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Implementation and Optimization of the Doubled Haploid Technology for Tropical Maize (*Zea mays* L.) Breeding Programs

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Contents

[1] Prigge V, Melchinger AE (2012) Production of haploids and doubled haploids in maize. In: Loyola-Vargas VM, Ochoa-Alejo N (eds.) Plant Cell Culture Protocols. Third edition. Humana Press - Springer Verlag, Totowa, New Jersey. In Press.

[2] Prigge V, Sánchez C, Dhillon BS, Schipprack W, Araus JL, Bänziger M, Melchinger AE (2011) Doubled haploids in tropical maize: I. Effects of inducers and source germplasm on *in vivo* haploid induction rates. Crop Science 51:1498–1506.

[3] Prigge V, Schipprack W, Mahuku G, Atlin GN, Melchinger AE (2012) Development of *in vivo* haploid inducers for tropical maize breeding programs. Euphytica. In Press.

[4] Prigge V*, Xu X*, Li L, Babu R, Chen S, Atlin GN, Melchinger AE (2012) New insights into the genetics of *in vivo* induction of maternal haploids, the backbone of doubled haploid technology in maize. Genetics 190:781-793. * Both authors contributed equally.

[5] Kleiber D*, Prigge V*, Melchinger AE, Burkard F, San Vicente F, Palomino G, Gordillo GA (2012) Haploid fertility in temperate and tropical maize germplasm. Crop Science 52:623-630. * Both authors contributed equally.

[6] Prigge V, Babu R, Das B, Hernández Rodriguez M, Atlin GN, Melchinger AE (2012) Doubled haploids in tropical maize: II. Quantitative genetic parameters for testcross performance. Euphytica. In Press.

Abbreviations

A_r	average number of alleles
BC_t	t^{th} backcross generation
CIMMYT	International Maize and Wheat Improvement Center
CML	CIMMYT maize line(s)
ΔG	response to selection
D	gene diversity
DH	doubled haploid(s)
DNA	deoxyribonucleic acid
FP	proportion of fertile haploids
F_t	t^{th} filial generation
HIR	haploid induction rate(s)
IND	inducer
IS	number of intact seeds
NARS	National Agricultural Research Systems
OP	open-pollinated population(s)
OPV	open-pollinated variety
QTL	quantitative trait locus/loci
SC	single cross(es)
SNP	single nucleotide polymorphism
SSR	simple sequence repeat
TCDMC	testcross dry matter content
TCGY	testcross grain yield
TIC	tropical inducer candidate(s)
$\bar{x}$	population mean

Chapter 1

General Introduction

Maize (*Zea mays* L.) is used for human and animal consumption, as a biofuel, and for a range of industrial purposes around the globe. The crop is a food staple and, alongside wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.), it is the main source of calories for the majority of people living in poverty-stricken areas of Africa, Asia, and America (Dowswell et al. 1996). Maize is currently produced on about 78 million hectares in more than 70 low-income and food-deficient countries (FAOSTAT 2011). However, in the near future irrigation water in these countries will become less due to alternative consumption (e.g., urban, industrial), and many small-holder farmers will be forced to grow rainfed maize instead of irrigated rice or wheat. This shift in combination with a steadily growing world population, projected to reach 9–10 billion people by 2050 (UN Population Division 2011), means that the demand for maize in the developing world is likely to double, and it is projected that maize becomes the highest production crop in developing countries by 2025 (Rosegrant et al. 2002).

Two major tools for meeting the future demands for maize and maize-based products are resource-conserving cropping systems and improved crop varieties that yield more with equal or less inputs than are currently used. In modern agriculture, farmers choose between two types of maize varieties – hybrids and open-pollinated varieties (OPV) – and their choice depends primarily on the prevalent environmental and economic situation as well as the availability of seed of the preferred variety type (Pixley and Bänziger 2004). Hybrid varieties are commonly developed by crossing two unrelated, homozygous inbred lines. Traditionally, the maize plants' cross-breeding nature required recurrent self-pollinations for 6–10 generations, i.e., 3–5 years when two seasons per year can be accomplished, to obtain sufficiently homozygous inbred lines (Hallauer et al. 2010). Hence, the key to increased genetic gains and accelerated development of improved varieties is reducing the time needed for inbred development. This can be most effectively achieved by application of the doubled haploid (DH) technology.

The Doubled Haploid Technology

Production of DH lines from heterozygous germplasm is surprisingly simple (Figure 1.1). In the first season, haploidy is induced in diploid maize plants, i.e., the chromosome pairs of the plants are reduced to single chromosomes. In the second season, the haploid chromosome set is duplicated, i.e., a copy of each single chromosome is made such that pairs of identical chromosomes are generated. The resulting diploid maize plant is called "doubled haploid" and it is 100% homozygous, because in each pair of chromosomes one chromosome is a copy of the other. Hence, completely homozygous DH lines can be produced in only two cropping seasons as opposed to 6–10 seasons using the traditional method of recurrent self-pollination.

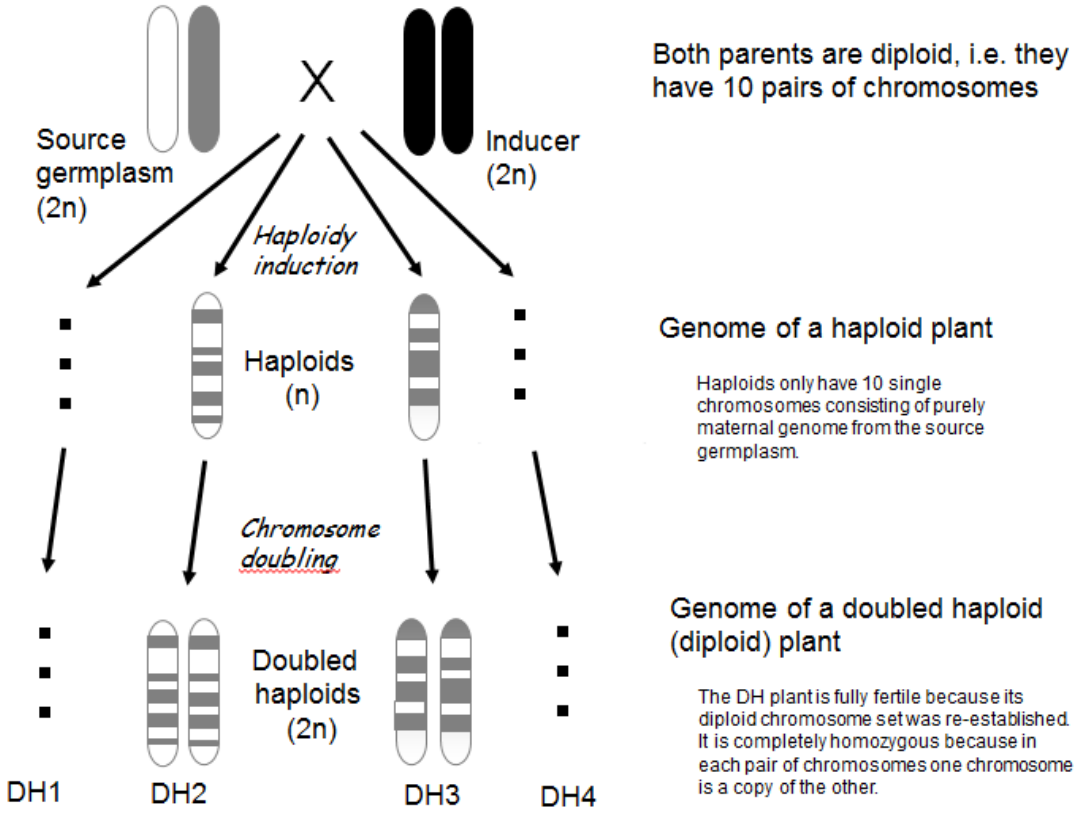

Figure 1.1. Schematic description of doubled haploid (DH) line development at the chromosomal level. The black shape represents inducer chromosomes, the white and grey shapes represent the chromosomes of the two inbred parents of a single cross source germplasm, respectively. Haploidy is induced by pollinating the heterozygous source germplasm with inducer pollen. Haploids contain the reduced gametic chromosome number (n=10). Their chromosome set is duplicated to obtain DH plants that are diploid (2n=20) and completely homozygous. DH1 to DH4 represent different DH lines, each with distinct genomic composition obtained from the source germplasm.

In addition to accelerating line development, adoption of the DH technology has several quantitative genetic, operational, logistic, and economic advantages (Nei 1963; Schmidt 2003; Melchinger et al. 2005; Röber et al. 2005; Seitz 2005; Smith et al. 2008; Geiger 2009): DH lines display the complete genetic variance at the beginning of the selection procedure, thus simplifying selection of superior genotypes; higher genetic variance results in higher heritability of lines *per se* and testcross evaluations, improving testing precision; 100% homozygosity implies that no residual heterozygosity is masking line performance thus ensuring that variety protection can be accomplished earlier; DH lines have higher *per se* performance for agronomic traits due to high selection pressure applied during the haploid phase; deleterious recessive alleles are effectively purged from germplasm pools as their negative effects cannot be compensated for in haploids; laborious testing of various generations of sub-lines is not necessary freeing resources that can be allocated to testcross evaluation; and identification of germplasm and its exchange between breeding programs is considerably simplified. In summary, DH technology allows breeders to evaluate significantly more hybrid combinations and these hybrids are based on completely homozygous inbred parents, thus facilitating increased genetic gains per cycle, increased efficiency of the breeding program, and reduced developmental costs.

DH technology may be considered the third most important methodological achievement for maize breeding, after hybrid technology and off-season nurseries (Seitz 2004). Accordingly, owing to its many advantages and relatively simple methodology, DH technology has rapidly replaced other methods of line development in many major maize breeding programs in Europe (Schmidt 2003), North America (Seitz 2005), and China (Chen et al. 2009). For example, Pioneer Hi-Bred generated more maize inbreds with the DH approach during 2010 than they had produced during the entire 80 year history using traditional methods in their breeding program (M. Albertsen 2011, pers. comm.). Many publicly funded breeding programs have also shifted their inbred development activities to take advantage of DH technology. For example, the maize breeding program at the University of Hohenheim, Germany, is almost 100% based on DH production in the dent pool (W. Schipprack 2011, pers. comm.) and in the Germplasm Enhancement of Maize (GEM) project (Pollak 2003) together with Iowa State University, USA, about 200–300 DH lines are produced every year (T. Lübberstedt 2011, pers. comm.). However, before the initiation of this thesis research, there had been no published accounts of DH technology adoption in non-temperate areas, let alone its large-scale application in national maize breeding programs and seed companies in developing countries, although the biological mechanisms underlying DH technology are expected to work independently of climatic conditions. Therefore, the establishment of DH technology for tropical and subtropical maize breeding programs was the focus of this thesis research.

Production of Haploids

Haploid organisms contain the gametic chromosome number in their somatic cells and occurrence of haploid plants has been observed in many species (for review, see Dunwell 2010). However, to more effectively exploit haploids for DH line development, haploidy can be induced artificially, either *in vitro* or *in vivo*. *In vitro* production of haploids involves the cultivation of male (anthers or microspores) or female (ovules) gametophytes on media under controlled conditions to induce embryogenesis leading to the development of haploid plants (Touraev et al. 2009). For example, microspore cultivation is the method of choice for haploid production in rapeseed (*Brassica napus*; Swanson et al. 1987). In maize, *in vitro* culture responsiveness is extremely genotype-dependent (Büter 1997; Tang et al. 2006) and, therefore, *in vitro* production of haploids has not become a routine technique in maize breeding.

