Chapter 2

Applications of Doubled Haploids in Plant Breeding and Applied Research

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Abstract

Manifold and diverse applications of doubled haploid (DH) plants have emerged in academy and in the plant breeding industry since the first discovery of a haploid mutant in the Jimson Weed (*Datura stramonium*), followed by the first reports about anther culture in the same species, maternal haploids by wide crosses in tobacco (*Nicotiana tabacum* L.) and barley (*Hordeum vulgare* L.), interspecific hybridization, ovary culture (gynogenesis), isolated microspore culture, and more recently the CENH3 approach in thale cress (*Arabidopsis thaliana* L.) and other species. Research and development efforts were and are still significant in both user groups. Luckily, often academic and industrial partners cooperate in challenging and sometimes voluminous projects worldwide. Not only to develop innovative DH protocols and technologies per se, but also to exploit the advantages of DH plants in a huge variety of research and development experiments. This review concentrates not on the DH technologies per se, but on the application of DHs in plant-related research and development projects.

Key words DH, Doubled haploids, Homozygosity, Molecular markers, Selection, Genetic variability, Recombination, Biostatistics, Genome editing, Genomic selection, Breeding strategy

1 Introduction

The first report about a haploid mutant was in *Datura stramonium* [1], and the first report of haploid production through anther culture was in a related species, *Datura innoxia* [2]. Maternal haploids by wide crosses were then described in tobacco [3] and barley [4]. Interspecific hybridization [5], ovary culture [6], isolated microspore culture [7, 8], and the use of haploid inducer lines, recently including the CENH3 approach [9] are the most prominent additional doubled haploid (DH) technologies. While the primary application of DHs was to fix genetic variability as fast as possible by immediately reaching full homozygosity (one "step" versus several selfing generations), this advantage of DHs was later and is still widely used in marker-assisted selection in academy. Later, with the development of cheaper and easier molecular

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biological and genomic tools and technologies, the link between DHs and marker applications in commercial breeding programs was accelerated as well. Today, DHs in many plant species are routinely used, often combined with diverse tool kits from the fields of genomics, transgenics (e.g. reverse breeding), bioinformatics, tissue culture, genome editing, epigenetics, but phenotyping and sophisticated field nursery technologies as well. This is often possible in species in which the efficiency of DH production was improved by optimization of several steps, being it either in vitro or in vivo. Those improvements (described in other chapters of this book) led mainly to accelerated in vitro haploid cell induction, better quality and quantity of organogenesis and regeneration, in vivo haploid induction, and genome doubling. Earlier reviews were published [10, 11].

Due to their genetic characteristics, DH plant lines are currently used in manifold applications in academic and commercial breeding programs, as well as in research and development (R&D) in plant biology, genetics, physiology, phenotyping, biostatistics, etc. Big data applications as genome sequencing, genomics, high throughput, or deep phenotyping are benefiting from DHs as well. Protocols for breeding of DH lines are available for almost 400 species (*see* Chapter 3 of Volume 1), and over 300 DH-derived cultivars have been developed in 12 species worldwide [12]. In maize, for example, methods for inducing, selecting, and doubling haploid plants are advanced and are in widespread use [13]. Some authors published studies about the epigenetic effects in DH lines. Especially, the developments in genomics (genome sequencing), the increasing speed and volume of data handling procedures, the new methods for hybrid mechanisms, and the improvement of DH protocols in formerly nonfunctional or minimally efficient DH protocols in "recalcitrant" crop species, has led to immense progress in selection and hybrid breeding.

This review article here starts with the use of DHs to analyze their genetics and agronomic characters and the comparison of DH populations with conventionally generated (selfed) populations under field conditions. In the following sections, the use of DHs in diverse genetic mapping studies and gene cloning approaches (and other genomic applications) is described, as well as their use in breeding and research of transgenic plants. The most recent reports from large biostatistical projects, genome editing, and phenotyping applications are mentioned too. In particular, the following applications of DHs in plant breeding and applied research will be discussed:

- Recombination and fixation of genetic variance.
- Use of haploid and DH technologies to develop wide crosses and use of hybridization.

