Genetic characterization of androgenic progeny derived from *Lolium perenne* x *Festuca pratensis* cultivars

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Summary

A successful androgenesis in amphidiploid *Festulolium* (*Lolium perenne* L. x *Festuca pratensis* Huds., 2n=4x=28) was obtained using PG-96 medium for embryo/callus induction. The green plant regeneration varied, and was 46 %, 35 % and 17 % for Bx350, Bx351 and Prior, respectively and over 800 green plants have been obtained. Androgenic progeny showed a large variation in freezing tolerance, 7 % of 292 progeny exceeding that of freezing hardy *F. pratensis* despite containing chromosomes of *L. perenne,* a more freezing-sensitive species. More than 60% of flowering 175 progeny produced dehiscent anthers with pollen stainability ranging from 5% to 85%.

Androgenic plants contained 14 or 28 chromosomes. There were 188 (56 %), 204 (77 %) and 114 dihaploids (81 %) from Bx350, Bx351 and Prior, respectively. However, the nuclear DNA content varied significantly even between plants with the same chromosome number. Variation in DNA content reflected the genetic variation inherent in androgenic populations.

High levels of chromosome pairing and recombination were observed due to close homology between genomes of *L. perenne* and *F. pratensis*.

Key words:

Androgenesis, *Festulolium*, Forage grass, freezing tolerance, Genome size, Genomic *in situ* hybridisation (GISH), pollen fertility

Introduction

Festuca L. and its closely allied genus *Lolium* L. have long fascinated agronomists, evolutionists, and plant breeders, and these genera are among the most widely studied of the non-cereal grasses. The *Festuca-Lolium* group is composed of some highly productive, nutritious, persistent, and well-adapted grasses which are widely used for agricultural and recreational purposes, and for stabilizing soils (Jauhar, 1993). Intergeneric hybrids between closely related *Lolium* and *Festuca* species are being used to broaden the gene pool and provide the plant breeder with options to combine high quality traits with broad adaptation to a range of environmental constraints (Humphreys *et al*., 2003). The complex of species has an enormous wealth of genetic variability and in hybrids high potential for genetic exchange, thus offering unique opportunities for the production of versatile hybrid varieties with new combinations of useful characters suited to modern grassland farming (Thomas *et al*., 2003). *Lolium* and *Festuca* species share valuable and complementary agronomic characters: for example *L. perenne*, offers good regrowth and nutritive value and is a good species for grazing whilst, *F. pratensis*, is more persistent and winter-hardy (Humphreys *et al*., 1998a).

Lolium and *Festuca* species hybridise naturally, and as hybrids regularly exchange genes at high frequency. Recently in Europe, USA and Japan, a range of *Lolium* x *Festuca* hybrids have been developed as novel temperate forage grasses (Humphreys *et al*., 2003; Momotaz *et al*., 2004; Pašakinskienė *et al*., 1997; Zwierzykowski *et al*., 1999). In support of this, androgenesis was found to be an effective procedure for selecting *Lolium-Festuca* genotypes comprising gene combinations rarely or never recovered by conventional backcross breeding programs (Leśniewska *et al*., 2001; Humphreys *et al*., 2003).

Successful androgenesis in *Lolium-Festuca* hybrids were reported (Humphreys *et al*., 1998b, 2000; Le_niewska *et al*., 2001; Zare *et al*., 1999, 2002; Zwierzykowski *et al*., 1998, 1999); for L. *multiflorum* x *F. arundinacea* hybrid, 40 green plants were induced per 100 cultured anthers (Zare *et al*., 2002), for *F. pratensis* x *L. multiflorum* amphidiploid cultivars, the frequency of green plant was 7% of anthers cultured (Le_niewska *et al*., 2001).

Nuclear DNA content (genome size) is a specific karyological feature that is very useful for systematic purposes and evolutionary considerations (Bennett & Leitch, 1995). Angiosperm DNA C-values are highly variable, differing over 1000-fold. Genome size is positively correlated to nuclear volume, cell volume, mitotic cycle time and the duration of meiosis. Flow cytometry has aided this research as it has been demonstrated to be a convenient and rapid method for estimating the nuclear genome size of plants.

Recent advances in the cytogenetics of the *Lolium-Festuca* complex provide new opportunities for understanding and manipulating complex physiological mechanisms. These advances rely on two exceptional properties of some of the more closely related species within the *Lolium-Festuca* complex. On the one hand, the chromosomes of the *Festuca* species have sufficient homology with the chromosomes of the *Lolium* species to pair freely and to recombine in hybrids. On the other hand, the chromosomes of *Lolium* and *Festuca* species can be discriminated using genomic *in situ* hybridisation (GISH) (Humphreys *et al*., 1997). The GISH technique provides the means to identify segments of alien chromosomes introduced into the recipient species, and has proved to be a powerful tool for determining their chromosome location.

In the current study, the genetic variation and numbers of detectable alien introgressions found amongst plants derived from amphidiploid *L. perenne* x *F.*

pratensis (2n=4x=28) following androgenesis was determined by genome size and GISH. The male fertility and freezing tolerance of the *Festulolium* anther culture-derived progeny was also analysed. The androgenesis techniques have led to novel genotypes rarely observed as outcomes of breeding programmes. *Lolium* x *Festuca* hybrids display promiscuous chromosome recombination enabling genes from one species to be transferred readily to homoeologous chromosome regions where they are expected to function normally and to remain stable. The ultimate objective of this study was to develop a novel grass that combines favourable attributes of *L. perenne* and *F. pratensis*.

Materials and Methods

Plant materials

The accessions of amphidiploid *Festulolium* hybrids between *Lolium perenne* L. and *Festuca pratensis* Huds. Prior, Bx350 and Bx351 (2n=4x=28) investigated in this study were provided by Prof. Mervyn O. Humphreys from the Institute of Grassland and Environmental Research, Aberystwyth, UK. Plants were grown in soil in a greenhouse under controlled conditions of 25°C/18°C and a 16 h photoperiod. Seedlings were vernalized in a cold room (at 3°C, 8h photo period with the light density approximate 30 $\mu molm^{-2}s^{-1}$) for 12 weeks, and the plants were then transferred into a glasshouse.

