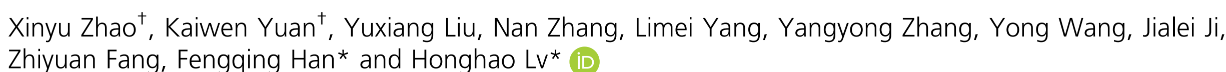
Plant Biotechnology Journal Open Access

aab SEB

Plant Biotechnology Journal (2022) **20**, pp. 2242–2244 doi: 10.1111/pbi.13934

Brief Communication

In vivo maternal haploid induction based on genome editing of *DMP* in *Brassica oleracea*



Xinyu Zhao[†], Kaiwen Yuan[†], Yuxiang Liu, Nan Zhang, Limei Yang, Yangyong Zhang, Yong Wang, Jialei Ji, Zhiyuan Fang, Fengqing Han* and Honghao Lv*

Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, Beijing, China

Received 18 August 2022;
revised 21 September 2022;
accepted 22 September 2022.
**Correspondence* (Tel +86-010-621-35629; fax 010-62146160; email hanfengqing@caas.cn (F.H.); lvhonghao@caas.cn (H.L.))
[†]These authors contributed equally to this work.

Keywords: *Brassica oleracea*, *BoC03.DMP9*, doubled haploid technology, maternal haploid induction, CRISPR-Cas9.

Brassica oleracea is an important plant species that includes many globally cultivated vegetable crops (cole crops), such as cabbage, broccoli, cauliflower, kale and Brussels sprouts. These plants provide human beings with not only plentiful nutrients such as carotenoids, minerals, vitamins A and C, dietary fibre but also unique health-promoting compounds like glucosinolates (Xu *et al*., 2014).

