Doubled Haploids

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Abstract Doubled haploid (DH) maize lines can be produced by *in vitro* and *in vivo* techniques. The *in vitro* approach is focused on anther and microspore culture. However, most maize genotypes proved to be highly recalcitrant. Genetic analyses showed that *in vitro* androgenetic response is under complex multifactorial control. Despite good results with specific genotypes, the technique has not yet become a routine tool in maize research or breeding. In contrast, *in vivo* procedures could be improved considerably and have been widely applied during the last 10 – 15 years. *In vivo* induction of paternal haploids based on the 'indeterminate gametophyte' mutant has become a standard technique for transferring elite seed parent lines into cytoplasms that condition male sterility. Similarly, the induction of maternal haploids by pollination with specific inducer genotypes has become a routine procedure for large-scale DH line production. Both *in vivo* techniques are much less affected by donor genotypes than the *in vitro* procedures.

Progress achieved in the induction of maternal haploids pertains to the induction rate, easily screenable markers for haploid identification, chromosome doubling procedures, and handling of seedlings which survived chromosome doubling. In research, DH lines are mainly being used for mapping purposes and in breeding they are progressively replacing conventional inbred lines. Various DH line-based breeding schemes have been suggested, and computer software has been developed for optimizing the dimensioning of the schemes and for determining the relative merits of alternative breeding strategies. DH lines feature important advantages regarding quantitative genetic, operational, logistic, and economic aspects. The DH-line technology can therefore be considered as one of the most effective tools in modern maize genetics and breeding. The mechanism of *in vivo* induction of gynogenetic haploids is not yet fully understood. Most likely, one of the two inducer sperm cells is not fully functional yet fuses with the egg cell. During subsequent cell divisions,

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a degeneration process starts and the chromosomes get fragmented and finally are eliminated from the primordial cells leaving only maternal chromosomes. The second sperm cell fuses with the central cell leading to a regular triploid endosperm and a normal-sized functional seed.

1 Introduction

The first haploid maize plant was described by Stadler and Randolph (1929, cited by Randolph 1932). About two decades later, Chase (1947) found haploids at low frequency (about 1 in 1000) in various US Corn-Belt materials. He recognized the great potential of haploids for the genetics and breeding of maize and consequently devoted all of his professional career to promote research and development in this field. Further milestones were the detection of colchicine as a chromosome doubling agent, the successful application of *in vitro* anther and microspore culture techniques (Section 2), and the detection of specific genotypes suited for *in vivo* haploid induction (Section 3). Various studies have shown that both *in vitro* and *in vivo* haploid induction are polygenically controlled characters and QTL (quantitative trait loci) analysis have identified genomic regions affecting haploid induction on almost all chromosomes (Section 4). Today, doubled haploids are widely applied in many fields of maize research and are worldwide used in commercial hybrid maize breeding (Section 5). In research, doubled haploid (DH) genotypes are a valuable tool in structural and functional genomics, proteomics, metabolomics, marker-trait association studies, marker-based gene introgressions, molecular cytogenetics, genetic engineering, etc. In breeding, DH lines allow an increase in the efficacy of selection, reduction in the length of a breeding cycle, simplification of the logistics, and saving time in commercializing a new breeding product.

2 *In vitro* Techniques

Encouraged by the successful application of *in vitro* culture techniques for the production of haploids in many crop plants (Kasha 1972), researchers in many countries tried to establish this approach in maize as well (for reviews see Büter 1997; Pret'ová et al. 2006). However, maize turned out to be an extremely recalcitrant plant species, with only a very few genotypes displaying satisfactory *in vitro* embryogenesis. Petolino and Thompson (1987) analyzed anther culture response in diallele crosses between four US Corn-Belt lines (H99, LH38, Pa91, FR16) known to have haploid regeneration aptitude. Significant differences between the six crosses were observed, ranging from 0.0 to 6.3% anther response (percentage of plated anthers displaying embryo-like structures). Both general and specific combining ability effects were significant. This clearly demonstrated the heritable nature of anther culture responsiveness. The findings were corroborated by Spitkó et al.

(2006) who studied the F_1 generation of a cross between a responsive Chinese line and a recalcitrant Hungarian ('Iodent') line, the first backcross (BC_1) of this cross to the recalcitrant parent, and two selected DH lines obtained from each of the two generations. Both F_1 and BC_1 plants had a higher anther culture ability than the Chinese parent and three of the four selected DH lines ranged between the F_1 resp. BC_1 and the Chinese parent. In addition, these lines displayed acceptable agronomic performance. These results demonstrated that high anther culture ability and agronomic performance can be combined by breeding.

