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Genetic improvement of anther culture response in maize: relationships with molecular, Mendelian and agronomic traits

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Abstract Two cycles of androgenetic in vitro doubled haploid (DH) plant production and intermating were implemented in an experimental synthetic population of maize. In vitro traits, including androgenetic embryo production, the regeneration potential and the frequency of spontaneous chromosome doubling, were studied. The success of the regenerated plants to self pollinate was also observed. Impressive genetic progress is reported for all the steps of the androgenetic process including seed set. Differential genetic progress is recorded according to the trait measured. Using a set of Mendelian and molecular markers that mapped to the different maize chromosomes, we were able to characterize the variation in the genetic variability in the population. Progress in the in vitro response was not found to be associated with any noticeable decline in global genetic variability. In addition, the QTL chromosomic regions tested, which were involved in androgenetic response, were not found to be subjected to a strong variation during the breeding experiment. Some phenotypical and morphological traits were also evaluated, and these showed that there was no depreciation effect in the agronomic value of the population. DH plant production and intermating the regenerated plants may be considered for the introduction and use of androgenesis in material which responds poorly.

Key words Anther culture · Maize · Breeding · Molecular markers · Agronomic traits

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Introduction

Anther or microspore in vitro culture provides a rapid method for inducing homozygosity in plants which are of interest for the production of breeding lines. During the last decade, many publications have reported that anther responsiveness and general tissue culture ability are genotype-dependent in numerous species (see review of Henry et al. 1994). Althrough anther culture represents a method by which large numbers of haploid individuals can theoretically be produced, applications of the technique to maize breeding are hampered by the small number of germplasms which present a significant level of response, i.e. the frequencies of androgenetic embryo induction and plant regeneration.

The possibility of breeding for various in vitro traits, such as regeneration, as a means to overcome these genetic limitations has been proposed (Foroughi-Wehr et al. 1982) and demonstrated in different crops, such as maize (Beckert and Qing 1984), alfalfa (Ray and Bingam 1989) and wheat (Landridge et al. 1991) and also for somatic embryogenesis in maize (Rosati et al. 1994). In particular, Petolino et al. (1988) and Barloy et al. (1990) pointed out that doubled haploid (DH) plant production itself may significantly enhance the level of response of the derived material to anther culture. This was closely observed only for the first steps of anther culture response and for a very narrow genetic basis. Althrough the initial induction of androgenesis, through stress pretreatment of the anthers or microspores, is probably under the control of few genes, numerous other genes are expressed during the whole in vitro androgenesis regeneration pathway including plant regeneration. This has been confirmed through intensive molecular analyses of different samples of recombinant lines where major quantitative trait loci (QTL) have been identified for diverse characters on nine out of ten chromosomes of the maize plant

(Cowen et al. 1992; Murigneux et al. 1994; Beaumont et al. 1995).

To date, little is known about the consequences of such genetic improvement through breeding for the in vitro androgenetic response trait on other agronomic or morphological traits. The main purposes of the research described here were therefore (1) to measure, on a wider maize synthetic population, the real genetic progress of anther culture responsiveness, including all the steps to be taken into account for practical breeding, and (2) to estimate the relationships between this in vitro trait improvement and the frequencies of some well-distributed Mendelian and molecular markers and diverse agronomic and morphological traits.

Materials and methods

Plant material and selection methodology

The synthetic population was developed by INRA at Clermont-Ferrand. The eight inbred lines used as parents in this synthetic population were H99, Pa91, Fr16, Oh43d, DH5, DH7, BMS and DBTS. They were chosen either for their known capacity for anther culture response or for their agronomic potential (Petolino and Thompson 1987; Barloy et al. 1989; Genovesi 1990). In particular, Oh43d is an elite agronomic line derived from the Lancaster group. Following a pyramidal cross, the synthetic population was intermated twice using more than 100 unselected male and 100 unselected female plants, at each generation, to constitute the C0 population.