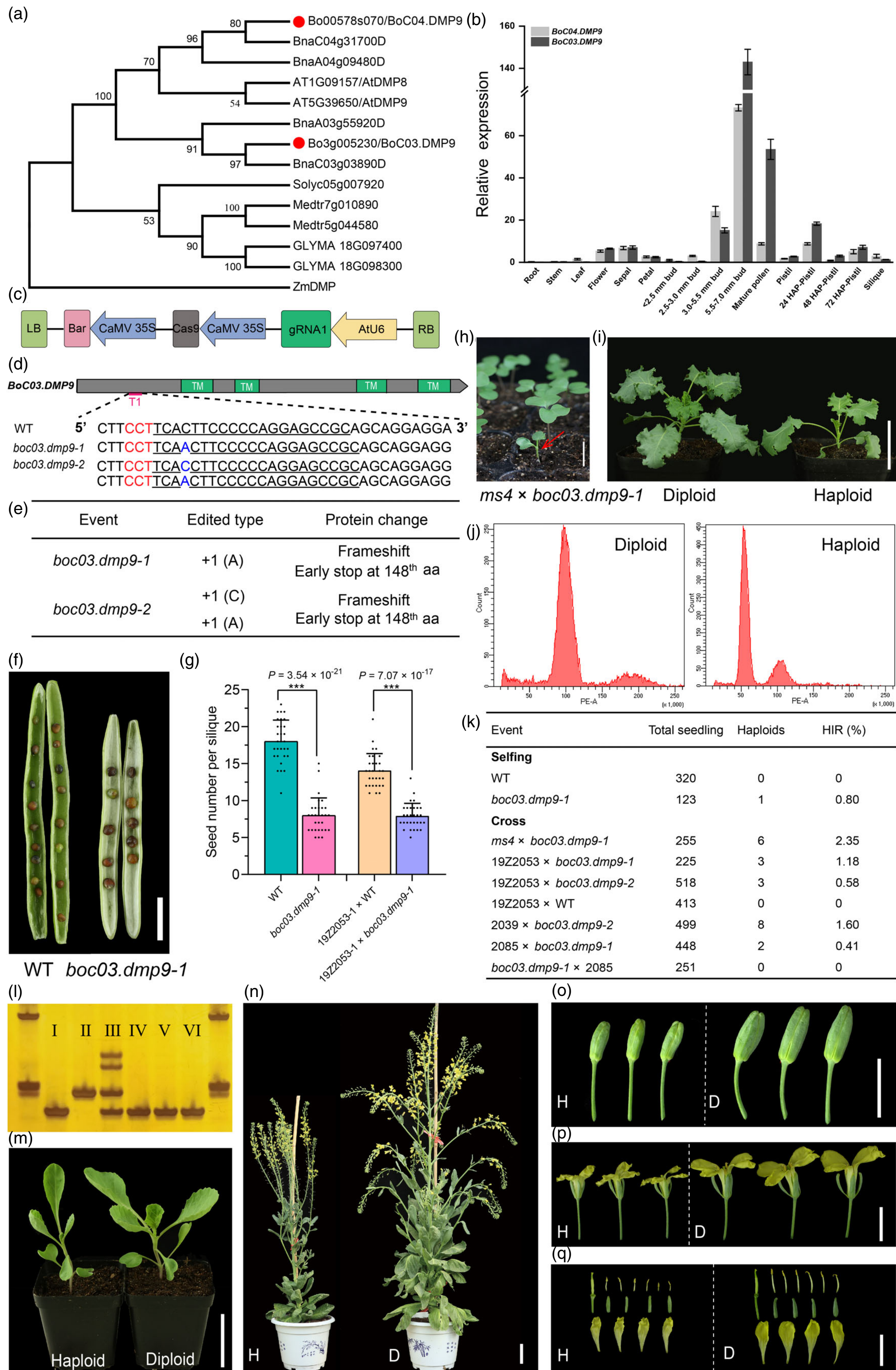
Heterosis utilization in crops, including cole vegetables, requires the development of homozygous lines usually generated by multiple rounds of selfing or backcrossing (Zhong *et al*., 2019). Doubled haploid (DH) technology enables the generation of complete homozygous lines within two generations, dramatically accelerating the breeding progress (Zhong *et al*., 2020). However, traditional haploid induction (HI) in *Brassica oleracea* depends on an *in vitro* anther/microspore cultivation approach, which is not only complicated but also highly limited by plant genotype. In recent years, *MTL/NLD/ZmPLA1*, *ZmDMP* and *ZmPOD65* were found to be responsible for inducing *in vivo* maternal haploid embryos in maize (Jiang *et al*., 2022 and references therein). Although orthologues of *MTL/NLD/ZmPLA1* have not been found in dicots, *ZmDMP-like* genes are present in dicots and have been demonstrated to trigger *in vivo* maternal HI in *Arabidopsis*, *Medicago truncatula*, tomato, rapeseed and tobacco (Li *et al*., 2022; Wang *et al*., 2022; Zhong *et al*., 2020, 2022a,b). However, it is still not known whether this approach can be applied to cole crops.

We found fifteen putative DMP-like proteins in the *Brassica oleracea* genome. Among the proteins identified above, BoC04.DMP9 and BoC03.DMP9 were highly similar to ZmDMP, with 61% and 60% sequence identity, respectively, and they were assigned to a subclade together with AtDMP9 and AtDMP8 (Figure 1a). qRT–PCR analysis indicated that both *BoC03.DMP9* and *BoC04.DMP9* are highly expressed in pollen and flower buds, with *BoC03.DMP9* being more highly expressed (Figure 1b). We cloned *BoC03.DMP9* and *BoC04.DMP9* from multiple cabbage inbred lines. Intriguingly, *BoC04.DMP9* was lost in these cabbage lines due to a 1-bp deletion in exon. We further investigated *DMPs* in the *Brassica* genus, which showed that *DMP8* was completely lost, and *DMP9* experienced duplication and then lost. Both A and B genomes retained two normal *DMP9* genes, whereas in the C genome, differential *DMP9* orthologues were lost, including *BoC04.DMP9* in *B. oleracea* and *BnaC03g03890D* in *B. napus*, indicating that the loss of *DMP9* is a recent event after the formation of *B. napus*.

We employed the CRISPR/Cas9 approach to knock out *BoC03.DMP9* in the cabbage 'MW' background. A CRISPR/Cas9 construct with a specific guide RNA sequence targeting the exon of *BoC03.DMP9* was generated and introduced into cabbage by *Agrobacterium*-mediated transformation (Figure 1c). We obtained 8 lines with mutations in the target region, among which two homozygous or biallelic *boc03.dmp9* mutants with deletions/insertions that led to frameshift and premature termination were selected for further studies (Figure 1d,e). Upon selfing or serving as pollen donors for crossing, the *boc03.dmp9* mutants showed significantly reduced seed sets (Figure 1f,g).

