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SHORT RESEARCH ARTICLE

Rapid creation of CENH3-mediated haploid induction lines using a cytosine base editor (CBE)

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ABSTRACT

- Haploid induction (HI) can create true-breeding lines in a single generation, which can significantly accelerates the breeding process. In recent years, scientists have developed a variety of new techniques to induce haploids through manipulation of CENH3, a variant of the centromere-specific histone H3. One alternative approach is based on CENH3 point mutations derived from EMS/TILLING, which is not lethal and yet is responsible for inducing haploids. However, most residues have been obtained by EMS mutagenesis over a long period of time.
- Recently, a new approach called 'base editing' was developed for plants. Here, we report a new method that uses a cytosine base editor (CBE) to create a point mutation of CENH3 as a haploid induction line, which substitutes adenine (A) for guanine (G).
- As proof of the extreme simplicity of this approach to create haploid-induced lines, we identified an L130F substitution within the histone fold domain in *Arabidopsis thaliana*. Subsequently, we tested the haploid-inducing potential of homozygous L130F plants by pollinating them with Col-0, and obtained 2.9% paternal haploid plants.
- In brief, our innovative technology provides a new perspective for the promotion of CENH3-mediated haploid induction in crops, and also provides a variety of options for breeders. Such conserved point mutations as L130F could be developed into a general instrument for haploid induction in a wide range of plant species. Extending these systems would represent a major advance over haploid production.

INTRODUCTION

In conventional breeding, high levels of homozygosity are produced through inbreeding, selfing for seven to nine generations, whereas this can be achieved in the first generation through double haploids (Dwivedi *et al.* 2015). In recent years, researchers have exploited a variety of genes to create haploid plants, such as *CENH3* (Lv *et al.* 2020; Wang *et al.* 2021), *MTL/PLA1/NLD* (Gilles *et al.* 2017; Kelliher *et al.* 2017; Liu *et al.* 2017), *DMP* (Zhong *et al.* 2019) and *PLD3* (Li *et al.* 2021). In 2010, breakthrough progress reported that haploid plants can be efficiently induced through the introduction of a single genetic alteration in the centromere histone H3 (CENH3) (Ravi & Chan 2010). Subsequent studies demonstrated that haploids can be induced using three biotechnological strategies to manipulate CENH3. First, a transgenic line of *GFP-tailswap* was constructed as either female or male (Ravi & Chan 2010; Kelliher *et al.* 2016). This line was created by replacing the N-terminal of endogenous CENH3 with the GFP-fused N-terminal of H3.3 (Wang *et al.* 2019). Crossing *GFP-tailswap* (female) with the wild type (male) produces a remarkable 25–45% paternal haploid progeny (Ravi & Chan 2010). However, 12 years since the first publication, there has onmly been one reported instance in maize, at a relatively low HI (0.065–0.86%) (Kelliher *et al.* 2016), suggesting that this method may not be suitable for all plants. The second method revealed that various single amino acid substitutions in the CENH3 α-N-helix or CATD (chromosome targeting domain) can also induce haploids in *Arabidopsis* (Kuppu *et al.* 2015; Kuppu *et al.* 2020). In addition, the applicability of the point mutation method to produce haploids in many species was summarized in a review by Kalinowska *et al.* (2019). Finally, a new study shows that CRISPR/Cas9-mediated deletions/recombinations in the α-N-helix or N-terminal can induce haploids in wheat (Lv *et al.* 2020). It should be noted that this *+/cenh3* mutant is not a completely functional loss mutant, but an N-terminal deletion/recombination, while the C-terminal sequence is invariable. In addition, the heterozygotes triggered higher haploid induction rates than homozygotes (Lv *et al.* 2020).