- DHs for mapping and diverse range of MAS procedures and strategies.
- DHs for genomic selection and genomic prediction.
- Haploid tissues used for genetic transformation and development of stable transgenic lines and DHs for breeding with transgenic lines.
- Use of haploid cells and tissues for increasing genetic variation by mutation.
- Genome editing by the use of DH technologies.
- Epigenetics and DHs.
- Reverse Breeding.

2 Recombination and Fixation of Genetic Variance

The agronomic and molecular comparison of DH and conventional lines started as soon as the first relatively efficient DH production methods were at hand. For example, this was the case in the late 1970s and beginning of the 1980s in barley. It was found that the traits of barley DH lines distributed as in single-seed descent (SSD) lines. These traits included grain yield, heading date, and plant height [14, 15]. The results indicated that although the SSD method has more opportunities for genetic recombination than the DH method, it did not produce a sample of recombinants significantly different from the DH sample; thus, both methods were equally efficient to derive homozygous lines from F_1 hybrids in a relatively short time. One example is that barley SSD and DH progenies were used to develop lines with high kernel weight, but low β-glucan content [16]. Since then, hundreds if not thousands of registered varieties were developed worldwide, not only in barley. Despite the fact that the influence and share of DH inbred lines in hybrid breeding is significant, detailed data and lists of varieties developed by the use of DHs are not available due to trade secrets or nonpublic data at variety registration authorities and breeding companies. The COST Action 851 of the European Commission started to collect those data [17] but there was no continuation of this effort, unfortunately.

Theoretically, in a recurrent selection program, the use of DHs can increase the genetic advance per unit of time. To evaluate the efficiency expected from the use of DHs for grain yield improvement in a maize population, two recurrent selection programs for testcross performance were initiated, using testcross progenies from DH lines and S_1 families. Several selection cycles using DH and S_1 families were carried out and testcross genetic variance was twice as high among DH lines as among S_1 families. A year advantage of 29% for the S_1 family method over the DH method with a

cycle of four years was calculated while in a 3-year cycle for the DH method, both methods were expected to be equivalent. With a 3-year cycle for the DH method, the advantage would have been in favor of DH method. Furthermore, the DH method has the advantage of simultaneously producing lines that are directly usable as hybrid parents. Thus, if the genetic advance per unit of time is evaluated at the level of developed varieties even with the same or lower genetic advance in population improvement, the DH method appears to be the most efficient [18].

Although there is still a lot of space for improvements and cost reduction, a range of different routine and highly efficient DH production systems are currently at hand in barley, wheat, triticale, several *brassica* species and in maize. In maize, for example, a comparison between the frequency of recombination events and genetic variance in DH F_1 and F_2 populations generated by haploid inducer lines revealed that DHF_2 showed a higher mean recombination [19]. It was shown that for several traits, the means between DH F_1 and DH F_2 lines did not differ, while the genetic variance was higher among DH F_2 lines than among DH F_1 lines for one trait. The ratio of repulsion to coupling linkages was higher among DH F_1 lines than among DH F_2 lines for one of the analyzed traits. These results indicated that the decision of inducing DH lines from F_1 or F_2 plants should be made from considerations other than the performance of the resulting DH F_1 or DH F_2 lines [19].

In maize, the F_1 generation has been the most frequently used for haploid induction, due to the facility in the process. However, using F_2 generations would be a good alternative to increase genetic variability owing to the additional recombination in meiosis. The effect of F_1 and F_2 generations on DH production in tropical germplasm was explored, evaluating the expression of the R1-navajo anthocyan marker in seeds, the working steps of the methodology, and the genetic variability of the DH lines obtained by assessing haploid induction rate, inhibition seed rate, and diploid seed rate [20]. Estimates of population parameters in DH lines from F_1 were higher than from F_2. Furthermore, it was shown that one additional generation was not enough to create new genotype subgroups. Additionally, the relative efficiency of the response to selection in the F_1 was higher than in the F_2 due to the number of cycles that are used to obtain the DH. The results showed that in tropical maize, the use of the F_1 generation is recommended due to a superior balance between time and genetic variability. It can be concluded that joint R&D projects between academic and commercial partners can lead to useful results for both parties. It is without doubt that DHs in the breeding of self- and cross-pollinating plant species have many advantages in terms of both time and data quality.