Anther culture and green plant regeneration

Spikes were harvested when substantial numbers of the microspores were in the middle- to late-uninucleate developmental stage and stored at 4°C in the dark with the stalks in water for different durations of cold treatment. Prior to excision of the anthers, the microspore developmental stage was checked microscopically. Spikes were surface sterilized with 2% sodium hypochlorite solution for 15 minutes, followed by repeated rinses in sterile water. The excised anthers were plated in a Petri dish (diameter 90 mm) containing 45 ml Gelrite (Wako, Osaka, Japan) -solid medium (0.4%, w/v). PG-96 medium (Guo *et al*., 1999; Guo & Pulli, 2000a, b) with 2.0 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mgl^{-1} 6-furfurylaminopurine (kinetin) and Ward medium (see Zare *et al.*, 2002) were used as the embryo (callus) induction medium. The Petri dishes were sealed with Parafilm, and incubated at 25°C in darkness. Every test was repeated at least three times with five replicates per treatment. After 10 weeks of culture, calli (1-2 mm in size) were counted and transferred to the solid medium 190-2 (Wang & Hu, 1984) containing 3% sucrose and 0.4% (w/v) Gelrite, supplemented with 0.1 mgl^{-1} 2,4-D, 1.5 mgl^{-1} kinetin for green plants regeneration. Subcultures were incubated at a 25°C in 16 h / 8 h day / night photoperiod with the light density approximate 80 $\mu molm^{-2}s^{-1}$. After shoots (2-3cm) had developed well, the plantlets were then transferred to Magenta boxes containing solid 190-2 medium with 3% sucrose and 0.4% (w/v) Gelrite, without growth regulators for root development.

Ploidy level test of progeny

Reagent kit Partec CyStain UV precise P was carried out for nuclei extraction and nuclear DNA from plant tissue was stained with 4',6-diamidino-2-phenylindole (DAPI) in order to determine the ploidy level by Partec PAS flow cytometry

(Münster, Germany) equipped with an HBO lamp for UV excitation. Leaf blade was chopped with a sharp razor blade in a Petri dish in about 0.5 ml of the commercial Partec "High resolution DNA kit, solution A: nuclei isolation". The suspension was filtered through a 50 μm mesh nylon filter and mixed with a 5-fold volume of the Partec "High resolution DNA kit, solution B: staining" with DAPI. The nuclear suspension was analysed on a Partec PAS.

Fertility estimation of progeny

The male fertility of androgenic *Festulolium* plants was analysed during the first growing season. Male fertility was measured by staining pollen with 1% acetocarmine (1% carmine in 45% aqueous acetic acid) and counting the frequency of stainable pollen grains under a light microscope. The fertile (F) and partially fertile (PF) plants were recorded with pollen stainability ranging from 5% to 85%. Releasing visible pollens from anthers in touching by a finger was recognized as dehiscent anthers. Male sterile (MS) plants were recorded if they had no pollen or very little stainable pollen (under 5%).

Freezing test

Plant materials chosen randomly for a freezing test included 292 anther culture-derived *Festulolium* progeny, three *Festulolium* parental accessions (Bx350, Bx351, Prior) , two accessions of *L. perenne* (Yatsugatake D-12 and Yatsugatake D-13, 2n=2x=14) and two cultivars of *F. pratensis* (Tomosakae, Harusakae, 2n=2x=14). Three tillers from each genotype of the androgenic progeny with the control cultivars were transplanted into a plastic box (28.2 x 28.2 x 5.2 cm) containing 16

cells filled with commercial soil on 25th August 2003 and were propagated vegetatively. Plants were grown outdoors for natural hardening. Twenty crown tissues with several tillers of 3 cm length per genotype were taken for freezing treatment on 10th December. In each genotype, sample with ten crown tissues was randomly placed in a programmed freezer with two replicates. After ice nucleation at -3 ^{o}C for 8 hours, samples were cooled to -17 ^{o}C at a temperature decrease of 1^{o}C / hour. They were maintained at -17 ^{o}C for 6 hours and the samples then thawed at 2 ^{o}C overnight. After regrowth for 4 weeks in the glasshouse, the numbers of surviving plants were counted.

Nuclear DNA content (genome size) measurement

Nuclear DNA content was determined using a Partec PAS flow cytometry (Münster, Germany) equipped with a 488 nm argon laser to measure the relative fluorescence intensities from propidium iodide (PI) stained nuclei. For comparative DNA measurements, every nuclear isolation was jointly carried out for one individual of the test material and one of the internal standard (*Hordeum vulgare* L. cv. Sultan, 2C = 11.12pg, Bennett *et al.,* 2000), thereby ensuring identical conditions. Forty-eight anther culture-derived *Festulolium* plants from Prior, 50 plants from Bx350 and 50 plants from Bx351 were selected randomly for measurements. For the isolation of nuclei, leaf blade was chopped with a sharp razor blade in a Petri dish in 0.5 ml nuclei-isolation buffer A of the Partec "High resolution DNA kit" to which per ml 0.25 mg RNAse was added. After adding 1.5 ml PI solution (50 mg/1 in water) the suspension with nuclei was filtered through a 50 μm mesh nylon filter. For each

sample, 5,000 – 10,000 nuclei were analysed using a Partec PAS. Three measurements for each plant were employed.

GISH analysis

Nine androgenic-induced *Festulolium* progeny were analysed by GISH. They were 350-184, 350-177, 350-216 and 351-160 (all 2n=28) and 350-11, 350-21, 351-111, Prior-57 and Prior-67 (all 2n=14).

