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Improvements of the Doubled Haploid Technology in Maize

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Chapter 1

General introduction

In vivo doubled haploid (DH) production has revolutionized the process of obtaining homozygous lines in maize. Compared to traditional line development by recurrent selfing, which takes about 6 generations to reach acceptable levels of homozygosity, the DH technology results in fully homozygous lines within only two generations. Furthermore, logistic requirements in line development are reduced as well as costs of labor and consumables, making line development faster and more efficient. These advantages of the DH technology enable breeders to react more quickly to new market requirements in development of hybrid varieties.

By reducing breeding cycle length and enabling increased selection intensities, the DH technology has the potential to lead to higher selection response per unit of time. Release of the full genetic variance from the source germplasm with the DH technology (Melchinger et al., 2005) increases the heritability and thus power to detect QTL (Orsini et al., 2012), as well as the prediction accuracy in genomic selection. Therefore, DH lines are widely used in breeding (Schmidt, 2003; Seitz, 2005) and in genomic selection studies (Albrecht et al., 2011; Riedelsheimer and Melchinger, 2013; Lian et al., 2014; Albrecht et al., 2014). Thus, the DH technology holds numerous advantages and applications for breeding and research.

The efficiency with which DH lines can be produced has driven the widespread use of genomic selection, because with increasing numbers of untested lines, the number of candidate hybrids increases exponentially. Further increases in DH production efficiency have potential to enable new breeding schemes with shorter cycle length or with early testing prior to DH production, which would increase selection gain based on theoretical considerations (Longin et al., 2007). Since production costs per DH line have a larger impact on selection gain than testcross production costs (Marulanda et al., 2016), increasing the efficiency of DH production is vital to increasing selection gain.

To produce DH lines by the *in vivo* method, four simple steps are required, reviewed by Molenaar and Melchinger (2019). These steps are i) induction of maternal haploid seeds with inducer genotypes, ii) sorting of haploid from diploid seeds, iii) chromosome doubling of D_0 seedlings, and iv) selfing of doubled haploids to produce D_1 ears with seed set. Recent advances

in the first two steps have enabled large numbers of haploid seeds to be simply produced and identified.

Haploid induction

Maternal haploids are induced by pollinating the source germplasm with an inducer genotype. Due to the monoecious floral biology of maize, induction crosses can be simply made by manual pollination or on a larger scale in isolation plots as used for hybrid seed production. The resulting induction-cross seed comprises normal hybrid seeds and maternal haploid seeds.

The genetic basis for haploid induction was recently revealed independently by three research groups. Within the 243 kb genomic region of quantitative trait locus (QTL) *qhir1* fine mapped by Dong et al. (2013), the three research groups cloned the single gene mandatory for haploid induction and named it: MATRILINEAL (Kelliher et al., 2017), NOT LIKE DAD (Gilles et al., 2017) and ZmPLA1 (Liu et al., 2017). Haploid induction is however governed by at least seven further QTL (Deimling et al., 1997; Barret et al., 2008; Prigge et al., 2012b).

The first haploid inducer genotype, Stock 6, possessed an induction rate of 2-3% (Coe, 1959). Breeding efforts have increased the haploid induction rate to 10% in modern inducer genotypes (Melchinger et al., 2016a), which all trace back to Stock 6 (Hu et al., 2016). To develop inducers with high induction rates, the pedigree method with selection of highly heritable traits in single plants of the F_2 followed by family-based selection for haploid induction rate was demonstrated to be an effective method (Prigge et al., 2012a). Backcrossing and marker assisted selection using the *qhir1* locus in combination with phenotyping have also been used to improve adaptation and develop new inducers (Dong et al., 2014; Chaikam et al., 2018). Altogether, the simple production of induction cross seeds and sufficiently high rate of haploid induction enable large numbers of haploid seeds to be produced at relatively low cost (Melchinger et al., 2016b).

Identification of haploids

Generally, markers or traits inherent to the inducer genotype are expressed in F_1 seeds but not in haploid seeds, thus making discrimination between haploid and diploid seeds possible. The standard method for sorting haploid and diploid seeds is by visual classification based on the *R1-nj* or “red crown” marker. However, in tropical and Flint germplasm that often carry inhibitor genes such as *C1-I*, or in germplasm where expression of the *R1-nj* marker is completely masked due to red or blue coloration of the pericarp, the standard visual sorting based on the *R1-nj* marker is not possible.

Sorting of haploid seeds from diploid crossing seeds in germplasm where the *R1-nj* marker is inefficient has witnessed substantial progress during the last decade. Firstly, new markers such as the red root marker (Chaikam et al., 2016) and transgenic green fluorescent protein (Yu and Birchler, 2016) or traits such as oil content (Melchinger et al., 2013) have been incorporated in inducers making haploid identification more reliable. Secondly, high throughput platforms have been developed which enable automatic sorting of haploid from diploid seeds using oil content (Wang et al., 2016; Melchinger et al., 2018). As a result, it has become possible to identify haploids in most maize germplasm, even automatically with minimal labor requirements.

Chromosome doubling

The chromosome doubling step in DH production currently involves artificial chromosome doubling treatment because on average ≤1% of untreated D_0 haploid plants are fertile in temperate and tropical germplasm (Kleiber et al., 2012). Problems in reductional cell division during meiosis in haploids generally makes haploids sterile, although untreated D_0 haploid plants can produce fertile pollen (Wu et al., 2017). This phenomenon is referred to in the literature as "haploid male fertility" or "spontaneous chromosome doubling."

Chromosome doubling represents a bottleneck in DH production because in both spontaneous and chemically induced chromosome doubling the majority of D_0 haploid plants are sterile and thus cannot be selfed to result in DH lines. This bottleneck is important because the rate of artificial chromosome doubling determines how many seeds, seedlings or plants must be processed in each production step and thus has a strong influence on the total production cost per DH line (Melchinger et al., 2016b).