Anthers were plated for more than 350 individual donor plants of this C0 population. A set of 30 dihaploid R0 fertile regenerated plants, obtained after spontaneous chromosome doubling, were intermated twice to derive the C1 population. This was repeated for the C1 population to obtain the C2 population, but for this later step, progenies of easily manually selfed DH were intermated twice. These regenerated dihaploid, obtained from different donor plants, were not selected for any morphological characteristics.

Anther culture procedure

The donor plants were all grown in a greenhouse in the spring. The general procedure, methods and media which promote direct embryogenesis are those reported by Barloy and Beckert (1993). After direct plating of the anthers on the G0 medium the petri dishes were stored at 7°C in the dark for 2 weeks and then placed at 28°C. After another 3-week period, the anthers were transferred onto the E0 medium to promote direct regeneration.

The three populations were re-evaluated together, in spring 1996, after in vitro selection to confirm their intrinsic value as well as to access the direct and indirect response to this selection. To determine genetic progress for tissue culture traits, we tested 71, 75 and 142 individual donor plants of the C0, C1 and C2 populations, respectively. To control environmental variations, we plated samples of approximately 200 anthers derived from a single tassel in two petri dishes for each donor plant. Overall data are indicated in Table 1. Different parameters, related to successive steps of androgenesis, were recorded throughout the process: AC (Anther Culturability) is the ratio of the number of anthers that produced at least one macroscopic androgenetic structure relative to the total number of plated anthers, ELS is the number of macroscopic embryo-like structures relative to the total number of plated anthers, PLA is the number of regenerated plants compared to the total number of plated anthers, F2N is the frequency of spontaneous diploid plantlets after cytometric observations made at the three-leaf stage of the young regenerated plant. These observations were recorded as recently reported in Antoine-Michard and Beckert (1997).

Mendelian and allozyme loci observation

The segregation of Mendelian traits in the experimental populations was analyzed and allele frequencies were determined. The *gl1*, *su1*, *Y1* and *P1* alleles, which control the glossy appearance of young leaves, the sugary and white color phenotype of kernels and the red color of the cob, respectively (Coe and Neuffer 1977), were analyzed. A sample of 1,000 plantlets were germinated, and 200 selfed ears were collected randomly from each population for observation. Some isozyme markers were also scored for another sample of 200 individual plants per population. Starch gel electrophoresis analyses were conducted following the established procedures of Stuber et al. (1988), and the systems were chosen for their polymorphism between the parents. Allelic forms were also determined following the guidelines of Stuber et al. (1988), and using classical control lines. Allelic frequencies were determined by direct counting and standard statistics were computed using the BIOSYS 1 program (Swofford and Selander 1981). The corresponding genes of the isozymes and morphological traits are located on different chomosomes and some are linked to chromosome segments carrying QTL for androgenesis. They are listed in Table 3.

Experimental design and agronomic observations

Different agronomic traits were characterized for 150 S1 families for the C0 and C2 populations, respectively. The entries were tested for 1 year in one location. Classical agricultural practices were followed. The trial was machine-planted with a density of 66,000 plants/ha. The experimental design was a randomized block with two repetitions. Each plot was a 5-m row. Of the 20 plants composing a line 10 were measured for the following traits: plant height (from the ground to flag leaf), ear height (from the ground to the insertion node), total number of leaves and the ear insertion node number. A global evaluation of the line for lodging resistance and number of tillers was made. Both characters were assessed on a scale from 0 to 5. Days to anthesis, yield and grain moisture content at harvest were also recorded. As a supplementary approach, sets of 60 and 137 DH lines derived from the C0 and C1 populations, respectively, were also crossed with the complementary flint line F2 as a top cross. The one-way hybrid structures were evaluated as described previously for dry kernel yield.

General statistical analysis

General analyses of variances were done using SAS (SAS Institute 1991) software. The procedures used were either GLM for parametrical tests or NPARIWAY for non-parametrical tests. The percentage data were transformed with the function "Arcsin sqrt(x)".

