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Overview of *in vitro* and *in vivo* doubled haploid technologies

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Overview of In Vitro and In Vivo Doubled Haploid Technologies

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i. Chapter Title: Overview of *in vitro* and *in vivo* doubled haploid technologies

ii. Abstract

Doubled haploids (DH) have become a powerful tool to assist in different basic research studies, and also in applied research. The principal (but not the only) and routine use of DH by breeding companies is to produce pure lines for hybrid seed production in different crop species. Several decades after the discovery of haploid inducer lines in maize and of anther culture as a method to produce haploid plants from pollen precursors, the biotechnological revolution of the last decades allowed to the development of a variety of approaches to pursue the goal of doubled haploid production. Now, it is possible to produce haploids and DHs in many different species because when a method does not work properly, there are several others to test. In this chapter, we overview the currently available approaches used to produce haploids and DHs by using methods based on *in vitro* culture, or involving the *in vivo* induction of haploid embryo development, or a combination of both.

iii. Key words: Androgenesis, Embryogenesis, Gynogenesis, Doubled haploid, Haploid, Haploid inducer, Tissue culture.

Introduction

As opposed to animals, plants have a remarkable developmental plasticity. This is reflected in the totipotency of differentiated plant cells that can undergo a transition towards an undifferentiated, proliferative growth, forming callus masses, and/or switch towards other developmental programs, different from their original one. This way, new plants from individual cells can be regenerated through organogenesis, promoting the successive formation of all their organs. Alternatively, the expression of the embryonic program (embryogenesis) can be promoted, thereby transforming plant cells into functional zygote-like structures which will become embryos and eventually plants. Virtually any plant cell type can be used, if optimal conditions (experimental treatments) are found to promote organogenesis and/or embryogenesis, which opens up a wealth of biotechnological possibilities. Among these cell types, the cells of the germ line (gametes or their precursors) are one of the most interesting from a biotechnological perspective, because they contain half of the chromosomes found in somatic cells, and are thus haploid.

Haploid generally refers to the product of meiosis, which, in a cell with a complete set of chromosomes ($2n$) leads to gametes with only half of this set (n). By convention “ n ” and “ $2n$ ” refer to the gametic and sporophytic chromosome numbers, respectively, and “ x ” denotes the number of sets of chromosomes (Figure 1A). Depending on the ploidy level of the organism, a haploid tissue/organism may have more than one copy of each homologous chromosome (Figure 1A). However, haploidy is not restricted to gametes in the plant kingdom. The life cycle of bryophytes (Figure 1B), for instance, relies mostly on the gametophyte which is the haploid phase with a short-lived $2n$ sporophyte dependent on the n gametophyte [1]. During evolution, plants progressively reduced their haploid phase. Haploid tissues (Figure 1B) were restricted spatially and temporally and became dependent for development and nutrition on $2n$ sporophytic tissues [1]. The extended life cycle proportion of the sporophyte provided

evolutionary advantages [2]. For instance, deleterious alleles, hence characters, might be hidden by the dominant ones held on homologous chromosomes.

From haploid cells, the artificial generation of new haploid individuals, which may become doubled haploid (DH), can be induced. To produce a DH, haploid cells (genetically unstable in essence) may undergo duplication of their genome at any time during their proliferation, becoming diploid with no need for additional treatments. Genome duplication is typically achieved by nuclear fusion after incomplete cytokinesis [3]. Since the second chromosome set is identical to the original one, they are true DHs, 100% homozygous. This is commonly known as *spontaneous* genome doubling, in the sense that nothing is done to specifically promote genome doubling, aside of the treatments applied to induce haploid embryogenesis itself, which may also have an indirect side effect on genome doubling. This is how DHs are produced in many instances, where the percentage of unaided genome doubling is high enough to make the direct disposal of haploid individuals cost-effective, keeping only those that effectively doubled their genome by themselves. However, different genetic backgrounds are differently prone to undergo such phenomenon without the application of additional treatments for genome doubling. In other cases, this percentage is very low, which makes mandatory the implementation in the DH protocol of such a treatment to stimulate doubling of haploid embryos. Among them, the most effective and widely used is by far the application of colchicine, an antimetabolic drug. Chapter 9 of this book compiles the principal methods used for chromosome doubling applied to DH production.

Haploids themselves are useful experimental systems to study, for example, the effects of recessive mutations, since their phenotypes are not masked by dominant alleles. However, their principal utility is to serve as the starting point to obtain DH lines. DHs are individuals whose diploid genome comes from a haploid set of chromosomes that has been duplicated, so that all their loci contain the same alleles. They are 100% homozygous individuals, and represent a

valuable tool for basic and applied research, including breeding programs, where they are often used as pure lines for hybrid seed production. Chapter 2 of this book compiles the principal applications of DHs in both applied research and plant breeding.

