Review article

Chromosome Doubling Methods in Double Haploid and Haploid Inducer-Mediated Genome-Editing Systems in Major Crops

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Abstract

The double haploid technique aims to generate pure inbred lines for basic research and as commercial cultivars. The double haploid technique first generates haploid plants and is followed by chromosome doubling, which can be separated in time or overlapped, depending the procedure for each species. For a long time much effort has been focused on haploid production via androgenesis, gynogenesis or parthenogenesis. Obtaining haploid plants is the first step and because this has frequently required more optimization, research in chromosome doubling methods has lagged behind. Nevertheless, chromosome doubling has recently been of renewed interest to increase the rates and efficiency of double haploid plant production through trialing and optimizing of different procedures. New antimitotic compounds and application methods are being studied to ensure the success of chromosome doubling once haploid material has been regenerated. Moreover, a haploid inducer-mediated CRISPR/Cas9 genome-editing system is a breakthrough method in the production of haploid plant material and could be of great importance for species where traditional haploid regeneration methods have not been successful, or for recalcitrant species. In all cases, the new deployment of this system will demand a suitable chromosome doubling protocol. In this review, we explore the existing double haploid and chromosome doubling methods to identify opportunities to enhance the breeding process in major crops.

Keywords: Double haploid, Androgenesis, Gynogenesis, Parthenogenesis, Haploid Inducer, CRISPR/Cas9, Chromosome doubling, Antimitotic.

Introduction

Plant breeding has attempted over many decades to increase crop yield and improve variety traits. The purpose of plant breeding is to boost agronomical traits such as: disease and insect plant resistance; tolerance to abiotic stresses like drought, extremes of temperature and salinity; and to increase yield while at the same time enhancing or maintaining nutritional quality (Poehlman et al. 1995).

Conventional breeding by backcrossing is a method to improve an elite line by adding a new trait. By crossing the elite line with a donor line, carrying the trait of interest, an F1 hybrid is obtained and backcrossed with the elite line again. The subsequent offspring is recurrently backcrossed with the elite line again, until the 5th to 8th generations. The final backcrossed line contains the new trait of interest and the characteristics of the elite line, and the genotypic background of the donor line has been cleared (Forster et al. 2007). Backcrossing is effective, but obtaining a stabilized line with the trait of interest is extremely time consuming due to the number of generations needed to be crossed and evaluated (**Fig. 1**). Instead, the emergence of doubled haploid technology in the second half of the 20th century has dramatically reduced the time required to generate pure homozygous lines.

Double haploid (DH) lines are pure and genetically homozygous individuals produced when spontaneous or induced chromosome duplication of haploid cells occurs. DHs are one of the leading achievements in plant breeding because completely homozygous plants can be produced within a year. DH production includes two major steps: haploid induction and chromosome doubling. Haploid induction attempts to regenerate haploid or spontaneous DH plants, which can be achieved by androgenesis, gynogenesis or parthenogenesis, depending on the species. The chromosome-doubling step is mandatory when spontaneous DHs are not regenerated and is achieved by using antimitotic compounds to double the ploidy level of haploid plants.

The haploid inducer (HI)-mediated genome-editing system is a promising approach for DH production that is still under development for the majority of crops. Nowadays, genome-editing technologies are of major importance in many research areas, plant breeding included. Since the discovery of CRISPR/Cas9 in 2012, and its first use in plants in 2013, many applications of genome-editing technology have been boosted thanks to the efficiency and versatility of CRISPR/Cas9 in comparison to previous genome-editing technologies such as targeted induced local lesions in the genome (TILLING), zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALENs). The CRISPR/Cas9 system is a genome-editing technology that enables an unprecedented control over the mutation process. CRISPR/Cas9 technology consists of a Cas9 nuclease, guided by a 20-nt sequence (gRNA), which induces DNA double-strand breaks (DSBs). DSBs are repaired by either non-homologous end-joining (NHEJ) or homology directed repair (HDR), generating insertion and deletion events (INDELs) in a precise DNA target sequence. CRISPR/Cas9 has features over its predecessors that make its use and application easier and more efficient (Belhaj et al. 2015).

Many reviews have been published summarizing haploid production and the best methodologies for each species. Many crops have excellent specific reviews focused on the DH techniques for a plant family or single species like the *Fabaceae* (Croser et al. 2007), the *Solanaceae* (Seguí-Simarro et al. 2011), the *Cucurbitaceae* (Dong et al. 2016), bell pepper (Irikova et al. 2011), wheat (Niu et al. 2014), rice (Mishra and Rao 2016), and sorghum (Teingtham and La Borde 2017). In addition, the use of antimitotic agents for plant tissue polyploidization has been reviewed by Dhooghe et al. (2011), and the current and novel technologies for haploid induction have been reviewed by Ren et al. (2017). However, an evaluation is still needed of the best methodologies for DH production that combine HI technology with the application of antimitotic agents for chromosome doubling in major crops. Moreover, the opportunities that HI-mediated genome-editing offers have not yet been reviewed for recalcitrant crop species.

In this review we discuss the following aspects in major crop species: the methods of DH production; the availability of chromosome-doubling methods to obtain DH lines; the opportunities for HI-mediated genome-editing systems in DH technology, and, finally, we focus on the development of the DH technique, HIs, antimitotic agents and chromosome-doubling protocols for the future of plant breeding.

