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Abstract

Mixing maternal and paternal genomes in embryos is not only responsible for the evolutionary success of sexual reproduction but a cornerstone of plant breeding. However, once an interesting gene combination is obtained, further genetic mixing is problematic. To rapidly fix genetic information, doubled-haploid plants can be produced: haploid embryos having solely the genetic information from one parent are allowed to develop and chromosome doubling generates fully homozygous plants. A powerful path to doubled-haploids production is based on haploid inducer lines. A simple cross between a haploid inducer line and the line with gene combinations to be fixed will trigger haploid embryo development. However the exact mechanism behind *in-planta* haploid induction remains an enduring mystery. The recent discoveries of molecular actors triggering haploid induction in the maize crop and the model *Arabidopsis thaliana* pinpoint an essential role of processes related to gamete development, gamete interactions and genome stability. These findings enabled translation of haploid induction capacity to other crops, and the use of haploid inducer lines to deliver genome editing machinery into various crop varieties. These recent advances not only hold promise for the next generations of plant breeding strategies, but it also provides a deeper insight into the fundamental bases of sexual reproduction in plants.

Introduction

In the plant breeder's toolbox, the "doubled haploid" (DH) method is used to rapidly produce homozygous plants. Progenies of DH plants are thus genetically homogeneous material, allowing breeders to evaluate their traits of interest on genetically fixed material at an early stage of the breeding cycle, thus increasing breeding efficiency [1–3]. The DH technology relies on two main steps: (1) a haploid induction system to generate haploid embryos/plantlets (Box 1), and (2) a chromosome doubling (copy/paste) step to restore diploidy of these plantlets. In other words, this technology entails the production of a variety of haploid plantlets which undergo whole genome duplication to produce a set of perfectly homozygous plants [1,4].

Although chromosome doubling steps remain challenging, the main limitation to a generalized use of DH technology in plant breeding is the availability of methods to induce haploidy in crop plants [5,6]. **Table 1** lists the main crops and vegetables for which a haploid induction system is used in breeding, as well as some economically important species lacking a robust haploid induction system. The starting point for DH breeding was the discovery in the 1970s that haploid embryos and plantlets can be produced by culturing anthers of *Datura* [7]. Indeed, still today the most popular methods for haploidization (Box 1) rely on the *in vitro* culture of haploid gametophytic tissues, more frequently from male gametophytic tissues (androgenesis *in vitro*), than from female gametophytic tissues (gynogenesis *in vitro*) [4,8,9] (see examples in **Table 1**). These *in vitro* methods depend on appropriate *in vitro* culture conditions that allow the haploid gametophytic tissues to be reprogramed into embryonic development, resulting in haploid embryos/plantlets. Although extensive research has greatly improved the efficiency of these techniques, these *in vitro* protocols remain labor intensive and limited to a small number of plant species, and often to particular genotypes within a species [8,10,11]. Alternative techniques involving crosses between different species (inter-specific crosses) or pollen treatments have been successfully developed for some crops such

as wheat and melon respectively [8,12] (see examples on **Table 1**). Nevertheless, these techniques usually still necessitate *in vitro* steps to rescue the immature embryos of the seeds that have a tendency to abort, generally due to a lack of development of the neighboring fertilization product, the endosperm. Lastly, haploid induction lines represent a unique *in planta* system without any tissue culture steps, which is able to produce haploid embryos/plantlets by simple crossing (intra-specific crosses), but is limited to a few plants species (**Table 1**) and has only been effectively used in maize breeding so far [3].

The present perspective focuses on recent advances made on *in planta* haploid induction systems. Indeed, the elucidation of the genetics and the identification of some molecular players in maize and *Arabidopsis thaliana* enable now the translation of these *in planta* induction systems to other crops. The gained knowledge also sheds new light on fundamental biological questions concerning the mechanisms involved in plant reproduction in general, and double fertilization in particular, as well as in genome (in)stability and aneuploidy (Box 1). In addition, combination of the *in planta* haploid induction systems with gene editing tools such as CRISPR/Cas9 allows the plant breeder's toolbox to be extended towards faster and more precise plant breeding.

Haploid inducer lines used for *in planta* haploid induction

Haploid inducer lines (Box 1) have the unique property of generating viable seeds with haploid embryos that can then easily give rise to haploid plantlets following germination [3,13]. It has to be noted that this trait is not fully penetrant: only a proportion of the seeds derived from a cross with haploid inducer have haploid embryos, while a large proportion of seeds have normal diploid embryos. Furthermore, the trait is also accompanied with a variable proportion of aborted seeds. The relative simplicity of this system is based on the fact that only crosses between the haploid inducer lines with plants of interest are necessary, as the development of the haploid embryos occurs within the plant without labor intensive *in vitro* culture steps. Nevertheless, four key points must be met for this method to be useful to breeders: (1) the possibility for large scale out-crossing, (2) a sufficiently high haploid induction rate, *i.e.* a ratio of haploid/diploid embryos ideally >10%, (3) a system to sort the desired seeds with haploid embryos *vs* classical seeds with diploid embryos, and (4) a homogeneous response as regards to the genetic background of the breeder (no or limited genotype interaction). Two main types of haploid inducer lines will be discussed in this review: the maize maternal haploid induction system and the centromere engineering system elaborated in *Arabidopsis thaliana*.