Induced haploid embryos/plantlets have only one of the two copies of the genome of the male or female donor plant they come from. Due to this, it is common to refer to them as “male/paternal” or “female/maternal” haploids, respectively. Theoretically, haploid cells can only have two origins. They must come:

- Either from male haploid nuclei derived from meiosis of microspore mother cells. The origin of these haploid nuclei would potentially include meiotic products, still within the tetrad, not released as microspores, individual microspores once released from the tetrad, or any of the different cells of the pollen grain, the vegetative cell, the generative cell, or the sperm cells (the male gametes) produced from the latter.
- Or from female haploid nuclei derived from meiosis of megaspore mother cells. The origin of these haploid nuclei would potentially include the functional megaspore, either included or released from the meiocyte, and any of the haploid cells of the embryo sac: synergid cells, antipodal cells, and the egg cell, the female gamete.

In practice, only some of these haploid cells have been demonstrated to produce haploid/DH plants. They include microspores at different stages, young pollen grains and egg cells. However, we cannot rule out the possibility of other haploid cells being able to be induced [4]. These cells can be induced to produce haploid and/or DH plants by means of different techniques, which exploit a series of phenomena observed to occur in plants under natural or experimental conditions. These techniques can be grouped into two main categories, *in vitro* and *in vivo* methods, depending on whether they include exclusively *in vitro* procedures, or there are stages of *in vivo* development of haploid/DH individuals (Figure 2). In this chapter, we will review the known *in vitro* and *in vivo* methods to produce DH individuals.

2. *In vitro* methods

The most widely extended approaches to obtain DHs have traditionally been based on the use of haploid cells of male and female origin to induce their development as haploid embryos by the application of different stresses *in vitro* and their subsequent *in vitro* culture. They are the so-called *in vitro* approaches (Figure 2). The production of haploid/DH plants from male haploid cells is commonly known as induction of *in vitro androgenesis*, whereas production of haploid/DH plants from female haploid cells is commonly known as induction of *in vitro gynogenesis*. The different strategies have in common the blockage of the normal development of these cells, whose natural fate is the production of functional gametes or accessory cells, and their *in vitro* reprogramming towards a different developmental fate, which is to become embryos without fertilization. This way, haploid and/or DH individuals can be produced *in vitro*.

In the last six decades, haploid cells of both male and female origins have been used to produce DHs *in vitro*, although with different success rates. In general, the haploid cells where *in vitro* haploid/DH induction has been most successful are male microspores and female egg cells. In particular, *in vitro* production of androgenic DHs has been more successful than production of gynogenic DHs due to several reasons:

- First, male haploid cells are by far more abundant. In a given hermaphrodite flower, thousands of microspores or pollen grains are present in each of the several anthers of a flower, whereas there is only one functional megaspore, which gives rise to six haploid cells per embryo sac, including the egg cell, per ovule. Different species may have different number of ovules per flower, and in some cases there may be up to hundreds of ovules, but

this number will never be comparable to the enormous number of microspores/pollens produced by the same flower.

- Second, female haploid cells are confined in the interior of ovules, surrounded by layers of nucellar and tegument tissues, all this being included within the ovary. This confines the newly formed haploid embryos within a very small space that does not enlarge in parallel to the embryo, because the ovule and ovary do not receive the necessary developmental cues to develop in parallel to the haploid embryo. On the other hand, androgenic embryos will only have to overcome the barrier imposed by anther walls, which are naturally programmed to dehisce and open along the dehiscence lines or pores. Thus, gynogenic embryos will have it more difficult to emerge from the surrounding tissues, which will account for the reduced rates of success.
- Third, in nearly all cases, the individuals regenerated from female haploid cells remain haploid, which makes always mandatory a step of genome doubling.

Both androgenic and gynogenic embryos may be produced through different *in vitro* experimental approaches, as explained next.

2.1 Methods based on *in vitro* androgenesis

Methods based on androgenesis exploit the possibility of switching the developmental fate of pollen precursors towards embryogenesis. The most used pollen precursors are, by far, microspores/young pollen grains. Microspore embryogenesis (also known as pollen embryogenesis) is by far the most used and efficient way to produce DHs *in vitro*. This experimental pathway was first discovered by Guha and Maheswari in 1964 [5], while working with *in vitro* cultured anthers of *Datura innoxia*. Later on, many different research groups have reproduced their findings in many other species and genera, making this experimental

phenomenon a powerful and widespread tool to produce DHs. However, not all the species respond equally to the induction of this process. Some species, considered models for the study of this phenomenon, respond fairly well. This is the case of certain lines of rapeseed (*Brassica napus*), tobacco (*Nicotiana tabacum*), or barley (*Hordeum vulgare*). Others, considered recalcitrant, present a low or very low response, and in other cases, a protocol to efficiently induce this process is still pending to be developed, as for scientifically or agronomically important species such as *Arabidopsis thaliana* or tomato (*Solanum lycopersicum*), respectively. Many other species are in between these two extreme situations, being possible to induce microspore embryogenesis, but with yet improvable protocols. Woody species are good examples of materials where some success has been achieved, but there is still a large room for improvement (reviewed in [6-8]). Chapter 3 of this book includes a list of species where production of haploids/DHs has been assessed by this approach.