Double haploid uses

DH lines are highly important for plant breeding due to their complete homozygosis, making qualitative and quantitative phenotypic selection more efficient. Since the very first attainment of DHs in *Brassica napus* (Thompson 1972), many publications have reported the development of DH lines in more than 250 species (Maluszynski 2003). Following the research conducted in the 70s and 80s that demonstrated the ability to generate DHs in many cereal, vegetable and horticultural crops, the focus in recent decades has been optimizing and

assaying different ways to enhance DH production in each species and genotype by introducing changes in every step of the DH programs.

DHs have been of great importance for: establishing chromosome maps and whole genome sequencing in the vast majority of genetically mapped and sequenced species; bulked segregant analysis (BSA), which is used for detecting markers associated with traits in segregation populations; and, mapping quantitative trait loci (QTLs) (Forster et al. 2007). These implications and application of DHs in basic research are usually further used for variety improvements. Furthermore, DHs have a great potential as new commercial cultivars, avoiding classical breeding methods to obtain stabilized and non-segregant lines, which are used as commercial cultivars such as stabilized homozygous lines or as parental lines to produce F1 hybrid lines.

Strategies for the production of double haploid material

Gametic haploid cells are the initial material used to obtain DH lines. Gametes from meiotic cells allow the generation of plantlets when cultured *in vitro* or when pollinated with irradiated pollen. The haploid step can be either a microspore from an anther or an ovule from an ovary, depending on the species. The usual methods to induce haploids are as follows: androgenesis, gynogenesis and parthenogenesis (**Fig. 1**). Plantlet regeneration from microspores or ovaries is a two-step protocol if a callus step is required prior to plantlet regeneration, or a one-step protocol if it directly induces an embryo or regenerates a plantlet. On the other hand, gametic cells from meiosis can be developed into haploid embryos, via false fecundation. Thus, a DH process always requires a gametic haploid step from which haploid or DH plantlets will be regenerated. .

In vitro culture techniques for gametic cells in androgenesis and gynogenesis allow the original gametophytic pathway of the gamete to be redirected towards a sporophytic pathway where plantlets can be regenerated. Androgenesis is the most common method to produce DHs (**Table 1**). Isolated microspores or microspores contained in anthers are cultured in specific induction media to induce the formation of callus. Subsequently, these calluses are cultured in regeneration media to regenerate fully formed plantlets. In most cereal species, androgenesis is the only known method for DH generation. Androgenesis is the best method to obtain DHs, with a high rate of spontaneous doubling, in species such as rice (Hooghvorst et al. 2018), oat (Kiviharju et al. 2017) and bell pepper (Keleş et al. 2015). Gynogenesis stimulates *in vitro* embryogenesis development of the unfertilized haploid egg cells. In this process, a two-step protocol is usually carried out to induce callus formation from the female ovules in induction medium and to regenerate plants from callus in regeneration medium. For onion (Fayos et al. 2015) or beet (Hansen et al. 1995), gynogenesis is the best method for DH production. The ploidy level of the androgenetic and gynogenetic regenerated plants can differ depending on cell events related to spontaneous or induced chromosome doubling (see below). Haploids, double haploids, mixoploids and tetraploids can be produced during the *in vitro* DH process (**Fig. 1**). In androgenesis and gynogenesis it is desirable that the regenerated plantlets originate from microspore or ovule cells; nonetheless, somatic embryogenesis from anther or ovary tissues can take place. This process is defined as the regeneration of a whole plant from undifferentiated somatic cells in *in vitro* culture. The ploidy of these plantlets is diploid, and the genomic background is identical to the line from which the DHs are expected to be generated (**Fig. 1**). Parthenogenesis methodology allows the formation of an embryo from an egg cell without fertilization. Egg cells can be induced to develop into haploid embryos following *in situ* pollination with irradiated pollen, and these embryos only inherit the maternal set of chromosomes due to false fecundation. Such embryos germinate *in vitro* and develop mostly haploid plants, but sometimes also mixoploid or spontaneously chromosome doubled haploid plants. In the *Cucurbitaceae*, parthenogenesis is the only successful approach to obtain DH plant material (Dong et al. 2016).

Conventional DH technology has had great importance in some species to produce pure homozygous lines. Nonetheless, in all reported species there is a high genotypic dependency on the efficiency of the method, with some cultivars adapted to the existing protocols and some others very recalcitrant to the process. Publications on rice, wheat and maize show correlation with the substantial progress in haploid technology, attainable given the intensive research efforts (Croser et al. 2007). The majority of crops have acceptable DH protocols from which DH lines are produced successfully, but they require a significant time investment that ranges from 5 months to 2 years, substantial personnel and equipment needs, and always have the inevitable variability in efficiency, depending on the genotype used. There are even crops of great economic importance, including tomato species (Seguí-Simarro et al. 2011) and members of the *Cucurbitaceae* and *Fabaceae* families (Croser et al. 2007; Dong et al. 2016), that lack a successful protocol for DH production.

Approaches and process of the chromosome-doubling step

Every DH program starts with the haploid gametophytic phase to efficiently obtain DH plants. During the latter part of the *in vitro* process, haploid plant material needs to undergo chromosome duplication to finally

obtain a fertile plant, from which DH seeds are recovered. The original chromosome set, whether maternal (gynogenesis and parthenogenesis) or paternal (androgenesis), must go through a spontaneous or induced duplication. The effective duplication of the haploid material is essential for the success of the DH process, because haploid plants are infertile. The chromosome doubling can be spontaneous or induced. Induced chromosome duplication may be feasible at different stages of the process: at the first pollen mitotic division of microspore cells, at the callus stage when growing *in vitro*, or at the plant stage when regenerated (**Table 1**). Earlier duplication is the ideal for avoiding mixoploid plants, or fully haploid plants, and to ensure a battery of DH plants.