In vivo production of haploids can be achieved by: (i) inter-specific crossing, which is widely applied for barley (*Hordeum vulgare*; by pollination with *H. bulbosum*; Kasha and Kao 1970) and wheat (by pollination with maize; Laurie and Bennett 1986); (ii) crossing with pollen treated with irradiation, heat, and/or chemicals (for review, see Dunwell 2010); or (iii) crossing with a specific haploid inducer genotype (Chase 1952b). The latter is the method of choice for maize breeding and research (for review, see Geiger 2009). Employing the inducer as female will yield paternal haploids (Kermicle 1969), while employing the inducer as male will yield maternal haploids (e.g., Röber et al. 2005). The proportion of haploids among the total induction cross progeny denotes the haploid induction rate (HIR) of the inducer employed.

In vivo haploid induction ability in maize is a selectable trait and significant progress in the development of new inducers has been made during the last two decades. Public inducer genotypes for maternal haploids were developed at various international institutions and cover a range of HIR, such as: 2% for Stock6 (Coe 1959), the first germplasm ever reported to induce haploidy and ancestor of all current inducers, and CAUHOI (Li et al. 2009); 2–5% for WS14 (Lashermes and Beckert 1988); 3% for ZMS (Tyrnov and Zavalishina 1984, cited in Chebotar and Chalyk 1996); 5% for MHI (Eder and Chalyk 2002); 6% for PK6 (Barret et al. 2008) and KEMS (Shatskaya et al. 1994), and 8–10% for RWS (Röber et al. 2005), HZI (Zhang et al. 2008), PHI (Rotarenco et al. 2010), and UH400 (W. Schipprack 2011, pers. comm.). However, all of the above inducers were developed from temperate germplasm and principally evaluated for HIR and agronomic performance under temperate climatic conditions. Hence, it is not clear whether they can be readily employed for induction of haploidy in tropical maize breeding programs.

In general, inducers with increased HIR allow maize breeders to reduce the amount of nursery space required for inducer and source germplasm plantings for induction

crosses. Further, marker-assisted transfer of haploid induction ability to different genetic backgrounds would enable the rapid development of haploid inducers adapted to various agro-ecological zones, such as the tropics and subtropics. Availability of haploid inducers that are adapted to different agro-ecologies promises to greatly accelerate the adoption of DH technology in non-temperate maize breeding programs. However, marker-based breeding approaches require the availability of reliable molecular markers that are associated with quantitative trait loci (QTL) affecting HIR. Previous studies identified QTL for HIR on chromosomes 1 and 2 (Deimling et al. 1997; Röber 1999; Barret et al. 2008). However, at the initiation of this thesis research, none of the modern haploid inducers with HIR>8% had been subjected to genome-wide QTL analyses. Further, high-throughput single nucleotide polymorphism (SNP) marker chip technology (Yan et al. 2010) offered the possibility to substantially increase the power of QTL detection.

How to Detect Maize Seeds With Haploid Embryo?

An effective system for distinguishing haploids from the remainder of the normal diploid F_1 crossing seeds is essential, due to the common low level of HIR. Most of the widely used inducers carry a dominantly inherited marker gene, *R1-nj*, that causes purple coloration of the scutellum and the aleurone of seeds (Nanda and Chase 1966; Neuffer et al. 1997), and can be used as an embryo- or endosperm-specific marker when identifying putative haploids. Normal maize endosperms are triploid as they arise from the fusion of two maternal polar nuclei and one paternal sperm cell. Normal maize embryos are diploid as they arise from the fusion of the maternal egg cell and the remaining paternal sperm cell. Therefore, as the purple *R1-nj*-encoded coloration is dominantly inherited, only seeds with a haploid embryo have a non-pigmented scutellum, while seeds with diploid embryos have purple-colored scutellum. The limitation of this system is its variable expression of color and presence of inhibitor genes (Coe 1962) in some genotypes, which can lead to the misclassification of diploids as haploids and vice versa (Röber et al. 2005). The presence of inhibitor genes in tropical germplasm and the effectiveness of the purple seed color marker for haploid identification in tropical DH breeding programs had only been studied in one tropical genotype (Belicuas et al. 2007).

For research purposes, the most reliable system of haploid identification is based on mutants carrying recessive morphological characteristics such as *liguleless* or *glossy* (Neuffer et al. 1997). Specific mutant stocks are used as females and are pollinated with inducer pollen. The resulting testcross progeny is grown until the four-leaf stage and visually monitored for the mutant phenotype: due to the recessive nature of the mutations, haploids show the mutant phenotype, while diploids show the wild-type

phenotype. The *liguleless* and *glossy* testers have been widely employed for monitoring of HIR during genetic studies as well as inducer development and maintenance activities (e.g., Lashermes and Beckert 1988; Bordes et al. 1997; Deimling et al. 1997; Röber et al. 2005; Barret et al. 2008). However, currently available mutant stocks are mainly adapted to temperate areas and it is not clear whether they can be used for efficient determination of HIR under tropical conditions.

From Haploids to Doubled Haploids via Duplication of Chromosomes

The majority of haploid plants are sterile due to disrupted gamete formation (Tang et al. 2009). Hence, duplication of the haploid chromosomes is necessary to facilitate self-pollination for seed increase and maintenance of the genotype. Jensen (1974), Rao and Suprasanna (1996), and Kasha (2005) provide comprehensive reviews on suitable methods of chromosome duplication in plants. In maize, the most common chromosome doubling agent, colchicine, is an alkaloid obtained from meadow saffron (*Colchicum autumnale* L.) and it acts as a mitotic inhibitor. Mitosis is the process of nucleus division in somatic cells: after DNA replication, the microtubules pull the duplicated chromatids toward the two poles and the cell divides into two daughter cells. Colchicine disrupts mitosis by binding to tubulin. In this way the formation of microtubules and the polar migration of chromosomes is inhibited and the result is single cells with duplicated chromosome number (Wan et al. 1989).

Several colchicine-based protocols suitable for large-scale chromosome duplication have been developed for *in vivo* (Gayen et al. 1994; Deimling et al. 1997) and *in vitro* (Wan et al. 1989; Barnabás et al. 1999) production of maize DH lines. Alternative methods include treatment of haploids with nitrous oxide (Kato 2002) or with herbicides having anti-microtubule effects (Wan et al. 1991; Beaumont and Widholm 1993). However, regardless of the method, artificial chromosome doubling of maize haploids is costly, requires special facilities, and/or involves noxious substances. Further, the common approach of *in vivo* production of maize haploids does not allow direct treatment of the egg cell for artificial chromosome duplication; instead the haploid seedlings are treated, thus resulting in chimeras exhibiting different ploidy levels in different tissues. Hence, increased levels of natural haploid fertility could help to circumvent the above limitations and allow abandoning artificial treatments. The search for alternative artificial treatments was the subject of a companion study (Häntzschel 2011), while a portion of this research investigates the options for exploitation of haploids' natural fertility for DH line production.

Objectives

The aim of my thesis research was to further improve the *in vivo* DH technology and facilitate its implementation in tropical maize breeding programs. In particular, the objectives were to

(1) present a detailed, step-by-step documentation of the production of haploids and DH lines using *in vivo* DH technology that can be used for capacity building purposes (Chapter 2),

(2) examine the agronomic performance and haploid induction ability of temperate haploid inducers under tropical conditions and investigate the response to haploidy induction shown by diverse tropical source germplasm (Chapter 3),

(3) investigate the efficacy of the most widely used haploid identification system (i.e., *R1-nj*-encoded purple embryo coloration) in tropical maize germplasm (Chapter 3),

(4) design and conduct a breeding program for development of haploid inducers that are well adapted to tropical environments and discover novel sources of haploid induction ability (Chapter 4),

(5) elucidate the genetic architecture of *in vivo* haploid induction ability and determine the stability of QTL across various genetic backgrounds (Chapter 5),

(6) study variation of male fertility of haploids derived from genetically diverse germplasm and to explore options for exploiting this natural fertility of maize haploids to avoid artificial chromosome doubling during DH line production (Chapter 6), and

(7) evaluate tropical DH lines for testcross performance under field conditions to estimate quantitative genetic parameters and identify potential parental components for tropical hybrid and synthetic varieties (Chapter 7).

Chapter 2

A Recipe for *in vivo* Production of Doubled Haploids [1]

Vanessa Prigge and Albrecht E. Melchinger

Abstract

The *in vivo* haploid induction approach offers several advantages compared to the *in vitro* induction approach and recurrent self-pollination. It is currently used for inbred line development in many commercial maize breeding programs. We describe the *in vivo* approach for generation of maternal doubled haploids (DH). It involves four steps: (i) inducing haploidy by pollinating source germplasm with pollen of a haploid inducer; (ii) identifying putative haploid seeds (seeds with a haploid embryo) using a seed coloration marker system; (iii) duplicating chromosomes of putative haploids by treating the seedlings with a mitotic inhibitor; and (iv) self-pollinating doubled haploid plants to multiply their seed. An accompanying video providing a detailed description of the above steps is available at http://www.youtube.com/watch?v=V2jOEuZjjrg.

[1] Prigge V, Melchinger AE (2012) Production of haploids and doubled haploids in maize. In: Loyola-Vargas VM, Ochoa-Alejo N (eds.) Plant Cell Culture Protocols. Third edition. Humana Press - Springer Verlag, Totowa, New Jersey. In Press.

Chapter 3

Performance of Temperate Haploid Inducers in the Tropics [1]

Vanessa Prigge, Ciro Sánchez, Baldev S. Dhillon, Wolfgang Schipprack, José Luis Araus, Marianne Bänziger, and Albrecht E. Melchinger

The original publication is available at https://www.crops.org/publications/cs (DOI: 10.2135/cropsci2010.10.0568).

Abstract

The adoption of the doubled haploid (DH) technology in tropical maize (*Zea mays* L.) breeding programs is slow due to a lack of tropical haploid inducers and reliable information on the performance of temperate inducers under non-temperate conditions. The objective of this study was to determine the *in vivo* haploid induction ability of three temperate inducers crossed to a diverse set of tropical maize source germplasm under tropical conditions. Three experiments were conducted employing inducers as male parents to pollinate 120 source germplasm in three environments. Haploid induction rates (HIR) obtained under field conditions were determined with two different haploid identification systems. We detected significant genotypic differences among inducers and source germplasm for HIR but no interactions between the two factors. Mean HIR under tropical conditions were similar to those reported for evaluations under temperate conditions suggesting that temperate inducers can be employed for initiation of DH breeding programs in the tropics. Misclassification of diploids as haploids resulted in inflated HIR, particularly in highly variable source germplasm such as landraces or when expression of the identification marker was weak. We conclude that induction of haploidy is not a limiting factor for DH line production in tropical maize, yet the development of well-adapted tropical inducers will be beneficial.

[1] Prigge V, Sánchez C, Dhillon BS, Schipprack W, Araus JL, Bänziger M, Melchinger AE (2011) Doubled haploids in tropical maize: I. Effects of inducers and source germplasm on *in vivo* haploid induction rates. Crop Science 51:1498–1506.

Chapter 4

Development of Tropical Haploid Inducers [1]

Vanessa Prigge, Wolfgang Schipprack, George Mahuku, Gary N. Atlin, and Albrecht E. Melchinger

The original publication is available at http://www.springerlink.com/content/102881/ (DOI: 10.1007/s10681-012-0657-5).