Even within a species, there will be varieties, lines and even individuals that respond differently. This strong influence of the genotype, together with the fact that this trait is transmitted across generations and segregates in the hybrids offspring [9,10], indicates that it is under genetic control [11-17]. Furthermore, it was proposed that, at least for *Brassica napus*, the embryogenic competence of microspores is controlled by two loci with additive effects [10]. The gene or genes involved, however, remain to be elucidated.

In microspore embryogenesis, androgenic haploids/DHs are typically produced upon deviation of microspores towards embryogenesis or callus formation. However, not all species can be induced by isolation and culture of microspores at the same stage (Figure 3). Indeed, for few species it has been shown that male-derived haploid and DH plants can be regenerated from meiocyte-derived callus [18-20]. This is a very exceptional route, much less frequent and studied than microspore embryogenesis. Under certain circumstances, meiocytes at late meiosis stages, always after recombination but before the release of the four individual microspores,

can be induced to proliferation (Figure 3, Route 1), forming undifferentiated haploid callus masses where haploid/DHs can be regenerated from. Meicyote-derived callogenesis has been documented in *Arabidopsis thaliana*, *Vitis vinifera*, *Digitalis purpurea* and *Solanum Lycopersicum* [21-32]. In addition to its rare occurrence and the scarcity of studies, the practical use of this alternative is hampered by a very low frequency of cases, since many of the regenerants produced come from the fusion of two meiotic haploid products separated by defective, incomplete, or absent cell walls (Figure 3, Route 1) [25]. This will give rise to a majority of non-DH, useless plants that must be identified as such and then discarded. Altogether, these limitations make that in practice, this *in vitro* alternative is not used.

For some species, mostly cereals, the early stages of microspore development have been described as the only stage where embryogenesis can be induced [33]. However, the majority of works point to the fact that the inducible developmental window revolves around the first pollen mitosis (Figure 3, Route 2), which means that vacuolated microspores but also young, just divided pollen grains would also be inducible (reviewed in [33,19,34]. The identification and isolation of these stages is essential for the success of this process, since in the vast majority of species, they are the most sensitive to the induction treatments [35]. Beyond these stages, induction has only exceptionally been reported [36].

To be induced to embryogenesis, microspores/pollens must be stressed. The need for application of physicochemical stress treatments seems common to all inducible species. The variety of responses, depending principally on the genotype but also on the developmental stage of the microspore/pollen, makes that each species has its own specific inductive treatments to trigger the developmental switch. Some of these stresses (heat, cold or starvation) are common to many species, whereas others need more specific stressors or combinations of them [37]. As a rule of thumb, the more recalcitrant a species is, the more combined and more intense stresses are needed. Typically, induction of microspore embryogenesis produces microspore-derived

embryos. However, there are some species where microspores proliferate in an undifferentiated manner, producing callus masses (Figure 3, Route 2; reviewed in [19]). In other cases, embryos cannot progress as such and proliferate as callus masses [38-40]. As pointed out previously [19], this may be due the absence of developmental regulation in this type of *in vitro* embryogenesis, devoid of endosperm and other seed tissues that may interact with the embryo. When suboptimal culture conditions do not compensate for this, abnormal embryos or even calli may arise.

The process of microspore embryogenesis and the different factors, mentioned above, that influence its success, may be implemented in practice using two *in vitro* approaches: anther culture and isolated microspore culture.

2.1.1 Anther culture

Anther culture is the most universal method to produce DHs. It is technically simple, consisting basically of the following steps: (1) flower bud collection, (2) isolation of anthers from flower buds, (3) inoculation and *in vitro* culture in agar-based culture medium, (4) isolation of embryos, (5) regeneration of plants, and (5) analysis of regenerants. Few weeks (months in many cases) after, microspore-derived embryos may be seen to emerge from anther walls, in parallel to the degradation and necrosis of these walls. In general, a given anther under optimal culture conditions may give rise to several tens of microspore-derived embryos during several months of culture. The presence of these walls (the tapetum principally) during the first stages of anther culture may protect and help microspores to undergo the first stages of haploid development, in a way similar to how they assist normal microspore development *in vivo*. Perhaps, this is the reason why anther culture works in many different species, including those where other DH methods do not work (See chapter 3).

