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Production of haploids and double haploids in annual (*Lolium multiflorum*) and perennial (*L. perenne*) ryegrasses

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Abstract. Anthers from 32 genotypes from one line of annual ryegrass (*Lolium multiflorum*) and 229 genotypes from 15 cultivars of perennial ryegrass (*L. perenne*) were tested for the production of haploids and double haploids. Six (23%) annual and 71 (31%) perennial ryegrass genotypes produced calli ranging from 1.3-16% and 0.8-12.2% respectively. However none of the annual ryegrass genotypes produced green plants and only one genotype (Option WH-1) of perennial ryegrass produced 16 (1.8%) green plants. The remaining produced only albino regenerates. Twelve of the 16 green plants were evaluated cytologically for chromosome numbers. Four were haploids thus confirming their origin from haploid pollen. The remaining eight were all diploids. One haploid and one diploid were tested for their homozygous condition using seven simple sequence repeat (SSR) markers and were found to be homozygous at all seven loci. In order to increase the recovery of green regenerates, we have used the responsive genotype Option WH-1 in crosses with three other perennial ryegrass genotypes and the progenies are currently been grown for anther culture.

Introduction

Ryegrasses (*Lolium multiflorum* L. and *L. perenne* L.) are the most important temperate forage grasses in the world. They are heavily cross pollinated in nature and therefore highly heterozygous and genetically non-uniform. Most agronomically interesting traits in ryegrasses are polygenic and interactions with environmental factors complicate breeding and genetic studies. Development of haploids and double haploids is a genetic tool which may alleviate some of these problems and allows better expression of recessive alleles.

The first result with anther culture of perennial ryegrass was reported by Zenkteler (1977) and Niizeki (1977) who obtained multicellular pollen and calli, respectively. Pollen derived albino plants of *L. perenne* were obtained by Stanis *et al.* (1983) and the first green regenerants from anther culture of *L. perenne* were reported by Stanis and Butenko (1984) but at a very low frequency. In particular, the capacity to form green plants is restricted to a few clones (Olesen *et al.* 1988, Boppenmeier *et al* 1989). Olesen *et al.* (1988), Bante *et al.* (1990), Halberg *et al.* (1990) and Madsen *et al.* (1995) reported the role of genetic determination in the ability to form callus and embryo and subsequent regeneration of green plants from cultured anthers of *L. perenne.*

Marker assisted selection (MAS) techniques for breeding new cultivars of ryegrasses (*L. multiflorum* and *L. perenne*) require construction of genetic linkage maps. Due to the outcrossing nature of ryegrass, our current genetic linkage maps are constructed using an F_1 population derived from heterozygous parents. While it is possible to develop genetic maps from populations derived from heterozygous material, homozygous parents are a more informative and easily analysed mapping population structure which simplifies map development. We are, therefore, developing haploid and

double haploid materials via anther culture to generate homozygous parent material for incorporation into our mapping populations.

Materials and methods

The plant material consisted of 32 genotypes of *L. multiflorum*, line 97649 and 229 genotypes from 15 different cultivars of *L. perenne* (Table 1). All plant materials were obtained from the Margot Forde Germplasm Centre, Palmerston North, New Zealand. *L. perenne* cultivars were selected to include both European and New Zealand cultivars. Seedlings from each cultivar/line were grown in 30x40 cm individual plastic trays in the glasshouse and 4 weeks old seedlings were transplanted into pots and grown in the glasshouse. Three-months-old plants were vernalised at 7°C for 6 weeks and transferred back to the glasshouse for flowering.

Selection of spikes containing microspores at mid to late uninucleate stage of development was carried out as described by He and Ouyang (1984) for wheat anther culture. Before culture the spikes were cold-treated at 3-5°C for three days. Spikelets containing desirable florets were surface sterilised by immersion in 70% ethanol for 1 minute and rinsing with sterile water for 1 minute. Spikelets were then further sterilised with 0.1% mercuric chloride for 8 min and rinsing twice with sterile water.

