Haploid Embryos: Being Like Mommy or Like Daddy?

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ABSTRACT:

In planta haploid embryo induction is a powerful plant breeding tool, but is limited to few crops. Two studies reported seed-based haploid systems producing paternal haploid embryos by engineering CENTROMERIC-HISTONE3. Along with recent translation of maize maternal haploid induction ability in wheat and rice, it extends haploid inducer lines collection.

Background

Haploid embryos contain only one set of parental chromosomes (n), instead of the classical situation with two sets of chromosomes (2n), one from the mother and one from the father. Nevertheless, haploid embryos and their subsequent haploid plantlets represent the basis of the Doubled Haploid (DH) technology which is a significant plant breeding tool [1,2]. DH technology could be simply summarized by: (i) the production of haploid embryos, and (ii) duplication (copy-paste) of the haploid genome to recover normal ploidy state. DH technology allows efficient plant breeding cycles, mainly by shortening the time for creating fixed genetic material (inbred line), since only two generations are needed to obtain homozygous plants, instead of six or more generations when using conventional crosses [1,2]. Thus, DH pipelines can offer rapid evaluation of phenotypic traits in

homogenous progeny, and the production of haploid embryos is central to this process. Although *in vitro* methods are most widely used to produce haploid embryos starting from the maternal or paternal germline (gametes or their precursors), they are costly and genotype-dependent [1]. The use of haploid inducer lines represents an attractive way to induce haploid embryos entirely *in planta*. These *in planta* methods produce haploid embryos within seeds, circumventing a major bottleneck of both *in vitro* and wide-hybridization methods, namely embryo rescue. A simple intraspecific cross between a plant of interest and a haploid inducer line allows the production of viable seeds, among which a certain proportion have haploid embryos (**Figure 1**). The uniparental haploid embryos lack the haploid inducer genome, making either paternal- or maternal haploid (**Figure 1**). Two main types of *in planta* haploid inducer lines were reported: (i) the CENH3-based induction system based on engineering of Centromeric Histone 3 (CENH3) (**Figure 1A**), and (ii) the maternal maize haploid induction ability which is based on mutation of the *MATRILINEAL (MATL) / NOT LIKE DAD (NLD) / PHOSPHOLIPASE-A (PLA)* gene (**Figure 1B**) [1–5]. Few additional haploid inducer lines were reported in sorghum and potato for example, but the molecular bases remain unknown [1].

A long-awaited discovery: efficient CENH3-based haploid induction in crops

The CENH3-based haploid induction system was born in 2010 thanks to a discovery using the model plant *Arabidopsis thaliana* [6]. Although this pioneering work has held great potential for plant breeders, efforts to translate this technology to crops have been hindered by the low haploid induction rate [1]. However, by engineering CENH3 genes in both maize and wheat crops, two recent studies reported paternal haploid induction with a rather high haploid induction rate (~5-8%) (**Figure 1A**) [7,8]. Wang *et al.* [7] initially failed to translate *Arabidopsis* CENH3-based haploid induction system to maize, but then surprisingly identified a new and simple way to induce haploid embryos. Crossing a female maize line heterozygous for the *cenh3* null mutation (*cenh3/*WT) with wild-type pollen led to ~5% of seeds with haploid embryos [7]. In wheat the situation is more complex since there are two *CENH3* genes (α and β), and because bread wheat is a hexaploid crop with three sub-genomes (named A, B and D), making a total of 12 *CENH3* alleles. Thanks to huge efforts in wheat transformation (more than 1150 events screened!) and rigorous analyses of gene editing events, Lv *et al*. were able to isolate a specific mutagenic scenario that led to haploid embryo induction [8]. They found that a specific mutation type on *CENH3α-A*, called restored frameshift (RFS), coupled with knockout alleles for both *CENH3α-B* and *CENH3α-D* triggered haploid induction (the 6 *CENH3β* alleles were left at wild-type state). The RFS consists of a wild-type protein sequence with the replacement of few amino acids from the N-terminal domain by unrelated ones [8]. They demonstrated the RFS on *CENH3α-A* gene is critical for haploid induction but, unexpectedly, the haploid inducer lines heterozygous for *CENH3α-A* RFS (RFS/WT) outperformed the homozygous ones (RFS/RFS) [8]. To sum-up, good paternal haploid induction systems based on CENH3 engineering were obtained in both maize and wheat (**Figure 1A**). Of note, maternal haploid embryos were reported at a low rate (0.5%) using the maize CENH3-based induction system [7], although the maternal origin was not double-checked.