However, anther cultures are not devoid of limitations. Perhaps, the main limitation comes from the fact that microspores are cultured together with anther walls. Anther walls (the tapetal layer mostly) may secrete molecules that may protect microspores or promote their growth, but it may also secrete inhibitory or even toxic compounds, as is the case of necrosing anther tissues. In any case, this secretory effect is uncontrollable in essence, and makes difficult a strict control of culture conditions. Moreover, when exposed to growth regulators, these walls are able to proliferate *in vitro*, producing calli. Indeed, some parts of the anther, such as the filament insertion, are especially prone to form calli when *in vitro* cultured. Therefore, we cannot rule out the possibility of occurrence of somatic embryos (very rare but possible) and calli (much more frequent) from anther walls. This implies that for every single plant confirmed as diploid (2C DNA content) by flow cytometry, we should check its origin. For this, the most reliable approach is the use of molecular markers previously confirmed as heterozygous in the donor plants used (see Chapter 10). However, for very well-known cultivars, where repeated analyses have shown that no somatic embryos are produced, this step may be skipped simply by discarding all calli produced and using only embryos.

2.1.2 Isolated microspore culture

Microspore embryogenesis can also be induced using microspore isolated from anthers. This alternative is more complex than anther culture because a step of microspore isolation and inoculation into liquid medium must be implemented to the protocol. In addition, the absence of anther tissues makes that proper microspore growth and development will exclusively depend on medium composition. Thus, in order to develop an efficient protocol for microspore culture, medium composition must include all the elements needed by microspores and must be adjusted to the particularities of the microspores of each species. The use of liquid culture

media increase the risk of contamination compared with anther cultures, which use to be performed in agar-based, semisolid media.

Together with these limitations, isolated microspore cultures have also some advantages that, in some cases, largely surpass the limitations. Microspore cultures avoid the uncontrollable contribution of anther walls, and the potential toxicity of anther wall degradation products. In addition, all nutrients and active compounds of the medium are easily available by microspores, suspended in the liquid medium instead of confined within the anther locule. These features are likely behind the fact that microspore cultures are notably faster than anther cultures. Indeed, in some model species where protocols are optimized, few weeks are needed produce hundreds of embryos from the microspores inoculated in a single dish [41], see Chapter 18. In addition to this, microspore cultures avoid the routine, time-consuming procedure of checking the haploid origin of all the diploid plants obtained. Since microspores are isolated from all other anther tissues and only microspores are inoculated into the dishes, the only possible origin for embryos or calli must be the microspores. In other words, all diploid regenerants obtained will be DHs. All these advantages considered, isolated microspore culture is the method of choice in those materials where efficient protocols are well established.

2.2 2.1 Methods based on *in vitro* gynogenesis

Gynogenesis would exploit the ability of egg cells to develop in the embryo sac as a haploid zygote without fertilization (Figure 4, Route 4). This alternative to the normal development of the megagametophyte was first described *in vitro* in 1976 by San et al. [42]. It would therefore be a form of female haploid parthenogenesis (from the Greek words *parthenos*, meaning “virgin” and *genesis*, meaning “origin”). In some species, the gynogenic embryo is believed to originate from antipodal or synergid cells, but in the vast majority of cases the gynogenic

embryo is derived from the egg cell (reviewed in [4]). Gynogenic embryos are mostly haploid, which implies that in order to obtain the desired double haploid, the application of additional treatments for chromosome duplication should be considered in nearly all cases. As in the case of androgenesis, colchicine is the most effective and therefore the most widely used antimitotic (Chapter 9).

The success of gynogenesis induction is influenced by many different factors, including the developmental stage of the embryo sac and the *in vitro* culture conditions. However, the genotype is the most important, even more than for microspore embryogenesis. In fact, this is the most limiting factor for the practical application of this technique, since there are very few responsive genotypes, much less than those that respond to microspore embryogenesis. Other limitations include a low efficiency, much lower than microspore embryogenesis (there are much less egg cells than microspores in a flower), a very low rate of spontaneous duplication of the genome, and low levels of embryo regeneration, perhaps due to the instability of haploid genomes, prone to chromosomal alterations.

All these limitations make *in vitro* gynogenesis-based approaches a secondary alternative, used in a reduced range of species where other *in vitro* approaches (microspore embryogenesis) have proven ineffective. Chapter 3 of this book includes a list of species where production of haploids/DHs has assessed by this approach. Among them, the model species for the study of this process is onion (*Allium cepa*) [43,44] See Chapter 13.

