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Genetic control of androgenetic response in *Lolium perenne* L.

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Abstract In a study of androgenesis in 90 Norwegian genotypes of perennial ryegrass (*Lolium perenne* L.), heritabilities ranged from $h_b^2 = 0.46$ to 0.80. Very high or completely positive genotypic correlations were found between most characters of androgenetic response (e.g. embryo-like structures per 100 anthers, plants per 100 embryo-like structures, albino plants per 100 anthers, green plants per 100 anthers). Three genotypes , 2 Norwegian (7-5 and 9-5) and 1 Danish (245), which had significantly different androgenetic responses were selected to study the genetic control of the processes. Genotypes 7-5 and 9-5 were highly embryogenic, 7-5 and 245 were relatively high producers of green plants, while 9-5 was unable to produce green plants. The six possible reciprocal crosses between these three genotypes were made, and 10 or 11 F_1 plants from each cross were used for anther culture experiments. The cross 7-5×245 showed average superiority over both parents for total plant regeneration and green plant production, results not previously reported. The phenotypic correlations estimated among progenies from the crosses ranged from r=–0.99*** to 0.81***. These considerable changes, relative to the results of the screening experiment, are most likely the result of changed allele frequencies caused by the strong selection of parents in these crosses, and a relatively simple genetical control. This is also inferred from the large transgressive segregation observed.

Key words Anther culture · Albinism · *Lolium perenne* Heritability · Genotypic correlations

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Abbrevations *ANT* anthers · *ELS* embryo-like structures *ALB* albino plants · *GRP* green plants *DH* doubled haploid plants

Introduction

A number of studies in several species of *Poaceae* have shown nuclear genetic control of the anther culture response, with mainly additive, but also some dominance and epistatic effects (Agache et al. 1988; 1989; Tuvesson et al. 1989). However, maternal, cytoplasmic and nuclear×cytoplasmic effects have also been reported (Powell 1988; Halberg et al. 1990; Sági and Barnabás 1989; Ekiz and Konzak 1991). Androgenetic response is determined by three genetic processes: (1) the induction of embryo-like structures (ELS), (2) the ability to regenerate plants and (3) the frequency of albino plants produced (Henry and de Buyser 1985). The last factor is particularily important in several of the in vitro techniques applied on species of the grass family where albinism is the main problem. Restriction fragment length polymorphism (RFLP) studies have shown mainly co-linearity between chromosomes of different grass species (Moore et al. 1993), and molecular studies have revealed similarities between closely related transcripts expressed in many species within *Poaceae,* e.g. wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), perennial ryegrass and rye-brome (*Bromus secalinus* L.) (Goldmark et al. 1992; Aalen et al. 1994). It is therefore possible to relate results from anther culture of perennial ryegrass to similar studies in wheat and barley. Results from genetic analyses of anther culture response in other species of the grass family are somewhat conflicting. It is still unknown whether the three processes are independently inherited or genetically correlated (Agache et al. 1988; 1989; de Buyser et al. 1992a), but a strong positive genetic correlation between, for example the ability to regenerate plants and green plant production (r=0.81**) has been reported (Tuvesson et al. 1989). It should also be mentioned that Deaton et al. (1987) reported a non-significant

negative genetic correlation (r=–0.24) between embryogenic induction and the regeneration of green plants.

There are three main causes of correlations between characters; pleiotropy, linkage and environmental effects. The degree of correlation arising from pleiotropy expresses the extent to which the characters are influenced by the same genes. However, little is known about the genetic or environmental control of these processes or of their relationships so far (Agache et al. 1989).

Since androgenesis is largely determined by genotype, intercrossing genotypes with the ability to produce green plants with interesting breeding materials could reduce the problem of low androgenetic response (Wenzel et al. 1977). This procedure has been applied in perennial ryegrass by Halberg et al. (1990): among 55 hybrids they found 6 to be superior to their parents in androgenetic response. Since the genetic control of androgenetic response is still unknown, the results from such crosses are unpredictable. More basic research is needed in order to be able to understand and explain the genetic basis of androgenesis. In this respect, somatic embryogenesis has been studied more extensively than regeneration (Higgins and Bowles 1990; Carman 1990). A high frequency of albino plants is the main problem in regeneration from both anther and microspore cultures of most species of the *Poaceae* family (Day and Ellis 1984, 1985; Harada et al. 1991; Dunford and Walden 1991). Regeneration would be a fruitful topic of research, and increased knowledge in this direction may help us to understand and solve the albino problem.

