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Brief Communication

Direct modification of multiple gene homoeologs in *Brassica oleracea* and *Brassica napus* using doubled haploid inducer-mediated genome-editing system

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The diploid *Brassica oleracea* and allopolyploidy *Brassica napus* are predominant members of commonly consumed vegetables and plant oil, respectively. *B. oleracea* vegetables mainly include Broccoli, Cauliflower, Cabbage, Brussels sprouts, and Kohlrabi. The complex genome structure and gene function redundancy are the main obstacles for gene stacking through the traditional cross-breeding approach. To solve this problem, high-efficiency CRISPR/Cas9 genome-editing technologies have been established (Li *et al*., 2018). However, an open question is that most of these established approaches employed *agrobacterium*-mediated T-DNA transformation to deliver CRISRP/Cas9 components into plant cells, which would unavoidably introduce exogenous large DNA fragments. Moreover, *Agrobacterium*-based strategy deeply relies on transformation efficiency of the recipient genotype, which extremely restricts rapid application of CRISRP/Cas9 in the majority of elite commercial verities.

Utilization of intraspecific crosses to trigger maternal haploidization event has been extensively applied in maize breeding programs. Stock 6 is the first reported maize haploid inducer (HI), and its causative mechanism has been recently uncovered that a 4-bp insertion occurred in a pollen-specific phospholipase gene, namely *MATRILINEAL* (*MATL*, also termed as *ZmPLA1* and *NOT LIKE DAD*) (Gilles *et al*., 2017; Kelliher *et al*., 2017; Liu *et al*., 2017). However, *MATL* does not have orthologue in dicot plants. Fu *et al*. reported two maternal doubled haploid inducers (DHIs) in *Brassica* crops, Y3560 and Y3380, that were generated by artificial synthesis of *Brassica* alloctaploid (AAAACCCC, 2n = 8X = 76) (Fu *et al*., 2018).

Given that post-fertilization elimination and fragmentation of male chromosomes, HI has been employed to deliver CRISPR/Cas genome-editing reagents for directly genome editing of elite breeding materials in maize, wheat, and *Arabidopsis* (Budhagatapalli *et al*., 2020; Kelliher *et al*., 2019; Wang *et al*., 2019). However, the application of HI-assisted breeding technology in other plant species is limited and the potential value for induction of genome editing of *Brassica* crops by CRISPR/Cas9-engineered DHI is still unrevealed.

To assess the efficacy for generating targeted mutagenesis of multiple homoeologs, the expression cassettes of a CRISPR/Cas9 vector harboring two sgRNAs were designed for targeting three and two homoeologous copies of *FAD2* gene in *B. napus* and *B. oleracea*, respectively (Figure 1a). After *agrobacterium*-mediated genetic transformation in Y3560 and Y3380 (Figure 1b), 195 plants were regenerated from 150 Y3380 seeds, and 8 of them were transgenic plants, whereas only 20 plants were regenerated from 150 Y3560 seeds and 4 of them were transgenic plants. Those transgenic plants were further evaluated by RT-PCR analysis on the expression of *Cas9*. The results showed that three plants from Y3380 (Y3380-15, Y3380-62, and Y3380-112) were detected with expression of *Cas9* gene (Figure 1c). Targeted mutation was achieved in Y3380 transgenic plants (Figure 1d). These data suggested that Y3560 is more recalcitrant to transformation compared with Y3380.

The ploidy level was determined in T_1 generation by flow cytometry. Four out of five Y3380-15 T_1 plants, five out of nine Y3380-62 T_1 plants, two out of seven Y3380-112 T_1 plants are octaploid/tetraploid mosaics (Figure 1e and 1f), indicating that a certain percentage of tetraploid cells may be separated from octaploid Y3380 donor material during tissue culture process. Y3380-15 T_0 plant was crossed with breeding material X1 of *B. oleracea* and ZS7 of *B. napus*. Nine *B. oleracea* plants and 34 *B. napus* plants were obtained after pollinating with the putative CRISPR/Cas9-engineered DHI line Y3380-15. These offspring were further determined by PCR using CRISPR/Cas9 vector-specific primers. No CRISPR/Cas9 transgene vector fragment was present in all nine *B. oleracea* offspring plants and ten out of 34 *B. napus* offspring plants (Figure 1g).