GISH was carried out according to Mizukami *et al*. (1998) and Salvo-Garrido *et al*. (2001) with some modifications. Root tips were taken from young seedlings for metaphase chromosome analysis. Total genomic DNA of *L. perenne* cv. Yatsugatake D-12 was used as probe, and total genomic DNA of *F. pratensis* cv. Harusakae as blocking DNA, after shearing in an autoclave at 105 ^{o}C for 10 minutes. The probe DNA was labelled with Digoxigenin -11-dUTP using Dig-High prime (Roche, Basel, Switzerland). The hybridisation mixture contained 100 ng probe DNA of *L. perenne*, 1 μg blocking DNA of *F. pratensis* in 50% formamide, 2×SSC and 10% dextran sulfate. The mixture was denatured at 92 ^{o}C for 10 min and immediately cooled in chilled water. A 70 μl aliquot of the denatured hybridisation mixture was dropped onto each chromosome preparation. The chromosome preparation was denatured at 70 ^{o}C for 6 minutes on a thermal block, and together with hybridisation incubated in a humid chamber at 37 ^{o}C over night. The washing and detection procedure was as described by Salvo-Garrido *et al*. (2001). Digoxigenin labelled probe were detected Anti-Digoxigenin-FITC conjugate (Sigma-Aldrich, St. Louis, MO, USA) and chromosomes counterstained with DAPI or PI. The FITC and DAPI/PI labelled chromosomes were visualised using an Olympus BX50 fluorescence microscope (Olympus, Tokyo, Japan), and images

captured separately with CCD camera (CoolSnap cf, Roper Scientific, Tucson, AZ, USA) as grey images, and then merged and pseudo-coloured using computer imaging software (RS Image Express, Roper Scientific).

Results

Anther culture response

Procedures were developed for androgenesis from *L. perenne* L. x *F. pratensis* Huds. amphidiploid cultivars. Embryos (calli) and green plants were obtained from all three accessions, but the genotype responses were different with accessions Bx350 and Bx351 more conducive to androgenesis than Prior. The anther culture response in PG-96 induction medium was 58.2 %, 44.3 % and 7.7 % in genotypes from Bx350, Bx351 and Prior, respectively (Fig. 1). The anther culture response from PG-96 was significantly higher than that from the modified Ward medium (Fig. 1).

Fig. 1

Visible calli developed following 5-6 weeks of anther culture. Green plant regeneration followed transfer of embryos/calli onto 190-2 regeneration medium containing cytokinin. In some instances, green shoots were obtained directly from calli on the induction medium when light was provided (Fig. 2). The green plant regeneration (green plants / 100 embryos) varied between Bx350, Bx351 and Prior, and was 46.3 %, 35.3 % and 16.6 %, respectively. Albinos were also recovered but their frequency was less than 10 %. Over 800 androgenic-derived green plants have been obtained thus far.

Fig. 2

Ploidy level of androgenic progeny

The ploidy level of androgenic *Festulolium* progeny was analysed by Partec PAS flow cytometry with DAPI staining. Among 742 progeny, plants with 14 chromosomes predominated. There were 188 dihaploids (56.1%), 204 (76.7%) and 114 (80.9%) from Bx350, Bx351 and Prior, respectively (Table 1).

Table 1

Male fertility of androgenic progeny

In anther culture-derived progeny of *Festulolium* Bx350, Bx351 and Prior, 54, 76 and 45 flowering plants were examined cytologically for pollen stainability as an indicator of male fertility. Of the plants examined, 33 plants (61.1%) amongst the Bx350 progeny were fertile or partially fertile with dehiscing anthers and pollen stainability ranging between 5% to 85%. For Bx351 and Prior, the male fertility and partial fertility percentages were 71.1 %(54 plants) and 66.7 %(30 plants), respectively (Table 2). For Bx350, the male fertile and partial fertile percentages in 14 chromosome progeny and 28 chromosome progeny were 46.3% and 14.8%; for Bx351, they were 56.6% and 14.5%; for Prior, they were 57.8% and 8.9%. For all three accessions, the male fertile percentage in 14 chromosome plants was 3-6 times higher than that in 28 chromosome plants (Table 2).

Table 2

Freezing tolerance of androgenic progeny

Two hundred and ninety-two anther culture-derived *Festulolium* progeny were assessed for survival during a freezing treatment. Statistical significant difference for survival rate in the freezing treatment was observed among androgenic genotypes. They displayed wide variation in freezing-tolerance with 148 progeny (50.7%) superior to the freezing-susceptible *L. perenne* accessions and 19 progeny (6.5%) superior to the freezing tolerant *F. pratensis* cv. Tomosakae. The elite freezing-tolerant plants comprised 4 dihaploid genotypes from Bx350, 1 dihaploid from Bx351, and 6 dihaploids derived from Prior which along with one 28 chromosome plant and *F. pratensis* cv. Harusakae showed 100% survival of the crown tissues (Fig. 3). The survival percentages for the parental *Festulolium* accessions Prior, Bx350 and Bx351 were 0, 0, 10-20 %, respectively. Among the anther culture-derived progeny, the dihaploid 14 chromosome plants showed higher freezing tolerance compared with the 28 chromosome plants (Fig. 3).

Fig. 3

Genome size of androgenic progeny

Hordeum vulgare cv. Sultan (2C = 11.12 pg) was used as an internal standard for flow cytometric measurements (Bennett *et al*., 2000). The nuclear DNA contents were calculated by direct comparison between the modal position of the control and test samples. The nuclear DNA content of 14 and 28 chromosome plants was

indicated by a single peak which indicated a uniform plant chromosome number and no chimaeras.

The nuclear DNA content (genome size) varied significantly within the 14 and 28 chromosome plants (Table 3). The standard deviation of nuclear DNA content in anther culture-derived plants was higher than those in cultivar/ strain plants. This DNA content difference reflected the genetic variation of anther culture-derived 14 and 28 chromosome plants.

Table 3.

GISH analysis of androgenic progeny

Nine anther culture-derived *Festulolium* plants were analysed by GISH, of which five were 14 chromosome plants and four were 28 chromosome plants.

Fig. 4

High levels of intergeneric translocations were observed, for example, in fertile Bx350-177 with a 28-chromosome genome comprising approximately equal amount of *Lolium* and *Festuca* DNA, there were 9 intergeneric translocations, some of which had two breakpoints in one arm (Fig. 4a, arrows). For dihaploid, Prior-88 with a 14 chromosome genome comprising predominant *Lolium* DNA, there were 8 intergeneric translocations (Fig. 4b). The presence of chromosomes from both parental species in each of the androgenic progeny and the frequent intergeneric *Lolium-Festuca* translocations with up to two breakpoints per chromosome show that introgressions in amphiploid hybrids between *L. perenne* and *F. pratensis* are common.

