Chapter 15
Barley and Wheat Doubled Haploids in Breeding

J. Weyen

Abstract The application of doubled haploids is a routine in barley and wheat breeding today. While in barley anther and isolated microspore culture are efficient technologies in wheat wide crosses with maize and subsequent phytohormone treatment and embryo rescue are better established due to genotype dependency of wheat in androgenic technologies. Although a quite significant number of DH lines can be produced in breeding programmes there are still some limiting factors which are related to technical and genetic factors. The actual status of DH lines in barley and wheat breeding and some of the limiting factors are listed and described below. Furthermore some aspects of R&D programmes to circumvent those limitations are described, followed by an outlook.

Keywords Doubled haploids, cereals, breeding, anther culture, microspore culture, wide crosses

Introduction

First reports about the regeneration and production of doubled haploid plants in barley and wheat were published more than 30 years ago. The regeneration of green plantlets from excised anthers in barley was reported by Clapham (1973) and in wheat by Ouyang et al. (1973). Barclay (1975) reported high frequencies of haploid production in wheat by chromosome elimination. Early reports concerning successful protocols of isolated microspore culture in barley were published by Kao et al. (1991) and in wheat by Datta and Wenzel (1987) and Hunter (1988).

Since then significant improvements resulting in more or less efficient technologies were published by many authors and generally the different technologies of anther culture (AC), isolated microspore culture (IMC) and wide crosses (WC) have become "routine" in public and private breeding and research programmes today worldwide. A collection of the most recent protocols for doubled haploid production in a range of different species, including barley and wheat can be found

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A. Touraev et al. (eds.) *Advances in Haploid Production in Higher Plants*,

in Maluszynski et al. (2003). Nevertheless there are still some obstacles and further R&D programmes are necessary to overcome constraints such as genotype dependency, albino regeneration and low spontaneous or incomplete fertility after induced doubling. As doubled haploid technologies *per se* become more and more efficient at the research laboratory level, the technologies and logistics in practical breeding programmes and the interaction with molecular marker technologies have to be aligned and adapted significantly. The production and deployment of fertile doubled haploid lines are still considered expensive and require speedy seed multiplication, multi locational field testing and a high quality data generation, collection and analysis system.

Barley

Doubled Haploid Technologies in Barley

Anther culture and the *Hordeum bulbosum* technologies were described earlier than isolated microspore culture in barley. Each of the technologies has major advantages and disadvantages. While *H. bulbosum* technology is relatively independent from high quality equipment both androgenic technologies require more sophisticated and stable glasshouse capacities (cooling, illumination) and also more attention to the issue of laboratory sanitary conditions. Nevertheless today the *H. bulbosum* technology is not used much, because the efficiency is significant lower than established AC and IMC programmes. One of the breakthroughs in barley androgenesis came with the patented use of maltose described by Hunter (1987, see also chapter by Dunwell, "Patents and haploid plants") and further improvements by many other authors are being developed in many laboratories and breeding companies.

As a precondition for successful androgenic doubled haploidy technology, optimal growing regimes for the donor plants must be at hand. In addition to optimal protection against diseases such as mildew and insects (aphids, thrips, spider mites) optimal abiotic conditions must be provided. Quality and quantity of light, water supply, fertilization, growth substrates, air humidity and carbon dioxide concentration are important factors that need careful maintenance. The optimal stage of microspore development (mid to late uni-nucleate) is another prerequisite which is essential for success in AC and IMC. The most common pre treatments which were described in successful protocols are cold treatment of tillers (in water or wrapped in watered paper towels and foil) or in a 0,3 M mannitol solution for a varying number of days. Some of these and further detailed protocols are published in Maluszynski et al. (2003).