The maize haploid inducer line

Two types of maize haploid inducer lines were discovered to induce paternal and/or maternal haploids *in planta* [14,15] . Here we will focus on the male haploid inducer line that can induce maternal haploidy, since this line and its derivatives are currently broadly used in maize breeding programs [16,17]. In Europe and the USA, all major breeding companies intensively use DH technology in their routine maize breeding programs. Thus an estimated significant proportion of hybrid maize seeds sold in Europe and the USA involves haploid inducer lines in its production scheme. Maternal haploid induction is triggered by the pollinator (male) parent [15]: crosses using pollen from this male haploid inducer line, lead to the development of the egg cell

into a haploid embryo, containing solely the maternal chromosome set/genome, whereas the endosperm is normally fertilized [3,15] (**Fig. 1**). The birth of this maize haploid inducer line dates back to the 1950s, when Ed Coe identified a spontaneous mutant (named "stock 6") able to induce about 2-3% haploid embryos [15]. This genetic trick was then introduced into different genetic backgrounds to improve the haploid induction rate by breeding. Numerous derivatives were developed leading to a complex pedigree of haploid inducer lines [18]. The derivatives with the highest haploid induction rates (>8%), such as the broadly used line "RWS" [16], are called "modern haploid inducer lines" and have made possible the large scale use of DH in maize breeding. The polygenic nature of haploid induction suggested by these successful breeding efforts was confirmed by quantitative trait loci (QTL) analyses [19], and ultimately by QTL cloning [20–23].

Centromere engineering to create haploid inducer lines

As well as utilizing natural variation to create haploid inducer lines, biotechnological manipulations can be employed to the same end. Modification of a centromeric variant of histone 3 (CENH3), also known as centromere protein A (CENP-A) in vertebrate, led to the discovery of a new type of haploid inducer line in *A. thaliana* [24]. Replacement of the CENH3 N-terminal (N-ter) tail by a GFP fused with the N-ter tail of conventional Histone 3.3 partially complements the lethal phenotype of a *cenh3* null mutant [24]. Remarkably this transgenic "GFP-tailswap" line, when used as female parent receiving wild-type pollen, led to the induction of ~30% of haploid embryos with only the male (wild-type) genome. The female chromosomes labeled by the GFP-tailswap are lost during early embryo divisions, creating a high frequency of paternal haploid embryos, together with aneuploid (~30%) and normal diploid embryos (**Fig. 2**) [24,25]. This tailswap strategy was successfully reproduced in maize, but with an average haploid induction frequency too low (below 1%) for breeding applications [26]. A related strategy was also successful in creating a haploid inducer line: instead of complementing the *A. thaliana cenh3* null mutant with the "GFP-tailswap", unaltered CENH3 variants from distantly related Brassica species were used [27]. This two-step strategy, requiring initially the creation of a *cenh3* knock-out mutant and subsequently the complementation of this mutant by a transgenic construct, has recently been simplified [28,29]. The idea behind this alternative one-step strategy is to screen for polymorphism at the *CENH3* locus to identify point mutations that lead to alterations of CENH3 function that result in the production of haploid embryos when crossed with wild-type plants. Critical amino acid changes within CENH3 have been identified/created that present haploid induction rates ranging from 0.6% to 12% depending on the mutation [13,28–31]. Among others, the *A. thaliana* TILLING (Targeting Induced Local Lesions IN Genomes) mutant A86V (Alanine #86 mutated in Valine) was able to induce 2.7% of paternal haploid plants [29]. However, a barley TILLING mutant with a mutation of a conserved amino acid (L92F) did not induce haploidy [28]. Recent patents also claim the creation of haploid inducer lines in some crops using single amino acid changes in CENH3 (review in [13]). However, only low haploid induction capacity (from 0% to ~2%) was obtained [13,32,33]. Altogether, these results open the door for the creation of new types of *in planta* haploid inducer lines based on CENH3 manipulation. In addition to the production of DH plants, CENH3-based haploid inducer lines could also be very useful for breeders to transfer a nuclear genome of interest into a different cytoplasm. Indeed, although the genome of the engineered CENH3 female is eliminated, its cytoplasm is maintained (**Fig. 2b**). This is of interest for the establishment of cytoplasmic male sterility (CMS), a frequently used tool in hybrid seed production which

facilitates crosses by avoiding emasculation [34]. CENH3-based haploid inducer systems could provide one-step CMS conversion and thus simplify hybrid crop management.

Nevertheless, to our knowledge, CENH3-based systems are not currently used in breeding programs, mostly due to the low haploid induction rate obtained in crop species [13]. As the rate of haploid induction is often a major limitation to the utility of this technology for breeding applications, a better understanding of the underlying mechanisms is clearly needed to overcome these barriers.

Molecular mechanism behind maize haploid inducer line

Two main hypotheses are found in the literature to explain the mechanism behind the maize haploid inducer line:

(1) Normal double fertilization with male and female gamete fusion, followed by selective male chromosome elimination during early embryogenesis. This hypothesis is mainly supported by the detection of incomplete paternal inducer chromosome segments in some fertilization products, either embryo or endosperm [35–40]. These observations suggest that the genetic information of the haploid inducer parent is transmitted and then progressively lost through mitotic cell divisions in the embryo, leading to haploid embryos and some cases of aneuploid/mixoploid (Box 1) embryos.

(2) Impaired double fertilization due to defects in pollen development or in gamete fusion [41–45]. This is first supported by the existence of pollen grains with a pair of morphologically different sperm cell nuclei [41]. A study also reported abnormal pollen grains composed of bi-nucleate pollen instated of normal tri-nucleate, but the low frequency could not account for anomalies leading to haploid induction [46]. These old studies need to be interpreted with care considering the specific and complex genetic background of the different haploid inducer lines, since near isogenic lines were not available to be used as controls in these experiments. A recent study used a technical breakthrough consisting of single nucleus sequencing within the pollen grain. Genomic instability, resulting in fragmented chromosomes were found in about 30% of the sperm cells of the haploid inducer line [44]. These defective male gametes could then lead to haploid embryos but also to both the failure of double fertilization and the production of aneuploid embryos and/or endosperms which abort. Interestingly, failed egg cell–sperm cell fusion, but normal central cell–sperm cell fusion, was observed when using pollen from haploid inducers [43,45]. These types of single fertilization could lead to endosperm development and stimulate the development of the haploid egg cell into a haploid embryo.