Endomitosis and nuclear fusion are the main causes of spontaneous duplication. These processes have been studied extensively in barley and inferred in other species (Kasha, 2005). During mitosis, chromosome multiplication and separation of cells usually occurs. Instead, in endomitosis, multiplication occurs but the cell fails to divide and one nucleus with two sets of chromosomes is restored. During nuclear fusion, two or more synchronized nuclei divide and develop a common spindle. Spontaneous chromosome doubling capacity during the process depends on the species and genotype. For example, the frequency of spontaneous DH androgenic bell pepper plants is 30-55% (Irikova et al. 2011; Keleş et al. 2015); in rice, it ranges between 8 to 30% of the regenerated plants (Alemanno and Guiderdoni 1994; Hooghvorst et al. 2018; López-Cristoffanini et al. 2018). In species whose spontaneous doubling rate is high, the processes of induced chromosome doubling have not been explored to any great extent for obvious reasons. Nevertheless, despite this some species are more likely to regenerate spontaneous DHs than others because all *in vitro* processes are genotypically dependent, including duplication. Indeed, species with generally high rates of spontaneous DH still need efficient protocols to induce doubling with antimitotic compounds because some genotypes within such species have low rates of spontaneous DH generation or no spontaneous production at all, meaning that antimitotic application is still essential.

A proportion of the so-called 'spontaneous' duplication reported in the literature is actually induced via chromosome doubling by means of pre-treatments that do not involve antimitotic compounds. Temperature stress, like heat or cold pre-treatment, is usually applied during androgenesis and gynogenesis prior to *in vitro* culture. Many of these pre-treatments are initially meant to increase microspore induction, but they usually increase the frequency of chromosome doubling due to the destabilization of microtubules and microfilament elements that form the cytoskeleton (Kasha, 2005). In microspores, cold pre-treatment is related to failure of cell wall formation leading to multinucleate structures (known as coenocytic structures, see Testillano et al. 2002), which result from nuclear division without cytokinesis. However, in spite of the basic research that has related pre-treatment protocols to increased numbers of microspores at the optimal stage, once the microspore culture is started and plants regenerate, it is difficult to demonstrate that the pre-treatment is the causal agent of the increase in frequency of DHs beyond the determination of the best microspore stage for embryogenesis.

The use of antimitotic compounds is mandatory when spontaneous chromosome doubling is absent or very low. A specific type of endomitosis, known as C-mitosis, takes place when antimitotic compounds are used to destabilize the cell cycle, perturbing not only mitosis but also arresting cells during interphase (Lu et al. 2012). In interphase, DNA is replicated and each replicated chromosome forms sister chromatids that are bound by the centromere's spindle tubules. When C-mitosis occurs, the antimitotic compound interacts with tubulin subunits destabilizing and inhibiting their assembly. Antimitotic treatment depends on the species and the protocol used for obtaining DH plants. Some key features considered are the antimitotic agent, its concentration, the exposure time and the treatment stage, which are thoroughly reviewed by Dhooghe et al. (2009).

In androgenetic protocols for DH production, free-microspores or microspores contained in anthers are usually cultured in colchicine-supplemented medium. By this means, chromosome doubling is achieved earlier, ensuring the success of the process. In addition, an increased level of microspore induction has been attributed to the presence of colchicine (Alemanno and Guiderdoni, 1994; Barnabás et al. 1999; Hooghvorst et al. 2018; Iqbal et al. 1994). Haploid plants as explant material are another important source for recovering DHs. Most of the DH protocols apply antimitotic compounds to plants grown either *in vitro* or *in vivo* to achieve chromosome doubling (**Table 1**). Immersion of the whole *in vitro* plantlet or the apical meristem *in vivo* are two approaches that usually yield good chromosome doubling results in many plant species. *In vitro* treatments usually take longer and the antimitotic concentration is lower, whereas *in vivo* treatments have shorter exposure times with higher concentrations of compound. Nevertheless, in some species like onion, the apical meristem of adult plants is inaccessible, impeding chromosome doubling *in vivo* (Bohanec 2002). Meanwhile, during propagation of regenerated haploid plants chromosome doubling has also been achieved by adding antimitotic compounds to the culture medium. Breeders sometimes discard androgenetic and gynogenetic haploid plants when spontaneous double regenerants are considered acceptable. Despite their great potential, the lack of a successful protocol for chromosome doubling in grown plants means that they frequently go to waste. In contrast, the parthenogenetic process usually depends on whole plants or embryos as the source of material for DH recovery (**Table 1**).

Several antimitotic agents have been used for chromosome doubling of haploid plants. Colchicine is the most popular antimitotic agent used for DH studies in most species because it has a high chromosome doubling ability. Yet there are many different chemicals with antimitotic effects such as amiprophosmethyl (AMP), pronamide, propham, oryzalin and trifluralin, which have similar effects and mechanisms of action to colchicine (Bartels and Hilton 1973). Colchicine has been historically the most used antimitotic agent for DH plant production. This compound is a toxic natural product extracted from plants of the *Colchicum* genus. It is known to inhibit mitosis in a wide variety of plant and animal cells by interfering with the structure of the mitotic spindle (Eigsti and Dustin 1955). Furthermore, research shows that the colchicine-binding protein is a subunit of microtubules. It has been reported that low dosages of the compound can effectively halt cell division for a small period of time, thus producing a doubling of the genetic load in some cells (Borisy and Taylor 1967). Nevertheless, colchicine has a highly toxic effect on plant and animal cells, being a hazardous compound to use in the laboratory. Moreover, when plants are treated with colchicine to induce doubling, the mortality rate is usually high, and is dependent on the concentration, time of exposure and species. Consequently, to optimize protocols, breeders need to balance dose and exposure time to ensure successful chromosome duplication while limiting the mortality rate.