From a methodological point of view, this technique is implemented by *in vitro* culture of ovules [45,46], ovaries [47] or even full immature flowers [43], not yet open and therefore unpollinated, until the embryo sac matures and the gynogenic embryo develops. In some species, mere *in vitro* culture seems not enough, and an "extra" factor must be applied to trigger the process. Examples of this factor include include pollination before ovary excision and *in vitro* culture [48], *in vitro* pollination with mentor pollen from other species [49], with pollen

irradiated with gamma or X rays to inactivate its fertilization capability [50] or with triploid pollen, still able to germinate and stimulate the egg cell, but not to fertilize. *In vitro* pollination can be done at the apical part of the stigma of entire pistils isolated from the flower and cultured *in vitro* [51]. After several months, gynogenic embryos will be visible. Alternatively, pollen may be applied by placental pollination, which implies isolating the ovules from the ovary, but maintaining a fragment of the placenta to help the viability of the egg cell. In many cases of gynogenesis in cucurbits and fruit trees, treated pollen is applied directly *in situ*, on the emasculated flower in the plant [52-56]. Then, seeds or haploid embryos are rescued and *in vitro* cultured (see also Section 3.2). These approaches would be half way between the *in vitro* approaches and the *in vivo* approaches described next (Figure 2).

3. *In vivo* approaches

Alternatives to the *in vitro* approaches to generate haploid plantlets are *in vivo* approaches (Figure 2). Overall, *in vivo* methods are less numerous as compared to *in vitro* methods and thus less plants/crops are concerned. *In vivo* methods look attractive because they appear to be “simpler”, since the plant is mainly doing the job instead of labor intensive *in vitro* work. Nevertheless, improvement and optimization are usually needed between the discovery of an *in vivo* induction system and its application in breeding scale. Indeed, long standing *in vivo* haploid induction methods exist [57,33,58] and some have been improved and are currently used on a routine basis in breeding programs. Examples of these methods include the use of maize haploid inducer lines (see Chapter 6) or the so-called *wide crosses*. More recently (10 years ago), new *in vivo* methods have been discovered [59], and are currently tested for translation to crops (*e.g.* centromere engineering, see Chapter 7). *In vivo* haploid induction methods could be divided into two main broad categories: (1) the “wide-crosses” and (2) the intra-specific crosses (Figure 2).

3.1 Haploidization by wide-crosses

Wide crosses consist to force crosses between species spanning wide taxonomic boundaries. It could thus involve inter-generic or inter-specific pollinations, and often concern crosses between a cultivated crop and a wild-relative species. Wide crosses are also named *wide hybridization*, and imply to overcome pre-fertilization and post-fertilization barriers [60]. They have been reported more frequently in monocotyledonous species as compared to dicotyledonous [57].

Two different outcomes, both useful for breeders, need to be distinguished from these wide crosses. Firstly, in case of successful hybridization, which could be helped by embryo rescue or other techniques [60], the production of hybrid embryos is a starting material in order to introduce agronomic traits of interest (disease resistance, stress tolerance...etc.) across species. Secondly, it could be used for the production of haploid embryos. Due to the unstable nature of hybrid embryos generated by joining two different genetic materials, chromosome elimination from one parent occurs during early embryogenesis in some “wide-crosses” [57,61,58,19]. Although the paternal chromosomes are eliminated in most of the cases to give rise to maternal haploid embryos (Figure 4, route 5), some rare cases were reported in which the paternal genome remains, being the maternal genome eliminated (Figure 3, route 3) (reviewed in [19]). A pioneering discovery in haploidization by wide-crosses was the *Bulbosum method* (Chapter 25), which has been well studied and is now widely used in barley breeding [57,62]. Cross of cultivated barley (*Hordeum vulgare*) using pollen from its wild relative *Hordeum bulbosum* leads to the production of *H. vulgare* haploid embryos [63,64]. The success of barley haploid embryo production thanks to wide crosses was then extended to other species, especially using maize pollen which appears to display low-intensity fertilization barriers [65,57]. For example, wheat and triticale haploid embryos are currently obtained in some plant breeding programs by

the following crosses: wheat × maize, and triticale × maize respectively [66,57,67]. Overall, wide crosses are limited to some crops at breeding scale, but once they are well established methods, they have the advantage to be effective across a wide range of genotypes, as opposed to the *in vitro* approaches, which are highly genotype-dependent within a given species. Nevertheless, a limitation of the wide crosses method is that it is not fully *in vivo* (Figure 2), since it necessitates to include a stage of *in vitro* tissue culture to prevent embryo abortion. Indeed, successful seed development relies on the correct development of the two fertilization products: the embryo and the endosperm. The endosperm tightly interacts, both physically and chemically, with the embryo and it is thus of vital importance to sustain embryo development [68-70]. In haploid embryo-producing wide crosses, the endosperm fails to develop properly, probably due to selective parental chromosome elimination occurring in this tissue as well, and consequently haploid embryos must be rescued by *in vitro* culture.