3 Use of Haploid and DH Technologies to Develop Wide Crosses and Use of Hybridization

As a DH technology, isolated microspore culture is often used on material with meiotic instability, such as interspecific hybrids. As a result of chromosome missegregation and homologous exchanges, DH progenies might lose their homozygous status. For example, the fertility, meiosis, and genetic variability were assessed in a self-pollinated progeny set (the MDL2 population) resulting from first-generation plants (the MDL1 population) derived from microspores of a near-allohexaploid interspecific hybrid from the cross (*Brassica napus* × *B. carinata*) × *B. juncea*. Seed fertility and viability decreased substantially from the MDL1 to the MDL2 generation. In the MDL2 population, 87% of individuals differed genetically from their MDL1 parent. These genetic differences resulted from novel homologous exchanges between chromosomes, chromosome loss and gain, and segregation and instability of pre-existing karyotype abnormalities. Novel karyotype change was extremely common, with 2.2 new variants observed per MDL2 individual. Significant differences between progeny sets in the number of novel genetic variants were also observed. Thus, meiotic instability clearly has the potential to dramatically change karyotypes (often without detectable effects on the presence or absence of alleles) in putatively homozygous, microspore-derived lines, resulting in loss of fertility and viability [21].

4 DHs for Mapping and Marker-Assisted Selection

Since the beginning of DH technology, DHs have been used for mapping and marker-assisted selection. Full homozygosity and fixation of recombination events offer extreme advantages in mapping and many marker-assisted selection projects, at both the academic and commercial levels. The number of reports about the use of DH lines in mapping studies is therefore enormous, covering a diverse range of traits, phenotyping, and genotyping technologies (*see* Chapter 3 of Volume 2), which gives an idea of how useful DHs are for mapping. Besides the exceedingly early mapping studies (*see* above) some newer reports are mentioned next. Marker-aided breeding and DH technology have been used to improve host plant resistance in barley, rice, and wheat [22]. RILs (recombinant inbred lines) and DHs induced by an engineered haploid inducer in *Arabidopsis thaliana* were developed for QTL mapping [23]. DHs in triticale were used for exemplary QTL mapping of days to heading, revealing loci on chromosomes 2BL and 2R responsible for extended vernalization requirement, and identifying candidate genes [24]. DHs have also been described as advantageous in a

pyramiding strategy of several BaMMV/BaYMV resistance genes in winter barley. For pyramiding of resistance *genes rym4*, *rym5*, *rym9*, and *rym11*, two different crossing strategies were applied and compared. Besides DH plants carrying all possible two-gene combinations, 20 DH plants out of 107 analyzed were found to carry *rym4*, *rym9*, and *rym11* in homozygosity, and 27 out of 187 tested were found to carry *rym5*, *rym9*, and rym11 in homozygosity [25]. There are many other reports in a significant range of species and for a huge diversity of traits.

Most frequently, the reasons to invest in the production of DHs include the easier phenotyping in DHs or the number of samples to be analyzed for certain genetic compositions of genes or haplotypes. For example, mapping of breeding traits using DH lines in oat was described in another study [26]. The root system architecture in seedlings of a population out of 300 maize DHs was phenotyped and significant single nucleotide polymorphism associations could be identified [27]. The application of DH melon lines for the development of multiple virus resistances was also described [28]. DH lines obtained from F_1 hybrids of reciprocal crosses between yellow- and black-seeded lines in *Brassica napus* were used to analyze tocopherols, plastochomanol-8, and phytosterols [29, 30], observing significantly positive correlations between the seed color and PC-8 content. According to the range of genetic variation among DHs of two populations, selected DH lines may be good parents for further breeding programs focused on increasing the amount and improving the quality of oilseed rapeseed oil. Furthermore, DHs in tobacco were reported to combined resistance to black root rot fungus and tomato spotted wilt virus (TSWV) [31].

DHs have also been proved useful to gain knowledge on the genetics of male fertility. A genome wide association study in two DH maize populations derived from a diversity panel (481 inbred lines) crossed with two parental lines was performed to analyze the genetics of haploid male fertility [32]. It was found out that this trait has a complex quantitative genetic structure (only one association larger than 11%), concluding that recurrent phenotypic selection coupled with marker-assisted selection for individual QTL might be the best strategy to improve haploid male fertility. On the other hand, a large-scale genome-wide association study to analyze spontaneous chromosome doubling in haploids derived from tropical maize inbred lines [33] concluded that the presence of large variation for both haploid male fertility and haploid fertility can be potentially exploited to improve the efficiency of DH derivation in tropical maize germplasm.