Abstract

Lack of adapted haploid inducers impedes adoption of the doubled haploid (DH) technology in tropical maize breeding programs. Our objective was to generate inducers with improved adaptation to tropical conditions. We developed segregating generations from crosses between temperate inducers having haploid induction rates (HIR) of 8–10% and tropical CIMMYT maize lines (CML; HIR=0%) and evaluated these for HIR and agronomic performance under tropical lowland field conditions. The applied pedigree breeding scheme comprising mass selection on individual F_2 plants for highly heritable and visually scorable traits, followed by family-based selection for HIR and other agronomic characteristics in advanced selfing and backcross (BC) generations seems suitable for breeding improved haploid inducers with adaptation to different agroecologies. The most advanced tropical inducer candidates (TIC) combine HIR of up to 10% with improved pollen production, disease resistance, and plant vigor compared to temperate inducers under tropical conditions. Agronomic characteristics were significantly improved in the BC to CML compared to BC to inducers, while mean HIR of both populations were similar, indicating that BC to the adapted parent are suitable to improve adaptation of new inducers without sacrificing high HIR. When screening random open-pollinated maize accessions, HIR of up to 3% were observed, suggesting that novel genetic variation may be present in maize accessions that could be exploited to improve HIR in maize. In conclusion, tropical inducer development proceeds well, but evaluation of TIC in multi-environment trials needs to be completed before large-scale dissemination can commence.

[1] Prigge V, Schipprack W, Mahuku G, Atlin GN, Melchinger AE (2012) Development of *in vivo* haploid inducers for tropical maize breeding programs. Euphytica. In Press.

Chapter 5

Comparative QTL Mapping for *in vivo* Haploid Induction Ability [1]

Vanessa Prigge, Xiaowei Xu, Liang Li, Raman Babu, Shaojiang Chen, Gary N. Atlin, and Albrecht E. Melchinger

The original publication is available at http://www.genetics.org/ (DOI: 10.1534/genetics.111.133066).

Abstract

Haploids and doubled haploid (DH) inbred lines have become an invaluable tool for maize genetic research and hybrid breeding, but the genetic basis of *in vivo* induction of maternal haploids is still unknown. We report results of comparative quantitative trait locus (QTL) analyses of *in vivo* haploid induction ability in maize. Haploid induction rates (HIR) were determined in testcrosses of a total of 1061 progenies of four segregating populations involving two temperate haploid inducers, UH400 (HIR=8%) and CAUHOI (HIR=2%), one temperate and two tropical inbreds with HIR=0%, and up to three generations per population. Mean HIR of the populations ranged from 0.6 to 5.2% and deviated strongly from the midparent values. One QTL (*qhir1*) explaining up to $\hat{p} = 66\%$ of the genetic variance was detected in bin 1.04 in the three populations involving a non-inducer parent and the HIR-enhancing allele was contributed by UH400. The segregation ratios of loci in bin 1.04 were highly distorted against the UH400 allele in these three populations, suggesting that transmission failure of the inducer gamete and haploid induction ability are related phenomena. In the CAUHOI×UH400 population, seven QTL were identified on five chromosomes, with *qhir8* on chromosome 9 having $\hat{p} > 20\%$ in three generations of this cross. The large-effect QTL *qhir1* and *qhir8* will likely become fixed quickly during inducer development due to strong selection pressure applied for high HIR. Thus, marker-based pyramiding of small-effect and/or modifier QTL influencing *qhir1* and *qhir8* may help to further increase HIR in maize. We propose a conceptual genetic framework for inheritance of haploid induction

[1] Prigge V*, Xu X*, Li L, Babu R, Chen S, Atlin GN, Melchinger AE (2012) New insights into the genetics of *in vivo* induction of maternal haploids, the backbone of doubled haploid technology in maize. Genetics 190:781-793. * Both authors contributed equally.

ability, which is also applicable to other dichotomous traits requiring progeny testing, and discuss the implications of our results for haploid inducer development.

Erratum to Genetics 190:781-793 (DOI: 10.1534/genetics.111.133066)

We regret to not have acknowledged the dissertation of Dr. Frank K. Röber (1999), which represents the more detailed account of the QTL study first reported by Deimling et al. (1997). We apologize for any inconvenience this mistake may have caused.

Chapter 6

Haploid Fertility in Temperate and Tropical Germplasm [1]

Daniel Kleiber, Vanessa Prigge, Albrecht E. Melchinger, Florian Burkard, Félix San Vicente, Guadalupe Palomino, and G. Andrés Gordillo

The original publication is available at https://www.crops.org/publications/cs (DOI: 10.2135/cropsci2011.07.0395).

Abstract

Doubled haploid (DH) technology facilitates rapid development of homozygous inbred lines for hybrid breeding in maize (*Zea mays* L.). However, the required artificial chromosome duplication step, which commonly involves toxic and costly chemicals, represents a bottleneck. Exploiting the haploids' natural fertility may enable abolishment of artificial treatments and enhance efficiency of line development. We screened haploid populations derived from U.S. Corn Belt and tropical germplasm for the proportion of fertile haploids (FP) and the number of intact seeds (IS) on selfed ears and examined the effects of environments, heterotic groups, maturity groups, and population types on FP and IS. FP ranged from 0 to 20% under field and from 0 to 70% under greenhouse conditions. Tropical elite germplasm had higher median FP and mean IS than tropical landrace accessions. The Corn Belt heterotic group Stiff Stalk had higher median FP than Iodent and Lancaster, while early germplasm showed higher median FP than the other maturity groups. Significant ($p<0.01$) genetic variance for FP was observed among elite Corn Belt materials and heritability was 0.79, indicating that recurrent selection to increase FP is promising. We propose that artificial chromosome duplication is not necessary for DH line production from germplasm with high FP. This seems particularly relevant to enable small maize breeding programs in developing countries to adopt the DH technology for line development.

[1] Kleiber D*, Prigge V*, Melchinger AE, Burkard F, San Vicente F, Palomino G, Gordillo GA (2012) Haploid fertility in temperate and tropical maize germplasm. Crop Science 52:623-630. * Both authors contributed equally.

Chapter 7

Testcross Performance of Tropical DH Lines [1]

Vanessa Prigge, Raman Babu, Biswanath Das, Marta Hernández Rodriguez, Gary N. Atlin, and Albrecht E. Melchinger

The original publication is available at http://www.springerlink.com/content/102881/ (DOI: 10.1007/s10681-012-0626-z).

Abstract

Single crosses (SC) of elite inbreds and open-pollinated populations (OP) are suitable source germplasm for doubled haploid (DH) line development in hybrid maize breeding, given that they combine a high population mean ($\bar{x}$) with adequate response to selection (ΔG). We report testcross grain yield (TCGY) and dry matter content (TCDMC) evaluations of 131 DH lines developed from ten tropical source germplasm comprising five OP (OP1-OP5) and five SC (SC1-SC5). Gene diversity (D) and the average number of alleles (A_r) per locus was estimated for DH lines based on 24 simple sequence repeat (SSR) markers. No significant differences between $\bar{x}$ of OP- and SC-derived DH lines for TCGY and TCDMC were detected when analysed across three environments. Significant genetic variance for both traits was only detected among OP-derived DH lines, which may be explained by a larger number of segregating quantitative trait loci (QTL) as suggested by higher D and A_r values than in SC-derived DH lines. The usefulness criterion ($U = \bar{x} + \Delta G$) was higher for the OP-derived DH lines for TCDMC, but higher for SC-derived DH lines for TCGY. DH lines from OP1, OP2, and OP3 showed high TCGY, suggesting that they may be useful in tropical hybrid breeding. We conclude that tropical OP represent a valuable source of untapped genetic variation that can efficiently be exploited with DH technology for hybrid maize breeding.

[1] Prigge V, Babu R, Das B, Hernández Rodriguez M, Atlin GN, Melchinger AE (2012) Doubled haploids in tropical maize: II. Quantitative genetic parameters for testcross performance. Euphytica. In Press.

Chapter 8

General Discussion

Experiences from Establishing the DH Technology at CIMMYT

CIMMYT's Global Maize Program began *in vivo* DH line development in 2007 and the involved processes have since been optimized and adapted for local conditions and CIMMYT facilities. The temperate inducers UH400, RWS, and RWS×UH400 have been successfully employed for induction of haploidy in CIMMYT's tropical and subtropical source germplasm from 2007 to 2011, despite being poorly adapted to tropical lowland conditions (Prigge et al. 2011). Further, more than 4000 tropical DH lines have been produced between 2007 and 2010 (Mahuku et al. 2010) following the protocol described by Prigge and Melchinger (2012). This indicates that established protocols for *in vivo* DH line development can be readily applied to tropical maize breeding programs, although specific amendments owing to institutional and/or environmental conditions may be necessary.

One of the obstacles during initiation of any DH production service involves reliable detection of haploids among the induction cross progeny (Röber et al. 2005; Belicuas et al. 2007). In our study with tropical source germplasm, misclassification of diploids as haploids was particularly problematic in highly variable germplasm such as landraces and OPV, and poor embryo color expression complicated haploid identification in some genotypes (Prigge et al. 2011). However, these experiments were conducted in CIMMYT's first season of large-scale haploidy induction and classification of the resulting seeds had been performed by newly recruited technical staff. Hence, employment of experienced staff is essential and has already led to significantly improved accuracy of haploid identification (G. Mahuku 2011, pers. comm.). Further, recycling of DH lines for generating new source germplasm is expected to gradually improve expression of seed coloration due to selection against color-suppressing genes.

For high-throughput applications in breeding programs, Rotarenco et al. (2007) proposed to identify haploids based on kernel oil content, the determination of which could be automated using analytical methods, such as nuclear magnetic resonance. For example, Li et al. (2009) developed CAUHOI, a Stock6-derived inducer with HIR of approximately 2% and high kernel oil content (78 g kg^{-1}), that allows identification of haploids based on both, lack of *R1-nj*-encoded embryo coloration as well as low embryo oil content. While this new approach is gaining recognition in the seed industry, its reliability and applicability to large-scale DH production remains subject to further investigation.

Transplanting the colchicine-treated seedlings to the field can be accomplished more efficiently with planting machines than via manual labor. At CIMMYT, we employed

a two-row vegetable planter with rotating cones; as the cones rotate, an individual pot containing the seedling is delivered into the soil. With this machine, 6000 seedlings can be planted by five employees in three hours, while manual transplanting required eight hours and 15 employees for the same number of seedlings. However, the heavy soils at CIMMYT's main DH facility in Agua Fría, Mexico, proved problematic for mechanized planting, because: (i) the machine's cones did not rotate and open reliably when soils where wet, which was a serious constraint during the rainy summer seasons; and (ii) the biodegradable 'jiffy' pots required for mechanized transplanting did not degrade rapidly enough, thus hindering the penetration of seedlings' roots, which presumably led to inappropriate nutrient and water supply during plant establishment.