Until now, most studies have focused on the second approach, which creates point mutations in the conserved domain of CENH3. However, most of the point mutations were derived from ethyl methane sulfonate (EMS) or TILLING approaches, which are costly and time-consuming processes. In this study, we introduce a cytosine base editing system (CBE), which enables direct and irreversible conversion of one target base into another without double-strand breaks or a donor template (Komor *et al.* 2016; Lu & Zhu 2017). This method has been used for the creation of carrot CENH3 point mutants and proved to be highly feasible (Meyer *et al.* 2022). Our new research might provide additional options for the development of CENH3-mediated haploid induction technology.

MATERIAL AND METHODS

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used as a wild-type control in this study, and the plants were grown in soil with white light of 100 μmol $m^{-2}·s^{-1}$ and a 16-h light/8-h dark cycle at 22 °C.

Plasmid construction and plant transformation

The gene *AtCENH3*(AT1G01370) was selected for editing using the CBE system. The plasmid of CBE, namely pCSGAPO01, was a kind gift from Professor Jiankang Zhu. First, we amplified the U6 promoter from *A. thaliana* genomic DNA to replace the local OsU6 promoter. According to the protocol for pCSGAPO01, a pair of primers was designed to form a short 20-bp DNA fragment with the exact overhangs: forward primer 5′-TGTGTAGCTCTTGTTGCTCTTCAAG-3′, reverse primer 5′-AAACCTTGAAGAGCAACAAGAGCTCA-3′. We expected mutational cytosine to be in the fifth place because the efficient 'deamination window' (C → T) is typically from positions 4 to 8 within the protospacer. Two *Bsa*I endonuclease sites were incorporated to flank the protospacer of gRNA and enable one-step construction of the base-editing plasmid for *Agrobacterium*-mediated transformation (Lu & Zhu 2017). The resulting vectors were transformed into wild-type Col plants using the floral dip method.

Extraction of DNA, genotyping and sequence analysis

Genomic DNA was prepared using a cetyl trimethylammonium bromide (CTAB) protocol. The PCR products were sent to Sangon Biotech (Shanghai, China) for sequencing, and the endonuclease *Hind III* was used for identification. The sequencing primers were as follows: forward primer ATCGTCAGTGATGTGGAAAT, and reverse primer ACTGAAAAAT GTCTTCTTTT.

Chromosome spreads

Chromosome analysis was modified as previously described (Wang *et al.* 2021). Young leaves were collected from individual plants, incubated in a chamber with 8-hydroxyquinoline for 2 h, and fixed with stationary liquid (formic acid:acetic acid = 3:1). Young leaves were cut with a razor blade and digested in an enzyme solution (mixture of pectolyase and cellulase) at 37 °C for 30 min. The leaf sections were washed in ethanol and then transferred to the stationary liquid. A small pestle was used to crush the leaf, and a moderate amount of the cell suspension was dropped onto microscope slides. We used the *Arabidopsis* centromeric tandem-repeat sequence cen180 (May *et al.* 2005; listed below) to label digoxin as a probe for FISH (fluorescence *in situ* hybridization). Slides were imaged on an Olympus BX63 epifluorescence microscope (Olympus, Japan). Primers cen180 forward: ACCATCAAAGC CTTGAGAAGCA; cen180 reverse: CCGTATGAGTCTTTGT CTTTGTATCTTCT were used.

RESULTS AND DISCUSSION

To date, 20 substitution alleles induce haploids on outcrossing wild-type *A. thaliana* at frequencies ranging from 1.1% to 44.0% (Karimi-Ashtiyani *et al.* 2015; Kuppu *et al.* 2015; Kuppu *et al.* 2020). To determine which point mutations are easiest to