What can we learn from maize and wheat CENH3-based haploid inducer lines?

The novelty brought by these two studies is the importance of heterozygosity to induce haploid embryos, indicating that one copy of the CENH3 wild-type allele is required to obtain the highest haploid induction rates [7,8]. However, the wheat situation differs from what has been reported in maize, since the CENH3α-A allele in a "knock-out/wild-type" configuration does not induce haploid embryos, but needs to be heterozygous in a "RFS/wild-type" state [8]. This contradictory situation could lie in the fact that the "RFS/wild-type" wheat haploid inducer line still has three copies of CNEH3β gene in a wild-type state, knowing that wheat has a complex centromere organization with the two versions of CENH3 (α and β) localizing in complementary centromeric domains.

Interestingly, Wang *et al*. [8] took advantage of the relative large size of maize kernels to genotype endosperm from seeds with haploid embryos. They were able to deduce that haploid

induction occurred when the embryo sacs (and thus egg cells) carried the *cenh3* null mutation [8]. The proposed working model is that these *cenh3* null embryo sacs have a diluted quantity of the wild-type CENH3 protein leading to defective centromeres. Indeed, just before meiosis, the diploid megaspore mother cell is heterozygous in a *cenh3* null/WT state. After meiosis, half of the resulting megaspores have the *cenh3* null allele in their haploid genome, but should have nevertheless inherited wild-type CENH3 protein in their centrosomes. This initial CENH3 protein pool is then diluted by the three post-meiotic cell divisions, creating egg cells with defective centromeres. Thus, when these "CENH3 diluted" egg cells are fertilized by wild-type sperm cells, it could lead to postzygotic maternal genome incompatibility that generates paternal haploid embryos [7]. A similar scenario could be envisioned in the wheat CENH3 engineered system, but with RFS allele instead of *cenh3* null allele. Nevertheless, the mode of action of wheat RFS alleles remains to be investigated. Since the RFS alters putative histone post-translational modification sites, it can be speculated that these CENH3 modifications have an important role in centromere stability. Experimental testing of RFS mutation on diploid plants with a single-copy *CENH3* gene should also help to clarify the situation.

Maize and wheat now have many ways to induce haploid embryos and multiple breeding applications

The discovery of efficient CENH3-based paternal haploid inducer lines places maize and wheat in a unique situation because different methods are now available to induce both maternal and paternal haploid embryos. The two historical wheat haploidization methods are: interspecific crosses, and *in vitro* culture of male haploid cells, the latter method being also reported for maize [1]. Furthermore, wheat, together with rice, have recently benefited from successful translation of maize *in planta* haploid induction system [9,10] (**Figure 1B**). Whereas all these methods could be used to speed-up the production of fixed material (DH lines), the CENH3-based haploid induction system offers the possibility to obtain an unusual cellular arrangement in which the paternal genome is surrounded by maternal cytoplasm. This allows cytoplasm swapping in a single-step, simplifying hybrid crop management [1].

Finally, haploid inducer lines have recently been elegantly used to deliver genome edit machinery to crop genotypes that are recalcitrant to genetic transformation [11,12]. This *trans* editing system allows the production of genome edited elite cultivars that are both transgene-free plants (because CRISPR-cassette is unstable like the haploid inducer genome) and homozygous (because they are DH plants).

To sum-up, *in planta* haploid induction systems allow not only convenient production of haploid embryos within viable seeds, but represent the entry point for increasing numbers of breeding applications, not forgetting that haploid inducer lines also represent entry points to better characterize plant reproduction.

Figure 1. Two different kind of haploid inducer lines to trigger either paternal (A) or maternal (B) haploid embryos through seeds in wheat.

(**A**) Engineering of CENH3 allows creation of haploid inducer lines which are able to generate viable seeds containing embryos with a paternal haploid genome. These CENH3-based haploid inducer lines trigger paternal haploid embryos when used as a female parent in outcrossing with wild-type pollen. (**B**) Translation of maize haploid induction system to wheat allows induction of viable seeds containing embryos with a maternal haploid genome. In this situation, the haploid inducer line is used as a male and outcrosses with wild-type female. In all cases, the haploid embryos lack the genome from the haploid inducer line. The main breeding advantages of these *in planta* inducer lines are (i) shortening the creation of inbred lines, (ii) shortening the creation cytoplasm swapping (for CENH3-based haploid inducer lines only) and (iii) allowing *trans* editing of non-inducer genome. This figure was created using BioRender (https://biorender.com/).