Results

The evolution from the C0 to the C2 population is presented in Table 1 for the different in vitro traits evaluated. It is clear that for a consistent number of donor plants as well as a large number of plated

Table 1 Genetic progress for androgenetic response through recurrent selection [*GP* genetic progress (C2/CO)]

	Plants[a]		PLA[b]	AC[c]			ELS[d]			PLT[e]			F2N[g]	Seed set[h]	DH[c]
	NDP	NUP		Mean[f]	RV[f]	Med[f]	Mean	RV	Med	Mean	RV	Med			
C0	71	2	12,248	13.0	0-58	7.9	56	0-364	26	5.0	0-31	2.7	34.4	20.5	0.47
C1	75	0	12,719	29.5	2-81	10.3	147	0-567	125	11.4	1-29	9.2	37.3	23.0	0.98
C2	142	0	24,284	31.5	1-79	14.3	217	0-830	195	22.7	0-66	21.2	40.8	46.6	4.62
GP				2.4			3.9			4.5			1.2	2.3	9.8

[a] NDP, Number of donor plants evaluated; NUP, Number of unresponsive plants
[b] PLA, Number of plated anthers
[c] AC, Anther culturability
[d] ELS, Number of embryo-like structures per 100 plated anthers
[e] PLT, Number of regenerated plantlets for 100 plated anthers
[f] Mean, percentage for 100 plated anthers; RV, range of variation, (plant-to-plant variation); Med, median (plant to plant variation)
[g] F2N, Frequency of spontaneous diploids
[h] Seed set, percentage of regenerated plants giving seeds after selfing
[i] DH%, selfed progeny for 100 plated anthers

anthers the process of intermating some of the regenerated DH plants had a very strong effect on these in vitro traits. The mean percentages of responsive anthers, numbers of embryo-like structures developed or regenerated plantlet production increased by factors of 2.4, 3.9 and 4.5, respectively. More interestingly, the variation ranges of the same traits were also wider in the C1 and C2 populations and the median value also increased significantly, indicating that the results are not only the consequence of a few extremely responsive individual donor plants. The percentage of spontaneous chromosome doubling also increased, but to a lesser extent than the other characters. This significant increase confirms that genes play a role in the expression of this character and suggests that this aspect might be improved using the DH plant production technique.

The next traits concern the selfing success of the diploid regenerated plants as being mainly due to the quality of their development. Limited progress was recorded from the C0 to the C1 population where only R0 plants were intermated, while from the C1 to the C2 population greater genetic progress was observed. This increased success may be the consequence of intermating DH selfed progenies rather than direct regenerated R0 DH plants themselves. The efficiency of the technique, i.e. the number of DH selfed progenies obtained per 100 plated anthers, is a global genetic progress close to a factor 10. The heritability of the AC variable measured as a ratio of genetic against phenotypic variance changed very little from the C0 ($h^2 = 0.85$) to the C2 ($h^2 = 0.84$). The genetic progress for in vitro traits did not seem to be associated with a reduction in its genetic variation within the C2 population.

Table 2 reports the variation in the correlation coefficients between the in vitro traits from the C0 to the C2 population. There is a marked decrease in the strong and positive correlation between the AC and ELS traits as well as between AC and the percentage of regenerated plantlets (PLT). A similar variation pattern between ELS and PLT was observed, but to a lesser extent. A comparison of the regression slopes for the level of production of regenerated plantlets with the percentage of responsive anther did not reveal any statistical differences (data not shown).

Table 2 Variation in the correlations between the in vitro traits

Traits[a]	Populations	AC	ELS	PLT
AC	C0	1	0.94	0.82
	C2	1	0.65	0.65
ELS	C0	–	1	0.89
	C2	–	1	0.82
PLT	C0	–	–	1
	C2	–	–	1

[a] See Table 1 for abbreviations

Table 3 reports the expected frequency of allelic composition and its variation for some Mendelian and molecular markers characterized in the populations. We recorded a slight but clear deficit in heterozygote frequency for all the markers. This may be the consequence of a non-panmictic mating system in the constitution of the populations. However, there were no statistical differences between the expected and observed frequencies of these alleles. The first 4 alleles of column 1 corresponded to natural mutations. The frequency of the *glossy 1* allele of the DH7 line was not modified through the process, although DH7 was one of the parents showing the highest response in the population and the corresponding gene is localized close to a QTL for androgenesis described in this line. The *su1* allele, introduced with the BMS parental line, appeared to decrease significantly. This could be related to the non-neutrality of the trait conferring very poor maturation of kernels and germination efficiency.