Recent breakthroughs have enabled the identification of the two main molecular players responsible for maize *in planta* haploid induction. The first key gene behind maternal haploid induction, named *MATL/NLD/ZmPLA1* (*MATRILINEAL* / *NOT LIKE DAD* / *ZmPHOSPHOLIPASE-A1*), was discovered simultaneously by three independent groups [20–22,47]. *MATL/NLD/ZmPLA1* is a pollen specific gene encoding for a predicted patatin-like phospholipase A2. A survey of many different haploid inducer lines showed that in all of them *MATL/NLD/ZmPLA1* carries a 4-bp insertion. This mutation is causing a frameshift and a truncated protein, which is mis-localized and unstable [20,21]. Gene knockout and complementation confirm that loss of function of *MATL/NLD/ZmPLA1* is responsible for the haploid induction trait [20–22]. The molecular effects of the *matl/nld/Zmpla1* mutation on sperm cells remain enigmatic, but the coincidence between

the appearance of chromosomal instability in haploid inducer lines [44] and the onset of *MATL/NLD/ZmPLA1* expression when sperm cells are formed [20] suggests that the effect of MATL/NLD/ZmPLA1 occurs during sperm cell formation, and not during earlier stages of pollen development, *i.e.* microspore development or first pollen mitosis.

A second critical factor for maize haploid induction is the recently characterized membrane protein ZmDMP (DUF679 Membrane Protein) [23]. *ZmDMP* gene knock out is able to increase 2 to 6-fold the haploid induction rate when combined with the *matl/nld/Zmpla1* mutation [23]. Interestingly, *ZmDMP* knockout on its own has at a very low haploid induction rate (~0.15%) [23]. This synergistic effect of *matl/nld/Zmpla1* and *Zmdmp* mutations implies that at least two distinct pathways are behind the high haploid induction rate observed in "modern" haploid inducer lines. The recent characterization of the two *A. thaliana* orthologous genes *AtDMP8* and *AtDMP9* [48], allows us to speculate on *ZmDMP* function in haploid induction. Indeed, both the single *Atdmp9* mutant and, more strongly, the *Atdmp8/Atdmp9* double mutant have double fertilization failures. Although 40% to 80% of the fertilizations are normal (=double fertilization), both complete absence of fertilization and single fertilization of the central cell without fertilization of the egg cell were observed (up to 40%) [48,49]. Based on these results in *A. thaliana*, we can speculate that in maize the membrane protein ZmDMP is involved in male/female gamete interaction necessary to achieve a correct double fertilization (**Fig. 1a**), although sperm cell localization of ZmDMP has not been investigated. This hypothesis is consolidated by observations of single fertilization of the central cell during crosses with haploid inducer lines [43], although it was not related directly to *ZmDMP* since the *Zmdmp* mutation had not been identified at that time. Moreover, a single fertilization phenotype could explain the high hetero-fertilization (Box 1) rate observed in crosses with haploid inducer lines [43]: an initial failure of egg cell fertilization could be rescued via fertilization by sperm cells from another pollen grain which does not produce this phenotype.

In light of these recent discoveries on the molecular players behind maize haploid induction, we propose possible molecular and cellular events which unify the two preceding hypotheses. The mutation in *MATL/NLD/ZmPLA1* leads to partial genome instability (by mechanisms yet to be determined) and thus to aneuploidy and chromosome fragmentation in some sperm cells [44]. This would create a scenario of "defective" pollen grains with chromosome fragmentation in either one or both sperm cells, among a majority of normal pollen grains [44] (**Fig. 1b**). Consequently, three main situations need to be considered when pollen from haploid inducer lines mutated only in *MATL/NLD/ZmPLA1* arrives at a wild-type embryo sac: (1) "normal" pollen (the majority) containing sperm cells with intact genome, which achieves normal double fertilization leading to the formation of normal seed (=diploid embryo and triploid endosperm) (**Fig. 1b**), (2) "defective" pollen with chromosome fragmentation in only one sperm cell, which leads either (50%) to aborted seeds if the defective sperm cell gives rise to the endosperm, or to (50%) seeds with a haploid embryo (and normal triploid endosperm) if the defective sperm cell fuses with the egg cell (**Fig. 1b**), and (3) "defective" pollen with chromosome fragmentation in the two sperm cells, which leads to aborted seeds (**Fig. 1b**). In these scenarios, the fragmented paternal chromosomes would continue to be degraded throughout the first mitotic divisions of the embryo. In summary, mutation of *MATL/NLD/ZmPLA1* alone, in an unfavorable genetic background for haploid induction and notably a wild-type *ZmDMP* allele, leads to 0.5-3% of haploid induction [15,20,23]. This low haploid rate is increased in haploid inducer lines having

mutations in both *MATL/NLD/ZmPLA1* and *ZmDMP* [23,50]. This observation could be interpreted by the fact that *Zmdmp* mutation impairs the double fertilization, creating some cases of single fertilization events (fertilized central cell and non-fertilized egg cell) (**Fig. 1c**). Thus additional haploid embryos can arise directly from these central cell single fertilization events but also from subsequent hetero-fertilization events, in which only the egg cell but not the central cell has a chance to meet a sperm cell with fragmented chromosomes (**Fig. 1c**). Although other cases of hetero-fertilization can be envisioned, the examples described above readily explain the fact that the *Zmdmp* mutation is able to boost the haploid induction rate when combined to the *matl/nld/Zmpla1* mutation, whereas the *Zmdmp* mutation alone has a very low haploid induction rate [23].