Nowadays, oryzalin and trifluralin are being widely employed as substitutes for colchicine. These compounds are dinitroaniline herbicides reported to bind to plant tubulin, which in turn confers an antimitotic effect similar to colchicine. Unlike colchicine, dinitroanilines have no effect on microtubules in vertebrate cells, which are resistant to its depolymerizing effects. It has been demonstrated that oryzalin has a high-affinity interaction with plant tubulin, binding rapidly and reversibly while forming a tubulin-oryzalin complex (Hugdahl and Morejohn 1993). The properties of dinitroaniline binding to tubulin are different from colchicine. In fact, dinitroaniline binding is time independent and the tubulin-dinitroaniline complex dissociates completely, unlike colchicine, which has been reported to bind slowly to tubulin and the tubulin-colchicine complex does not easily dissociate. Furthermore, oryzalin has been reported to have a much higher affinity for unpolymerized tubulin than the polymerized form (Borisy and Taylor 1967; Hugdahl and Morejohn 1993). Scientific interest lies in the fact that dinitroanilines can be much less hazardous for humans than colchicine and equally effective at lower doses. Key features commented above for a successful chromosome doubling need to be determined empirically for each species. More work is required concerning the chromosome-doubling step in many species and genotypes, describing the best results of different treatments (**Table 1**).

The antimitotic compound is usually dissolved in DMSO (dimethyl sulfoxide). DMSO has a double utility, as a solvent to dissolve the antimitotic and to increase cell permeability by allowing an increase in absorption of the agent into the plant (Hamill et al. 1992). However, DMSO may increase plant mortality due to its relative toxicity (Dhooghe et al. 2011). Other solvents can be used instead, such as NaOH or 70% ethanol for oryzalin, acetone for trifluralin, or even water (Dhooghe et al. 2011). If chromosome doubling is performed by immersion of some part of the plant in an antimitotic solution, detergent or surfactants can be added too to enhance the surface contact. In contrast, if the treatment is applied to a specific area on the plant, such as the lateral or apical meristems, lanolin paste can be used to localize a dose of solution.

CRISPR/Cas9 a new actor in DH technology

The haploid induction strategy is based on intraspecific crossing to obtain haploid progeny through an HI line. HI lines have the ability to produce haploid embryos upon pollination of a receptor line. Due to a mutation in a specific gene, which is essential for the normal fertilization of female gametic cells, fertilization is impeded, and egg cells develop into haploid embryos. Natural HI lines have been used in wheat (Laurie and Bennett 1988), maize (Coe 1959), tobacco (Burk et al. 1979) and barley (Kasha and Kao, 1970) since the beginning of modern breeding. For instance, in maize, HI lines were discovered to carry a 4-bp insertion in the carboxy terminus of the MATRILINEAL (MATL) gene, also known as NOT LIKE DAD (NLD) (Gilles et al. 2017) or PHOSPHORYLASE A1 (PLA1) (Liu et al. 2017), which encodes a pollen-specific phospholipase determined as the causal factor in the haploid induction process (Prigge et al. 2012). Nevertheless, few species have natural HI lines; the production of HI lines in the laboratory through genome-editing techniques is a major challenge in haploid technology to improve the DH process. As previously mentioned, targeting induced local lesions in genomes (TILLING), zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) are potential genome-editing tools to produce positive mutants. Despite this, the random mutations of TILLING, and the complex, time-consuming engineering and unwanted off-target mutations of ZFNs and TALENs have meant that the CRISPR/Cas9 system has become genome-editing system of choice.

Substantial progress has been made in DH technology in cereals, resulting in great advances in DH production, which is achieved mainly via androgenesis. Recently, MATL has become a target gene for genome

editing in rice and maize with the CRISPR/Cas9 system, taking advantage of gene mutation during haploid induction. In maize, maternal haploids were obtained with an efficiency of *ca.* 6.7% using the HI technique (Kelliher et al. 2017) and in rice, the average haploid induction rate was *ca.* ~6% (Yao et al. 2018). These rates of haploid production represent a breakthrough that has the potential for further improvement. This new DH-generation pathway could be implemented for other secondary cereals like barley, oats, rye or triticale because existing DH protocols are less efficient than in species like rice, maize or wheat. With many genetic transformation methods validated and whole genome sequencing available, breeders should be able to take advantage of HI validated technology to increase the number of DHs and the efficiency of DH production in recalcitrant species via HI-mediated genome-editing technology, avoiding the need to test *in vitro* androgenic protocols for a range of genotypes.

Concerning dicotyledons, the main target for the HI technique has been the CENH3 (centromeric histone 3) gene, which is a histone present in all plants that determines the position of the centromere, and thus plays a major role in chromosome segregation during mitosis. Since its discovery, much research has been conducted to elucidate its function and capacity during haploid induction in dicot species. Nevertheless, the specific mechanism and functions are still not clear. In 2010, major progress was achieved when studying the function of CENH3 in *Arabidopsis thaliana* (Ravi and Chan 2010). This work demonstrated that a CENH3-null mutant line underwent haploid induction when crossed with a CENH3 wild type line by eliminating chromosomes of the mutant line. Hence, the MATL and CENH3 genes are the current targets for HI-mediated genome-editing systems in monocots and dicots, respectively. However, other genes related to chromosome segregation during mitosis or pollen development have potential as target genes for HI technology using CRISPR/Cas9, as reviewed by Ren et al. (2017).