State of the art

Colchicine treatment of seedlings is currently the standard method for chromosome doubling (Chaikam and Mahuku, 2012; Prigge and Melchinger, 2012). Briefly, putative haploid seeds are germinated in a climate chamber on germination paper and seedlings that reach a shoot length of approximately 2cm are removed for treatment on usually three successive days until only non-germinated or crippled seedlings remain which will be discarded. On each day, the tip of each seedling shoot is cut to facilitate uptake of the treatment solution, and seedlings are submerged in an aqueous solution of 0.06% colchicine and 0.5% dimethyl sulfoxide for eight hours. After treatment, seedlings are thoroughly rinsed to remove traces of the treatment

solution, and planted in small pots in a greenhouse for cultivation until seedlings are ready to be transplanted into the DH nursery.

Chromosome doubling treatment increases the proportion of fertile plants due to economically acceptable doubling rates of approximately 10 to 30%, depending on the germplasm and environment (Eder and Chalyk, 2002; Chaikam and Mahuku, 2012). Treatment with colchicine inhibits formation of microtubules during cell division so that chromosomes are not pulled apart during mitosis. As a result, the number of chromosomes in the cell is duplicated (Bartels and Hilton, 1973; Wan et al., 1991; Chaikam and Mahuku, 2012). With current treatment protocols, the majority of treated D_0 plants are still sterile after treatment and cannot be selfed to produce DH lines. Thus, increases in artificial chromosome doubling rates would be desirable.

Furthermore, disadvantages of colchicine make alternative chemicals desirable. The high toxicity of colchicine is a concern for workplace safety. For example, the gap between beneficial and lethal effects of medical colchicine is small, with 100% mortality at concentrations above 0.8 mg kg^{-1} body weight (U.S. Food and Drug Administration, 2009). Therefore, people handling colchicine must be trained in correct personal protection measures, handling and storage. After treatment, the solution must be properly disposed of, which is expensive and difficult to implement in developing countries (B.M. Prasanna, personal communication, 2015). Alternative chemicals to colchicine would increase worker safety and make the chromosome doubling step of DH production simpler to implement in developing countries.

Chromosome doubling: potential improvements

When considering improvements of the chromosome doubling step in DH production, two main paths are possible: *i*) chromosome doubling by alternative chemical treatments or *ii*) chromosome doubling without treatment by spontaneous chromosome doubling. The more immediate path is artificial chromosome doubling treatment, because this simply involves a modification of the current standard treatment protocol. The alternative path, spontaneous chromosome doubling, would simplify DH production by eliminating working steps such as treatment of seedlings, but would also require genetic improvement of spontaneous chromosome doubling ability in most germplasm prior to implementation.

One potential option for improving spontaneous chromosome doubling in haploid source germplasm is recurrent selection. Although this has been suggested by various authors (Kleiber et al., 2012; Wu et al., 2017; Ma et al., 2018) because of high heritabilities for this trait

(0.68-0.91), peer-reviewed studies have not been published to the best of my knowledge. Little information was available about the type of gene action involved in the expression of this trait, and research on the genetic architecture of this trait would benefit the design of breeding programs for improving this trait.

For chemically induced chromosome doubling, choice of chemicals and choice of treatment method is important for the toxicity of a treatment, its success rate, and efficiency. Multiple anti-mitotic chemicals are known which have lower acute toxicities than colchicine. Various anti-mitotic herbicides inhibit microtubule assembly and organization (Vaughn and Lehnen, 1991) and have been tested *in vitro* as alternatives to colchicine. For example, amiprophos methyl, pronamide, trifluralin, oryzalin and chlorpropham have been tested for their suitability as doubling agents in maize callus culture (Wan et al., 1991; Beaumont and Widholm, 1993) and in maize root tips (Häntzschel and Weber, 2010). However, a systematic study for identifying the optimal dosage of these antimitotic agents for *in vivo* DH production was missing in the literature.

An essentially non-toxic alternative to colchicine is nitrous oxide (N_2O) gas. Chromosome doubling effects of some gases such as nitrogen (N_2), N_2O and propane were reported by Ferguson et al. (1950). This effect of N_2O has since been observed in various grain and ornamental crops such as *Begonia* (Dewitte et al., 2009), *Hordeum vulgare* L. (Dvorak et al., 1973), *Phalaenopsis* (Wongprichachan et al., 2013), *Triticum* spp. (Kihara and Tsunewaki, 1960), and *Z. mays* (Kato, 2002). Little research has been carried out regarding the mode of action of the above anti-mitotic gases, but Kitamura (2009) found that N_2O inhibits polymerization of microtubules thereby causing an increase in ploidy.

Kato (2002) used N_2O treatment to induce chromosome doubling of maize plants in the floral primordial stage. A disadvantage of this method is that it involves treatment of potted plants with fully developed leaves, which would require huge treatment chambers for treatment of thousands of seedlings for large-scale DH production. Research on methods for large-scale application of N_2O treatment in DH production and influence of various treatment factors was still missing in the literature.

A potential reason for the lack of literature regarding improvement of chromosome doubling protocols are the great resource and time requirements for such research. Such research requires measuring the success rate of different treatments, currently only possible by cultivating maize plants after treatment until male and female fertility can be assessed. Therefore, new methods for predicting fertility/ploidy would be desirable to accelerate research on alternative chromosome doubling methods.

Diagnosis of ploidy: state of the art

Monitoring of ploidy is inherent to DH production because this type of line development involves transitions between the haploid and diploid level, in contrast to traditional line development by recurrent selfing that only involves diploid seeds and plants. The gold standard for determining if a plant is haploid (H), DH or diploid crossing (C) is a field score (Mahuku, 2012; Melchinger et al., 2013; Wu et al., 2014a; Chaikam et al., 2015; Liu et al., 2017). A D_0 plant is classified as H if it displays the characteristic short weak phenotype with narrow upright leaves. In contrast a plant is classified as DH if it displays the H phenotype but produces enough pollen for self-pollination and seed set, and as C if it displays a vigorous tall phenotype with wide sprawling leaves.