Although DH lines are generally expected to represent a random gametic array of the source germplasm (Lashermes et al. 1988), natural selection may impose a pressure during DH development, for example as a result of (i) assortative mating during haploidy induction, due to flowering synchrony between only some of the source germplasm genotypes and the haploid inducer, and/or (ii) high mutational load of landraces (Wallace 1970; Pacheco et al. 2002). For example, from 3000 putative haploid seeds obtained from 'BRAZIL1546', a Brazilian open-pollinated landrace accession used in the study of Prigge et al. (2012c), only 33 (1.1%) viable DH lines were generated (V. Prigge, unpubl. data). In contrast, on average 3-5% viable DH lines were produced from tropical single cross source germplasm (V. Prigge, unpubl. data), and this figure may rise to more than 10% once DH production processes are fully optimized, as observed in commercial production pipelines (G. A. Gordillo, 2011, pers. comm.). The lower rate of return for landrace accessions can mainly be attributed to higher mutational load exposed by the lack of compensation for the negative effects of deleterious recessive alleles in haploids (Gallais 1990). However, Wilde et al. (2010) reported that the rate of return was 6% for three European landraces when DH lines were produced following proprietary commercial protocols, indicating that optimization of the production processes may lead to increased rates of return independent of the population type.

CIMMYT's DH line production is currently centralized in Mexico, with regional breeding programs from Africa, Asia, and South America providing seeds of their source germplasm and DH lines generated from these source germplasm being returned to them. In theory, breeders should receive their DH lines two seasons after provision of the source germplasm. However, international seed exchange regulations require phytosanitary examination, which entails destructive analyses of at least 10 seeds per DH line (M. Mezzalama 2010, pers. comm.). Since many DH plants yield few seeds upon self-pollination after artificial chromosome doubling, an additional season of seed multiplication may be required to facilitate simultaneous phytosanitary testing and seed shipment, so that 3–4 seasons may pass until regional breeding programs receive DH lines. Hence, establishment of DH production pipelines at CIMMYT's regional maize breeding hubs in Kenya, Zimbabwe, India, and/or Colombia may help increase throughput of DH line production. These hubs may also serve as service providers to national maize breeding programs in neighboring countries.

Genetic Analysis and its Implications for Inducer Breeding

This study reports for the first time results of comparative QTL mapping conducted with modern inducers and temperate and tropical germplasm. The QTL *qhir1* in bin 1.04 was repeatedly detected in different populations and generations in the present

study (Prigge et al. 2012b) and previously (Deimling et al. 1997; Röber 1999; Barret et al. 2008). Likewise, *qhir8* in bin 9.01 was stable across all generations of the CAUHOI×UH400 cross. Hence, these two QTL represent target locations for fine-mapping and marker-based selection for HIR. However, the mapping populations studied by Prigge et al. (2012b) displayed variable mean HIR ranging from 0.6 to 5.2%. The authors primarily attributed these differences to the presence of modifier genes or QTL by genetic background interactions. Further, interaction of specific maternal and paternal factors such as parent-specific gene transcription levels during early embryogenesis (Meyer and Scholten 2007; Autran et al. 2011) could be responsible, given that fertilization does occur during haploidy induction in maize. Similarly, such processes could be responsible for the variable response to haploidy induction shown by diverse tropical germplasm (Kebede et al. 2011; Prigge et al. 2011). Gene expression studies will be necessary to compare transcript levels and origins determined in seeds obtained from haploid induction crosses to verify that varying parental contributions are causal of variable HIR levels.

To identify modifier loci acting on *qhir1* and other small-effect QTL for HIR, genotyping with a high-resolution marker coverage would be required. While novel methods such as genotyping-by-sequencing (Elshire et al. 2011) nowadays permit the rapid obtainment of high-density genotyping information at reasonable cost, high-throughput phenotyping of HIR remains laborious and time-consuming, regardless of the haploid identification system employed (*liguleless* vs. *R1-nj*-encoded embryo coloration). As outlined in detail by Prigge et al. (2012a, b), phenotyping of haploid induction ability is complicated by variable error variances arising from variable HIR levels of inducers and variable numbers of testcross seeds used to determine these HIR levels. Employment of equal numbers of testcross seeds for all inducer families seems the only option available to enable researchers to improve accuracy of HIR estimates, since HIR levels are a genetic characteristic. The number of testcross seeds required to detect significant differences between two inducers can be determined analytically (see Appendix A). For example, using a one-sided test, detection of HIR differences of 1% requires 2814 and 5644 testcross seeds if the reference inducer has HIR=5% and 10%, respectively (Table A.1). Hence, in mapping populations of reasonable size, considerable resources will have to be spent on testcross seed production and evaluation. Transgenic inducers expressing green fluorescence protein may be amenable to automation and could, thus, potentially facilitate reliable high-throughput detection of haploids for genetic research.

Strongly distorted allelic frequencies were observed at several chromosomal regions in the populations studied for HIR by Prigge et al. (2012b). Segregation distortion is a common phenomenon in maize (e.g., Lu et al. 2002). However, highly significant segregation distortion against the UH400 allele was observed in bin 1.04, which harbored a major QTL for HIR (Barret et al. 2008; Prigge et al. 2012b), suggesting that haploid induction ability is disfavored by natural selection. Hence, HIR levels must be constantly monitored during haploid inducer maintenance, because without breeders' selection, HIR levels are expected to gradually diminish. Further, the presumably recessive nature of the trait in combination with segregation distortion against inducer allele(s) suggests that novel genetic variation for haploid induction ability may potentially exist in open-pollinated maize populations, as the recessive mutations could hide from natural selection in heterozygous genotypes. Preliminary results from screening 10 South American maize accessions for HIR support this hypothesis (Prigge et al. 2012a). Hence, more maize accessions should be screened for haploid induction ability. Accessions with confirmed HIR could be integrated into haploid inducer breeding

activities by: (i) direct use of the accession as open-pollinated inducer and detection of haploids based on kernel oil content; (ii) recombination of the accession with established inducer stocks and selection of superior inducer genotypes from the segregating progeny; and/or (iii) production of DH lines from the accession such that reproducible units are available that can systematically be evaluated for HIR.

Backcrossing to the CML parents appeared a valuable option for obtaining tropical inducers with improved adaptation without sacrificing high levels of HIR (Prigge et al. 2012a). However, besides HIR and agronomic characteristics, the expression of purple color in aleurone and scutellum tissues are of utmost importance for tropical inducers to allow detection of haploids based on endosperm and embryo coloration patterns. In fact, several of the BC_1F_3-CML families contained plants showing red aleurone color rather than purple; some plants also contained some seeds with red and some with purple aleurone on the same ear. Similar phenotypes were observed when studying the *Pr1* locus of the anthocyanin biosynthesis pathway (Sharma et al. 2011), which is a complex system regulated by several genes depending on the tissue type (Chandler et al. 1989). The presence of the recessive *pr1* allele in homozygous state leads to red aleurone pigmentation (Sharma et al. 2011), caused by accumulation of pelargonidin rather than cyanidin (the latter is needed for purple-pigmented aleurone cells). It is possible that BC_1F_3-CML individuals with red endosperm are homozygous for the *pr1* allele. Due to this recessive inheritance, such genotypes would not permit detection of haploids among testcross progeny and, thus, would not be suitable as inducers.

The Mechanisms of *in vivo* Haploid Induction Remain Obscure

Similar to the phenomenon of heterosis (Hallauer et al. 2010), the concept of *in vivo* induction of haploidy in maize is being exploited extensively in global hybrid maize breeding, even though the underlying biological mechanism(s) are poorly understood. In fact, it is possible that different mechanisms act in different inducer genotypes. Geiger (2009, p. 649) summarized the two major hypotheses for potential mechanisms as follows:

> "(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."

Fertilization of the egg cell and subsequent elimination of paternal chromosomes is the common mechanism observed when wide crosses are used for *in vivo* induction of haploidy. Prominent examples include the "bulbosum method" for barley (Kasha and Kao 1970) and the "maize method" for wheat (Laurie and Bennet 1986). In maize seeds generated from intra-specific inducer by source germplasm crosses, micronuclei were

observed in the cytoplasm of cells of embryonic tissue (Wedzony et al. 2002; Zhang et al. 2008), which is generally considered an indicator of chromosome elimination (Kasha and Kao 1970). Further, using SSR markers, several researchers observed a variable proportion of paternal chromosomal fragments in haploid maize plants generated from pollination with a haploid inducer (Fischer 2004; Zhang et al. 2008; Li et al. 2009), thus supporting the first hypothesis formulated by Geiger (2009).

Incomplete or delayed elimination of paternal chromosomes could be one reason for presence of inducer fragments in the genome of haploids. In haploid barley embryos produced by the bulbosum method, the number of cells containing the regular seven chromosomes increased with increasing age of the embryos (Subrahmanyam and Kasha 1973). Examining *in vivo*-produced maize haploids, Wedzony et al. (2002) observed that elimination of micronuclei did not start before embryogenesis reached the globular phase. These observations led Röber (1999) to the supposition that gradual elimination of paternal chromosomes may hinder spontaneous duplication of haploids' chromosomes, as low rates of spontaneous chromosome duplication were often observed in haploids produced with *in vivo* methods in potato (Caligari et al. 1988), barley (Subrahmanyam and Kasha 1973), and maize (Chase 1952a). In contrast, presence or absence of paternal chromosomal segments at loci controlling male fertility-related processes such as anther emergence or pollen production could also be responsible for variable degrees of male fertility in haploids, a phenomenon observed in temperate and tropical maize germplasm alike (e.g., Geiger and Schönleben 2010; Kleiber et al. 2012). High-density genome-wide marker analyses of haploid plants displaying pollen-shedding anthers should be conducted to study the involvement of paternal chromosome segments in haploid fertility.

Extensive studies of male gametophytic mutants and their role in double fertilization events common in flowering plants have been conducted particularly in *Arabidopsis* (for review, see Borg et al. 2009). Of interest could be the Chromatin Assembly Factor-1 (CAF-1) pathway mutants *fas1*, *fas2*, and *msi1*, which display a range of phenotypes owing to success or failure of pollen mitotic division at different stages in *Arabidopsis* (Chen et al. 2008). The authors found that, apart from normal tricellular pollen (i.e., one vegetative nucleus and two sperm cells), CAF-1-deficient mutants also produce a fraction of bicellular pollen (i.e., the vegetative nucleus and only one fully differentiated sperm cell) which is able to successfully fertilize either the egg cell or the central cell but can obviously not perform the regular double fertilization.

This principle can be adapted to the second hypothesis suggested by Geiger (2009) for the mechanism underlying *in vivo* haploidy induction in maize, i.e., failure of fertilization of the egg cell and subsequent parthenogenetic development of the reduced egg into a haploid embryo, which is supported by several experimental studies (Sarkar and Coe 1966; Chalyk et al. 2003; Barret et al. 2008). If the single sperm cell fertilizes the central cell, the egg cell remains unfertilized and the embryo becomes haploid; if the egg cell is fertilized by the single sperm cell, the central cell remains diploid and cannot develop into a functional endosperm, potentially leading to abortion and/or resulting in phenotypes similar to defective kernel mutations. Rotarenco et al. (2009) reported that HIR of an inducer was positively correlated with the frequencies of both embryoless and endospermless kernels resulting from its self-pollination. CAF1-deficient mutants of *Arabidopsis* generally only exhibit a small fraction (6%) of pollen containing a single sperm cell (Chen et al. 2008) and, thus, in the majority of cases regular double fertilization will occur. This agrees well with the relatively low average HIR

(8–10%) commonly observed for inducer genotypes in maize. Further, reduced male transmission was reported for CAF-1 deficient mutants (Chen et al. 2008) leading to segregation distortion at the corresponding loci, a phenomenon also reported for chromosomal regions harboring major QTL for HIR in maize (Barret et al. 2008; Prigge et al. 2012b). However, Sarkar and Coe (1966) reported that all of 3830 studied pollen grains of maize inducer Stock6 were tricellular, none were bicellular.