Parthenogenetic approaches of haploid production via HI lines obtained by CRISPR/Cas9 can increase the chances of DH plant generation and ease the usual time-consuming and labor-intensive processes of androgenesis, gynogenesis and parthenogenesis (**Fig. 1**). Further, the genotype dependency of many of the *in vitro* steps like callus induction and plant regeneration, or *in vivo* steps like pollination with irradiated pollen might be avoided using an HI approach, and this may also enable haploid plant material to be obtained from recalcitrant genotypes. To obtain MATL- or CENH3-mutated lines for use as HIs, the transformation should be optimized in at least one genotype for each species because the regeneration and mutation processes are mandatory. This HI mutated genotype could be used to pollinate many genotypes of interest for haploid embryo generation, avoiding the need to optimize the process for recalcitrant varieties. With efficient production of haploid plants in place using HI approaches, only the chromosome-doubling step at the haploid plant stage will become the bottleneck in achieving an efficient production of DH lines (**Fig. 1**).

The HI-mediated genome-editing approach for DH production for breeding purposes is a major discovery. Apart from improvements in the application of HIs as discussed above, for a number of crop species the HI technique might be the best way, if not the only way, to produce DHs. Much research needs to be done on this aspect to confirm the ease of work in parallel with better results.

It is worth noting that DH production in crop species like those from the *Solanaceae* could be greatly improved thanks to the HI technique. Members of the *Solanaceae* are very recalcitrant to *in vitro* DH processes and a methodology has not been established yet for a number of species, making it difficult for breeders to use DH technology on a routine basis (Seguí-Simarro et al. 2011). For instance, DH lines can be obtained efficiently in eggplant and bell pepper through anther culture (**Table 1**). Nevertheless, to our knowledge there is currently no suitable DH method available for tomato, despite all the efforts invested in DH production in this major horticultural crop. Thus, classical breeding is the only method to obtain new commercial tomato varieties, complicating the advances of breeding selection. Fortunately, delivery of CRISPR/Cas9 by *Agrobacterium*-mediated transformation has been reported several times in tomato (Van Eck 2018). Therefore, the generation of HI CENH3-mutant tomato lines via genome editing could represent a considerable breakthrough. This could lead to a new era for tomato breeding, avoiding the current six to eight generations of self-pollination that are still required to produce inbred lines for use in hybridization. CRISPR/Cas9 has been successfully applied in tomato with mutation efficiencies of 80-100% for applications such as studying mutation stability of heredity in later generations (Pan et al. 2016) obtaining parthenocarpic tomato fruits (Ueta et al. 2017), and increasing plant resistance to powdery mildew (Nekrasov et al. 2017).

In the *Fabaceae*, attempts to produce DHs have been reported in many species (soybean, field pea, chickpea, peanut and common bean), mainly via androgenesis. Leguminous species are particularly important for low input and sustainable cropping due to their ability to fix nitrogen and as a dietary protein source for human food and animal feed (Croser et al. 2007). Nonetheless, not much progress has been made with DH technology applications because there has been little research undertaken on these species, and the induction and regeneration rates are inherently low. Some DH lines have been produced thanks to spontaneous doubling and high rates of

somatic regeneration (Croser et al. 2007; Ochatt et al. 2009). However, successful induction of chromosome doubling has not been widely reported in this family because of the scarcity of the obtained haploid material. Significantly, the CRISPR/Cas9 system has been applied in soybean multiple times (Chilcoat et al. 2017; Sun et al. 2015) and should be extended to other *Fabaceae* species. The HI-mediated CRISPR/Cas9 genome-editing technique presents a great opportunity to produce DHs.

Another important family where HI-mediated genome-editing system could be advantageous is the *Cucurbitaceae*. Parthenogenesis via pollination with irradiated pollen is the best-known method to obtain haploid material among these species. However, the efficiency of the process is usually impeded by: (i) high genotypic dependency; (ii) low production of haploid embryos; and (iii) difficulty to induce chromosome doubling of haploid plants due to mortality, hyperhidricity and a high ratio of haploid and mixoploid plants (Dong et al. 2016; Gonzalo et al. 2011; Lim and Earle 2008, 2009). All this makes the process of DH production time-consuming and inefficient. The HI approach in the *Cucurbitaceae* would be similar to parthenogenesis via irradiated pollen due to the initial pollination of a donor plant with haploid-inducer pollen and irradiated pollen, respectively, and the rest of the process will be the same. Additional research is necessary to confirm whether the use of an HI line results in increased production of haploid embryos, as the actual ratio stands between 0 and 5% of seeds containing haploid embryos. There are a few reports of the CRISPR/Cas9 genome-editing system being applied to cucumber, watermelon and melon, with mutation efficiencies ranging from 42 to 100% (Chandrasekaran et al. 2016; Hu et al. 2017; Tian et al. 2017; Hooghvorst et al. 2019). In other important cucurbit species, such as *Cucurbita maxima*, *C. moschata* and *C. pepo*, there are no reports of success with the CRISPR/Cas9 method or generation of DHs through parthenogenesis via irradiated pollen. Nevertheless, HI-mediated genome editing should afford great opportunities for breeding in these species as well.