Discussion

Androgenesis

In the current study, amphidiploid *Festulolium* accessions Bx350, Bx351 and the cultivar Prior (all 2n=4x=28) proved to be very responsive to androgenesis, and it was possible to generate large numbers of dihaploid (n + n = 14) and 28 chromosome androgenic plants. Compared with *L. perenne* x *F. pratensis*, androgenesis in *L. perenne* is more genotype-dependent (Olesen *et al.* ,1988; Opsahl-Ferstad *et al.* , 1994; Madsen *et al*., 1995; Spangenberg *et al*., 1998) and androgenesis in *F. pratensis* was very recalcitrant (data not shown). Androgenesis is easier in polyploids than diploids (e.g. Humphreys *et al*., 2000). In the current study, PG-96 medium was used as the androgenic embryo/callus induction medium. The PG-96 medium was composed of relatively complex organic acids and vitamin compounds (Guo *et al*., 1999). In previous studies, modified PG-96 induction medium promoted regeneration from isolated microspores of timothy and rye (Guo & Pulli, 2000a, 2000b).

Androgenesis from *Festuca-Lolium* complex had been studied using different parental hybrids such as *F. pratensis* x *L. multiflorum* (Leśniewska *et al*., 2001; Rapacz *et al*., 2004) and *L. multiflorum* x *F. arundinacea* (Humphreys *et al*., 1997, 1998b; Pašakinskienė *et al*., 1997; Zwierzykowski *et al*., 1999; Zare *et al*., 1999). In androgenesis of *F. pratensis* x *L. multiflorum*, over 80% of the androgenic plants derived from amphidiploid donor plants (2n=4x=28) had 14 chromosomes and were likely to be dihaploids with a single genome of *Lolium* and *Festuca* (Leśniewska *et al*., 2001). Androgenic plants derived both from F_8 hybrids of *F. pratensis* x *L*

multiflorum (2n=4x=28) and from pentaploid F_1 hybrids of *L.multiflorum* x *F. arundinacea* (2n=5x=35), contained rare gene combinations that contributed to drought and/or freezing tolerance in excess of the parental genotypes (Humphreys *et al*., 2000). In androgenic populations derived from a *F. arundinacea* x *L. multiflorum* hybrid, Zwierzykowski *et al*. (1998) reported chromosome variation to be much wider than that transmitted by conventional backcrossing.

Fertility of androgenic progeny

In the current study, we found more than 60% of anther culture-derived *Festulolium* progeny from Bx350, Bx351 and Prior to have male fertile or partial male fertility. This was particularly evident amongst the dihaploids where 46-58 % were fertile compared with 9-15 % of the 28 chromosome plants (Table 2). On backcrossing these dihaploid plants showing good freezing tolerance as female parents with *L. perenne* (2n=2x=14), seed set was normal indicating that they were also female fertile (data not shown). The lower fertility found amongst the 28 chromosome plants compared with the dihaploid plants was surprising. Infertility is associated generally with irregular meiosis in synthetic autopolyploids. A consequence of these irregularities is the failure of equal separation of the chromosomes at anaphase I and gametes with chromosomes deviating from the haploid number are formed. This in turn gives rise to aneuploidy in filial generations of the autopolyploids (Thomas, 1993). Leśniewska *et al.* (2001) assumed that 28 chromosome plants had arisen primarily following spontaneous doubling in culture from dihaploid genotypes but realised some could have the products of unreduced gametes. A detailed GISH analysis would confirm the origin of the 28 chromosome plants.

Unbalanced chromosome combinations may have resulted in gene duplications and/or deletions and explain, at least in part, the high sterility found amongst the androgenic genotypes as described by Humphreys *et al.* (2000). With the exception of *L. multiflorum* x *L. perenne*, interspecific and intergeneric amphiploids of the *Lolium-Festuca* complex have generally been considered genetically unstable because of interspecific and intergeneric chromosome pairing. Characteristically they show irregular meiosis and polysomic inheritance, leading to a shift towards one or other of the parental types, and loss of the favourable gene combinations of the parental species (Canter *et al*., 1999; Humphreys *et al*., 2003).

However, a high frequency of bivalent formation and regular meiosis as well as fairly stable chromosome pairing were reported for Prior (see Canter *et al*., 1999). The cultivar Prior is one of several intergeneric amphidiploids created at the Welsh Plant Breeding Station in the early seventies, and is the product of crosses made between colchicine-induced autotetraploids of the parental species *L. perenne* and *F. pratensis*. In genotypes of an advanced generation (F_8) in Prior, more than the expected 14 *Lolium* chromosomes were observed by GISH (Canter *et al.*, 1999). The Bx350 and Bx351 were bulk seeds from F_1 amphidiploids crossed between tetraploid *L. perenne* and *F. pratensis* cultivars (personal communication, Prof. M. O. Humphreys). *L. perenne* and *F. pratensis* are very closely related among species of *Festuca* and *Lolium* by protein electrophoretic analysis, RAPD, ITS and SSR analysis (Jauhar, 1993; Stammers *et al*., 1995; Šiffelová *et al*., 1997; Gaut *et al*., 2000; Momotaz *et al*., 2004). The high level of recombination observed in triploid F_1 hybrids between *L. perenne* and *F. pratensis* suggests conservation of gene order (King *et al*., 1998). The genome of *L. perenne* and *F. pratensis* share close homology, and tend to regular chromosome pairing and development of balanced and viable gametes. As a consequence, chromosome rearrangements between *L.*

perenne and *F. pratensis* that arise in the deletion of gene sequence of *Lolium* would be expected to be compensated for by the presence of homoeologous *Festuca* sequence or vice versa. It is likely as a consequence that dihaploid *L. perenne* x *F. pratensis* androgenesis-derived plants represent relatively-balanced genomes and perform as functional diploids.

