Original article

Haplodiploidization of maize (*Zea mays* L) through induced gynogenesis assisted by glossy markers and its use in breeding

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Summary — Reliable detection of haploids at the seedling stage is a prerequisite for the use of haplodiploidization through in situ gynogenesis in maize breeding. The use of recessive alleles of glossy genes as markers makes this detection possible in various materials. Two synthetics with a wide genetic basis: flint *glossy1* CGT and dent *glossy6* DGT were studied. After pollination with the FIGH 1 inducer, they produced haploid seedlings at a rate of 0.64 and 0.93%, respectively. The rate is increased (0.94% on average) for the CGT (C_0) synthetic after selfing, in S_1 progenies. The distribution of 203 S_1 families showed a significant deviation in comparison with a Poisson distribution. The DGT synthetic showed an increase in induction rate when a new synthetic was formed with doubled haploid (DH) lines, suggesting that 'inductibility' may have some genetic effects. No spontaneous doubling was shown by the haploid seedlings of either synthetic. Colchicine treatment allowed a recovery of male fertility in 30 to 60% of detected haploids according to the experiments. The progeny recovery rate was influenced by the genetic basis, as the DGT synthetic gave better results than the CGT synthetic. The growing period also had an influence upon the recovery of fertile ears. Hybrids produced from heterotic DH lines showed an agronomic potential similar to standard hybrids. It is concluded that in situ gynogenesis assisted by the use of glossy markers can now be used in maize breeding.

maize breeding / in situ haploids / diploidization / colchicine / glossy

Résumé — Haplodiploïdisation du maïs (*Zea mays* L) par gynogenèse induite assistée par marqueurs glossy et son application en sélection. L'utilisation effective de l'haplodiploïdisation par gynogenèse in situ en sélection du maïs nécessite une détection fiable des haploïdes au stade plantule. Le marquage génotypique par des allèles récessifs des gènes glossy permet cette détection. Deux « synthétiques » à base large : CGT (cornée glossy1 tardive), DGT (dentée glossy6 tardive), ont été étudiées. Pollinisées par l'inducteur FIGH 1, elles ont produit des plantes haploïdes à des taux respectifs de : 0,64 et 0,93 %. Pour la synthétique CGT (C_0), ce taux est augmenté (0,94 % en moyenne) lorsque l'on passe de la première génération aux descendances S_1. L'analyse de la distribution de l'induction de 203 S_1 de CGT montre une déviation significative par rapport à une distribution de Poisson. Dans la population DGT, le taux d'induction de plantes haploïdes s'est accru par la constitution d'une « synthétique » à partir de lignées haploïdes doublées (HD). Cela suggère des effets génétiques pour « l'inductibilité ». Les plantules haploïdes provenant de CGT et DGT n'ont pas présenté de diploïdisation spontanée. Le traitement à la colchicine permet la restauration de la fertilité mâle dans 30 à 60 % des cas selon les essais. Le taux de descendances diploïdes fertiles obtenues par rapport au

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nombre d'haploïdes traités varie en fonction du matériel génétique (DGT est supérieure à CGT) et des périodes de culture des plantes traitées colchicine. Des hybrides réalisés à partir de lignées HD complémentaires possèdent un potentiel agronomique intéressant, parfois proche des témoins. La gynogenèse in situ assistée par marqueurs glossy est envisageable en sélection.

maïs / haploïdes in situ / diploïdisation / colchicine / glossy

INTRODUCTION

We have concentrated our studies on the examination of two techniques of haplodiploidization: use of maternal haploids issued from in situ gynogenesis and in vitro androgenesis. Anther culture can produce high numbers of haploids from particular genotypes, and genotypic effects (QTL) have been demonstrated (Murigneux et al, 1994). Androgenetic haploids are often characterized by a high rate of spontaneous chromosome doubling. Spontaneous maternal haploids were used with some difficulty owing to the low rate of occurrence (0.1%) (Chase, 1952, 1974). The discovery of the high inducing line Stock 6 (Coe, 1956) and the availability of the improved inducer WS 14 (Lashermes and Beckert, 1988) offer very attractive prospects for the use of in situ gynogenesis independent of genotype.

