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Modification of centromere structure: a promising approach for haploid line production in plant breeding

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Abstract: Breeding based on doubled-haploid approaches has recently become a common tool for accelerating crop improvement in many plant species. However, many plant species do not have a reliable method for haploid induction. A promising new approach involving centromere engineering has recently been proposed to overcome this limitation. Here we provide a perspective of this novel method for the production of haploid plants, which was originally described in the model plant species *Arabidopsis thaliana*. Centromeres, known to be critical for the accurate distribution of chromosomes in every cell division, have now become a novel target for crop improvement by enabling doubled-haploid technologies.

Key words: CENP-A, CenH3, histone, GFP, kinetochore, agronomy

1. Introduction

The breeding of many plant species requires the development of fully homozygous (i.e. inbred) lines. The classical approach starts with several generations of self-pollination until an acceptable level of homozygosity has been reached, depending on the plant species under investigation. This is a slow process due to the many (usually 8 to 10) generations required to reach nearly complete homozygosity. In some species, it is possible to induce haploid (1n) sporophyte individuals from a highly heterozygous parent plant. The haploid can then be induced to double its chromosome number, resulting in a completely homozygous sporophyte plant with the normal chromosome number (2n). This plant, termed a doubled-haploid, is genetically inbred, but can be derived much more rapidly.

Unfortunately, many plant species do not have an established system for developing doubled-haploid lines. Production of haploid plants through centromere engineering is one promising method to overcome this barrier, and is one of the most promising modern plant breeding approaches in recent years. Although this approach has been received with great excitement in the plant breeding community (Chan, 2010; Copenhaver and Preuss, 2010; Ravi and Chan, 2013; Comai, 2014), currently there is no example of haploids developed through centromere engineering in any crop plant. However, the centromeric histone H3 gene is now functionally characterized in many agronomically important plant species, which may provide new possibilities for using this approach in some of these species.

2. Classical methods for haploid plant production

Classical methods for haploid plant production generally range from sporadic in vivo occurrence to experimentally in vitro induction of haploid plants (Dunwell, 2010). Here we do not intend to cover the literature in detail, but instead refer the reader to some excellent recent reviews (Forster et al., 2007; Dunwell, 2010).

A common method for the production of haploid lines relies on the regeneration of microspores through anther culture (Taşkın et al., 2013a, 2013b; Baktemur et al., 2014; Olszewska et al., 2014). A second common way is through interspecific hybridization, in which a domesticated crop is crossed to an inducer line to generate haploid offspring (Kasha and Kao, 1970; Barclay, 1975; Savaskan et al., 1997; Chaudhary et al., 2005). Such inducer lines have been identified for a limited number of species (Forster et al., 2007). Neither of these common haploid induction methods is universally applicable to all major agricultural crops. Therefore, there is a need to develop an easy and effective way of haploid production for many plant species.

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3. The centromere as a new target for plant improvement

Every eukaryotic chromosome must have a centromere for proper cell division during mitosis and meiosis. The centromere serves as the spindle fiber attachment region known as the kinetochore (Figure 1). The packaging of DNA within eukaryotic nuclei includes a canonical histone H3 protein, which contributes to the formation of the canonical nucleosome. While the canonical nucleosome is typical in most eukaryotic chromatin, the nucleosomes in eukaryotic centromeres are known to also include a centromere-specific histone H3 variant, called CENH3 (Figure 2). CENH3 defines the functional centromeres upon which the kinetochore is established (Perpelescu and Fukagawa, 2011). Any alteration of the centromere structural components could have devastating effects on the fidelity of cell division and overall organismal viability. Incorporation of a centromere-specific histone H3 variant into the nucleosomes is a key determinant for specifying centromere identity. CENH3 is a universal protein present in all eukaryotes, including agronomically important crops.

There are some key features to the CENH3 protein that are highly conserved across taxa. There are 2 critical domains: the N-terminal tail and the C-terminal histone fold domains (Figure 2). A longer and divergent amino terminal domain distinguishes CENH3 proteins from canonical H3 proteins (Henikoff et al., 2001). Meanwhile, the C-terminal histone fold domain is highly conserved among plant CENH3 proteins (Tek et al., 2014).