A small experiment was conducted to find out the best available media for our anther culture experiment. Five different induction media were evaluated using anthers from three different genotypes, each from perennial and annual ryegrasses. These included MS, B5, R2M, 190-2 and PII. PII was found to be the most effective media for callus induction, so in the subsequent experiment only PII medium of Wang and Hu Han (1984) solidified with 0.3% agar and pH adjusted to 5.8 was used for callus induction.

Anthers were dissected from desirable florets and plated in 9 cm Petri dishes containing PII media. The anthers were cultured in dark at 30°C for the first 3 days and then incubated in dark at 26°C for the rest of the culture period. After 4-7 weeks of anther culture pollen embryos and calli were transferred to regeneration medium 190-2 (Wang and Hu Han 1984) and incubated at 26°C in continuous white fluorescent light for regeneration of plants. Regenerated green plants were raised to a size of 4-5 cm and were then transferred to a jiffy pot containing MS salt. Once the root tips of an individual plant emerged from the jiffy pot, they were transferred to 10 cm plastic pots containing potting mix and grown under glasshouse conditions. Somatic chromosome counts were made from root tips squashes using the method previously reported by Hussain *et al.* (1997). Two anther derived plants (a haploid and a diploid) and the parent line (Option WH-1), from which they were derived were analysed using eight perennial ryegrass EST-SSR markers (simple sequence repeat markers derived from expressed sequence tags), selected from across the ryegrass genome. PCR and genotyping were conducted as in Faville *et al.* (2004).

Results and discussion

A total of 22,607 anthers from 32 genotypes of one *L. multiflorum* line, and 229 genotypes from 15 cultivars of *L. perenne* were cultured on PII media (Table 1). Only 6 (23%) annual and 71 (31%) perennial ryegrass genotypes responded to callus production within 28-50 days of culture. Pollen embryos or calli from responding anthers were transferred to the regeneration medium within 10-15 days of their formation. The majority of *L. perenne* and *L. multiflorum* genotypes will produce

almost entirely albino regenerates (Andersen 2003). All of the 77 genotypes except one genotype of cultivar Option (designated as Option WH-1), produced albino plants. Genotype Option-WH-1 produced 16 green plants from approximately 450 anthers. The success rate of green plants regeneration in this experiment was lower than previously reported (Olesen *et al*. 1988, Halberg *et al*. 1990). However, all previous experiments encountered a very high albino regeneration. Ryegrass genotypes with higher capacity to regenerate green plants are very rare among common material. Genotypes with higher response for green regenerates can be obtained through hybridisation and recombination (Halberg *et al*. 1990). When high responsive genotypes are crossed with random genotypes from other cultivars, the hybrid progeny generate 2-5% green plants (Madsen *et al*. 1995). We have therefore reciprocally crossed Option WH-1 with three other genotypes from cv. Kingston and the progeny are currently being grown for anther culture. Further crosses with a wide range of cultivars are being made.

Twelve of the 16 green plants produced from Option WH-1 anthers were evaluated cytologically for somatic chromosome numbers from root tip squashes. Four were haploids with seven chromosomes (Figure 1) thus confirming their origin from haploid pollen. The remaining eight were all diploids (14 chromosomes, Figure 1). Four newer green plants have yet to be confirmed. Green plants derived from ryegrass pollens generally have high frequency of spontaneous chromosome doubling. In some cases 50-70% of the plants are double haploids (Halberg *et al*. 1990, Bante *et al.* 1990). A low percentage may double twice spontaneously thus producing tetraploids (Bante *et al.* 1990). For these reasons plants derived from ryegrass anther culture do not generally need to be artificially chromosome doubled.