Flow cytometry is used in *in vitro* DH production of cereals and canola for determining the ploidy status of plants in early growth stages (Germanà, 2011; Saaten-Union Biotec GmbH, 2018). For larger scale applications, a platform has been developed which enables 1,000 samples to be analyzed per day (Erich Pollähne GmbH, 2019; Saaten-Union Biotec GmbH, 2018). Using a flow cytometer to produce the frequency distributions of reflectance from stained cell nuclei that correspond to their relative DNA content, ploidy can be classified visually. Diploid cells have twice the reflectance of haploid cells, and mixaploid tissue from doubled haploids show reflectance distributions of both ploidy levels (Bohanec, 2003). In maize *in vivo* DH production research, flow cytometry has been used to discriminate H and C plants (Belicuas et al., 2007; Couto et al., 2013; Wu et al., 2014b) and to compare success rates of different chromosome doubling protocols (Couto et al., 2015). However, the effectiveness of flow cytometry for predicting the seed set of a D_0 plant was not evaluated in previous studies.

A difference in stomata size between diploid and tetraploid maize was already observed by Randolph (1935). Choe et al (2012) proposed to use stomata guard cell length for determining a D_0 maize plant's ploidy status because they found significant differences in stomata length between H, DH and C plants in growth stages V2 to V8. However, there is no information available on variances of individual plant mean stomata length, which is necessary for discriminant analysis in a mixture distribution. Information on the influence of other factors such as chromosome doubling treatment or genotype on the mixture distributions of plant stomata length are also lacking.

Diagnosis of ploidy: potential applications

In addition to the potential benefit for research on alternative chromosome doubling agents as a fast and cheap method for predicting the success rate of a treatment, methods for determining

a plant's ploidy would have additional applications in DH production. One application would be to help discard undesirable H and C plants before transplanting to the DH nursery for a potentially vast reduction in the required size of the DH nursery. Although progress has been made in discriminating C seeds, a proportion of C seeds, depending on sorting method, source germplasm and inducer is still planted in the DH nursery and requires roguing. Since the majority of D_0 plants are sterile without seed set, a method for detecting DH plants would greatly reduce the number of useless plants.

DH production facilities would also benefit from a method for detecting DH plants, because this would enable them to predict the number of lines produced by the end of the season. If it is clear early in the season that the production goal will not be met, then another batch of seedlings could be treated or new induction crosses could be initiated at an early stage.

Objectives

The main goal of this thesis was to improve the chromosome doubling step of *in vivo* DH technology in maize through:

1. Establishing alternative chromosome doubling protocols with reduced toxicity
2. Evaluating the gene action involved in spontaneous chromosome doubling
3. Evaluating the response to selection for spontaneous chromosome doubling
4. Comparing methods for diagnosing ploidy of plants in DH production.

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Chapter 2

Colchicine alternatives for chromosome doubling in maize haploids for doubled-haploid production

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Abstract

DH maize lines are commonly produced by *in vivo* chromosome doubling using colchicine, a highly toxic chemical. This study was conducted to evaluate the efficacy of various alternative chromosome doubling treatments i) alone or in combination in varying concentrations, and ii) in different methods of application to the meristems. Several antimitotic herbicides with different modes of action were evaluated either alone or in combination with two phytohormones in different concentrations in four experiments. Survival rate (SR), measured as the proportion of germinated seedlings surviving until pollination, reproduction rate (RR), measured as the proportion of D_0 plants with seed set, and overall success rate (OSR), measured as the proportion of germinated seedlings yielding plants with seed set after self-pollination were recorded for all treatments. Amiprophos-methyl and pronamid applied by the seedling soaking treatment method yielded higher OSR than all treatments except colchicine. Cost per D_0 plant with seed set was approximately 10% higher using the best alternative treatment than the colchicine control. In conclusion, APM in combination with an optimum concentration of pronamid is a promising alternative treatment in view of the lower toxicity and similar OSR.

Chapter 3

Nitrous oxide-induced chromosome doubling of maize haploids

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Abstract

Chromosome doubling is a major cost factor of DH line development. The standard chromosome doubling protocol requires colchicine, a highly toxic antimitotic chemical requiring special disposal. In comparison, N_2O is a relatively safe antimitotic gas with simple disposal requirements. Therefore, the objective of this study was to compare N_2O-based chromosome doubling treatment of maize seedlings with a standard colchicine-based treatment and a promising antimitotic herbicide-based alternative treatment. Various treatment factors, including different pressures and concentrations of N_2O, O_2, and air in the treatment chamber, imbibition duration, and treatment duration, were evaluated for their effect on i) SR, measured as the proportion of haploid (D_0) plants surviving treatment, ii) RR, measured as the proportion of D_0 plants with fertile pollen which could be selfed, and iii) OSR, measured as the proportion of D_0 plants with seed set obtained from the germinated seedlings. The OSR of the best N_2O treatment ranged from 6.4 to 33.3%, depending on the year and environment, and did not differ significantly from herbicide treatment. Similarly, the OSR of colchicine ranged from 5.0 to 28.1%. In conclusion, N_2O treatment is a competitive alternative to colchicine and herbicide treatments for chromosome doubling in maize DH production, offering safety and waste disposal-related benefits.

Chapter 4

Haploid male fertility and spontaneous chromosome doubling evaluated in a diallel and recurrent selection experiment in maize

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Abstract

Although the DH technology is a cornerstone of line development in temperate maize breeding programs, the low success rate of DH production leaves much room for improvement. Currently, the majority of haploid plants do not produce enough fertile pollen for self-pollination after standard colchicine treatment nor after herbicide or N_2O treatment. Improvement of haploid male fertility (HMF) by selection for a higher spontaneous chromosome doubling rate (SDR) has the potential to increase DH production efficiency. In order to evaluate the gene action governing SDR in two breeding populations, we adapted the quantitative genetic model of Eberhart and Gardner (1966) for the case of haploid progeny from ten DH lines and corresponding diallel crosses. In addition, we carried out three cycles of recurrent selection for SDR in two additional populations to evaluate the effect of single plant selection for this trait. While additive genetic effects predominated in both diallel crosses, epistatic effects played a significant, but smaller role. Heritability of SDR was high, exceeding 0.91 on an entry-mean basis, however, the single-plant heritability relevant for selection was low, ranging from 0.11 to 0.19. Nonetheless, recurrent selection increased SDR from approximately 5% to 50%, suggesting an oligogenic inheritance. This improvement is greater than that due to standard colchicine treatment, which yields at maximum 30% fertile haploids in germplasm with normal rates of HMF. Altogether, the results show the great potential of selection for spontaneous chromosome doubling to streamline development of DH lines to a degree which may enable new breeding schemes with more efficient allocation of resources.

