**Brief Communication**

**In planta** haploid induction by genome editing of **DMP** in the model legume **Medicago truncatula**

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Double haploid (DH) technology, based on in vivo haploid induction, enables the fixation of recombinant haplotypes within two generations, thereby greatly increasing crop breeding efficiency (Jacquier et al., 2020). Although haploid plants can be produced from some legumes via an in vitro anther/microspore culture approach (Croser et al., 2006), an in vivo (seed-based) haploid induction system has not yet been established for this family, hindering the application of DH technology. Here, we report the successful generation of haploid plants through seeds by editing DMP (DOMAIN OF UNKNOWN FUNCTION 679) homologues in *Medicago truncatula*, a well-characterized model legume.

Mutations in ZmDMP were shown to enhance haploid induction in maize (*Zea mays*) when combined with mutations in *MTNL/ZmPLA1* (Gilles et al., 2017; Kellher et al., 2017; Liu et al., 2017; Zhong et al., 2019). Although MTNL/ZmPLA1 is not conserved in dicots, DMP is conserved in both monocots and dicots (including legumes), and loss of function ZmDMP orthologues in the dicot Arabidopsis (*Arabidopsis thaliana*) trigger maternal haploid induction (Zhong et al., 2020), opening the possibility of applying the DMP-triggered in vivo haploid induction system to leguminous plants. In agreement with previous reports (Zhong et al., 2019, 2020), phylogenetic analysis showed that ZmDMP has homologues in several legumes, including soybean (**Glycine max**), alfalfa (**Medicago sativa**) and *M. truncatula* (Figure 1a). Using *M. truncatula*, we explored whether the mutation of DMP homologues might be used for haploid induction in legumes.

We searched the *M. truncatula* genome (v4.0) using a Basic Local Alignment Sequence Tool for Protein (BLASTP) analysis and ZmDMP as query. When using a minimum protein sequence identity of 40%, we identified six putative DMP-like proteins. Phylogenetic analysis showed that MtDMP8 (Medtr7g010890) and MtDMP9 (Medtr5g044580), which are most similar to ZmDMP (63.9% and 62.8% sequence identity, respectively), cluster together with ZmDMP in a separate subclade that includes Arabidopsis DMP8 and DMP9 (Figure 1a). MtDMP8 and MtDMP9 both contained four putative transmembrane domains.

Consistent with this prediction, both proteins colocalized with the PIP2A (At3g53420)-based plasma membrane marker pm-GFP (Zhu et al., 2020) when MtDMP8 and MtDMP9 were transiently expressed as red fluorescent protein (RFP) fusions in Arabidopsis leaf protoplasts (Figure 1b). RT-qPCR analysis revealed that both MtDMP8 and MtDMP9 are highly expressed in mature anthers and pollen, with MtDMP9 being more highly expressed, suggesting that MtDMP8 and MtDMP9 function during the late stages of gametophyte development (Figure 1c).

To assess the role of MtDMP8 and MtDMP9 in haploid induction in *M. truncatula*, we generated single and double knockout mutants in MtDMP8 or MtDMP9 (Figure 1d) using the pDIRECT_22C vector of the CRISPR-Cas9 toolkit (Cermak et al., 2017) and two pairs of specific guide RNA sequences (gRNAs, each pair targeting one gene). After Agrobacterium (*Agrobacterium tumefaciens*)-mediated transformation of *M. truncatula* accession R108 (Zhu et al., 2020), CRISPR mutants with deletions and insertions that led to translational frame shifts were found at MtDMP8 and/or MtDMP9 in the T₁ generation (Figure 1d). Pollen development was normal in the T₁ progeny of mtDMP8 and mtDMP9 single mutants, but pollen viability was reduced in mtDMP8 mtDMP9 double mutants (Figure 1e). Furthermore, seed set was slightly reduced in both mtDMP8 and mtDMP9 single mutants, but mtDMP8 mtDMP9 double mutants showed drastically reduced seed set (Figure 1f), confirming previously reported defects in seed set and putative roles for MtDMP8 and MtDMP9 in fertilization. Haploid *M. truncatula* plants, which exhibit typical haploid characteristics of reduced stature, as well as small ovules and sterile pollen, were identified amongst the self-pollinated progenies of mtDMP8 mtDMP9 mutants (Figure 1g-i). The average haploid induction rate (HIR) ranged from 0.29% to 0.82% among the T₂ progeny of mtDMP8 mtDMP9 mutant lines (Figure 1j). However, not a single haploid plant was identified among the T₂ progeny from selfing mtDMP8 and mtDMP9 single mutants or wild-type plants (Figure 1j). To investigate whether mtDMP8 mtDMP9 mutants could induce haploid embryos in different female parents, the *M. truncatula* ecotype Jemalong A17 was pollinated with pollen from mtDMP8 mtDMP9-1. We identified three haploids among 550 plants from this crossing, whereas no haploids were found among the 620 plants resulting from the cross using wild-type R108 as pollen donor (Figure 1j). The haploid plants were morphologically similar to the female parent A17 (Figure 1k). Thus, the simultaneous inactivation of MtDMP8 and MtDMP9 can trigger in vivo maternal haploid induction in *M. truncatula*.

Our successful haploid induction in *M. truncatula* provides a promising starting point for legume haploid gene editing and...
Genome editing of DMP triggers Medicago haploid induction

(d) MSAD_260941
MSAD_215960
Medtr7g010860/MdMMP8
MSAD_261085
Medtr5g044580/MdMMP9
MSAD_213258
Glyma_18G097400
Glyma_18G098300
AtDMP8
AtDMP8
ZmDMP
Medtr4g078870
Medtr4g088335
Medtr2g014520
Medtr2g014550

(b) RFP
pm-GFP
Merged
Bright field

(c) Relative expression

WT
mtDMP8-1
mtDMP8-2
mtDMP8-3
mtDMP8 mtDMP9-1
mtDMP8 mtDMP9-2
mtDMP8 mtDMP9-3

MTDMP9
ATG

(i) Event
Total seedling
Haploids
HiR(%) Selfing
WT
500
0
0 mtDMP8-1
522
0
0 mtDMP8-2
513
0
0 mtDMP8-3
433
0
0 mtDMP8-1
471
0
0 mtDMP8-2
425
0
0 mtDMP8-3
457
0
0 mtDMP8 mtDMP9-1
585
2
0.34 mtDMP8 mtDMP9-2
368
3
0.82 mtDMP8 mtDMP9-3
350
1
0.29 Cross
A17 x mtDMP8 mtDMP9-1
550
3
0.55 A17 x WT
620
0
0

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mechanistic studies of haploid induction in legumes. Future work will extend the range of applications of DMP-triggered \textit{in vivo} haploid induction to crops and forages such as soybean and alfalfa, paving the way for the deployment of DH technology in legume breeding.

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**Conflict of interest**

The authors declare no conflict of interest.

**Author contributions**

N.W., L.N. and H.L. designed the research. N.W., X.X, T.J., L.L. and P.Z. performed the experiments and analysed the data. H.C. and K.W. provided technical support. H.L. wrote the manuscript.

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