In our study the proportion of defective kernels was termed embryo abortion rate (EAR), because the kernels showed a phenotype of "completely collapsed kernels appearing as embryoless pericarps" (Prigge et al. 2012b). However, this term may be misleading, because there was no cytological confirmation of missing embryos conducted and, therefore, presence of degenerated embryos cannot be excluded. Such embryos and degenerated kernels in general, by the above second hypothesis, may have resulted when the single available sperm cell fertilized the egg cell. Hence, the second hypothesis of haploidy induction put forward by Geiger (2009) received support by two observations reported by Prigge et al. (2012b): (i) joint presence of QTL for HIR and EAR in bin 1.04, and (ii) *dek* loci, leading to degenerated embryo and endosperm tissue in mutant carriers (Sheridan and Neuffer 1980), mapped to the same bins (1.04, 5.04, 9.01) as QTL for HIR explaining more than 10% of the genotypic variance in different populations. Without further genetic mapping or gene expression studies, it remains speculative if and how CAF-1 pathway mutants interfere with HIR but they certainly merit further investigation to elucidate the biological basis of *in vivo* haploid induction in maize.

Integration of Haploids and Doubled Haploids in Tropical Maize Breeding Programs

In recent years, a vast body of literature has emerged dealing with the optimum allocation of resources in hybrid maize breeding programs employing DH lines (e.g., Bouchez and Gallais 2002; Longin et al. 2006, 2007a, b; Gordillo and Geiger 2008a, b; Wegenast et al. 2008, 2009, 2010). It is beyond the scope of this research to provide a comprehensive review, let alone outline suitable strategies for tropical maize breeding programs aiming at implementing DH technology. However, one novel breeding scheme repeatedly mentioned is early testing, which combines selection between and within families from segregating populations (e.g., Longin et al. 2007b; Wegenast et al. 2008). Yet, to avoid extended breeding cycles, large numbers of DH lines need to be produced from individual F_2 plants, which would require the availability of inducers with HIR notably increased beyond 10%. Such inducers have not been reported thus far, but strategies outlined by Röber (1999), Prigge et al. (2012b, c), and in this chapter may contribute to their development. Alternatively, paternal haploids produced by pollinating a paternal inducer (Kermicle 1969) with pollen from individual F_2 plants could facilitate the production of large numbers of DH lines for early testing.

In vivo haploid induction may also play a vital role in improving agronomic characteristics of germplasm pools and, consequently, increase their value for maize breeding. Chalyk and Rotarenko (1999) propose to include haploid plants in recurrent selection programs by capitalizing on the generally high degree of female fertility of maize haploids (Chase 1952a; Chalyk 1994; Geiger et al. 2006). Their "haploid recurrent selection" scheme involves: (i) inducing haploids from a population to be improved

(cycle C_0); (ii) growing haploids and pollinating those featuring sufficiently fertile female flowers with pollen of the original (diploid) population; (iii) storing half of the seeds (C_1) harvested from haploids in a cold room while inducing haploidy in the other half anew; (iv) pollinating the new haploids with pollen of the improved generation C_1 to produce C_2 seeds; and so on (Chalyk and Rotarenco 1999). In this way, deleterious alleles are gradually removed from the population because there is no compensation for such alleles in the haploid phase (Gallais 1990).

Dominance and overdominance are not apparent in haploids. Taking the haploid plant as a selection unit thus enables the selection for useful genes with additive and epistatic effects, which form the basis for the improvement of populations in maize (Hallauer et al. 2010). In addition, due to significant genetic correlations between haploid lines (obtained by inducing haploidy in DH lines) and their corresponding DH lines and testcrosses for several agronomic traits, selection at the haploid level is expected to result in positively correlated genetic gain at the DH and the testcross level (Geiger 2011). Genetic variation for natural male fertility of maize haploids (e.g., Geiger and Schönleben 2011; Kleiber et al. 2012) further suggests that DH line production could be accomplished without artificial chromosome doubling in the long run, if the majority of source germplasm proves amenable to recurrent selection for increased haploid fertility. This seems particularly relevant to enable small national maize breeding programs and seed companies in developing countries to adopt the DH technology, but it will also help to increase efficiency of advanced seed companies.

CIMMYT has a long history in developing drought-tolerant and nutrient-efficient maize varieties (e.g., Edmeades et al. 1999). Further, CIMMYT populations have been continuously improved for general and specific combining ability to produce special purpose trait populations and broad-based complementary gene pools (Ortiz et al. 2011). The enormous variation contained in such populations can efficiently be captured in DH lines (Wilde et al. 2010; Prigge et al. 2012c) and made available to breeders as reproducible units that can be tested in replicated experiments. The required processes for DH production from tropical source germplasm have been established at CIMMYT, supported strongly by this research (Prigge et al. 2011, Kleiber et al. 2012; Prigge and Melchinger 2012; Prigge et al. 2012a, b, c). To fully extend its impact to resource-poor farmers, DH technology now needs to be transferred from CIMMYT to national agricultural research systems (NARS) and local seed companies in developing countries.

There are two basic options for NARS and small seed companies to implement a DH program into their breeding activities (Atlin 2010). First, NARS could produce DH lines from their own breeding populations. This may happen by (i) sending F_1 seed of their source germplasm to CIMMYT's central DH facility and receiving DH lines in return; or (ii) conducting haploidy induction at the NARS station and sending haploid seeds to the central facility for artificial chromosome duplication; or (iii) conducting both haploidy induction and chromosome duplication at the NARS site. Second, NARS could receive proprietary DH lines generated from CIMMYT source germplasm. Ordering the required number of DH lines, either from one's own or from CIMMYT's germplasm, may be the most promising option to quickly benefit from DH technology, particularly for small public and private maize breeding programs. An example of a successfully operating service provider is the maize DH facility at Iowa State University, USA (http://www.plantbreeding.iastate.edu/DHF/DHF.htm), that currently produces DH lines for several seed companies from within and outside the U.S. (T. Lübberstedt 2011, pers. comm.). A similar service could be established in regional hubs to facilitate adoption and dissemination of DH lines and thereby increase efficiency of tropical maize breeding programs.

Conclusions

In vivo production of maize haploids and DH lines was documented through a detailed protocol and a 15-min video. The video is publicly accessible at http://www.youtube.com/watch?v=V2jOEuZjjrg and may proof very valuable for capacity building.

This research demonstrated that induction of haploidy is not a limiting factor for DH line production from tropical source germplasm, even if conducted with haploid inducers of temperate origin.

We showed that it is possible to combine high HIR with adaptation to tropical climate and developed inducers with HIR of up to 10% and excellent agronomic characteristics under tropical lowland conditions. Further, this study revealed that maize genetic resources held in germplasm banks could potentially be useful as novel genetic components to enhance HIR.

QTL mapping for *in vivo* haploid induction ability suggested that the trait is controlled by one or few major QTL and several small-effect and/or modifier QTL. We identified target regions for map-based cloning and provided a conceptual genetic framework for inheritance of *in vivo* haploid induction ability, that can also be used for heredity studies in other dichotomous traits. Furthermore, we showed that natural selection presumably disfavors haploid induction ability and, therefore, monitoring of HIR during inducer maintenance breeding is of utmost importance.

This research confirmed that notable genetic variation exists for male fertility and seed development capacities in haploids of temperate and tropical origin. Hence, the current levels of fertility may be further increased by recurrent selection, thus potentially superseding artificial chromosome doubling methods for DH line production in the long term.

We developed DH lines from tropical open-pollinated populations that excelled in testcross performance, suggesting that they may be useful in tropical hybrid breeding. As the genetic base of elite material is constantly being narrowed due to recycling breeding, these DH lines may also be used to broaden the base of elite germplasm pools.

Finally, the *in vivo* DH technology was successfully implemented at CIMMYT and it is expected that tropical DH lines will proof very valuable to exploit the genetic variation currently locked in gene banks and to accelerate the arrival of improved varieties to farmers' fields.

Chapter 9

Summary

Doubled haploid (DH) technology is currently the fastest way to achieve homozygosity in maize and it offers numerous quantitative genetic, operational, logistical, and economic advantages. In temperate areas, one can hardly imagine hybrid maize breeding without DH lines anymore, yet adoption of this technology is still to be realized in tropical areas. Therefore, the main goal of my thesis project was to establish and validate the DH technology for tropical maize breeding programs at the International Maize and Wheat Improvement Center (CIMMYT) in Mexico.

In vivo production of maternal haploids and DH lines involves four steps: (i) inducing haploidy by pollinating source germplasm with pollen of a haploid inducer; (ii) identifying those seeds with haploid embryos based on a visually scorable morphological marker; (iii) duplicating chromosomes of putative haploids by treating the seedlings with a mitotic inhibitor; and (iv) self-pollinating DH plants to multiply their seed. To impart knowledge of the materials required and the methodology applied during each of the above steps, we compiled a detailed protocol and produced a publicly available video. These two comprehensive learning tools will be very useful for capacity building.

Lack of reliable information on the performance of temperate inducers under non-temperate conditions may be one reason for the slow adoption of DH technology in tropical maize breeding programs. Therefore, we assessed haploid induction rates (HIR) and agronomic performance of two temperate inducer lines (RWS and UH400) and one hybrid (RWS×UH400) in tropical lowland environments in Mexico. HIR obtained under tropical conditions were similar (8–10%) to those previously reported from evaluations under temperate conditions, indicating that temperate inducers can be used for initiation of DH breeding programs in the tropics. However, the inducers showed symptoms of poor adaptation such as poor pollen production, poor seed set, and strong susceptibility to tropical leaf diseases. Hence, better adapted inducers would be advantageous for large-scale induction of haploidy in tropical DH programs.

To develop better adapted haploid inducers, segregating populations were generated from crosses between the abovementioned temperate inducers and eight tropical CIMMYT maize lines (CML) from Mexico and Zimbabwe. Mass selection of individual F_2 plants was conducted for visually scorable and highly heritable traits, followed by family-based selection for HIR and complex agronomic traits. Several tropical inducer candidates (TIC) were identified with HIR of up to 10% and notably improved agronomic performance under tropical lowland conditions. Compared to backcrosses to the inducers, backcrosses to the CML showed similar HIR combined with a significantly

later anthesis date and improved plant vigor. Hence, backcrossing to the adapted parent may be a suitable approach to improve adaptation of new inducers while maintaining high HIR levels. Furthermore, we screened randomly chosen South American maize accessions and observed HIR of up to 3%, suggesting that novel sources of haploid induction ability may be present in CIMMYT's vast germplasm collection.

Although extensively exploited in DH line production, the genetic mechanisms underlying *in vivo* induction of maternal haploids in maize are still largely unknown. Therefore, we conducted comparative quantitative trait locus (QTL) mapping for HIR to explore the genetic architecture of this phenomenon. Segregating populations were generated from four crosses composed of two temperate haploid inducer lines (UH400 and CAUHOI) and one temperate and two tropical non-inducer lines. One major QTL on chromosome 1 (*qhir1*; bin 1.04) explaining up to 66% of the genotypic variance was detected in the three populations involving non-inducer lines. Hence, bin 1.04 represents an interesting region for map-based cloning. Further, *qhir1* was affected by strong segregation distortion against the inducer allele, indicating that natural selection disfavors haploid induction ability. Seven QTL with smaller effects were detected in the CAUHOI×UH400 population. Our results suggest that marker-based pre-selection for *qhir1* among progeny segregating for HIR, followed by phenotypic or genomic selection for HIR and agronomic characteristics among pre-selected families permits pyramiding of small-effect and/or modifier QTL. Further, we proposed a conceptual genetic framework for inheritance of *in vivo* haploid induction ability in maize.