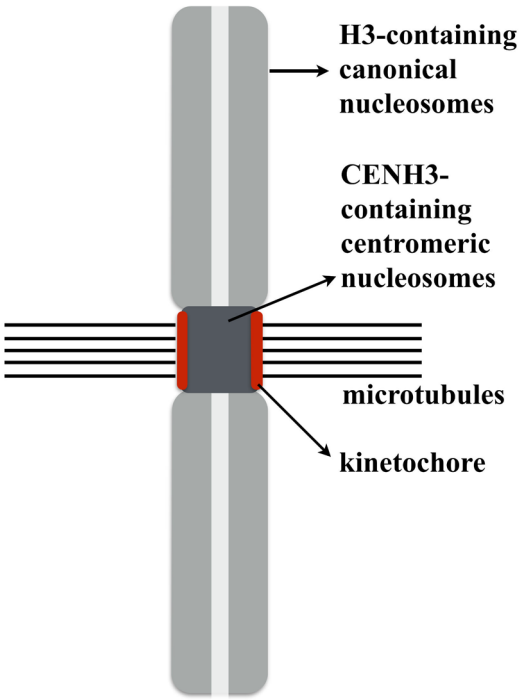

Figure 1. A schematic representation of a eukaryotic metaphase chromosome and centromere structure.

4. Modification of Arabidopsis centromere structure for haploid line production

Using *Arabidopsis thaliana* as a model system, Ravi and Chan (2010) developed a novel approach for haploid production that specifically modified the function of the CENH3 protein (Figure 3). First, they obtained knockout CENH3 mutant lines, which were lethal in the homozygous mutant state. Second, they rescued the lethal effect of the CENH3 mutation by transforming a modified CENH3 transgene, in which the N-terminus was fused to a green fluorescent protein (GFP) domain (Figure 2).

In fact, 2 types of CENH3 transgenes were successful for inducing haploids by genome elimination (Ravi and Chan, 2010). These 2 constructs are shown as GFP-CENH3 and GFP-tailswap in Figure 2. The GFP-CENH3 construct simply fused GFP to the CENH3 N-terminus, while the GFP-tailswap construct included GFP and also replaced the amino-terminal tail domain of CENH3 with a canonical H3 domain. It is not known if other modifications of CENH3 with or without GFP tagging could also lead to uniparental genome elimination. Whether these modifications and recombinant proteins are sufficient for induction of haploid lines in other crops remains to be tested.

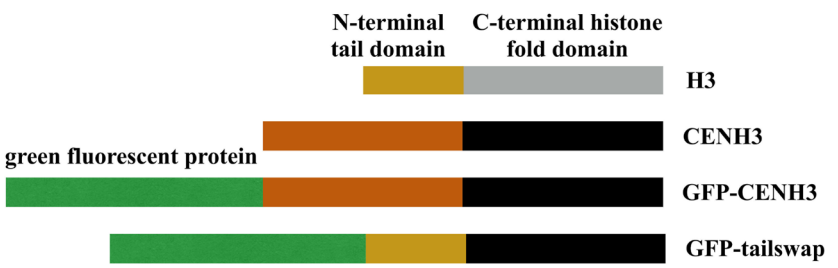

Figure 2. Canonical histone H3, centromere-specific histone H3 (CENH3) and modified recombinant CENH3 constructs. Canonical histone H3 is a highly conserved protein within eukaryotes.

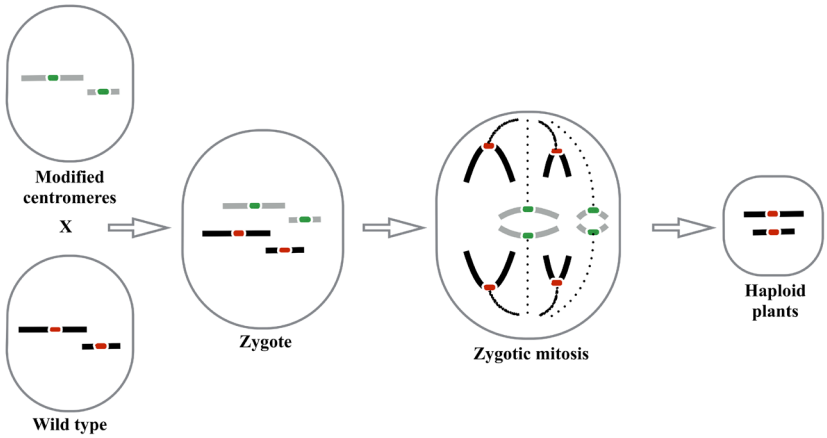

Figure 3. A diagrammatic representation of haploid production with modified centromeres. The figure is simplified and redrawn from Chan (2010).