3 *In vivo* Techniques

If maize plants are crossed with specific genotypes, so-called inducers, a certain fraction of kernels possess a haploid rather than a regular diploid F_1 embryo. This phenomenon is called *in vivo* (or *in situ*) haploid induction. Generally, kernels with a haploid embryo have a regular triploid endosperm. Therefore, such kernels display the same germination rate and vigor as those with a diploid embryo (Coe and Sarkar 1964).

Two methods of *in vivo* haploid induction are known in maize leading to paternal (androgenetic) and maternal (gynogenetic) haploids, respectively. In induction crosses aiming at paternal haploids, the inducer is used as female and the donor plant as male parent. Thus the cytoplasm of paternal haploids originates from the inducer but the chromosomes exclusively from the donor plant. For production of maternal haploids, on the other hand, the inducer is used as pollinator, leading to haploids carrying both cytoplasm and chromosomes from the donor. Different inducers are used for induction of paternal and maternal haploids (Sections 8.3.1 and 8.3.2). Both methods of *in vivo* haploid induction are much less dependent on the donor genotype than current *in vitro* techniques (Röber et al. 2005, Spitkó *et al*. 2006).

3.1 Induction of Paternal Haploids

The induction of gynogenetic haploids rests on properties of the mutant 'indeterminate gametophyte' caused by the recessive gene *ig* (Kermicle 1969). Multiple embryological abnormalities have been observed in homozygous *ig* plants. In some embryo sacs not all nuclei divide a third time, leading to various cytological irregularities including egg cells without a nucleus. After fusion with one of the two paternal sperm cells, such an egg cell may develop into a haploid embryo possessing the maternal cytoplasm and only paternal chromosomes. In selected inducer lines, the haploid induction rate ranges from 1 to 2% (Kermicle 1994; Schneerman et al. 2000).

Paternal haploids have gained considerable importance for the creation of cytoplasmic male-sterile (CMS) analogues of seed parent lines in commercial hybrid breeding. For this purpose, induction lines in various CMS-inducing cytoplasms have

been developed (Pollacsek 1992; Schneerman et al. 2000). Using these CMS inducer versions, the transfer of new breeding lines into the CMS cytoplasm requires only a single induction cross rather than multiple backcross generations.

3.2 Induction of Maternal Haploids

Chase (1952) reported spontaneous haploid induction rates in US Corn-Belt materials of about 0.1%. This value was far too low for a commercial application of the DH technology, as suggested by the author. A great step forward was the detection of inbred line Stock6 which had a 10 - 20 times higher induction rate (Coe 1959). Stock6 became the "Mother" of all subsequently developed inducers. Considerable progress was reported from groups working in India (Sarkar et al. 1994), Russia and Moldova (Tyrnov and Zavalishina 1984; Chalyk 1994; Shatskaya et al. 1994), France (Lashermes and Beckert 1988; Bordes et al. 1997), and Germany (Deimling et al. 1997; Röber et al. 2005). In the course of time, more effective techniques were developed for rapid identification of haploid embryos or seedlings, for chromosome doubling and for raising and selfing large numbers of doubled haploid plants (generation D_0) in the field. As a consequence, the so-called DH technology has meanwhile become a standard tool in modern maize research and breeding (Seitz 2005; Röber et al. 2005; Presterl et al. 2007).

3.2.1 Induction Rate

According to literature reports, the presently most effective inducer is line RWS developed at the University of Hohenheim, Stuttgart, Germany (Röber *et al*. 2005). It was derived from a cross between the Russian inducer synthetic KEMS (Shatskaya et al. 1994) and the French inducer line WS14 (Lashermes and Beckert 1988) and is adapted to the temperate climate of Central Europe but is effective also in tropical environments (Röber et al. 2005). Averaged across a wide range of donors and environments, it has an induction rate of about 8%. A sister line, RWK-76, developed from the reciprocal cross (WS14 x KEMS) even reached an average induction rate of 9 - 10% (unpubl. data). The same rate was observed for the cross RWS x RWK-76. Although having related parents, this cross is much more vigorous and a better pollen shedder than each of its parents and therefore easier to handle, particularly in adverse environments.