Chapter 5

Early diagnosis of ploidy status in doubled haploid production of maize by stomata length and flow cytometry measurements

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Abstract

In *in vivo* DH production, haploid seeds are induced in source germplasm from which DH lines are desired. After germination and treatment, a large number of putative haploid (H) seedlings are planted in a nursery, which give rise to a relatively small number of fertile plants. Reliable discrimination of sterile H plants, fertile DH plants and undesirable crossing (C) plants in early growth stages could greatly increase DH production efficiency by saving a large portion of resources wasted on planting and cultivation of the undesired plants. The objective of this study was to evaluate the effectiveness of flow cytometry and stomata length measurement for classification of plants in growth stages V3-V4 as H, DH or C. As reference classification, we used a field score based on plant phenotype, which is commonly applied in DH production and research. Our results show that identification of misclassified C seeds is possible with these methods because stomata length distributions of H&DH and C plants overlap only marginally, and the flow cytometry-based classification is associated with the reference field score. In contrast, stomata length distributions for H and DH overlap substantially. Therefore, the main application we see for these classification methods in early growth stages is the identification of C seedlings.

Chapter 6

General discussion and future perspectives

Artificial chromosome doubling

In two series of experiments, first evaluating anti-mitotic herbicide alternatives to colchicine, then evaluating the anti-mitotic gas N_2O, we were able to narrow down the experimental alternatives to one herbicide treatment and one N_2O treatment with similar success rates as colchicine. We decided to evaluate anti-mitotic herbicides because they are less toxic than colchicine and can be implemented using the same equipment and facilities as standard seedling submersion treatment with colchicine. N_2O as an alternative to colchicine treatment also has the advantage of low toxicity and additionally does not require chemical waste disposal.

Treatment safety

In contrast to colchicine which is highly toxic, the anti-mitotic herbicide pronamide has a low oral lethal dose (LD_{50} > 2500 mg kg^{-1}) and is an approved herbicide in the European Union according to EC Regulation 1107/2009 (University of Hertfordshire, 2013a; European Commission, 2015). The anti-mitotic herbicide APM also has relatively low acute toxicity, with an oral LD_{50} of 309 mg kg^{-1} in rats (University of Hertfordshire, 2013b). APM is not an approved herbicide in the EU, and detailed information on toxicity and environmental fate of APM is lacking (Tomlin, 2009; University of Hertfordshire, 2013b). Nonetheless, APM has been evaluated as a chromosome doubling agent in maize anther culture (Wan et al., 1991) and is available through tissue culture suppliers such as Duchefa Biochemie B.V..

N_2O's lack of acute toxicity is demonstrated by its use as a food packaging gas (World Health Organization, 2018) and the extensive documentation on workplace safety of personnel applying this gas as an inhalation anesthetic in surgery (Becker and Rosenberg, 2008). However, negative health effects have been associated with long term exposure to this gas at elevated concentrations (Clark and Brunick, 2015). The maximum concentration of N_2O allowed in a workspace in Germany is 0.194 mg L^{-1} (The Linde Group, 2011) and personnel handling N_2O, as well as herbicide and colchicine treatments, need to be trained in safe handling of this gas.

Alternative treatment factors

In order to increase the overall success rate (OSR), measured as the proportion of treated seedlings with successful chromosome doubling (seed set upon self-pollination), chemical concentrations in herbicide treatment should balance the chromosome doubling effect and the phytotoxicity of treatment. The OSR is limited by the survival rate (SR) of treated seedlings, and is further reduced by low reproduction rate (RR), measured as the proportion of seedlings surviving treatment which have seed set upon self-pollination. Trends in success rates in the herbicide treatments evaluated by Melchinger et al. (2016) indicate that further improvements in OSR beyond those of colchicine treatment are not promising with APM and pronamide. The linear decrease in SR, coupled with a linear increase in RR as the pronamide concentration increased, lead to a maximum OSR within the concentration range evaluated. While further increases of pronamide concentration would most likely reduce the treatment OSR, OSR improvements with different concentrations of APM cannot be excluded.

In alternative herbicide treatment, the choice of herbicide mode of action was essential to developing an effective chromosome doubling treatment. Evaluation of flufenacet, which blocks mitosis by inhibiting the synthesis of very long chain fatty acids (Weed Science Society of America, 2011), did not show chromosome doubling effects in the concentration tested. In contrast, herbicides which inhibit microtubule assembly, such as trifluralin and oryzalin (Weed Science Society of America, 2011), demonstrated chromosome doubling effects. However, addition of trifluralin and oryzalin had either neutral or negative effects on OSR compared to addition of pronamide. The possibility that oryzalin and trifluralin may be effective at other concentrations and combinations cannot be excluded.

In contrast to herbicide treatment, changing the optimum concentrations of nitrous oxide within the range evaluated by Molenaar et al. (2018) did not change the survival rate significantly, nor increase the RR i.e. only small and not significant negative effects on OSR were observed. The relatively high SR of N_2O treatment compared to herbicide treatment shows the lower phytotoxicity of this treatment in the concentrations evaluated, but the less effective chromosome doubling led to similar OSR. Due to the different trends in success rates between herbicides and N_2O, SR alone cannot be used as a criterion to screen different chemicals for chromosome doubling effects because this is not linked to phytotoxicity.

Additional factors in N_2O treatment with strong effects on OSR were changes in imbibition duration of seeds before treatment as well as treatment duration. Further improvement of OSR by changes in imbibition duration and treatment duration seem unlikely since any deviation from the best treatment resulted in lower OSR. In contrast, seedling shoot

length, measured with a caliper before treatment, and presence or absence of light during germination had no effect on OSR.