To further verify whether those transgene-free plants were purely derived from their female parents, Bnapus 50K Illumina Infinium SNP array was employed for genotyping analysis of the genetic similarity. Based on the screening of *B. oleracea* X1 female and Y3380 male parent genome, 2136 female parent-specific SNP were identified, and over 99.9% of them were

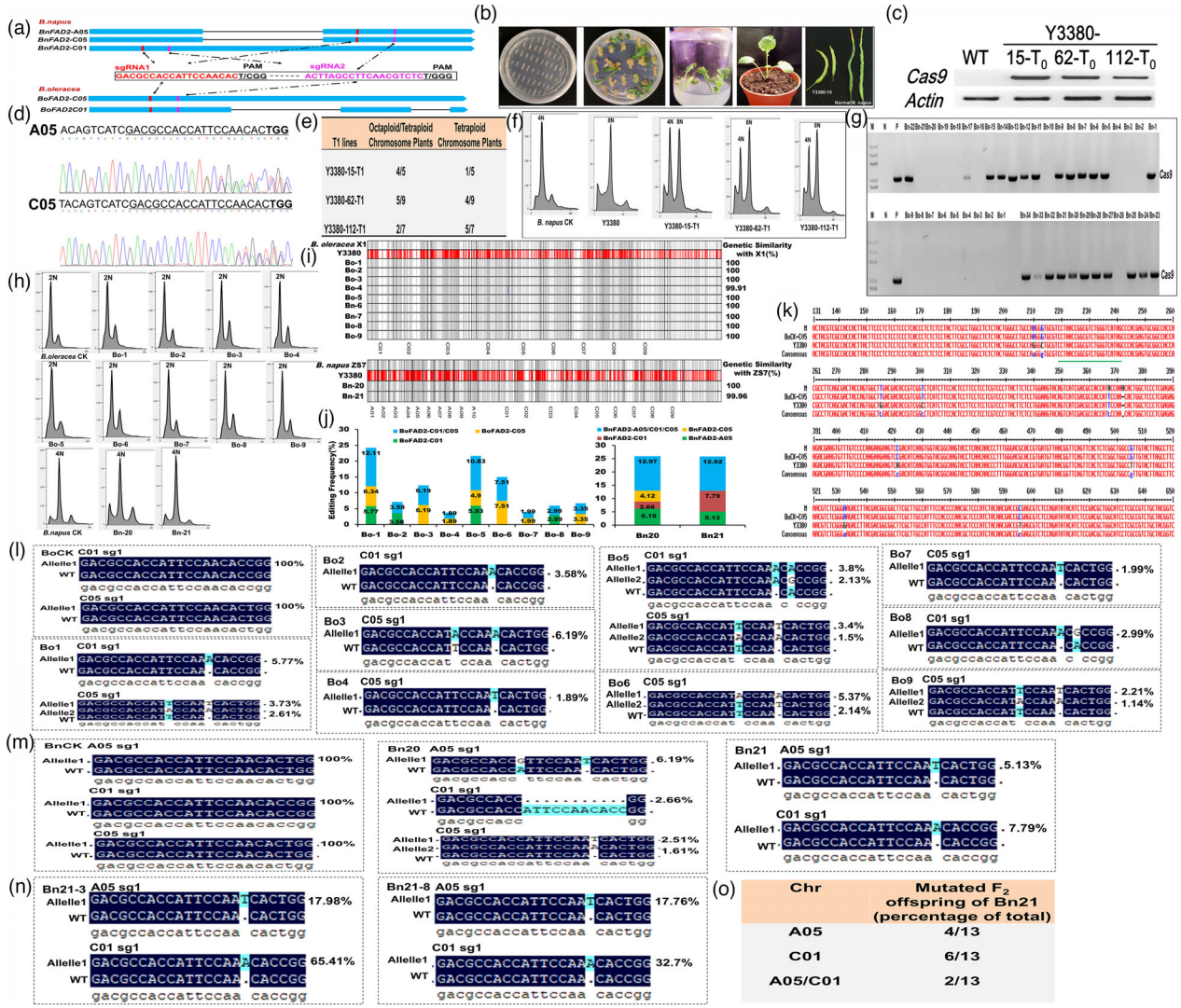

Figure 1 Doubled Haploid Inducer-Mediated Genome Modification of Multiple Gene Homoeologs in *Brassica oleracea* and *Brassica napus*. (a) Two sgRNAs were devised to target the conserved region of three *BnFAD2* and two *BoFAD2* homoeologs. (b) Genetic transformation of Y3380. (c) RT-PCR analysis of *Cas9* expression in three transgenic Y3380 lines. (d) Evaluation of the efficacy of this CRISPR/Cas9 system in Y3380-15 T_0 plant. (e) and (f) Flow cytometry analysis of Y3380-15, Y3380-62, and Y3380-112 T_1 plants. (g) Screening for putative doubled haploid offspring without CRISPR/Cas9 vector by PCR using *Cas9*-specific primers. (h) DNA quantification of doubled haploid lines. (i) Genotyping analysis of nine *B. oleracea* and two *B. napus* offspring using Bnapus 50K Illumina Infinium SNP array. Gray represents the loci with the same alleles as *B.oleracea* X1 or *B.napus* ZS7. Red and blue lines represent different and heterozygous SNP alleles, respectively. (j) Statistical analysis of editing frequency occurred in different *FAD2* homoeologs of *B. oleracea* and *B. napus* doubled haploid offspring. (k)Verification of Hi-Tom sequencing result by TA cloning method. Hi-Tom sequencing analysis of Y3380-CRISPR/Cas9 induced mutagenesis in *B. oleracea* (l), *B. napus* (m) and the next generation of Bn-21(n). (o) Summary of mutated F_2 offspring.