3.2 Haploidization by intra-specific crosses

Two main methods could be differentiated in order to induce haploid embryos via intra-specific crosses: (1) pollination with treated pollen, and (2) the use of haploid inducer lines. While methods based on treated pollen usually necessitate haploid embryo rescue due to early seed abortion [71,33], the haploid inducer lines present the advantage to be fully *in planta* because the output is the production of viable seeds containing haploid embryos (Figure 2) [72,73]. Depending on the methods and species considered, haploidization by intra-specific crosses could produce two different kinds of haploid embryos: maternal haploid embryos with the cytoplasm and nuclear genome from the female parent (Figure 4, route 4 and/or route 5), and paternal haploid embryos, having the cytoplasm of the egg cell (maternal) but the nuclear genome from the male parent (Figure 3, route 3). In the latter case, male haploid embryos produced by intra-specific crosses might be additionally useful for other biotechnological

purposes beyond conventional production of DH pure lines. Indeed, since mitochondrial defects are behind cytoplasmic male sterility (CMS), CMS is maternally inherited through the cytoplasm [74,75]. CMS is a valuable tool in hybrid seed production, since it avoids the time-consuming process of emasculation to prevent self-pollination [76]. This trait is traditionally transferred from one germplasm to another through multiple rounds of backcrossing. Obtaining a nuclear male genome within a “*maternal*” cytoplasm in just a single cross reduces CMS conversion to just one step, thus accelerating hybrid seed production.

Pollinations with treated pollen induce maternal haploid embryos (Figure 4, routes 4, 5). This method consists in the treatment of pollen, prior to pollination, with physical or chemical agents, irradiation being the most used treatment [71,33]. Although haploidization via pollen treatments has been reported in more than 15 species [71,33], it works ineffectively (low haploid induction rate). Thus, this method is used in plant breeding only when no alternative efficient methods are available, for example in melon and cucumber [77]. Haploid inducer lines could be seen as an exception since they exist in few species only [58,78,73]. Moreover, haploid inducer lines are routinely used in plant breeding in maize (Chapter 6) and potato [79]. Intensive researches are currently being done to extend the CENH3-based inducer line to others crops (Chapter 7). In addition, the identification of the causal genes leading to embryo haploid induction in maize allowed for the translation of this trait to two other crops: wheat and rice [80-82]. Lastly, recent patents and publications reported on the identification of sorghum haploid inducer lines [83,84].

In maize, two different types of haploid inducer lines have been reported, which are able to produce either maternal or paternal haploid embryos (Figure 4, route 5 and Figure 3, route 3, respectively) [85,86]. Chapter 6 details the properties and uses of these two maize haploid inducer lines. These two lines gained their haploid induction phenotypes due to mutations in genes involved in male and female gametophyte development, and in double fertilization.

In potato (*Solanum tuberosum* L.), the haploid induction system relies on a cross between a diploid male haploid inducer line (*S. tuberosum* Andigenum group, previously referred to as cultivar *S. Phureja*) with a tetraploid cultivated potato of interest used as female parent (Figure 4, route 5 [79,87-89]). It thus refers to an interploidy cross (4x potato × 2x haploid inducer line), and the haploid embryos found in some of the viable seeds of this cross are commonly called dihaploids to indicate that they contain two sets of chromosomes (from maternal origin). Such dihaploid plants are not homozygous, but allow breeders to work at the diploid level for simpler genetic analysis/mapping, or for introgression of valuable traits from the wild species. Since wild species are mostly diploid, they could be then crossed with the di-haploid by inter-specific hybridization [87,88].

The CENH3-based haploid inducer lines originate from the manipulation of the centromeric histone protein CENH3 in *Arabidopsis thaliana* [59]: it was reported that the genome of the parental having the engineered CENH3 is eliminated after the cross with wild-type plants with intact CENH3, creating haploid inducer lines (Chapter 7 and for recent reviews: [73,61,58,90]). These CENH3-based haploid inducer lines are thus able to induce either maternal or paternal haploid embryos (Figure 4, route 5 and Figure 3, route 3), although they seem more efficient in producing paternal haploid embryos, in *Arabidopsis* at least [59]. Although CENH3 is conserved across plant species, efficient translation of this haploid induction method to crops remain to be achieved, since very low haploid induction rates have been observed so far in crops [78,73,58].

To sum-up, haploidization by intra-specific crosses is attractive since haploid embryos are formed within a viable seed, but remain limited to few crops. Once haploid inducer lines have been reported or created, the main limitations/constraints for the use haploid inducer lines in breeding programs are the need for a relatively high haploid induction rate, and the existence of a system to identify the seeds having haploid embryos among the seeds having diploid

embryos . The history of the maize male haploid inducer line exemplifies the important need of research and development to achieve a “good” haploidization method by intra-specific crosses: indeed more than 20 years separate the discovery of the first maize haploid inducer line by Ed Coe [86] from its use in maize breeding programs [91,92]. The improvements of both haploid induction rate (from ~2-3% to ~10%) and color markers to accurately identify haploid embryos were some of the key steps in the successful use of maize haploid inducer lines in breeding programs (Chapter 6). Recently, the knowledge gained in the mode of action behind the maize *in vivo* haploid induction system [73,72], allowed for the successful translation of this feature to two new crops: rice and wheat [80-82].