Necessary Improvements

Although there are many published improvements, the production of several hundreds of green plantlets per genotype in breeding programmes is only possible by

the adaption of those protocols (working often with only a minor range of genotypes) to the specific conditions of each barley breeding station. Working on several hundred crosses per year in a doubled haploid laboratory requires extensive work and reliable protocols. Conducting conventional breeding alongside doubled haploid (DH) systems is logistically difficult and expensive. Stable and reliable numbers of doubled haploid plants are a pre requisite for successful DH breeding. Despite the significant improvements described above there are still three major obstacles in barley AC and IMC. One is the very genotype dependent occurrence of albino plantlets and the second is the more technical problem of contamination by bacteria and fungi in isolated microspore culture. Contamination is not a significant problem in AC, because losses are not too dramatic under normal circumstances. Nevertheless the use of antibacterial and anti-fungal substances is possible, but is a rather expensive solution to the problem.

Albino plants are still a problem in many cereal species (see also chapter by Torp and Andersen on "Albinism in microspore culture"). It is clearly a genotype dependent trait and other factors may also have an influence (light quantity and quality, media composition, stress treatment, etc.). Oleszczuk et al. (2006) described a relatively lower number of albinos in barley ICM after increased temperatures in a low temperature pre-treatment of isolated anthers. Other components of media were also often reported to have an impact on the ratio of green/albino plantlets, but the physiological status of the donor plants and the exact stage of microspore development remain the most important influencing factors. The significant genetic variation between barley genotypes in this respect is still not explained sufficiently. Often spring barley seems to be more prone to albinism.

While in barley AC and IMC the spontaneous doubling rates are often acceptable they are one of the major disadvantages in wheat. In our experience the average doubling percentage in spring and in winter barley was between 66% in field planted DH populations. Nevertheless the data varied between 20–95% per genotype, which proves a genetic control and dependency also of this trait, which has a significant economical impact. The biological processes for spontaneous doubling in barley were described by Shim et al. (2006). Plants should be grown under optimal conditions in order to maximise doubled haploidy. Flow cytometry can be used to select and transfer haploid plantlets to a chemical induction procedure. This process could be automated, but the costs are prohibitive and, usually, the spontaneous doubling rate in barley is high enough for breeding purposes.

Further technological improvements require studies on the influence of carbon dioxide fertilization and air humidity on the response of barley in AC and/or ICM. The quality and quantity of data in this respect is very low due to the costs of such experiments. Plant physiological status is also dependent upon water and nutrient supply and culture substrate and these are all worthy for testing in future R&D programmes (see also chapter by Wędzony et al. on "Progress in doubled haploid technology in higher plants").

DHs in Barley Breeding

Worldwide several hundred if not thousands of doubled haploid barley varieties are registered. Unfortunately a complete overview does not exist. Nevertheless on the COST 851 website (www.scri.ac.uk/assoc/COST851/Default.htm) a list of several spring and winter barley varieties is provided (see also chapter by Devaux and Kasha on "Overview of barley doubled haploid production"). It is quite obvious that every year an impressive number of new DH varieties are listed and registered worldwide (mainly in EU, Canada, US, Australia). One very good example of the usefulness of the DH technology is the successful breeding of varieties with different resistance genes against the Barley Mild Mosaic Virus/Barley Yellow Mosaic Virus (BaMMV/BaYMV) complex (Werner et al. 2000). Other pyramiding projects are running for BYDV resistance genes and significant efforts have been made in Australian breeding programmes to combine genes for quality, abiotic stresses and biotic stresses by combining doubled haploidy with molecular marker assisted breeding (P. Langridge 2008, personal communication).

Advantages of DH breeding programmes were often published and discussed worldwide. Accelerated breeding has been brought about by instant access to homozygote material, faster and easier recognition of quantitative traits and easier maintenance of material. These factors can act in concert with conventional programmes, but there are examples where the whole breeding programme has been converted to DH breeding. Quality data of quantitative traits such as malting and brewing quality are more reliable if they are generated with doubled haploid material as this allows repeated sampling and replication. After accelerated multiplication of DH progenies it is easy to test resistance and even yield data in replicated experiments on more than one location in a very short time interval. By this kind of breeding strategy the number of years for testing is replaced by the number of locations and often lines can be notified for the official testing procedures after 4–5 years of breeding (Forster and Thomas 2005).