Table 3 Variation in Mendelian marker frequencies between populations

Marker allele		Chromosome	Parent	Expected frequency	Populations			C0/C2[a] evolution		QTL authors[b]	
					C0	C1	C2				
Glossy 1	1	7	DH7	0.13	0.11	0.11	0.09	–	ns	Yes	b, c
Su 1	1	4	BMS	0.13	0.09	-na	0.02	Decrease	**	Yes	b
Y 1	1	6	DH7, BMS	0.25	0.18	-na	0.18	–	ns	no	–
P 1	1	1	DH5, Pa91, DBTS	0.38	0.48	-na	0.44	–	ns	Yes	a, b, c, d
Dia1	8	2	DH5, DH7, Pa91, H99, DBTS	0.63	0.55	0.69	0.75	Increase	**	Yes	a, b, c, d
Mdh 2	6	6	Oh43d, DBTS, BMS	0.37	0.33	0.36	0.38	–	ns	No	–
Idh 2	4	6	Fr16, DBTS, Pa91	0.37	0.45	0.44	0.58	Increase	*	No	–
Mdh1	1	8	DH5, Fr16	0.25	0.34	0.29	0.32	–	ns	Yes	b, c
Acp1	4	9	Fr16, Pa91, Oh43d, BMS	0.50	0.42	0.39	0.39	–	ns	Yes	a, b
Pgd1	3.8	6	DH5, DH7, H99, BMS	0.50	0.46	0.42	0.38	Decrease	*	No	–
Pgm2	4	5	DH7, H99, Fr16, DBTS, Oh43d, Pa91	0.75	0.79	0.75	0.78	–	ns	Yes	b, c
Pgm2	8	5	DH5	0.13	0.16	0.23	0.19	–	ns	Yes	b, c

[a] C0/C2 variation: decrease or increase: * significant at 5% level, ** significant at 1% level; ns, no statistical difference between C0 and C2
[b] Authors: a, Cowen et al. (1992), b, Murigneux et al. (1993), c, Beaumont et al. (1996), d, Armstrong et al. (1992)

Table 4 Evaluation of the variation for some agronomic traits

Characters	Mean value of Populations		C.V. trial	Statistical comparison		
	C0	C2		Median	Mean	Dispersion
Days to anthesis	96.40	95.41	6%	S	S	NS
Number of leaves	19.30	18.80	5%	NS	NS	NS
Height of plant	163.91	152.81	6%	S	S	NS
Number of ear node	12.90	12.80	7%	NS	NS	NS
Relative height of ear	0.49	0.51	11%	NS	NS	NS
Lodging resistance	0.52	0.52	10%	NS	NS	NS
Number of tillers	1.38	1.97	7%	S	S	S
Grain yield (S1 families)	37.00	36.00	29%	NS	NS	NS
Grain yield (DH × Testor)	60.00	58.00 (C1)	17%	NS	NS	NS

[a] S, Significant at 5% level; NS, not significant

The frequencies of the other two characters did not vary according to the population, although the *P1* allele was carried by highly androgenetic responding lines and was also located in an androgenetic QTL domain.

For the isozyme alleles, we recorded slight variations of frequency within the breeding process depending on the marker gene considered. For instance, in the Diaphorase system, the allele 8 obtained from the DH5 and DH7 highly responsive inbred lines, and other parents, expanded through the recurrent cycles. The corresponding gene map in a QTL region for androgenesis was described by Murigneux et al. (1993). The *Pgd1* system was not located in or close to a QTL region and we recorded a decrease in the frequency of the allele *3.8*, although this allele also came from the DH5 and DH7 lines.

Table 4 reports the general comparison of the populations for the agronomic traits evaluated. The median, mean and dispersion of the different families were evaluated for each trait. Some traits seemed to be influenced by the breeding process for their median and mean but to a lesser extent. Tillering increased, the height of the plant was reduced slightly and the earliness and lodging resistance of the C2 population were increased. A comparison of the dispersion of agronomic traits, through non-parametrical tests, did not reveal any strong variation.