Common methods for artificial duplication of haploid chromosome sets mostly involve toxic and costly reagents and are extremely labor-intensive. This in turn leads to serious bottlenecks during DH line development. When screening haploid populations derived from 260 diverse temperate and tropical source germplasm, we observed significant genetic variation for fertility-related traits, suggesting that haploid fertility can be effectively improved by recurrent selection. This may facilitate abolishment of artificial chromosome doubling during DH production, which seems particularly relevant for enabling small national maize breeding programs and seed companies in developing countries to adopt the DH technology.

Various types of populations can be used to extract DH lines as parental components for hybrid breeding, under the premise that they combine a high population mean with sufficient response to selection. We developed 131 DH lines from five tropical elite single crosses (SC) and five tropical open-pollinated populations (OP) and evaluated them for testcross performance in three environments in Mexico. While testcross grain yield means of the two population types did not differ significantly, significant genetic variance was only revealed for OP-derived DH lines. Several DH lines from OP excelled in testcross performance and may be useful for tropical hybrid breeding programs. In addition, tropical OP may harbor valuable untapped genetic variation that can effectively be exploited with DH technology.

Experimental results of this thesis work demonstrate that established protocols for *in vivo* DH line development can be readily applied to tropical maize breeding programs. Adoption of the DH technology promises to greatly increase the efficiency of breeding programs by shifting resources from labor-intensive inbred development to more thorough testing of inbreds. Production of DH lines is also an exciting tool to (i) immortalize genetic resources so that they can be reproduced, characterized, and conserved more easily and serve as a storage unit for valuable allelic combinations or

rare variants; (ii) conduct high-resolution genetic analyses of important traits, and perhaps most importantly; (iii) accelerate the arrival of improved varieties to farmers' fields.

Chapter 10

Zusammenfassung

Doppelhaploide (DH) Linien stellen den schnellsten Weg zur Erzeugung homozygoter Inzuchtlinien bei Mais dar und bieten darüberhinaus erhebliche Vorteile unter quantitativ-genetischen, logistischen und wirtschaftlichen Aspekten. In den gemäßigten Breiten ist Hybridmaiszüchtung ohne DH-Linien nicht mehr denkbar, doch in tropischen Maiszüchtungsprogrammen ist diese Technologie bisher kaum verwendet worden. Ziel der vorliegenden Arbeit war es daher, die DH-Technologie für den Einsatz in tropischen Maiszüchtungsprogrammen am Internationalen Mais- und Weizenforschungszentrum (CIMMYT) in Mexiko zu etablieren.

Die *in vivo* Produktion von Haploiden und DH-Linien umfasst vier Arbeitsschritte: (i) Haploideninduktion durch Bestäubung des Ausgangsmaterials mit Pollen eines Induktorgenotyps; (ii) Identifizierung der Körner mit haploidem Embryo mit Hilfe eines visuell bonitierbaren, morphologischen Markers; (iii) Verdoppelung des Chromosomensatzes haploider Pflanzen durch Behandlung mit Mitosehemmstoffen; und (iv) Selbstbestäubung der doppelhaploiden Pflanzen zur Saatgutvermehrung. Im Rahmen dieser Thesis wurde eine detaillierte Methodenbeschreibung dieser Arbeitsschritte vorgenommen und für Schulungsmaßnahmen zusätzlich als Video veröffentlicht.

Unter der Annahme, dass der Mangel an geeigneten Induktorgenotypen ein Grund für den bisher äußerst geringen Einsatz der DH-Technik in den Tropen ist, wurde die Eignung der Induktorlinien RWS und UH400 sowie der Induktorhybride RWS$\times$UH400, die allesamt an die klimatischen Bedingungen Zentraleuropas angepasst sind, für den Einsatz in tropischen Gebieten geprüft. Die Haploideninduktionsraten (HIR) dieser drei Genotypen unter tropischen Bedingungen lagen durchschnittlich zwischen 8 und 10% und entsprachen somit den unter europäischen Bedingungen ermittelten HIR. Dies zeigt, dass die geprüften Induktoren zur Initiierung von DH-Programmen auch in tropischen Gebieten eingesetzt werden können. Allerdings litten die europäischen Induktoren stark an tropischen Blattkrankheiten und zeigten verminderte Wüchsigkeit, schwache Pollenproduktion und geringen Kornansatz. Deshalb scheint es längerfristig empfehlenswert, besser an tropische Bedingungen angepasste Induktorgenotypen zu entwickeln.

Solch besser angepaßte Genotypen wurden aus spaltenden Populationen von Kreuzungen der oben genannten Induktoren mit acht CIMMYT-Maislinien (CML) aus Mexiko und Simbabwe erzeugt. Mittels Massenselektion auf hochheritable, visuell bonitierbare Merkmale an F_2-Einzelpflanzen und anschließender familien-basierter Selektion auf HIR und agronomische Merkmale konnten tropische Induktorkandidaten entwickelt werden, die HIR von bis zu 10% sowie verbesserte agronomische Eigenschaften

unter tropischen Bedingungen aufwiesen. Im Vergleich zu Familien, die durch Rückkreuzung zum Induktorelter entstanden sind, wiesen aus Rückkreuzungen zur CML entstandene Familien ein signifikant späteres Blühdatum sowie verbesserte Wüchsigkeit bei unverändert hohem HIR-Niveau auf. Rückkreuzungen zum adaptierten Elter bieten daher eine gute Möglichkeit, verbesserte Anpassung und hohe HIR in neuen Induktoren zu kombinieren. Desweiteren wurden bei der Prüfung von südamerikanischen Genbank-Akzessionen HIR von bis zu 3% festgestellt. Dies deutet an, dass neuartige genetische Variation für das Merkmal Haploideninduktionsfähigkeit möglicherweise in solchen Akzessionen zu finden ist.

Die *in vivo* Produktion von maternalen Haploiden wird intensiv zur DH-Linienerzeugung genutzt, doch die ihr unterliegenden genetischen Mechanismen sind weitgehend unbekannt. Im Rahmen der vorliegenden Arbeit wurde daher eine vergleichende Analyse der verantwortlichen Genorte (QTL = quantitative trait loci) durchgeführt, um die genetische Architektur des Merkmals Haploideninduktionsfähigkeit zu untersuchen. Aus zwei Induktorlinien (UH400 und CAUHOI), einer chinesischen und zwei tropischen Inzuchtlinien mit HIR=0% wurden vier Kartierungspopulationen erzeugt. In den drei Induktor × Nicht-Induktor-Populationen wurde ein Haupt-QTL (*qhir1*) auf Chromosom 1 (Abschnitt 1.04) detektiert, das bis zu 66% der genetischen Varianz erklärte. Diese chromosomale Region erscheint daher sehr interessant für Feinkartierungs- und Klonierungsansätze. Die zu Ungunsten des Induktorallels signifikant vom Mendelschen Aufspaltungsverhältnis abweichenden Allelfrequenzen an den *qhir1*-flankierenden Loci weisen ferner darauf hin, dass natürliche Selektion gegen das Merkmal Haploideninduktionsfähigkeit agiert. In der CAUHOI×UH400-Population wurden sieben weitere QTL mit kleineren Effekten detektiert. Zur Pyramidisierung mehrerer QTL erscheint es daher sinnvoll, zuerst eine marker-gestützte Vorselektion auf *qhir1* in spaltenden Populationen durchzuführen und anschließend phänotypische oder genomische Selektionsverfahren auf dieser vorselektierten Fraktion anzuwenden. In Rahmen dieser Arbeit wurde zudem ein genetisches Konzept für die Vererbung der Haploideninduktionsfähigkeit vorgestellt.

Die üblicherweise eingesetzten Methoden zur künstlichen Chromosomensatzverdoppelung von Haploiden basieren auf gesundheitlich bedenklichen Chemikalien und sind sehr kosten- und arbeitsintensiv, so dass dieser Produktionschschritt meist als Nadelöhr während der DH-Linienentwicklung gilt. Daher wurden im Rahmen dieser Arbeit haploide Maispopulationen von 260 genetisch diversen, tropischen und gemässigten Ausgangsmaterialien auf Fertilitätsmerkmale geprüft. Hierbei wurde signifikante genetische Variation beobachtet, so dass rekurrente Selektionsverfahren zur Erhöhung der Haploidenfertilität aussichtsreich scheinen. Dies könnte inbesondere kleineren nationalen Maiszüchtungsprogrammen und Saatgutfirmen den Einsatz der DH-Technologie ermöglichen, da es langfristig den Einsatz künstlicher Chromosomenaufdoppelungsmethoden überflüssig macht.

Die DH-Technik erlaubt eine erhebliche Beschleunigung der Erzeugung elterlicher Komponenten und verschiedene Populationstypen können als Ausgangsmaterial genutzt werden. Die Feldevaluierung von 131 DH-Linien, die aus tropischen Einfachkreuzungen und offen-stäubenden Populationen (OP) entwickelt wurden, zielte daher auf die Prüfung der Testkreuzungsleistung dieser Linien in drei Umwelten in Mexiko ab. Die mittleren Testkreuzungskornerträge der beiden Populationstypen unterschieden sich nicht signifikant. Signifikante genetische Varianz konnte hingegen nur bei den aus OP entwickelten DH-Linien beobachtet werden. Zudem zeigten einige aus OP entwickelte

DH-Linien hervorragende Testkreuzungsleistungen. Dies zeigt, dass genetische Variation in OP mit Hilfe der DH-Technik effektiv für die Maiszüchtung nutzbar gemacht werden kann. Sollten diese Ergebnisse bei Versuchen in den Zielumwelten bestätigt werden, so könnten die im Rahmen dieser Arbeit entwickelten DH-Linien direkt als Elternkomponenten verbesserter Hybridsorten für tropische Gebiete eingesetzt werden.

Die experimentellen Ergebnisse dieser Arbeit zeigen, dass die zuvor für Züchtungsprogramme der gemäßigten Zonen etablierte Methodik der DH-Technik ohne weiteres in tropischen Regionen eingesetzt werden kann. Ihre Verwendung in tropischen Maiszüchtungsprogrammen dürfte zu erheblichen Effizienzsteigerungen und damit verbundenem gesteigertem Züchtungsfortschritt führen. Des weiteren eignen sich DH-Linien zur Erschließung genetischer Ressourcen für die Maiszüchtung, zur genetischen Analyse wichtiger Merkmale, sowie vorrangig als Elternkomponenten verbesserter Maissorten.

Chapter 11

References

Atlin GN (2010) Possible models for use of DH technology in African maize breeding and the DTMA project. Presentation held at the Drought Tolerant Maize for Africa (DTMA) Annual Project Meeting, 13–18 Sept., 2010, Nairobi, Kenya.

Autran D, Baroux C, Raissig MT, Lenormand T, Wittig M, Grob S, Steimer A, Barann M, Klostermeier UC, Leblanc O, Vielle-Calzada J-P, Rosenstiel P, Grimanelli D, Grossniklaus U (2011) Maternal epigenetic pathways control parental contributions to *Arabidopsis* early embryogenesis. Cell 145:707–719.

