Original Paper Plant Biotechnology, **16** (4), 291-296 (1999)

Diploidization and Genetic Variation in Anther Cultured Populations of *Lolium perenne* L.

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Received 4 February 1999; accepted 21 June 1999

Abstract

Four hundred and six anther culture developed progeny plants of eight different families of *Lolium perenne* L. were evaluated for their ploidy level, genetic variation at isozyme loci and performance at field level. Seventy six percent of the total progeny plants were found to be diploids, (2n=14) but the rate of diploidization varied from family to family. The segregations of eight isozyme loci among the progeny families and their level of heterozygosity were assessed. The heterozygosity was found to be low for all, only one family segregated at four isozyme loci and the rest were even lower. The field performance of the artificial homozygous naturally out-breeding species was evaluated and none could survive to the extreme environmental conditions over the winter, however they grew under controlled conditions.

Introduction

The genetic improvement of a crop generally depends on the development of homozygous lines. The development of such lines by conventional pedigree methods and single seed descent is a time consuming and tedious job and many problems are associated with the assessment and segregating of populations. These problems can be overcome by the use of homozygous doubled haploid lines (DH) with desired gene combinations from a heterozygous parent and can be achieved by the haploidization of diploid and subsequent chromosome doubling (Powell *et al.*, 1990). The production of DH from an early generation of a diploidization program has been widely accepted in many crops as an efficient alternative, but the rate of success of this method is dependent on many factors of which Snape *et al.* (1988) identified three as: I) frequency of doubled haploid production, II) the logistic requirements of this alternative system, and III) the type and range of genetic variation produced. The last criterion is largely concerned with the theoretical and practical utilization of DH. Powell *et al.* (1986) and Caligari *et al.* (1987) reported that the range of variability obtained from DH differ in many respects from that of others. Again the greater additive genetic variation in DH resulting from the absence of within family segregation enhances the utilization of this approach to relate variations detected at the nucleic acid level to whole plant phenotypic variation.

Lolium perenne L. is one of the most agriculturally important *Lolium* species. Originating in the Mediterranean region now widely distributed throughout the temperate regions of the world. Together with *Lolium multiflorium* this species accounts for over 80% of agricultural grass seed sold and is regarded as the basis for grassland improvement in the U.K (Breese and Tyler, 1986). *L. perenne* is an out-breeder and diploid (2n=14) and a fairly efficient self-incompatibility system exists in this species (Cornish *et al.*, 1980). In the out-breeding perennial ryegrasses homozygous material is not usually available and selfing is often difficult or impossible (Cooper, 1959). Traits with low heritability are very difficult to transfer from parent to progeny, thus anther culture is the best alternative to develop a generation with traits in the homozygous state.

The rapid fixation into diploid, spontaneously or artificially, makes the DH an alternative tool for both plant breeders and geneticists. Perennial ryegrass is a relatively underdeveloped crop and a knowledge of the genetic architecture of this forage species is a necessary adjunct to the breeding program (Breese and Hayward 1972). Since *Lolium*

perenne is an out-breeders, it is naturally heterozygous and consequently equivalent to F_2 in terms of breeding. The present study has been considered to estimate the extent of spontaneous diploidization in the anther cultured populations of *Lolium perenne*. To screen the population for genetic variability, a number of isozyme systems have been assayed to estimate the genetic variation and a field trial was performed to evaluate the agronomic performance of the doubled haploid families.

Materials and Methods

Plant Materials

The anther culture plants of *Lolium perenne* L. were developed by the Genetics Group of the Institute of Grassland and Environmental Research (IGER), Aberystwyth, U.K over the years 1988 to 1991. In November 1991, they were transferred into 3.5" pots filled with levington compost. At the end of December they were transferred into 5.0" pots and grown in a controlled glass house, and after January 1992 three of the four replicates were left outside for cold treatment before transplanted into the field. The developed plants were grouped into eight progeny families based on the donor parents, these are:

255: Developed from a plant of the cultivar **Verna** and consisted of 26 plants.

P2: Developed from a plant of the cultivar **Parcour** and consisted of 96 plants.

55F: Developed from a hybrid of the cross between **Talbot 75** and **Talbot 24** and consisted of 24 plants.