To sum-up, mutation in *MATL/NLD/ZmPLA1* alone leads to a fraction of pollen with defective sperm cells because of their fragmented chromosomes. Independently the mutation of *ZmDMP* could be linked to central cell single fertilizations observed in haploid inducer lines with high haploid induction rate. These single central cell fertilization events create at least two favorable situations to produce additional haploid embryos: (1) they could promote directly the development of egg cells into haploid embryos without sperm cell / egg cell fusion, and (2) they lead to subsequent hetero-fertilizations that favor the occurrence of cases in which an egg cell that receives a defective sperm cell has more probability to be accompanied by an endosperm that is correctly fertilized, ensuring proper seed development (**Fig. 1**). Since all scenarios imply at least one fertilization event, they all fall in the category of sperm cell-dependent parthenogenesis (Box 1), also called gynogenesis (Box 1) [51]. To summarize, in maize haploid inducer lines, high haploid embryos rate are due to the combination of defective sperm cells and fertilization defects. More experimental work is needed to test this unified hypothesis, and also to understand the links between the *bona fide* phospholipase activity of MATL/NLD/ZmPLA1 and chromosome instability in sperm cells.

Molecular mechanism behind CENH3-based haploid induction system

The mechanism behind haploidization via engineering of CENH3 has been detailed and discussed in many recent reviews [12,13,30,52–54]. Centromeres are the region of chromosomes that provide the site for microtubule attachment, allowing chromosome movement and segregation during mitosis and meiosis. CENH3 is part of the protein complex that is essential to proper centromere structure and function. Thus, mutations impairing CENH3 function such as the “GFP-tailswap” described above result in defective centromeres. Wang and Dawe proposed a relationship between centromere size and haploid formation [53]. In situations where “CENH3-defective” plants were crossed with wild-type plants, the chromosomes harboring defective or smaller centromeres are progressively lost (**Fig. 2**). Thus, chromosome segregation fails during early embryogenesis resulting in uniparental genome elimination and haploid embryo formation (**Fig. 2**). Although the exact mechanism of chromosome elimination in CENH3-based haploid induction system has not been resolved yet (recently reviewed in [54]), this model explains why haploids are not found when CENH3-defective plants are self-fertilized, probably because defective centromeres are not in competition with normal or larger centromeres. Remarkably, haploid induction in barley interspecific crosses (using the ‘bulbosum’ method, Box 1) also involves a mechanism that is CENH3-dependent [55].

Curiously, the CENH3-based haploid induction system does not work well through the male, *i.e.* when a wild-type female parent receives CENH3 engineered pollen, the rate of maternal haploid induction is very low [13]. The reasons for this preference remain to be elucidated.

Haploid induction systems as a building stone for the engineering of crops.

The benefits offered by haploid induction systems to crop breeding programs are diverse, as DH technology can be used in conjunction with several different molecular techniques to overcome various constraints to crop improvement. One example is the recent use of haploid induction systems to expand the application of genome editing technologies to crops. The past decade has seen a rapid expansion of genome editing technologies, most notably due to the development of Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR)/Cas genome editing systems. The key innovation of CRISPR/Cas technology is that the nuclease responsible for inducing DNA modifications can be easily targeted to almost any genomic locus by virtue of a synthetic guide RNA that is co-delivered with the Cas nuclease. Due to its low cost and ease of design, this technology has already become a standard tool for molecular biology research and offers great potential for agricultural applications [56]. However, to utilize the full potential of these genome editing systems for crop improvement, several obstacles have yet to be overcome. One major obstacle is the fact that a large number of the elite cultivars used in commercial breeding are recalcitrant to genetic transformation, thus rendering the delivery of CRISPR/Cas systems for editing of the elite cultivar difficult or impossible. One way around this constraint is to introduce the genome editing system into a genetically transformable variety and to then introgress the desired edits by recurrent backcrosses into the elite cultivar. To avoid this time consuming process and readily combine the agronomic traits of whatever elite cultivars with a genome editing trait, two different research groups elegantly used the benefit of *in planta* haploid inducer lines [57,58]. They combined haploid induction along with CRISPR/Cas technology to directly deliver genome edits to crop varieties that are recalcitrant to genetic transformation and to avoid the presence of the transgene triggering the edits in the genome of the elite material [57,58]. Both systems, Haploid Inducer (HI)-Edit [57] and Haploid-Inducer Mediated Genome Editing (IMGE) [58], involve the introduction of a transgenic CRISPR/Cas cassette into a haploid inducer line that is then used for a cross with an elite cultivar (**Fig. 3**). The haploid progeny is then screened for CRISPR/Cas-induced mutations and genome doubling is subsequently induced to produce diploid, transgene free, genome-edited elite cultivars. Both systems rely on the fact that the CRISPR/Cas tool is expressed or present within the zygote prior to the elimination of the haploid-inducer genome, carrying the transgene necessary to achieve genome editing of the non-transgenic haploid genome *in trans*. Hence, the efficiency of the system relies not only on the penetrance of haploid induction but also on the relative timing of the expression/elimination of the haploid-inducer genome. Therefore, further research into the mechanisms of haploid induction will also serve to improve the efficiencies of HI-Edit/IMGE systems in the future. The achievement of this technique using maize haploid inducer lines [57,58] also informs us on the mechanism behind maize haploid induction (**Fig. 3**). The fact that some maternal haploid plants were edited by paternal material implies that fusion of the sperm- and egg-cells occurs to trigger those haploid embryos. More precisely (**Fig. 3b-c**) the sperm cell from a haploid inducer could bring to the egg cell either: (1) the Cas9

ribonucleoprotein (assembly of Cas9 protein and guide RNA) which are already expressed in sperm cell (**Fig. 3b**), as exemplified by the HI-Edit system in which pollen-specific promoters seem to be more efficient as compared to constitutive promoters; and/or (2) the transgene DNA inserted in the haploid inducer genome, which is then expressed in the fertilization product before being eliminated throughout zygotic divisions (**Fig. 3c**). Whatever the scenario is, it rules out the hypothesis that haploid embryos develop without gamete fusion, at least for the subset of edited haploid plants (~3%). It thus shows that egg cell / sperm cell fusion occurs but delivers an unstable paternal genome, which is sufficient to trigger embryo development, and thus gynogenesis.