Roux (1995) tested lines Stock6, WS14, and W23*ig* for their ability to induce maternal haploids. Line W23*ig* is an isogenic form of dent line W23 (developed in Wisconsin, USA) except for the *ig* gene that enables the induction of paternal haploids (Section 8.3.1). The non-converted W23 line induces neither paternal nor maternal haploids. In agreement with this, the induction rate of W23*ig* for maternal haploids did not significantly deviate from the frequency of spontaneously occurring haploids. The induction rates were 0.2% for W23*ig*, 2.0% for Stock6, and 7.3% for WS14.

Significant differences between donor genotypes were observed for the induction rate (Roux 1995; Eder and Chalyk 2002; Röber et al. 2005). However, the range of variation was small compared to that reported for anther or microspore culture response (Section 8.2). Environmental conditions also influence the success of *in vivo* haploid induction. Using KEMS and RWS as inducers and a donor genotype marked with the recessive mutant 'liguleless', Röber et al. (2005) obtained an average induction rate of 2.0% in the most adverse and a rate of 16.4% in the most favourable field environment. Optimizing the growing conditions by minimizing biotic and abiotic stress generally raises the induction success (personal communications from various breeders).

3.2.2 Haploid Identification

A key issue in applying the *in vivo* haploid induction approach on a commercial scale is an efficient screening system allowing one to differentiate between kernels or seedlings generated by haploid induction and those resulting from regular fertilization. The most efficient haploid identification marker is the 'red crown' or 'navajo' kernel trait encoded by the dominant mutant allele *R1-nj* of the 'red color' gene *R1*. In the presence of the dominant pigmentation genes *A1* or *A2* and *C2*, *R1-nj* causes deep pigmentation of the aleurone (endosperm tissue) in the crown (top) region of the kernel. In addition, it conditions pigment in the scutellum (embryo tissue). Pigmentation may vary in extent and intensity depending on the genetic background of the particular donor and inducer. In conjunction with additional color genes (*B1*, *Pl1*), *R1-nj* also conditions pigmentation of the coleoptile and root of the seedling.

Nanda and Chase (1966) and Greenblatt and Bock (1967) first used the red crown mutant as a selectable marker in haploid-induction experiments. To be effective, the donor has to have colorless seeds and the inducer needs to be homozygous for *R1-nj* and the aforementioned dominant color genes. A kernel resulting from haploid induction then has a red crown (regular triploid endosperm) and an unpigmented scutellum (haploid maternal embryo), whereas a regular F_1 kernel displays pigmentation of both aleurone and scutellum (Figures 1 and 2). If only the egg cell but not the central cell is fertilized, the kernel has a pigmented (diploid) embryo and a non-pigmented, diploid maternal endosperm and aborts during the early kernel development phase (see "tooth gaps" in Fig. 2). Kernels resulting from (unintended) selfing or outcrossing with other colorless donors don't show any pigmentation. The red crown marker does not work if the donor genome is homozygous for *R1* or for dominant anthocyanin inhibitor genes such as *C1-I*. These genes seldom occur in commercial US Corn-Belt germplasm (Seitz, pers. comm.) but may be a problem in European flint (Röber et al. 2005) or tropical (Belicuas et al. 2007) materials. If *R1-nj* is poorly expressed in the scutellum, a validation of the putative haploids is possible in the early seedling stage by means of the coleoptile and root color gene *Pl*.

An unambiguous distinction between maternal haploid and regular F_1 plants was accomplished by means of a dominant herbicide resistance marker by