5. Applicability of centromere engineering to agronomically important crops

The exact mechanism underlying the uniparental chromosome elimination is not known. However, zygote-specific CENH3 dynamics have been suggested to play an important role following the zygote formation (Chan, 2011). For example, centromeric loss of CENH3 protein has been suggested to lead to the *Hordeum bulbosum* genome elimination in early developmental stages of interspecific barley hybrids, driven by a mitosis-dependent process (Sanei et al., 2011).

In theory, it is possible to utilize centromere engineering to generate haploid lines in agronomically important crops. As an initial step, the CenH3 gene in the species of interest must be identified and functionally characterized with an exclusive localization at centromeres (Figure 4). Here both the endogenous CENH3 and the GFP-tagged CENH3 transgene are incorporated at the centromeres. So far, the CENH3 proteins have been identified in over a dozen of plant species with agronomical importance (Table 1). In these studies, a specific polyclonal antibody raised against the CENH3 corresponding to the species was used to functionally characterize the centromeres. Subsequently, the antibody was used to clone and determine the DNA sequences underlying the centromeres. Therefore, the availability of DNA and protein sequences of CenH3 genes provides a valuable initial resource in a diverse group of agronomically important crop species.

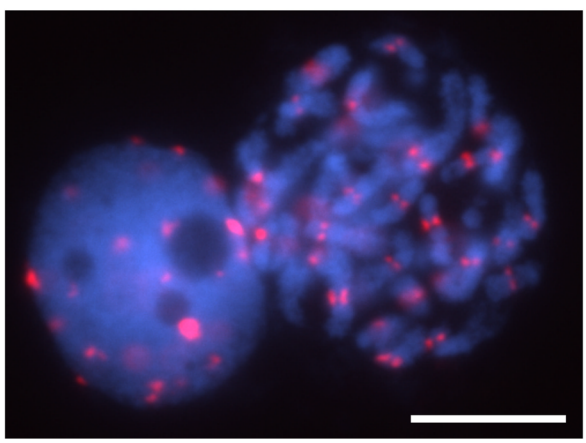

Figure 4. *Lotus japonicus* interphase (left) and metaphase (right) chromosomes tagged with GFP-LjCENH3. DAPI stained chromosomes are shown in blue. GFP tagged recombinant LjCENH3 is shown in red. Both endogenous and GFP-tagged LjCenH3 are expressed in this image. For details, see Tek et al. (2014). The scale bar is 10 µm.

Currently there are several groups attempting to generate haploid inducer lines in several plants (Table 2), using the principles described by the Ravi and Chan (2010). One should consider that Arabidopsis has only 5 chromosome pairs, which are relatively small. Conversely, most crop species have many more chromosome pairs, including many species with large chromosomes and variable levels of polyploidy. It may be more difficult to modify the behavior of larger numbers of chromosomes, as compared to the work demonstrated in Arabidopsis. Furthermore, Ravi and Chan (2010) reported a high rate of anueploidy, in addition to haploid formation, in Arabidopsis. One might imagine that the relative rate of aneuploid individuals may be increased in crop species with higher numbers of chromosomes. Therefore, while the concept of genome elimination by centromere engineering may translate to crop species, it is possible that there are additional details and complications that need to be addressed to organize the behavior of chromosomes in larger, more complex crop genomes.

It is unlikely that the list in Table 2 covers all of the ongoing projects in this area of research. Furthermore, it is likely that there will be more groups utilizing this

Table 1. CenH3 genes have been identified in several agronomically important crop species.