There are two main reasons why the use of maternal haploids is restricted: i) spontaneous chromosome doubling is rare (Chase, 1952; Zabirova et al, 1993) and its induction by colchicine treatments is relatively inefficient (Khvatova, 1976; Botez and Panfil, 1979); ii) haploid detection is difficult. Different marker genes were tested. After pollination with the high haploid inducer carrying the *ACR* dominant gene complex for embryo pigmentation, haploids were identified from non-colored embryos in the female parent. However, the level of anthocyanin expression can be negatively influenced by a poor maturation process, the presence of the dominant inhibitory *C1-I* gene (Chumak, 1979) or the masking effect of strongly colored pericarps due to some of the alleles of the *P* locus. No known dominant marker combines all the requirements that would allow perfect detection at the seedling stage, ie, independent expression of the background in heterozygous plants, no lethality at the homozygous state in the background of the haploid inducer strain, early expression during the vegetative phases required for the compulsory colchicine treatment, and easy and cheap screening. It has been suggested (Lashermes and Beckert, 1988; Beckert, 1994) that a heterologous gene with a strong expression in plants might be introduced into maize by genetic transformation.

The use of a dominant herbicide resistant marker from genetic transformation was presented by Geiger et al (1994). This procedure still presents difficulties in everyday application since the haploid seedlings are susceptible to the herbicide.

In the absence of a useful dominant gene in maize, we propose an alternative procedure for breeding programs by using recessive markers in the material subjected to haploid production. Two of the appropriate genes have already been tested: i) the liguleless genes (Emerson et al, 1935) suppress the ligule and the blade junction making the leaves erect, and ii) the glossy genes (Hayes and Brewbaker, 1928; Bianchi and Marchesi, 1960), which suppress the epicuticular waxes. The leaves become shiny and water adheres to them. Nevertheless, the recessive allele of the liguleless genes presents many disadvantages in mature plants linked to the erect leaves: yield decrease due to the reduction of photosynthetic leaf area, poor weed control, root lodging susceptibility and difficult detasseling. The use of the recessive alleles of the glossy genes (*gl1*, *gl2*, *gl6*) expressed at the seedling stage is more valuable. Their use was limited to genetic studies of production or detection of haploids (Coe, 1961; Rhodes and Green, 1979; Pollacsek, 1992).

The objectives of our study were to investigate: (i) the usefulness of glossy markers for haploid detection; (ii) their influence on haplodiploidization parameters (inductibility, in relation to the female parent, the potential of induction related to the pollinator, detection, spontaneous or artificial diploidization); (iii) the agronomic value of the breeding material and the haplodiploidized lines (DH lines) first produced.

MATERIAL AND METHODS

Plant material

Induced material

This material originates from recurrent selection programs maintained at the Inra Plant Breeding Station in Clermont-Ferrand and consists of:

—i) a flint glossy late broad base synthetic (CGT), produced by intermating 20 S_8 families from a genetic pool including 25 flint European open pollinated varieties crossed with an inbred line, which was crossed successively with a mix of 27 European flint inbred lines, then with the F 492 × F 712 flint single cross. The recessive allele of the glossy 1 gene (*gl1*) in homozygous state is from the Inra inbred lines F 160, F161 and F564. The bulk of the S_0 plants and each S_1 family were pollinated by the haploid inducer. The induction rate was calculated on 900 kernels per family;

—ii) a dent glossy late broad base synthetic (DGT) formed with 28 S_8 families proceeding from selection within representative elementary synthetics of the main North-American genetic groups ('Iodent', 'Minnesota', 'Stiff Stalk Synthetic') crossed with isogenic glossy (*gl6*) inbred lines introduced in the initial synthetics. The bulk of S_0 plants corresponded to the C_0 cycle level (DGT C_0). From the intermating of 48 DH lines from the DGT C_0, we obtained a new synthetic DGT C_1^{HD}.