Different authors compared the level and variation of yield, resistance and other agronomic traits in populations derived by SSD, DH and conventional breeding schemes. Some of them concluded the loss of genetic variation in DH populations out of crosses between parents with a significant genetic diversity, while crosses between similar genetic material were not affected. However such publications are rather old now and with the improved technologies and often excellent numbers of green plantlets per genotype, including exotic crosses in IMC, these problems seem to be of little concern to practical breeders. Nevertheless major disadvantages in respect to yield, yield stability, agronomic performance or resistance/tolerance to biotic and abiotic stresses were not described.

Traditional objections against doubled haploids as somaclonal variation, loss of genetic variation and lower stability of resistance to diseases are still under discussion. Nevertheless there are more and more winter and spring barley breeding programmes which are completely reliant upon doubled haploidy and these are becoming more and more successful on the markets worldwide.

Success on the seed markets was only possible by integrating DH laboratories with field breeding methodologies. This interaction is absolutely important in making DH technology a success. Issues here are mainly concerned with the timing of production and planting or culturing the donor plants and subsequent DH populations. Due to the fact that the culture and the maturing of DH populations (typically, up to 60,000 individual plants for a breeding programme per cycle) is not feasible (financially) under glasshouse conditions, field planting systems have to be established and optimized, and these vary considerably between the two, winter and spring barley, crop types. Optimal preparation of the field and further control of the DH nurseries against abiotic stresses (mainly drought, but also hail, wind, heavy rain showers), biotic stresses (fungi, soil borne and insect transmitted viruses, birds) are necessary. Due to, the often, intercalating time regimes of DH planting and their location (often separate or isolated) the populations can be very sensitive to insects and birds. Regular watering and pesticide applications should be provided when necessary, bird nets and protection against rabbits, mouse and deer are often necessary.

It should be noted that the use of doubled haploids is only a minor technical step in a total breeding procedure. The intellectual investment in planning a crossing programme and the quality of the breeding programme in total, the marketing and a good portion of luck in the official and unofficial field experiments are all essential inter-related factors.

Wheat

Doubled Haploid Technologies in Wheat

As in barley wheat doubled haploids were described early in the 1970s. Today in routine breeding programmes the maize pollination method in combination with a phytohormone treatment (dipping, spraying, injection etc.) and a subsequent embryo rescue and regeneration step is the method of choice. This is due to significant genotype dependency, high numbers of albinos and work load involved in AC and IMC in wheat. Nevertheless there are some institutions still working with AC, but the efficiencies remain quite low in comparison to the maize/wheat system. But AC remains still an option for specialized groups with a relatively uniform genetic pool and may take the lead in the future once more efficient methods are developed.

Significant improvements in wheat ICM are now possible by the application of inducer chemicals (Zheng et al. 2001), ovary co-culture (Mejza et al. 1993; Puolimatka et al. 1996) and arabinogalactanproteins (Letarte et al. 2006). Hansen and Andersen (1998a, b) described the use of antimitotic agents as colchicine, amiprophosmethyl and trifluralin in ICM and Navarro-Alvarez et al. (1994) described this approach in wheat anther culture too.

There are many publications that describe stress treatments such as temperature treatments, osmotic treatments and starvation, or even combinations thereof, of spikes, tillers or even isolated anthers or microspores. Touraev et al. (1996) reported

the successful regeneration after heat shock treatment on anthers and Shariatpanahi et al. (2006) reported successful regeneration without any stress pre-treatment and the use of a nutrient free induction medium.

Recently the number of publications concerning DH protocols for durum wheat has increased (Cistue et al. 2006; Labbani et al. 2007). The main improvements which are described by them are a mannitol pretreatment and the use of colchicine *in vitro*. The production of doubled haploids by maize durum technology is routine today, but unfortunately not very efficient and therefore expensive.