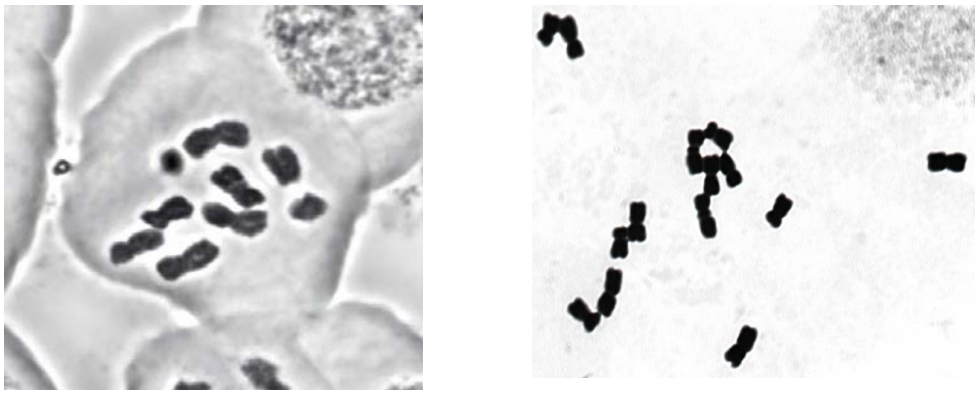
Two anther derived plants, one haploid (H1) and one diploid (DH1) and the Option WH-1 parent, were analysed using seven perennial ryegrass SSR markers, selected from across the ryegrass genome (Faville *et al.* 2004). Parental line (Option WH-1) showed a heterozygous profile for all EST-SSR markers (Table 2). By contrast both lines H1 and DH2 were apparently hemizygous at each locus, carrying single alleles of the same sizes as observed in Option WH-1. This is strong evidence that H1 and DH2 are haploid and double haploid derivatives respectively of Option WH-1. Different alleles were inherited by lines H1 and DH2 at four of the seven loci, indicating that they are genetically distinct.

Table 1. Results from anther culture with different genotypes of annual and perennial ryegrasses

Line/cv	No of genotypes screened	Total number of anthers cultured	Mean anthers/ genotype	Range	No of genotypes producing callus	Callus/ 100 anthers	Green plants
97649	32	2756	86.1	65-113	6	1.3-16.0	
Bronsyn	16	1796	112.3	70-137	3	0.8-1.5	
Meridian	22	1587	72.1	56-90	18	1.3-11.0	
Marathon	15	972	64.8	50-82	2	0.5-0.8	
Yatsyn	15	1456	97.1	80-108	2	0.3-0.7	
Kingston	12	1523	126.9	80-130	5	2.3-6.8	
GA 120	12	1189	99.1	92-105	--	--	
Samson	18	1239	68.8	60-85	1	0.2	
Impact	20	1179	59.0	50-72	--	--	
Pastel	16	1537	96.1	80-115	11	3.7-11.6	
Premium	16	1257	78.6	60-87	3	0.8-3.6	
Magyar	16	1235	77.2	50-90	7	1.1-2.6	
Mara	15	1606	107.1	85-124	9	2.5-9.3	
Option	12	2875	239.8	90-450	9	1.6-12.2	16
LP 9425	12	420	35	30-38	--	--	
LP 9335	12	380	31.7	25-40	1	0.6	
Total	261	21205			77		16

Table 2. Allele sizes (bp) at seven EST-SSR loci in two anther derived green plants (H1 and DH1) and the parental Option WH-1from which they were derived.

	EST-SSR allele profile		
EST-SSR	*Option WH-1*	*H1*	*DH1*
pps0080	220	220	220
	224		
pps0094	136	136	
	137		137
pps0149	346	346	
	347		347
pps0210	212		212
	214	214	
pps0255	263	263	
	264		264
pps0373	323		
	333	333	333
pps0433	190		
	198	198	198



A B

Figure 1: Haploid and Double haploid derived from *L. perenne* anther culture. A = 7 chromosomes (haploid) and B = 14 chromosomes (diploid).

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