Barret P, Brinkmann M, Beckert M (2008) A major locus expressed in the male gametophyte with incomplete penetrance is responsible for in situ gynogenesis in maize. Theor Appl Genet 117:581–594.

Barnabás B, Obert B, Kovács G (1999) Colchicine, an efficient genome doubling agent for maize (*Zea mays* L.) microspores cultured in anthero. Plant Cell Rep 18:858–862.

Beaumont VH, Widholm JM (1993) Ploidy variation of pronamide-treated maize calli during long term culture. Plant Cell Rep 12:648–651.

Belicuas PR, Guimarães CT, Paiva LV, Duarte JM, Maluf WR, Paiva E (2007) Androgenic haploid and SSR markers as tool for the development of tropical maize hybrids. Euphytica 156:95–102.

Bordes J, Dumas de Vaulx R, Lapierre A, Pollacsek M (1997) Haplodiploidization of maize (*Zea mays* L.) through induced gynogenesis assisted by glossy markers and its use in breeding. Agronomie 17:291-297.

Borg M, Brownfield L, Twell D (2009) Male gametophyte development: a molecular perspective. J Exp Bot 60:1465-1478.

Bouchez A, Gallais A (2007) Efficiency of the use of doubled-haploids in recurrent selection for combining ability. Crop Sci 40:23–29.

Büter B (1997) In vitro haploid production in maize. p. 37–71. In: Jain SM, Sopory SK, Veilleux RE (eds.) In Vitro Haploid Production in Higher Plants. Kluwer Academic Publishers, Dordrecht.

Caligari PDS, Powell W, Liddell K, de Maine MJ, Swan GEL (1988) Methods and strategies for detecting *Solanum tuberosum* dihaploids in interspecific crosses with *S. phureja*. Ann Appl Biol 112:323–328.

Chalyk ST (1994) Properties of maternal haploid maize plants and potential application to maize breeding. Euphytica 79:13–18.

Chalyk S, Baumann A, Daniel G, Eder J (2003) Aneuploidy as a possible cause of haploid-induction in maize. Maize Genet Newsl 77:29-30.

Chalyk ST, Rotarenco VA (1999) Using maternal haploid plants in recurrent selection in maize. Maize Genet Newsl 73:56–57.

Chandler VL, Radicella JP, Robbins TP, Chen J, Turks D (1989) Two regulatory genes of the maize anthocyanin pathway are homologous: isolation of B using R genomic sequences. Plant Cell 1:1175–1183.

Chase SS (1952a) Monoploids in maize. p. 389–399. In: Gowen JW (ed.) Heterosis. Iowa State College Press, Ames, IA, USA.

Chase SS (1952b) Production of homozygous diploids of maize from monoploids. Agron J 44:263–267.

Chebotar OD, Chalyk ST (1996) The use of maternal haploids for genetic analysis of the number of kernel rows per ear in maize. Hereditas 124:173–178-

Chen Z, Tan JLH, Ingouff M, Sundaresan V, Berger F (2008) Chromatin assembly Factor 1 regulates the cell cycle but not the cell fate during male gametogenesis in *Arabidopsis thaliana*. Development 135:65–73.

Chen S, Li L, Li H (2009) Maize Doubled Haploid Breeding (in Chinese). China Agricultural University Press, Beijing.

Coe EH (1959) A line of maize with high haploid frequency. Am Nat 93:381–382.

Coe EH (1962) Spontaneous mutation of the aleurone color inhibitor in maize. Genetics 47:779–783.

Deimling S, Röber FK, Geiger HH (1997) Methodik und Genetik der in-vivo-Haploideninduktion bei Mais. Vortr Pflanzenzüchtg 38:203–224.

Dowswell CR, Paliwal RL, Cantrell RP (1996) Maize in the Third World. Westview Press, Oxford.

Dunwell JM (2010) Haploids in flowering plants: origins and exploitation. Plant Biotech J 8:327–424.

Eder J, Chalyk ST (2002) In vivo haploid induction in maize. Theor Appl Genet 104:703–708.

Edmeades GO, Bolanos J, Chapman SC, Lafitte HR, Bänziger M (1999) Selection improves drought tolerance in tropical maize populations. 1. Gains in biomass, grain yield and harvest index. Crop Sci 39:1306–1315.

Elshire RJ, Glaubitz JC, Sun Q, Poland JA, Kawamoto K, Buckler ES, Mitchell SE (2011) A robust, simple genotyping-by-sequencing (GBS) approach for high diversity species. PLoS One 6(5): e19379. doi:10.1371/journal.pone.0019379.

FAOSTAT (2011) Statistical databases and data-sets of the Food and Agriculture Organization of the United Nations. http://faostat.fao.org/default.aspx (accessed July 2011).

Fischer E (2004) Molekulargenetische Untersuchungen zum Vorkommen paternaler DNA-Übertragung bei der *in-vivo*-Haploideninduktion bei Mais (*Zea mays*). Dissertation, University of Hohenheim, Germany.

Gallais A (1990) Quantitative genetics of doubled haploid populations and application to the theory of line development. Genetics 124:199–206.

Gayen P, Madan J, Kumar R, Sarkar K (1994) Chromosome doubling in haploids through colchicine. Maize Genet Newsl 68:65.

Geiger HH (2009) Doubled Haploids. p. 641–657. In: Bennetzen JL, Hake S (eds.) Maize Handbook - Volume II: Genetics and Genomics, Springer Science and Business Media, New York.

Geiger HH (2011) Genetic correlations between haploids, doubled haploids, and testcrosses in maize. p. 65. In: Conference Book of the 22nd EUCARPIA Maize and Sorghum Conference, June 19–22, Opatija, Croatia.

Geiger HH, Schönleben M (2011) Incidence of male fertility in haploid elite dent maize germplasm. Maize Genet Newsl 85:22–32.

Geiger HH, Braun MD, Gordillo GA, Koch S, Jesse J, Krützfeldt BAE (2006) Variation for female fertility among haploid maize lines. Maize Genet Newsl 80:28–29.

Gordillo GA, Geiger HH (2008a) Optimization of DH-line based recurrent selection procedures in maize under a restricted annual loss of genetic variance. Euphytica 161:141–154

Gordillo GA, Geiger HH (2008b) Alternative recurrent selection strategies using doubled haploid lines in hybrid maize breeding. Crop Sci 48:911–922

Hallauer AR, Carena MJ, Miranda Filho JB (2010) Quantitative Genetics in Maize Breeding. Springer Science and Business Media, New York.

Häntzschel KR (2011) Bestimmung und Optimierung von Colchicin-Alternativen für die Doppelhaploiden-Technik bei Mais (*Zea mays* L.). Dissertation, University of Hohenheim, Germany.

Hartung J, Elpelt B, Klösener K-H (2005) Statistik: Lehr- und Handbuch der angewandten Statistik, 14th edn. Oldenbourg Verlag, Wien.

Jensen CJ (1974) Chromosome doubling techniques in haploids. p. 153–190. In: Kasha KJ (ed.) Haploids in Higher Plants: Advances and Potential. University of Guelph, Guelph.

Kasha KJ (2005) Chromosome doubling and recovery of doubled haploid plants. p. 123–152. In: Palmer CE, Keller WA, Kasha KJ (eds.) Biotechnology in Agriculture and Forestry, Vol. 56 Haploids in Crop Improvement II. Springer, Berlin.

Kasha KJ, Kao KN (1970) High frequency haploid production in barley (*Hordeum vulgare* L.). Nature 225:874–876.

Kato A (2002) Chromosome doubling of haploid maize seedlings using nitrous oxide gas at the flower primordial stage. Plant Breed 121:370–377.

Kebede AZ, Dhillon BS, Schipprack W, Araus JL, Bänziger M, Semagn K, Alvarado G, Melchinger AE (2011) Effect of source germplasm and season on the in vivo haploid induction rate in tropical maize. Euphytica 180:219–226.

Kermicle JL (1969) Androgenesis conditioned by a mutation in maize. Science 166:1422–1424.

Kleiber D, Prigge V, Melchinger AE, Burkard F, San Vicente F, Palomino G, Gordillo GA (2012) Haploid fertility in temperate and tropical maize germplasm. Crop Sci 52:623–630.

Lashermes P, Beckert M (1988) Genetic control of maternal haploidy in maize (*Zea mays* L.) and selection of haploid inducing lines. Theor Appl Genet 76:405–410.

Lashermes P, Gaillard A, Beckert M (1988) Gynogenetic haploid plants analysis for agronomic and enzymatic markers in maize (*Zea mays* L.). Theor Appl Genet 76:570–572.

Laurie DA, Bennett MD (1986) Wheat by maize hybridization. Can J Genet Cytol 28:313–316.

Li L, Xu X, Jin W, Chen S (2009) Morphological and molecular evidences for DNA introgression in haploid induction via a high oil inducer CAUHOI in maize. Planta 230:367–376.

Longin CFH, Utz HF, Reif JC, Schipprack W, Melchinger AE (2006) Hybrid maize breeding with doubled haploids: I. One-stage versus two-stage selection for testcross performance. Theor Appl Genet 112:903–912.

Longin CFH, Utz HF, Melchinger AE, Reif JC (2007a) Hybrid maize breeding with doubled haploids: II. Optimum type and number of testers in two-stage selection for general combining ability. Theor Appl Genet 114:393–402.

Longin CFH, Utz HF, Reif JC, Wegenast T, Schipprack W, Melchinger AE (2007b) Hybrid maize breeding with doubled haploids: III. Efficiency of early testing prior to doubled haploid production in two-stage selection for testcross performance. Theor Appl Genet 115:519–527.

Lu H, Romero-Severson J, Bernardo R (2002) Chromosomal regions associated with segregation distortion in maize. Theor Appl Genet 105:622–628.

Mahuku G, Martinez L, Prigge V, Kebede AZ, Araus JL (2010) Progress in doubled haploid production at CIMMYT. Presentation held at the Drought Tolerant Maize for Africa (DTMA) Annual Project Meeting, 13–18 Sept., 2010, Nairobi, Kenya.

Melchinger AE, Longin CFH, Utz HF, Reif JC (2005) Hybrid maize breeding with doubled haploid lines: quantitative genetic and selection theory for optimum allocation of resources. p. 8–21. In: Proc. of the 41st Annual Illinois Corn Breeders School 2005, Urbana-Champaign, IL. 7–8 Mar. 2005. Univ. of Illinois at Urbana-Champaign.

Meyer S, Scholten S (2007) Equivalent parental contributions to early plant zygotic development. Curr Biol 17:1686–1691.

Nanda DK, Chase SS (1966) An embryo marker for detecting monoploids of maize (*Zea mays* L.). Crop Sci 6:213–215.

Nei M (1963) The efficiency of haploid methods of plant breeding. Heredity 18:95–100.

Neuffer MG, Coe EH, Wessler SR (1997) Mutants of Maize. CSHL Press, New York.

Ortiz R, Taba S, Chávez Tovar VH, Mezzalama M, Xu Y, Yan J, Crouch JH (2010) Conserving and enhancing maize genetic resources as global public goods – A perspective from CIMMYT. Crop Sci 50:13–28.

Pacheco CAP, dos Santos MX, Cruz CD, Parentoni SN, de Oliveira Guimarães PE, Comes e Gama EE, da Silva AE, Lemos de Carvalho HW, Vieira Júnior PA (2002) Inbreeding depression of 28 maize elite open pollinated varieties. Genet Mol Biol 25:441–448.