Plant material used in glossy versus non-glossy comparisons

We compared:

—i) the *gl1* CGT and *gl6* DGT synthetics described above;

—ii) the 'non-glossy' versions of CGT and DGT, represented by all the S_8 lines introduced in the CGT and DGT formation;

—iii) a set of 145 hybrids made from DH lines according to a factorial design between 29 dent lines from DGT C_0 as female parents and five flint lines from CGT as male parents.

Haploid inducer

The inducing strain FIGH 1 (French inducer gynogenetic haploids 1) derived from the WS 14 inducer (Lashermes and Beckert, 1988) was used. It was developed and maintained from a S_2 family by full sibs regarding high pollen shedding and a good seed set production. The *ACR* gene complex initially present in the line and referred to as Stock 6 was inherited.

Haplodiploidization

Gynogenetic induction

The breeding nurseries were located in normal 'Limagne' field conditions. The plants were detasseled in order to avoid contamination by casual selfing during manual crossing operations. They were then pollinated with FIGH 1 within a time-limit of 3 days after silking.

Detection of haploid plants and ploidy control

At sowing time, it is possible to check whether the grains originate from a cross with FIGH 1 by the presence of the aleurone pigmentation due to the xenia effect, except when the inhibitory *C1-I* allele is present in female parents. This color can also be absent in a few cases of heterofertilization with two pollen grains (Sprague, 1929). Seedling screening was carried out in plastic multi-cell (96) plaques, Ø 35 mm (Puteaux, Versailles, France) and after that were sown two to three kernels per cell in a vermiculite substrate. Glossy seedlings were visually recognized when the first ligulate leaf appeared. A spray was used to test the adherence of water to the leaves. The evaluation of initial spontaneous diploidization was carried out with a flow cytometer apparatus Partec II CA GmBH, (De Laat et al, 1987).

Artificial chromosome doubling

Before the colchicine treatment, the glossy seedlings were grouped into homogeneous sets and placed individually in multi-cell (35) plaques (Ø 45 mm). The vermiculite substrate received a nutrient solution twice a week. Bunches of 10 to 25 seedlings at the three ligulate leaf stage were immersed from the bottom to the middle of the first leaf sheath in an aqueous colchicine solution of 1.5 g/l^{-1} (SIGMA ref 9754) for 3 h at room temperature.

Plant growth after colchicine treatment

The seedlings were transferred into small pots filled with a mixture of peat and vegetable mold (1:1) before regrowth. Viable plantlets were placed in 12-L pots with a pouzzolane substrate plus peat, receiving the nutrient solution or in a plastic greenhouse. The pollen shedding plants were selfed daily, until exhaustion of pollen.

Field experiments

The synthetics DGT and CGT with their non-glossy counterparts were compared at Clermont-Ferrand in 1989 and 1990 for the conventional material and in 1995 for the new synthetics. The visual comparison using a box plot representation (Tukey, 1977) was performed with the S-Plus User's Guide, 1993. The 145 hybrids obtained by crossing DH lines were compared in a multilocal network with nine of the best performing commercial hybrids. The experimental design was a randomized complete block with replications carried out at the Inra Plant Breeding Stations at Clermont-Ferrand, Lusignan and Mons en Chaussée for grain yield evaluation.

Table I. Comparison of inductibility for the dent (DGT) and flint (CGT) synthetics.

Synthetics	DGT C_0	CGT C_0	CGT S_1
Number of seeds sown	16 100	49 500	241 600
Number of haploids detected	149	318	2284
Percentage of haploids	0.93	0.64	0.94

χ^2_1 DGT C_0 / CGT C_0 = 14.7 (P = 0.011)
χ^2_1 CGT C_0 / CGT S_1 = 38 (P = 0.011)

RESULTS AND DISCUSSION

Induction and inductibility

During the year of manipulation, the significantly different rates of induction by FIGH 1 ranging from CGT C_0 (0.64%) to DGT (0.93%) showed a variation in 'inductibility' according to the genetic material (table I).