255.37: Developed from a hybrid of a polycross between some anther culture responsive plants and **255** and consisted of 24 plants.

94A, 94B and 94D: These three families were developed from three different self plants of **Talbot 75** and consisted of 10, 80 and 67 plants, respectively.

27B: This progeny family is consisted of 82 plants and was developed from a hybrid of the cross between **Talbot 54** and **Talbot 24**.

Chromosome Counting

Three young tillers from each of the plants were cleaned and placed in a root tip culture tank. After 7 -10 days tips of the roots were collected and pre-treated with cold water (1 ℃) for 16 hours. The root tips were fixed in a freshly prepared solution of acetic-alcohol (1:3) for a minimum of 2 hours. The root tips were hydrolized in 1N HCl at 60 ℃ for 10 minutes and stained in Feulgen solution. Squashes were prepared in 45% acetic acid solution and chromosomes were counted at the metaphase stage of mitosis.

Isozyme Analysis

Starch gel electrophoresis was performed to determine the isozyme genotypes of eight isozyme systems - Phosphoglucoisomerase (PGI), Glutamate Oxaloacetate Transaminase (GOT), Acid Phosphatase (ACP), Superoxide Dismutase (SOD), Diaphorase (DIA), Esterase (EST) and Benzoyl Arginine Aminopeptidase (BAP).

The gel buffer of the starch gel was prepared by mixing buffer A (0.05M Tris adjusted to PH 8.2 with 1M Citric acid) and buffer B (0.2M Boric acid adjusted to PH 8.2). For each 250m*l* gel buffer A and B were mixed in 1:9 proportion and 30gm of starch was added to it. The mixture was heated with continuous stirring until it become translucent. The heated solution was degassed and poured into a mould and covered immediately with glass plate to prevent the formation of any air bubbles.

A single, fresh, young and healthy leaf from each of the individual plants was crushed with 1-2 drops of crushing buffer and sample solution was soaked up with a wick of Whatman filter paper. The wicks were placed in a slit cut across the gel and electrophoresis was performed for about five hours at 250V and 25A current at 4 ℃. The gel was sliced into three and stained in appropriate staining solution.

Field Trial

The field trials for all the progeny families were carried out in an experimental field of the IGER, Aberystwyth, U.K. The plants were transplanted in a completely randomized way with three replications on the 10th of June 1992 with 20.0"x 20.0" spacing. Each row consisted of 20 plants and the layout was surrounded by guards. Shortly after transplanting the plants suffered from a period of drought and were watered artificially. The fertilization and weeding were provided when necessary.

Results

Chromosome Counting

Out of 406 plants chromosomes were counted for 351 plants from eight different families. Seventy six percent of the total progeny plants were found to be diploids (2n=14). In the family 255.37 all were found to be diploids, 82 out of 96 plants of the P2 family were counted as diploids (85%) and in the 27B, 66% of the plants had 14 chromosomes, only one triploid was found in the 94D family. Four plants of the P2 family and 1 of the 27B family were counted as tetraploids, (**Fig. 1**). The results are

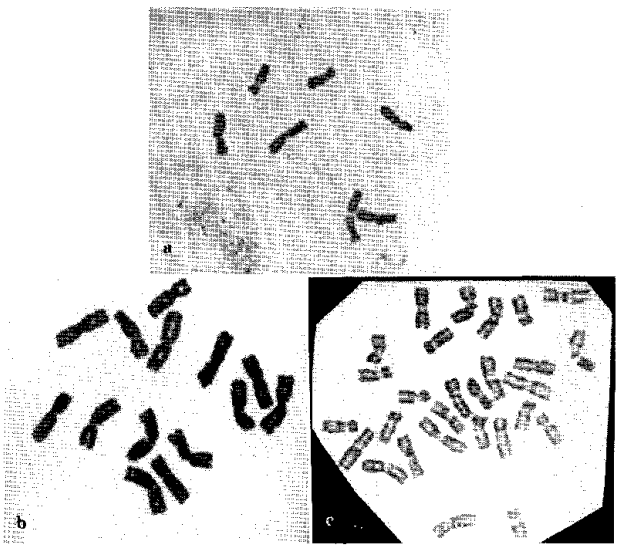

Fig. 1 Somatic metaphase chromosomes of *Lolium perenne* at different ploidy level. a) Haploid cell (n=7) b) Diploid cell (2n=14) and c) Tetraploid cell (4n=28).