Figure 1 *boc03.dmp9* mutants trigger maternal haploid induction. (a) Phylogenetic analysis of DMP homologues in *Brassica oleracea* (Bo), *Arabidopsis* (At), *Brassica napus* (Bna), soybean (Glyma), *Medicago truncatula* (Medtr) and *Solanum lycopersicum* (Soly). BoC03.DMP9 and BoC04.DMP9 are indicated with red dots. A neighbour-joining phylogenetic tree (1000 bootstrap replications) was constructed using MEGA7 software. (b) Relative expression levels of *BoC03.DMP9* and *BoC04.DMP9* in cabbage tissues. Three independent biological replicates were performed. Error bars represent mean ± SD. HAP, hours after pollination. (c) Schematic diagram of the CRISPR/Cas9 construct targeting *BoC03.DMP9*. *Bar*, bialaphos resistance gene; AtU6, *Arabidopsis* U6-26 promoter. (d) Schematic of *BoC03.DMP9*. Grey blocks, gene coding region; green blocks, predicted transmembrane domains (TMs); red lines, the region (T1) targeted by sgRNA. Sequences from the wild-type (WT) and mutants are shown below the overview. The target sequences are underlined. Insertions are highlighted in blue, and PAM sequences are highlighted in red. (e) Two representative plants edited at the target region of *BoC03.DMP9*. (f) Representative siliques from selfed MW (WT) and *boc03.dmp9* mutants. (g) Seed number per silique of selfed WT and *boc03.dmp9* mutants. Bars represent mean ± SD (n = 30); asterisks indicate significant differences (***$P < 0.001$, Student's t-test). (h) Six-day old seedlings from *ms4* × *boc03.dmp9-1*. Purple plants are typical F_1 hybrids. Red arrow indicates a haploid with non-purple phenotype similar to *ms4*. (i) Diploid and haploid from the tester line *ms4*. (j) Flow cytometry analysis verification of putative haploids. (k) HIR of *boc03.dmp9* determined by selfing or crossing. (l) Putative haploids were genotyped with molecular markers. Left and right lanes, DNA marker; I-III, PCR bands of the 19Z2053, *boc03.dmp9* mutant, a F_1 hybrid from 19Z2053 × *boc03.dmp9-1*, representatively; IV-VI, PCR bands of three haploids from 19Z2053. (m–q) Phenotypes of haploid and diploid 19Z2053. H, haploid; D, diploid. Scale bars: 1 cm (f, h, o, p and q), 5 cm (i, m and n).

© 2022 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd.
This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.



(e)

Event	Edited type	Protein change
boc03.dmp9-1	+1 (A)	Frameshift Early stop at 148th aa
boc03.dmp9-2	+1 (C) +1 (A)	Frameshift Early stop at 148th aa

(k)

Event	Total seedling	Haploids	HIR (%)
Selfing			
WT	320	0	0
boc03.dmp9-1	123	1	0.80
Cross			
ms4 × *boc03.dmp9-1*	255	6	2.35
19Z2053 × *boc03.dmp9-1*	225	3	1.18
19Z2053 × *boc03.dmp9-2*	518	3	0.58
19Z2053 × WT	413	0	0
2039 × *boc03.dmp9-2*	499	8	1.60
2085 × *boc03.dmp9-1*	448	2	0.41
boc03.dmp9-1 × 2085	251	0	0

© 2022 The Authors. *Plant Biotechnology Journal* published by Society for Experimental Biology and The Association of Applied Biologists and John Wiley & Sons Ltd., **20**, 2242–2244

To test whether *boc03.dmp9* mutants could induce the production of haploids, we crossed *boc03.dmp9* mutants (as male parents) with the tester line *ms4*, a curly kale male-sterile line. *ms4* is an ideal HI testing material owing to its two characteristics: (i) completely green, a natural phenotype resulting from the abolishment of anthocyanin accumulation (Figure 1h), and (ii) male sterility, which prevents the occurrence of selfing. We found that six out of 255 progenies exhibited a completely green phenotype (Figure 1i). Flow cytometry analysis revealed that all six non-purple plants were true haploids, which corresponded to a haploid induction rate (HIR) of 2.35% (Figure 1j,k).