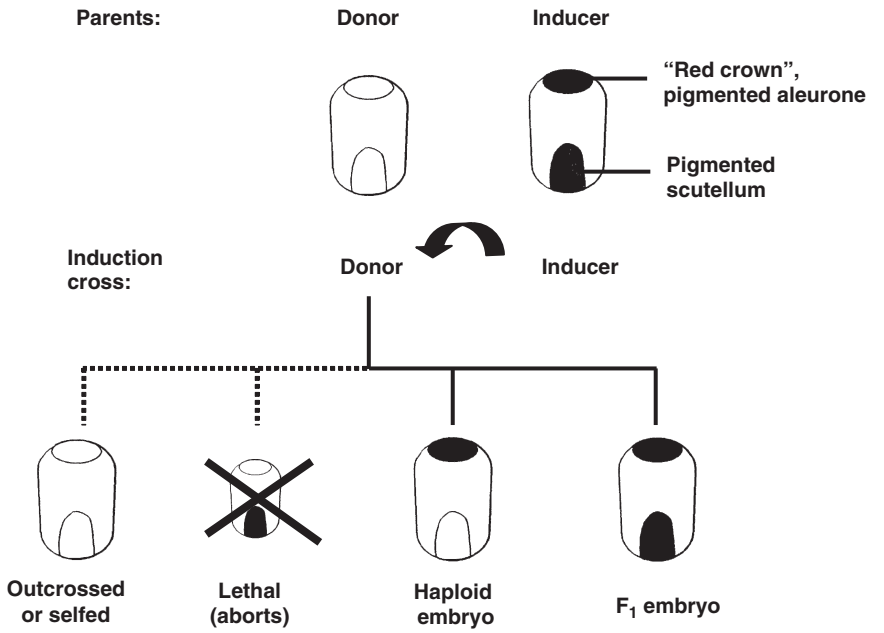

Fig. 1 Graphical representation of the dominant maize pigmentation marker 'red crown' and its use for identifying kernels with haploid embryo resulting from haploid induction crosses

Geiger et al. (1994). The authors had transferred the phosphinotricin acetyl transferase gene *pat* to the inducer line RWS. Using this transgenic line as inducer, the induced haploid seedlings are sensitive and the F_1 plants resistant. Applying the herbicide to a small leaf region leads to a clear distinction between the two types of seedlings without killing the susceptible haploids. However, for large-scale applications, the method is too laborious.

More recently, Belicuas et al. (2007) showed that microsatellite markers are a reliable and universally applicable haploid identification tool. But the cost of genotyping and the need for adequate high-throughput marker facilities are presently limiting the applicability of this approach.

A cheap and fast solution was suggested by Rotarenko et al. (2007). The authors observed that kernels with a haploid embryo have a significantly lower oil concentration than those with a diploid F_1 embryo. This is due to the reduced size of haploid compared to diploid embryos. Averaged across eight donors, the oil concentrations of the haploid and diploid embryos amounted to 46.7 and 55.6 g kg^{-1}, respectively. The authors did not show single-kernel data which would allow one to judge the precision achievable with this approach. Anyway, because a fast, inexpensive and non-destructive assessment of oil concentration is feasible with single-kernels at high-throughput, the approach deserves further investigation. Inducers with a high oil concentration should be best suited for this approach.

Fig. 2 Ears of a colorless dent single cross pollinated with inducer line RWS featuring the 'red crown' kernel marker (regular triploid F_1 endosperm). Gaps indicate aborted kernels (diploid maternal endosperm) (Photographed by Silvia Koch)

3.2.3 Properties of Haploids

Haploid plants are smaller and less vigorous than corresponding DH or inbred lines (Chase 1952; Chalyk 1994). They are also much more sensitive to any kind of stress. Most haploid plants display a certain degree of female fertility if pollinated by diploids. In a study by Chalyk (1994), 96% of the haploids derived from a synthetic dent population produced ears carrying at least a few kernels. The highest kernel number per ear was 107 and the average 27. Even higher values were obtained by Geiger et al. (2006). The authors analyzed haploid progenies of three elite dent

single crosses and all three of them showed some degree of fertility. In the most fertile progeny, the average number of kernels per plant amounted to 80.

On the contrary, most haploids lack male fertility. Early studies of Chase (1952) revealed fertile sections of the tassel in about one percent of the haploid plants studied. In the aforementioned materials, Chalyk (1994) observed some pollen in 3% of the haploids. Results of Zabirova et al. (1993) demonstrate a great influence of the donor genotype on the frequency of pollen-shedding haploids. The authors detected a donor line from which 33% of the induced haploids could successfully be selfed. The line resulted from four cycles of selection for this trait.

In the above reports, none of the authors provide cytological data on the ploidy level of the selfed plants. So they may have been completely haploid or may have undergone spontaneous, possibly sectorial, chromosome doubling during the pre-anthesis phase of development. At Hohenheim, leaves and immature anthers of putative haploids derived from current breeding materials were analyzed by flow cytometry and only haploid ones were bagged for selfing (Geiger and Schönleben, unpubl.). Twenty five out of 390 plants (6.4%) produced between 1 and 11 (average 3.8) seeds per selfed ear. However, three plants (0.8%) yielded 16, 23, and 40 seeds, respectively. Taken together, data indicate that there is promising genetic variation for spontaneous male fertility of haploids in various breeding materials.

