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Double haploid production & breeding approaches

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Abstract
Doubled haploid (DH) production has become an important tool in plant breeding largely due to its capacity to produce completely homozygous plants in one generation. Doubled haploids (DHs) are plants derived from a single pollen grain and doubled artificially to form homozygous diploids. A DH individual has two identical homologs, so that the amount of recombination information is equivalent to a backcross. Homozygosity at a particular locus is always desirable feature for crop improvement programs. Breeding is one of the approaches to attain this but it requires minimum 6-7 cropping years. Use of haploid to produce double haploid is one of the potent approaches to overcome this lacuna. Haploid can be either produce through androgenesis or gynogenesis, androgenesis being most used approach. Not only are traits fixed for selection but the multiple generations of inbreeding required using traditional breeding methods are circumvented. However, prior to implementing a DH breeding program, the breeder should consider factors such as the potential for linkage drag, types of crosses to be used and whether production resources are sufficient to produce the DH populations necessary for success. Doubled haploid technology can be integrated with marker-assisted breeding for greater efficiency and to craft the DH population for particular traits. The technology can also be used to accelerate development of germplasm with new genes of interest and to generate cytogenetic stocks. The future for DH breeding is promising because robust DH protocols are available for an ever-growing number of crops and future applications will see a closer integration with molecular-marker and gene-splicing technologies.

Keywords: Triclosan, TCS, determination, detection, sensor

Introduction
A doubled haploid (DH) is a genotype formed when haploid cells undergo chromosome doubling. Artificial production of doubled haploids is important in plant breeding. Haploid cells are produced from pollen or egg cells or from other cells of the gametophyte, then by induced or spontaneous chromosome doubling, a doubled haploid cell is produced, which can be grown into a doubled haploid plant. If the original plant was diploid, the haploid cells are monoploid, and the term doubled monoploid may be used for the doubled haploids. Haploid organisms derived from tetraploids or hexaploid are sometimes called dihaploids (and the doubled dihaploids are, respectively, tetraploid or hexaploid). Conventional inbreeding procedures take six generations to achieve approximately complete homozygosity, whereas doubled haploidy achieves it in one generation (Jain *et al.*, 1996) [21]. Dihaploid plants derived from tetraploid crop plants may be important for breeding programs that involve diploid wild relatives of the crops. Haploids are plants (sporophytes) that contain a gametic chromosome number (n). They can originate spontaneously in nature or as a result of various induction techniques. Spontaneous development of haploid plants has been known since 1922, when Blakeslee first described this phenomenon in *Datura stramonium* (Blakeslee *et al.*, 1922) [3]. this was subsequently followed by similar reports in tobacco (*Nicotiana tabacum*), wheat (*Triticum aestivum*) and several other species (Forster *et al.*, 2007) [15].

However, spontaneous occurrence is a rare event and therefore of limited practical value. The potential of haploidy for plant breeding arose in 1964 with the achievement of haploid embryo formation from *in vitro* culture of *Datura* anthers (Guha and Maheshwari, 1964, 1966) [17, 20]., which was followed by successful *in vitro* haploid production in tobacco (Nitsch and Nitsch, 1969) [29]. Many attempts have been made since then, resulting in published protocols for over 250 plant species belonging to almost all families of the plant kingdom (reviewed in Maluszynski *et al*., 2003) [23]. In fact, under optimal conditions, doubled haploids (DH) have been routinely used in breeding for several decades, although their common use is still limited to selected species.

Production of double haploids

Haploids produced from diploid species (2n=2x), known as monoploids, contain only one set of chromosomes in the sporophytic phase (2n=x). They are smaller and exhibit a lower plant vigor compared to donor plants and are sterile due to the inability of their chromosomes to pair during meiosis. In order to propagate them through seed and to include them in breeding programs, their fertility has to be restored with spontaneous or induced chromosome doubling. The obtained DHs are homozygous at all loci and can represent a new variety (self-pollinated crops) or parental inbred line for the production of hybrid varieties (cross-pollinated crops). In fact, cross pollinated species often express a high degree of inbreeding depression. For these species, the induction process *per se* can serve not only as a fast method for the production of homozygous lines but also as a selection tool for the elimination of genotypes expressing strong inbreeding depression. Selection can be expected for traits caused by recessive deleterious genes that are associated with vegetative growth. Traits associated with flower fertility might not be related and should be eliminated by recurrent selection among DH lines. The production of pure lines using doubled haploids has several advantages over conventional methods. Using DH production systems, homozygosity is achieved in one generation, eliminating the need for several generations of self-pollination. The time saving is substantial, particularly in biennial crops and in crops with a long juvenile period. The main factors affecting haploid induction and subsequent regeneration of embryos are: genotype of the donor plants, physiological condition of donor plants (i.e. growth at lower temperature and high illumination), developmental stage of gametes, microspores and ovules, pre-treatment (i. e. cold treatment of inflorescences prior to culture, hot treatment of cultured microspores), composition of the culture medium (including culture on "starvation" medium low with carbohydrates and/or macro elements followed by transfer to normal regeneration medium specific to the species) and physical factors during tissue culture (light, temperature).