5 DHs for Genomic Selection and Genomic Prediction

Fast and affordable genome sequencing technologies and powerful software tools, in combination with high-speed processing units, made it possible to develop new biostatistical approaches in academia and plant breeding industry. The integration of genomic resources with DH technology provides new opportunities for the improvement of selection methods, maximizing selection gains and accelerating the development of new cultivars. Just as an example, it is now possible to estimate the additive variance for line value and the variance of additive by additive epistasis for line value from an experimental design with several lines per DH plant randomly taken from a population. Variances of higher-order epistasis can be estimated with a two-factor mating design in which a cross is replaced by the population of lines that can be derived from it. With a diallelic or a factorial design, a direct test for the presence of homozygous by homozygous epistasis is possible. A brief consideration of these expressions leads to the conclusion that recurrent selection of single DH descents will be one of the most efficient methods for low heritability, together with a rapid development of DH lines [34]. Another example of a more complex combination of genomics and DHs is the use of microsatellite marker analysis of DH progenies to predict heterosis in snowball cauliflower [35]. It was evident that the development of DH lines could broaden the genetic base of any crop through creating more diversity in the existing population. In addition to this, the study further suggested that genetic distances based on genomic and EST-SSRs can be used as a predictor of heterosis for commercial traits in CMS and DH-based F_1 cauliflower.

DHs have also been used in genomic selection strategies using both phenotypes and genotypes [36]. It was also shown that there exist additional opportunities to combine genomic prediction methods with the creation of DHs. The authors proposed an extension to genomic selection, optimal haploid value (OHV) selection, which predicts the best DH that can be produced from a segregating plant. This method focuses selection on the haplotype and optimizes the breeding program toward its end goal of generating an elite fixed line. The authors rigorously tested OHV selection breeding programs, using computer simulation, and showed that this approach results in more genetic gain than genomic selection. OHV selection preserved a substantially greater amount of genetic diversity in the population than genomic selection, which is important to achieve long-term genetic gain in breeding populations.

It was argued that due to the large estimates of genotypic variance among DH lines derived from maize landraces, individual lines with superior performance for agronomic and morphological

traits can be selected and introgressed into the elite material [37]. Further, the improvement of seed set and other traits related to fitness in synthetic populations suggest that the DH technique might help in purging detrimental alleles present in landraces, apparently without strongly affecting the phenotypic diversity. Creation of DH lines from landraces shows great promise to broaden and improve the genetic basis of the Elite Flint breeding material without necessarily introducing negative agronomic features present in the landraces. Furthermore, the rapid decay of linkage disequilibrium (LD) together with the high genotypic variances and absence of population structure within the populations of DH lines derived from landraces make these lines an ideal tool for high-resolution association mapping. Genomic prediction within and among doubled haploid libraries from maize landraces was reported as well [38]. Altogether, the DH technology combined with genomic prediction offers a powerful approach to exploit the idle genetic diversity within landraces, but substantial investments are needed to mine this "gold reserve" for future breeding. It was concluded, that selected DH lines averaged similar testcross performance as their original landraces, and the best of them approached the yields of elite inbreds, demonstrating their potential to broaden the narrow genetic diversity of the flint germplasm pool. As to trait correlations of DH lines, correlation of test cross performance (TP) with line per se (LP) performance was zero for grain yield, underpinning the need to evaluate TP in addition to LP. For all traits, the authors observed substantial variation for TP among the DH lines and the best showed TP yields similar to the elite inbreds. Their results demonstrate the high potential of landraces for broadening the narrow genetic base of the flint heterotic pool and the usefulness of the DH technology for exploiting genetic resources from gene banks.

The use of DH maize lines for hybrid maize breeding was explored in a series of papers. In hybrid maize breeding, DHs are increasingly replacing inbreds developed by recurrent selfing. The authors analyzed the optimum allocation of the number of lines, test locations, as well as the number and type of testers in hybrid maize breeding using DHs. The production costs of DHs had only a minor effect on the optimum number of locations and on values of the optimization criteria [39]. In a second paper the authors stated that the optimum allocation of test resources is of crucial importance for the efficiency of breeding programs [40].