Variation in success rates

In DH production, various factors affect the OSR, including environment and genetic background of the source population. Therefore, it is difficult to predict the success rate of a treatment, and to plan for DH production. For example, within Experiment 1 in Molenaar et al. (2018), the OSR in the field across three planting date-by-genotype combinations ranged from 3.1 to 14.5% for colchicine treatment, and from 0.6 to 16.7 for N_2O treatment T1. In Experiment 2 the strong variation across planting dates for bulks treated with colchicine and the best N_2O treatment T5 resulted in large 95% confidence intervals for OSR of 3.5-7.1% and 4.6-9.0%, respectively (Molenaar et al., 2018). The strong effect of the environment on OSR is also demonstrated by the consistently higher OSRs in the greenhouse, which agree with the literature (Eder and Chalyk, 2002). OSRs of colchicine, N_2O treatment T5, and the best herbicide treatment from Melchinger et al. (2016) in the greenhouse, ranged from approximately 20-30%.

Spontaneous chromosome doubling

The approximately ten-fold increase in spontaneous chromosome doubling rate (SDR) after three cycles of selection for spontaneous chromosome doubling shows the vast potential for increasing a germplasm's innate ability to produce seed set without chemical treatment (Molenaar et al., 2019b). This marks a paradigm shift in the chromosome doubling step of DH production in maize: while with artificial chromosome doubling treatments the majority of D_0 plants are sterile, with spontaneous chromosome doubling a scenario becomes realistic where the majority of D_0 plants are fertile.

Single plant selection is a promising approach in breeding for improving spontaneous chromosome doubling rate. In contrast to selection based on entry means across multiple environments, single plants may be selected already in the D_0 nursery three seasons before selection based on entry means, which requires seed multiplication, and haploid induction of DH lines to produce sufficient haploid seed for evaluation of SDR in multiple environments. A prerequisite for single-plant selection is sufficient single-plant heritability, which was not considered in previous studies.

Gene action and variance components

The single-plant heritability for SDR or pollen score, which was between 0.10 and 0.20 in two diallel crosses evaluated by Molenaar et al. (2019b), is promising for selection gain. This relatively high single-plant heritability may mainly be due to the predominance of additive genetic effects found in the diallel analysis of Molenaar et al. (2019b). Using a selection intensity of $\alpha \approx 15\%$ ($i_\alpha \approx 1.5$), selection gain across three cycles of selection for SDR and seed set of D_0 ears aligned well with theoretical expectations in one biparental population. In the second biparental population, selection gain showed a convex trend, suggesting deviation from a purely additive inheritance of SDR due to positive epistatic interactions between favorable alleles at a small number of loci (Hansen, 2013).

Diagnosis of ploidy in DH production

The evaluation of flow cytometry and stomata length for diagnosis of ploidy in DH production clarified the potential applications of these ploidy-monitoring methods (Molenaar et al., 2019a). The analysis of stomata length revealed significant chromosome doubling treatment effects, as well as significant individual plant variance. However, despite significant differences in mean stomata length of H, DH and C plants, accounting for treatment effects does not enable the discrimination of DH and H plants.

A potential application of stomata length measurement is detection of C plants before transplanting to the field if many C seeds are present. Based on mixture distribution analysis of stomata length, the misclassification rate for C plants decreases with increasing proportions of C plants. Detection of C plants is less promising for flow cytometry because this method only demonstrated a high association between prediction method and gold standard classification for untreated material. The utility of C-plant identification in DH production with untreated material should be subject to a cost-benefit analysis, because this would require seedling cultivation in pots and subsequent transplanting. However, one has to bear in mind that adopting this approach would eliminate a major benefit of spontaneous chromosome doubling based DH production, namely low labor requirements due to direct sowing of H seeds

Identification of DH versus H plants in growth stages V3 and V4 is not possible with flow cytometry because of the poor association between the predicted ploidy and the gold standard classification. A likely explanation for this poor association is the chimeric nature of treated D_0 plants and that the lineage of cells in different parts of the adult plant traces back to different regions of the apical meristem (Coe and Neuffer, 1978; McDaniel and Poethig, 1988). Thus, the presence of doubled haploid tissue in a leaf from an early growth stage is a poor

indicator of a future doubled haploid sector in the tassel, which would confer haploid male fertility. Therefore, use of a diagnostic tool for predicting seed set in D_0 plants in order to reduce the number of plants in the D_0 nursery to only those of interest for DH production, is not promising.

Economics of DH production

The analysis of variable costs in Melchinger et al. (2016) revealed the great importance of the chromosome doubling step and overall success rate in DH production for the final cost per DH line. Since variable costs per unit processed are the same across treatments, except for chemical costs, which make up less than 2% of the total costs, most of the differences in costs across treatments is due to differing numbers of seeds required in each stage of production. Therefore, OSR is of utmost importance for determining the total cost per DH line and comparing alternative treatments.

Treatment costs are dependent on environmental conditions because the success rates, which are mainly responsible for the treatment costs, are environment-dependent. In Molenaar et al. (2018) the N_2O treatment T5 had the lowest variable costs, but the difference to the best herbicide treatment from Melchinger et al. (2016) and to a standard colchicine treatment was small, and success rates were not significantly different. Therefore, these three treatments can be considered as having similar variable costs which, however, may change - possibly even leading to a rank shift, depending on the year. Overhead costs such as from the N_2O treatment chamber as well as laboratory and disposal costs for the liquid chemical treatments should be considered for a DH-production-program specific cost analysis.

Spontaneous chromosome doubling based DH production has potential to greatly reduce the cost of DH lines in two ways: 1) by reducing labor and material requirements for seedling treatment and transplanting and 2) by increasing OSR of DH production to levels beyond those reached by standard chemical treatments. Obviously, when SD rates are greater than or equal to chemically induced OSR, spontaneous chromosome doubling would be preferable, because of the lack of costs due to treatment, nursing of seedlings in the greenhouse, and transplanting to the field. Instead, H seeds could simply be sown in the DH nursery instead of being transplanted. In fact, lower SD rates may already be economical because the reduction in treatment costs account for more than 20% of the total costs.

Resume and outlook

Based on the results of this thesis, it seems most likely that future improvements in chromosome doubling rates will rather be reached through increases in haploid male fertility than through improvements in chromosome doubling protocols, unless a great technological leap occurs. With the chemicals evaluated in this thesis for seedling submersion treatment, relatively small changes in OSR compared to SR occurred. Therefore, higher concentrations would quickly lead to increased mortality, which obviously reduces the number of plants considerably that remain for transplanting in the DH nursery.