Another important approach to the HI technique is the HI-Edit system, where successful one-step genome editing is achieved. Kelliher et al. (2019) crossed HI lines carrying the CRISPR/Cas9 cassette targeting genes for phenotypical evaluation with inbred lines to test the ability to produce positive genome-edited mutants in the haploid offspring. The intraspecific crossing in maize led to a mutant haploid descendance of 2 to 8%, depending on the target gene. The interspecific crossing between a wheat inbred line and a maize line homozygous for the Cas9 gene resulted in a 1.8% rate of mutant haploid production. Moreover, in *Arabidopsis*, 17% of the offspring were mutant haploids when crossing a CRISPR/Cas9-derived HI line with an inbred line. Wang et al. (2019) applied the same system in maize and obtained 10 positive genome-edited plants among the 245 haploids produced. This new system enables direct editing of elite inbred lines via a single crossing, thus overcoming recalcitrance.

Despite all the other improvements in DH line production that have emerged, chromosome doubling is one step that has been inherited from the classical DH approach (**Fig. 1**). It is therefore of major importance to adapt and optimize new chromosome-doubling protocols via antimitotic compounds to increase the number of DH lines derived from the improved HI protocols. Some species, such as sorghum and bell pepper, do not have an optimized well-described chromosome-doubling protocol because of their high level of spontaneous DH regenerants. For instance, in rice, the spontaneous chromosome-doubling rate is usually very high, ranging from 30 to 80% (**Table 1**), and induced chromosome doubling has been ignored since the generation of the first DHs (Niizeki and Oono 1968). Nevertheless, before 2019 no reliable reports on doubling rice plants were published, authors treated androgenic haploid plants with a recovery of 35%. On the other hand, species whose DH production is impeded by poor performance in tissue culture do not have a reliable described method for chromosome doubling; this is the case of rye, watermelon, other secondary cucurbit species, tomato, leguminous species, etc. (REFERENCES?).

Another important aspect of HI-CRISPR/Cas9-based technology is the legislation put in place to regulate the development and commercialization of GMOs in the EU, and which handles issues of uncertainty and safety (Sprink et al. 2016). The Directive 2001/18/EC defines a GMO as an organism "in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination", and GM techniques are further described as "recombinant nucleic acid techniques involving the formation of new combinations of genetic material". In our opinion, haploid production through CRISPR/Cas9 mutants used for HI needs to be exempt from this regulation because the resultant GMO-free haploid line would never have recombinant DNA incorporated into its single progenitor cell. Nevertheless, as pointed out by Abbott (2015), the EU criterion in the current legislation is based on the process and the product, and a process that uses recombinant technology could create controversy for regulatory institutions, despite the impossibility of tracking down its origin.

Ploidy-level identification of plant material

Ploidy identification of the plants produced during the DH process is essential before the chromosome-doubling treatment to determine whether the plant material has undergone spontaneous duplication, and afterwards to check whether or not the antimitotic treatment has successfully doubled the plants' chromosomes. There are multiple methods to check ploidy level: cytologically, morphologically, via marker-assisted selection or via flow cytometry methods.

Cytological procedures for ploidy level determination can be carried out by counting chromosomes or examining the epidermal tissue of the leaves. Chromosome counting usually uses root tip cells, which are fixed, and the chromosomes are then stained and observed for counting (Maluszynska 2003). Cytological analysis of leaves correlates chloroplast number, stomatal dimensions and size with ploidy level. However, applying chromosome-counting methods to species with small chromosome size is very time-consuming and difficult, and chloroplast and stomatal analysis is species and genotype-dependent. Despite this, results from both cytological procedures are extremely accurate and sample preparation and staining is easy and fast.

Haploid and diploid plants can also be distinguished according to morphological observations of the plant material. Morphological observation is based on comparing plant traits of the donor plants and regenerants, such as: height, vigor, leaf shape, flower development, fertility and presence of pollen. This methodology does not require special equipment, but it is sometimes unreliable and subject to environmental effects. In the *Cucurbitaceae*, leaf morphology, flower shape and size, pollen production, stem length or node number, are phenotypic variations that can be analyzed for ploidy determination (Dong et al. 2016). Couto et al. (2013) correlated haploid levels with small plant size and brittle leaves, but excluded ploidy determination of haploid seeds via morphometric parameters due to the great variability in the ???. Yuan et al. (2015) detected haploid individuals in two *Brassica* species due to the weak growth of the haploids and the small size of the plants, as well as the presence of smaller flower buds, the absence of pollen, and a lack of stamens in the flowers.

The use of segregating alleles in the donor parent is another methodology for ploidy level determination via marker-assisted selection. Simple sequence repeats (SSR), random amplification of polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) marker analysis techniques are ideal to identify ploidy-level as well as homozygosity in spontaneous DH regenerants or heterozygosity in diploid somatic regenerants. For instance, in soybean, 114 androgenic embryo-like structures were analyzed for the Satt418 SSR marker, and 74% were found to be heterozygous, originating from somatic anther tissue (Rodrigues et al. 2004). In pumpkin, 23 SSR markers were screened in 253 parthenogenetic diploid plants and the results showed no spontaneous chromosome doubling (Košmrlj et al. 2013). In oat, (Kiviharju et al. 2017) DNA markers were used for selection and this indicated that 3.4% of regenerated androgenic plants were heterozygous.