Selection of freezing-tolerant *Festulolium* progeny

Androgenesis was found to be an effective procedure for selecting *Lolium-Festuca* genotypes comprising gene combinations rarely or never recovered by conventional backcross breeding programs. Androgenesis provides a vast source of genetic variation that may be recovered through the gametes. It may also initiate desirable epigenetic and pleiotropic effects (Thomas *et al*., 2003; Humphreys *et al*., 1998b). An androgenic population showed extreme diversity in freezing-tolerance in *L. multiflorum* x *F. arundinacea* (Zare *et al*., 1999), and showed variation for winter survival, freezing tolerance and resistance to cold-induced photoinactivation of photosystem II (PSII) in *F. pratensis* x *L. multiflorum* (Rapacz *et al*., 2004).

In the current study, 51 % of *Festulolium* androgenic progeny showed higher freezing tolerance than susceptible *L. perenne* cv. Yatsugatake D-12 and D-13, and 7 % of *Festulolium* progeny showed higher freezing tolerance than the freezing tolerant *F. pratensis* cv. Tomosakae (Fig. 3). This result is consistent with the data of Zare *et al*. (1999) in which 6 % of the androgenic plants (from *L. multiflorum* x *F. arundinacea*) were more freezing resistant than the freezing-tolerant *Festuca* parent.

Genome size of androgenic progeny

Nuclear DNA C-value and genome size are important biodiversity characters with fundamental biological significance and many uses (Bennett & Leitch, 1995; Bennett *et al.*, 2000). It is clear that this variation in nuclear DNA content has played important roles in plant evolutionary processes. The increase in DNA content, which is caused by polyploidization, unequal crossing-over and transposition, leads to duplication of gene loci, potential increase in gene products and diversification of gene function (see Sugiyama *et al*., 2002). Intraspecific variation in angiosperm genome size has been reported in addition to variation within a single plant (see Turpeinen *et al*., 1999).

In the current study, the standard deviations of nuclear DNA content in anther culture-derived plants were apparently bigger than those in cultivar/ strain standard plants (Table 3). In both dihaploid and 28 chromosome populations derived by androgenesis, the genome size of single plants showed a significant difference (data not shown). This is likely due to polyploidization, duplication and/or deletion and aneuploid effects resulting from genome reorganisation during chromosome recombination and pairing. Androgenesis provides a vast of sources of genetic variation. The nuclear DNA content of 14 chromosome plants was less than 50 % of that in 28 chromosome plants (Table 3). It is logical to suppose that DNA content increases in proportion to ploidy levels and that the genome size of amphidiploids is the sum value of its parents. DNA diminution per basic genome with increase ploidy level has been reported in many species and a positive correlation between genome size and polyploidy has been also shown in many cases (Ohri, 1998). The reason for discrepancy was unknown. The androgenic plants carried intact and recombinant chromosomes of both *Lolium* and *Festuca* species in different numbers

with the consequence that their genomes were highly unbalanced (Thomas *et al*., 2003).

Conclusion

More than 800 androgenic-derived *Festulolium* plants were produced from Bx350, Bx351 and Prior. The dihaploids and 28 chromosome plants showed considerable freezing tolerance variation. Fertile dihaploid progeny with good freezing hardening can be used to produce new amphidiploid cultivars with fixed desirable gene combinations as homozygotes by chromosome doubling or alternatively can be backcrossed with *Lolium* to combine desirable traits by introgression breeding. These valuable breeding materials will be used in *Festulolium* breeding programmes to speed up the breeding process and provide novel robust new forage grasses.

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References

Bennett MD, Leitch IJ. 1995. Nuclear DNA amounts in angiosperms. *Annals of Botany* **76**:113-176.

Bennett MD, Bhandol P, Leitch IJ. 2000. Nuclear DNA amounts in angiosperms and their modern uses - 807 new estimates. *Annals of Botany* **86**: 859-909.

Canter PH, Pašakinskienė I, Jones RN, Humphreys MW. 1999. Chromosome substitutions and recombination in the amphiploid *Lolium perenne* x *Festuca pratensis* cv Prior (2n=4x=28). *Theoretical and Applied Genetics* **98**: 809-814.

Gaut BS, Tredway LP, Kubik C, Gaut RL, Meyer W. 2000. Phylogenetic relationships and genetic diversity among members of the *Festuca-Lolium* complex (Poaceae) based on ITS sequence data. *Plant Systematics and Evolution* **224**: 33-53.

Guo YD, Sewón P, Pulli S. 1999. Improved embryogenesis from anther culture and plant regeneration in timothy. *Plant Cell, Tissue and Organ Culture* **57**: 85-93.

Guo YD, Pulli S. 2000a. An efficient androgenic embryogenesis and plant regeneration through isolated microspore culture in timothy (*Phleum pratense* L.). *Plant Cell Reports* **19**: 761-767.

Guo YD, Pulli S. 2000b. Isolated microspore culture and plant regeneration in rye (*Secale cereale* L.). *Plant Cell Reports* **19**: 875-880.

Guo YD, Yli-Mattila T, Pulli S. 2003. Assessment of genetic variation in timothy (*Phleum pratense* L.) using RAPD and UP-PCR. *Hereditas* **138**: 101-113.

Humphreys M, Thomas HM, Harper J, Morgan G, James A, Ghamari-Zare A, Thomas H. 1997. Dissecting drought- and cold-tolerance traits in the *Lolium-Festuca* complex by introgression mapping. *New Phytologist* **137**: 55-60.

Humphreys MW, Pašakinskienė I, James AR, Thomas H. 1998a. Physically mapping quantitative traits for stress-resistance in the forage grasses. *Journal of Experimental Botany* **49**: 1611-1618.

Humphreys MW, Zare AG, Pasakinskiene I, Thomas H, Rogers WJ, Collin HA. 1998b Interspecific genomic rearrangements in androgenic plants derived from a *Lolium multiflorum* x *Festuca arundinacea* (2n=5x=35) hybrid. *Heredity* **80**:78-82.