Early testing prior to DH production is a promising approach in hybrid maize breeding. In this third report of the series the authors determined the optimum allocation of the number of S (1) families, DH lines, and test locations for two different breeding schemes, compared the maximum selection gain achievable under both breeding schemes, and investigated limitations in the current method of DH production. Different assumptions were made

regarding the budget, variance components, and time of DH production within S(1) families. The large potential of early testing prior to DH production was indicated. Substantial increases in haploid induction and chromosome doubling rates as well as reduction in costs of DH production would allow for early testing of S(1) lines and subsequent production and testing of DH lines in a breeding scheme that combines high selection gain with a short cycle length [41].

Parental selection influences the gain from selection and the optimum allocation of test resources in breeding programs. Two hybrid maize breeding schemes with evaluation of testcross progenies were analyzed. One with DH lines in both stages (DHTC) and the second with S(1) families in the first stage and DH lines within S(1) families in the second stage (S(1)TC-DHTC). The breeding Scheme S(1)TC-DHTC led to a larger selection gain but had a longer cycle length than DHTC. However, with further improvements in the DH technique and the realization of more than two generations per year, early testing of S(1) families prior to production of DH lines would become very attractive in hybrid maize breeding [42]. DHs may be developed directly from S(0) plants in the parental cross or via S(1) families. The superiority of S(1)TC-DHTC was increased when the selection was done among all DH lines ignoring their cross and family structure, and using variable sizes of crosses and S(1) families. In DHTC, the best selection strategy was to ignore cross structures and use uniform size of crosses [43].

DH lines were used in a comparison of five different genomic selection strategies for grain yield. Different variables were considered, including the available budget, the costs for DH (DH) line and hybrid seed production as well as variance components for grain yield in a wide range. A nursery selection for disease resistance just before genomic selection (GS) on grain yield was included. Owing to the extremely high number of test candidates entering breeding strategies with GS, the costs for DH line production had a larger impact on the annual selection gain than the hybrid seed production costs. A specific genomic selection procedure for cereals was concluded [44].

Assuming a finite number of unlinked loci and a given total number of individuals to be genotyped, three methods of marker-assisted selection (MAS) for gene stacking in DH lines derived from biparental crosses were compared by theory and simulations [45]. With best linear unbiased prediction (BLUP), information from genetically related candidates is combined to obtain more precise estimates of genotypic values of test candidates and thereby increase progress from selection. The breeding schemes involved selection for testcross performance either of DH lines at both stages or of S(1) families at the first stage and DH lines at the second stage [46].

6 DHs and Genetic Transformation

Easier inheritance and faster fixation of transgenes in homozygosis are the reasons to combine the use of genetic transformation and DH technologies. Physiological and genetic parameters of DNA synthesis in barley microspores were analyzed to optimize the particle bombardment technology. By maintaining the temperature low during the 4 h osmotic adjustment period following the cold plus mannitol pre-treatment, it was expected to find a frequency of homozygous DHs higher than following a 21-day cold pre-treatment. The best procedure for obtaining transgenic barley plants from this study was to pre-treat the cultures at either 4 °C or 25 °C during 4 h, using the actin promoter and adding arabinogalactan proteins in the microspore culture medium [47, 48]. Beyond this, anther culture-derived haploid embryos were used as explants for *Agrobacterium*-mediated genetic transformation of bread wheat to develop stable drought-tolerant transgenics. Stable transgenic DH plants showed faster seed germination and seedling establishment, and better drought tolerance in comparison with nontransgenic DH plants [49]. Embryogenic pollen cultures of barley were also used for *Agrobacterium*-mediated genetic transformation to achieve transgene homozygosity immediately. The routine application of the method based on cultivar 'Igri' over a period of over 10 years has achieved an average yield of about two transgenic plants per donor spike. The whole procedure from pollen isolation to nonsegregating transgenic, mature grain takes less than 12 months [50]. Genetic transformation with isolated wheat microspores and microspore-derived embryos has been described as well [51]. Thus, DH technologies have the potential to facilitate and simplify breeding of transgenic lines and registered varieties as in nontransgenic breeding. Back crossing programs and pyramiding and stacking several transgenes by crossing may benefit from DH technology as well.