Discussion and conclusion

Our results clearly demonstrate that for a significant number of the different donor plants tested and anthers plated DH plant regeneration and intermating increased the global level of response for in vitro androgenetic plant production in maize. This response was first demonstrated by Petolino et al. (1988), but only for androgenetic embryo production and for

a narrow genetic basis. We established here that embryo production and regeneration potentials are affected. After having observed that the spontaneous chromosome-doubling frequency also had a genetic basis (Antoine Michard and Beckert 1997) we demonstrated that it is also improved by regeneration but to a lesser extent. The final step of the process, i.e. the easiness of seed recovery from the selfed DH regenerated plant, also seems to be improved. This improvement is characterized by a global enhancement of response affecting a large proportion of the plants, and the genetic variation of the populations, for the in vitro characters, did not decline.

Different and independent genetic control of the induction and regeneration phases of androgenesis was postulated some years ago (Henry and De Buyser 1985). By means of different genetic tools, diverse chromosomes or chromosomes segments have been shown to be genetically involved in various crop species. The decline of correlation coefficients within the in vitro traits through the selection procedure is statistically significant and might be in relation to the manipulation of these different genetic systems. However, if we want to screen donor plants for a higher level of androgenetic response, in practice, the percentage of responsive anthers and the number of embryo like-structures produced per anther sample are excellent predictors of final production in regenerated plantlets.

Identical observations were recently made by Rosati et al. (1994) for somatic embryogenesis in maize, where recurrent selection has proved to be very efficient for the genetic improvement of a specific character in another population. Surprisingly, they also recorded that the genetic variability did not decline, but even increased for the total number of shoots regenerated per callus. They hypothesized the possible role of somaclonal variation for the creation of specific new alleles. In our case, the regeneration of specific gametes from a very large initial population may select particular and extremely rare recombinant events. This kind of gametic selection may have a very strong efficiency, but part of the genetic control of the androgenetic induction is probably at the sporophytic level through the evolution of the cells of the anther wall and the selection was not made only at this gamete level (Murigneux et al. 1993).

The most interesting result is that this high level of regeneration improvement was not related to a global decrease in the genetic variability of the population. This variation was also not related to any strong depreciation of the agronomic value. Tillering was the only character that was clearly influenced. We do not have any clear explanation for this modification and the slight reduction in the height of the plants. This has to be confirmed through other experiments on unrelated material. The number and genetic localization of molecular markers which have been observed in our work do not represent all genomic regions. We cannot exclude that other chromosome regions might be involved in a stronger reduction of genetic variability. In a very narrow genetic basis, androgenetic fitness plays a major role in the observation of markers with segregation distortion, as has been reported in numerous studies including both high and poor responding parents (Murigneux et al. 1993; Foisset and Delourme 1996). The manipulation of larger genetic populations might reduce the magnitude of the consequences of differential anther culturability. The lack of genetic variability decline was also partly related to crossing DHs derived from different donor plants, even if the DH were selected from a very large gamete population within each donor plant. These general observations confirm the use of in vitro DH intermating for increasing androgenetic response levels without altering agronomic value, before the final in vitro production of elite agronomical DH lines.

Other synthetic populations have also been tested following the same general protocol. We recorded the same level of improvement in androgenetic response, and agronomical observations are underway.

The donor or regenerated plants have never been selected for their own induction reactivity level or their androgenetic embryo regeneration capacity. We certainly were able to observe a greater genetic progress for in vitro traits with a direct selection on this traits and by intermating higher responding DH plants derived from different germplasms. This direct selection will probably derive more reactive material of interest for the identification of genes expressed during androgenesis induction or for diverse biotechnical applications.