Haploid production through pollination with pollen of the same species

Maternal haploid induction in maize (*Zea mays* L.) is a result of legitimate crossing within one species with selected inducing genotypes (line, single cross or population). It results in a majority of regular hybrid embryos and a smaller proportion of haploid maternal embryos with normal triploid endosperms. The first recognized inducer line was the genetic strain Stock 6, with an haploid induction rate of up to 2.3% (Coe, 1959) [11], which was subsequently improved by hybridization and further selection. Today, modern haploid inducing lines display high induction rates of 8 to 10% (Geiger & Gordillo, 2009) [18]. They are routinely used in commercial DH-line breeding programs due to their high effectiveness and lower genotype dependence.

The production of maternal haploids stimulated by irradiated pollen requires efficient emasculation, which has in some cases been shown to limit its use because the method is too laborious. To overcome such an obstacle in onion, for instance, only male sterile donor plants were used as donor plants, but such lines, possessing cytoplasmically inherited male sterility, are of very limited practical use. An increase in the irradiation dose causes a decrease in the total number of developed embryos but the obtained regenerants are mostly of haploid origin. For most plant species, *in vitro* embryo rescue is necessary to recover haploid plants. The collection of mature seeds has only been reported for kiwifruit (Pandey *et al*., 1990; Chalak & Legave, 1997) [31, 6], onion (Dore & Marie, 1993) [13], mandarin (Froelicher *et al*., 2007) [16]. and species of the genus *Nicotiana* (Pandey & Phung, 1982) [30]. Even for the aforementioned species, *in vitro* germination of seeds enhanced the recovery of haploid plants.

Hploid production through Wide hybridization

In barley, haploid production is the result of wide hybridization between cultivated barley (*Hordeum vulgare*, 2n=2x=14) as the female and wild *H. bulbosum* (2n=2x=14) as the male. After fertilization, a hybrid embryo containing the chromosomes of both parents is produced. During early embryogenesis, chromosomes of the wild relative are preferentially eliminated from the cells of developing embryo, leading to the formation of a haploid embryo, which is due to the failure of endosperm development. A haploid embryo is later extracted and grown *in vitro*.

Paternal chromosome elimination has also been observed after interspecific crosses between wheat (*Triticum aestivum*) and maize. After pollination, a hybrid embryo between wheat and maize develops but, in the further process, the maize chromosomes are eliminated so that haploid wheat plantlets can be obtained. Such haploid wheat embryos usually cannot develop further when left on the plant, because the endosperm fails to develop in such seeds. By applying growth regulator 2,4-dichlorophenoxyacetic acid *in planta*, embryo growth is maintained to the stage suitable for embryo isolation and further *in vitro* culture. potato Haploid production in cultivated potato (*Solanum tuberosum* L. ssp. *tuberosum*, 2n=4x) can be achieved by inter-generic pollination with selected haploid inducer clones of *S. phureja* (2n=2x). The tetraploid female *S. tuberosum* produces an embryo sac containing one egg cell and two endosperm nuclei, all with the genetic constitution n=2x, while the diploid pollinator *S. phurea* produces two sperms of the genetic constitution n=x or 2x. After pollination, dihaploid (2n=2x) embryos can develop from un-fertilized egg cells, which are supported by a 6x endosperm formed by the fusion of polar nuclei with both reduced sperm cells. The frequency of dihaploid seeds is low; they have to be selected from hybrid seeds containing 3x or 4x embryos developed from egg cells (n=2x) fertilized with haploid (n=x) or diploid (n=2x) sperm cells. (Maine, 2003).