In order to assess the natural variability for androgenetic response in perennial ryegrass, nine Norwegian populations (90 genotypes) were screened in anther culture. On the basis of the results of the screening experiment, 3 genotypes, 7-5, 9-5, and 245, were selected because they exhibited differences in all processes of androgenetic response. A detailed genetic analysis was performed on the progenies (63 F_1 plants) from all possible crosses between these genotypes. Reciprocal crosses were included in order to identify the possible effects of the different chloroplast genomes (cytoplasmic inheritance) or maternal effects. The present paper reports the results from this investigation.

Materials and methods

Plant material

From each of nine local populations of perennial ryegrass collected on the west coast of Norway (Collection nos. ABY–BA[1] 10103, 10104, 10106, 10107, 10108, 10109, 10111, 10112 and 10113), 10 genotypes were randomly selected, yielding a total of 90 Norwegian genotypes to be screened for androgenetic response. In addition, 3 Danish genotypes, 175 (cv 'Sisu'), 245 (cv 'Verna') and 255 (cv 'Verna'), were used as reference clones, these have been used by several other groups and are known to have a fairly good androgenetic response (Olesen et al. 1988). Plants of the genotypes 7–5 and 9–5 were selected from this screening experiment together with plants of genotype 245. These genotypes were crossed in the six possible combinations, including reciprocals, and 10 or 11 F_1 plants were taken from each reciprocal cross. The genetic study of androgenetic response was based on the resulting 63 F_1 progenies.

[1] Collections stored at the Genetic Resources Unit, AFRC/IGER, Aberystwyth, Wales

Plant growth conditions

One set of donor plants used in the screening experiment (93 clones) was grown at a 21°/15 °C day/night temperature cycle under controlled greenhouse conditions, with natural daylight (May) in addition to 115 $\mu Em^{-2} s^{-1}$ from light tubes (Hy Pressure Mercury Halogen), while an identical set of clones was grown in the field. The 63 F_1 mother clones were vernalized and then grown at 15°/12 °C day/night temperatures (12 h cycles) in natural daylight (August), with additional light under greenhouse conditions as in the screening experiment.

Anther culture procedure

Spikes were harvested when the microspores were at the mid- to late-uninucleate stage (He and Ouyang 1984). They were surface sterilized with 0.1% mercury chloride for 8 min. Anthers (ANT) from the five mid flowers of about nine spikes were plated on solid 'potato II' induction medium with maltose (Wang and Hu 1984; Hunter 1987) and 0.3% gelrite, in a 'triple-dish-design' (Lyne et al. 1986). Each petri-dish contained 8 ml medium and 30 anthers. The anthers were incubated at 33 °C for 3 days before being transferred to 26 °C (Ouyang et al. 1983) and low light intensities; 15 $\mu Em^{-2} s^{-1}$ continuous white fluorescent light (Bjørnstad et al. 1989). Embryo-like structures (ELS) were transferred three times to regeneration medium '190-2' (Wang and Hu 1984) 4–8 weeks after the incubation of anthers. Plants were grown further on rooting medium '190-2' (without growth regulators) before being transplanted into soil. The anther culture procedure was slightly altered for the study of the F_1 progeny. In this case, induction medium '190-2' was used, and the anthers were incubated directly at 26 °C. This can be justified since these changes were found to be insignificant for androgenetic response (Opsahl-Ferstad et al. 1994).

Observations and statistical analysis

The number of embryo-like structures (#ELS), and albino (#ALB) and green plants (#GRP) produced were recorded. On the basis of these observations, six characters were calculated from these observations: numbers of ELS, ALB and GRP per 100 anthers plated (#ELS/100 ANT, #ALB/100 ANT, #GRP/100 ANT), total plant regeneration (#PL/100 ELS), percent albino (%ALB/PL) and percent green plants (%GRP/PL). Heritabilities and genetic and phenotypic correlations were calculated according to standard methods. Residual plot analysis showed that the data were not normally distributed. The calculated variables were therefore square root transformed before the analyses of variance were carried out using the SAS/STAT GLM procedure (SAS 1987). Differences between means were tested by Duncans Multiple Range Test (SAS 1987).