Great improvements were made by this research in reducing the toxicity of chromosome doubling treatment. First, Melchinger et al. (2016) developed a herbicide treatment in which the components have several hundred fold lower oral toxicity than colchicine, but this treatment still requires proper chemical waste disposal. Second, Molenaar et al. (2018) developed an N_2O treatment which has no disposal requirements since N_2O can simply be released into the atmosphere in a well ventilated area. Moreover, this treatment showed similar success rates as both the best herbicide treatment from Melchinger et al. (2016) and a standard colchicine treatment.

Although success rates were similar in the best alternative treatments compared to the control, there is still room for improvement of treatment success rates, working steps, or environmental conditions influencing chromosome doubling rate. For example, environmental conditions had a strong impact in these experiments, with OSR in the greenhouse being substantially higher than in the field, but there is no study to my knowledge that has focused on optimizing environmental conditions. Treatment success rates may be improved by chromosome doubling of the embryo while still developing on the source germplasm (Barton et al., 2014), however, no research has been published optimizing such treatments. Also conceivable are other labor-saving treatments such as spraying of approved herbicides onto D_0 plants in the DH nursery. Such treatment would save labor because H seed could simply be sown into the nursery, instead of prior greenhouse cultivation and transplanting, while treatment would simply involve spraying with a common field sprayer instead of handling each seedling individually. Another simple labor saving change would be to directly plant treated seedlings into the DH nursery where environmental conditions allow, to eliminate transplanting from pots into the DH nursery. In N_2O treatment, a higher density of seedlings in the treatment chamber should be tested to reduce treatment chamber costs.

Although increases in success rates of DH production were more promising with genetic improvement of spontaneous chromosome doubling, chemical treatment will still be necessary

in some cases. Obviously, where spontaneous chromosome doubling rates are too low, chemically induced chromosome doubling will be required. This would especially be the case with non-elite material but also when introducing new elite breeding material into a breeding program in which SDR have been improved to a level acceptable for DH production. For example, with standard production of DH libraries from landraces, which were proposed by (Melchinger et al., 2017) for broadening the genetic diversity of elite material, OSR is less than half that in elite material.

In order to evaluate the suitability of new treatments for application in breeding, and to improve success rates, we proposed a two-stage testing scheme (Melchinger et al., 2016). First, new treatments should be evaluated with bulks of haploid seeds from diverse source germplasm in order to determine the average treatment effect independent of genotype. Approximately 200-400 seeds should be sufficient for this purpose, as suggested by Prigge et al. (2012) for early testing of haploid induction rate. In a second step, selected treatments should be tested in combination with H seeds from multiple source germplasm to enable estimation of genotype-by-treatment effects for selecting treatments that are least dependent on genetic background.

Methods for diagnosing the ploidy in DH production in order to streamline research on alternative chromosome doubling agents were not promising (Molenaar et al., 2019a). DH plants could not be distinguished effectively from H plants, neither by flow cytometry, nor by stomata length. Therefore, we do not suggest these methods for measuring success rates in DH production. However, one potential application of stomata measurements is removal of false positive C plants in situations where misclassification rates in detecting H seeds lead to high proportions (>50%) of true C plants in the DH nursery. Improvements in measurement methods would be required to make this a viable option compared to simple rogueing in the field. Such improvements are within reach if relatively cheap portable microscopes are used to acquire images of stomata from leaves instead of from varnish imprints on slides. These images should then be immediately measured with specially tailored machine learning algorithms, as implemented in Vialet-Chabrand and Brendel (2014), so that results are available on site.

Results from the diallel analysis and the selection experiment in Molenaar et al. (2019b) show that in only three cycles of recurrent selection for haploid male fertility traits, SDR can be improved to levels exceeding the OSR of standard colchicine treatment and the best alternative herbicide (Melchinger et al., 2016) and N_2O treatments (Molenaar et al., 2019b). Our analysis of first and second-degree statistics showed that results can be population specific, which means that caution should be exercised in generalizing QTL mapping results from bi-parental population. This agrees with the lack of overlap in QTL across studies found by Ma et

al. (2018). Furthermore, the sample size of future genome-wide association studies should be sufficient to detect epistatic effects.

We proposed two recurrent selection schemes for increasing haploid fertility and seed set (Molenaar et al., 2019b), depending on the number of winter generations possible. With one available winter generation, only pollen traits can be selected, while seed set can additionally be selected if two generations are available. In both scenarios, selection would be carried out in the summer generation and haploid seeds of recombined populations would be induced in the winter generation. If only one winter generation is possible, D_0 plants with fertile tassels, and possibly with above average pollen shed, could be intermated in a chain crossing design. The resulting heterozygous plants would be induced in the winter season. If two winter generations are possible, D_0 plants with fertile pollen, and possibly above average pollen shed, could be selfed. In the first winter generation, the homozygous plants could be intermated to produce heterozygous plants for haploid induction in the second winter generation. A benefit of the latter approach would be that DH lines, although relatively few in number, would become available earlier than in DH production after pre-breeding.

Once a sufficient SDR is reached by pre-breeding for economical DH production, the cost per DH line will continue to decrease due to the inherent selection for this trait during SD-based DH production. In addition to increasing the efficiency of DH production, SD-based DH production may also improve seed set as observed in this study (unpublished data). This could have an effect on breeding schemes if selection on pollen production increases seed set to > 50 seeds per ear. Such seed set rates would enable a reduction in cycle length of hybrid breeding schemes involving seed multiplication before nursery assessment. Selection gain could also be increased if improvements in SDR lead to production of > 50 DH lines, coupled with strong increases in haploid induction rates, enable early testing before DH production (Longin et al., 2007).

Conclusions

For the chromosome doubling step in DH production, we developed different alternative treatments based on herbicides and N_2O, which demonstrated similar success rates as colchicine. However, these rates were not significantly different due to the large confidence intervals of mean OSR, despite large sample sizes evaluated. These new treatments are less toxic than colchicine treatment and, in addition, N_2O treatment eliminates the need for chemical waste disposal, which is particularly useful for implementation of DH technology in developing countries. We anticipate that genetic increases in SDR of untreated material might lead to a

paradigm shift in the production of DH lines because recurrent phenotypic selection for haploid fertility increased the proportion of DH plants recovered from H seedlings to a greater extent than standard colchicine treatment.