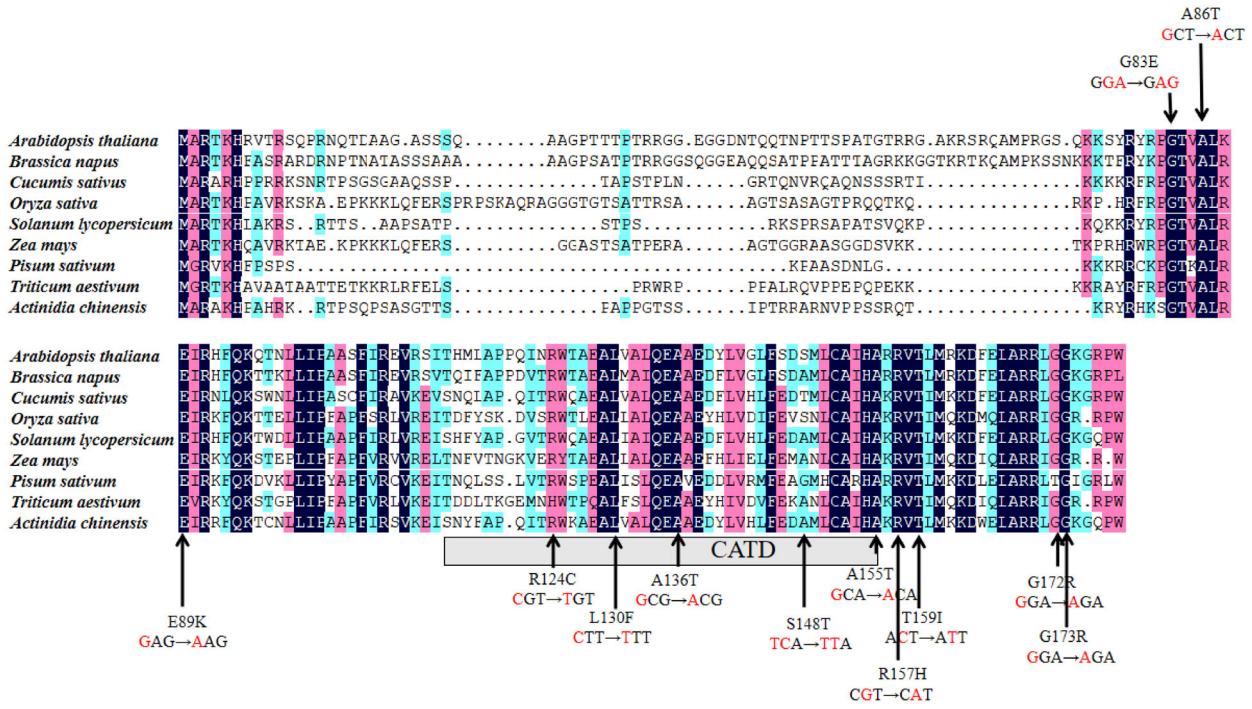

Fig. 1. Multiple protein sequence alignment of CENH3. The red arrow indicates the point mutations that results in the induction of haploids at a frequency of 4.8% or higher.