presented in **Table. 1.**

Isozyme Analysis

The genotypes of the anther culture responsive parents at different isozyme loci were evaluated and the level of heterozygosity varied from one to four loci. The parent of the P2 family found heterozygous at four isozyme loci, the lowest heterozygosity (heterozygous at one isozyme locus) was found in the parents of 94A, 94D and 27B families (**Table 2**). Consequently, of the eight progeny families only P2 was found segregating at four isozyme loci (PGI, GOT2, DIA and EST). Progenies of 55F segregated at three isozyme loci, that of the families 255, 255.37 and 94B at two isozyme loci, the rest at only one locus. None of the family was found to be segregating at SOD locus. The segregation of P2 and 94B families at PGI and BAP loci respectively are presented in **Fig. 2.** The χ^2 analysis was performed to test the fit of the segregation for the expected 1:1. Significant deviation from 1:1 was found at PGI in 255 and P2, at GOT3 in 255.37, at ACP in 55F, 255.37 and 94B, at DIA locus in P2 and 27B and at BAP in 94B family. The results are presented in **Table 3**.

Field Trial

The growth of the plants in the field was found to be very poor compared with their respective parents and other varieties surrounding the plot transplanted at the same time. At the beginning of the winter in 1992 the plants started to die off and after winter, except for the parents, all the plants were found to be dead (**Fig. 3**).

Discussion

About 76% of the anther cultured progeny were found to be diploid and the diploidization occured spontaneously. In the family P2, 85% of the progeny plants were found to be diploid and segregated at four isozyme loci. In the 94B, 75% of the progeny plants were found to be diploid and segregated at two isozyme loci ACP and BAP. Progenies of the family 55F and 255.37 segregated at three and two isozyme loci, respectively, and their diploidization rates were 45% and 100%. In the rest of the families, the diploidization rates ranged from 66% to 80% and variation at isozyme loci was found to be very low.

In our study the diploidization rate among the

Table 1. Ploidy level of another cultured plants of *Lolium perenne*

Family	Plant Total	Plant Counted	Haploid	Diploid (%)	Triploid	Tetraploid
255	26	26	6	20(77)	–	–
P2	96	96	10	82(85)	–	4
55F	21	11	6	5(45)	–	–
255.37	24	24	–	24(100)	–	–
94A	10	10	2	8(80)	–	–
94B	80	56	14	42(75)	–	–
94D	67	57	13	43(75)	1	–
27B	82	71	23	47(66)	–	1

Table 2. The isozyme genotypes of the donor plants of anther culture for the DH progeny families.

Family	Parent	PGI	GOT2	GOT3	ACP	SOD	DIA	EST	BAP
255	Var. Verna	ab	aa	cc	aa	bb	ss	an	–
P2	Var. Parcour	ab	bn	bb	bb	bb	sf	an	–
55F	a hybrid of Tal. 75xTal. 24	ab	bb	bb	ac	bb	sf	aa	–
255.37	a hybrid from 255 polycross	bb	aa	cd	ac	bb	ff	aa	–
94A	a selfed progeny of Tal. 75	ab	bb	cc	aa	bb	ss	–	–
94B	a selfed progeny of Tal. 75	aa	aa	bb	ab	bb	ff	–	sf
94D	a selfed progeny of Tal.75	aa	aa	bb	bb	bb	sf	–	–
27B	a hybrid of Tal. 54xTal. 24	bb	bb	bb	cc	bb	sf	aa	–

– No isozymic band was revealed in the respective enzyme systems

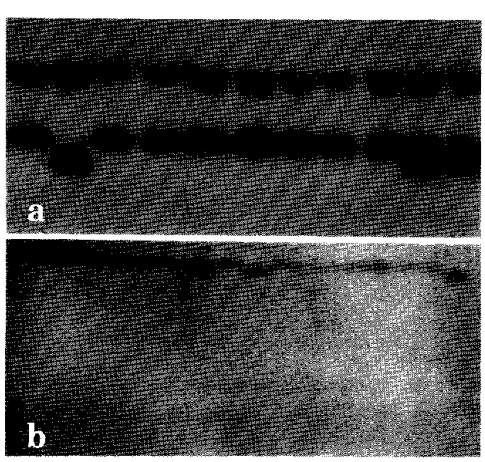

Fig. 2 Segregation of progeny families at isozyme loci. a) P2 family at PGI, ▽ ▽ Parents and b) 94B at BAP. ▽ Parent