Despite all the methods described above for ploidy-level determination, flow cytometry is the most commonly used method for a wide range of species because of its convenience and rapidity in estimating the nuclear genome size in plants (Doležel et al. 1998). Estimation of ploidy-level via nuclear suspensions of plant cells and chromosome staining has been used in DH programs for routine laboratory analysis. Much optimization has been invested in increasing the numbers of nuclei in suspension using extraction methods such as chopping and bead beating, alongside numerous modifications to isolation buffers and staining procedures (Doležel and Barto, 2005; Hooghvorst et al. 2019). Moreover, in addition to haploid and diploid cells, flow cytometry is especially useful for detecting triploid, tetraploid and mixoploid plants. On the other hand, flow cytometry requires highly specialized equipment that was not required with earlier methods of ploidy determination.

Conclusions

The attempt of plant breeding and DHs to increase productivity and other important plant traits makes it necessary to continue refining existing and new methods to finally achieve a more sustainable production according to social needs. We have undertaken an analysis of DH production methods coupled with chromosome-doubling protocols for production of DH plants in major crops. We have drawn general conclusions about the success of different approaches to DH generation and the implications of the existing technologies of DH production and chromosome doubling for future research. DH technology has been a major boost for plant breeding, reducing the time and labor required to derive new breeding varieties. Among the double haploid processes, chromosome doubling is often overlooked due to the importance of the haploid induction step or the high frequency of spontaneous DHs, and this is reflected in the absence of DH protocols or a lack of efficiency in those that exist. Androgenesis via anther culture is the most common protocol for haploid and DH plant production, being the predominant method in vegetable and horticultural crops, and the only method used in cereals. The chromosome-doubling step is far from being settled and there is a need to continue investigating new protocols based on new or existing antimitotic compounds in order to reduce toxicity-related mortality and to attain higher frequencies of chromosome doubling. Induced chromosome doubling has a genotypic dependency, and even species with a high rate of spontaneous doubling should not be ignored when developing efficient chromosome-doubling methods because some genotypes are unable to regenerate spontaneously. Inducing haploids using genome editing via the CRISPR/Cas9 system could revolutionize the whole process of haploid generation and DH production, and should have an impact on plant breeding in the coming years to parallel the early days of the DH technique. For crops with a short progress on haploid technology, CRISPR/Cas9 HI approach could open a new insight, allowing the production of pure homozygous lines. For many of the vegetable crops and secondary cereals, HI systems will allow researchers to avoid the need for wide ranging adaptation of protocols to different genotypes, which is a highly time-consuming pathway requiring much trial and error. The only adaption required to the tissue culture technique will be the regeneration of CRISPR/Cas9 genome-edited plants, which will be a much easier task because only one genotype will be sufficient as the pollen donor for haploid induction across a diversity of receptor genotypes of the same species. In the future, HI-mediated genome-editing CRISPR/Cas9 system should be exempted from GMO legislation due to the presence of GMO-free parental material and haploid descendancy. The uprising CRISPR/Cas9-based gene targeting approach for haploid induction will make the chromosome-doubling step inexorable, because of the low or absent spontaneous chromosome doubling in haploid induction. Therefore, new *in vitro* or *in vivo* chromosome-doubling protocols will be needed for species where haploid induction has not yet been reported due to low regeneration efficiency or a complete lack of regeneration success.

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Contribution Statement

In this article, Isidre Hooghvorst proposed the idea and wrote the manuscript. Salvador Nogués helped to write and revise the article. The authors declare no competing interests.

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Figure legends

Figure 1: Schematic representation of three possible methods for producing purely homozygous lines: backcrossing breeding, double haploid technology and the haploid inducer-mediated CRISPR/Cas9 genome-editing system. Chromosome representation shows the genetic ploidy level according to number (one chromosome for haploids and two chromosomes for diploids) and the genetic background according to the color (black for elite receptor lines, pink for donor lines, green for genotypes optimized by genome transformation, yellow for the allele of interest and red for the CRISPR/Cas9 cassette targeting the MATL or CENH3 genes). Backcrossing breeding can take from six to eight generations depending on the species and possible MAS coupled. DH technology can take from six months to two years depending on the species. An HI-mediated genome-editing system can take one year. DH technology and HI-mediated genome-editing system can start with BC_1 to BC_4 plants.

Table legends

Table 1. Overview of the most commonly used methods for double haploid production and chromosome doubling and their efficiency in major crops.