A second area in which haploid inducer systems can be used for agricultural applications is the creation of artificial apomixis (a form of asexual reproduction) in hybrid varieties to allow the hybrid genome to be propagated clonally through seeds. The phenomenon of heterosis, which is defined as increased vigor of an F1 hybrid relative to the mid-parental value, has been used frequently in modern agriculture. However, production of F1 hybrids is a laborious and time-consuming process and F1 seed stocks must be continuously replenished by crossing parental varieties because the heterotic effect is lost due to genetic segregation during meiosis in the F1 plant. Hence, methods to clonally propagate F1 seeds would be a great asset to agriculture. Efforts to artificially engineer apomixis have largely focused on combining mutations that disrupt meiotic divisions, such that meiosis is replaced by mitotic-like cell divisions whereby all daughter cells receive the same genome. These “mitosis instead of meiosis” (*MiMe*) genotypes have been successfully created in *A. thaliana* [59] and rice [60]. However, because the gametes of the *MiMe* plants are diploid there is an increase in ploidy for each subsequent generation which can be detrimental to plant growth. To overcome this problem, mutations associated with haploid induction can be introduced into the *MiMe* genetic backgrounds. This concept was recently demonstrated by creating a hybrid rice genotype that can be clonally propagated through its seeds, thus fixing the heterotic genotype [61]. In this study, three mutations were introduced by CRISPR/Cas technology at the *REC8 (MEIOTIC RECOMBINATION PROTEIN8)*, *PAIR1* (*HOMOLOGOUS PAIRING ABERRATION IN RICE MEIOSIS1*) and *OSD1 (OMISSION OF SECOND DIVISION*1) loci to generate a *MiMe* genotype in an elite hybrid cultivar. A fourth mutation at the *MATL/NLD/ZmPLA1* locus was also introduced to induce the formation of haploid, clonal gametes. While this approach clearly demonstrated that the combination of mutations could result in a fixation of the hybrid genotype by clonal propagation through seeds, the frequency of apomitic seeds remain low. In addition, the introduction of the *matl/nld/zmpla1* mutation was associated with a dramatic decrease in the seed setting rate. Hence, future research into the mechanism(s) of haploid induction will be necessary to uncouple or reduce the haploid induction and fecundity-loss phenotypes [62], thus enabling this strategy to become commercially viable.

Limitations and perspectives

The two *in planta* haploid induction systems described here have opposite history with regard to their use. The maize system was first intensively used by breeders before discovering the cause of the haploid induction phenotype, whereas the haploid induction capacity of the CENH3 system was a by-product of basic science, which has subsequently been applied to breeding. Thus the knowledge that is

(and will be) generated to decipher the molecular and cellular mechanisms, will impact both basic science (pollen development, double fertilization, gamete interactions, genome stability) and applied science (breeding methods). Once sufficiently understood, a longer term conceivable perspective could be to mimic the mechanisms by altering other molecular actors. For example, impairing centromere proteins, other than CENH3, could also destabilize the centromere and thus theoretically lead to haploid induction. Sperm cell chromosome fragmentation triggered by other means than MATL/NLD/ZmPLA1 may allow higher haploid induction rates.

A more tangible perspective, which is currently intensively developed, is the translation of these two haploid induction systems to (other) crops. As mentioned earlier, the haploid induction system based on *MATL/NLD/ZmPLA1* has recently been successfully translated to rice and wheat [63–65]. Nevertheless, translation to dicotyledonous species is still challenging due to difficulties identifying the functional orthologs of *MATL/NLD/ZmPLA1* [20]. Regarding the CENH3 haploid induction system, efforts have been made to translate it from *A. thaliana* to different crops, but with minimal success due to the low haploid induction rate observed in the crops tested so far (review in (Kalinowska et al., 2019)).

Having a high haploid induction rate (of at least 10%) or increasing the haploid induction rate represents an essential condition for routine uses in breeding programs. In both the maize haploid inducer line and the *A. thaliana* CENH3 engineering system, additional mutations have been found to enhance the haploid induction rate, *ZmDMP* and *AtLIG4* (DNA Ligase IV) respectively [23,25]. These additional actors need to be taken into account to design translation strategies to other species.

Another important limitation for efficient utilization of *in planta* haploid induction systems is the mode of sexual reproduction of the plants/crops. Autogamous species (plants that self-fertilize) are more difficult to handle compared to allogamous species (species that cross-fertilize), because emasculation of flowers (to leave only the female part) often requires massive efforts. Although in laboratory conditions flower emasculations are feasible to evaluate the haploid induction rate, it remains challenging at the breeders' scale. This limitation could be by-passed using strategies involving chemical emasculation of female genotypes, or using inducible male sterility in the lines used as females.

The artificial doubling process of the chromosomes of haploid plantlets represents another critical step in DH technology [66]. Currently, chemical treatments (for example colchicine) that impair mitosis are used. They act like a 'copy–paste' function of the haploid genome to then produce a diploid genome. At the breeder's scale, this step becomes very quickly labor intensive (treatments, germination, transplanting…) with the need of special facilities which further increase the costs. In maize, different studies have identified genetic traits that are able to improve spontaneous chromosome doubling [67–71]. Thus combining *in planta* haploid induction with spontaneous doubling could give rise to fully *in planta* doubled haploid production. Although spontaneous doubling presents a great opportunity to avoid the artificial chromosome doubling steps, its limitation is that this trait has to be present in the genetic material from the plant that will give rise to the DH, and not the haploid inducer line (since its genome is not transmitted). Another related interesting application could be to introduce this spontaneous chromosome doubling trait into haploid inducer lines in order to increase their doubling capacity. Selfing haploid inducer

lines should give rise to a higher number of viable seeds (rescuing the haploid one) and thus improve the propagation of the haploid inducer line.

To conclude, although *in planta* haploid induction is still limited to few crops, the recent discoveries described in this perspective, open new possibilities to extend the plant breeder's toolbox. In addition, deciphering haploid induction mechanisms will bring a greater understanding to plant reproduction.