detected in the nine *B. oleracea* offspring (Bo-1 to Bo-9, Figure 1i). In contrast, 2782 *B. napus* female parent-specific SNP were found, and two *B. napus* offspring (Bn-20 and Bn-21) exhibited over 99.9% SNP similarity with the female parent ZS7 (Figure 1i). DNA quantification by flow cytometry demonstrated that the ploidy level of all these plants was consistent with their *B. oleracea* or *B. napus* female parents, respectively (Figure 1h). Thus, these nine diploid *B. oleracea* and two tetraploid *B. napus* plants should be doubled haploids induced by CRISPR/Cas9-engineered Y3380 (termed as Y3380-CRISPR/Cas9).

To evaluate the potential mutation efficacy caused by Y3380-CRISPR/Cas9 inducer, all devised genome targeting sites were examined by Hi-Tom high-throughput sequencing assay. As shown in Figure 1j,1l, and 1m, two out of nine *B. oleracea* offspring, one out of two *B. napus* offspring were detected with targeted mutagenesis in all designed two homoeologs of *B. oleracea* and three homoeologs of *B. napus*, respectively. The editing frequency in nine *B. oleracea* offspring plants ranged from 1.89% to 12.11%, whereas that in two *B. napus* lines ranged from 12.92% to 12.97% (Figure 1j). To further confirm Hi-Tom sequencing data, several SNPs were identified at the BoFAD2C05 homoeolog between *B. oleracea* X1 female and Y3380 male parent (Figure 1k). Then, TA cloning method was employed to isolate the mutation types from *B. oleracea* offspring. After analyzing plenty of TA cloning results, a mutation type was eventually detected and confirmed that mutagenesis events

precisely occurred at the target site of the female genome (Figure 1k). In addition, the mutation types were faithfully inherited to the next generation (Figure 1n and 1o). It was reported that both homogenous and heterozygous haplotypes were identified using maize *MLT* and Arabidopsis *CENH3* based HI-Edit systems, indicating that HI induced genome editing can occur after the zygotic S phase (Kelliher *et al*., 2019). Compared with the mutation efficiency (less than 10% edited offspring) generated by maize haploid inducer-mediated genome-editing methods (Kelliher *et al*., 2019; Wang *et al*., 2019), all doubled haploid lines induced by Y3380-CRISPR/Cas9 were detected with targeted mutagenesis. However, homozygous mutation event was not found, implying a distinct mechanism might be underlying this Y3380-CRISPR/Cas9 genome-editing system.

In summary, we successfully used *Brassica* doubled haploid inducer Y3380 as CRISPR/Cas9 carrier to generate site-specific mutagenesis in *B*. *oleracea* and *B*. *napus* without *Cas9* transgene integration. Compared with existing HI genome-editing approaches, the mutated progenies caused by Y3380-CRISPR/Cas9 pollen are already doubled haploid plants without the requirement of additional artificial doubling processes. Furthermore, our sequencing data demonstrated that Y3380-CRISPR/Cas9 genome-editing system is qualified for simultaneous genome modification of multiple homoeologous gene copies in two main *Brassica* crops, *B*. *oleracea* and *B*. *napus*. Although the chimeric mutation always needs several generations of self-pollination to create homozygous mutant lines, this approach is undoubtedly able to break the limitation of the tissue culture process and direct genome editing of important breeding materials. Future improvement of its editing efficiency would be achieved by employing gamete-preferential promoters and heat stress treatment at 37°C to increase Cas9 activity during fertilization (Kelliher *et al*., 2019; LeBlanc *et al*., 2018). This is the first demonstration of doubled haploid inducer-mediated genome editing in *Brassica* crops, shedding new light on rapid application of CRISPR/Cas technology in a wide range of plant species.

Conflict of interest

No conflict of interest was declared.

Author contributions

C.L, Q.H. designed the experiments and wrote the paper; S.-F.S, M.-D.S, Y.-Q.S, X.-Z.H, M.-Y.H, W.C. H.-Y.Z performed the experiments; L.F, H.W, W.-X.W, J.L analyzed data; H.-T.C, J.L, D.-S.M, and B.-H.Z revised the manuscript. F.-S.H, Y.-J, and L.-Y provided the material and participated in discussion. All authors read and approved the manuscript.

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