7 Use of Haploid Cells and Tissues for Increasing Genetic Variation by Mutation

DH lines can also be used to accelerate and simplify the handling of mutations, as these are easier to handle in homozygous, DH individuals. DH lines can be generated from mutated parental lines, but haploid cells are optimal targets for direct mutagenesis as well. For example, anther culture in a japonica rice variety was used with donor plants derived from EMS-mutagenized fertilized egg cells. The authors were able to generate stable mutants for a diverse range of traits and many of those lines showed the same yield as the original variety "Mankeumbeo" [52]. Flower buds of Chinese cabbage were soaked in a range of EMS solutions at different

concentrations, isolated microspore culture was applied, and plants regenerated therefrom. Embryo production rate and seedling rate were evaluated in five of the genotypes. Mutations in four color-related genes were identified. In total, 142 mutants with distinct variations in leaf shape, leaf color, corolla size, flower color, bolting time, and downy mildew resistance were identified from 475 DH lines [53].

Many more examples are published and definitely DHs are a very useful tool to generate variability by mutation technologies and to accelerate the development of advanced breeding lines with new, useful traits. Indeed, routine DH breeding with mutated genetic material is the daily operation of many plant breeders.

8 Genome Editing Using DH Technologies

The benefits of DH lines or haploid and later doubled cell and plant material in genome editing experiments is identical as the benefits of DH material in the development of mutations by more traditional technologies. Interestingly, genome editing in haploid cells or cells in vitro can generally be applied to further explore new basic processes in in vitro development of plant cells. Optimized methods for genome editing of haploid microspores and production of DH plants by microspore culture has been reported for wheat [54]. Many plant species and/or genotypes are still recalcitrant to conventional transformation methods. This, together with the long generation time of crop plants, poses a significant obstacle to effective application of gene editing technology, as it takes a long time to produce modified homozygous genotypes. As an alternative, the haploid, single-celled microspores are an attractive target for gene editing experiments, as they enable generation of homozygous DH mutants in one generation.

A different strategy to deliver edited genomic information into haploid cells was used in field and sweet corn and wheat [55]. The aberrant reproductive process of haploid induction was co-opted to induce edits in nascent seeds of diverse monocot and dicot species, which enables direct genomic modification of commercial crop varieties. The technology was tested in field and sweet corn using a native haploid-inducer line and extended to dicots using an engineered CENH3 system. They also recovered edited wheat embryos using Cas9 delivered by maize pollen and their data indicated that a transient hybrid state precedes uniparental chromosome elimination in a maize haploid inducer system. Edited haploid plants lack both the haploid-inducer parental DNA and the editing machinery. Therefore, edited plants could be used in trait testing and directly integrated into commercial variety development.

When combined, DHs and genome editing are two powerful game-changing technologies to generate pure inbred lines with

multiple desired traits: DHs for acceleration and genome editing for new genetic variability. This is the case of the Haploid-Inducer Mediated Genome Editing (IMGE) approach [56], which utilizes a maize haploid inducer line carrying a CRISPR/Cas9 cassette targeting for a desired agronomic trait to pollinate an elite maize inbred line and to generate genome-edited haploids in the elite maize background. Homozygous pure DH lines with the desired trait improved could be generated within two generations, thus bypassing the lengthy procedure of repeated crossing and backcrossing used in conventional breeding [56].

9 Epigenetics and DHs

Epigenetic factors play an important role in gene regulation. It is interesting to find out the impact of DH technologies on epigenetics during haploid embryogenesis and vice versa. Indeed, tissue culture-induced genetic and epigenetic variation in triticale has been described [57]. Regenerated plants out of androgenesis and somatic embryogenesis were analyzed by metAFLP and RP-HPLC in four distinct genotypes and it was shown that regeneration via in vitro culture was error-prone and affected DNA sequence and methylation patterns, irrespective of the culture method used. Similar results have been reported in chrysanthemum, an important ornamental species. Its highly heterozygous state complicates molecular analysis, so it is interesting to derive haploid forms. A total of 2579 nonfertilized chrysanthemum ovules pollinated by *Argyranthemum frutescens* were cultured in vitro to isolate a haploid progeny [58]. 105 calli were produced, and in three of them, one single regenerant emerged. Only one of them was a true haploid. Nine DH derivatives were subsequently generated by colchicine treatment of 80 in vitro-cultured haploid nodal segments. Morphological screening showed that the haploid plant was shorter than the DHs, developed smaller leaves, flowers, and stomata, and only few of its pollen grains were able to germinate, although they were abnormal. Both the haploid and the DHs produced yellow flowers, whereas those of the donor cultivar were mauve. Methylation-sensitive amplification polymorphism (MSAP) profiling showed 52.2% of cytosine-methylated amplified fragments in the donor genome, whereas in haploid and DH genomes these percentages were 47.0 and 51.7%, respectively. In other words, there was a reduction in global cytosine methylation caused by haploidization, and a partial recovery following chromosome doubling.

