrid was similar to the frequency of Lm ($4x$) × Fg ($4x$) F_1 associations reported in the current study. However, in contrast to the results presented here, Chen and Sleper (1999) recovered no chromosome recombinants among their 14 chromosome backcross populations.

In the current study, nine BC_2F_1 populations derived from the initial Lm-Fg F_1 hybrid P188/48-1 were screened for their ability to withstand periods of prolonged drought. The BC_2F_1 plants that performed best under the drought stress contained Fg alien introgressions and generally originated from backcrosses involving Lm parents with poor drought resistance. However, the BC_2F_1 genotype that performed best overall under drought was P194/208-19. This plant derived from a backcross involving a Lm cv. AberComo genotype that had poor drought resistance. P194/208-19 was found to contain a single terminal Fg segment on the NOR arm of chromosome 3. Genes for resistance to severe drought had been transferred previously onto Lm chromosome 3 from a genome of *F. arundinacea* (Humphreys and Pašakinskienė 1996). However, in that case, the *Festuca* genes were derived from the *F. pratensis*-derived genome of hexaploid *F. arundinacea* and the introgression involved sequences at a median position on the non-satellite arm of chromosome 3.

The drought resistance of P194/208-19 was tested again during the summer of 2002 and produced a result consistent with that obtained in 2001. Over both the 2001 and 2002 drought trials, the re-growth of P194/208-19 following drought exceeded that of both the Fg parent and the control genotypes. While Fg can be characterised as having good drought and heat tolerance, its establishment is poor, its growth rate is slow and it regularly enters quiescence during the summer months (Humphreys et al. 1997). However, despite the presence of Fg genes in P194/208-19, the growth rate of the introgression line was always superior under drought stress to that of both the Lm and Fg parents. The ability to continue growth and the production of high-quality forage during times of mild drought and over large periods of the year will be an essential prerequisite for future cultivar development. A third drought test during 2003 on the BC_3F_1 population P201/32 coincided with extreme high temperatures during the glasshouse experiment (exceeding 50°C) and as a result the stress incurred was both rapid and extreme. The BC_2F_1 parent P194/208-19 is heterozygous (*LmFg*) for the Fg genes introgressed onto chromosome 3. Consequently, in the BC_3F_1 population, which arose from the cross P194/208-19 (*LmFg*) × Lm (*LmLm*), plants with Fg genes would be expected at a 1:1 ratio. The severity of the stress on the BC_3F_1 population resulting from a combination of drought and extreme heat led to heavy mortality, with survival and recovery in only 8% of the population tested. However, in all of the BC_3F_1 survivors, the entire set of the ten Fg-derived molecular markers was present. Marker screens on a substantial part (78%) of the BC_3F_1 population had shown earlier that the complete set of ten Fg markers was present in 43% of the BC_3F_1 population and that the markers were present in reduced numbers in a further 10% of the BC_3F_1population. The occurrence among the BC_3F_1 survivors of only plants containing a complete set of ten Fg markers was evidence that it was undesirable to reduce further the size of the introgressed Fg sequence as this was likely to impact on the overall effectiveness of the drought resistance. The development of a 411-bp STS marker, Fg71673, that co-segregated with the Fg genes for drought resistance in the Lm populations described provides an opportunity to screen large populations for the presence of the Fg genes during cultivar development. One of the Fg71673 primers has since been labelled fluorescently for use on the ABI 3100 Genetic Analyser (unpublished results) making it better adapted for inclusion in the "breeders' toolkit". It would be advantageous to develop a similar STS or equivalent marker associated with the proximal region of the translocated Fg chromosome sequence in order that the targeted Fg genes are bracketed with markers on either side.

We have yet to determine which physiological mechanism relevant to drought resistance has been altered following the incorporation into Lm of the Fg genes. However, during another drought treatment using an alternative BC_3F_1 population derived from the P194/208-19, a putative association was observed between the presence of the Fg markers and a change in osmotic adjustment more typical of the Fg than the Lm parent genotypes (J. Humphreys, unpublished). Further research is required to confirm whether or not this change in osmotic adjustment is the underlying mechanism responsible for the improved drought resistance found in the introgression line compared with its Lm parent.

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