Humphreys MW, Zwierzykowski Z, Collin HA, Rogers WJ, Zare A-G, Liesniewska A. 2000. Adrogenesis in grasses - methods and aspects for future breeding . In: Biotechnological Approaches for Utilization of Gametic Cells, Proceedings of the COST 824 final meeting, Bled, Slovenia, 1-5 July 2000, p 5-13.

Humphreys MW, Canter PJ, Thomas HM. 2003. Advances in introgression technologies for precision breeding within the *Lolium-Festuca* complex. *Annals of Applied Biology* **143**:1-10.

Jauhar PP. 1993. Cytogenetics of the *Festuca-Lolium* Complex. Monographs on Theoretical and Applied Genetics 18, Berlin Heidelberg, Germany: Springer-Verlag.

King IP, Morgan WG, Armstead IP, Harper JA, Hayward MD, Bollard A, Nash JV, Forster JW, Thomas HM. 1998. Introgression mapping in the grasses. I. Introgression of *Festuca pratensis* chromosomes and chromosome segments into *Lolium perenne*. *Heredity* **81**: 462-467.

Leśniewska A, Ponitka A, Ślusarkiewicz-Jarzina A, Zwierzykowska E, Zwierzykowski Z, James AR, Thomas H, Humphreys MW. 2001. Androgenesis from *Festuca pratensis* x *Lolium multiflorum* amphidiploid cultivars in order to select and stabilize rare gene combinations for grass breeding. *Heredity* **86**: 167-176.

Madsen S, Olesen A, Dennis B, Andersen SB. 1995. Inheritance of anther culture response in perennial ryegrass (*Lolium perenne* L). *Plant Breeding* **114**: 165-168.

Mizukami Y, Sugita S, Ohmido N, Fukui K. 1998. Agronomic and cytological characterization of F_1 Hybrids between *Lolium multiflorum* Lam. and *Festuca arundinacea* var. *glaucescens* Boiss.. *Grassland Science* **44**: 14-21.

Ohri D. 1998. Genome size variation and plant systematics. *Annals of Botany* **82** (Supplement A): 75-83.

Olesen A, Andersen SB, Due IK. 1988. Anther culture response in perennial ryegrass (*Lolium perenne* L.). *Plant Breeding* **101**: 60-65.

Opsahl-Ferstad H-G, Bjørnstad Å, Rognli OA. 1994. Genetic control of androgenetic response in *Lolium perenne* L.. *Theoretical and Applied Genetics* **89**: 133-138.

Pašakinskienė I, Anamthawat-Jonsson K, Humphreys MW, Jones RN. 1997. Novel diploids following chromosome elimination and somatic recombination in *Lolium multiflorum* x *Festuca arundinacea* hybrids. *Heredity* **78**: 464-469.

Pašakinskienė I, Griffiths CM, Bettany AJE, Paplauskiene V, Humphreys MW. 2000. Anchored simple-sequence repeats as primers to generate species-specific DNA markers in *Lolium* and *Festuca* grasses. *Theoretical and Applied Genetics* **100**: 384-390.

Rapacz M, Gasior D, Zwierzykowski Z, Lesniewska-Bocianowska A, Humphreys MW, Gay AP. 2004. Changes in cold tolerance and the mechanisms of acclimation of photosystem II to cold hardening generated by anther culture of *Festuca pratensis* x *Lolium multiflorum* cultivars. *New Phytologist* **162**: 105-114.

Salvo-Garrido H, Travella S, Schwarzacher T, Harwood WA, Snape JW. 2001. An efficient method for the physical mapping of transgenes in barley using *in situ* hybridisation. *Genome* **44**: 104-110.

Śiffelová G, Pavelková M, Klabouchová A, Wiesner I, Naśinec V. 1997. Computer-aided RAPD fingerprinting of accessions from the ryegrass-fescue complex. *Journal of Agricultural Science* **129**: 257-265.

Spangenberg G, Wang Z-Y, Potrykus I. 1998. Biotechnology in Forage and Turf Grass Improvement. Monographs on Theoretical and Applied Genetics 23, Berlin Heidelberg, Germany: Springer-Verlag.

Stammers M, Harris J, Evans GM, Hayward MD, Forster JW. 1995. Use of random PCR (RAPD) technology to analyze phylogenetic-relationships in the *Lolium-Festuca* complex. *Heredity* **74**: 19-27.

Sugiyama S, Yamaguchi K, Yamada T. 2002. Intraspecific phenotypic variation associated with nuclear DNA content in *Lolium perenne* L. *Euphytica* **128**: 145-151.

Thomas H. 1993. Chromosome manipulation and polyploidy. In: Hayward MD, Bosemark NO, Romagosa I, eds. Plant Breeding, Principles and prospects. London, UK: Chapman & Hall, 79-92.

Thomas HM, Morgan WG, Humphreys MW. 2003. Designing grasses with a future - combining the attributes of *Lolium* and *Festuca***.** *Euphytica* **133**: 19-26.

Turpeinen T, Kulmala J, Nevo E. 1999. Genome size variation in *Hordeum spontaneum* populations. *Genome* **42**: 1094-1099.

Wang X, Hu H. 1984. The effect of potato II medium for Triticale anther culture. *Plant Science Letter* **36**: 237-239.

Zare AG, Humphreys MW, Rogers WJ, Collin HA. 1999. Androgenesis from a *Lolium multiflorum* x *Festuca arundinacea* hybrid to generate extreme variation for freezing-tolerance. *Plant Breeding* **118**: 497-501.

Zare AG, Humphreys MW, Rogers WJ, Mortimer AM, Collin HA. 2002. Androgenesis in a *Lolium multiflorum* x *Festuca arundinacea* hybrid to generate genotypic variation for drought resistance. *Euphytica* **125**: 1-11.

Zwierzykowski Z, Lukaszewski AJ, Lesniewska A, Naganowska B. 1998. Genomic structure of androgenic progeny of pentaploid hybrids, *Festuca arundinacea* x *Lolium multiflorum*. *Plant Breeding* **117**: 457-462.

Zwierzykowski Z, Zwierzykowska E, Slusarkiewicz-Jarzina A, Ponitka A. 1999. Regeneration of anther-derived plants from pentaploid hybrids of *Festuca arundinacea* x *Lolium multiflorum*. *Euphytica* **105**: 191-195.