4. Concluding remarks

In this chapter we have revised the principal approaches currently available to produce haploids and DHs (Figure 2) for different purposes, principally focused on the rapid generation of pure lines to accelerate hybrid seed production or CMS conversion and to simplify breeding programs by producing di-haploids, as for potato. These approaches imply the use of methods exclusively based on *in vitro* culture, *in vivo* induction of haploid development, or a combination of them to induce haploid embryos *in vivo* and then rescue them *in vitro*. Practical examples of the application of these methods to particular species or varieties are presented throughout the book. The choice of the best performing approach will depend on the species used, and to what extent these methods have been developed and adapted to this species. Together, these approaches illustrate how a given goal can be accomplished by different biotechnological means, and are a good example of the power of combining different biotechnological for solving specific applied problems of industry and in general, of society.

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Figure legends

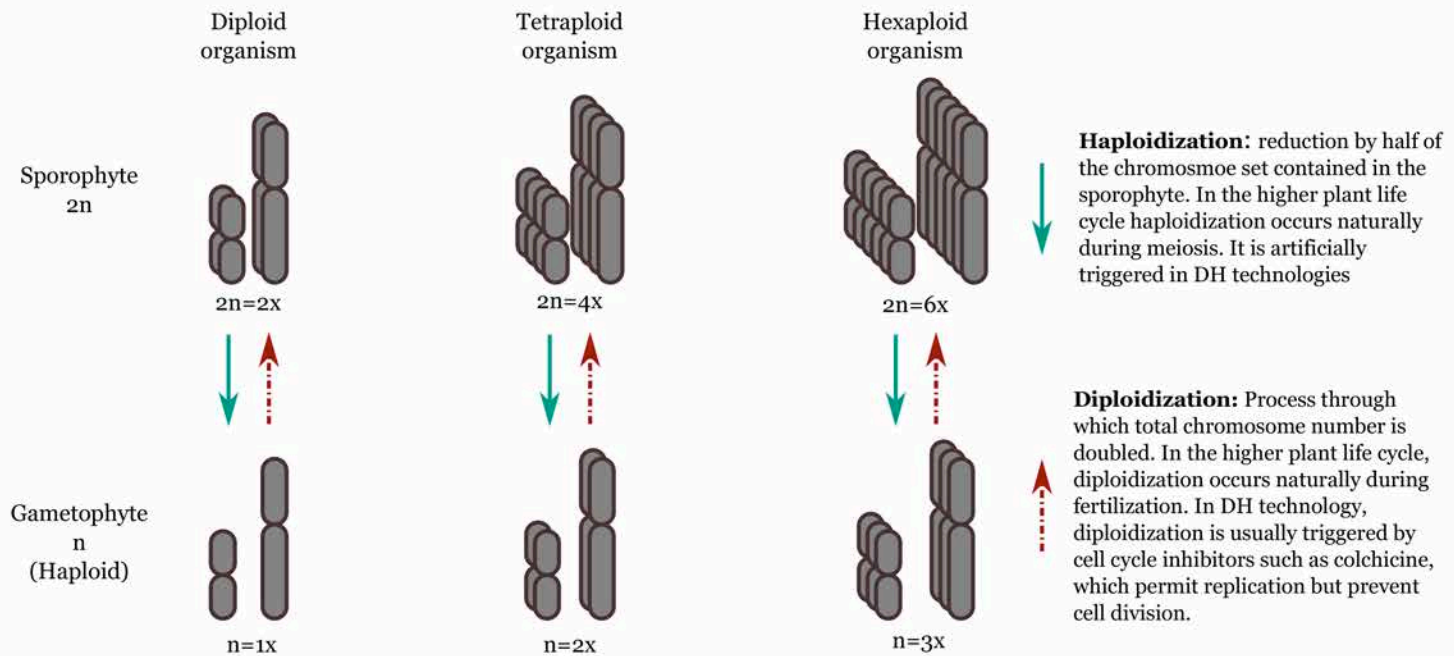
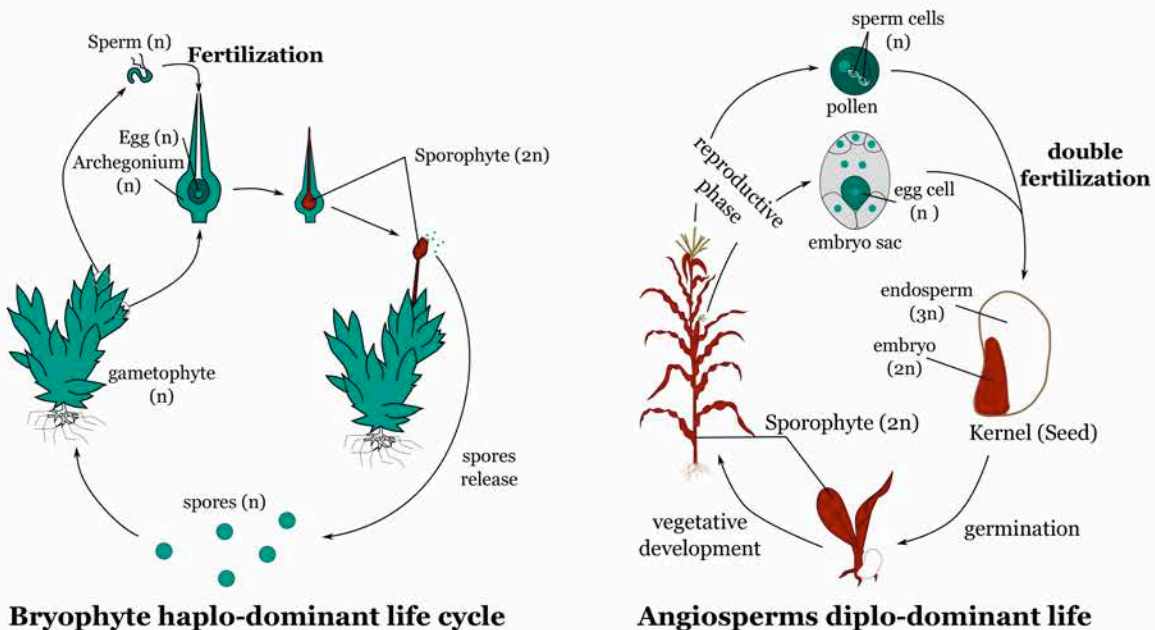
Figure 1. Basic concepts concerning haploidy. **A.** *Haploid* generally refers to a cell (or organism) containing half the chromosome number found in somatic cells. However, according to the ploidy level of the considered species, a haploid individual may harbor one or several versions of each chromosome. Examples are shown for a theoretical diploid, tetraploid and hexaploid, each with a set of two different, non homologous chromosomes ($x = 2$). **B.** Simple representation of the contrasted life cycles of bryophytes, left and angiosperms, right, using maize as example. Turquoise color refers to the gametophytic (haploid) stage, and dark red to the sporophytic (diploid) stage. Note that some organisms' life cycle relies mostly on the haploid gametophytic stage. On the other hand, angiosperms drastically reduced their haploid phase to a few cells embedded in the diploid tissue.