We further carried out a set of tests using *boc03.dmp9* mutants to cross cabbage materials, including two inbred lines (19Z2053 and 2039) and one hybrid (2085). Molecular markers showing InDel polymorphism between *boc03.dmp9* and the female parents were developed to screen all the progenies. Haploid would show genotype identical to the corresponding female (Figure 1l). Potential haploids identified by molecular markers were further confirmed by cytometry analysis and plant phenotyping. The HIRs ranged from 0.41% to 1.60% (Figure 1k). Haploids derived from the 19Z2053 × *boc03.dmp9* and 2039 × *boc03.dmp9* crosses were morphologically similar to the corresponding female parent (Figure 1m,n) but had smaller organs and were sterile (Figure 1o–q). We also tested the HI ability by the use of the *boc03.dmp9* as a female, but no haploids were identified after crossing (Figure 1k).

In summary, we demonstrated that *boc03.dmp9* mutants could induce *in vivo* maternal haploids in cole crops. The reported *DMP*-based *in vivo* HI system offers a novel, simple and cost-effective DH technology without genotype recalcitrance. Importantly, this system is applicable to one-step creation of homozygous male-sterile lines for hybrid seed production. In summary, this HI system could accelerate cultivar improvement and genetic studies of these important vegetable crops and provides reference information for extending this system to other dicotyledonous crop species.

Acknowledgements

This work was supported by grants from the Science and Technology Innovation Program of the Chinese Academy of Agricultural Sciences (CAAS-ASTIP-IVFCAAS) and China Agriculture Research System of MOF and MARA (CARS−23).

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

F.H. and H.L. conceived and designed the work. X.Z., K.Y., Y.L. and N.Z. performed the experiments. F.H. and X.Z. wrote and revised the manuscript. L.Y., Y.Z., Y.W., J.J. and Z.F. analysed the data and revised the manuscript. All authors have read and approved the final manuscript.

References

Jiang, C., Sun, J., Li, R., Yan, S., Chen, W., Guo, L., Qin, G. *et al*. (2022) A reactive oxygen species burst causes haploid induction in maize. *Mol. Plant*, **15**, 943–955.

Li, Y., Li, D., Xiao, Q., Wang, H., Wen, J., Tu, J., Shen, J. *et al*. (2022) An *in planta* haploid induction system in *Brassica napus*. *J. Integr. Plant Biol.* **64**, 1140–1144.

Wang, N., Xia, X., Jiang, T., Li, L., Zhang, P., Niu, L., Cheng, H. *et al*. (2022) *In planta* haploid induction by genome editing of *DMP* in the model legume *Medicago truncatula*. *Plant Biotech. J.* **20**, 22–24.

Xu, F., Zheng, Y., Yang, Z., Cao, S., Shao, X. and Wang, H. (2014) Domestic cooking methods affect the nutritional quality of red cabbage. *Food Chem.* **161**, 162–167.

Zhong, Y., Liu, C., Qi, X., Jiao, Y., Wang, D., Wang, Y., Liu, Z. *et al*. (2019) Mutation of *ZmDMP* enhances haploid induction in maize. *Nat. Plants*, **5**, 575–580.

Zhong, Y., Chen, B., Li, M., Wang, D., Jiao, Y., Qi, X., Wang, M. *et al*. (2020) A *DMP*-triggered in vivo maternal haploid induction system in the dicotyledonous *Arabidopsis*. *Nat. Plants*, **6**, 466–472.

Zhong, Y., Chen, B., Wang, D., Zhu, X., Li, M., Zhang, J., Chen, M. *et al*. (2022a) *In vivo* maternal haploid induction in tomato. *Plant Biotech. J.* **20**, 250–252.

Zhong, Y., Wang, Y., Chen, B., Liu, J., Wang, D., Li, M., Qi, X. *et al*. (2022b) Establishment of a *dmp* based maternal haploid induction system for polyploid *Brassica napus* and *Nicotiana tabacum*. *J. Integr. Plant Biol.* **64**, 1281–1294.