3.2.4 Artificial Chromosome Doubling

For decades, artificial chromosome doubling was a serious constraint of haploid induction in maize since, in contrast to most other crop plants, the seedling reaction was highly genotype specific to the usual colchicine treatment. A breakthrough was accomplished by Gayen et al. (1994) who applied the colchicine at the coleoptile stage, 2-3 days after germination. The authors cut off the tip of the coleoptiles and immersed the whole seedling into a 0.06% colchicine solution plus 0.5% DSMO (dimethyl sulfoxide) for 12 hrs at 18°C. Deimling et al. (1997) further increased the efficacy of the method by reducing the roots to 20-30 mm and placing the immersed seedlings in the dark. After the colchicine treatment, the seedlings are carefully washed in water and subsequently grown (during the first days under high humidity) in the greenhouse to the 5- to 6-leaf stage. A few weeks later the plants are transferred to the field. Eder and Chalyk (2002) applied the method to a broad range of donor genotypes and achieved an average doubling rate of 49%. For comparison, the authors tested a colchicine injection method applied in the 3- to 4-leaf stage and reached a doubling rate of only 16%. With both methods, 50 - 60% of the pollen-shedding plants could be selfed.

A gentler method of chromosome doubling was developed by Kato (2002). He treated haploid plants in the flower primordial stage with nitrous oxide gas ($N0_2$) for 2 days at 600 kPa. Averaged across donor genotypes, 44% of the treated plants produced seed after self-fertilization. However, a very strong influence of the donor genotype on the doubling rate was observed. Furthermore, the method is very laborious and requires special equipment (safe gas chambers) and, therefore,

is not easily adaptable to high-throughput applications. On the other hand, the high recovery rate makes it attractive for approaching specific cytogenetic and other scientific problems.

3.2.5 Possible Mechanisms

Principally, two mechanisms leading to maternal haploids have been conceived. (1) One of the two sperm cells provided by the inducer is defective yet able to fuse with the egg cell. During subsequent cell divisions, the inducer chromosomes degenerate and are eliminated stepwise from the primordial cells. The second sperm cell fuses with the central cell and leads to a regular triploid endosperm. (2) One of the two sperm cells is not able to fuse with the egg cell but instead triggers haploid embryogenesis. The second cell fuses with the central cell as under the first hypothesis. At any rate, kernel abortion is expected if the functional sperm cell fuses with the egg cell and the defective one fuses with the central cell or if the central cell remains unfertilized (Fig. 2).

Experimental data in support of the first hypothesis come from the studies of Wedzony et al. (2002). The authors fixed ovaries of selfed RWS plants at intervals during the first 20 days after pollination. Eighteen out of 203 embryos contained micronuclei in every cell of the shoot primordia. Micronuclei varied in number and diameter, displaying the typical characteristics of metabolically inactive chromatin. In some equatorial plates, chromosome fragments were observed. Micronuclei elimination started during the globular state of embryogenesis. These observations are indirectly corroborated by the results of Fischer (2004). The author used microsatellite markers to check for a strictly maternal origin of haploids induced by RWS. Among 624 haploid plants and 309 DH lines (generation D_1), 1.4% of the genotypes possessed one or, rarely, several inducer chromosome segments. Generally, these segments had replaced the homologous maternal segments.

Observations supporting the second hypothesis were reported by Chalyk et al. (2003). The authors found 10 -15% aneuploid microsporocytes in the haploid induction lines MHI and M471H. From this, the authors concluded that part of the viable pollen of these inducers may be aneuploid and will result in sperm cells which only fuse with the central cell.

Another abnormality of MHI was described by Rotarenko and Eder (2003). The authors detected a three times higher heterofertilization rate in crosses with MHI compared with regular inbred lines. This correlated with a higher frequency of haploids induced by a single controlled pollination, compared to open-pollination under topcross conditions. Similar observations have not been made with other inducers.

Taking all of this information together, the mechanism of haploid induction is still not fully understood. Most likely, several reproductive abnormalities are involved and different inducers may vary in this regard. At any rate, researchers should keep in mind that maternal haploids might possess small fractions of the inducer genome.

4 Genetics of Haploid Induction

Both, *in vitro* and *in vivo* haploid induction are under polygenic control. Segregating generations derived from crosses between parents contrasting in haploid induction ability revealed continuous variation for various induction-associated traits (Lashermes and Beckert 1988; Deimling et al. 1997; Röber et al. 2005).