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7 Summary

The *in vivo* doubled haploid (DH) technology in maize carries many advantages over traditional line development by recurrent selfing and has played an integral role in numerous breeding programs since the early 21st century. Briefly, the DH technology involves fours main steps: (i) induction of maternal haploid seeds by pollination with an inducer genotype, (ii) sorting of haploid (H) from diploid crossing (C) seeds, (iii) chromosome doubling treatment of haploid seedlings, and (iv) self-pollination of fertile D_0 plants. Many improvements have been made during the last decade, such as the development of new haploid inducers equipped with alternative marker systems allowing haploid discrimination in many tropical and temperate Flint germplasm, where this was previously impossible. Furthermore, high throughput platforms were developed which enable automated sorting of haploid from diploid crossing seeds.

A bottleneck in the DH technology is still the success rate of chromosome doubling treatment, which has a strong influence on the costs of DH production. Currently, only a minority (~10%) of treated D_0 haploid plants result in DH lines. Improvement in the chromosome doubling step of DH production would not only make DH lines cheaper, but could also change the optimum allocation of resources in hybrid breeding. In addition, the development of treatments using alternative doubling agents to colchicine, which is toxic to humans, would improve worker safety and simplify waste disposal issues for developing countries to benefit from the DH technology. Initiating such developments is the goal of this thesis.

In a first step, we evaluated anti-mitotic herbicides with different modes of action as alternatives to colchicine for reducing the toxicity of chromosome-doubling treatment and for potentially increasing the success rates. In a series of experiments, we evaluated anti-mitotic herbicides with different modes of action in different concentrations and combinations. Based on the results of the initial experiments, we chose a specific concentration of amiprophos-methyl for evaluation in combination with varying concentrations of pronamide in a further experiment. This revealed the optimal concentration of pronamide in combination with the chosen concentration of amiprophos-methyl. However, this less-toxic treatment showed slightly lower success rates and slightly higher costs per DH line as compared to the standard colchicine treatment.

In a second step after evaluating anti-mitotic herbicides for seedling treatment, we evaluated gaseous treatments using nitrous oxide (N_2O), an anti-mitotic gas, in varying concentrations and combinations with air and pure oxygen. In two years of evaluation, we found an N_2O treatment which had similar success rates as colchicine. The major benefit of such treatment is that this gas can simply be released into the atmosphere, eliminating the difficulty of proper chemical waste disposal, which is difficult to secure in developing countries. The only requirement is a treatment chamber, in contrast to the laboratory facilities required for handling colchicine.

Besides research on chromosome doubling protocols, we also evaluated methods for diagnosing the ploidy of maize D_0 plants. There are important potential applications of such diagnostic methods in DH production, for example speeding up research on alternative chromosome doubling as well as managing the production of a desired number of DH lines. We evaluated the suitability of flow cytometry and stomata length measurement for discrimination of H, DH, and C plants in growth stages V3-V4. Therefore, we evaluated the importance of factors such as genotype, treatment, and measurement error variance, which were missing in the literature, but are integral for discriminant analysis of individual-plant mean stomata length. For both methods, only discrimination of C from H and DH plants was promising. Detection of DH plants was difficult, presumably because of their chimeric nature, which may reduce the correlation between the results of the diagnostic method and the reference field score determined in a later growth stage.

In a third step, we evaluated the potential of spontaneous chromosome doubling (SCD) as an alternative to chemical treatment-based chromosome doubling. Although previous studies found significant genetic variation and high heritability for SCD, a classical quantitative genetic analysis, elucidating the type of gene action governing this trait, and a selection experiment for improving SCD was missing in the literature. We found a predominance of additive genetic effects compared to epistatic effects, and a large selection gain after three cycles of recurrent selection for SCD to levels far beyond those reached by standard colchicine treatment. This indicates the great potential of SCD to improve the DH technology.

The approximately ten-fold increase in spontaneous chromosome doubling rate (SDR) reached in our recurrent selection experiment marks a paradigm shift in the chromosome doubling step of DH production in maize. DH production efficiency can be greatly increased by the vast improvement in SDR, and production can be further simplified to enable even higher throughput. Instead of chromosome doubling treatment, which involves much handling of

seedlings, haploid seeds from germplasm with a high innate ability to produce seed set without chemical treatment can be simply seeded in the DH nursery, eliminating the most costly production steps. Thus, this thesis has provided new opportunities to increase worker safety and reduce toxic waste in DH production, and further provided a proof of concept for genetic improvement of spontaneous chromosome doubling, which has great prospects for increasing the efficiency of DH production in maize.

8 Zusammenfassung

Die *in vivo* Methode zur Erzeugung von Doppelhaploiden (DH) bei Mais bietet wesentliche Vorteile gegenüber der traditionellen Produktion von reinerbigen Inzuchtlinien mittels rekurrenter Selbstung und ist mittlerweile integraler Bestandteil vieler Maisuchtprogramme in den gemäßigten Anbauzonen. Die DH-Technologie umfasst im Wesentlichen vier Schritte: (i) die Induktion von maternalen, haploiden Samen durch Bestäubung des Ausgangsmaterials mit Pollen von Induktor-Genotypen, (ii) die Identifizierung haploider (H) und diploider Kreuzungs-Körner (C) im Erntegut der Induktionskreuzungen, (iii) die Behandlung haploider Keimlinge zur Aufdopplung des Chromosomensatzes, und (iv) die Selbstung fertiler D_0 Pflanzen. Innerhalb der letzten 10 Jahre wurden viele methodische Verbesserungen zur Identifizierung haploider Samen erzielt, wie beispielsweise die Entwicklung neuer Induktor-Genotypen mit alternativen Markern, welche die Bestimmung von Haploiden im tropischen und im gemäßigten Flint-Genpool erlauben. Weiterhin wurden Hochdurchsatz-Plattformen entwickelt, die eine automatische Sortierung von haploiden und diploiden Samen ermöglichen.