Common name	Species	CenH3 gene copy number*	Reference
Barley	*Hordeum vulgare*	2	Sanei et al., 2011
Carrot	*Daucus carota*	1	Dunemann et al., 2014
Chinese cabbage	*Brassica rapa*	1	Wang et al., 2011
Common bean	*Phaseolus vulgaris*	1	Iwata et al., 2013
Corn	Zea mays	1	Zhong et al., 2002
Cotton	*Gossypium hirsutum*	1	Luo et al., 2012
Garlic	*Allium sativum*	1	Nagaki et al., 2012
Onion	*Allium cepa*	1	Nagaki et al., 2012
Pea	*Pisum sativum*	2	Neumann et al., 2012
Potato	Solanum tuberosum	1	Gong et al., 2012
Rice	*Oryza sativa*	1	Nagaki et al., 2004
Soybean	*Glycine max*	1	Tek et al., 2010
Sugarcane	*Saccharum officinarum*	2	Nagaki and Murata, 2005
Tobacco	*Nicotiana tabacum*	2	Nagaki et al., 2009
Wheat	Triticum aestivum	3	Li et al., 2012

*In some species, the copy number of transcribed CenH3 gene is inconclusive.

technology in other plant species in the future. Nevertheless, this list provides evidence for the importance of this research and its potential impact on crop improvement. Hopefully, we will soon see promising results in some of the agronomically important plant species.

6. Conclusions and perspective

Centromere-mediated genome elimination is a fairly recent advancement in the production of haploid plant lines. Although it was initially discovered in Arabidopsis, the approach has received a tremendous amount of attention in a short period of time, due to its dramatic potential for applications in plant breeding. Here we briefly summarized the original work performed in Arabidopsis (Ravi and Chan, 2010) and cataloged a subset of studies that are pursuing this methodology in crop species. Because of this work, centromeres are no longer viewed simply as an epigenetic mark of fundamental importance, but also a potential site for important agricultural applications. The centromere DNA repeat-sequences and the protein-encoding sequences of CenH3 genes have already been identified in a taxonomically diverse group of plant species. These initial studies provide valuable resources for researchers pursuing the development of haploid inducer lines that will greatly aid in the breeding programs of agronomically important crops.

Table 2. Ongoing research projects attempting to produce haploid lines using centromere engineering.

Plant species	Principal investigator	Name of the project
Banana	Anne Britt University of California, Davis, USA Leena Tripathi International Institute of Tropical Agriculture (IITA), Uganda	BREAD: fast breeding for slow cycling crops: doubled haploids in Cassava and Banana/ Plantain
Barley	Jochen Kumlehn IPK, Gatersleben, Germany Andreas Houben IPK, Gatersleben, Germany	Production of haploids by means of uniparental genome elimination
Brachypodium	Eduardo Blumwald University of California, Davis, USA Christian Tobias USDA Agricultural Research Service, USA	Expanding the breeder's toolbox for perennial grasses
Cassava	Anne Britt University of California, Davis, USA Paul Chavarriaga International Center for Tropical Agriculture (CIAT), Colombia	BREAD: fast breeding for slow cycling crops: doubled haploids in cassava and banana/ plantain
Cotton	Allen Van Deynze University of California, Davis, USA	Development of a doubled haploid inducer resource in cotton
Lotus japonicus	Kiyotaka Nagaki Okayama University, Japan	Centromere engineering
Rice	Kiyotaka Nagaki Okayama University, Japan	Centromere engineering
Soybean	Robert Stupar University of Minnesota, USA	Development of a haploid- induction system for soybean
Sugarbeet	Jochen Kumlehn IPK, Gatersleben, Germany Andreas Houben IPK, Gatersleben, Germany	Production of haploids by means of uniparental genome elimination
Switchgrass	Eduardo Blumwald University of California, Davis, USA Christian Tobias USDA Agricultural Research Service, USA	Expanding the breeder's toolbox for perennial grasses
Tobacco	Kiyotaka Nagaki Okayama University, Japan	Centromere engineering
Tobacco	Jochen Kumlehn IPK, Gatersleben, Germany Andreas Houben IPK, Gatersleben, Germany	Production of haploids by means of uniparental genome elimination

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