Results

Screening experiment

Of the 90 genotypes screened, 74 (82%) produced ELS, 64 (71%) produced albino plants, but only 15 (17%) produced green plants. The total production was 8451 ELS, 2971 albino and 90 green plants from 34 290 anthers plated, an average of 24.6 ELS, 8.7 albino and 0.3 green plants per 100 anthers. The combined analysis of variance over populations showed only a few significant differences (Table 1), while highly significant differences were found between genotypes for all six characters tested. Among the

Table 1 Mean squares (MS) from the analysis of variance of the screening experiment, where 90 genotypes were tested, in two replicates, for six characters determining androgenetic response

Source	*df*	Characters						
		#ELS/100 ANT	#PL/100 ELS	#ALB/100 ANT	#GRP/100 ANT	*df*	%ALB/PL	%GRP/PL
Populations	8	28.29	18.64*	18.45*	0.32***	8	2.18	3.22
Genotype/ population	81	22.45***	7.95***	7.76***	0.26***	57	1.69***	3.27***
Error	81	4.63	1.83	1.82	0.07	44		

*, **, *** Significant at the 5%, 1% and 0.1% level, respectively

Table 2 Genotypic correlations (r) and broad-sense heritabilites (h_b^2) estimated for the characters of androgenetic response, from the screening experiment

Characters	# ELS/100 ANT	# PL/100 ELS	# ALB/100 ANT	# GRP/100 ANT	% ALB/PL	h_b^2
# ELS/100 ANT						0.80
# PL/100 ELS	0.66					0.61
# ALB/100 ANT	1.00	0.67				0.79
# GRP/100 ANT	0.69	0.18	0.59			0.71
% ALB/PL	0.69	1.00	0.71	0.32		0.78
% GRP/PL	0.53	0.02	0.44	0.94	(0.16)	0.46

Table 3 Mean squares (MS) from the analysis of variance of the 63 F_1 donor plants resulting from the three crosses between the selected genotypes 7-5, 9-5 and 245. Six characters determining androgenetic response were tested, as in the initial screening experiment

Source	*df*	Characters					
		#ELS/100 ANT	#PL/100 ELS	#ALB/100 ANT	#GRP/100 ANT	%ALB/PL	%GRP/PL
Crosses (C)	2	69.9	28.4 **	25.7 *	35.9 ***	31.1 **	101.5 ***
Reciprocals (R)/C (R/C)	3	10.0	0.9	4.7	2.7	2.3	9.2
Genotypes/R/C (error)	57	26.3	3.7	5.2	3.6	4.1	6.8

*, **, *** Significant at the 5%, 1% and 0.1% level, respectively

genotypes which produced green plants, a restricted analysis of variance showed significant differences between genotypes only for total regeneration (#PL/100 ELS) and albino plant production (#ALB/100 ANT).

Broad sense heritabilities for the characters and their genotypic correlations are presented in Table 2; phenotypic correlations were similar (data not shown). Table 2 shows high heritabilities for all characters controlling androgenetic response, except for percent green plant production (h_b^2=0.46). Genotypic correlations between embryogenic ability (#ELS/100 ANT), total regeneration, and albino production were highly positive or complete. In contrast, genotypic correlations between total regeneration and green plants produced, and percent albino and per cent green plants produced, were low or insignificant.

Crosses

From 24 264 anthers plated in the crossing experiment, 20 021 ELS, 2894 albino plants and 1391 (32%) green plants were generated, an average of 82.5 ELS, 11.9 albino and 5.7 green plants per 100 anthers. The crosses were not significantly different for induction of ELS. The ability to develop ELS was present in 62 of the 63 F_1 donor plants derived from these crosses. Of the 62 F_1 plants generating ELS, 54 were able to regenerate plants and 20 produced green plants. The means of the F_1 donor plants from each of the three crosses were significantly different for total regeneration (PL/100 ELS) and albino and green plant production (Table 3). Reciprocal effects were not statistically significant, although there were indications that there were reciprocal effects concerning green plant production in cross 1 and cross 3. These effects might have been significant if clonal replicates of the F_1 plants had been used. There was, however, extensive variation among F_1 progenies within each cross (data not shown).