Figure legends

Fig. 1. Effect of induction medium and genotype on anther culture response in three *Festulolium* accessions. Means standard deviation are from 5 replicates.

Fig. 2. Androgenic calli with high regeneration potential induced via anther culture of *Festulolium* Bx351.

Fig. 3. Freezing tolerance test of androgenic plants derived from three *Festulolium* plants. (FL: *Festulolium*; Lp: *Lolium perenne*; Fp: *Festuca pratensis*)

Fig. 4. GISH image of androgenic *Festulolium* progeny. The DNA of *L. perenne* was used as probe, and shown as blue color. The chromosomes of *F. pratensis* were shown as pale blue color. Translocation breakpoints are indicated by arrows. (a) Bx350-177 with fertile pollen, a 28-chromosome genome comprising approximately equal amount of *Lolium* and *Festuca* DNA with 9 intergeneric translocations, some of which have two breakpoints in an arm (arrows). Bar: 20 μm. (b) Prior-88, a 14-chromosome genome with intergeneric translocations. Bar: 10 μm.

Table 1 Ploidy levels in androgenic *Festulolium* progeny determined by flow cytometry.

Accessions	Dihaploids (2n=2x=14)	28 chromosome plants (2n=4x=28)
Bx350	188 (56.1 %)	147 (43.9 %)
Bx351	204 (76.7 %)	62 (23.3 %)
Prior	114 (80.9 %)	27 (19.1 %)

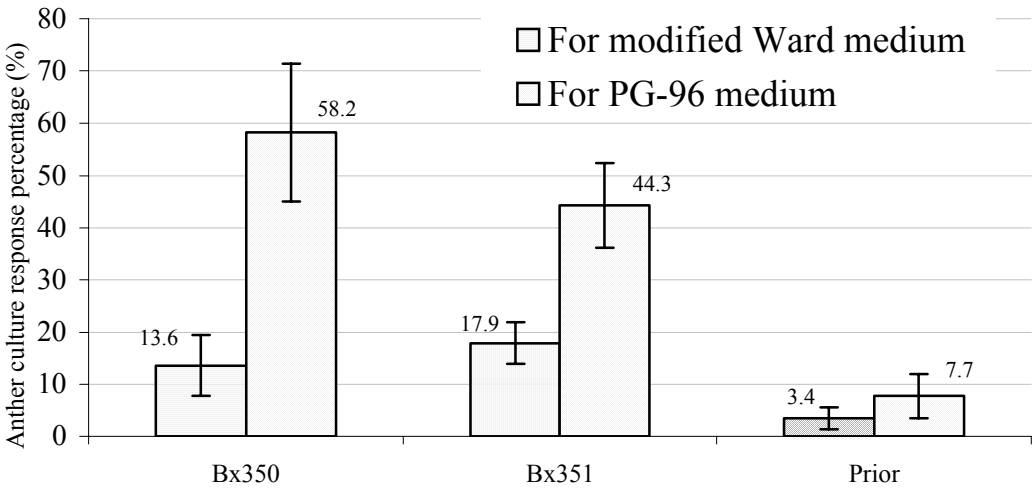
Table2 Pollen fertility in androgenic *Festulolium* progeny. F & PF, fertile and partial fertile, with pollen stainability ranging from 5% to 85%. MS, male sterile, no pollen or very few pollens (under 5%) were stained.

Bx 350				**Bx 351**				**Prior**			
2n=2x=14		2n=4x=28		2n=2x=14		2n=4x=28		2n=2x=14		2n=4x=28	
F & PF	MS	F & PF	MS	F & PF	MS	F & PF	MS	F & PF	MS	F & PF	MS
25 (46.3 %)	12 (22.2 %)	8 (14.8 %)	9 (16.7 %)	43 (56.6 %)	19 (25.0 %)	11 (14.5 %)	3 (3.9 %)	26 (57.8 %)	11 (24.4 %)	4 (8.9 %)	4 (8.9 %)

Table 3 Genome size (pg) variation in androgenic *Festololium* progeny**.**

Accessions	Prior (12.27±0.092)		350 (11.99±0.107)		351 (11.99±0.090)	
	Dihaploids	28 chromosome plants	Dihaploids	28 chromosome plants	Dihaploids	28 chromosome plants
DNA contend	5.42±0.207	12.01±0.291	5.36±0.232	12.9±0.322	5.33±0.222	12.79±0.314
(Range)	(4.69-5.71)	(11.65-12.26)	(4.97-5.84)	(12.36-13.23)	(5.04-5.73)	(12.30-13.14)

（ pg ± SD)