The significant increase in the induction rate for the group of S_1 families (0.94%) in comparison with the C_0 level (0.64%) for the CGT synthetics could be explained by an inbreeding effect. This has already been suggested by Seaney (1955) and Lashermes (1987). Lashermes and Beckert (1988) found a 2.1% minimum induction rate up to 4.6% in various single crosses in the material. Such a discrepancy may be due to the difference in genetic basis or a loss of modifier genes during the fixation process from WS 14 (level S_2 during the tests) to FIGH 1. A year effect (Lashermes, 1987) might also offer another explanation.

The low occurrence of haploids may be analyzed by taking a Poisson's distribution curve as a limit of the binomial distribution as a model. The distribution of the CGT S_1 families does not fit this model well where n = 203 (χ^2 = 88.7; P < 0.001). The frequency of families with a low and high rate induction is higher than predicted (fig 1). This phenomenon suggests a genetic effect that can only be confirmed in the S_2 generation.

As shown in table II, the DGT induction rate is significantly increased between the C_0 level (0.93%) and the C_1 ^{HD} (1.31%) level. This feature tended to demonstrate the positive effect of the cycle of haplodiploidization. These results are in agreement with those of Chase (1952, 1974) and
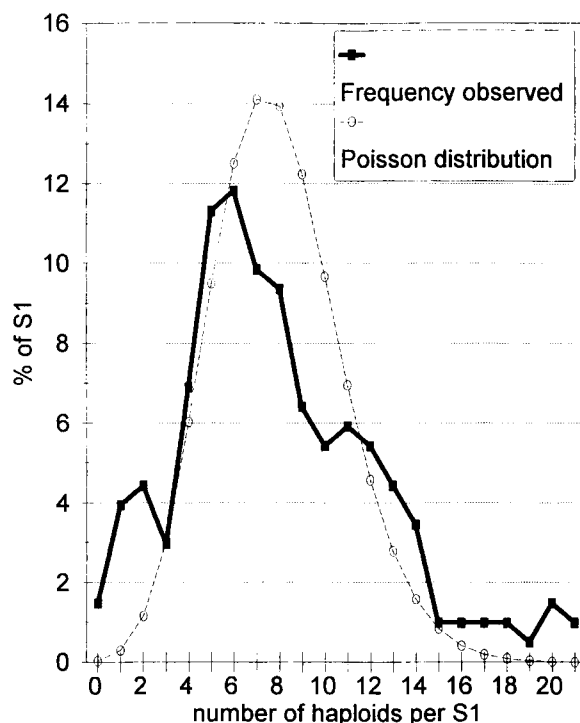
Fig 1. Measured percentage polygon of haploids produced within 203 S_1 CGT families compared to a Poisson distribution of the same mean. The S_1 were grouped according to their ability to produce haploids, ie, six haploids were produced by 11.8% of the 203 S_1 families.

Table II. Comparison of haploid obtained from the original synthetic DGT C_0 and the synthetic DGT C_1^{HD}.

Synthetics	DGT C_0	DGT C_1^{HD}
Number of seeds sown	16 100	25 800
Number of haploid seedlings obtained	149	339
Percentage of haploids	0.93	1.31

χ^2_1 = 13.0 (P = 0.04)

Khoklov et al (1975). Conversely, Khvatova (1976) did not find a significant increase in the haploid plants produced after a first haplodiploidization cycle.

Chromosome doubling and progeny recovery

Chromosome doubling was judged by the tassel pollen shed. In the absence of colchicine treatment for the two synthetics (table III) no spontaneous chromosome doubling was observed, which is correlated with the lack of pollen pro-

Table III. Effect of the colchicine treatment and production progenies.