manipulate and are conserved among other plant species, we analysed 12 substitutions that induce haploids among >4.8% of progeny, based on previous studies (Karimi-Ashtiyani *et al.* 2015; Kuppu *et al.* 2015; Kuppu *et al.* 2020). We found that most substitutions are conserved in several plant species, suggesting their potential applicability to other crops (Fig. 1). Next, we found that the positions R124C, L130F, T159I (C to T) and A86T, E89K, A136T, A155T, R157H, G172R and G173R (G to A) could likely apply to the CBE system because of the potential C•G to T•A substitution (Fig. 1). In addition, there was no A•T to G•C conversion in these 12 substitutions, so the ABE system is not suitable for CENH3-mediated haploid induction. A CBE system has been reported previously (Lu & Zhu 2017); in this CBE system, the efficient 'deamination window' (C converted to T) is typically from positions 4 to 8 within the protospacer (Lu & Zhu 2017). Based on this limit, we found that only L130F was a potential target site, which is highly conserved in higher plants. Accordingly, one CRISPR/Cas9 target site for *AtCENH3-L130F* was designed for the intended editing (Fig. 2a). In addition, the L130F of *CENH3* was selected as a potential mutation site because the mutated base sequence (AAGCTTTT) can be digested by *Hind* III endonuclease to benefit mutant screening (Fig. 2a). The T_1 generations were selected as hygromycin resistance, which we then verified using the *Hind* III endonuclease to *AtCENH3-L130F* alleles from 92 transgenic seedlings. We found that five plants were heterozygous at the site of L130F (Table 1). The frequency of conversion of cytidine (C) to thymine (T) was 5.43%, as previously reported (Lu & Zhu 2017). Homozygous mutants were selected from the T_1 segregated progeny (Fig. 2b, 4#). The phenotype of the homozygous mutants was not significantly different from that of wild-type plants. According to a previous study, independently derived transgenic lines display similar levels of HI (Kuppu *et al.* 2020); therefore, we chose only a single transgenic line, representing L130F as females, crossed with wild-type plants. We first eliminated most of the non-haploid plants according to the plant size of the hybrid progeny and then used FISH to count chromosomes of the candidate plants (number of haploid chromosomes is only half of a normal diploid) and, finally, we obtained about 2.9% of haploid progeny (Fig. 3, Table 2). The haploid plants were shorter in height, had smaller organs, many infertile siliques and half number of chromosomes (Fig. 3). However, the reciprocal cross and self-cross did not generate haploid plants (Table 2).

Table 1. Genotypic analysis of independently derived transgenic line.

line (T_1)	total seeds	genotype at endogenous CENH3 (T_1)			mutation rate (%)
		WT (+/+)	het (+/−)	homo (−/−)	
L130F	94	89	5	0	5.43

In this study, we combined CBE- with CENH3-mediated haploid induction technology to induce *in vivo* paternal haploid formation in *A. thaliana*. The technology of this base editor would be more convenient than EMS-induced methods to create point mutations In particular, the CBE system can perform site-directed mutagenesis of target bases, which greatly reduces the time required to obtain a point mutation. In addition, the CBE system can also screen offspring that do not contain genetically modified components (GM-free), which provides a variety of options for future agricultural applications. Our results show that the CBE system can also create stable haploid plants, similar to EMS, and has a similar level of HI. Our study aims to provide breeders with more options for haploid induction. However, only L130F was successfully created by this system because of the limitations of the PAM (canonical NGG motif). In future, other non-canonical PAM motifs, including NG, AGC and TGA PAM, together with NGG PAM, in principle will enable targeting of other point mutations. In addition to the CBE, the adenine base editor (ABE) can also be used to create point mutations, which substitute adenine (A) for guanine (G) (Hua *et al.* 2020). However,