Species	Common name	Double haploid method	Induced chromosome doubling method					Chromosome doubling efficiency[b]		Reference
			Stage	Application	Antimitotic compound	Concentration	Exposure time[a]	Spontaneous	Induced	
Cereal crops										
Avena sativa	Oat	Anther culture	*In vitro* plantlets	Immersion	Colchicine	0.05% suppl. 2% DMSO	5 h	37.42%	88.17%	Kiviharju et al. 2017
Hordeum vulgare	Barley	Microspore culture	-	-	-	-	-	>90%	88.3-93.5%	Li and Devaux, 2003
Hordeum vulgare	Barley	Anther culture	*In vivo* plantlets	Immersion	Colchicine	0.05% suppl. 2% DMSO	5 h	-	-	Jacquard et al. 2003
Sorghum bicolor	Sorghum	Anther culture	-	-	-	-	-	95.3%	-	Kumaravadivel and Sree, 1994
Secale cereale	Rye	Anther culture	-	-	-	-	-	13-67%	-	Tenhola-Roininen et al. 2005
Triticum aestivum	Wheat	Microspore culture	Tillers of *in vivo* plants	Immersion	Colchicine	0.10%	5-8 h	-	95.60%	Niu et al. 2014
Triticum aestivum	Wheat	Microspore culture	Internode of pollinated spikes	Injection	Colchicine	1% suppl. 100 ppm 2,4-D	48 and 72 h	0	33-100%	Sood et al. 2003
Oryza sativa	Rice	Anther culture	Anthers	Induction medium	Colchicine	250 mg·L^{-1}	48 h	31%	65.50%	Alemanno and Guiderdoni, 1994
Oryza sativa	Rice	Anther culture	Anthers	Induction medium	Colchicine	300 mg·L^{-1}	48 h	0-0.18 DH green plantlets per 100 anthers	0.75 DH green plantlet per 100 anthers	Hooghvorst et al. 2018
Oryza sativa	Rice	Anther culture	*In vitro* plantlets	Immersion	Colchicine	500 mg·L^{-1}	5 h	-	35%	Hooghvorst et al. (data not published)
Triticum x *Rye*	Triticale	Microspore culture	Embryos	Microspore culture	Colchicine	0.3 mM	24 h	30%	50-55%	Würschum et al. 2012
Zea mays	Maize	Anther culture	Anthers	Induction medium	Colchicine	0.2 g·L^{-1}	3 d	19%	20%	Obert and Barnabás, 2004
Zea mays	Maize	Anther culture	Microspores	Induction medium	Colchicine	1,250 µM	1 w	40%	93.75% DH plantlets for 100 anthers	Antoine-Michard and Beckert, 1997
Vegetable and horticultural crops										
Apiaceae										
Daucus carot	Carrot	Microspore culture	*In vivo* plants	Immersion	Colchicine	0.34%	1.5 h	50%	-	Ferrie et al. 2011
Pastinaca sativa	Parsnip	Microspore culture	*In vivo* plants	Immersion	Colchicine	0.34%	1.5 h	50%	-	Ferrie et al. 2011
Brassicaceae										
Brassica napus	Rapeseed	Microspore culture	Microspores	Induction medium	Colchicine	500 mg·L^{-1}	15 h	45-64.3%	83-91%	Zhou et al. 2002
Brassica oleracea var. *capitata*	Cabbage	Microspore culture	Rooted *in vitro* plantlets	Immersion	Colchicine	0.2% suppl. 2% DMSO	9-12 h	0-76.9%	58.3-75%	Yuan et al. 2015
Brassica oleracea var. *italica*	Broccoli	Microspore culture	Rooted *in vitro* plantlets	Immersion	Colchicine	0.05% suppl. 2% DMSO	6-12 h	50.6-100%	54.5-58.3%	Yuan et al. 2015
Cucurbitacea										
Cucumis melo	Melon	Parthenogenesis	*In vivo* plants	Immersion	Colchicine	0.50%	2 h	-	46.03%	Solmaz et al. 2011
Cucumis melo	Melon	Parthenogenesis	*In vivo* plants	Immersion	Colchicine	0.50%	2 h	23%	20.93% DH and 9.30% mixoploids and	Hooghvorst et al. (data not published)
Cucumis sativa	Cucumber	Parthenogenesis	*In vitro* microcuttings	Culture medium	Colchicine	500 mg·L^{-1}	48 h	0%	30% DH and 55% mixoploid	Claveria et al. 2005
Cucumis sativa	Cucumber	Parthenogenesis	*In vitro* nodal explants	Immersion	Oryzalin	50 mg·L^{-1}	18 h	-	86.21%	Ebrahimzadeh et al. 2018
Solanacea										
Capsicum annuum	Bell pepper	Anther culture	*In vitro* axillary buds	Lanolin paste in axillary buds	Colchicine	1%	-		n/a	Gyulai et al. 2000

Capsicum annuum	Bell pepper	Anther culture	-	-	-	-	-	22.2-53.4%	-	Keleş et al. 2015
Solanum melongena	Eggplant	Anther culture	*In vitro* axillary buds	Lanolin paste in axillary buds	Colchicine	0.50%	-	60%	25%	Corral-Martínez and Seguí-Simarro, 2012
Solanum tuberosum	Potato	-	Nodal segment	Immersion	Oryzalin	25 µM	8 h	20-78%	10.10%	Greplová et al. 2009
Other important plant crops										
Allium cepa	Onion	Gynogenesis	Embryos	Solid elongation medium	APM	25 µM	24 h	-	35%	Fayos et al. 2015
Allium cepa	Onion	Gynogenesis	*In vitro* plantlets	Culture medium	Colchicine	10 mg·L^{-1}	3 d	1%	46%	Campion et al. 1995
Allium cepa	Onion	Gynogenesis	*in vitro plantlets*	Media culture	Oryzalin	10 µM	3 d	-	67%	Jakše and Bohanec, 2003
Asparagus officinalis	Asparagus	Anther culture	*In vitro* shoot tips	Apical lanolin application	Colchicine	1.20%	-		21-97%	Tsay, 1997
Beta vulgaris	Beet	Ovule culture	Ovule	Culture medium	Colchicine	0.40%	2.5 h	7.10%	4 DH per 100 ovules	Hansen et al. 1995
Beta vulgaris	Beet	Ovule culture	Ovule	Culture medium	AMP	100 µM suppl. 1.5% DMSO	5 h	6.60%	4.7 DH per 100 ovules	Hansen and Andersen, 1998
Beta vulgaris	Beet	Ovule culture	*In vitro* plantlets	Agarose culture medium	Trifluralin	3.4 mg·L^{-1}	36 h	0-10%	62.50%	Gürel et al. 2000
Nicotiana tabacum	Tobacco	Anther culture	*In vivo* plant	Root dipping	Colchicine	0.50%	24 h		21-32%	Sood et al. 2013

[a]*h* hours, *d* days and *w* weeks

[b]If not specified, percentages refer to chromosome doubled material relative to the initially treated material