In a QTL study, Cowen *et al*. (1992) analyzed 98 F_3 lines derived from a cross between two contrasting US Corn-Belt lines (B73, recalcitrant; 139/39-05, responsive) for anther culture response, taking the number of embryo-like structures per 100 plated anthers as response criterion. Seventy five RFLP clones were used to genotype 98 F_3 lines. Fifty seven percent of the phenotypic variance among F_3 lines could be explained by the joint effects of two major and two minor QTL. The two major QTL reside on chromosomes 3 and 9. At both loci the alleles for responsiveness are recessive and show strong complementary epistasis. Results indicate that marker-assisted introgression of QTL for responsiveness shows promise for improving the anther culture ability of breeding materials. Further progress can be expected from the rapidly increasing knowledge in functional genomics of microspore embryogenesis in other plant species (for review see Hosp et al. 2007).

Murigneux et al. (1994) conducted QTL analyses in three DH-line populations derived from three crosses between contrasting dent parent lines. In two populations, the DH lines were evaluated *per se* and in the third population as testcrosses. The populations comprised 48, 96, and 95 DH lines, respectively, and were genotyped for at least 100 polymorphic RFLP markers. In each cross, three to four QTL were found for percentage of responding anthers and zero to four for number of embryos per 100 anthers. The QTL are scattered over chromosomes 3, 4, 5, and 7 - 10. QTL positions did not agree between populations in any case. Jointly, the QTL explained only 30 - 40% of the phenotypic variance for percent responding anthers.

Röber (1999) evaluated a population of 211 F_3 plants derived from the cross W23*ig* x Stock6 (the parents of WS14, see Section 8.3.2) for *in vivo* induction of maternal haploids. QTL analysis with 84 polymorphic RFLP markers revealed two QTL located on chromosomes 1 and 2 jointly explaining 17.9% of the phenotypic and 40.7% of the genotypic variance for induction rate. The positive QTL allele on chromosome 1 is dominant and originates from Stock6 (the "high" parent) whereas the one on chromsome 2 is additive and originates from W23*ig* (the "low" parent). Interestingly, no QTL for *in vitro* haploid induction was detected on chromosomes 1 and 2 in the two foregoing anther culture studies.

Because *in vivo* haploid induction could be considered a detrimental trait from the evolutionary point of view, one might expect that the F_1 of a cross between two unrelated inducers would furnish a lower induction rate than the better parent. In an induction experiment with WS14, KEMS, WS14 x KEMS, and KEMS x WS14, Röber et al. (2005) obtained induction rates of 2.0%, 6.9%, 3.9%, and 4.6%, respectively. No significant difference existed between the mid-parent and the mean F_1 value whereas KEMS significantly surpassed all other entries. In another experiment (Geiger, unpublished), RWS, RWK-76, and their cross were compared. In this case,

the F_1 reached the same induction rate as the better parent, RWK-76. Thus, neither of the two experiments supports the hypothesis above.

5 Using Doubled Haploids in Breeding

During the last decade, *in vivo* haploid induction has developed into a routinely used tool in hybrid maize breeding. While the induction of paternal haploids has proven to be an efficient tool for converting high combining seed parent lines to isogenic CMS analogues (Pollacsek 1992; Schneerman et al. 2000), the induction of maternal haploids is used for the rapid development of homozygous lines in mainstream breeding and in exploiting genetic resources (Röber et al. 2005). The following three sections will focus on the use of DH lines in recurrent selection (RS) and in hybrid parent line development (LD).

5.1 Quantitative Genetic Aspects

As is well known from quantitative genetics, the gain from selection for performance traits depends on (1) the selection intensity, (2) the heritability coefficient, (3) the genetic correlation between selection criterion and gain criterion, and (4) the genetic standard deviation for the gain criterion (Hallauer and Miranda 1981; Falconer and Mackay 1996). The gain criterion, *i.e*. the criterion for evaluating the selection response, is the GCA (general combining ability) with one or more heterotic group(s). Selection criteria are the performances of the candidate lines *per se* and of their testcrosses.

Strong selection increases the short-term response to selection but reduces the effective population size and thus leads to a steady decline of the genetic variance in the course of medium- to long-term RS and LD programs. To keep this decline within adequate limits, a minimum number of selected lines needs to be saved for starting a new breeding cycle (Gordillo and Geiger 2008a). Since the loss of genetic variance increases with the inbreeding coefficient (F), *i.e*. with the degree of homozygosity of the candidate lines, more lines need to be saved when selecting among DH lines (completely homozygous) compared to early-generation selfed lines (F = 0.5, 0.75, 0.875, … in generations S_1, S_2, S_3, …, respectively). Thus, all other things being equal, selection intensity needs to be more restricted when using DH lines.