		No treatment		Colchicine treatment			
		$DGT\ C_0$	$CGT\ C_0$	$DGTC_0$		$CGT\ C_0$	$CGT\ S_1$
		1994	1994	1994	1995	1994	1995
Haploid seedlings	(a)	76*	172*	74*	63*	146*	2284
Surviving plants			66	57	125	1966	
Lethality (%)			10.8	9.5	4.4	13.9	
Plants shedding pollen	(b)	0	0	40	39	68	661
Chrom. doubling (%)	(b/a)	0	0	54.1	61.9	46.6	28.9
Progenies	(c)	0	0	19	26	0**	70
Progenies (%)	(c/a)	0	0	25.7	41.3	0	3.1

* Flow cytometry measurements ; ** post flowering damage (overheated greenhouse).

duction. Sometimes filiform anthers were present, but these never caused pollen to be shed. This colchicine treatment was compulsory. Over the whole experiment a rate of 4.4 to 13.9% lethality followed the colchicine treatment, yet 28.9 to 61.9% of tassels were fertile. The restoration of male fertility reached at least 50% for the DGT synthetic. The lowest percentage shown by the CGT synthetic could be attributed to the effect of stress during the growth period. The CGT syn-

Fig 2. S_1 synthetic CGT. Effect of growth period from detected haploids until progeny were obtained; 44 000 kernels were sown for the January and February sets, 66 000 for the March and April sowing dates.

thetic showed many cases of protandry and also ears without silking. Progeny were produced by 0.8 to 8.1% (fig 2) of the haploids detected during the growth period. Sowing at the beginning of spring was less detrimental for the pre- and post-floral stages. Even at diploid level, the majority of European synthetics showed some difficulty in selfing when inbreeding increased. During 1995, 15 S_5 families out of 208 were unselfable. On the other hand, the DGT synthetic was more manageable and according to the year, 25.7 to 41.3% of detected haploids produced progeny following colchicine treatment. Chase (1952) reported obtaining progeny with 10% of putative haploids (not methodologically checked); this may be due to a specific ability for spontaneous chromosome doubling in that material. Without checking the initial ploidy level, Zabirova et al (1993) also reported the same feature. These authors had a 33% success rate in a specific genotype evaluated by the plant frequency that could be selfed. This trait was heritable according to Shatskaia et al (1994).
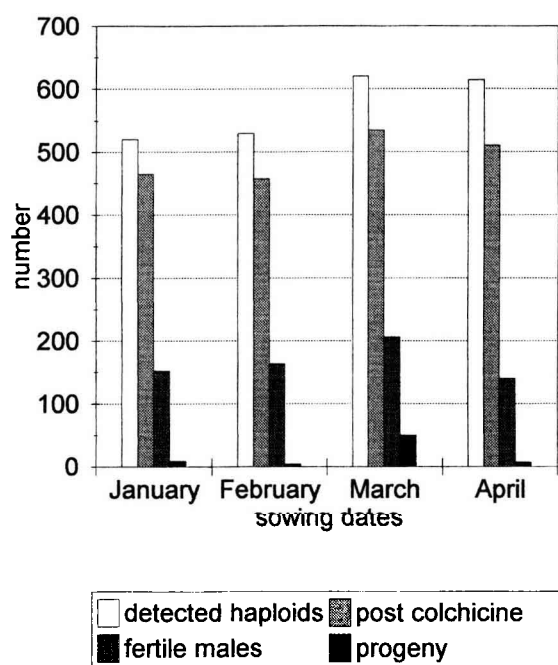
Effect of glossy utilization