Fig. 3 Comparison of field performance of DH with those of their respective parents and other varieties of *Lolium perenne* L. a) DH b) Parent c) Other varieties

eight DH families varied from 45% to 100%. In 94A, 94B and 94D (all derived from Talbot 75), similar diploidization rates have been observed (75 ~ 80%). Lower rates of diploidization (45% and 66%) have been observed in 55F (derived from Talbot 75 × Talbot 24) and 27B (derived from Talbot 54 × Talbot 24). 100% diploidization was observed in the family 255.37, which was developed from a hybrid of a polycross between some anther culture responsive plants and 255. Thus, it has been clearly indicated that in *Lolium perenne* the diploidization rate is genotype dependent, and the lower rates of diploidization in 55F and 27B could be dependant on the genotype of the parent, Talbot 24.

The level of variation at different isozyme loci was found to be low. Of the eight families only one (P2) segregated at four isozyme loci and in the rest were lower than this. The lower level of heterozygosity in the anther culture responsive parents and consequently in their progeny families may reflect the tendency of responsive genotypes to be homozygous.

The segregations at the majority of the isozyme loci were found to be distorted. This type of segregation has also been reported in the DH populations of many crops including *Lolium perenne* (Hayward *et al.*, 1990), barley (Powell *et al.*, 1986; Thompson *et al.*, 1991), and rice (Guiderdoni *et al.*, 1989). One

Table 3. Segregation of progeny families at different isozyme loci with χ^2 value

Isozyme loci with genotypic class		Progeny families with genotypic classes at the segregating loci							
		255	P2	55F	255.37	94A	94B	94D	27B
PGI	aa	18	70	7		3	80	67	
	bb	8	26	14	24	7			62
	χ^2	3.85*	20.2***	2.33		1.6			
GOT2	aa	26			24		80	67	
	bb		54	21		10			82
	nn		42						
	χ^2		1.5						
GOT3	bb		96	21			80	67	82
	cc	26			22	10			
	dd				2				
	χ^2				16.7***				
ACP	aa	26		6	5	10	22		
	bb		96				58	67	
	cc			15	19				82
	χ^2			3.9*	6.0**		16.2***		
SOD	bb	26	96	21	24	10	80	67	82
DIA	ss	26	26	8		10		28	30
	ff		70	13	24		80	39	52
	χ^2		20.2***	1.2				1.8	5.9*
EST	aa	11	56	21	24	—	—	—	82
	nn	15	40						
	χ^2	0.23	2.67						
BAP	ss	—	—	—	—	—	16	—	—
	ff						64		
	χ^2						28.8***		

*P<0.05**P<0.01***P<0.001

of the possible causes of distorted segregation could be that the neutral and co-dominant markers are linked to non-neutral genes in the heterozygous state. The double recessive progeny resulted from anther culture may be under represented or more calli from the parents of dominant genotypes may be differentiated into plantlets. Alternatively, it is possible that there is selection among the plantlets themselves. In a study Hayward *et al.* (1990) indicated that a genetic component is responsible for disturbed segregations in *Lolium*. They explained the allele in excess as being linked to the viability genes affecting the generating ability and described them as lethal genes which usually occur in high frequency in naturally out-breeding species. The loci with distorted segregation in this study could also be linked with such lethal genes and cause disturbance in segregation through gametophytic selection. Hayward and McAdam (1977) found similar segregation at PGI and GOT loci in *L. perenne*.

It has been reported that the natural diploid population of *L. perenne* shows a much higher tolerance to cold temperature. Sugiyama (1998) and Bingham (1980) reported that the performance of plants under stress depends on the level of their heterozygosity. Perennial ryegrass is naturally an out-breeder and heterozygous and the populations

under study were made homozygous artificially. The homozygous state of this species could make it vulnerable to extreme environmental conditions and may be the cause of the death of these plants under field conditions.

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