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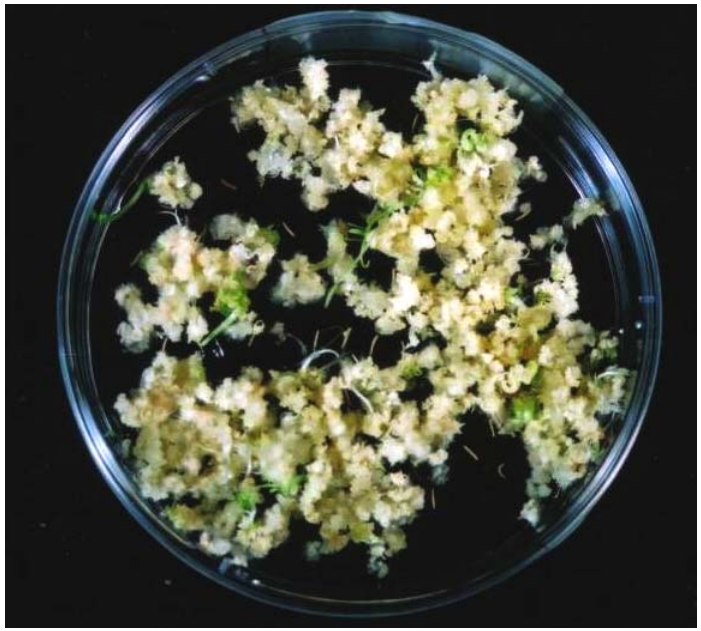
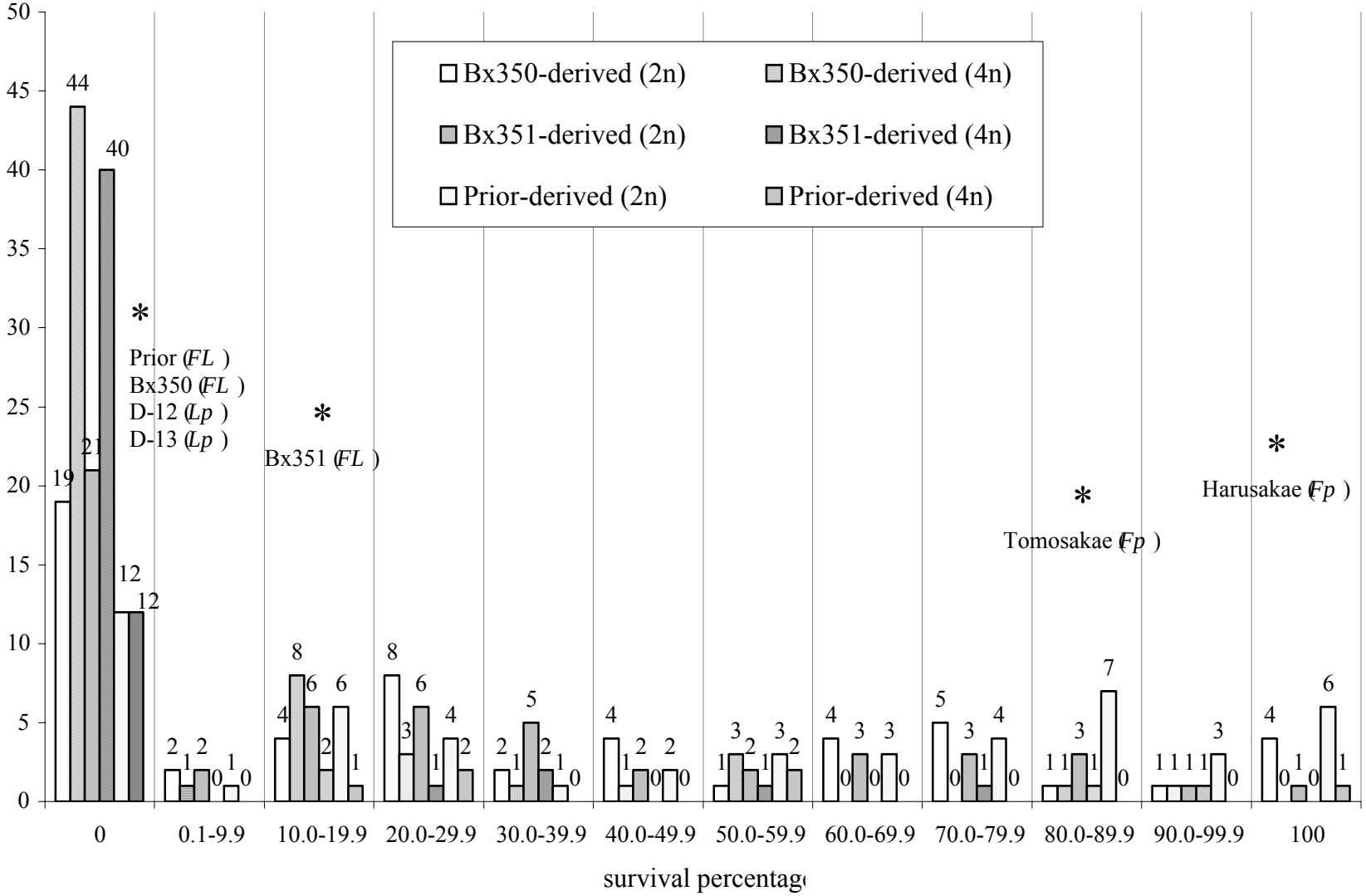


Fig. 2. Androgenic calli with high regeneration potential induced via anther culture of *Festulolium* Bx351.



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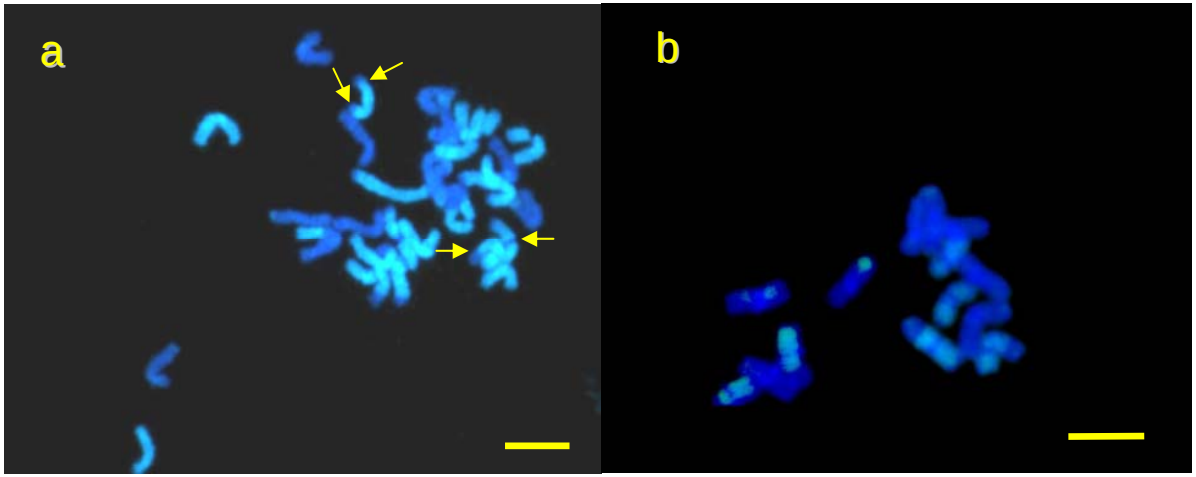
(*FL: Festulolium; Lp: Lolium perenne; Fp: Festuca pratensis*)



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