Figure 2. Overview of *in vitro* and *in vivo* haploidization methods in plants. Intersection depicts *in vivo* methods that necessitate *in vitro* step(s) to prevent haploid embryo abortion.

Figure 3. Different alternatives to produce male-derived DHs. Microsporogenesis and microgametogenesis are natural pathways that normally take place to give rise to the male gametophyte (pollen grain) and male gametes (sperm cells). Male-derived haploids or DHs may arise from deviations of these pathways at three different levels: (1) diverting the meiocyte, (before microspore release) towards proliferation to induce *in vitro* the formation of callus, from which haploids and DHs, but also heterozygous diploids, can be produced by organogenesis (Route 1); (2) reprogramming the vacuolate microspore or young pollen grain towards embryogenesis (or alternatively callus formation + regeneration through organogenesis) by the *in vitro* application of a stress treatment and subsequent *in vitro* culture (Route 2); and (3) *in vivo* elimination of the female genome after egg fertilization by a sperm cell (Route 3). See text for further details.

Figure 4. Different alternatives to produce female-derived haploids and DHs. Megasporogenesis and megagametogenesis are pathways that normally take place to give rise to the female gametophyte (embryo sac) and female gametes (egg cell and central cell). Female-derived haploids or DHs may arise by two main means: (1) Reprogramming of egg cell

development into haploid embryogenesis, either by *in vitro* induction or *in vivo* pollination with haploid inducer lines (Route 4); and (2) the sperm cell fertilizes the egg cell, but the male genome is progressively eliminated during early embryogenesis. Some methods lead to viable seeds with haploid embryos and are thus fully *in vivo*, whereas other methods need additional steps of *in vitro* culture.

A**B**

 haploid tissue/cells

 diploid tissue/cells

***In vitro* methods**

***In vitro* culture of:**

- microspores
- anthers
- ovaries
- ovules
- Flower buds

***In vivo + in vitro* methods**

- Wide crosses
- Pollen treatments

***In vivo* methods**

- Haploid inducer lines
(including CENH3-based haploid inducer lines)