Haplod production through gynogenesis

It can be achieved with the *in vitro* culture of various un-pollinated flower parts, such as ovules, placenta attached ovules, ovaries or whole flower buds. Although gynogenetic regenerants show higher genetic stability and a lower rate of albino plants compared to androgenetic ones, gynogenesis is used mainly in plants in which other induction techniques,

such as androgenesis and the pollination methods above described, have failed. Gynogenic induction using un-pollinated flower parts has been successful in several species, such as onion, sugar beet, cucumber, squash, gerbera, sunflower, wheat, barley etc. (Bohanec, 2009 and Chen *et al*., 2011) [4, 8]. but its application in breeding is mainly restricted to onion and sugar beet.

Haplod production through androgenesis

Androgenesis is the process of induction and regeneration of haploids and double haploids originating from male gametic cells. Due to its high effectiveness and applicability in numerous plant species, it has outstanding potential for plant breeding and commercial exploitation of DH. It is well established for plant breeding, genetic studies and/or induced mutations of many plant species, including barley, wheat, maize, rice, triticale, rye, tobacco, rapeseed, other plants from *Brassica* and other genera (for protocols, see Maluszynski *et al*., 2003) [23]. Its major drawbacks are high genotype dependency within species and the recalcitrance of some important agricultural species, such as woody plants, leguminous plants and the model plant *Arabidopsis thaliana*. Another culture was the first discovered haploid inducing technique of which efficiency was sufficient for plant breeding purposes (Maluszynski *et al*., 2003) [23].

Genetics of double haploid

In DH method only two types of genotypes occur for a pair of alleles, A and a, with the frequency of ½ AA and ½ aa, while in diploid method three genotypes occur with the frequency of ¼ AA, ½ Aa, ¼ aa. Thus, if AA is desirable genotype, the probability of obtaining this genotype is higher in haploid method than in diploid method. If n loci are segregating, the probability of getting the desirable genotype is (1/2)n by the haploid method and (1/4)n by the diploid method. Hence the efficiency of the haploid method is high when the number of genes concerned is large. Studies were conducted comparing DH method and other conventional breeding methods and it was concluded that adoption of doubled haploidy does not lead to any bias of genotypes in populations, and random DHs were even found to be compatible to selected line produced by conventional pedigree method (Winzeler, 1987) [38].

Applications of doubled haploids in plant breeding

The induction and regeneration of haploids followed by spontaneous or induced doubling of chromosomes are widely used techniques in advanced breeding programs of several agricultural species. They have been successfully used for commercial cultivar production of species such as asparagus, barley, *Brassica juncea*, eggplant, melon, pepper, rapeseed, rice, tobacco, triticale, wheat and more than 290 varieties have already been released. Using DH technology, completely homozygous plants can be established in one generation thus saving several generations of selfing comparing to conventional methods, by which also only partial homozygosity is obtained. Another feature that should be considered is the breeding strategy. Within the breeding process, DH lines can be induced as soon as from F1 generation (note that gametes on F1 plants represent the F2 generation), although some breeders prefer to induce DH lines from later generations. Induction in the F2 generation was proposed as an option because lines originated from F3 generation gametes had passed through another recombination cycle. However, Choo *et al*. (1982) [9], comparing DH and single seed descent methods showed that there was no difference in the sample of recombinants.

Mapping quantitative trait loci

Most of the economic traits are controlled by genes with small but cumulative effects. Although the potential of DH populations in quantitative genetics has been understood for some time, it was the advent of molecular marker maps that provided the impetus for their use in identifying loci controlling quantitative traits. As the quantitative trait loci (QTL) effects are small and highly influenced by environmental factors, accurate phenotyping with replicated trials is needed. This is possible with doubled haploidy organisms because of their true breeding nature and because they can conveniently be produced in large numbers. Using DH populations, 130 quantitative traits have been mapped in nine crop species (Forster & Thomas, 2003) [34]. In total, 56 DH populations were used for QTL detection (Maluszynska *et. al.*, 2003) [25].