On the same genetic basis, it can be seen (fig 3) that the distributions of CGT and DGT glossy synthetics were similar to the series of normal families as far as yield was concerned. The use of glossy alleles for haploid recognition was not detrimental to the agronomic value of the experimental genetic material. Many DH × DH hybrids reached the yield of recent commercial hybrids belonging to the French A, B, C and E groups of
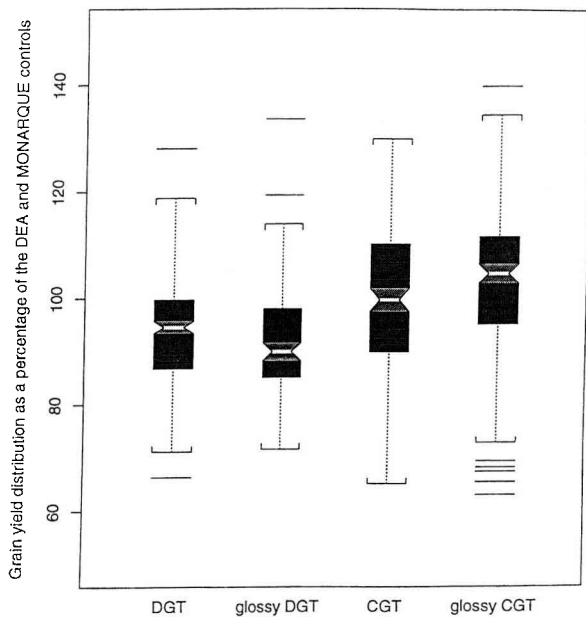
Fig 3. Box plot design for the yield distribution of the normal synthetics and their glossy counterparts. The center line through each box represents the median of the data. The dark shaded regions represent the 'interquartile distance', which is the difference between the third quartile of the data and the first quartile. The whiskers (the dotted lines extending from the top and bottom of the box) extend to the extreme values of the data. The horizontal lines outside of the whiskers indicate possible deviant data points. The dotted area gives the confidence limits of the median.
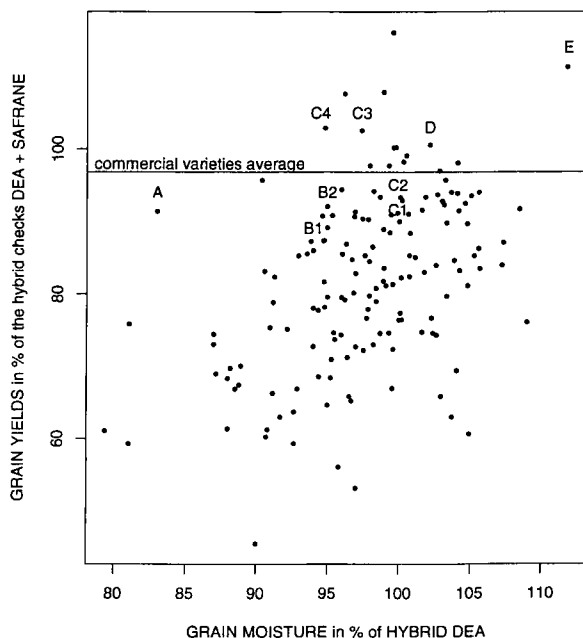
earliness classification and 10% of them equaled or exceeded the standard average (fig 4). This preliminary study showed that the genetic material is agronomically valuable, whether the glossy allele is present or not. If different glossy alleles are assigned to different heterotic groups, ie, *gl1* for the flint group or *gl6* for the dent group, the resulting hybrids become 'non-glossy'. In the case of the appearance of a negative effect unknown until now, ie, susceptibility to a fungus in wet conditions, the trouble would be restricted to the basic seeds.

In conclusion, in spite of the absence of a dominant marker placed in the inducing strain, detection can be solved with the use of glossy recessive alleles. Currently, *gl2*, *gl3* and *gl5* offer other opportunities for marking different genetic groups. The DH lines produced by this procedure can be directly subjected to a process of conversion for cytoplasmic male sterility by the use of in situ androgenesis (Chase, 1963), a system that also requires marker assistance. Management of the agronomic conditions of the plants after treatment appears to be an area needing improvement. Inducing potential needs to be increased, but henceforth the present data allow a practical use in breeding schemes.

Fig 4. Relationship between grain yield (percentage of the hybrid checks DEA and SAFRANE) and grain moisture (percentage of the hybrid check DEA) for the dent DH lines × flint DH lines hybrids. Some commercial hybrids belonging to the French earliness groups (A, B, C, D, E) are included (A = cv GRANAT; B1 = cv DK 250; B2 = cv MAGISTER; C1 = cv ADONIS; C2 = cv DEA; C3 = cv DK 300; C4 = cv SAFRANE; D = cv FURIO; E = cv ETALON).

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