Inhibition of Histone Deacytelase activity is sufficient to induce embryogenic growth in cultured pollen of *B. napus* and *Arabidopsis* [59]. Proteins in the range of 10–25 kD were differentially acetylated after TSA treatment compared with a control. Thus, in this

respect, the deregulation of HDACs or HDAC-mediated pathways by stress and the accompanying changes in histone acetylation status could provide a single, common regulation point for the induction of haploid embryogenesis.

In conclusion, more research will be necessary to identify and to understand the role of epigenetic factors and to be able to influence them to improve DH technologies even more.

10 Reverse Breeding

Reverse breeding [60] is defined as the combination of a technology to control genetic recombination in hybrids through engineered meiosis, followed by DH production from nonrecombinant gamete precursors, in order to generate perfectly complementing homozygous parental lines, useful to reconstruct the hybrid background they come from. This is why it is called "reverse." The method is based on reducing genetic recombination in the selected heterozygote by eliminating meiotic crossing over. Male or female spores obtained from such plants contain combinations of nonrecombinant parental chromosomes which can be cultured in vitro to generate homozygous DH plants (DHs). From these DHs, complementary parents can be selected and used to reconstitute the heterozygote in perpetuity. The advantages and possibilities of such a method for on-demand reconstruction of hybrid backgrounds in the context of private breeding companies are evident.

11 Future Perspectives

Although in many plant species the progress in DH technology to produce DHs and to link DHs with other research and breeding tools was immense in the last decades, there is still a lot of space for further R&D, in both basic academic studies and more applied projects in commercial breeding companies. The use of DH populations to study genetic effects and inheritance of breeding traits will increase with new improved DH protocols and new DH technologies. Mapping and marker-assisted development using DHs are routine today in breeding companies and will increase, if phenotyping and data handling and processing technologies develop in parallel. New software tools are also necessary for this, and new algorithms co-developed by using artificial intelligence and machine learning will surely help. DHs would then in parallel serve as very suitable genetic material for complex biostatistical R&D projects in academia, industry, or both in joint consortia. For example, the prediction of yield in self and cross- pollinating species would be simplified. By having new hybrid breeding

technologies, the genetic analysis of heterosis, genetic diversity, pool development, etc., will be easier even in self-pollinating crops. Sugar beet, sunflower, and rice are only a few of the important crops in which DH routines are highly demanded. Many vegetables, herbs, fruits, nuts, and ornamental species are so far difficult to handle in androgenic or gynogenic protocols. Haploid inducer lines, either natural or engineered, will support DH technology development in those species mainly for academic purposes, but in part for industrial applications as well. Tree breeding will benefit as well.

It is without doubt that DHs will accelerate the breeding of new registered varieties in many plant species. Even in species where DH technologies work "routinely" today as in barley, wheat, triticale, and rapeseed, there is much to improve, as there is still a strong genotype dependency in some of the key steps in the procedures. In so far recalcitrant crops or crops in which several genotype-dependent tissue culture steps as microspore or egg cell induction, regeneration, rooting, and even more importantly ploidy doubling new DH technologies would of course lead to a significant step forward in the breeding process. The development of new DH protocols and improvements in already functional protocols depend very much on the analysis of the genetics and physiology of gametophytic cell development in vivo and in vitro. Not only the sequencing and bioinformatic analysis of genomes but also other technologies will support those. Microscopic technologies, cell phenotyping and image analysis, new software algorithms, and automation are under steady development also in the plant field. Both, academic and industrial R&D projects, separate or in joint efforts, public or protected by trade secrets or patents will lead to a continuous growth of knowledge, and this will lead to more registered improved plant varieties, which are and will be desperately needed under the future climate conditions.

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