The parental plants of the crosses were significantly different in most of the characters studied (Table 4). Parental plant 7-5 produced significantly more ELS, had a higher total regeneration and produced more albino plants than the other two parental plants, but was equal to 245 in green

Table 4 Comparisons between the means of the characters of androgenetic response for parental plants and the 21 F_1 donor plants from the respective crosses

Source	Characters					
	#ELS/100 ANT	#PL/100 ELS	#ALB/100 ANT	#GRP/100 ANT	%ALB/PL	%GRP/PL
Parental plant						
7-5 (P_1)	174.1 [a]	19.3 [a]	31.0 [a]	2.0 [a]	93.3 [a]	6.0 [b]
9-5 (P_2)	115.8 [ab]	3.6 [b]	3.9 [b]	0.0 [b]	100.0 [a]	0.0 [c]
245 (P_3)	73.2 [b]	7.2 [b]	3.5 [b]	1.7 [a]	70.7 [b]	26.3 [a]
Crosses						
Cross 1 ($P_1 \times P_3$)	82.5 [ab]	31.8 [a]	11.1 [ab]	15.2 [a]	42.8 [b]	56.8 [a]
Cross 2 ($P_2 \times P_3$)	58.9 [b]	7.9 [b]	4.3 [b]	0.0 [b]	100.0 [a]	0.0 [b]
Cross 3 ($P_1 \times P_2$)	117.9 [a]	19.5 [b]	21.3 [a]	1.6 [b]	88.0 [a]	8.7 [b]

[a] Entries with the same letters are not significantly different at $P \geq 0{,}05$

Table 5 Phenotypic correlations calculated for the characters of androgenetic response from the 21 F_1 donor plants from cross 1 and cross 3 producing green plants

Variable		#ELS/100 ANT	#PL/100 ELS	#ALB/100 ANT	#GRP/100 ANT	%ALB/PL
#PL/100 ELS	Cross 1	0.40				
	Cross 3	0.51*				
#ALB/100 ANT	Cross 1	0.80***	0.27			
	Cross 3	0.91***	0.66**			
#GRP/100 ANT	Cross 1	0.72***	0.81***	0.29		
	Cross 3	0.29	0.26	0.21		
%ALB/PL	Cross 1	−0.22	−0.75***	0.23	−0.72***	
	Cross 3	0.03	−0.09	0.01	−0.77***	
%GRP/PL	Cross 1	0.48*	0.75***	0.11	0.70***	−0.91***
	Cross 3	0.10	0.18	0.01	0.82***	−0.99***

*, **, *** Significant at the 5%, 1% and 0.1% level, respectively

plant production. F_1 progenies from cross 1 (7-5×245) were significantly better than progenies from the other crosses with respect to total regeneration ability and green plant production (Table 4). When comparing parent and progeny means, the embryogenic responses in the progeny were intermediate or at the level of the lower parent (Table 4). F_1 plants from cross 1 demonstrated a significant average superiority for total regeneration ability and green plant production, while the progeny means in cross 3 (7-5×9-5) were at the level of the best parent for the same characters (Table 4). Average superiority of F_1 progenies over parents has not been previously reported in perennial ryegrass.

Green plants were only produced from crosses 1 and 3, and significantly more from cross 1, while the 21 F_1 donor plants of cross 2 only produced albino plants (Table 4). From cross 1, 7 of the 21 F_1 hybrid plants were superior to their parents in green plant production, with 10–118 GRP/100 ANT (30–94% GRP/PL), while only 2 of the F_1 hybrid plants in cross 3 were superior with 4-32 GRP/100 ANT (39–49% GRP).

The phenotypic correlations (Table 5) between the components of androgenetic response estimated in cross 1 and cross 3 were in most cases lower than the genotypic correlations previously estimated in the screening experiment from which the parental plants were selected (Table 2). The most extreme reduction was observed in cross 1 for the correlation between total regeneration and percent albino plants (r=0.69 vs. –0.75***), number of green plants and percent albino plants produced (r=0.32 vs. –0.72***) and percent green plants and percent albino plants produced (r=0.16 vs. –0.91***). However, the correlations between regeneration (PL/100 ELS) and number of green plants produced (r=0.81***) as well as percent green plants regenerated (r=0.75***) increased considerably in cross 1, which produced the highest number of green plants (Table 5). Cross 3 had higher correlations for characters including albinism because of more albino and fewer green plants (Table 5).