Backcross breeding

In backcross conversion, genes are introgressed from a donor cultivar or related species into a recipient elite line through repeated backcrossing. A problem in this procedure is being able to identify the lines carrying the trait of interest at each generation. The problem is particularly acute if the trait of interest is recessive, as it will be present only in a heterozygous condition after each backcross. The development of molecular markers provides an easier method of selection based on the genotype (marker) rather than the phenotype. Combined with doubled haploidy it becomes more effective. In marker assisted backcross conversion, a recipient parent is crossed with a donor line and the hybrid (F1) backcrossed to the recipient. The resulting generation (BC1) is backcrossed and the process repeated until the desired genotypes are produced. The combination of doubled haploidy and molecular marker provides the short cut. In the back-cross generation one itself a genotype with the character of interest can be selected and converted into homozygous doubled haploid genotype (Thomas *et al.*, 2003) [34]. Chen *et al.* (1994) [5]. used marker assisted backcross conversion with doubled haploidy of BC1 individuals to select stripe rust resistant lines in barley.

Bulked segregant analysis

In bulked segregant analysis, a population is screened for a trait of interest and the genotypes at the two extreme ends form two bulks. Then the two bulks are tested for the presence or absence of molecular markers. Since the bulks are supposed to contrast in the alleles that contribute positive and negative effects, any marker polymorphism between the two bulks indicates the linkage between the marker and trait of interest. BSA is dependent on accurate phenotyping and the DH population has particular advantage in that they are true breeding and can be tested repeatedly. DH populations are commonly used in bulked segregant analysis, which is a popular method in marker assisted breeding (Ardiel *et al.*, 2002) [1]. This method has been applied mostly to rapeseed and barley.

Genetic maps

Genetic maps are very important to understand the structure and organization of genomes from which evolution patterns and syntenic relationships between species can be deduced. Genetic maps also provide a framework for the mapping of

genes of interest and estimating the magnitude of their effects and aid our understanding of genotype/phenotype associations. DH populations have become standard resources in genetic mapping for species in which DHs are readily available. Doubled haploid populations are ideal for genetic mapping. It is possible to produce a genetic map within two years of the initial cross regardless of the species. Map construction is relatively easy using a DH population derived from a hybrid of two homozygous parents as the expected segregation ratio is simple, *i.e*. 1:1. DH populations have now been used to produce genetic maps of barley, rapeseed, rice, wheat, and pepper. DH populations played a major role in facilitating the generation of the molecular marker maps in eight crop species (Maluszynska *et al.*, 2003) [25]. Genetic ratios and mutation rates can be read directly from haploid populations. A small doubled haploid (DH) population was used to demonstrate that a dwarfing gene in barley is located chromosome 5H. In another study the segregation of a range of markers has been analyzed in barley (schon *et al.*, 1990) [32].

Although QTL analysis has generated a vast amount of information on gene locations and the magnitude of effects on many traits, the identification of the genes involved has remained elusive. This is due to poor resolution of QTL analysis. The solution for this problem would be production of recombinant chromosome substitution line, or stepped aligned recombinant inbred lines (Kearsey *et al.*, 2002) [22]. Here, backcrossing is carried out until a desired level of recombination has occurred and genetic markers are used to detect desired recombinant chromosome substitution lines in the target region, which can be fixed by doubled haploidy (Thomas *et al.*, 2000) [35]. In rice, molecular markers have been found to be linked with major genes and QTLs for resistance to rice blast, bacterial blight, and sheath blight in a map produced from DH population (wang *et al.*, 2001) [38].

Traditional breeding methods are slow and take 10-15 years for cultivar development. Another disadvantage is inefficiency of selection in early generations because of heterozygosity. These two disadvantages can be overcome by DHs, and more elite crosses can be evaluated and selected within less time. Uniformity is a general requirement of cultivated line in most species, which can be easily obtained through DH production. There are various ways in which DHs can be used in cultivar production.