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References

Antoine Michard S, Beckert M (1997) Spontaneous versus colchicine induced chromosome doubling in maize anther culture. Plant Cell. Tissue Organ Cult 48 : 203–207

Armstrong CL, Romro-Severson J, Hodges TK (1992) Improved tissue culture response of an elite inbred through backcross breeding, and identification of chromosomal region important for regeneration byRFLP analysis. Theor Appl Genet 84 : 755–762

Barloy D, Beckert M (1993) Improvement of regeneration ability of androgenetic embryos by early anther transfer in maize. Plant Cell Tissue Organ Cult 33 : 45–50

Barloy D, Denis L, Beckert M (1989) Comparison of the aptitude for anther culture in some androgenetic doubled haploid maize lines. Maydica 34 : 303–308

Beaumont VH, Rocheford TR, Widholm JM (1995) Mapping the anther culture response genes in maize (*Zea mays* L.). Genome 38 : 968–975

Beckert M, Qing CM (1984) Result of a diallele trial and a breeding experiment for in vitro aptitude in maize. Theor Appl Genet 68 : 247–251

Coe EH, Neuffer MG (1977) The genetics of corn. In: Sprague GF (ed) Corn and corn improvement. American Society of Agronomy, Madison, pp 111–210

Cowen NM, Johnson CD, Armstrong K, Miller M, Woodsley A, Pescitelli S, Skokut M, Belmar S, Petolino JF (1992) Mapping genes conditioning in vitro androgenesis in maize using RFLP analysis. Theor Appl Genet 84 : 720–724

Foisset N, Delourme R (1996) Segregation distortion in androgenetic plants. In: Jain SM, Sopory SK, Veilleux RE (eds) In vitro haploid production in higher plants, vol 2. Kluwer Academic Publ, Dordrecht, pp 189–201

Foroughi-Wehr B, Freidt W, Wenzel G (1982) On the genetic improvement of androgenetic Haploid Formation in Hordeum vulgare L. Theor Appl Genet 62 : 233–239

Genovesi AD (1990) Maize: In vitro production of haploids. In: Bajaj YPS (ed) Biotechnology in agriculture and forestry vol 12: haploids in crop improvement. Springer, Berlin Heidelberg New York, pp 176–202

Henry Y, De Buyser J (1985) Effect of the 1B/1R translocation on anther culture ability in wheat (*Triticum aestivum*). Plant Cell Reports 4 : 307–310

Henry Y, Vain P, De Buyser J (1994) Genetic analysis of in vitro plant tissue culture responses and regeneration and capacity. Euphytica 79 : 45–58

Langridge P, Lazzeri P, Lörz H (1991) A segment of rye chromosome 1 enhances growth and embryogenesis of calli derived from immature embryos of wheat. Plant Cell Rep 10 : 148–151

Murigneux A, Baud S, Beckert M (1993) Molecular and agromorphological evaluation of doubled haploid lines in maize. 2. Comparison with single seed descent lines. Theor Appl Genet 87 : 278–287

Murigneux A, Bentolila S, Hardy T, Baud S, Guitton C, Jullien H, Ben Tahar S, Freyssinet G, Beckert M (1994) Genotypic variation of quantitative trait loci controlling in vitro androgenesis in maize. Genome 37 : 970–976

Petolino JF, Thompson SA (1987) Genetic analysis of anther culture response in maize. Theor Appl Genet 74 : 284–286

Petolino JF, Jones AM, Thompson SA (1988) Selection for increased anther culture response in maize. Theor Appl Genet 76 : 157–159

Ray IM, Bingham ET (1989) Breeding diploid alfalfa for regeneration from tissue culture. Crop Sci 29 : 1545–1548

Rosati C, Landi P, Tuberosa R (1994) Recurrent selection for regeneration capacity from immature embryo-derived calli in maize. Crop Sci 34 : 343–347

SAS Institut (1991) SAS/STAT R user's guide: release 6.03. SAS Institut, Cary, N.C.

Stuber CW, Wendel JF, Goodman MM, Smith JSC (1988) Techniques and scoring procedures for starch gel electroporesis of enzymes from maize (*Zea mays* L.) North Carolina Agricultural Research Service, North Carolina State University, Raleigh

Swofford DL, Saalander RB (1981) BIOSYS 1: a Fortran program for the comprehensive analysis of electrophoretic data in population genetic and systematic. J Hered 72 : 81–83