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Author contributions

T.W. led the writing of the manuscript. N.M.A.J., D.E.P. and T.W. contributed to the critical reading of the manuscript, provided suggestions and contributed to the writing of specific sections. L.M.G, P.M.R. and J-P.M. contributed to the critical reading of the manuscript and provided suggestions. L.M.G composed the figures with help from N.M.A.J. T.W. initiated and coordinated the project.

Competing interests

N.M.A.J, L.M.G., and J-P.M. are employees of LIMAGRAIN Europe. Pending patent applications PCT/EP2016/060202 (published as WO2016177887), EP3091076.

Figures:

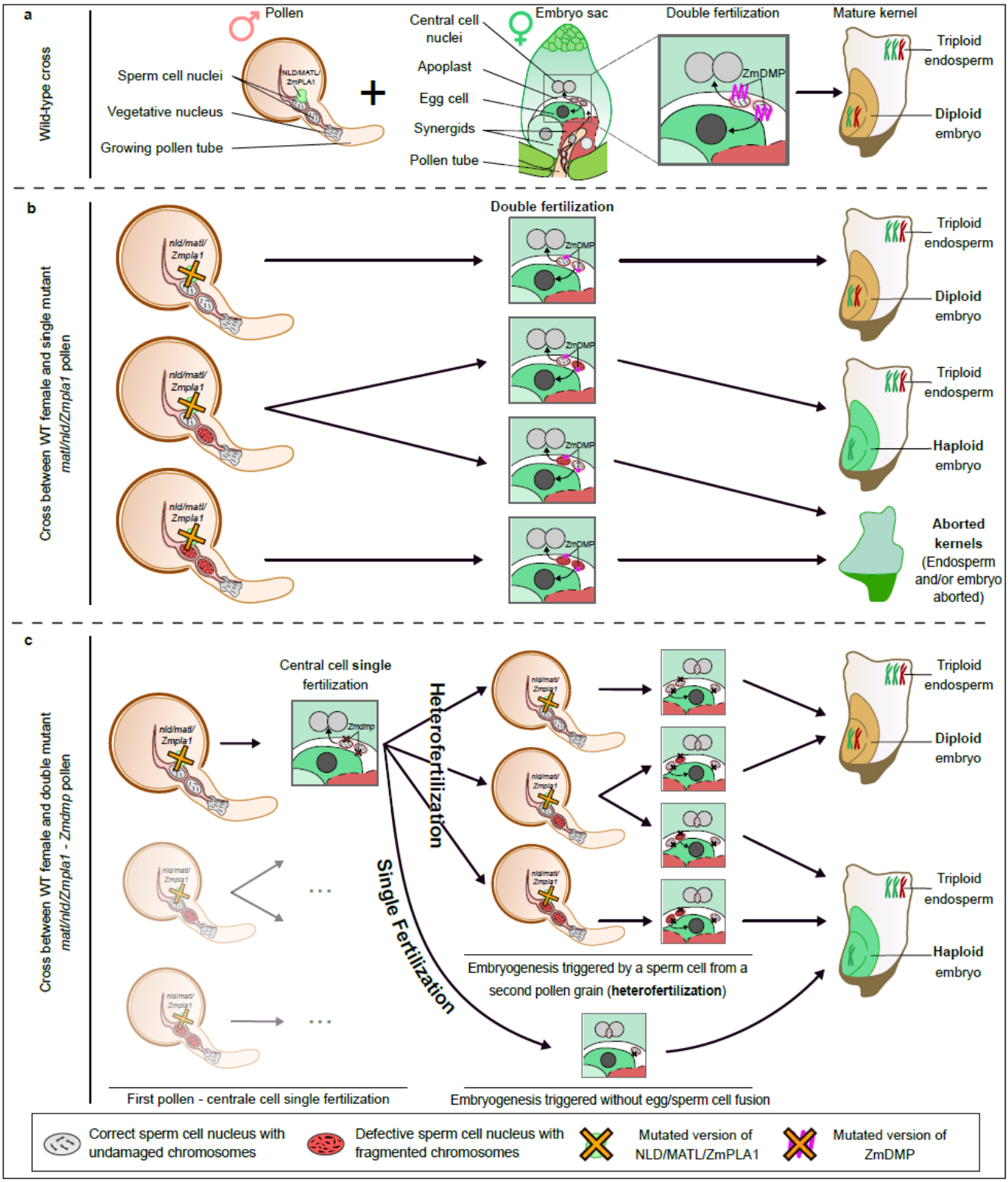

Fig. 1 Hypothetic mechanistic model of *in planta* haploid induction in maize. a, Double fertilization in a classical wild-type cross resulting in a diploid embryo and a triploid endosperm. **b**, In a cross with *matl/nld/Zmpla1* mutant pollen, three main types of pollen grains co-exist, containing zero, one or two defective sperm cell with fragmented chromosomes. Fertilization of a wild-type female could thus lead to: normal kernels, kernels with a haploid embryo,

and to aborted kernels. **c**, Selected outcomes of a cross with *matl/nld/Zmpla1* and *Zmdmp* double mutant pollen illustrating the boosting effect of the *Zmdmp* mutation on the haploid induction rate. In addition to what is described in (b), two situations could explain the production of new haploid embryos. Firstly, single fertilization of the central cell by some non-defective sperm cell (in terms of chromosome fragmentation) would allow a second pollen grain to deliver, via hetero-fertilization, defective sperm cell. Secondly, the single central cell fertilization could be sufficient to trigger egg cell development without fertilization of the egg cell.