Necessary Improvements

Although there are wheat genotypes that exhibit a significantly higher response in anther or microspore technique, and sometimes there are also small pools of genotypes with satisfactory response levels, the reproducibility and the transfer of published protocols has not been reported to be successful to date with respect to breeding. Glasshouse conditions, laboratory specific technologies and the experience and the "green finger" are still very important factors to transfer published protocols. The exchange of knowhow by exchange of people/expertise seems one of the most promising and efficient ways to bring tissue culture successful to routine operations.

Isolated microspore cultures with the Bulgarian winter wheat variety 'Svilena' are often reported to be excellent. The identification of genomic regions which are responsible for this trait would be scientifically very interesting. There is, theoretically, also a possible exploitation for wheat breeders, but due to economic restrictions and strong competition such a breeding programme is very difficult to perform by a private plant breeding company.

Similarly it has not been possible to identify the signals or molecules which are emitted by, or exchanged between, wheat ovaries and wheat pollen *in vitro*. Although there are reports that certain arabinogalactanproteins (AGPs) might be enhancing factors, physiological and technical proof is lacking. The pursuit of genetic explanations is also required.

Spontaneous doubling in wheat haploid plantlets is normally low. While after isolated microspore culture higher values can be observed in some genotypes after wide crosses, the rate does not exceed 10%. Therefore the application of anti-mitotic agents on plantlets, microspores or even on the donor plants is necessary. *In vitro* application of colchicine, APM and trifluralin on freshly isolated microspores of wheat was described by Hansen and Andersen (1998a, b). The best doubling success was achieved after 48 hours application of 10 µM APM (74%) and with trifluralin (65%) and after 24 hours application of 1 mM colchicine (53%). The number of embryos was lower, but the number of green plantlets and doubled haploid plantlets per spike was higher than the control. Other anti-mitotic agents as caffeine (Thomas et al. 1997) have also been reported.

Normally the procedure of chemical induced doubling is performed on plantlets which were already potted in soil. Soil residues are removed from plantlets which are at the 3–5 leaf stage, roots are shortened and plantlets are dipped to a colchicine solution for some hours. By thorough washing with tap water the excess colchicine is removed and the plantlets are re-potted. After keeping the air humidity high in the first days the recovery rate can be increased. Normally only a small percentage of the plantlets die in this treatment. Nevertheless the doubling rate is very variable from year to year and chimeras often occur. Complete fertility is seldom observed due to the, often, chimeric nature of such plants. The doubling success is also very dependent on the environment in which the plantlets are cultured and matured.

The injection of colchicine, sometimes together with a phytohormone, to the internodes of spikes which are then subsequently pollinated with maize pollen has been described (Sood et al. 2003). Nevertheless due to the toxicity of colchicine such treatments are often difficult with respect to health and safety issue of workers in glasshouses and laboratories.

DH in Wheat Breeding

Many wheat doubled haploids were also listed by COST851 (http://www.scri.ac.uk/assoc/COST851/Default.htm). Exact data about the application of doubled haploid technologies in breeding programmes worldwide do not exist. It is estimated by the author that several hundred thousands of doubled haploid lines are produced per year worldwide (EU, Canada, Australia, China and elsewhere). It is most probable that the majority of the plantlets are produced by maize wheat pollination (J. Thomas 2007, P. Davies 2007, personal communication). Nevertheless from time to time there are interesting publications on wheat microspore culture, and it is not known what is happening in the biotechnology laboratories of the wheat breeding companies concerned.

The utilization of doubled haploids in wheat breeding is not as common as in barley. For example, most of the recently registered barley varieties in Germany are doubled haploids and 50% of the seed propagation acreage in barley was taken up with doubled haploid varieties in spring and in winter barley in Germany in 2006/07. Other data from other EU countries or worldwide are not known. In wheat these numbers are significantly lower in the EU. But exact data are not known.

Generally, it seems to be possible to apply for variety protection and registration after 4 or 5 years, also in winter types. Due to the lack of years the number of tested environments must be adjusted.

Specific examples for specific traits in registered varieties which were developed by a doubled haploid technology are not known by the author. Nevertheless interesting registered doubled haploid wheat varieties have been on the market for many years and the economical advantages of DH breeding in wheat is becoming more and more visible.

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