Novel approaches combining DHs and molecular genetics

A simplified scheme for backcrossing has been proposed (Forster *et al*., 2007) [15], aimed at shortening the period needed for the introduction of a particular trait from donor to recipient germplasm. According to the scheme, DHs are produced from the BC1 generation. Segregation of parental chromosomes into the filial generation is followed by molecular markers to identify lines with only recipient chromosomes. The gene of interest should thus be introduced into the recipient chromosome by a random crossing over event in the BC1 generation. A protocol for "reverse breeding" was proposed by Wijnker *et al.* (2007) [40]. According to this invention, superior hybrid genotypes are first identified among the segregating population. Using genetic transformation, a gene for induced suppression of meiotic recombination is then introduced, and several DH lines are produced. Segregation of chromosomes is followed by chromosome specific molecular markers and a final combination of two lines represents complementary sets constituting the original heterozygous superior hybrid. Both methods described above are at present predominantly theoretical and one of their obvious limitations is the number of chromosomes of a particular species. As described by Dirks (2009) [12], the probability of finding two lines with a complete set of homologous chromosomes sharply decreases with the number of chromosomes of a particular species. For instance, the probability of identifying such complementary lines is 1 in 47 for *Arabidopsis* (n=x=5) but as high as 1 in 532 in a species with n=x=12. Genetic transformation at haploid level has been studied in several ways. The most common approach has been for haploid plants to be transformed using established transformation methods. To give just one example, Chauhan *et al*. (2011) [7]. transformed haploid bread wheat with an HVA1 gene to obtain drought tolerance. Chromosome doubling thus enabled stable fixation of the integrated gene, and this feature was tested for 14 generations. Another approach has been for haploid cells themselves, mainly microspores, to be targets of transformation prior to haploid induction. Touraev *et al*. biolistically transformed tobacco microspores, induced maturation and pollinated to achieve transformed progeny. Eudes and Chugh (2008) [14]. and Chugh *et al*. (2009) [10]. transformed triticale microspores using the coupling of cell-penetrating peptides with plasmid DNA and regenerated haploid transformed plants.

A completely novel approach for haploid induction has recently been developed by the genetic engineering of the centromeric region (Ravi & Chan, 2010) [32]. Centromeres are chromosomal regions in which DNA sequences serve as binding sites for kinetochore proteins, on which spindle microtubules bind during mitosis and meiosis. In this novel method, a kinetochore protein (CENH3) was first disabled by mutation and the altered version was then inserted by genetic transformation. In such plants, this novel CENH3 protein is also disabled but only to such an extent that its chromosome segregating function is maintained, while defective kinetochores cause elimination of this chromosomal set during mitotic divisions in zygotic cells. To achieve haploid induction, therefore, the method requires inactivation of the endogenous *CENH3* gene by mutation or RNAi interference and the insertion of an additional gene coding for the *CENH3* variant. This method has some resemblance to the genome elimination described previously in wide crosses or in the case of maize intra-specific crosses and potentially allows its use in any plant species. The authors claim that another feature of this system is that the 'inducer line' (line with the altered centromeric gene) can be used to induce either maternal or paternal haploids by crossing the mutant with female or male wild-type plants. The procedure has so far been demonstrated in *Arabidopsis thaliana*, causing up to 50% of the F1 progeny to be haploid. At the same time, this protocol is the first demonstration of haploid induction in this model species, which has been recalcitrant to all haploid induction protocols available so far. Attempts to test this procedure are currently ongoing in other species. Protocols involving genetic engineering in agricultural applications have given rise to opposition in several countries, thus limiting their availability in breeding programs. It should be noted that, in the case of the presented 'transgenic inducer technology', the final haploid line would not possess any transgenic elements, because the chromosomes of the inducer line are outcompeted by a wild-type parent. It remains to be resolved how such a process will be regulated under GMO legislation. At least at the EU level, legal

regulation/deregulation of such new techniques is already under discussion.

Conclusion

Technological advances have now provided DH protocols for most plant genera. The number of species amenable to doubled haploidy has reached a staggering 250 in just a few decades. Response efficiency has also improved with gradual removal of species from recalcitrant category. Hence it will provide greater efficiency of plant breeding. Doubled haploidy is and will continue to be a very efficient tool for the production of completely homozygous lines from heterozygous donor plants in a single step. Since the first discovery of haploid plants in 1920 and in particular after the discovery of *in vitro* androgenesis in 1964, techniques have been gradually developed and constantly improved. The method has already been used in breeding programs for several decades and is currently the method of choice in all species for which the technique is sufficiently elaborated. Species for which well-established protocols exist predominantly belong to field crops or vegetables, but the technique is gradually also being developed for other plant species, including fruit and ornamental plants and other perennials. It should be mentioned that, in addition to breeding, haploids and doubled haploids have been extensively used in genetic studies, such as gene mapping, marker/trait association studies, location of QTLs, genomics and as targets for transformations. Furthermore, the haploid induction technique can nowadays be efficiently combined with several other plant biotechnological techniques, enabling several novel breeding achievements, such as improved mutation breeding, backcrossing and hybrid breeding.

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