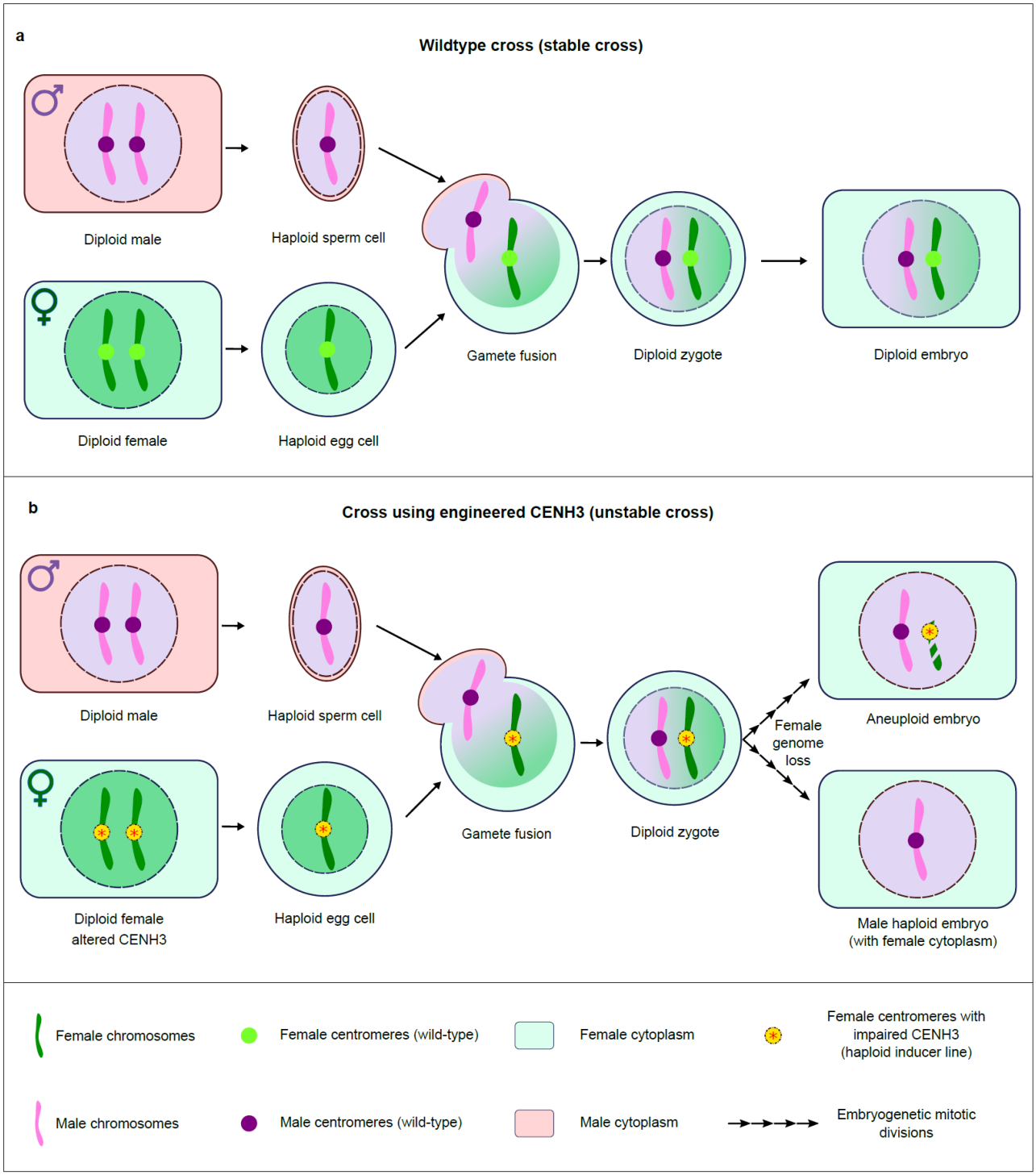

Fig. 2 Model of the uniparental chromosome elimination in the CENH3-based haploid inducer system. **a**, In a cross between two wild-type lines, the haploid egg cell fuses with a haploid sperm cell during the gamete fusion to form a diploid zygote, then a diploid embryo containing both a female and a male genome. **b**, In a cross between a haploid inducer line with defective CENH3 as female and a wild-type line as male, the female chromosomes harboring altered centromeres are eliminated during the first divisions of embryogenesis due to a defect in chromosome segregation. This process triggers the formation of a haploid embryo containing only the male genome and the parental female cytoplasm, as well as some aneuploid embryos.