Contrary to the effective population size, the genetic variance of the selection criterion and consequently the heritability coefficient increase as F increases. This leads to better differentiation among highly homozygous lines *per se* and among their testcrosses (Griffing 1975; Röber et al. 2005; Gordillo and Geiger 2008b). Seitz (2005) compared three sets of S_2 lines and corresponding DH lines evaluated for testcross grain yield with the same testers and in the same environments. On average, the estimated genetic variance among the DH lines was 2.1 times higher

than among the S_2 lines. In an analogous experiment with S_3 and DH lines, a 1.6 times higher variance was obtained for the DH lines. These findings were in principal confirmed by Bordes et al. (2007), who compared S_1 lines and single-seed descent (SSD) lines (in S_5) with corresponding DH lines. Combined across locations, the estimated genetic variance for testcross grain yield was 1.6 times higher among the DH lines than among the S_1 lines and 1.2 times higher than among the SSD lines. The greater estimates of the variance between the DH lines compared to the SSD lines (F = 0.97) was unexpected and might be attributable to unconscious selection during the selfing phase of the SSD lines.

The genetic correlation between selection criterion and gain criterion also increases with the homozygosity of the candidate lines, *i.e.* the closer the genotypes of the candidate lines agree with those of the homozygous lines to be derived thereof. This again favors the use of DH lines in breeding.

Epistatic gene action may positively or negatively affect hybrid maize performance (Lamkey and Edwards 1999). Selection for positive epistasis is most effective among uniform selection units such as DH or completely inbred lines. Negative epistatic effects are frequently observed in three-way and double-cross hybrids (Melchinger et al. 1986) and are to be expected in progenies of selected lines that are intercrossed to start a new RS or LD cycle. This decline of performance can be explained by a disruption of coadapted gene arrangements accumulated in elite breeding lines. To limit such negative effects, a balance between genetic recombination and fixation of gene arrangements is needed irrespective of whether the breeding method is based on DH or inbred lines.

5.2 Breeding Schemes

Various DH line-based schemes have been suggested for hybrid maize breeding (Gallais 1988; Bordes et al. 2007; Longin et al. 2006 and 2007; Gordillo and Geiger 2008a and b). To ensure long-term breeding progress, the gene pools used for line development need to be continuously improved by RS (Hallauer and Miranda 1981). Most efficiently, this can be accomplished by combining LD and RS in one integrated and comprehensive breeding scheme (Gordillo and Geiger 2008b). However, whereas LD aims at maximizing short-term success, RS is geared towards raising the genetic potential of the breeding population in the long run. This means that, in RS, considerably more lines must be selected as parents for the next cycle than are needed for establishing experimental hybrids in LD. The breeder therefore must decide which weight he or she wants to give to short- and long-term goals when allocating resources.

An example of a breeding scheme using DH lines is given in Figure 3. The dimensioning example refers to a breeding budget of 500,000 EUR. The figures approximately correspond to optimum values as determined by the computer software MBP (version 1.0) developed by Gordillo and Geiger (2008c). In brief, the scheme comprises the following steps:

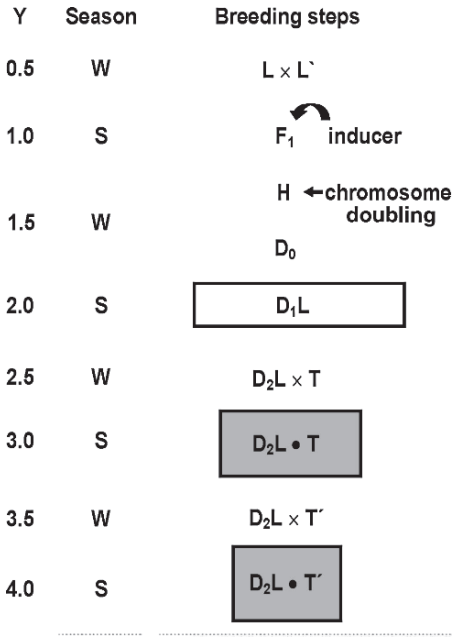

Fig. 3 Scheme for an integrated recurrent selection (RS) and line development (LD) procedure based on doubled haploid (DH) lines. RS cycles are completed after the first stage of testcross evaluation in year 3 and LD cycles after the second stage in year 4. The dimensioning example refers to a breeding budget of 500,000 EUR per cycle and was determined by model calculations applying the optimization software MBP (version 1) (Gordillo and Geiger 2008c) assuming equal weights for RS and LD; W = winter, S = summer, L = line, H = haploid seedling, D_x = DH generation x, T = tester, N_T = number of testers)