Pixley KV, Bänziger M (2004) Open-pollinated maize varieties: A backward step or valuable option for farmers? p. 22–29. In: Friesen DK, Palmer AEF (eds.) Integrated approaches to higher maize productivity in the new millennium. Proc. of the 7th Eastern and Southern Africa Regional Maize Conf, Nairobi, Kenya. 5–11 Feb. 2004.

Pollak LM (2003) The history and success of the public-private project on germplasm enhancement of maize (GEM). Advances in Agronomy 78:45–87.

Prigge V, Melchinger AE (2012) Production of haploids and doubled haploids in maize. In: Loyola-Vargas VM, Ochoa-Alejo N (eds.) Plant Cell Culture Protocols. Third edition. Humana Press - Springer Verlag, Totowa, New Jersey. In Press.

Prigge V, Sánchez C, Dhillon BS, Schipprack W, Araus JL, Bänziger M, Melchinger AE (2011) Doubled haploids in tropical maize: I. Effects of inducers and source germplasm on *in vivo* haploid induction rates. Crop Sci 51:1498–1506.

Prigge V, Schipprack W, Mahuku G, Atlin GN, Melchinger AE (2012a) Development of *in vivo* haploid inducers for tropical maize breeding programs. Euphytica. In Press.

Prigge V, Xu X, Li L, Babu R, Chen S, Atlin GN, Melchinger AE (2012b) New insights into the genetics of *in vivo* induction of maternal haploids, the backbone of doubled haploid technology in maize. Genetics 190:781–793.

Prigge V, Babu R, Das B, Hernández Rodriguez M, Atlin GN, Melchinger AE (2012c) Doubled haploids in tropical maize: II. Quantitative genetic parameters for testcross performance. Euphytica. In Press.

Rao PS, Suprasanna P (1996) Methods to double haploid chromosome numbers. p. 317-339. In: Jain SM, Sopory SK, Veilleux RE (eds.) In Vitro Haploid Production in Higher Plants, vol 1. Kluwer Academic Publishers, Dordrecht.

Röber FK (1999) Fortpflanzungsbiologische und genetische Untersuchungen mit RFLP-Markern zur in-vivo-Haploideninduktion bei Mais. Dissertation, University of Hohenheim, Germany.

Röber FK, Gordillo GA, Geiger HH (2005) *In vivo* haploid induction in maize – performance of new inducers and significance for doubled haploid lines in hybrid breeding. Maydica 50:275–283.

Rosegrant MW, Cai X, Cline SA (2002) World Water and Food to 2025. International Food Policy Research Institute, Washington, D.C., available at http://www.ifpri.org/sites/default/files/pubs/pubs/books/water2025/water2025.pdf (accessed July 2011).

Rotarenco VA, Kirtoca IH, Jacota AG (2007) Possibility to identify kernels with haploid embryo by oil content. Maize Genet Newsl 81:11.

Rotarenco V, Dicu G, Sarmaniuc M (2009) Induction of maternal haploids in maize. Maize Genet Newsl 83:15–17.

Rotarenco V, Dicu G, State D, Fuia S (2010) New inducers of maternal haploids in maize. Maize Genet Newsl 84:15.

Sarkar KR, Coe EH (1966) A genetic analysis of the origin of maternal haploids in maize. Genetics 54:453–464.

Schmidt W (2003) Hybridmaizüchtung bei der KWS SAAT AG. p. 1-6. In: Bericht über die Arbeitstagung der Vereinigung der Pflanzenzüchter und Saatgutkaufleute Österreichs, Gumpenstein, Österreich, 25.–27. November.

Seitz G (2004) Interview published in Farmjournal, link provided at: http://www.greatlakeshybrids.com/performance/research-information/doubled-haploid-breeding-technology/ (accessed July 2011).

Seitz G (2005) The use of doubled haploids in corn breeding. p. 1–7. In: Proc. of the 41st Annual Illinois Corn Breeders' School 2005. Urbana-Champaign, Illinois, USA.

Sharma M, Cortes-Cruz M, Ahern KR, McMullen M, Brutnell TP, Chopra S (2011) Identification of the *Pr1* gene product completes the anthocyanin biosynthesis pathway of maize. Genetics 188:69–79.

Shatskaya OA, Zabirova ER, Shcherbak VS, Chumak MV (1994) Mass induction of maternal haploids in corn. Maize Genet Newsl 68:51.

Sheridan W, Neuffer MG (1980) Defective kernel mutants of maize. II. Morphological and embryo culture studies. Genetics 95:945–960.

Smith JSC, Hussain T, Jones ES, Graham G, Podlich D, Wall S, Williams M (2008) Use of doubled haploids in maize breeding: implications for intellectual property protection and genetic diversity in hybrid crops. Mol Breed 22:51–59.

Subrahmanyam NC, Kasha KH (1973) Selective chromosomal elimination during haploid formation in barley following interspecific hybridization. Chromosoma 42:111–125

Swanson EB, Coumans MP, Wu SC, Barsby TL, Beversdorf WD (1987) Efficient isolation of microspores and the production of microspore-derived embryos from *Brassica napus*. Plant Cell Rep 6:94–97.

Tang F, Tao Y, Zhao T, Wang G (2006) *In vitro* production of haploid and doubled haploid plants from pollinated ovaries of maize (*Zea mays*). Plant Cell Tissue Organ Cult 84:233-237.

Tang Q-L, Feng Y-C, Han X-L, Zheng M-M, Rong T-Z (2009) Study on haploid inducing and its meiotic abnormality in maize. Agricultural Sciences in China 8:1159–1165, doi:10.1016/S1671-2927(08)60325-9

Touraev A, Forster B, Jain SM (2009) Advances in Haploid Production in Higher Plants. Springer Science and Business Media, New York.

UN Population Division (2011) World Population Prospects: The 2010 Revision. http://esa.un.org/unpd/wpp/Other-Information/Press_Release_WPP2010.pdf (accessed July 2011)

Wallace B (1970) Genetic Load. Prentice-Hall, Inc., Englewood Cliffs, New York.

Wan Y, Petolino J, Widholm J (1989) Efficient production of doubled haploid plants through colchicine treatment of anther-derived maize callus. Theor Appl Genet 77:889–892.

Wan Y, Duncan DR, Raybun AL, Petolino JF, Widholm JM (1991) The use of antimicrotubule herbicides for the production of doubled haploid plants from anther-derived maize callus. Theor Appl Genet 81:205–211.

Wedzony M, Röber F, Geiger HH (2002) Chromosome elimination observed in selfed progenies of maize inducer line RWS. p. 173. In: VII Intern. Congress on Sexual Plant Reproduction. Maria Curie-Sklodowska University Press, Lublin, Poland.

Wegenast T, Longin CFH, Utz HF, Melchinger AE, Maurer HP, Reif JC (2008) Hybrid maize breeding with doubled haploids: IV. Number versus size of crosses and importance of parental selection in two-stage selection for testcross performance. Theor Appl Genet 117:251–260.

Wegenast T, Dhillon BS, Utz HF, Longin CFH, Maurer HP, Melchinger AE (2009) Comparison of selection strategies and optimization criteria in hybrid maize breeding with doubled haploids. Maydica 54:343–352.

Wegenast T, Utz HF, Longin CFH, Maurer HP, Dhillon BS, Melchinger AE (2010) Hybrid maize breeding with doubled haploids: V. Selection strategies for testcross performance with variable sizes of crosses and S_1 families. Theor Appl Genet 120:699–708.

Wilde K, Burger H, Prigge V, Presterl T, Schmidt W, Ouzunova M, Geiger HH (2010) Testcross performance of doubled-haploid lines developed from European flint maize landraces. Plant Breed 129:181–185.

Yan J, Yang X, Shah T, Sánchez-Villeda H, Li J, Warburton M, Zhou Y, Crouch J, Xu Y (2010) High-throughput SNP genotyping with the GoldenGate assay in maize. Mol Breeding 25:441–451.

Zhang ZL, Qiu FZ, Liu YZ, Ma KJ, Li ZY, Xu SZ (2008) Chromosome elimination and in vivo haploid induction by stock 6-derived inducer line in maize (*Zea mays* L.). Plant Cell Rep 27:1851–1860.

Appendix A

Minimum Number of Testcross Seeds

Based on a binomial distribution (induction of haploidy yes/no), we can formulate the hypothesis that two inducers belong to the same population, namely the population that encompasses all genotypes having a certain haploid induction rate (HIR). We test whether two inducers have the same HIR ($H_0 : p_0 = p_1$) using a two-sided test. We estimate the number of testcross seeds N required to detect significant differences between p_0 and p_1 with this two-sided test using the following formula (Hartung et al. 2005, p. 206):

$$N \geq \left(\frac{\sqrt{p_0(1-p_0)}u_{1-\alpha/2} + \sqrt{p_1(1-p_1)}u_{1-\beta}}{p_1 - p_0} \right)^2 \tag{A.1}$$

where N = required number of testcross seeds, p_0 and p_1 = HIR of two inducers, α = Type I error rate, β = Type II error rate, and u = quantile of the standard normal distribution. The assumption of $\alpha = \beta = 0.1$ results in $u_{1-\alpha/2} = 1.6449$ and $u_{1-\beta} = 1.2816$ for the two-sided test (Hartung et al. 2005, p. 891).

If we want to test whether one inducer exhibits a higher HIR than a reference inducer, then a one-sided test is appropriate ($H_0 : p_1 \geq p_0$). To estimate N we use the formula:

$$N \geq \left(\frac{\sqrt{p_0(1-p_0)}u_{1-\alpha} + \sqrt{p_1(1-p_1)}u_{1-\beta}}{p_1 - p_0} \right)^2 \tag{A.2}$$

where the symbols are as above. The assumption of $\alpha = \beta = 0.1$ results in $u_{1-\alpha} = u_{1-\beta} = 1.2816$ for the one-sided test (Hartung et al. 2005, p. 891).

Hence, if we want to be able to detect 1% ($p_1 - p_0 = 0.01$) differences between the HIR of two inducers, and if the average HIR of one inducer is 4% ($p_1 = 0.04$), then a minimum of $N = 2828$ testcross seeds is required following the two-sided approach (Table A.1). This number is reduced to $N = 2207$ when applying the one-sided approach. If the average HIR of inducers is 10%, a minimum of $N = 7314$ and $N = 5644$

testcrosss seeds are required to detect significant differences of 1% with the two-sided and one-sided tests, respectively.

Table A.1. Minimum number of testcross seeds N required to detect significant differences ($p_1 - p_0$) between haploid induction rates of two inducer genotypes with two-sided ($H_0 : p_0 = p_1$) and one-sided ($H_0 : p_1 \geq p_0$) tests assuming different p_1 of one of the two inducers.

	p_1 (Haploid induction rate)					
$p_1 - p_0$	0.01	0.02	0.03	0.04	0.05	0.10
	N, two-sided test					
0.005	2373	5757	9042	12252	15392	30053
0.01	–	1178	2106	2828	3620	7314
0.02	–	–	366	580	784	1726
0.05	–	–	–	–	–	221
	N, one-sided test					
0.005	1900	4495	7013	9475	11881	23121
0.01	–	973	1585	2207	2814	5644
0.02	–	–	300	464	620	1341
0.05	—	–	–	–	–	177

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