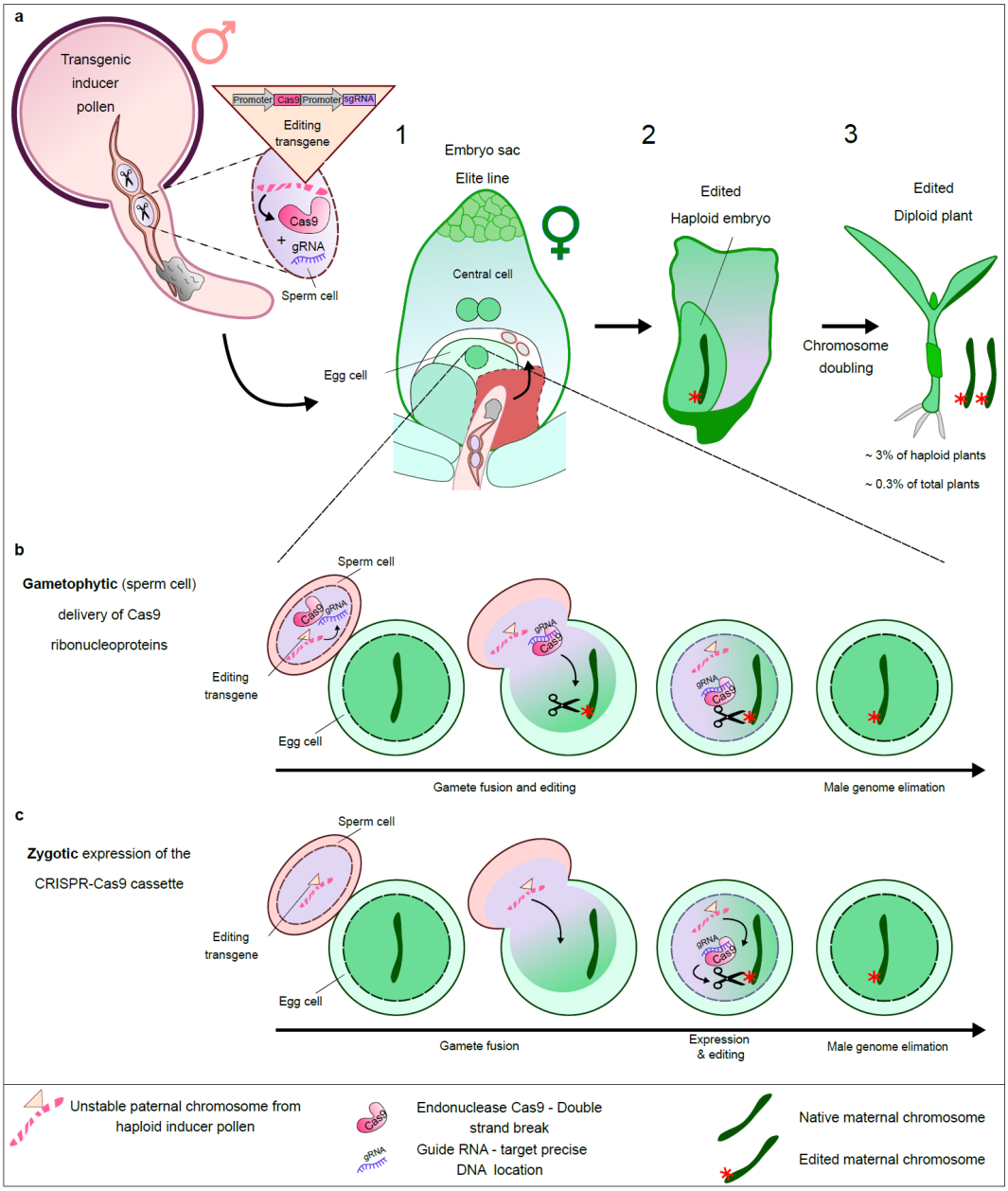

Fig. 3 Example of *trans*-editing using maize haploid inducer line. a, Schematic view of *trans* editing using *in planta* maize haploid inducer line: 1/ Pollen from haploid inducer line containing a CRISPR/Cas9 cassette (transgene needed

for genome editing) designed to target gene(s) of interest is used to pollinate maize elite line recalcitrant to genetic transformation; 2/ About 3% of the maternal haploid embryos are edited, without carrying the paternal transgene in their genome; 3/ Chromosome doubling (spontaneous or artificial) allows recovery of a homozygous (DH), edited diploid elite plant. Two non-exclusive scenarios allow *trans* editing depending on the promoter used to drive the Cas9: **b**, Sperm cell delivers directly the Cas9 protein and guide RNA (Ribonucleoprotein complex) in the egg cell, and/or **c**, the fragmented haploid inducer genome is transmitted and allows expression of Cas9 and guide RNA in zygotic tissues, before being eliminated.

Table

	Main methods used to produce haploid plantlets					
Plant	**anther or microspore culture**	**Ovary or Ovule culture**	**Irradiated pollen**	**interspecific cross**	***in planta* haploid inducer line**	**Ref**
maize					**x**	17,66
wheat	**x**			**x**	**v**	4,8,10,64,65
barley	**x**			**x**		4,8,10
ryegrass	**x**					4,72
triticale	**x**			**x**		4,8
flax	**x**					4
rape plant	**x**					4,8
melon			**x**			73
cucumber		**x**	**x**			11,73
sugar beet		**x**				4,8,10,11
chilli pepper / sweet pepper	**x**					10
eggplant	**x**					11
onion		**x**				4,8,11
carrot	**x**					8,10
asparagus	**x**					4
cauliflower / cabbages / broccoli	**x**					4,10
potato	**x**				**x**	4,8,11
sorghum					**v**	74,75
rice	**x**				**v**	4,8,10,63
sunflower*						
tomato*						
soybean*	**x**					11

Table 1. Example of crop plants and vegetables in which haploid plantlet production is used in breeding, together with main haploid induction system used. This table is not exhaustive, but illustrates main methods to

produce haploid plantlets. * Important crop lacking efficient haploid induction system. **V** *in-planta* haploid inducer lines recently available (from translation of maize system for rice and wheat, and by discovery of a new inducer line for sorghum).

Box 1. Glossary

Haploid embryos/plants: Plants having only a single set of each homologous chromosome (*i.e.* the gametic chromosome number, "n") in their somatic cells. Generally somatic cells are diploid (2n) with a set of chromosomes from the father and a set of chromosomes from the mother and haploid cells are only found in the male (pollen) and female (embryo sac) gametophytes.

Haploidization: Obtaining a haploid (n) cell or organism from a diploid (2n) one.

Haploid inducer line: Plant line having the property to trigger the *in planta* production of haploid embryos/plantlets after a conventional intraspecific cross with a line of interest. The uniparental haploid progeny lacks the haploid inducer line genome.

Aneuploid: Aneuploid organisms or cells have a numerical change in part of the chromosome set: they have a chromosome number either greater or smaller than that of the wild type. Certain authors extend the notion to large chromosomal deletions and insertions.

Mixoploid: An organism having an unequal number of chromosomes in adjacent cells or tissues.

Hetero-fertilization: Fertilization in which the egg cell and central cell are fertilized by sperm cells from different pollen grains.

'Bulbosum' method: After a successful fertilization between cultivated barley (*Hordeum vulgare*) and a wild relative (*Hordeum bulbosum*), uniparental genome elimination of *Hordeum bulbosum* is observed leading to a haploid barley plantlet after embryo rescue.

Parthenogenesis: Form of asexual reproduction which leads to the development of an organism containing only maternal genetic information without fertilization.

Gynogenesis: Form of parthenogenesis in which the egg cell development needs to be induced by sperm cells. In gynogenesis, the sperm serves to trigger embryogenesis but paternal genetic information is not found in the somatic part of organism.