- Creating variation by intercrossing 79 selected lines (recombination units).
- *In vivo* haploid induction of maternal haploids in generation F_1.
- Artificial chromosome doubling in the seedling stage, raising and selfing fertile plants (Generation D_0).
- Evaluation of 6400 D_1 lines in single-row observation plots for visually scorable traits at 2 locations.
- Production of testcrosses of 3200 D_2 lines with 1 tester.
- Evaluation of the testcrosses in yield trials at 7 locations.
- Intercrossing the best 79 D_2 lines to commence the next RS cycle and testcrossing of the best 43 lines with 5 testers.
- Evaluation of the testcrosses in yield trials at 15 locations (15 x 5 = 75 plots per line).
- Selection of the 5 best DH lines for production of experimental hybrids.

In the described scheme, the RS cycle extends over six generations and the LD cycle over eight. This takes three and four years, respectively, if sufficient off-season capacities are available. The LD cycle could be reduced to three years by renouncing

the second stage of testcross evaluation. The expenses saved in year 4 would then be available for boosting the budget in years 1 - 3. This reduces the expected selection response per cycle but increases the response per year (Longin et al. 2006; Gordillo and Geiger 2008a and b). However, a second stage of testcross selection still seems to be advisable in order to diminish bias due to genotype x year interactions. Moreover, model calculations of Gordillo and Geiger (2008b) revealed that, in the long run, the annual genetic gain from two-stage selection can be further raised if the lines selected for commencing a new cycle are not only taken from the cycle under consideration but also from the first selection stage of the breeding cycle started one year later. This genetic interlinking of yearly staggered breeding cycles allows for a higher selection intensity and accelerates the decline of gametic phase disequilibrium in the respective gene pool.

Longin et al. (2007) investigated the efficiency of early testing for combining ability before *in vivo* haploid induction is applied. The authors compared the two-stage selection scheme in Figure 3 with a combined S_1/DH line scheme. At the first stage, S_1 lines and at the second stage DH lines derived from selected S_1 lines are evaluated for testcross performance. This scheme takes one year more time than the "pure" two-stage DH scheme. The maximum predicted genetic gain was about 10% larger, if computed per cycle, but 3% smaller on an annual basis. To reduce the cycle length of the S_1/DH line scheme, S_1 plants rather than S_1 lines would need to be crossed (as males) to a tester and additionally be used as females in haploid induction. Considerable improvement of the DH technology is needed to obtain a large enough number of DH lines from a single S_1 plant before this accelerated S_1/DH scheme will become applicable.

5.3 Operational, Logistic, and Economic Aspects

Developing maternal DH lines by *in vivo* haploid induction requires specific skills and equipment for (1) large-scale chromosome doubling, (2) raising unregulated seedlings in the greenhouse, (3) transplanting the surviving plantlets to the field, (4) avoiding stress during the growing period, and (5) selfing the adult plants. Under adequate conditions, about one to five DH lines emanate from one donor plant (Eder & Chalyk 2002; Röber et al. 2005). Improvement seems possible for each of the five foregoing steps. However, if several times more DH lines per S_1 donor plant are demanded, an androgenetic approach might, in the long run, be more promising.

Considerable savings are possible in line development by haploid induction compared to inbreeding. No continued selfing is needed for creating homozygous genotypes, and maintenance breeding can be kept to a minimum. The fact that DH lines are genetically fixed units also simplifies the logistics of seed transfer between main and off-season sites, since DH lines need to be shipped only once whereas, in a selfing program, new sublines arise in every segregating generation.

Since DH lines are homozygous and homogeneous from the very first, they fully meet the requirements for obtaining plant variety protection. Thus, DH lines allow the breeder to considerably reduce the time to commercialization which can be considered as the most significant economic advantage of the technology (Seitz 2005; Bordes et al. 2006). Moreover, haploid induction is a very helpful tool in stacking transgenic or other monofactorial traits in potential hybrid parents by marker-assisted backcrossing, since the segregation pattern is much simpler at the haploid than at the diploid level. This makes it feasible to fix a demanded stack in the shortest possible time and with the lowest possible genotyping expenditure.

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