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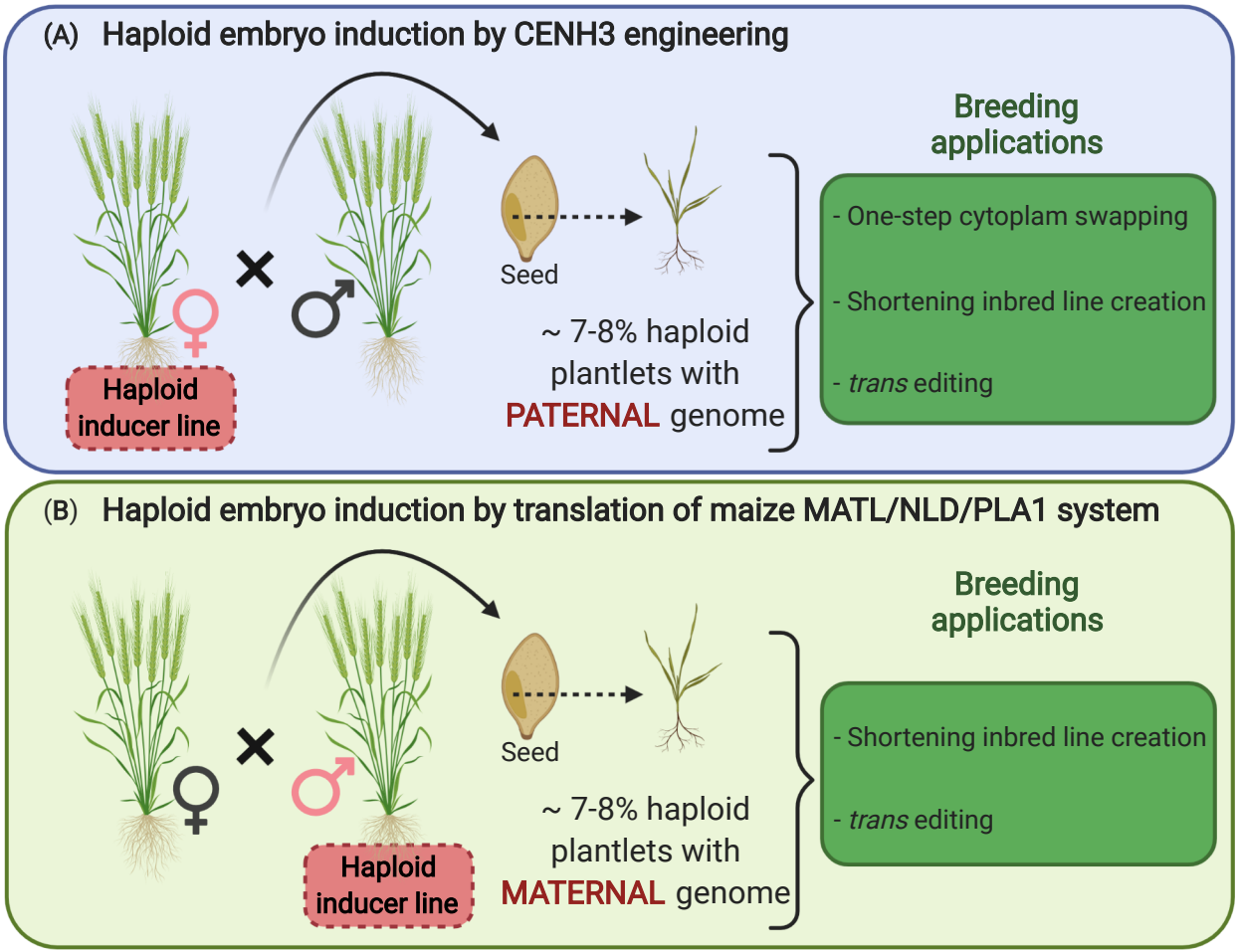
(A) Haploid embryo induction by CENH3 engineering
Haploid inducer line
Seed
~ 7-8% haploid plantlets with PATERNAL genome
Breeding applications
- One-step cytoplam swapping
- Shortening inbred line creation
- trans editing
(B) Haploid embryo induction by translation of maize MATL/NLD/PLA1 system
Haploid inducer line
Seed
~ 7-8% haploid plantlets with MATERNAL genome
Breeding applications
- Shortening inbred line creation
- trans editing