In der DH-Technologie besteht jedoch ein großer Bedarf, die Erfolgsraten bei der Behandlung zur Aufdopplung des Chromosomensatzes zu steigern, da bislang nur bei einem bescheidenen Prozentsatz (~10%) behandelter haploider Keimlinge fertile D_0-Pflanzen erzeugt werden können und dies substantiell die Kosten der DH-Produktion bestimmt. Verbesserungen dieses Schrittes der Produktion von DH-Linien haben neben einer Kostenersparnis auch Auswirkungen auf die optimale Allokation von Ressourcen in der Hybridzüchtung. Die Nutzung alternativer Aufdopplungsverfahren, insbesondere die Verwendung alternativer Wirkstoffe zu dem bislang üblichen hochtoxischen Colchicin, bergen erhebliche Vorteile für den Arbeitsschutz und eine einfachere Chemikalienentsorgung. Derartige Fortschritte könnten den Einsatz der DH-Technologie insbesondere in Entwicklungsländern befördern. Ziel der vorliegenden Arbeit war es, diese Entwicklungen durch Suche nach alternativen Aufdoppelungsverfahren voranzutreiben.

Als erster Schritt wurden in einer Versuchsreihe anti-mitotische Herbizide mit unterschiedlichen Wirkungsweisen und Konzentrationen als Alternative zu Colchicin untersucht, um einerseits die toxische Gefährdung bei der Behandlung zur Chromosomen-

Aufdopplung zu reduzieren und andererseits die Erfolgsrate zu erhöhen. Basierend auf den Ergebnissen aus ersten Experimenten wurde in einem weiteren Experiment eine Konzentrationssteigerung von Pronamid in Kombination mit Amiprophos-methyl untersucht und eine optimale Applikation beider Chemikalien gefunden. Diese zeigte eine nur marginal geringere Erfolgsrate bei kaum höheren Kosten pro erzeugter DH-Linie im Vergleich zur bisherigen Standardmethode mittels Colchicin.

Als zweiter Schritt wurden gasförmige Behandlungen mit Distickstoffmonoxid (N_2O), einem antimitotischem Gas, in verschiedenen Konzentrationen und Kombinationen mit Luft oder reinem Sauerstoff getestet. Mittels der zweijährigen Untersuchungsreihe konnte gezeigt werden, dass Behandlung mit N_2O eine ähnliche Erfolgsrate hat wie Colchicin. Der große Vorteil der Lachgasbehandlung gegenüber Colchicin besteht darin, dass das Gas nach der Nutzung in die Atmosphäre entlassen werden kann und keine aufwendige chemische Abfallentsorgung notwendig ist, was in Entwicklungsländern meist nicht garantiert werden kann. Zur Behandlung haploider Keimlinge ist lediglich ein Druckbehälter und das Gas erforderlich, jedoch anders als im Umgang mit Colchicin kein speziell ausgestattetes Labor.

Ein weiterer Schwerpunkt dieser Arbeit betraf die Evaluierung diagnostischer Methoden zur Bestimmung des Ploidiegrades von D_0-Pflanzen. Diese diagnostischen Methoden werden in der DH-Produktion beispielsweise dazu benötigt, um alternative Aufdopplungsverfahren schnell evaluieren zu können oder um die Produktion einer gewünschten Zahl von DH-Linien besser zu steuern. Hierzu wurden die Durchfluss-Zytometrie und die Längenmessung von Stomata, einer in der Literatur beschriebenen Methode, auf ihre Eignung zur Unterscheidung von H, DH und C Pflanzen in den Entwicklungsstadien V3 bis V4 geprüft. Dafür wurde die Bedeutung der Faktoren Genotyp, Behandlung und Messfehler analysiert, welche in der Literatur bislang ignoriert wurden, aber entscheidend für eine Diskriminanzanalyse der Stomata Länge sind. Mit beiden Methoden war nur die Diskriminierung von C gegenüber H und DH-Pflanzen erfolgsversprechend. Die Identifizierung von DH-Pflanzen erwies sich als schwierig, vermutlich aufgrund deren chimären Beschaffenheit. Die Korrelation zwischen den Ergebnissen der diagnostischen Methoden und der als Referenz dienenden Feld-Bonitur in späteren Entwicklungsstadien war zudem sehr niedrig.

In einem dritten Schritt wurde die spontane Chromosomen-Aufdopplung (spontaneous chromosome doubling; SCD) als Alternative zur chemischen Behandlung untersucht. Während bisherige Arbeiten große genetische Unterschiede sowie eine hohe Heritabilität für SCD fanden, fehlten bislang klassische quantitativ-genetische Untersuchungen, um die genetische

Architektur von SCD zu analysieren. In dieser Arbeit wurde gezeigt, dass SCD vornehmlich auf additiven Geneffekten beruht und weniger auf epistatischen Effekten. In einem erstmals durchgeführten Selektionsexperiment zur Verbesserung der SCD konnte bereits nach drei Generationen rekurrenter Selektion ein erheblicher Selektionserfolg erreicht werden. Damit wurde die spontane Aufdopplungsrate (spontaneous doubling rate; SDR) auf ein Niveau gebracht, welche den Erfolg der Standardmethode basierend auf der Behandlung mit Colchicin weit übertraf.

Die im Vergleich zu dem Ausgangsniveau des Zuchtmaterials um den Faktor 10 erhöhte SCD, die in unserem rekurrenten Selektionsexperiment erreicht werden konnte, markiert einen Paradigmenwechsel in einem für die DH-Technik wichtigen Schritt. Erheblich höhere SDR verbessern die Effizienz der DH-Produktion und vereinfachen diese zugleich. Denn anstatt der Behandlung von Keimlingen mit Colchicin können haploide Körner mit hoher SDR direkt im Zuchtgarten ausgesät werden und damit der arbeitsintensivste und zugleich teuerste Schritt der DH-Produktion umgangen werden. Insgesamt zeigt die vorliegende Arbeit verschiedene neue, erfolgsversprechende Möglichkeiten auf, um den obligatorischen Schritt der Chromosomen-Aufdopplung effizienter zu gestalten und somit die DH-Produktion von reinerbigen Linien in der Maiszüchtung zu verbessern.

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