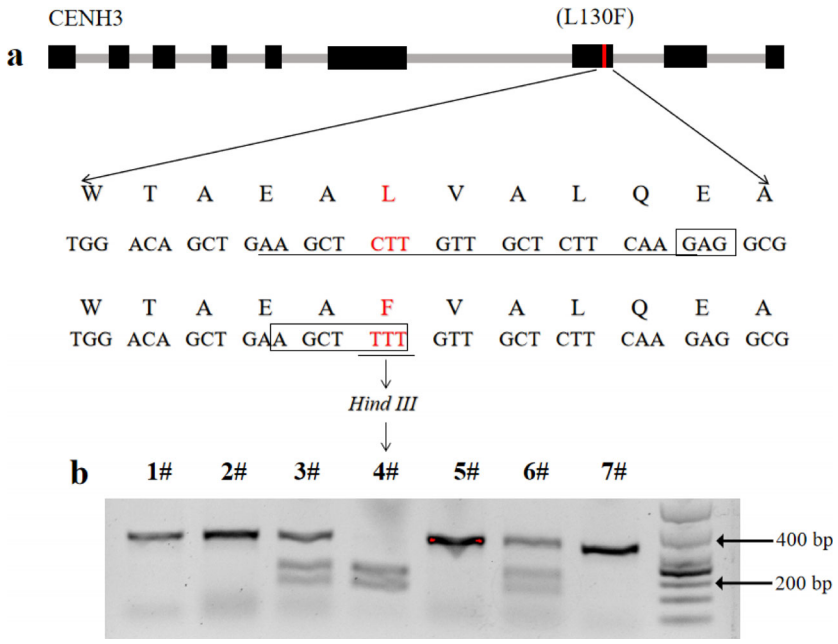

Fig. 2. Created haploid progeny to targeted base in *AtCENH3* using the cytosine base editor (CBE) system. (a) Base editing of cytosine residues in the base editor window at the CENH3 site L130F locus. (b) PCR products were digested with *Hind III*. 1#, 2#, 5# and 7# indicate Col-0; 3# and 6# indicate heterozygous progeny; 4# indicates homozygous plants.

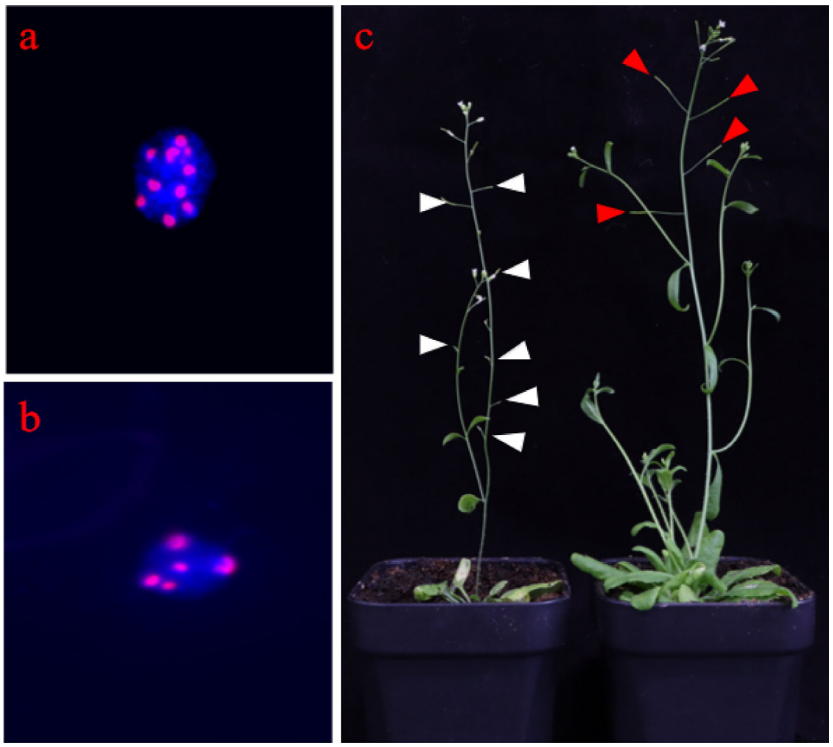

Fig. 3. Detection of haploid plants of *Arabidopsis thaliana*. (a and b) Chromosome number in interphase phase of haploid (b) and diploid (a) plants of the F_1 generation. (c) Haploid plants (left) have shorter stature than diploids plants (right). White arrows indicate haploid sterile pods; red arrows indicate normal fertile pods of diploids.

Table 2. *CENH3-L130F* results in haploid induction on outcrossing.

	ploidy			
cross (♀ × ♂)	haploid	other	total	HI rate (%)
L130F × Col-0	14	469	483	2.9
Col-0 × L130F	0	462	462	0
L130F × L130F	0	344	344	0

we did not find any single mutation with high HI efficiency suitable for the ABE system (Fig. 3). In addition to the ABE and CBE systems, the Prime Editor (PE) system has more powerful functions that can effectively convert any base without a double-strand break (DSB) or donor DNA (Hua *et al.* 2021).

AUTHOR CONTRIBUTIONS

SW conceived the project and wrote the manuscript, SW and KO performed the experiments.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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