Discussion

The characters of androgenetic response may be characterized by both qualitative and quantitative inheritance, since the abilities to produce ELS and regenerate plants are genetically determined, as are also the actual numbers and frequencies. The mean values and the distribution of the characters observed in these crosses indicate that more than one locus is involved in all the processes of androgenetic response in perennial ryegrass. The observed segregation

of these characters indicates quantitative genetic control, but simultaneously the considerable change in correlations following the strong selection of parental plants indicates that relatively few loci are involved. Even if there are only a few loci involved, the quantitative nature of the variation, caused by large environmental variation among different spikes, clones and genotypes, makes genetic interpretations of anther culture results difficult. However, it is clear that when studying F_1 donor plant progenies, dominance effects are absent in homozygote plants. Isozyme analysis of the green plants which were shown not to be heterozygous indicated homozygosity, since most of them were also diploid (DH). Directional selection of ELS should not be neglected; in vitro selection for isozymes of phosphoglucose isomerase (PGI-2) in DH of perennial ryegrass has been reported by Hayward et al. (1990), where green versus albino progenies segregated differently and deviated from Mendelian expectations. They speculated on a possible linkage between the marker loci and lethal genes, the latter are expected to occur at high frequencies in outbreeding species.

The ability to develop ELS seems to be controlled by additive gene effects, while total regeneration and green plant production seem to be controlled by dominance (Table 4).

There may be different genes with epistatic interactions that affect green and albino plant production, as has been shown in wheat by de Buyser et al. (1992b). These investigators found genetic factors on one wheat chromosome that increased albino plant production while another factor increased green plant production. In the present study the incomplete correlations found between albino (#ALB/100 ANT) and green plant (#GRP/100 ANT) production (r=0.29) as well as those found between #ALB/100 ANT and%ALB/100 ELS (r=0.23) may support such a model. This model may also explain why parental plant 7-5 was producing significantly more albino plants than the other genotypes as well as more green plants (together with 245) than 9-5 (Table 4). Differental gene action may also explain why as many as 7 out of the 21 F_1 plants from cross 1 produced between 50% and 100% percent green plants.

If albino and green plant production is controlled by different genes there should be a low or no genetic correlation between albino (#ALB/100 ANT) and green plant production (#GRP/100 ANT). This correlation was positive and rather strong (r=0.59) in the screening experiment, but insignificant in the crosses (r=0.29). When green plant production (#GRP/100 ANT) and percent albino plants (%ALB/PL) was compared, the correlation was weak (r=0.39) in the screening experiment, but highly negative (r=-0.72***) in the crosses. This correlation is expected to be highly negative if both albino and green alleles are present. When albino plant production is insignificantly correlated with percent green plant production (r=0.11), this may indicate that albinism is more influenced by the environment and less genetically controlled than green plant production, which is consistent with the general idea that albinism is caused by mutations induced by the in vitro conditions in addition to effects of nuclear genes not mutated during in vitro culture. This is also consistent with the high correlation between number and percent green plants produced (r=0.70***) and the insignificant correlation between number and percent albino plants produced (r=0.23).

Halberg et al. (1990) found a positive genetic correlation (r=0.397**) between ELS formation and percent green plants produced, which led them to conclude that the improved induction of embryogenesis was the main reason for the higher regeneration of green plants. However, this may be explained by their selection of green plant producers, which was not the case in our experiments, where the correlation between regeneration ability and green plant production in cross 1 was the highest (r=0.81**). The best producer of green plants was F_1 donor plant no. 25, which regenerated 117.8 GRP/100 ANT and 86.9% green plants. These figures are at the level of those obtained with high responsive wheat and barley genotypes. The cultivar 'Igri' has been reported to regenerate from 18.6 (Knudsen et al. 1989) to 590 GRP/100 ANT (Hunter 1987) in anther cultures, while a minimum of 10 GRP/ANT has been reported from microspore cultures (Ziauddin et al. 1991).

Our results in particular, and other results on a whole, indicate that the best androgenetic response will be obtained by crossing plants able to complement each other genetically. The application of new genetic markers is a promising tool for detecting and localizing genes or chromosome segments controlling embryogenic ability, total regeneration and green plant production in particular, for selecting suitable breeding materials and for constructing proper crosses. Progress in androgenetic response should also make haploid techniques more attractive in forage grass breeding in the near future.

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